Selective MicroRNA Suppression in Human Thoracic Aneurysms: Relationship of miR-29a to Aortic Size and Proteolytic Induction

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Background—Increasing evidence points to a direct role for altered microRNA (miRNA or miR) expression levels in cardiovascular remodeling and disease progression. Although alterations in miR expression levels have been directly linked to cardiac hypertrophy, fibrosis, and remodeling, their role in regulating gene expression during thoracic aortic aneurysm (TAA) development has yet to be explored.

Methods and Results—The present study examined miR expression levels in aortic tissue specimens collected from patients with ascending TAAs by quantitative real-time PCR, and observed decreased miR expression (miRs -1, -21, -29a, -133a, and -486) as compared with normal aortic specimens. A significant relationship between miR expression levels (miRs -1, -21, -29a, and -133a) and aortic diameter was identified; as aortic diameter increased, miR expression decreased. Through the use of a bioinformatics approach, members of the matrix metalloproteinase (MMP) family, proteins involved in TAA development, were examined for putative miR binding sites. MMP-2 and MMP-9 were identified as potential targets for miR-29a and miR-133a, respectively, and MMP-2 was subsequently verified as a miR-29a target in vitro. A significant inverse relationship between miR-29a and total MMP-2 was then identified in the clinical TAA specimens.

Conclusions—These findings demonstrate altered miR expression patterns in clinical TAA specimens, suggesting that the loss of specific miR expression may allow for the elaboration of specific MMPs capable of driving aortic remodeling during TAA development. Importantly, these data suggest that these miRs have biological and clinical relevance to the behavior of TAAs and may provide significant targets for therapeutic and diagnostic applications.

Key Words: aneurysm ▪ thoracic aorta ▪ microRNA ▪ MMP ▪ remodeling

Within the spectrum of cardiovascular diseases, thoracic aortic aneurysms (TAAs) continue to be one of the most dangerous and difficult to treat problems in cardiothoracic surgery. Although it is clear that aortic dysfunction and dilatation are a direct result of pathological remodeling of the aortic extracellular matrix (ECM) and that this process is mediated in part by the family of matrix metalloproteinases (MMPs), there remains a paucity of information regarding the upstream mechanisms that regulate these enzymes during TAA development. Recently, a novel class of small noncoding RNA molecules (microRNAs, miRs), 20–25 nucleotides in length, were shown to have important posttranscriptional gene regulatory functions.1 MicroRNAs target short nucleotide sequences within the 3’ untranslated region (UTR) of specific messenger RNAs (mRNAs) and function to induce message degradation, or, more typically, translational repression. To date, more than 1000 unique miRs have been identified within the human genome (miRBase statistics),2 and, based on computational methodology current predictions, suggest that approximately one-third of expressed human genes contain miR regulatory target sites.3 Moreover, a single miR is capable of targeting multiple miRNAs, and a single mRNA may contain multiple miR binding sites.4–6 Together, this suggests that a common set of miRs can fine-tune the protein abundance of a cassette of specific genes that together influence specific cellular functions.

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As it is becoming increasingly apparent, miRs are important determinants of disease within the cardiovascular system. Clear roles for altered miR expression have been implicated in the regulation of smooth muscle cell phenotype, angiogenesis, atherosclerosis, restenosis, and other vascular injury responses. Moreover, recent work has identified that changes in miR expression may contribute to the pathogenesis of aortic aneurysm or dissection. Liu et al profiled miR expression in a rat model of abdominal aortic aneurysm, using a miR microarray, and identified 15 dysregulated miRs with putative targets in multiple signaling pathways, including the mitogen activated protein kinase (MAPK) pathway, which may be important for AAA development. Elia et al examined the miR-143/miR-145 cluster in mouse model of atherosclerosis (ApoE knockout mouse) and reported markedly decreased expression levels in the knockout mice that were further reduced when the animals were fed a high fat diet. They then examined human biopsies from ascending aortic aneurysms and again observed a loss of expression for miR-143/miR-145 and suggested that the loss of these miRs induced structural modifications in the aorta, mediated in part through the incomplete differentiation of vascular smooth muscle cells. Last, Liao et al recently examined miR expression in aortic tissue from a small cohort of patients with thoracic aortic dissections, using a miR microarray. They likewise reported dysregulated miR expression (18 miRs upregulated, 56 miRs downregulated) with putative targets in the focal adhesion and MAPK pathways. Together, these reports suggest that dysregulated miR expression is a common feature of vascular disease and dysfunction and that targeting the regulation of miR expression/function may provide significant therapeutic advantages.

Accordingly, it is not unreasonable to postulate that alterations in miR expression may exert an important role in the aortic medial degeneration and dilatation associated with TAA development. Therefore, the present study measured miR expression levels in clinical TAA specimens and examined the coordinate relationships with indices of aneurysm progression.

Methods

Patient Demographics

The study population consisted of aortic tissue specimens obtained from ascending TAA patients with tricuspid aortic valves (n=30) at time of surgical resection. The study excluded specimens from patients with Marfan syndrome, bicuspid aortic valves, or documented aortic dissection. Results were compared with a reference control group consisting of nonaneurysmal aortic specimens (n=10) collected from the ascending aorta of heart donors and coronary artery bypass graft patients. There were no sex (control, 80% male; TAA, 83% male; P=0.81 χ²) or age differences (control, 57.±4 years; TAA, 62.±2 years; P=0.31) between control and ascending TAA patients. All aortic specimens were maintained in frozen storage (~80°C) until time of experimentation. These specimens are a part of a multi-institutional aortic tissue bank located at the Medical University of South Carolina. This study was approved by the institutional review boards of the Medical University of South Carolina, the University of Pennsylvania, and Yale University, and informed consent was obtained from all patients.

Sample Preparation and miR Expression Analysis

Resected aortic tissue specimens were homogenized in cell disruption buffer (mirVana PARIS miRNA Isolation Kit; Applied Biosystems/Ambion, Austin, TX), using a Qiagen TissueLyser (Qiagen, Valencia, CA) bead-mill homogenizer. Total RNA was isolated (mirVana PARIS miRNA Isolation Kit), and reverse transcribed (TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems). For quantitative PCR studies, the cDNA was preamplified according to the manufacturer recommendations (TaqMan PreAmp Master Mix; Applied Biosystems) and the products used with specific TaqMan MicroRNA Assays (hsa-miR-1, hsa-miR-21, hsa-miR-29a, hsa-miR-133a-1, hsa-miR-208, hsa-miR-486–5p, hsa-miR-760; and snRNA U6; internal control) (Applied Biosystems) to analyze specific miR expression on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA), using the following 2-step procedure: initial denaturation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C.

Microarray Analysis

Total RNA isolated from 4 normal aortic specimens and 4 large TAAs was used for miR expression analysis by microarray. The total RNA, containing low-molecular-weight RNA was examined on a Bioanalyzer 2100 (Agilent Technologies) with the Small RNA Chip to ensure low-molecular-weight RNA content and quality. Total RNA samples (100 ng) were labeled with the FlashTagTM Biotin RNA Labeling Kit (Genisphere LLC), following manufacturer recommendations. Biotin-labeled samples were hybridized to Affymetrix GeneChip miRNA Arrays that were then washed, fluorescently labeled, and scanned using Affymetrix instrumentation in accordance with Affymetrix and Genisphere protocols. The resulting hybridization data were processed with Affymetrix miRNA QC Tool software (Version 1.0.33.0). Processing settings were as follows: (1) detection scoring was applied; (2) background adjustment used the BC-CG Adjust algorithm; (3) normalization was done, using the quantile method; (4) the optional processes “added small constant” and “threshold” were used at default settings; (5) summarization was done using median polish. Resulting summarized hybridization data for the human miR content was then imported into dChip for comparative analysis. Differential expression was assessed as either a change in detection or a statistical change in magnitude between aneurysmal and control samples. Differential detection was assigned to miRs scoring entirely undetected in one group but detected in one of more samples of the other group; differential magnitude was assigned to miRs detected in one or more samples of both groups and differing by fold change >1.5, P<0.05 (Student t test, unpaired). False discovery for differential magnitude was estimated as the median number of human miRs discovered by 100 iterations of comparison with randomized sample assignments.

miR Target Prediction

As a first approach to identifying potential miR binding sites in MMP-2 and MMP-9, the full-length transcripts for MMP-2 and MMP-9 were used to search the TargetScanHuman database (version 5.1, http://www.targetscan.org/). The results identified target binding sites for miR-29a (conserved, 7mer-8; context score =−0.01, P<0.01; 0.82) and miR-133a (poorly conserved, 7mer-8; context score =−0.04, P<0.01) in MMP-2 and MMP-9, respectively. To confirm the initial TargetScan results for MMP-2, a series of other bioinformatics databases were also consulted. Of the 8 additional sites that were examined, a target binding site for miR-29a was identified in the 3’UTR of MMP-2 in 6 of 8 sites examined including: RNAdiary (http://bibiserv.tech.fak.uni-bielefeld.de/mnahybrid/), NBmiRTar (http://wotan.wistar.upenn.edu/NBmiRTar), PicTar (http://picTar.bio.nyu.edu), RNA22 (http://cbcrsv.watson.ibm.com/rna22.html), miRanda (http://www.microrna.org/microrna/home.do), and PITA (http://pita.mbc.nctu.edu.tw). Two sites, DIANA-microT (http://www.diana.plcbi.upenn.edu/cgi-bin/micro_t.cgi) and miTarget2 (http://mirdb.org/miRDB/) failed to identify a potential target binding domain.
Viral Transduction of Vascular Smooth Muscle Cells

Human primary aortic vascular smooth muscle cells (PromoCell, Heidelberg, Germany; Cat No. C-12533) were maintained in Smooth Muscle Cell Growth Medium 2 (PromoCell, Cat No. C-22062) with 10% heat-inactivated Fetal Bovine Serum (Cat No. 16000–044, Invitrogen, Carlsbad, CA), 5 μg/mL Amphotericin B (PromoCell, Cat No. C-42040) and 0.5 mg/mL Gentamicin (PromoCell, C-42060) at 37°C in a humidified 5% CO₂ incubator. The cells (2x10⁶ cells, passage No. <8) were seeded into T-25 culture flasks, grown to 70% confluence, and exposed to lentivirus (6.6x10⁶ PFU/mL) encoding either has-miR-29a (precursor form; pMRN1-Hsa-mir-29a, Cat No. CS970MRB-1, System Biosciences, Mountain View, CA), anti–miR-29a (pmiRZip-29a, Cat No. CS070MZ-1, System Biosciences) or a nontargeting mismatch control (pGreenPuro Scramble Hairpin Control, Cat No. MZIP000-VA-1, System Biosciences), using Transdux reagent (LV850A-1, System Biosciences) to improve transduction efficiency, or exposed to Transdux reagent alone (vehicle control). Five days after transduction, the has–miR-29a precursor or the has–anti–miR-29a, or treated with MZIP000-VA-1, System Biosciences, were transduced with lentiviral constructs, as described above, encoding the IL3. Aortic or cellular extracts (10⁵ cells, passage No. 20–201, Millipore, Billerica, MA) before analysis by gelatin zymography.

Gelatin Zymography

The relative abundance of MMP-2 and MMP-9 from the aortic tissue specimens or vascular smooth muscle cells was determined by gelatin zymography. The aortic tissue specimens were homogenized in cold acidic extraction buffer using the Qiagen Tissuelyser. Tissue and cell homogenates were centrifuged (4°C, 10 minutes, 1200g), and protein concentrations were determined (BCA Protein Assay, Pierce, Rockford, IL). Aortic or cellular extracts (10 μg total protein) were fractionated on nondenaturing 10% polyacrylamide gels containing 0.1% (w/v) gelatin (Invitrogen Corporation, Carlsbad, CA). The gels were then equilibrated and incubated in Zymogram Developing Buffer (Invitrogen) for 18 hours at 37°C. After staining with 0.5% Coomassie brilliant blue (2 hours, room temperature), the gels were destained to reveal regions of gelatin clearance. The relative abundance of the active and latent forms of MMP-2 and MMP-9 (as verified by recombinant MMP-2 and MMP-9 standards) were then determined by densitometry using the Gel-Pro Analyzer software package (v 3.1.14, Media Cybernetics Inc, Silver Spring, MD).

Confocal Microscopy

Human primary aortic vascular smooth muscle cells were seeded in 35-mm glass-bottom dishes (Cat No. P35GC-1.5–20-C; MatTek Corporation, Ashland, MA). When 70% confluence was reached, the cells were transduced with lentiviral constructs, as described above, encoding the has-miR-29a precursor or the has-anti-miR-29a, or treated with Transdux reagent (vehicle control) alone. Five days after transduction, the cells were fixed for 20 minutes with fresh 3.7% paraformaldehyde. The cells were then washed with PBS (6x5 minutes) and then permeabilized with 0.1% Triton X-100 for 10 minutes. After a second PBS wash (3x5 minutes), the cells were blocked in 10% normal goat serum for 1 hour. Human MMP-2 (active and latent forms) was then identified by incubating the cells with a rabbit polyclonal antibody (1:200, Cat No. ab37150, Abcam, Cambridge, MA) for 2 hours, followed by a 2-hour wash in PBS (5x3 minutes) and then incubated in secondary antibody (1:500, AlexaFluor647 goat-anti-rabbit IgG; Cat No. A-21244, Invitrogen Corporation) for 30 minutes at room temperature in the dark. Each viral vector carries a bicistronic copy of green fluorescent protein (GFP) under control of an independent promoter. Cells were then examined by confocal microscopy using a Zeiss LSM 510 Meta Confocal Microscope with a Plan-Neofluar 43X/1.3 oil objective. Dual fluorescence (GFP, ex=488 nm, em=band-pass 505–530 nm; AlexaFluor647, ex=633, em=long pass 650) was recorded and the images exported as tif files.

Data Analysis

To determine miR expression, the relative change in cycle threshold value (ΔCT) from the internal control, snRNAU6, was computed. Expression was then calculated for each normal and TAA specimen using the equation Expression = 2^(-ΔΔCT), which is premised on the fact that each CT value is in direct proportion to the amount of microRNA present at the beginning of the reaction. The results were articulated as a percent change from normal aorta to avoid any bias in total miR concentrations introduced by the preamplification step. The mean percent change in miR expression from normal aorta was then calculated. Specimens that did not cycle, or had Ct values higher than 35, were removed from analysis. Additionally, any expression values that fell more than 2 standard deviations away from the mean were considered outliers and removed from analysis so as not to bias the expression results. Results were expressed as mean±SEM.

Relative protein abundance of MMP-2 and MMP-9 was determined from densitometry of zymographic gels. Integrated optical densities were determined for each specimen and the mean percent change of the ratio of active:total MMP from normal aorta was calculated (outliers were identified as values falling more than 2 standard deviations away from the mean and removed from the data set), and the results are expressed as mean±SEM.

All statistical procedures were carried out using the Stata statistical package (Intercooled Stata v8.2; StataCorp LP, College Station, TX). Patient demographics were compared by χ² analysis (sex) or 1-way ANOVA (pcompw module) with Tukey post hoc analysis (age). Changes in miR expression and relative protein abundance were determined using 2-tailed, 1-sample mean comparisons versus normal aorta set at 100%, and differences between groups were determined using 1-way ANOVA (pcompw module) with Tukey post hoc analysis; in both cases values of P<0.05 were considered significantly different. Differences in miR expression between the normal and aneurysm groups, as determined by microarray, were calculated using a Student t test. Values of P<0.05 were considered to have a significant change in expression. Additionally, differences in miR detectability between the normal and aneurysm groups were calculated using χ² square analysis (tabi command, Stata v 8.2). Accordingly, if a given microRNA was detectable in 3 or more specimens of a single group when compared with no detection in the other group, the miR was considered to have significant detectability with P<0.005. Relationships between miR expression or MMP protein abundance and aortic size, as well as the relationship between miR expression and total MMP-2 protein abundance, were determined using linear least-squares regression analysis. Results were reported graphically and a correlation constant (r value) and probability value were determined (pwcorr and regress modules); relationships with P<0.05 were considered significant.

Results

Quantitative real-time polymerase chain reaction (QPCR) was used to determine the relative expression levels of miRs -1, -21, -29a, -133a, -208, -486–5p, and -760 as compared with snRNAU6, a ubiquitous small nuclear RNA component of the spliceosome, used as an endogenous control. The QPCR results revealed significantly reduced expression of 5 of the 7 miRs examined; miRs -1, -21, -29a, -133a, and -486–5p (Figure 1 and online-only Data Supplement S1). Expression of miR-760 was unchanged, and the α-myosin heavy chain–encoded (myocardial-restricted) miR-208 was not detected in our aortic specimens. To further verify that the loss of miR expression was due specifically to reduced miR levels and not dramatic changes in snRNAU6, a table of Ct values (mean±SEM) for each miR examined is provided as a part of the online-only Data Supplement Materials (Table S1).

To further examine miR expression in normal and TAA tissues, microarray analysis was performed. The results yielded 37 differentially expressed miRs between normal aorta and aneurysmal aorta, consisting of 4 miRs with increased expression in the TAA group and 33 miRs with decreased expression in the TAA group (Figure 2A). In addition, there were 106 miRs
that were differentially detected either in normal aorta or aneurysm but not both. These results yielded 54 miRs that had increased expression in the aneurysm group and 52 miRs that had decreased expression (Figure 2B). If a given microRNA was detected in 3 or more specimens from either group (normal or aneurysm), it was considered to have a significant change in expression ($\chi^2$ analysis, $P<0.05$). These data support the changes in miR expression determined by quantitative PCR for miR-1, miR-29a, miR-133a, and miR-486–5p. Functions for the 37 differentially expressed miRs were estimated based on published manuscripts reported in PubMed (online-only Data Supplement Figure S2). Accordingly, 27% target proliferation pathways, 16% target growth arrest pathways, 8% target ECM structure/function, 5% (each) target apoptosis, migration, and angiogenesis, 3% (each) target drug resistance, immune response, autophagy, and cell adhesion pathways, and 22% to date have no reported function.

The qPCR results were then stratified based on aortic diameter of the TAA specimens as compared with normal aorta. Mean percent change ($\pm$SEM) of miR expression in clinical TAA specimens versus normal aorta. All specimens were analyzed by quantitative PCR. The results for all cycling specimens are shown in the graph. Dashed line represents the results for normal aorta set at 100% for each miR examined ($n-$minimum of 8). Bars represent results for miR expression in cycling TAA specimens; miR-1 ($n=21$), miR-21 ($n=26$), miR-29a ($n=27$), miR-133a ($n=25$), miR-486–5p ($n=26$), and miR-760 ($n=26$); $*P<0.05$ versus 100%. Expression of the myocardial-specific miR-208 was not detected in our aortic specimens.

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Using linear least-squares regression modeling, miR expression was compared with aortic size for each of the TAA specimens. Several significant inverse relationships were identified: miR-1 ($r=-0.5433, P=0.0109, n=21$), miR-21 ($r=-0.4132, P=0.0359, n=26$), miR-29a ($r=-0.5364, P=0.0039, n=27$), and miR-133a ($r=-0.4247, P=0.0262, n=25$) (Figure 3B).

Potential biological targets were searched using the TargetScanHuman database for the presence of conserved nucleotide sequences (7–8 nucleotides in length) that significantly matched with the seed regions (nucleotides 2–7) of miRs -1, -21, -29a, and -133a.\(^3\) Potential binding sites for miR-29a (conserved, 7mer-8; context score $= -0.01, P_{CT}=0.82$) and miR-133a (low species conservation, 7mer-8; context score $= -0.04, P_{CT}<0.01$) were identified in MMP-2 and MMP-9, respectively (Figure 4A).\(^3,5,15\)

To investigate the relationship between MMP abundance and miR expression, the relative abundance of MMP-2 and MMP-9 protein levels was examined in the same clinical TAA specimens, using zymography. Both active and latent bands were identified by recombinant standards and total MMP was calculated (sum of active + latent), and the percent change in ratio of active:total forms as compared with normal aorta were stratified by aneurysm size, providing both a measure of MMP abundance and activational state. The data revealed an increased MMP-2 active:total ratio in each of the aneurysm size groups, whereas no difference in the MMP-9 ratio was observed (Figure 4B and 4C). Using linear least-squares regression modeling, the increase in active:total MMP-2 ratio displayed a significant positive relationship to aneurysm size ($r=0.4056, P=0.0262, n=30$), suggesting that the abundance and activation of MMP-2 increase with increasing aortic diameter.

Accordingly, to examine the relationship between MMP-2 abundance and miR expression, human primary aortic vascular smooth muscle cells were transduced with lentiviral constructs encoding the miR-29a precursor or the miR antagonist, anti-
Figure 3. MicroRNA (miR) expression and relationship to aortic size. A, miR expression was stratified into groups based on aortic diameter (thoracic aortic aneurysm [TAA] size) defined as small TAAs (4.0–5.0 cm), medium TAAs (5.1–6.0 cm), or large TAAs (6.1–7.5 cm), and results were compared with normal aorta (Normal). Expression levels were calculated as a percent change from normal aorta (set at 100%) and displayed as box plots showing the median (solid line), interquartile range (25th to 75th percentile; gray box), and the mean (dashed line), overlaid with a scatterplot of each value; *P<0.05 versus 100%, #P<0.05 versus small TAAs.

B, Linear least-squares regression analysis demonstrating several significant inverse relationships between miR expression in clinical TAA specimens and aortic diameter; miR-1 (r = −0.5433, P = 0.0108, n = 21), miR-21 (r = −0.4132, P = 0.0359, n = 26), miR-29a (r = −0.5364, P = 0.0039, n = 27), and miR-133a (r = −0.4247, P = 0.0344, n = 25).
miR-29a. Transduced cells were examined by confocal microscopy and gelatin zymography for changes in the abundance of latent and active forms of the MMP-2 protein. GFP, driven from an independent promoter on each viral construct, was used to identify transduced cells. The cells were stained with an antibody for total MMP-2, and dual fluorescence was recorded. As shown in the transduction vehicle controls, MMP-2 is localized to the periphery of the cell (identified by white arrows). To quantify the effects of modulating miR-29a on the abundance of total MMP-2 protein levels, cells were transduced with miR-29a precursor or anti–miR-29a and harvested for gelatin zymography for changes in the abundance of matrix metalloproteinase (MMP-2) in clinical thoracic aortic aneurysm (TAA) specimens. A significant miR binding sites identified using the TargetScanHuman database (v 5.1, released April 2009). The unique findings of this study were 4-fold.

First, within the focused set of miRs studied, 5 were found to have decreased expression in clinical ascending TAA specimens as compared with normal aorta (miRs -1, -21, -29a, -133a, and -486) as established by quantitative PCR. MicroRNA expression values were calculated from normalized C_T values (ΔC_T), using the equation (expression=2^(-ΔC_T)) for each specimen, premised on the fact that each C_T value is in direct proportion to the amount of microRNA present at the beginning of the reaction. Whereas factors such as PCR amplification efficiencies can influence the amount of product generated and thereby influence the sensitivity of an individual reaction, the distribution of measured miR values within the sampled cohort for this study (n=30 TAA specimens) provided sufficient power to discriminate a 50–80% reduction in miR concentration between the normal and TAA groups (corresponding to a C_T value increase of 1–1.2 cycles). The statistical power for comparing miR values between the 2 groups was greater than 0.92 for all miRs measured except miR-760, where the power was 0.29. Based on expectations of structural remodeling within the aortic wall in the aneurysm specimens, we anticipated that the protein levels of multiple MMPs and ECM proteins would be induced in response to TAA development.20 Because miRs

Discussion

TAA disease results as a consequence of pathological remodeling within the aortic vascular ECM. This remodeling process induces a progressive weakening of the ECM through a variety of mechanisms including altered collagen deposition/processing and the elevation of proteinase activity.17–19 These changes within the aortic wall result in decreased compliance and competence, culminating in aortic dilation and eventual rupture. In an effort to further understand the molecular pathogenesis of TAA development, this study examined the expression of a focused set of miRs in clinical TAA specimens compared with aortic expression levels in patients without aneurysm disease. It was hypothesized that TAA development would coincide with alterations in specific miR expression that could affect the induction of target proteins, which contribute to aortic vascular remodeling. This study focused on the expression of seven miRs that were selected because of their reported involvement in the regulation of specific protein targets within the cardiovascular system, including several MMPs and ECM components (collagens, elastin, and microfibrillar proteins).7 The unique findings of this study were 4-fold.
function to degrade mRNA or repress message translation, we were not surprised to observe a significant loss of miR expression, knowing that many of the putative targets would increase in response to the disease state. This loss of miR expression in vascular disease is consistent with other reports examining miR expression by microarray. For example, Liao et al profiled miR expression in a cohort of ascending aortic specimens by microarray, comparing normal patients with those with aortic dissection. They observed 74 miRs that were differentially expressed with a predominance of miRs (n/H11005/H11005 56) showing reduced expression in the dissected tissue.13

To further examine miR expression differences between the aneurysmal and normal aorta, microarray analysis was performed, and, similar to Liao et al, of the 37 differentially expressed miRs identified, 33 were found to have decreased expression. Included in the group of 33 miRs with decreased expression, were the miR-143/miR-145 cluster, previously reported by Elia et al to be decreased in human ascending aortic aneurysm biopsies,12 and miR-29a, identified in this study as having significantly decreased expression in TAA specimens. Based on previous literature, the majority of these differentially expressed miRs target pathways involved in proliferation, growth arrest, and ECM structure/function. This study suggests that the loss of specific miR expression may allow for the elaboration of specific protein targets that contribute to the aortic remodeling process during TAA development.

Second, several miRs displayed a significant relationship between the loss miR expression and the enhancement of aortic diameter (miRs -1, -21, -29a, and -133a). These results suggested that dynamic changes in miR expression levels probably occur during aneurysm progression and emphasize that the loss of ongoing translational repression may play a key role in modulating the determinants of vascular remodeling.

Figure 5. Modulation of microRNA (miR)-29a expression levels in human primary aortic vascular smooth muscle cells. A, Cells were exposed to lentiviral constructs, containing a bicistronic copy of green fluorescent protein (GFP), designed to overexpress miR29a, anti–miR-29a, or to the transduction reagent alone. Five days after transduction, the cells were stained with matrix metalloproteinase (MMP-2)-specific antisera, using an AlexaFluor647 secondary antibody. GFP (ex 488 nm/em 509 nm) was used to identify transduced cells; red fluorescence (MMP-2; ex 633 nm/em 670) showed the localization and abundance of the active and latent forms of MMP-2. MMP-2 was localized to the cell periphery in vehicle-treated cells (transduction vehicle control, top), whereas in the miR-29a-transduced cells, (middle panels), MMP-2 abundance was attenuated. In the anti–miR-29a-transduced cells (bottom panels), MMP-2 protein levels were enhanced. White arrows show regions of MMP-2 accumulation at the perinuclear region and cell periphery.

Figure 6. Relationship between total matrix metalloproteinase (MMP-2) abundance and microRNA (miR)-29a expression in clinical thoracic aortic aneurysm specimens. Linear least-squares regression analysis demonstrated a significant inverse relationship between miR-29a expression and total MMP-2 abundance (r = −0.4198, P = 0.0209, n = 30).

Red bar = 50 μm. B, Cells were exposed to the transduction vehicle alone, miR-29a lentivirus, or anti–miR-29a lentivirus. Five days after transduction, cells were harvested and examined by gelatin zymography. The results demonstrated that overexpression of miR-29a attenuated total MMP-2 protein abundance, whereas overexpression of anti–miR-29a enhanced total MMP-2 protein abundance (n = 3; *P < 0.05 versus control, #P < 0.05 versus miR-29a–transduced cells).
Third, because alterations in miR expression have been shown to modulate target protein abundance, a bioinformatics approach was used to identify putative miR target sequences in genes known to be involved in TAA formation and progression. As a first approach, the TargetScanHuman database was queried with the sequence of full-length transcripts for several MMPs and ECM structural proteins. Potential biological targets were screened for the presence of conserved nucleotide sequences that significantly matched the seed regions of miRs -1, -21, -29a, and -133a. Results identified a highly conserved miR-29a target sequence (7mer-8) in the 3’ UTR of MMP-2 and a less well-conserved miR-133a target sequence (7mer-8) in the 3’ UTR of MMP-9. Work by Bartel et al has suggested that conserved 7mer-8 target sequences are significant predictors for high efficacy miR-mediated message destabilization.4 The MMPs are a diverse family of proteases capable of degrading all components of the vascular ECM. In numerous studies using human specimens and animal models, increased protein levels of MMP-2 and MMP-9 have been directly implicated in aneurysm development in both the abdominal and thoracic aorta.16,21–29 Therefore, the presence of these predicted target sequences suggests that these proteases may be subject to post-transcriptional or translational regulation by miR expression.3,30,31 The protein levels of MMP-2 and MMP-9 were therefore examined in the clinical TAA specimens, and the indices of MMP-2 abundance were found to be elevated compared with normal aortic specimens. Moreover, a positive relationship was identified between the active:total MMP-2 ratio and aortic diameter, suggesting MMP-2 as an important mediator of aortic remodeling, consistent with previous findings from this laboratory and others.20,24,26,32–35 To demonstrate a direct relationship between miR-29a and MMP-2, lentiviral vectors were used to transduce human primary aortic vascular smooth muscle cells with either the miR-29a precursor, or an anti–miR-29a, designed to knock down cellular miR-29a levels. The transduced cells were then examined for changes in MMP-2 protein levels by confocal microscopy and gelatin zymography. The data demonstrated overexpression of miR-29a resulted in the attenuation of MMP-2 protein levels, whereas the overexpression of anti–miR-29a resulted in enhanced MMP-2 protein abundance. These results identify MMP-2 as one of the target proteins for miR-29a and support previous reports that have identified miR-29 as a posttranscriptional regulator of MMP-2.30,31

Lastly, to further implicate a role for miR-29a in aneurysm development, regression modeling was performed and a significant inverse relationship between miR-29a expression and total MMP-2 protein abundance was demonstrated. This unique observation suggested that the loss of aortic miR-29a levels may permit the elaboration of MMP-2 translation within the developing aneurysm and may identify a potential mechanism by which MMP-2 protein induction occurs during TAA development.

Although the present study has identified miR-29a as a potential regulator of MMP-2 in the aorta, it is important to note that each miR can target upward of 200 different mRNAs, many of which may affect TAA development. For example, in silico mapping also identified miR-29a target binding sites in type I and type III collagens, elastin, and fibrillin-1. Dysregulation of any of these putative targets may be deleterious to the structure and function of the thoracic aorta. Thus, altered miR expression and function within clinical TAAAs may play a principal role in aneurysm progression by fine-tuning the protein abundance of a cassette of specific genes that together can influence a tissue-specific response.

The unique results of the present study carry several significant clinical implications. First, miR expression profiles may provide significant insight into the identification of potential upstream mediators of aortic ECM remodeling and may reveal therapeutic strategies for the treatment of TAA disease. Second, the altered miR expression profiles identified in this study may be expanded to lay the foundation for the development of diagnostic or prognostic bioassays with the potential to define phases of disease progression, informing of the best time to intervene surgically or even indicating the potential for aortic rupture. Last, understanding the regulation of specific miR expression in relation to the pathophysiology behind TAA development may also provide novel therapeutic strategies aimed at modulating miR expression to arrest aneurysm development or even reverse dilatation.

The present study identified that the loss of miR expression during clinical TAA development may play a key role in exacerbating pathological remodeling by removing an inhibitory signal that normally attenuates MMP production. Importantly, these data suggest that these miRs have biological and clinical relevance to the behavior of TAAs and may provide significant targets for diagnostic and therapeutic applications.

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Disclosures
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References

December 2011
Although it is clear that the development of thoracic aortic aneurysms (TAA) is a direct result of pathological remodeling of the aortic extracellular matrix (ECM) and that this process is mediated in part by the family of matrix metalloproteinases, there remains a paucity of information regarding the upstream mechanisms that regulate these enzymes during TAA development. Recently, a novel class of small noncoding RNA molecules (microRNAs, miRs), 20–25 nucleotides in length, were shown to have important posttranscriptional regulatory functions. This study identified a loss of miR expression during clinical TAA development that may play a key role in exacerbating pathological remodeling by removing an inhibitory signal that normally attenuates MMP production. The unique results of this study carry several significant clinical implications. First, miR expression profiles may provide significant insight into the identification of potential upstream mediators of aortic ECM remodeling and may reveal therapeutic targets for the treatment of TAA disease. Second, the altered miR expression profiles identified in this study may be expanded to lay a foundation for the development of diagnostic or prognostic bioassays with the potential to define phases of disease progression, informing of the best time to intervene surgically, or even indicating the potential for aortic rupture. Last, understanding the regulation of miR expression in relation to the pathophysiology behind TAA development may also provide novel therapeutic strategies aimed at modulating miR expression to arrest aneurysm development or even reverse dilatation.
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**Table S1.** Raw Ct values (Mean±SEM) for each miR examined in this study.

<table>
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<th>Small RNA Control</th>
<th>Control</th>
<th>Small TAAs</th>
<th>Medium TAAs</th>
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<td>24.5±0.5</td>
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<td>26.6±0.5</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** Alterations in miR expression in clinical TAA specimens as compared to normal aorta. Representative plots of raw cycling data depicting the difference in $\Delta$Ct values on the x-axis and the change in relative fluorescence values on the y-axis, between normal aorta (solid line) and TAA aorta (dashed line) from quantitative real-time PCR results.

**Figure S2.** Functional characteristics of the 37 differentially expressed miRs between Aneurysm (n=4) and Normal (n=4) aorta. The functions for the 37 differentially expressed miRs were estimated based on published manuscripts reported in PubMed.; 27% target proliferation pathways, 16% target growth arrest pathways, 8% target ECM structure/function, 5% (each) target apoptosis, migration, and angiogenesis, 3% (each) target drug resistance, immune response, autophagy, and cell adhesion pathways, and 22% to date have no reported function.

**Figure S3.** Transduction of human primary aortic vascular smooth muscle cells with a non-targeting mismatch control virus. A. Cells were exposed to a non-targeting mismatch control lentivirus, containing a bicistronic copy of green fluorescent protein (GFP), or to the transduction reagent alone. Five days post-transduction the cells were harvested and cell homogenates were examined by gelatin zymography and immunoblotting. The results demonstrated no change in latent (72 kDa) or active (64 kDa) MMP-2 with lentiviral transduction of a non-targeting sequence (top).
Immunoblotting for GFP (*middle*) and β-actin (*bottom*) confirmed lentiviral transduction of the mismatch control (GFP), and equal lane loading (β-actin) respectively (representative blots shown, n=3). B. Quantitation of total MMP-2 protein abundance following viral transduction. The results demonstrated that overexpression of a non-targeting sequence had no effect on total MMP-2 protein levels (n=3; p=0.6047).
Figure S1.
Figure S2.
Figure S3.

A.

B. MMP-2 (Total) Abundance (percent change from vehicle control)

Vehicle Control    Mismatch Control

MMP-2

GFP

β-actin

72kDa

64kDa

25kDa

45kDa

Vehicle Control    Mismatch Control

MMP-2 (Total) Abundance (percent change from vehicle control)

0 20 40 60 80 100 120

Vehicle Control    Mismatch Control