miR-29b Participates in Early Aneurysm Development in Marfan Syndrome

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Rationale: Marfan syndrome (MFS) is a systemic connective tissue disorder notable for the development of aortic root aneurysms and the subsequent life-threatening complications of aortic dissection and rupture. Underlying fibrillin-1 gene mutations cause increased transforming growth factor-β (TGF-β) signaling. Although TGF-β blockade prevents aneurysms in MFS mouse models, the mechanisms through which excessive TGF-β causes aneurysms remain ill-defined. Given that miRNAs are known to influence cellular matrix (ECM) formation, and apoptosis, however, although TGF-β blockade inhibits aneurysm formation and growth in murine models of MFS, the molecular mechanisms by which excessive TGF-β signaling leads to aneurysm development remain unknown.

Methods and Results: Using quantitative polymerase chain reaction, we discovered that miR-29b, a microRNA regulating apoptosis and extracellular matrix synthesis/deposition genes, is increased in the ascending aorta of Marfan (Fbn1<sup>C1039G/+</sup>) mice. Increased apoptosis, assessed by increased cleaved caspase-3 and caspase-9, enhanced caspase-3 activity, and decreased levels of the antiapoptotic proteins, Mel-1 and Bcl-2, were found in the Fbn1<sup>C1039G/+</sup> aorta. Histological evidence of decreased and fragmented elastin was observed exclusively in the Fbn1<sup>C1039G/+</sup> ascending aorta in association with repressed elastin mRNA and increased matrix metalloproteinase-2 expression and activity, both targets of miR-29b. Evidence of decreased activation of nuclear factor κB, a repressor of miR-29b, and a factor suppressed by TGF-β, was also observed in Fbn1<sup>C1039G/+</sup> aorta. Furthermore, administration of a nuclear factor κB inhibitor increased miR-29b levels, whereas TGF-β blockade or losartan effectively decreased miR-29b levels in Fbn1<sup>C1039G/+</sup> mice. Finally, miR-29b blockade by locked nucleic acid antisense oligonucleotides prevented early aneurysm development, aortic wall apoptosis, and extracellular matrix deficiencies.

Conclusions: We identify increased miR-29b expression as key to the pathogenesis of early aneurysm development in MFS by regulating aortic wall apoptosis and extracellular matrix abnormalities. (Circ Res. 2012;110:312-324.)

Key Words: aneurysm • apoptosis • extracellular matrix • Marfan syndrome • microRNA

Marfan syndrome (MFS) is an autosomal-dominant systemic connective tissue disorder that affects 1 in 5000 individuals. Patients with MFS typically have development of ocular, musculoskeletal, and cardiovascular manifestations, but aortic root aneurysm and ensuing aortic dissection remain the leading cause of death. Although current management includes medical therapy, only surgical treatment with prophylactic replacement of the entire aortic root effectively increases life expectancy in patients with MFS. Recent studies have demonstrated that the underlying fibrillin-1 gene mutation in MFS increases the activity of transforming growth factor-β (TGF-β), a multifunctional cytokine that regulates proliferation, differentiation, inflammation, extra-
vascular wall proliferation, angiogenesis, differentiation, and apoptosis,6-7 we hypothesized that miRNAs may participate in the vascular remodeling and subsequent aneurysm development characteristic of MFS.

In the present study, we use transgenic Marfan mice (Fbn1<sup>C1039G/+</sup>) to determine if altered miRNA expression contributes to aortic wall remodeling and aneurysm formation. Increased expression of miR-29b, a miRNA known to regulate genes involved in apoptosis and ECM synthesis/ deposition, was observed in Fbn1<sup>C1039G/+</sup> ascending aorta (AS). Notably, elastin (ELN) levels in Fbn1<sup>C1039G/+</sup> AS aorta are decreased because of reduced early elastogenesis and increased elastin fragmentation. In vitro studies suggest that enhanced TGF-β signaling in the aortic wall of Fbn1<sup>C1039G/+</sup> mice may serve to reduce active nuclear factor κB (NF-κB), thereby causing a derepression of miR-29b. Finally, treatment of Fbn1<sup>C1039G/+</sup> mice with a silencing miR-29b antisense oligonucleotide inhibitor completely blocked aortic root dilation at 4 weeks. Taken together, our data suggest that the combination of miR-29b-mediated reduction in early ECM deposition and smooth muscle cell apoptosis initiates aneurysm formation in this Marfan mouse model. These data provide important new insights into the mechanisms of early aortic root enlargement in MFS and potentially allows for the development of new therapies in MFS and related disorders.

**Methods**

Mice

All animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University (http://labanimals.stanford.edu/) and followed the National Institutes of Health and United States Department of Agriculture Association Guidelines for the Care and Use of Animals in Research. All experiments were performed with both male and female Fbn1<sup>C1039G/+</sup> mice and C57BL/6J littermate wild-type (WT) controls. Experimental groups were as follows.

**Locked Nucleic Acid miR-29b Oligonucleotide Inhibitor**

Animals received a locked nucleic acid (LNA)-antimiR-29b inhibitor (mmu-miR-29b, miRCURY LNA microRNA Power inhibitor; Exiqon, Woburn, MA) either prenatally or postnatally. First, in prenatal studies, female Fbn1<sup>C1039G/+</sup> mice were time-mated with WT male mice and then treated 14.5 days postcoitum with a single tail-vein injection of: (1) LNA-antimiR-29b (8 mg/kg); (2) scrambled-control-miR (8 mg/kg) (miRCURY LNA microRNA inhibitor; Exiqon, Woburn, MA); or (3) phosphate-buffered saline (PBS; control; pH 7.4; Gibco, Invitrogen, Carlsbad, CA). Pups from these female mice were euthanized at age 4 weeks. In separate postnatal experiments, 2-week-old Fbn1<sup>C1039G/+</sup> mice and C57BL/6J littermate wild-type controls were euthanized at age 4 weeks. The 5’-3’ sequences for the LNA-antimiR-29b and LNA-scrambled-miR control were ACTGATTTCAAATGGTGCT and GTGTAACACGTACCAAGCCCA, respectively.

**Losartan**

Pregnant mice were treated beginning at 14.5 days postcoitum with oral losartan (0.6 g/L in drinking water; Merck KgaA, Darmstadt, Germany). Pups then received oral losartan until they were euthanized at age 4 weeks.

**TGF-β Neutralizing Antibody**

The 2-week-old mice were injected with either intraperitoneal pan-sensitive TGF-β neutralizing antibody or rabbit IgG (control; R&D Systems, Minneapolis, MN) for 3 consecutive days, followed by another injection 1 week later. Mice were euthanized at age 4 weeks.

**IκB-α Phosphorylation Inhibitor (BAY Inhibitor)**

The 6-week-old mice were injected with either 10 mg/kg intraperitoneal BAY 11-7082 (Calbiochem, Gibbstown, NJ) or DMSO/PBS (control) every other day and euthanized at age 8 weeks.

**Echocardiography**

Transthoracic echocardiography was performed on sedated mice by using 2% inhaled isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane; Baxter Healthcare Corporation, Deerfield, IL) delivered via nose cone. The aorta was imaged in the parasternal long-axis view. Measurements were obtained by two blinded investigators in triplicate at the level of the sinus of Valsalva.

**Magnetic Resonance Imaging**

A self-shielded General Electric microSigna 7.0 7-T MRI machine was used. Cardiac sequences were acquired while gating the cardiac and respiratory cycles. Mice were anesthetized using 2% isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane; Baxter Healthcare Corporation, Deerfield, IL) delivered via nose cone. The aorta was imaged in the parasternal long-axis view. Measurements were obtained by two blinded investigators in triplicate at the level of the sinus of Valsalva.

**Preparation of Aortic Tissue**

Mice were euthanized with an inhalation overdose of isoflurane. The aorta was dissected and then divided into three portions: (1) aortic...
root (distal to aortic valve), ascending aorta and arch (AS); (2) descending thoracic aorta (D); and (3) abdominal aorta (ABD).

Western Blot Analysis
Separated frozen sections of aorta were homogenized in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO). Using 10 μg of protein per well, Western blot (WB) was performed. Quantification by densitometry was performed using ImageJ software (National Institutes of Health). All experiments included at least three replicates per group.

Caspase-3 Fluorescent Activity Assay
To measure caspase-3 activity in protein extracts (prepared similarly to Western blotting), the Apo-ONE Homogeneous Caspase-3/7 assay (Promega, Madison, WI) was performed following manufacturer's protocol with 50 μg of protein. All experiments included at least three replicates per group.

Gelatin Zymography
To measure matrix metalloproteinase (MMP)-2 and MMP-9 activity in protein extracts, 10 μg of total protein were resolved by nondeaturing electrophoresis through a 10% gelatin gel (Invitrogen, Carlsbad, CA). The molecular sizes of gelatinolytic activities were determined using 10 ng of protein standards (MMP-2; Calbiochem, Gibbstown, NJ; and MMP-9; Abcam, Cambridge, MA). Quantification by densitometry was performed using ImageJ software (National Institutes of Health) on inverted images of gel. All experiments included at least three replicates per group.

RNA Isolation
Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). RNA then was quantified by Nanodrop (Agilent, Foster City, CA) and samples were included when the 260/280 nm ratio was >1.8.

Detection and Quantification of miRNAs by Quantitative Polymerase Chain Reaction
RNA was obtained as outlined. Diluted RNA was reverse-transcribed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Subsequently, the product was used to detect miRNA expression by quantitative polymerase chain reaction using TaqMan microRNA Assay kits (Applied Biosystems, Life Technologies) and confirmed by TaqMan real-time polymerase chain reaction assay. All experiments included at least four replicates per group. Values were normalized to sno202 (mouse) and were expressed as ΔΔCT (2^−(ΔΔCT[microRNA]−ΔΔCT[sno202])). All experiments included at least four replicates per group.

Gene Expression Via Quantitative Real-Time Polymerase Chain Reaction
Total RNA from the samples was converted to cDNA according to the manufacturer’s instructions using the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA was amplified in duplicates on the ABI PRISM 7900HT with primers and probes (Applied Biosystems, Life Technologies). Gene expression levels were normalized to corresponding 18S or glyceraldehyde 3-phosphate dehydrogenase internal controls and calculated as stated.

Histological Analysis
Histological staining was performed on AS and D. The aorta was dissected and fixed in 4% formalin. Isolated tissue then was dehydrated and embedded in Tissue-Tek OCT Compound Histo-mount (Sakura, Torrance, CA). Aortic tissue was then cut at 7-μm serial sections and stained with Accustain elastin Verhoff’s van Gieson kit according to manufacturer’s protocol (Sigma-Aldrich, St. Louis, MO).

Immunofluorescence Staining
Primary antibody against cleaved active caspase-3 (1:50; Cell Signaling Technologies, Danvers, MA) and NF-κB subunit p65 (1:25; Abcam, Cambridge, MA) was applied overnight to histological sections. Nuclei were stained with Hoechst reagent (bisBenzide H 33258; Sigma-Aldrich) using 1 μL per 10 mL PBS for 10 minutes. Immunofluorescence microscopy was used to detect active caspase-3 and active nuclear NF-κB (p65).

In Situ Hybridization
In situ hybridization for miR-29b was performed using the miRCURY LNA microRNA in situ hybridization optimization kit (Exiqon, Woburn, MA) and 5’-DIG and 3’-DIG labeled probes for mmu-miR-29b according to the manufacturer’s protocol. The sequence of the LNA miR-29b control probe was 5’-DIG/ AACACTGATTTCAAAATGGTGCTA/DIG-3’.

In Vitro Smooth Muscle Cell Studies
Aortic smooth muscle cells (SMCs) were derived from 4-week-old WT and Fbn1C1039G/+ mice, cultured in SMC media (Lonza, SmGM-2 Bullet Kit CC3182; Basel, Switzerland), and exposed to recombinant TGF-β1 (10 ng/mL; 100-B; R&D Systems, Minneapolis, MN) in chamber slides after starvation with 0.2% fetal bovine serum in SMC media for 24 hours. Cells were then cultured either with 2.5 μmol/L BAY 11-7082 or 10 μmol/L Iosartan (Merck KGaA, Darmstadt, Germany). Cells were then fixed in ice-cold 100% ethanol for 15 minutes and air-dried for 30 minutes before immunofluorescence staining. SMCs were stained for NF-κB subunit p65 as described.

Statistical Analysis
Statistics were calculated using SPSS 18.0/19.0 (SPSS, Chicago, IL). Data are presented as mean±SEM. Unless otherwise stated, results are compared to age-matched littermate WT controls. Groups were compared using the Student t test for parametric data or univariate ANOVA for group comparison. A value of P<0.05 was considered statistically significant.

Results
Early Aortic Root Aneurysm Development in the Fbn1C1039G/+ Marfan Mouse Model
To detect early aneurysm development, both transthoracic echocardiography and MRI were performed on Fbn1C1039G/+ and C57BL/6J (WT) littermate control mice at 2, 4, 6, and 8 weeks of age (Figure 1A). By transthoracic echocardiography, we found that aortic root aneurysms develop as early as 2 weeks of age in Fbn1C1039G/+ mice (Fbn1C1039G/+ 1.01±0.03 mm; WT 0.91±0.12 mm; P<0.001) and continue to increase in size over time. By 8 weeks, the aortic diameter reaches 1.58±0.09 mm in Fbn1C1039G/+ mice compared to 1.27±0.05 mm in WT controls (P<0.001). An identical pattern and time course were identified using cardiac MRI (data not shown).

miR-29b Expression Enhanced in the AS Aorta During Early Aneurysm Formation
The miRNA expression levels have not been previously examined during aneurysm development in MFS. Several miRNAs influence processes involved in the pathogenesis of aneurysm formation, including vascular wall proliferation, angiogenesis, differentiation, and apoptosis. Therefore, we next screened the Fbn1C1039G/+ and WT aortic wall segments for differential miRNA expression. The expression of miR-21, a known regulator of neointimal proliferation and apoptosis, was not different in Fbn1C1039G/+ mice as compared
to WT mice (data not shown). However, miR-29b, a miRNA involved in apoptosis and ECM synthesis and deposition, was significantly upregulated in the Fbn1C1039G/+ AS aorta beginning at 2 weeks (3.14 ± 0.40-fold; P = 0.008), peaked by 4 weeks (5.91 ± 0.06-fold; P = 0.023), and returned to baseline by 8 weeks (Figure 1B). Furthermore, this appeared to be unique to the AS aorta, because miR-29b expression in the Fbn1C1039G/+ AS was significantly greater compared to either D or ABD aortas (3.83 ± 0.50-fold over D, P = 0.034; and 3.72 ± 0.5-fold over ABD, P = 0.034; Figure 1B). Corroborating these findings, in situ hybridization for miR-29b showed more miR-29b in the 4-week-old Fbn1C1039G/+ AS aortic wall compared to either D or WT aortic segments (Online Figure I).

Aortic Wall Apoptosis Increased in Fbn1C1039G/+ Mice During Aneurysm Development

Among the putative functions for miR-29b is the promotion of apoptosis by repressing the expression of antiapoptotic factors. Although aortic wall apoptosis has been reported to participate in the pathogenesis of abdominal aneurysm formation, its role during aneurysm development in MFS remains controversial. In this study, apoptosis was evaluated by caspase-3 activity assays and WB analysis to detect proapoptotic (cleaved caspase-3, caspase-9) and antiapoptotic (Mcl-1 and Bcl-2) factors. Apoptotic measurements in Fbn1C1039G/+ aorta were compared to the corresponding segments in the WT aorta. Low levels of apoptosis were detected in all three aortic segments of WT controls using WB and caspase-3 activity analyses, likely representing normal vessel wall development and remodeling. Caspase-3 activity was elevated in Fbn1C1039G/+ AS aorta at all early time points (2–6 weeks vs WT), but peaked at 4 weeks (4.30 ± 0.78-fold Fbn1C1039G/+ vs WT; P = 0.012), began decreasing at 6 weeks, and returned to baseline by 8 weeks (Figure 2A). To confirm these findings, cleaved (active) caspase-3 and caspase-9 were measured by WB analysis. Mirroring the results from the caspase-3 activity assay, levels of cleaved caspase-3 and caspase-9 were elevated at 2 weeks in Fbn1C1039G/+ AS aorta compared to WT, reached significance by 4 weeks (caspase-3, 4.51 ± 0.32-fold, P = 0.007; and caspase-9, 8.27 ± 0.48-fold, P = 0.025), started to decrease at 6 weeks, and returned to baseline by 8 weeks (Figure 2A, B). Moreover, this increase in apoptosis at early ages in the AS aorta of the Fbn1C1039G/+ mice was associated with a significant reduction in the levels of the antiapoptotic proteins Bcl-2 and Mcl-1. By 8 weeks, Bcl-2 and Mcl-1 protein expression increased in Fbn1C1039G/+ AS aorta compared to WT (Bcl-2, 46.1% ± 36.4% increase over WT, P = 0.05; and Mcl-1, 95.8% ± 24.5% increase over WT, P = 0.03; Figure 2C, D), corresponding to the time point when excessive apoptosis was no longer apparent in the Fbn1C1039G/+ mice. Interestingly, although apoptosis was increased in the AS aortic segments from Fbn1C1039G/+ as compared to WT mice, apoptosis was not significantly greater in Fbn1C1039G/+ AS aorta as compared to D and ABD aortas, when quantified by WB (Figure 2B). This finding suggests that in addition to enhanced apoptosis, a second molecular mechanism must be involved to result in formation of aneurysms restricted to the AS segment.

Early Aneurysm Formation Associated With Reduced ECM in Fbn1C1039G/+ Mice

In addition to aortic wall apoptosis, the pathogenesis of aneurysm formation involves dysregulation of ECM synthesis, deposition, and remodeling. Although the role of ECM remodeling in aneurysm formation is acknowledged, the upstream signaling events that initiate this process are not well-studied. miR-29b regulates numerous genes coding for ECM proteins, including multiple collagens, fibrillins, and...
elastin. Moreover, MMPs, enzymes involved in proteolytic degradation of the ECM, are also known downstream targets of miR-29b. Therefore, we next evaluated if ECM synthesis and remodeling are dysregulated in the Fbn1C1039G/H11001 mice.

The gene expression of ELN was reduced in the AS aorta of the Fbn1C1039G/H11001 mice beginning at 2 weeks of age and significantly decreased at 4 weeks when compared to WT (90.405 ± 15.0% reduction; P = 0.03). This early suppression of ELN was followed by a significant increase in the Fbn1C1039G/H11001 AS aorta at 8 weeks (16.90 ± 0.25-fold increase vs WT; P = 0.03), corresponding with decreased miR-29b levels at this same time point (Figure 3A). Furthermore, the reduced ELN mRNA in the AS aorta of the Fbn1C1039G/H11001 mice at 4 weeks was significantly lower than that observed in the D and ABD segments at the same time point (18.81 ± 1.67-fold decrease from D, P = 0.004; 19.42 ± 1.63-fold decrease from ABD, P = 0.006). Histological analysis of Fbn1C1039G/H11001 AS aorta after elastin van Gieson staining demonstrated an overall decrease in the amount of medial elastin, as well as increased elastin fragmentation, when compared to WT mice (Figure 3B). This finding was in contrast to no appreciable difference between Fbn1C1039G/H11001 and WT in ELN quantity or integrity in the sections obtained from the D.

Proteolytic degradation of ELN within the aortic wall media by MMPs participates in aneurysm development. The increased fragmentation of ELN evident on histological examination of the AS segments from Fbn1C1039G/H11001 mice suggest that heightened proteolytic activity may be an additional mechanism explaining the aneurysm formation in the AS aorta of these mice. Quantitative polymerase chain reaction analysis revealed >3-fold increase in MMP-2 at 2 weeks in the Fbn1C1039G/H11001 AS aorta as compared to WT AS aorta (3.28 ± 0.50-fold increase compared to WT; P = 0.027), and a similar increase as compared to MMP-2 levels at 8 weeks (3.71 ± 0.86-fold increase; P = 0.021; Figure 3C). Similar to the dysregulation of ELN expression, these alterations were isolated to the AS, with MMP-2 levels in the AS aorta at 2 weeks significantly higher than those found in the Fbn1C1039G/H11001 D and ABD aortas at the same time point (3.31 ± 0.52-fold increase over D, P = 0.03; 3.57 ± 0.92-fold increase over ABD, P = 0.05). In addition to MMP-2 mRNA levels, MMP-2 activity by gelatin zymography was also significantly increased in the AS aorta of Fbn1C1039G/H11001 mice beginning at 2 weeks and peaking at 4 weeks (3.86 ± 0.44-fold increase over WT; P = 0.04). Correlating with the gene expression results, MMP-2 activity showed a significant decrease at 8 weeks in Fbn1C1039G/H11001 AS compared to 2 weeks of age (Figure 3D). Analysis of MMP-9 gene expression and activity showed similar trends but did not reach statistical significance (data not shown).
Repressors of miR-29b Decreased in Fbn1<sup>C1039G/+</sup> Mice

To begin to elucidate the mechanism leading to the increased miR-29b levels in the AS aorta during early aneurysm formation, the pathways of two known upstream repressors of miR-29b, NF-κB and c-Myc, were examined. The expression of c-Myc was significantly reduced in Fbn1<sup>C1039G/+</sup> AS aorta (63.2 ± 20.5% of WT; *P* = 0.021) at 4 weeks, corresponding to the time point of peak miR-29b expression, but increased to levels greater than WT levels by 8 weeks (3.65 ± 0.34-fold compared to WT; *P* = 0.007; data not shown).

NF-κB activation occurs via an IkB kinase complex (including IKK-α and IKK-β) that phosphorylates the NF-κB inhibitor (IkB), causing its degradation, and the subsequent translocation of NF-κB into the nucleus, where it can regulate gene expression. In our model, IKK-α was significantly reduced at 2 and 4 weeks in Fbn1<sup>C1039G/+</sup> AS aorta compared to WT (48.65 ± 27.26% reduction; *P* = 0.038; and 55.6 ± 27.1% reduction, *P* = 0.038; at 2 and 4 weeks, respectively; Figure 4A). In addition, IKK-β was significantly decreased in 4-week-old Fbn1<sup>C1039G/+</sup> AS aorta compared to WT AS aorta (79.2% ± 45.2% reduction; *P* = 0.05; Figure 4B). By 8 weeks, the time when miR-29b expression and aortic wall apoptosis had decreased, Fbn1<sup>C1039G/+</sup> AS IKK-α, IKK-β, and NF-κB p65 protein levels by WB were significantly elevated as compared to those at 4 weeks (IKK-α, 8.90 ± 0.61-fold, *P* < 0.001; IKK-β, 15.1 ± 5.56-fold, *P* = 0.01; NF-κB, 8.20 ± 0.48-fold, *P* < 0.001; Figure 4B, C). Because NF-κB p65 protein levels detected by
WB measures total cellular protein, and not exclusively the activated form present in the nucleus, aortic wall histological staining was performed. Immunohistochemistry demonstrated decreased nuclear staining of the NF-κB p65 subunits in Fbn1C1039G/H11001 AS aorta compared to both WT AS aorta and Fbn1C1039G/H11001 D at 4 weeks. This suppression of NF-κB activation was then followed by an increase at 8 weeks, at the time point when miR-29b levels had returned to normal (Figure 4D).

Finally, to confirm that NF-κB participates in miR-29b repression, both in vivo and in vitro studies were performed. First, animals were treated with a selective and irreversible IKK-α phosphorylation inhibitor (BAY inhibitor) beginning at 6 weeks of age, corresponding to the time period when NF-κB levels are initially elevated. Importantly, reduced NF-κB activation via BAY inhibitor treatment significantly increased miR-29b levels in the AS aorta (10.50 ± 0.48-fold compared to WT; *P = 0.008) at age 8 weeks (Figure 4E, Online Figure IIA). In separate in vitro experiments, aortic SMCs were treated with BAY inhibitor, which resulted in decreased NF-κB activation (Figure 4F). Similar to our in vivo findings, impeding NF-κB activation in Fbn1C1039G/+ SMCs with BAY inhibitor significantly increased miR-29b levels (65.03% ± 0.30% increase compared to control WT SMCs; *P = 0.008; Figure 4G). Therefore, the data suggest that decreased NF-κB activation secondary to reduced IKK-α and IKK-β expression could result in the increased miR-29b expression observed in the AS aorta of the Fbn1C1039G/+ mice at 4 weeks of age.

**TGF-β1–Treated Fbn1C1039G/+ Aortic SMCs Express Elevated miR-29b Levels**

Enhanced TGF-β signaling contributes to the pathogenesis of aneurysm formation in MFS; however, the molecular mechanism accounting for this effect is not known. TGF-β1 is known to inhibit NF-κB activation. Therefore, to define a mechanistic link between the increased TGF-β signaling reported in Marfan models and the elevation of miR-29b we observed, we performed in vitro studies using SMCs from the aorta of WT or Fbn1C1039G/+ mice. WT SMCs were used for...
this experiment to: (1) demonstrate that NF-κB is constitutively active in the aortic SMCs under control conditions; (2) identify if exposure to excessive TGF-β1, as is thought to occur in the mutant phenotype, will repress this constitutive activity; and (3) establish whether excessive TGF-β signaling inhibits NF-κB, thereby increasing miR-29b expression. Additional experiments were also performed on Fbn1^{C1039G/+} SMCs to determine if they respond differently to excessive TGF-β1 signaling compared to WT SMCs. We incubated SMCs with recombinant TGF-β1 or vehicle (control) and performed immunocytochemistry to detect the presence of nuclear (active) NF-κB. Vehicle-treated WT SMCs had prominent nuclear staining for the NF-κB subunit, p65, suggesting that NF-κB is constitutively active in the SMCs at this stage of development. TGF-β1 treatment for 24 hours markedly decreased NF-κB p65 nuclear staining (Online Figure III). A similar decrease in active NF-κB was detected in Fbn1^{C1039G/+} SMCs treated with TGF-β1 compared to vehicle control for 24 hours (Figure 5A). We then quantified miR-29b expression in both groups of cells after either TGF-β1 or vehicle treatment. Interestingly, TGF-β1 treatment did not significantly increase miR-29b expression in WT SMCs. In contrast, TGF-β1 stimulation began increasing miR-29b expression in Fbn1^{C1039G/+} SMCs at 24 hours and reached significantly elevated levels by 48 hours (3.12±0.13-fold compared to WT control cells; P=0.03; Figure 5B), suggesting that elevated TGF-β1 levels alone may not be sufficient to cause the mutant phenotype. Then, to determine how TGF-β modulates miR-29b expression in Fbn1^{C1039G/+} in vivo, mice were treated with a pan-sensitive TGF-β neutralizing antibody (TGF Nab). TGF-β neutralizing antibody treatment reduced early aeurysm formation in Fbn1^{C1039G/+} mice and suppressed miR-29b expression levels at 4 weeks compared to untreated animals (Figure 5C, 5D; Online Figure IIB). Taken together, these data strongly suggest that enhanced TGF-β signaling in the aortic wall of Fbn1^{C1039G/+} mice may serve to reduce active NF-κB, thereby causing a derepression of miR-29b.

Prevention of Early Aneurysm Formation, Reduction of Aortic Wall Apoptosis, and Increase in ECM by miR-29b Blockade

As biological proof of concept that miR-29b contributes to aortic root aneurysm formation in this Marfan mouse model, we then treated the Fbn1^{C1039G/+} mice with LNA-antimiR-29b inhibitor containing an antisense oligonucleotide that silences miR-29b. Mice were initially treated with a single dose of the LNA-antimiR-29b inhibitor prenatally and euthanized at 4 weeks. LNA-antimiR-29b inhibitor treatment resulted in a significant reduction in miR-29b expression levels measured by quantitative polymerase chain reaction, compared to untreated groups (Figure 6A, Online Figure IIC). We also confirmed with in situ hybridization that LNA treatment resulted in decreased miR-29b levels in Fbn1^{C1039G/+} AS aorta compared to untreated controls (Online Figure I). Notably, aortic root dilation was completely blocked at 4 weeks in LNA-treated Fbn1^{C1039G/+} mice, with aortic diameters not different from those of WT control (Figure 6B). Because early miR-29b expression peaks by 4 weeks, we also treated Fbn1^{C1039G/+} pups with LNA-antimiR-29b inhibitor postnatally and again noted early aneurysm prevention at 4 weeks (Figure 6B). Fbn1^{C1039G/+} mice treated with LNA inhibitor either prenatally or postnatally showed significantly less aortic dilation compared to untreated animals and animals treated with a scrambled mutated sequence. Therefore, these results confirm that miR-29b plays a key role in the pathogenesis of early aneurysm development. Coinciding with aneurysm prevention, treatment with the LNA miR-29b inhibitor reduced AS aortic wall apoptosis in the Fbn1^{C1039G/+} mice as evidenced by a significant reduction in cleaved caspase-3 and caspase-9 and an increase in Mcl-1 and Bcl-2 by WB (Figure 6C, D) compared to untreated groups. Importantly, these effects are specific to the LNA inhibitor sequence; we confirmed that treatment with the scrambled mutated LNA sequence did not change results from untreated animals (data not
shown). Moreover, ELN gene expression and protein levels were also significantly greater in mice receiving the miR-29b inhibitor than untreated Fbn1\(^{C1039G/+}\) mice (Figure 6E, 6F, Online Figure IV), demonstrating that the early increases in miR-29b expression serve to dysregulate ECM remodeling in Fbn1\(^{C1039G/+}\) mice. Supporting our WB and quantitative polymerase chain reaction data, aortic wall histology demonstrated diminished Fbn1\(^{C1039G/+}\) AS apoptosis (cleaved caspase-3; Figure 6G), increased elastin deposition, and decreased elastin fragmentation (Figure 6H) in LNA-treated Fbn1\(^{C1039G/+}\) mice compared to untreated control.

**Prevention of Early Aneurysm Formation and Reduction in miR-29b Expression by Losartan Treatment**

Losartan, an angiotensin II receptor 1 blocker recently reported to reduce aneurysm growth in Fbn1\(^{C1039G/+}\) mice, is currently being tested in clinical trials in pediatric patients with MFS. Angiotensin II receptor 1 blockade has been hypothesized to work by reducing the deleterious effects of amplified TGF-β signaling, although the exact mechanism is unknown. In this study, losartan (prenatal treatment until age of 4 weeks) reduced early aneurysm formation in Fbn1\(^{C1039G/+}\) pups and prevented the increase in miR-29b at 4 weeks, bringing levels down to those found in WT controls (Figure 7A, 7B, Online Figure IID). The normalization of miR-29b expression produced by losartan treatment in Fbn1\(^{C1039G/+}\) AS aorta was also associated with the prevention of apoptosis and a normalization of the levels of the ECM component, ELN (Figure 7C–E). To better understand the effect of losartan on miR-29b expression, we performed in vitro studies using aortic SMCs. We found that losartan treatment did not influence the nuclear activity of NF-κB (Figure 7F).

**Figure 6. Prevention of early Fbn1\(^{C1039G/+}\) aneurysm formation, reduction in aortic wall apoptosis, and increase in extracellular matrix (ECM) by locked nucleic acid (LNA) miR-29b blockade in vivo. A,** Fold difference in miR-29b expression by quantitative polymerase chain reaction (qPCR) in Fbn1\(^{C1039G/+}\) and wild-type (WT) ascending (AS) aorta comparing no treatment (Untx; n=6) and prenatal LNA miR-29b inhibitor treatment (LNA pre; n=3). **B,** Fold difference in aortic root diameter in 4-week-old Fbn1\(^{C1039G/+}\) and WT mice in vivo using transthoracic echocardiography (TTE) comparing no treatment (Untx), LNA scrambled mutated sequence control treatment (Scr), prenatal LNA treatment (LNA pre), and postnatal LNA treatment (LNA post; n=3–5). **C,** Fold difference in WB protein levels of cleaved caspase-3 and caspase-9. **D** antiapoptotic miR-29b targets (Bcl-2, Mcl-1), and **E** miR-29b ECM targets (ELN, Col3A1) in AS aorta of Fbn1\(^{C1039G/+}\) and WT mice comparing no treatment (Untx; n=6), prenatal LNA treatment (LNA pre; n=3), and postnatal LNA treatment (LNA post; n=3). **F,** Fold difference in elastin (ELN) expression by qPCR in Fbn1\(^{C1039G/+}\) and WT AS aortas comparing no treatment (Untx) and prenatal LNA treatment (LNA pre; n=3). **G,** Representative immunofluorescence images of cleaved (active) caspase-3 (red) with Hoechst nuclear counter stain (blue) in AS aortas at 4 weeks in WT, Fbn1\(^{C1039G/+}\), and Fbn1\(^{C1039G/+}\) treated with LNA prenatally, and Fbn1\(^{C1039G/+}\) treated with LNA postnatally. Scale bars represent 50 μm. **H,** Representative ELN histological staining of AS aortas at 4 weeks comparing WT, Fbn1\(^{C1039G/+}\), and Fbn1\(^{C1039G/+}\) treated with LNA prenatally, and Fbn1\(^{C1039G/+}\) treated with LNA postnatally. Scale bars represent 50 μm. Results presented as mean±SEM. *P<0.05, **P<0.01.
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Discussion

Although TGF-β blockade has shown benefit in MFS mouse models, the exact mechanisms through which TGF-β signaling leads to aneurysm development remain ill-defined. In this study, using Fbn1C1039G/− mice, we have demonstrated that: (1) aortic root dilatation forms as early as 2 weeks of age; (2) miR-29b expression is increased in Marfan AS aorta during early aneurysm development; (3) enhanced TGF-β signaling in the aortic wall of Fbn1C1039G/− mice may serve to reduce active NF-κB, thereby causing a derepression of miR-29b; (4) treatment with a miR-29b oligonucleotide inhibitor prevents early aneurysm formation in Marfan mice; and (5) Marfan aortic aneurysm development is most closely associated with reduced elastogenesis and increased elastin fragmentation.

Here, we report for the first time to our knowledge that miR-29b plays an important role in early aneurysm formation in a Marfan mouse model. The miR-29b expression was significantly increased in the AS aorta, but not in the D or ABD aortas. Enhanced miR-29b expression in the AS aorta correlates with the clinical scenario observed in MFS patients, in whom aneurysms are typically localized to the aortic root. Liao et al recently noted that other miR-29 family members, miR-29a and miR-29c, were underexpressed in patients with ascending aortic dissections, possibly related to increased aortic wall collagen deposition and stiffness. However, in that study patients with connective tissue disorders were excluded and miR-29 levels were measured after the dissection occurred. More convincingly, here we show that both prenatal and postnatal miR-29b blockade prevented early aortic root aneurysm development. The miR-29b reduction proved to be an effective strategy, whether targeted directly using an oligonucleotide inhibitor or indirectly via administration of either losartan, an angiotensin II receptor 1 blocker, or TGF-β neutralizing antibody. Because miR-29b targets hundreds of genes, its role in aneurysm development may be multifactorial. To betterdefine the pathophysiology of aneurysm formation, in this study we focus on two important processes, cell survival and ECM remodeling.

Intriguingly, miR-29b peak expression occurs early in aneurysm development, although aneurysm size has been reported to increase exponentially in older mice (6–8 months). We hypothesize that increased miR-29b expression in the AS aorta may represent the initiating event in aneurysm formation in MFS. Although a single dose of LNA-antimiR-29b inhibitor administered in utero significantly reduced aortic dissection occurred. More convincingly, here we show that both prenatal and postnatal miR-29b blockade prevented early aortic root aneurysm development. The miR-29b reduction proved to be an effective strategy, whether targeted directly using an oligonucleotide inhibitor or indirectly via administration of either losartan, an angiotensin II receptor 1 blocker, or TGF-β neutralizing antibody. Because miR-29b targets hundreds of genes, its role in aneurysm development may be multifactorial. To better-define the pathophysiology of aneurysm formation, in this study we focus on two important processes, cell survival and ECM remodeling.

Alternatively, it is possible that the mechanisms for aneurysm development in MFS may represent two distinct processes, an early miR-29b-dependent event and a late miR-29b-independent event. For example, whereas increased miR-29b levels are found in the abdominal aortic aneurysms induced in apolipoprotein E−/− mice by angiotensin II, miR-29b inhibition was only efficacious in blocking aortic dilations at 1 week after treatment and did not prevent late aneurysm growth after 4 weeks.

Several investigators have proposed a role for vascular SMC apoptosis in aortic aneurysm formation; however, the importance in MFS remains controversial. Because miR-
29b blockade prevented early aneurysm formation and was associated with decreased aortic wall apoptosis, a fundamental event during the pathogenesis of aneurysm development likely includes aortic wall apoptosis. Importantly, no link has been previously made between apoptosis, miRNA expression, and aneurysm formation in MFS. Although Nagashima et al. observed increased apoptosis in vascular SMC cultures derived from patients with MFS, Habashi et al. did not detect enhanced apoptosis in intermediate stages of aneurysm formation in Fbn1/C1039G/H11001 mice. In contrast to our study, Habashi et al. examined later time points, including in mice older than 8 weeks. In addition, more sensitive detection methods for apoptosis were utilized in our study. Because apoptosis was not significantly different between Marfan aortic segments, apoptosis alone cannot explain why aneurysms are localized to the AS aorta. Worthy of note, histology revealed scattered sections of apoptosis interspersed with unaffected areas within the aortic wall. We believe that the low levels of apoptosis detected in all three aortic segments of WT littermate controls represent normal vessel wall development and remodeling. These findings suggest that aortic root aneurysm development in MFS may be a primary failure of the normal vascular remodeling process that should occur early in development.

Because apoptosis alone cannot explain localized aneurysm growth, we characterized alterations in ECM proteins that occur during early aneurysm progression. Insoluble elastin, mainly synthesized during development and postnatal growth, is associated with fibrillin-1, the major component of extracellular microfibrils, and a regulator of elastogenesis. Whether aneurysm formation results from an early defect in elastogenesis or a later disruption of mature elastic fibers is unclear. In this study, we report that ELN levels are decreased early in Marfan mice, suggesting that a defect in elastogenesis contributes to the pathology in these mice. However, histological analysis also demonstrated increased ELN fragmentation. Several investigators have reported that enhanced MMP production promotes abdominal aneurysm formation by increasing ECM destruction. In this study, both MMP-2 expression and activity were specifically upregulated in the AS segment of the Marfan aorta only, explaining the disruption of medial wall ELN isolated to this area. MMPs are secreted by leukocytes and SMCs, and MMP-2 can degrade both elastin and collagen. The absence of aortic wall inflammatory cell infiltration in our model suggests that MMPs are secreted locally. MMP expression has been shown to be upregulated by miR-29b, so we believe that the transient increase in miR-29b expression results in enhanced MMP-mediated ELN degradation. We speculate that the combination of impaired ELN production, enhanced degradation, and aortic wall apoptosis initiates and perpetuates aneurysm growth (Figure 8). In addition to its structural properties, fibrillin-containing microfibrils participate in cell signaling. Because ECM provides signals that differentiate and stabilize SMCs, it is possible that a reduction in ECM proteins may instigate SMC apoptosis in our model system.

Although the precise mechanism remains to be determined, our results suggest that increased early miR-29b in Fbn1/C1039G/+ mice may be related to decreased levels of miR-29b inhibitors, including NF-κB and c-Myc. In our model, both IKK-α and

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**Figure 8. The miR-29b in early aneurysm development in Marfan syndrome: hypothesized model.** At baseline, nuclear factor κB (NF-κB) is bound to inhibitory IκB and sequestered in the cytoplasm, unable to affect gene transcription. IKK-α and IKK-β mediate IκB phosphorylation, label it for proteosomal degradation, which liberates NF-κB. In Fbn1/C1039G/+ ascending (AS) aorta, we hypothesize that an early initiating event, such as increased transforming growth factor (TGf)-β signaling, decreases Iκκ-α and IKK-β, and thereby decreases free active nuclear NF-κB levels. Without the transcriptional inhibition of NF-κB, miR-29b expression increases. The miR-29b regulates a wide variety of targets with roles in extracellular matrix (ECM) deposition and breakdown and apoptosis.
IKK-β were significantly reduced in Fbn1/C1039G+ aorta in parallel to the increase in miR-29b expression. We theorize that diminished IKK-α and IKK-β levels decrease the amount of active NF-κB, consequently leading to increased miR-29b expression (Figure 8). Moreover, the in vitro studies suggest that elevated TGF-β levels in Marfan mice may initiate this cascade of events via the reduction in active nuclear NF-κB. Additionally, blocking NF-κB in vivo increases miR-29b expression, whereas blocking TGF-β in vivo decreases miR-29b expression. Importantly, although miR-29b elevation is localized to the AS aorta, our WB analysis did not find significant differences in NF-κB among different segments of the aorta. Because WB measures cellular NF-κB (p65), which may not accurately reflect differences in active (nuclear) NF-κB (p65), we subsequently demonstrated histologically reduced active nuclear NF-κB p65 subunits in the Fbn1/C1039G+ AS aorta only. Fascinatingly, whereas TGF-β treatment decreased active nuclear NF-κB in both WT and Fbn1/C1039G+ derived SMCs, it only increased miR-29b expression in Fbn1/C1039G+ SMCs, suggesting that Marfan cells may respond physiologically differently to excessive TGF-β expression, whereas blocking TGF-β of active NF-κB in aortic root aneurysm paradigm in MFS from a “diagnose and therapeutic approach. Furthermore, this newly described mechanism suggests priming of apoptotic pathways. Carcinogenesis. 2010;31: 1882–1888.


Disclosures

D.R.M., R.C.R., and M.P.F. have applied for a patent relating to this work.

References


**Novelty and Significance**

**What Is Known?**

- Patients with Marfan syndrome, a connective tissue disorder, have development of potentially fatal aortic root aneurysms.
- Excess transforming growth factor-β (TGF-β) signaling contributes to aneurysm formation in animal models of Marfan syndrome, although the mechanism remains undefined.
- MicroRNA-29b (miR-29b) regulates genes involved in apoptosis and extracellular matrix synthesis and deposition.

**What New Information Does This Article Contribute?**

- The miR-29b levels are increased in the ascending aorta of Marfan mice during early aneurysm development.
- Inhibition of miR-29b in vivo reduces early aortic dilation, decreases aortic wall apoptosis, and increases extracellular matrix deposition.
- Enhanced TGF-β in the Marfan mice appears to reduce the activation of the miR-29b repressor, nuclear factor-κB (NF-κB), thereby allowing for pathological upregulation of miR-29b.

Aortic root aneurysms and subsequent aortic dissection/rupture are the leading causes of death in patients with Marfan syndrome. Because the microRNA miR-29b regulates genes involved in apoptosis and extracellular matrix remodeling, we investigated its role during early aneurysm development in a transgenic Marfan mouse model. This study shows for the first time to our knowledge that miR-29b expression is markedly increased in Marfan ascending aorta during early aneurysm development in association with reduced elastogenesis and increased elastin fragmentation. Blocking miR-29b in vivo prevents early aneurysm formation, reduces aortic wall apoptosis, and increases elastin deposition. Enhanced TGF-β signaling in Marfan mice decreases NF-κB activation, a known miR-29b repressor, and thus may explain the increased miR-29b expression observed in the ascending aorta. Finally, TGF-β blockade reduces miR-29b expression and abrogates aortic dilation. Taken together, the present report is the first to our knowledge to identify dysregulation of miR-29b as key to the pathogenesis of aneurysm formation in Marfan syndrome, and provides a new link to how excess TGF-β leads to aneurysm formation. Furthermore, these data suggest that pharmacological inhibitors that prevent miR-29b upregulation during early development may represent a novel therapeutic approach to prevent aneurysm development.
miR-29b Participates in Early Aneurysm Development in Marfan Syndrome


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Supplemental Material

Detailed Methods

Mice

All animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University (http://labanimals.stanford.edu/) and followed the NIH and USDA Guidelines for the Care and Use of Animals in Research. All experiments were performed with both male and female Fbn1C1039G/+ mice and C57BL/6J littermate wild type (WT) controls. Experimental groups were as follows:

(a) LNA miR29b oligonucleotide inhibitor: Animals received a locked nucleic acid (LNA)-anti-miR-29b inhibitor (mmu-miR-29b, miRCURY LNA™ microRNA Power inhibitor, Exiqon, Woburn, MA, USA) either pre- or postnatally. First in prenatal studies (LNA pre), female Fbn1C1039G/+ mice were time mated with WT male mice, then treated 14.5 days post-coitum with either a single tail vein injection of: (1) LNA-anti-miR-29b (8mg/kg); (2) scrambled-control-miR (8mg/kg) (miRCURY LNA microRNA inhibitor, Exiqon, Cambridge, MA, USA); or (3) phosphate buffered saline (PBS) (control) (pH 7.4, Gibco, Invitrogen, Carlsbad, CA, USA). Pups from these female mice were sacrificed at age 4 weeks. In separate postnatal experiments (LNA post), 2 week old Fbn1C1039G/+ mice and C57BL/6J littermate WT controls received a single intravenous injection of either (1) LNA-anti-miR-29b (8mg/kg); (2) scrambled-control-miR (8mg/kg); or (3) PBS control and sacrificed at 4 weeks. The 5’-3’ sequences for the custom-made LNA-anti-miR-29b and LNA-scrambled-miR control were ACTGATTTCAAATGGTGCT and GTGTAACACGTCTATACGCCCA, respectively.

(b) Losartan: Pregnant mice were treated 14.5 days post-coitum with oral losartan (0.6 g/L in drinking water) (Merck KGaA, Darmstadt, Germany). Pups also received oral losartan (0.6 g/L in drinking water) and were sacrificed at age 4 weeks.

(c) TGF-β neutralizing antibody: 2 week old mice were injected with either intraperitoneal (IP) pan-sensitive TGF-β neutralizing antibody (NAb) or rabbit IgG (control) (R&D Systems, Minneapolis), for 3 consecutive days, followed by another injection one week later. Mice were then sacrificed at age 4 weeks.

(d) IκB-α phosphorylation inhibitor (BAY 11-7082): 6 week old mice were injected with either 10mg/kg (dissolved in DMSO/ PBS) IP BAY 11-7082 (Calbiochem, Merck KGaA, Darmstadt, Germany) or DMSO/ PBS (control) every other day and sacrificed at age 8 weeks.

Echocardiography

Transthoracic echocardiography (TTE) was performed in sedated mice by using 2% inhaled isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) (Baxter Healthcare Corporation, Deerfield, IL, USA) delivered via nose cone. Animals were positioned supine and imaged with a Siemens-Acuson Sequoia C512 system equipped with a multifrequency (8 to 14 MHZ) 15L8 transducer (Siemens Healthcare, Erlangen, Germany). The aorta was imaged in the parasternal long axis view. Measurements were obtained in triplicate at the level of the Sinus of Valsalva. Two blinded investigators performed the echocardiography.

Magnetic Resonance Imaging

A self-shielded General Electric microSigna 7.0 7T magnetic resonance imaging (MRI) machine was used. Cardiac sequences were acquired while gating the cardiac and respiratory cycles. Mice were anesthetized using 2% isoflurane in humidified air with electrocardiogram (EKG) leads inserted percutaneously on the chest and respiratory pillow placed on the abdomen.
Preparation of aortic tissue

Mice were sacrificed with an inhalation overdose of isoflurane. Immediately following sacrifice the abdominal aorta was transected and flushed via the left ventricle with ice cold phosphate buffered saline (PBS; pH 7.4, Gibco, Invitrogen, Carlsbad, CA, USA). The aorta was then dissected from fat and connective tissue under the microscope, beginning with the aortic root at the level of the aortic valve and ending with the abdominal aorta terminus (at the bifurcation of the iliac arteries). The aorta was then divided into three portions: 1) aortic root (distal to aortic valve), ascending aorta and arch (AS); 2) descending thoracic aorta (D); and 3) abdominal aorta (ABD). Specimens were snap frozen individually in liquid nitrogen and stored at -80°C for further processing.

Western Blot Analysis

Separated frozen sections of aorta were homogenized in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined through the Bicinchoninic Acid assay (Thermo Scientific Pierce, Protein, Rockford, IL, USA), according to manufacturer protocol. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10 µg of protein per well. Western blot (WB) was performed with the following primary antibodies: mouse α Bcl-2 (BD, Franklin Lakes, NJ, USA), rabbit α c-Myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse α caspase-3, rabbit α caspase-9, rabbit α IKK-β, mouse α β-actin (all Cell Signaling Technologies, Danvers, MA, USA), mouse α Mcl-1, rabbit α IKK-α, rabbit α NF-kB p65 (all Abcam, Cambridge, MA, USA), rabbit α vinculin (Sigma-Aldrich, St. Louis, MO, USA). Either vinculin or actin was used as the loading control based on the molecular weight of the target protein. Using vinculin or actin did not result in significantly different results. Quantification by densitometry was performed using ImageJ software (NIH). WB analysis was performed with a minimum of n=3 for each genotype (C57BL/6 littermate control, Fbn1^C1039G/+), age (2, 4, 6, and 8 weeks), and treatment (losartan, miR-29b LNA).

Caspase-3 Fluorescent Activity Assay

To measure caspase-3 activity in protein extracts (prepared similarly to Western blotting), the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) was performed following manufacturer protocol on 50 µg of protein. The caspase-3 fluorescent activity assay was performed on n=3 for each genotype (C57BL/6 littermate control, Fbn1^C1039G/+ ) and age (2, 4, 6, and 8 weeks). Each data point contained 3 pooled aortas from littermates in order to achieve sufficient protein quantity.

Gelatin zymography

To measure MMP-2 and MMP-9 activity in protein extracts, 10 µg total protein for each sample was mixed with non-reducing SDS sample buffer and resolved by non-denaturing electrophoresis through a 10% gelatin gel (Invitrogen, Carlsbad, CA, USA). After being incubated 1 hour in renaturing buffer and 48 hours at 37°C in developing buffer, gels were stained in SimplyBlue SafeStain (Invitrogen, Carlsbad, CA USA). Proteinases were observed as clear bands against a dark background of intact substrate. The molecular sizes of gelatinolytic activities were determined using 10 ng of protein standards (MMP-2: Calbiochem, Gibbstown, NJ, USA; MMP-9: Abcam, Cambridge, MA, USA). Quantification by densitometry was performed using ImageJ software (NIH) on inverted images of gel. Zymography was performed on n=3 for each genotype (C57BL/6 littermate control, Fbn1C1039G/+ ) and age (2, 4, and 8 weeks). Each data point contained 3 pooled aortas from littermates in order to achieve sufficient protein quantity.
RNA Isolation
Total RNA was isolated using Trizol (TRIzol, Invitrogen, Carlsbad, CA) on aortic samples or cultured smooth muscle cells. RNA then was quantified by Nanodrop (Agilent, Foster City, CA, USA) and samples were included when the 260/280nm ratio was >1.6.

Detection and Quantification of miRNAs by Quantitative PCR
RNA was obtained as outlined above and diluted to a concentration of 2 ng/µl. Diluted RNA was reverse transcribed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Life Technologies Carlsbad, CA, USA) according to the manufacturer’s instructions. Subsequently, 5 µl of the product was used for detecting miRNA expression by quantitative PCR (qPCR) using TaqMan microRNA Assay kits (Applied Biosystems, Life Technologies Carlsbad, CA, USA) for the following miRNAs: hsa-miR-21, hsa-miR-29b and snoRNA202, and confirmed by TaqMan Real-time PCR (RT-PCR) assay. Values were normalized to sno202 (mouse) and are expressed as ∆∆CT (2^(-∆∆CT[miRNA]-∆∆CT[sno202])). All experiments included at least four replicates per group.

Gene expression via Quantitative Real-Time PCR
Total RNA from the samples was converted to complementary DNA (cDNA) according to the manufacturer’s instructions using the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, USA). The cDNA was amplified in duplicates on the ABI PRISM 7900HT with primers and probes (Applied Biosystems, Life Technologies Carlsbad, CA, USA). Gene expression levels were normalized to corresponding 18S or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal controls and calculated as stated above.

Histological Analysis
Histological staining was performed on each section of the aorta (AS, D, ABD). Mice were euthanized and perfused through the heart with constant pressure (100mmHg) using PBS followed by warm (37°C) agarose gel (Amresco, Solon, OH, USA) diluted in saline (3% w/v). After the agarose solidified, the aorta was dissected free from the surrounding tissue and fixed in 4% formalin. Isolated tissue was then dehydrated through a graded sucrose series and embedded in Tissue-Tek OCT Compound Histomount (Sakura, Torrance, CA, USA). Aortic tissue was then cut at 7 µm thick serial sections and stained with Accustain Elastin Verhoeff’s Van Gieson (EVG) kit according to manufacturer’s protocol. (Sigma Aldrich, St. Louis, MO, USA).

Immunofluorescence staining
Primary antibody against cleaved, active caspase-3 (Cell Signaling Technologies, Danvers, MA, USA 1:50) and NF-κB subunit, p65 (Abcam, Cambridge, MA, USA 1:25) was applied overnight after heat induced epitope retrieval using Universal Antigen Retrieval Reagent (R&D Systems, Minneapolis, MN, USA) and incubation with Sea Block blocking buffer (Thermo Scientific, Rockford, IL, USA). Samples were then incubated with secondary antibody for 1 hour at room temperature (Alexa Fluor 647, Invitrogen, Carlsbad, CA, USA: 1:200). Nuclei were stained with Hoechst reagent (bisBenzide H 33258) (Sigma-Aldrich, St. Louis, MO, USA) using 1 µl per 10 ml PBS for 10 minutes. Immunofluorescence microscopy was used to detect active caspase-3 and active nuclear NF-κB (p65).

In situ hybridization (ISH)
ISH for miR-29b was performed by using the miRCURYLNA microRNA ISH Optimization Kit (Exiqon, Cambridge, MA, USA) and 5’-DIG and 3’-DIG labeled d probes for mmu-miR-29b according to the manufacturer’s protocol. The sequence of the LNA miR-29b control probe was: 5’-DIG/AACACTGATTCTAAATGTTGCTA/DIG-3’.
**Smooth Muscle Cell Cultures**

Aortic smooth muscle cells (SMC) were derived from 5 pooled 4 week old WT or Fbn1 mouse aortas. The heart was flushed with 5 ml of PBS and the entire aorta harvested and placed in filtered ice cold SMC media (Lonza, Basel, Switzerland). After removing the surrounding tissue, including the adventitia, the samples were minced. The samples were then incubated in 5 ml Collagenase Ia (0.5mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 45 minutes at 37°C on an orbital shaker. To stop the collagenase activity, SMC media was added to the sample, filtered through a 100 mm strainer and spun down at 250 RCF for 5 minutes at room temperature. The supernatant was discarded and the pellet washed with 5 ml PBS and spun again at 250 RCF for 5 minutes. After discarding the supernatant, the pellet was resuspended with 1 ml SMC media and seeded in 4 wells of a 24 well plate.

**In vitro Smooth Muscle Cell Studies**

Aortic SMCs were derived from 4 week old WT and Fbn1 mice, as noted above, and exposed to recombinant TGF-β1 (10 ng/ml) (R&D Systems, 100-B, Minneapolis, MN, USA in chamber slides after starvation with 0.2% fetal bovine serum (FBS) for 24 hours (Lonza, SmGM-2 Bullet Kit CC3182, Basel, Switzerland). Or cells were treated with either 2.5 µM BAY 11-7082 (Calbiochem, Merck KGaA, Darmstadt, Germany) or 10 µM Losartan (Merck KGaA, Darmstadt, Germany). Cells were then fixed in ice cold 100% ethanol for 15 minutes and air-dried for 30 minutes prior to immunofluorescence staining. SMCs were incubated with a primary antibody against NF-κB subunit, p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA 1:250) and smooth muscle actin (Genetex, Irvine, CA, USA 1:100) at room temperature for 3 hours. Samples were then incubated with secondary antibody for 45 minutes (Alexa Fluor 568, Invitrogen, Carlsbad, CA, USA: 1:250). Nuclei were stained with Hoechst reagent (bisBenzide H 33258) (Sigma-Aldrich, St. Louis, MO, USA) using 1 µl per 10 ml PBS for 10 minutes. Immunofluorescence microscopy was used to detect active nuclear NF-κB (p65).

**Statistical analysis**

Statistics were calculated using SPSS 18.0/ 19.0 (SPSS Inc, Chicago, Ill, USA). Data are presented as mean±standard error (SEM). Groups were compared using the Students t-test for parametric data or univariate analysis of variance (ANOVA) for group comparison. A value of p<0.05 was considered statistically significant.
## Online Figures

### Online Figure I. *In situ* hybridization for miR-29b

Representative image of *in situ* hybridization for miR-29b (blue) in AS aorta at 4 weeks comparing WT, *Fbn1*<sup>C1039G/+</sup>, *Fbn1*<sup>C1039G/+</sup> treated with LNA prenatally, and *Fbn1*<sup>C1039G/+</sup> treated with LNA postnatally. U6 positive control illustrated. Scale bars represent 50 µm.
Online Figure II. Effect of BAY NF-κB inhibition, TGF-β1 blockade, prenatal LNA miR-29b blockade, and Losartan on miR-29b expression in SMCs, in vitro

Fold difference in miR-29b expression by qPCR in Fbn1<sup>C1039G/+</sup> and WT AS, D, and ABD aortas with the following treatments: (A) BAY NF-κB inhibitor (BAY); (B) TGF-β1 neutralizing antibody (TGF NAb); (C) prenatal LNA miR-29b inhibitor (LNA pre); and (D) Losartan (LOS) (n=3-6).

Results presented as mean±SEM. *p<0.05; **p<0.01
Online Figure III. Effect of TGF-β1 on NF-κB activation in WT SMCs, *in vitro*
Representative immunofluorescence staining of NF-κB p65 (red) in WT SMCs following TGF-β1 treatment for 24 hours. Blue represents nuclear Hoechst staining and pink co-localization represents nuclear (active) NF-κB p65. Scale bar represents 50 µm.
Online Figure IV. *In vivo* elastin gene expression in response to miR-29b blockade

Fold difference in ELN expression by qPCR in \( Fbn1^{C1039G/+} \) and WT AS, D, and ABD aortas at 4 weeks, comparing no treatment (untx) and prenatal LNA treatment (LNA pre) (n=3). Results presented as mean±SEM. *p<0.05; **p<0.01