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Progressive Aortic Dilation Is Regulated by miR-17–Associated miRNAs

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ABSTRACT

BACKGROUND Patients with a bicuspid aortic valve (BAV) are at increased risk for progressive aortic dilation associated with extracellular matrix (ECM) degradation by matrix metalloproteinases (MMP). However, the mechanisms responsible for initiating this process are unknown. In the heart, MMP activity is regulated by micro-ribonucleic acid-17 (miR-17)–related downregulation of tissue inhibitors of metalloproteinases (TIMP); a similar process may exist in the aorta.

OBJECTIVES This study sought to ascertain whether aortic matrix degradation in BAV patients progresses by miR-17–related miRNA regulation of TIMP-MMP.

METHODS To eliminate confounding patient-related factors, severely dilated and less dilated aortic tissue samples were collected from 12 BAV patients. Gene and protein expression levels were evaluated in paired tissue samples from the same patient and were compared to aortic samples from 16 patients with aortas that appeared to be normal.

RESULTS Gene expression analyses confirmed increased expression of miR-17–related miRNAs in less dilated compared with severely dilated tissue from the same patient or normal aortic sample. TIMP-1, -2, and -3 were significantly decreased, and MMP2 activity was significantly increased in less dilated samples, suggesting that this normal-looking tissue was in the early stages of ECM degradation. Smooth muscle cells isolated from normal or BAV aortas transfected with an miR-17 mimic had decreased TIMP-1 and -2 expression and increased MMP2 activity, whereas the opposite effects were seen with an miR-17 inhibitor, suggesting that miR-17 may control the TIMP-MMP balance in these tissues. Luciferase reporter assays demonstrated that miR-17 regulated TIMP-1 and -2 expression.

CONCLUSIONS Our in vitro and in vivo studies taken together confirm that miR-17 directly regulates TIMP-1 and -2. Less dilated aortic BAV tissue may be in the initial stages of dilation under the control of miR-17–related miRNAs. New therapies that inhibit these miRNAs may prevent aortic dilation. (J Am Coll Cardiol 2016;67:2965–77)

Bicuspid aortic valve (BAV), the most common cardiac congenital abnormality, occurs in 1% to 2% of the population (1) and is a significant risk factor for aortopathy and subsequent premature cardiovascular complications. BAV alters blood flow in the ascending aorta, resulting in increased wall shear stress that may contribute to progressive aortic dilation (1,2). Additionally, BAV patients have a higher incidence of genetic abnormalities that predisposes them to aortic dilation. Both abnormal blood flow and genetic predisposition may contribute to BAV aortopathy.

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ABBREVIATIONS AND ACRONYMS

BAV = bicuspid aortic valve

ECM = extracellular matrix

miRNA = micro ribonucleic acid

MMP = matrix metalloproteinase

SMC = smooth muscle cell

TIMP = tissue inhibitor of matrix metalloproteinases

Progressive aortic dilation, if untreated, can lead to aortic dissection and/or rupture, both of which are potentially fatal (3). Currently, aortic aneurysms in BAV patients are treated by dilated ascending aorta removal and graft replacement (4). However, the extent of the abnormal tissue is difficult to determine at surgery, and only dilated segments are removed. Adjacent aortic segments of normal diameter are left intact. Although the incidence of distal aortic complications after successful proximal aortic repair has not been conclusively established, the 2010 aortic disease guidelines (5) recognized the risk of progressive aortopathy after successful proximal repair in BAV patients and recommended yearly surveillance to detect recurrent aortic dilation. In reviewing the incidence of aortic dilation, dissection, or aortic reoperation following aortic valve replacement among BAV patients, Marfan syndrome patients, and control patients, Itagaki et al. (6) concluded that, at 15-year follow-up, BAV patients had a greater tendency for aortic dilation than controls but not as great as that found in Marfan syndrome patients. Therefore, the risk of recurrent aortic dilation in BAV patients after successful proximal aortic repair may be small but not negligible. If we are able to discover the mechanisms associated with aortic dilation, this information could provide a new target to treat BAV patients who undergo either valve and/or aortic surgery to prevent subsequent aortic dilation.

SEE PAGE 2978

Both cell death and extracellular matrix (ECM) degradation have been identified as hallmarks of BAV aortopathy and aneurysm formation (2,7). Progressive ascending aortic dilation has been associated with activation of matrix metalloproteinases (MMPs) and down-regulation of their endogenous inhibitors (tissue inhibitor of matrix metalloproteinases [TIMPs]), which results in degradation of the aortic wall matrix and decreased elasticity (2,3,7). However, the mechanism responsible for initiating this process in BAV patients has not been elucidated. Neither increased shear stress nor BAV-associated genetic abnormalities fully explain why some patients are more prone to progressive dilation, and the mechanisms initiating matrix degradation remain obscure. Effective strategies to prevent aortic dilation have not yet been developed, and new therapies will require a more complete understanding of the molecular mechanisms involved.

Micro-ribonucleic acids (miRNAs) are small cellular RNAs that regulate gene function in many types of cells, including those in the arterial wall (8). We previously reported that MMP activity is regulated by miRNAs in cardiac tissue (9); emerging evidence suggests that miRNAs contribute to the pathogenesis of aortic dilation (10–12). However, the specific miRNAs responsible have not been determined.

To explore the contribution of miRNAs to aortic dilation, we performed miRNA microarray screening using paired samples of aortic tissue from severely dilated and normal appearing, less dilated aortic segments from BAV patients.

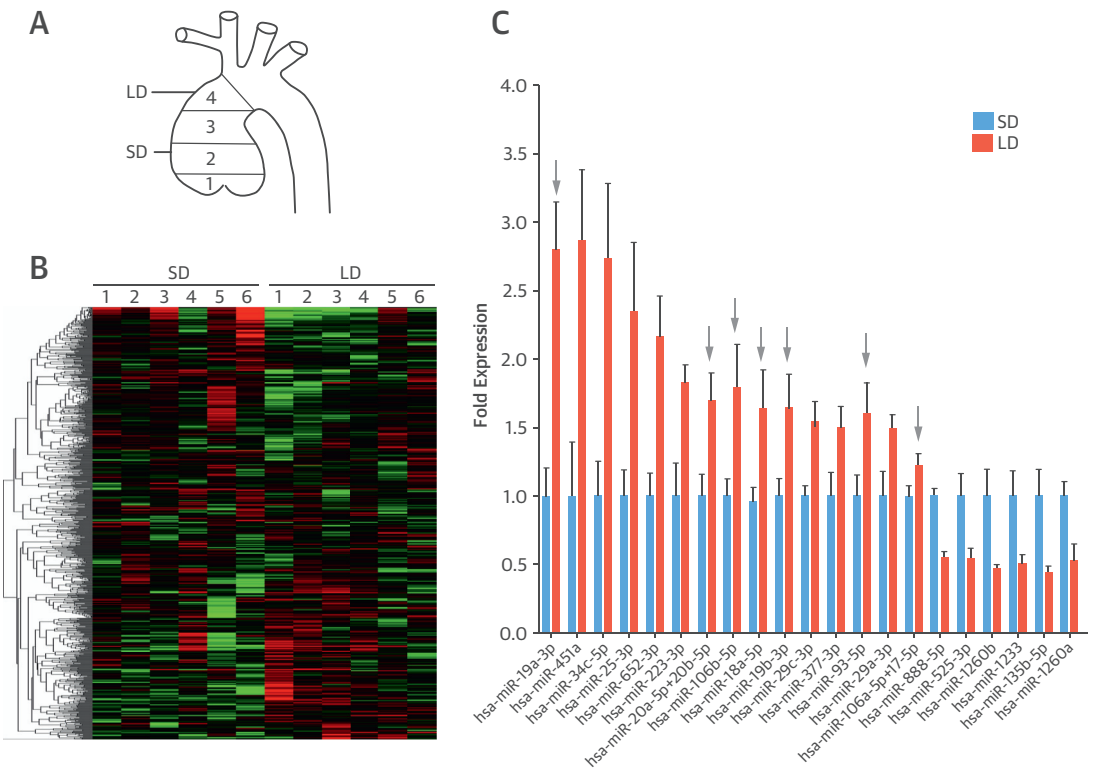
METHODS

PATIENT INFORMATION AND TISSUE COLLECTION. This study was approved by the Research Ethics Board of the University Health Network and complies with the Declaration of Helsinki. Aortic specimens were collected after patients provided informed written consent. Samples from severely dilated and less dilated sections of ascending aortas from 12 BAV patients (7 males and 5 females; 56.8 ± 9.7 years of age) were obtained during surgical removal of the ascending aorta at Toronto General Hospital (**Figure 1A**). Six of the 12 patients had a family history of some type of aortopathy; however, none of these 12 patients had any mutations documented on genetic testing. To avoid the confounding influence of patient-specific conditions and comorbidities, 6 same-patient paired samples were used for miRNA microarray analysis.

More definitive determination of miRNA upregulation was performed by real-time quantitative reverse transcription PCR (RT-qPCR) for selected miRNAs in the paired samples from all 12 patients (including paired samples from the 6 patients who underwent miRNA microarray analysis). Full-thickness control aortic specimens were also collected from 16 patients without BAV (10 males and 6 females; 59.7 ± 10.9 years of age) with normal-appearing ascending aortas at the site of proximal vein graft anastomoses during coronary artery bypass graft surgery, using an aortic punch. From the 16 samples, 6 samples were used to detect miRNAs, and samples from the remaining 10 patients were used for Western blotting determination of TIMPs and MMPs.

COMPONENT EXPRESSION AND ACTIVITY ASSAYS. TIMP-1, -2, -3, and -4, MMP-2 and -9 messenger RNA (mRNA), and protein expression levels were quantified in 12 pairs of severely dilated and less dilated aortic samples and 10 normal aortic samples by RT-qPCR (primers used for RT-qPCR are provided in Online Table 1) and Western blot analysis. Collagen type I

FIGURE 1 Differentially Expressed miRNAs



(A) Tissue was collected from segments 2 (severely dilated [SD]) and 4 (less dilated [LD]) as seen in this schematic of the location for the matched tissue segments collected from BAV patient surgical specimens. **(B)** Array heat map shows expression of ~800 miRNAs in paired less dilated and severely dilated aortic segments from 6 patients. **Green** = down-regulated miRNAs and **red** = up-regulated miRNAs, relative to each other. **(C)** Twenty-one miRNAs showed a trend for differential expression between less and severely dilated segments; 7 **(gray arrows)** are in the miR-17 gene cluster or have the same seed sequence as miR-17. BAV = bicuspid aortic valve; hsa = *Homo sapiens*.; miRNA = micro ribonucleic acid.

and elastin protein expression levels were evaluated in 6 pairs of severely and less dilated samples. Elastase enzymatic activity was evaluated in 9 paired severely and less dilated samples by using a fluorescence-based activity assay. Movat pentachrome staining was used to evaluate tissue structure in 1 normal aortic sample and in 1 pair of severely and less dilated samples from a single patient.

STATISTICAL ANALYSIS. Statistical analysis was performed using Prism version 6.0 (GraphPad Software Inc., San Diego, California). Values are mean ± SE. After confirming that data were normally distributed, using the Shapiro-Wilk statistic ($p > 0.05$), we compared less dilated to severely dilated tissue groups by paired Student *t* tests. Comparisons among 3 groups were evaluated by 1-way analysis of variance (ANOVA) followed by Tukey post-hoc tests when the F value of the ANOVA was significant. All other comparisons were analyzed by 2-way ANOVA, followed by Bonferroni post hoc tests. Statistical significance was $p < 0.05$.

RESULTS

We explored differential miRNA expression in less dilated and severely dilated tissues using a microarray screen. Differential expression of 847 miRNAs was compared in paired tissue samples from 6 BAV patients (Figure 1B). Twenty-one miRNAs had a trend for different expression between less and severely dilated segments (Figure 1C). Among these differentially expressed miRNAs were several members of the miR-17 miRNA gene cluster (miR-17, miR-18a, miR-19a/b) and several miRNAs with the same seed sequence as miR-17 (miR-20a/b, miR-93, miR-106a/b) (Figure 1C). We previously showed that miR-17 plays an important role in cardiac matrix remodeling (9), a process that might also be involved in aortic dilation.

Therefore, we focused on the role of these miR-17–related miRNAs in aortic dilation. Because the differential microarray screen for miRNA expression in paired severely and less dilated samples (**Figure 1B**) was an exploratory effort and the large number of miRNAs evaluated does not provide definitive results, we used an independent test, an RT-qPCR assay, to determine whether there were any true differences between the 2 aortic tissue segments. Results for other miRNAs implicated in aortic dilation are provided in Online Figure 1.

DIFFERENTIAL EXPRESSION OF miR-17 CLUSTER AND RELATED miRNA. RT-qPCR assay of less dilated and severely dilated aortic segment pairs from 12 BAV (including the same 6 paired BAV samples from the microarray screen) and 6 normal patients was used to determine the differential expression of miRNAs from the miR-17 gene cluster and miR-17–related miRNAs identified in our screen. Expression of the miR-17 cluster or with the same seed sequence as miR-17 showed significantly increased expression in less dilated aortic segments from BAV patients (**Figure 2A**); miRNAs with the same seed sequence as miR-17 (miR-17, miR-20a/b, miR-106a/b, miR-93) and from the miR-17 gene cluster (miR-18a, miR-19a/b) were also significantly increased in less dilated aortic segments from BAV samples compared to those from normal aortas ($p < 0.05$ vs. normal) (**Figure 2A**). All but miR-18a and miR-19b were significantly increased in less dilated samples compared to those in severely dilated tissues ($p < 0.05$) (**Figure 2A**). Moreover, using the ubiquitously expressed U6 small nuclear RNA as a positive control, we saw higher miR-17 expression in less dilated tissues by in situ hybridization (**Figure 2B**, Online Figures 2A and 2B). Cells found in the outer (tunica adventitia) and inner (tunica intima) layers of the aorta (**Figure 2C**, Online Figures 2C and 2D) also expressed miR-17. Taken together, these results suggest that differential regulation of several miR-17 cluster miRNAs and several miRNAs with the same seed sequence as miR-17 may play a role in BAV-related progressive dilation of aortic tissue.

TIMP1, -2, AND -3 EXPRESSION. We previously demonstrated that miR-17 was associated with down-regulation of TIMP-1 and -2 expression during cardiac matrix remodeling, and other studies have demonstrated that miR-17 regulates TIMP-3 (9,13). Based on this information, we hypothesized that progressive aortic dilation may be initiated by upregulation of miRNAs of the miR-17 cluster and other miR-17–related miRNAs, which decrease the expression of TIMPs, increasing MMP activation. This hypothesis suggests that the miR-17–TIMP–MMP signaling cascade accelerates progressive dilation of the aorta.

Using tissue pairs from 12 BAV patients, we evaluated TIMP mRNA and protein expression by RT-qPCR (**Figure 3A**) and Western blot analysis (**Figure 3B**). TIMP-1 and -2 mRNA expression ($p < 0.05$) and TIMP-1, -2, and -3 protein expression ($p < 0.01$ for TIMP-1; $p < 0.05$ for TIMP-2 and -3) (**Figure 3C**) were significantly decreased in less dilated aortic segments. No differences were found in levels of TIMP-4 mRNA or protein expression. These results suggest that increased expression of miR-17 and related miRNAs may precipitate decreased expression of TIMP-1, -2, and -3 in less dilated aortic segments and then initiate BAV-related aortic dilation.

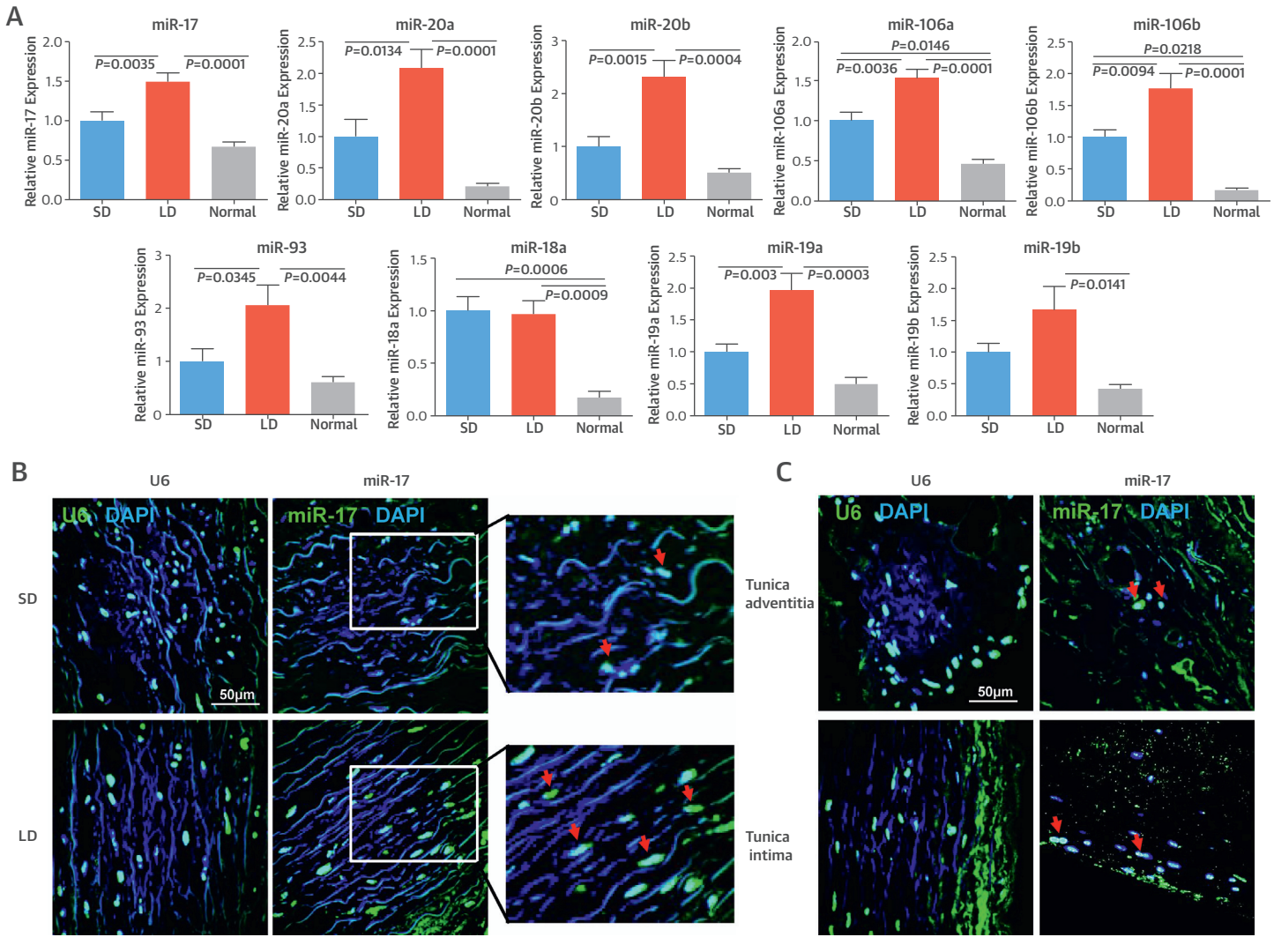
Previously, we demonstrated that miR-17 regulates TIMP-1 and -2 expression by interacting with either the protein coding region (for TIMP-1) or the 3′ untranslated region (3′-UTR) (for TIMP-2) with the mouse form of these genes (9). We confirmed that this holds true for the human form of TIMP-2 (Online Figure 3). Also, our in vitro data showed that miR-17 regulates TIMP1 through miRNA recognition elements in the TIMP-1 3′-UTR (Online Figure 4).

MMP2 ACTIVITY. MMP activity has been shown to contribute to matrix disruption and aortic dilation (14). However, we found no significant difference between less and severely dilated aortic segments in MMP-2 or -9 mRNA expression by RT-qPCR (**Figures 4A and 4B**) or total MMP-2 or -9 protein levels by Western blot (**Figures 4E and 4F**). We did observe significantly greater expression of the active form of MMP-2 (distinguishable from pro-MMP-2 by Western blot analysis) in less dilated than in severely dilated aortic segments ($p < 0.05$) (**Figures 4C and 4D**). Similarly, gelatin zymography demonstrated significantly greater MMP-2 activity in less than severely dilated aortic segments from the same patients ($p < 0.05$) (**Figures 4G and 4H**).

SEVERELY DILATED AORTIC SEGMENTS. Severely dilated aortic segments treated with Movat pentachrome stain showed disrupted elastin fiber structure, reduced smooth muscle cell (SMC) density, and increased mucopolysaccharide deposition in the medial aortic layer compared with less dilated or normal aortic segments (**Figure 5A**).

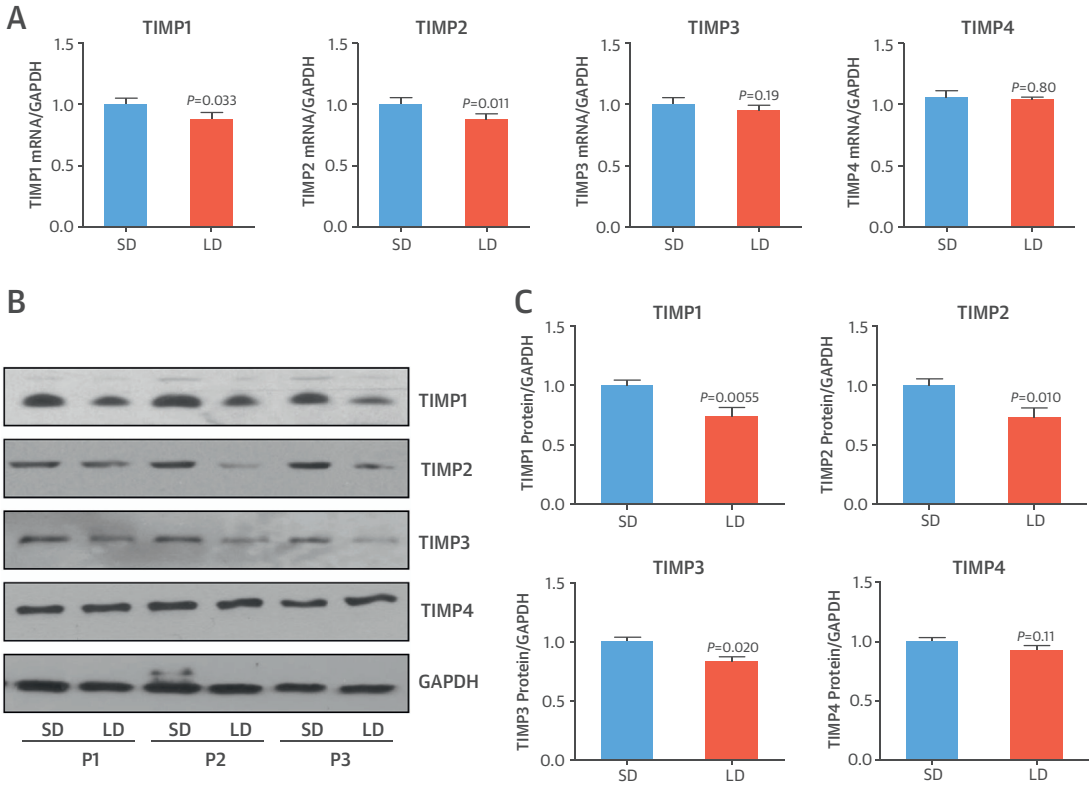
Expression levels of the mRNA encoding collagen type I were similar in both of the dilated tissues (data not shown), but its protein concentration was significantly higher in severely dilated segments than in matched less dilated aortic segments ($p < 0.05$) (**Figures 5B and 5C**). Elastin protein content was markedly reduced in the severely dilated

FIGURE 2 miRNAs with Increased Expression



In less dilated (LD **[orange bars]**) segments from BAV patients, miRNAs with the same seed sequence as miR-17 (miR-17, miR-20a/b, miR-106a/b, miR-93) and from the miR-17 gene cluster (miR-18a, miR-19a/b) were significantly increased compared to normal aortas ($p < 0.05$) **(A)**. All but miR-18a and miR-19b were significantly increased in less dilated compared to severely dilated (SD tissues **[blue bars]** ($p < 0.05$). **(B)** Confocal images of in situ hybridization confirmed higher miR-17 expression in less dilated than in severely dilated BAV patient tissue; target RNAs are **green (arrows),** and 4′, 6-diamidino-2-phenylindole (DAPI)-stained nuclei are **blue**. U6 small nuclear RNA was used as a positive control. **(C)** In situ hybridization showed miR-17 was also expressed in cells of the tunica adventitia and tunica intima of the less dilated segment. Abbreviations as in Figure 1.

FIGURE 3 Downregulation of TIMP



(A) TIMP mRNA expression was evaluated by RT-qPCR and normalized to that of GAPDH. TIMP-1 and -2 expression levels were significantly downregulated in less dilated (LD **[orange bars]**) than in severely dilated (SD **[blue bars]**) samples. **(B)** Expression levels of TIMP-1, -2, -3, and -4 protein were examined by Western blot analysis in 3 patient sample pairs (P1 to P3). **(C)** Levels of TIMP-1, -2, and -3 protein expression were lower in less dilated aortic tissue. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; RT-qPCR = real-time quantitative reverse transcription PCR; other abbreviations as in Figure 1.

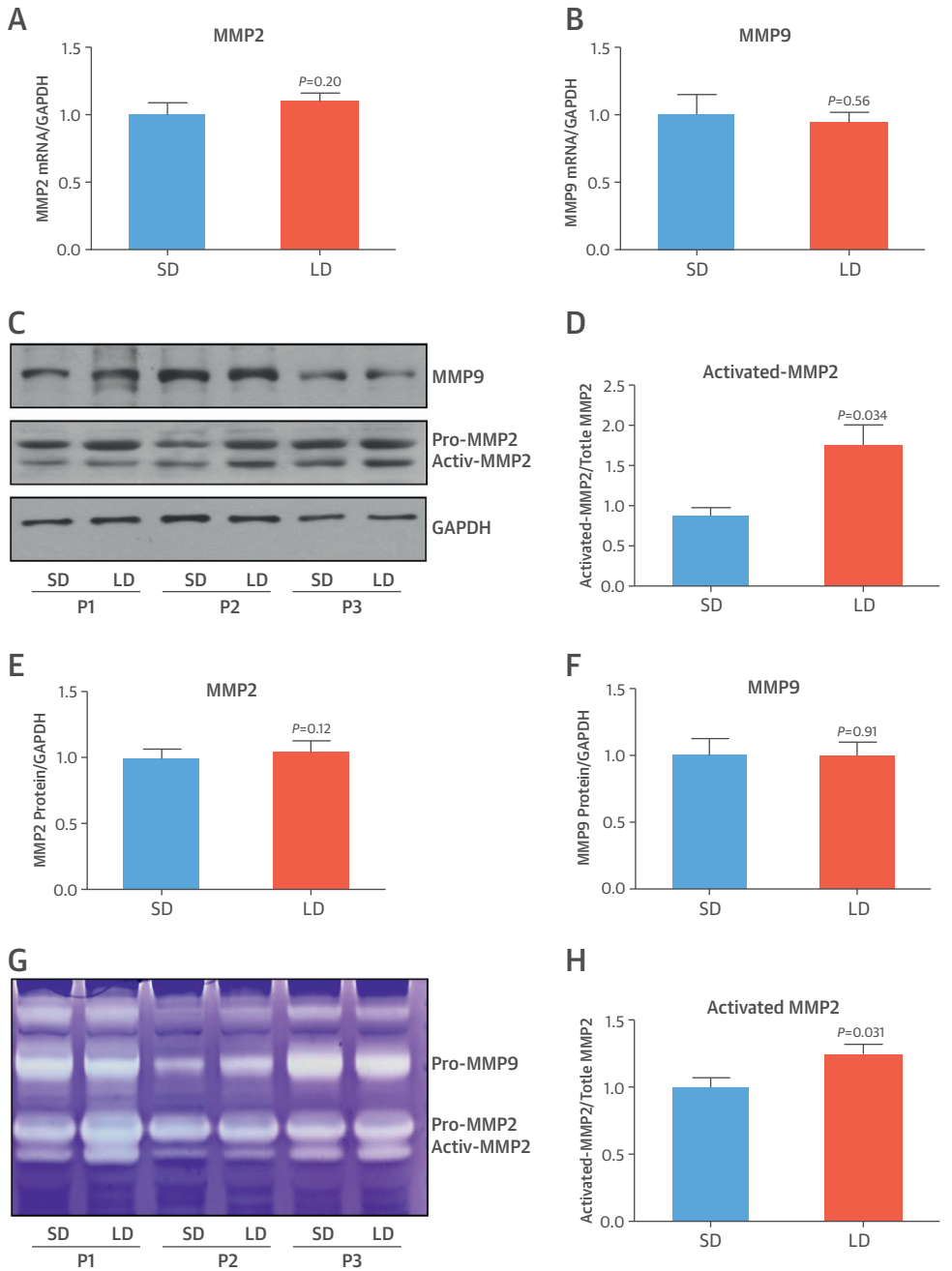
samples ($p < 0.05$) (Figure 5D), whereas expression of elastin-encoding mRNA remained unaltered (data not shown). Elastase enzymatic activity measured with fluorescence-labeled substrates was significantly higher in severely dilated segments than in matched less dilated segments ($p < 0.01$) (Figure 5E). Decreased elastin expression and increased elastase activity in the severely dilated aortic segments suggest advanced aortic dilation.

SMCs FROM BAV PATIENTS. SMCs are the main cellular components of the aortic wall (15). To test whether TIMP-1 and -2 were repressed by miR-17 expression in aortic SMCs from BAV patients, we isolated aortic SMCs from normal, less dilated, and severely dilated tissue. SMCs from these groups expressed several smooth muscle markers, confirming their SMC identity (Online Figure 5A).

To assess the effects of miR-17 on TIMP and MMP protein expression and activity, we analyzed cell lysates prepared from these SMCs transfected with a miR-17 mimic and a scrambled control by using Western blot analysis. TIMP-1 and -2 protein levels were significantly lower in cells transfected with the miR-17 mimic than in control cells ($p < 0.01$ in severely and less dilated SMCs with 5 nM of mimic) (Online Figures 5B to 5D). Additionally, BAV patient SMCs transfected with an inhibitor of miR-17-5p showed significantly increased TIMP-1 and -2 protein expression ($p < 0.01$ for severely and less dilated SMCs with 20 nM of inhibitor). These results suggested that miR-17 can regulate protein expression of TIMP-1 and -2.

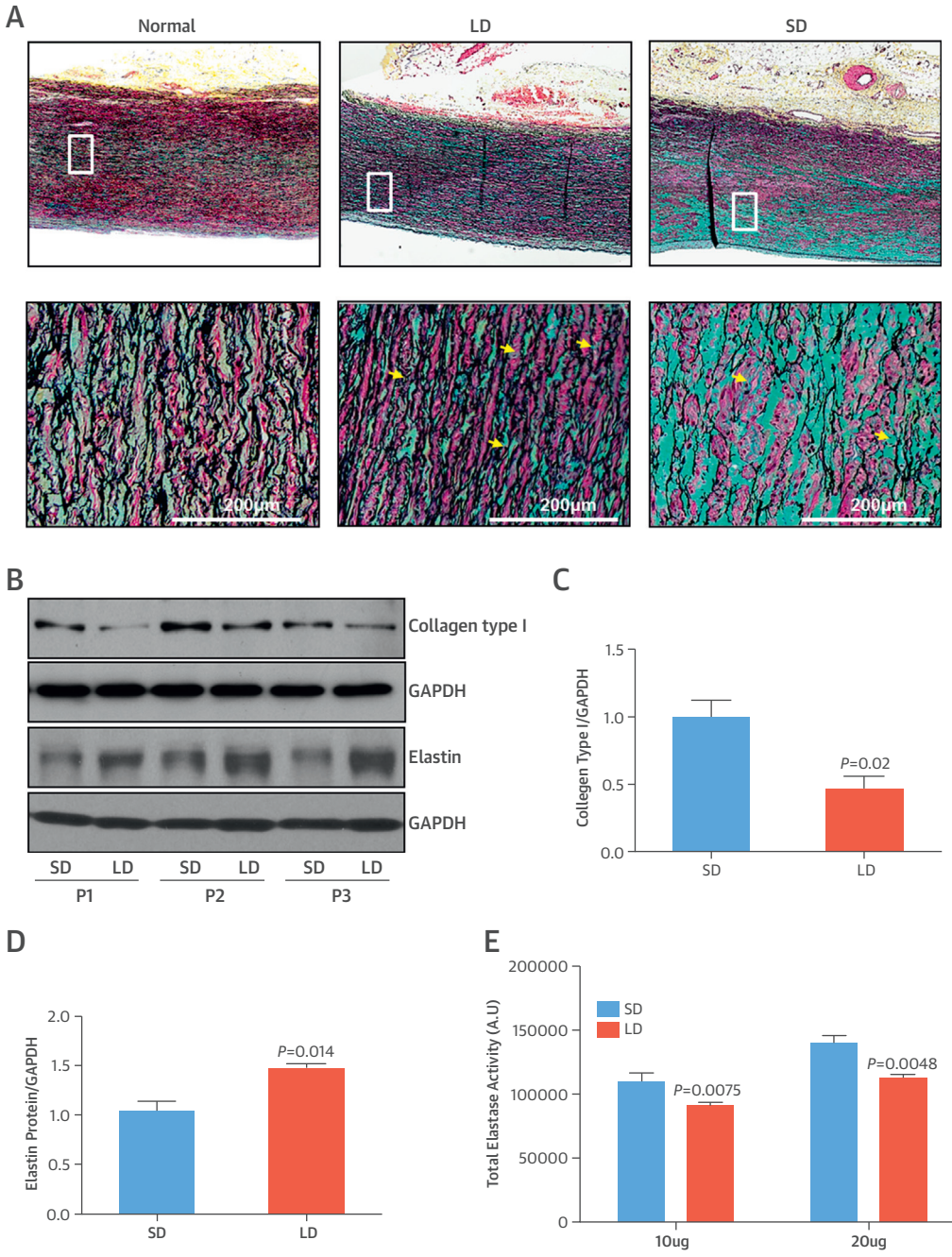
Next, we examined the effects of the miR-17 mimic and inhibitor on activity of MMP-2, which turned out to be significantly increased in SMCs transfected with the miR-17 mimic and significantly decreased in those transfected with the inhibitor ($p < 0.05$) (Online Figure 5H). These results demonstrated that

FIGURE 4 **MMP2 and MMP9 Activity**



There were no significant differences between expression levels of MMP-2 **(A)** and those of MMP-9 **(B)** mRNA in less dilated (LD **[orange bars]**) and severely dilated (SD **[blue bars]**) tissues. **(C)** MMP-2 and -9 expression levels were evaluated by Western blot analysis (50 μg of aortic protein/lane). **(D)** Expression of activated MMP-2 (easily differentiated from the larger pro-MMP-2 protein) was increased, whereas no differences were found in total MMP-2 **(E)** or MMP-9 **(F)** protein expression. **(G)** Activity levels of MMP-2 and -9 (10 μg of aortic protein/lane) were evaluated by gelatin zymography. **(H)** Activ-MMP-2 activity was significantly higher in less dilated aortic samples. P1 to P3 = patients 1, 2, 3; other abbreviations as in Figure 1.

FIGURE 5 Matrix Characteristics in Dilated Tissues



(A) In Movat pentachrome staining of normal, less dilated (LD), and severely dilated (SD) aortic samples, severely dilated tissue showed increased mucopolysaccharide **(turquoise [lower right])** staining and decreased cellularity. Smooth muscle cells **(lower left)** = **red**; elastin fibers **(lower middle)** = **black (yellow arrows)**. Collagen type I expression was significantly increased **(B,C)**, whereas elastin expression **(B,D)** was significantly decreased in severely dilated tissue (n = 6/group; paired samples from 6 patients; normalized to GAPDH expression). **(E)** Elastase enzymatic activity within equivalent amounts of total aortic protein was increased in severely dilated samples (n = 9/group; paired samples from 9 patients). AU = arbitrary unit of fluorescence; P1 to P3 = patients 1, 2, 3; other abbreviations as in Figure 1.

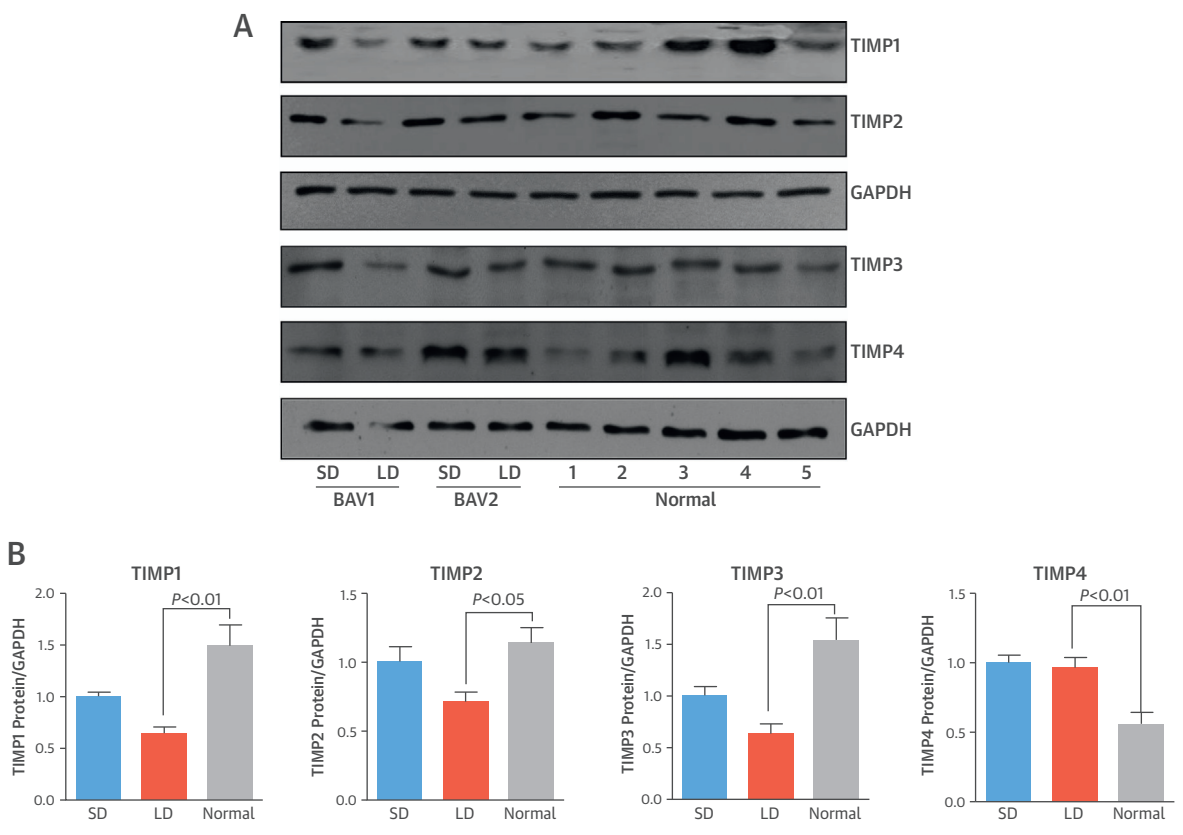
expression of TIMP-1 and -2 as well as MMP2 activity are associated with miR-17 function in SMCs from aortas with BAV-associated dilation.

Our data suggest that increased miR-17 expression in BAV aortas may contribute to decreased TIMP-1 and -2 expression and increased MMP-2 activity, contributing to ECM breakdown and dilation. To strengthen the support for this hypothesis, we compared activation of this pathway in BAV patient samples to that in tissue collected from normal aortas. Compared to normal tissue, TIMP-1, -2, and -3 protein expression levels were significantly lower in less dilated BAV aortic tissue ($p < 0.01$ for TIMP-1 and -3; $p < 0.05$ for TIMP-2) (Figure 6). However, TIMP-4 protein expression was significantly higher in less dilated BAV aortic tissues than in normal tissue ($p < 0.01$) (Figure 6). Expression levels of both the total and the activated MMP-2 protein were significantly lower in normal aorta tissue than in less dilated BAV tissue ($p < 0.05$), although MMP-9 protein expression was unchanged (Figures 7A and 7B). Taken together, these results imply that aortic dilation progresses through increased miR-17 expression in the early stages, resulting in decreased TIMP activity and subsequent activation of MMP2.

DISCUSSION

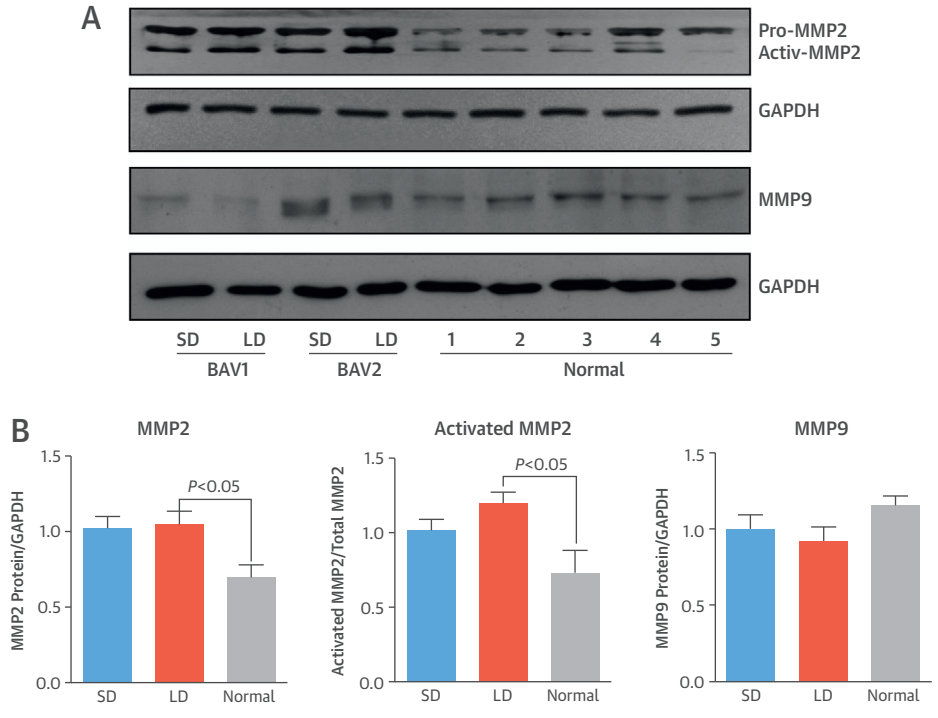
We collected paired severely dilated and normal appearing less dilated aortic samples from BAV patients and performed comprehensive miRNA profiling, followed by RT-qPCR validation. We found that differential regulation of miRNAs in the miR-17 gene cluster and other miR-17-related miRNAs was associated with histological evidence of early aortic disruption, which may predispose to dilation.

FIGURE 6 TIMP Protein Expression



(A) Representative Western blot images examined TIMP-1, -2, -3, and -4 protein expression in 5 normal aortas (1–5) and 2 BAV patient sample pairs. **(B)** TIMP-1, -2, and -3 protein levels were significantly lower in less dilated (LD) BAV aortic tissue than in normal aortic tissue, but TIMP-4 protein level was significantly higher in less dilated (LD) BAV patient aortic tissue than in normal aortic tissue, normalized to GAPDH expression (normal: n = 10; less and severely dilated: n = 12, paired samples from 12 patients). Abbreviations as in Figure 1.

FIGURE 7 MMP Levels



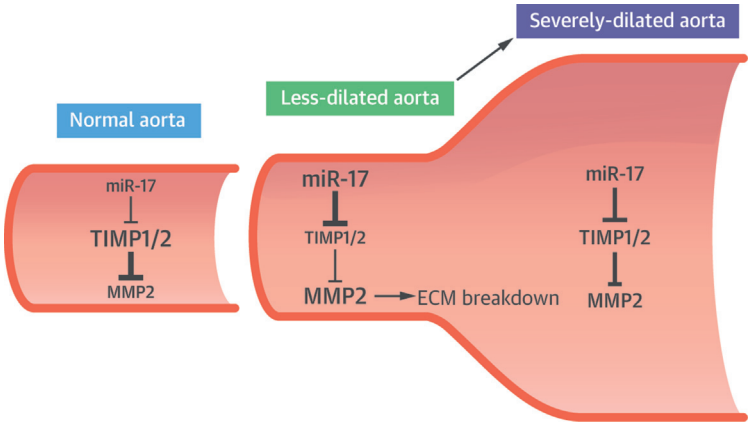
(A) Representative Western blot images examined MMP-2 and -9 protein expression in 5 normal aortas (1–5) and 2 BAV patient sample pairs. **(B)** Total and activated MMP2 expression levels were significantly increased, but total MMP-9 protein expression was unchanged in the less dilated BAV patient aortic tissue than in normal aortic tissue (normal: n = 10; less dilated **[LD]** and severely dilated **[SD]** samples: n = 12, paired samples from 12 patients). Abbreviations as in Figure 1.

These miRNAs appear to function through a TIMP-MMP pathway (Central Illustration).

Between-patient variation may obscure disease-related differences and result in false-positive and false-negative results. Additionally, previous studies were not able to compare aortic tissue in the early stages of dilation to those with extensive dilation. Our paired tissue approach, with 1 normal-appearing segment (<4 cm in diameter, less dilated) and the other from a substantially dilated (>4 cm in diameter, severely dilated) aorta segment from the same individual, eliminated between-patient variability.

To our knowledge, this type of paired comparison of aortic tissue samples has not been previously reported. Other studies have compared differential expression of miRNAs in animal models or compared dilated aortic tissue segments to tissue obtained from individuals with normal-sized aortas (16,17). Maegdefessel et al. (18), for example, used murine models of abdominal aortic aneurysm to investigate the role of miRNAs in aneurysm formation. They suggested that therapeutic manipulation of miR-29b and its target genes holds promise for limiting disease progression and protecting against rupture. In a study by Kin et al. (19), atherosclerotic abdominal aortic aneurysm and normal aortic wall tissues were obtained from patients undergoing surgery; the authors reported that miRNAs related to fibrosis (miR-29b), inflammation (miR-124a, miR-146a, miR-155, miR-223), and endothelium (miR-126, let-7 family members, miR-21) were significantly upregulated in aneurysmal tissue (19). As with our study, Kin et al. (19) found that miR-17-related miRNAs (miR-18a, miR-20a, miR-93) were upregulated in aneurysmal tissues compared with normal wall tissues. However, they did not evaluate the TIMP-MMP axis in relation to miR-17-related miRNAs. Ikonomidis et al. (20) examined the protein abundance of key MMPs (MMP-1, -2, -3, -8, -9), TIMPs (TIMP-1, -2, -3, -4), and miRNAs (1, 21, 29a, 133a, 143, 145) in ascending thoracic aortic aneurysm tissue and plasma specimens obtained from patients with BAV or tricuspid aortic valve at the time of surgical resection (20). In agreement with our study, they found increased

CENTRAL ILLUSTRATION **miR-17 Pathway Activation in Patients With BAV**



Wu, J. et al. J Am Coll Cardiol. 2016;67(25):2965–77.

In normal aortic tissue, expression of miRNAs of the miR-17 gene cluster and other miR-17–related miRNAs is low, allowing robust expression of their TIMPs. TIMPs strongly inhibit MMP-2 activity. During the early phases of BAV-associated dilation, these miRNAs are upregulated, strongly inhibiting their TIMP targets, which subsequently increases MMP-2 activity. Enhanced MMP-2 activity leads to progressive ECM breakdown, aortic dilation, and aneurysm formation. Once severe tissue dilation has occurred, miR-17 expression is reduced, TIMP expression increases, and MMP-2 activity decreases toward baseline levels. BAV = bicuspid aortic valve; ECM = extracellular matrix; miRNA = micro ribonucleic acid; MMP = matrix metalloproteinase; TIMP = target tissue inhibitor of MMP.

MMP-2 proteolytic activity in BAV relative to normal aorta. miRNAs 1, 21, 29a, 133a, 143, and 145 were also examined in our microarray analysis. The differential expression of these microRNAs in our paired dilated samples are summarized in Online Figure 1.

Genetic abnormalities frequently associated with BAV aortopathy (2) may contribute to the variation associated with measures of matrix remodeling. In the current study, 6 of the 12 BAV patients had a family history of some type of aortopathy, but none of the patients had an identified genetic mutation. Future studies will be necessary to determine the influence of any genetic abnormalities on matrix-remodeling induced aortic dilation.

We evaluated expression of 847 miRNAs in severely and less dilated aortic tissue and observed a substantial induction of members of the miR-17 gene cluster and other miRNAs that share the miR-17 seed sequence in normal-appearing BAV less dilated tissue. We hypothesize that this tissue was undergoing matrix remodeling and was in the early stages of aortic dilation. The BAV severely dilated tissue represents the end stage of this process. Therapeutic interventions during early stages of dilation might prevent progression, whereas the only intervention possible after aortic dilation is surgical excision.

We previously found that miR-17 plays a key role in regulating cardiac matrix remodeling by downregulating TIMP-1 and -2 expression (9). Other reports have suggested that miR-17 can also target and inhibit TIMP-3 (13) and that miR-106a and miR-20a, members of the miR-17 family, directly target TIMP-2 (21). Increased MMP:TIMP ratios in BAV patient aortic tissue were associated with increased proteolysis and matrix degradation (22,23). Both the MMP-2 and the MMP-9 activity have been implicated in BAV-associated aortic aneurysm formation (24–26). Here, we found that MMP-2 and -9 protein levels were unchanged but that MMP-2 activity was significantly higher and TIMP-1, -2, and -3 expression significantly lower in less dilated than in severely dilated or normal samples. We also showed that miR-17 can regulate TIMP-1 and -2 expression in primary SMCs derived from BAV or normal aortic tissues.

These results suggest that an increased activated MMP:TIMP ratio may be an early hallmark of BAV aortopathy-associated dilation and that miR-17-related miRNAs may influence this TIMP-MMP balance by regulating TIMP expression, thereby initiating progressive aortic aneurysm formation. After severe dilation is complete, miR-17-related miRNA expression gradually dissipates, consistent with our previous work on miR-17 expression in the post-MI myocardium (9).

Upregulation of miR-17-related mRNAs could represent a potential new therapeutic target.

Decreasing miR-17 expression during the early stages of dilation could prevent further progression. Our preliminary studies in SMCs indicate that inhibitors of miR-17 effectively prevented decreased TIMP and increased MMP expression. However, further experimentation is required to confirm this.

STUDY LIMITATIONS. BAV may be associated with genetic abnormalities that may contribute to matrix remodeling. All patients in this study underwent genetic testing for known abnormalities associated with aortopathy and none were found. Future studies will be required to determine the influence of any genetic contributors to matrix-remodeling. As all patients in the current study had BAV, our results cannot be extrapolated to patients with tricuspid aortic valve disease or any other aortopathy. Finally, a causal relation between inhibition of miR-17 and reduced matrix degradation will require validation in an animal model. We carefully considered all available mouse models of BAV aortopathy, but none adequately reproduce the clinical findings. Therefore, we developed an ex vivo model to test the effects of modulating miRNAs on the mechanisms responsible for matrix remodeling. Future studies will be required to establish a causal relationship between miR-17 inhibition and reduced matrix remodeling.

CONCLUSIONS

We provided the first evidence that the miR-17-TIMP-MMP pathway may regulate matrix degradation in progressive aortic dilation in BAV-associated aortic aneurysm. These results provided new insights into the mechanisms of early BAV-related aortic dilation and suggested future preventative strategies in the form of new small molecular therapeutic targets. Pharmacological inhibitors of miR-17 given during the early stages of dilation might prevent BAV aortopathy-related aneurysm formation.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In patients with a BAV, progressive aortic dilation is associated with ECM degradation induced by MMPs that are regulated by miR-17–related miRNAs. Inhibition of miR-17 decreased MMP activity, and genetic analyses confirmed a relationship between expression of miR-17–related miRNAs and the severity of aortopathy.

TRANSLATIONAL OUTLOOK: The next steps are to investigate whether inhibitors of miR-17 retard the rate of aortic dilation in animal models of aortic aneurysm and verify whether inhibition of miR-17 reduces ECM breakdown in the aortic smooth muscle cells of patients with BAV.

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KEY WORDS aorta, bicuspid aortic valve, MMP, TIMP

APPENDIX For expanded Methods and Results sections as well as a supplemental table and figures, please see the online version of this article.