

Altered MicroRNAs in Bicuspid Aortic Valve: A Comparison Between Stenotic and Insufficient Valves

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Background and aim of the study: Bicuspid aortic valve (BAV), the most common form of congenital heart disease, is a leading cause of aortic stenosis (AS) and aortic insufficiency (AI). AS is typically caused by calcific valve disease. Recently, microRNAs (miRNAs) have been shown to modulate gene expression. The study aim was to examine the miRNAs that were altered in the aortic valve leaflets of patients with AS compared to those in patients with AI. In-vitro experiments were also carried out to determine if these miRNAs could modulate calcification-related genes.

Methods: Aortic valve samples (fused and unfused leaflets) were collected from nine male patients (mean age 44.9 ± 13.8 years) undergoing aortic valve replacement (AVR). PIQOR™ miRxplore Microarrays containing 1,421 miRNAs were used and hybridized to fused leaflet samples labeled with Cy5; unfused samples were used as controls and labeled with Cy3. A quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to validate the miRNA array results. Cultured human aortic valve interstitial cells (AVICs) were treated with miRNA mimics, and qRT-PCR was

carried out to determine any changes in mRNAs.

Results: By microarray analysis, seven miRNAs were shown to be statistically different between the AS and AI patients. In the stenotic samples, the miR-26a and miR-195 levels were shown (by qRT-PCR) to be reduced by 65% and 59%, respectively ($p < 0.05$), and miR-30b to be reduced by 62% ($p < 0.06$). Human AVICs treated with miR-26a or miR-30b mimics showed decreased mRNA levels of calcification-related genes. miR-26a repressed *BMP2* by 36%, alkaline phosphatase (*ALPL*) by 38%, and *SMAD1* by 26%, while miR-30b reduced the expression of *SMAD1* by 18% and of *SMAD3* by 12%. In contrast, miR-195-treated AVICs had increased mRNA levels of calcification-related genes, such as *BMP2* by 68% and *RUNX2* by 11%.

Conclusion: miR-26a, miR-30b, and miR-195 were each decreased in the aortic valves of patients requiring AVR due to AS, compared to those requiring replacement due to AI. These miRNAs appear to modulate calcification-related genes in vitro.

Aortic valve calcification/stenosis has a significant clinical impact, as it is the third-leading cause of adult heart disease (1) and the most common form of acquired valvular disease in developed countries (2). The risk factor most closely linked to calcific aortic stenosis is a bicuspid aortic valve (BAV), which is present in 1-2% of the population (2). Both, the examination of human calcified aortic valve tissue and in-vitro

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experiments using aortic valve interstitial cells (AVICs) have demonstrated the presence of several gene pathways involved in calcification, including bone morphogenetic protein-2 (BMP-2) (3,4), NOTCH1 (5-8), and the SMADs (Mothers against decapentaplegic) (3,4,9).

The microRNAs (miRNAs) are a class of highly conserved small, non-coding RNAs that have key roles in the modulation of gene expression (10). miRNAs are primarily involved in repressing mRNA transcripts by binding specific sequences in the 3'-untranslated region (3'UTR) of mRNA. There is a rapidly growing body of literature examining the role of miRNAs in cardiovascular development and disease (11-16).

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Table I: Demographics and clinical characteristics of the patients (n = 9).

Parameter	Value
Age (years)*	45 ± 13.8
Gender ratio (M:F)	9:0
Body mass index (kg/m ²)*	26.4±3.0
Reason for AVR	
Valve insufficiency	5
Valve stenosis	4
Systemic disease	
Hypertension	0
COPD	0
Aneurysm of ascending aorta (mm)*	41.3 ± 7.3

*Values are mean ± SD.

AVR: Aortic valve replacement; COPD: Chronic obstructive pulmonary disease.

While miRNA profiling has been performed in hepatocellular (17) and neck carcinoma (18,19), sepsis (20), and heart failure (21), the present authors are unaware of any reports profiling miRNAs associated with aortic valve disease, and their possible role in calcification. Since BAV predisposed to both stenosis and insufficiency (regurgitation) in the present patient cohort, the study aim was to evaluate the differentially expressed miRNAs in BAV with aortic stenosis (AS) as compared to aortic insufficiency (AI). An additional aim was to determine whether these miRNAs could alter the expression of calcification-related genes.

Materials and methods

Aortic valve collection

Aortic valve samples (fused or unfused leaflets) were collected from nine male patients (mean age 44.9 ± 13.8 years) undergoing aortic valve replacement (AVR). Preoperatively, the functional state of the aortic valve was determined by echocardiography and/or angiocardiography, according to guidelines of the American Heart Association and the American College of Cardiology (22). All of the patients examined for altered miRNA expression had type 1 bicuspid aortic valve leaflets (23), with fusion of the right and left coronary leaflets, that was confirmed at the time of AVR. The morphology of the fused and unfused leaflets was inspected at the time of surgery, after which the leaflets were collected separately and frozen in liquid nitrogen. The patient demographics are listed in Table I.

The study protocol was approved by the institutional ethics committee, and written informed consent to participate in the study was obtained from all patients.

*Table II: Sample quality control (QC).**

Microarray	Reason for AVR	Sample QC
6170041	AV insufficiency	Bad
6620037	AV insufficiency	Good
6620029	AV insufficiency	Bad
6620030	AV insufficiency	Good
6620031	AV insufficiency	Good
6620032	AV stenosis	Good
6620033	AV stenosis	Good
6620034	AV stenosis	Good
6620035	AV stenosis	Bad

*Principal component and hierarchical clustering analyses excluded three microarrays from the statistical analysis, as these arrays were outliers.

AV: Aortic valve.

RNA isolation and miRNA array

RNA was isolated from the leaflets using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The RNA from the fused leaflet was labeled with Cy5, while the unfused leaflet RNA (which served as a control) was labeled with Cy3 using the PIQOR MiRXplore protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). The samples were hybridized to PIQOR miRXplore Microarrays (Miltenyi Biotech) according to the manufacturer's recommendations.

Statistical analysis of miRNA array data

Normalization

Image files were processed using GeneSpring 7.3.1 (Agilent). The files were normalized by dividing the Cy5 channel (fused leaflet) with its matched Cy3 channel (unfused leaflet), creating a ratio. The next step in the normalization was to scale the ratios from each matched set to the 50th intensity from the matched set. This calculation was done for each matched set, such that each matched set was scaled to the most representative intensity. As there were nine matched sets in total, this procedure was performed nine times, the aim being to ensure that systematic error was removed from the data.

Assessment of group homogeneity

A principal component analysis and hierarchical clustering (Spearman correlation, average linkage) were performed using the normalized ratio data. As a common result, three samples (microarrays 6170041, 3320029, 6620035) were reported as outliers from the respective sample group. (Table II). These samples were excluded from further analysis.

Table III: miRNA microarray results.*

Name	Fold change	Regulated	p-value
hsa-miR-130A	-1.48148	Down in AVS	0.00259
hsa-miR-30B	-1.83486	Down in AVS	0.00748
hsa-miR-497	-1.58983	Down in AVS	0.0119
hsa-miR-195	-1.9802	Down in AVS	0.0158
hsa-miR-26A	-1.62075	Down in AVS	0.0265
hsa-miR-16	-1.9685	Down in AVS	0.0353
hsa-miR-27A	-1.88679	Down in AVS	0.0406

*Seven miRNAs were found to have a statistically significant decrease in AS as compared to AI valve samples. AVS: Aortic valve stenosis.

Probe quality control

The next step was to filter the data based on the signal error within the data; this removed from the statistical analysis any miRNAs that had a large amount of signal noise. This filter was applied to all of the data, and miRNA were only passed if they achieved the specified signal-to-noise ratio in all samples. This ensured that the data used in the proceeding statistical analysis were sound. The total size of the miRNA array was 1,421, which is a combination of human, murine, viral and other species miRNAs. A total of 66 miRNAs remained after applying this filter.

Statistical analysis

The remaining six samples equated to three replicates in the stenosis and insufficient groups. The data were analyzed for significant differentially expressed miRNAs between the groups. The miRNAs used in this analysis were the 66 miRNAs remaining after the signal-to-noise ratio filter. The test applied to this data was Student's *t*-test, with a p-value cut-off of 0.05. No multiple testing was applied.

qRT-PCR

The miRNA qRT-PCR was performed on the fused leaflet valve samples used in the miRNA array experiment (AI group, n = 5; AS group, n = 4) to validate the miRNA microarray results. The TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with miRNA-specific primers was used according to manufacturer's protocol. The $\Delta\Delta\text{CT}$ values were calculated, and *U6* was used as an endogenous control.

Transfection of human AVICs

Human aortic valve leaflets were harvested from recipient hearts at the time of cardiac transplant, with Institutional Review Board approval. Human AVICs were cultured according to standard protocols (24,25). Fully confluent AVICs were transfected with either 100

pM miRNA mimic (Dharmacon, Lafayette, CO, USA) or Block-It, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols (8). As a transfection control, and to determine transfection efficiency, the cells were transfected with non-functioning siRNA (Block-It, Invitrogen) (26), and the RNA isolated using Trizol after 96 h. cDNA was prepared using Superscript III (Invitrogen). qRT-PCR was performed using TaqMan primers (Applied Biosystems) for a panel of calcification-related genes. The $\Delta\Delta\text{CT}$ values were calculated, and glyceraldehyde phosphate dehydrogenase (*GADPH*) served as the control.

Statistical analysis

The statistical significance of the differences between groups was determined with the unpaired *t*-test. A p-value ≤ 0.05 was considered to be statistically significant.

Results

Reduction of miR-26a, miR-30b and miR-195 in AS samples

miRNA microarrays

In order to identify possible miRNAs (the expression levels of which were altered in BAV), a miRNA array analysis was performed. A statistical analysis of the array data showed that seven miRNAs (miR-16, miR-26a, miR-27a, miR-30b, miR-130, miR-195, and miR-497) were altered in BAV (Table III). All seven miRNAs were down-regulated in the AS group as compared to the AI group, of which three miRNAs from the same family (miR-16, miR-195, and miR-497) were found to be significant for their difference between the AS and AI phenotypes.

miRNA qRT-PCR

To validate the miRNA arrays results, miRNA qRT-PCR was performed on the fused leaflets from both groups. As the fused leaflet tends to be the predominant focus of disease (data not shown), the unfused leaflet was used as an internal control.

Both, miR-26a and miR-195 levels were reduced by 65% and 59%, respectively (p < 0.05), in the stenotic leaflets, as compared to the insufficient leaflets by qRT-PCR. Likewise, miR-30b was shown to be reduced by 62% in the stenotic samples (again using qRT-PCR), with a p-value (<0.06) that approached significance (Fig. 1). The remaining miRNAs were not altered in any statistically significant manner.

Modulation of calcification-related genes by miR-26a, miR-30b, and miR-195

In order to determine if miR-26a, miR-30b, and miR-195 have roles in modulating calcification-related

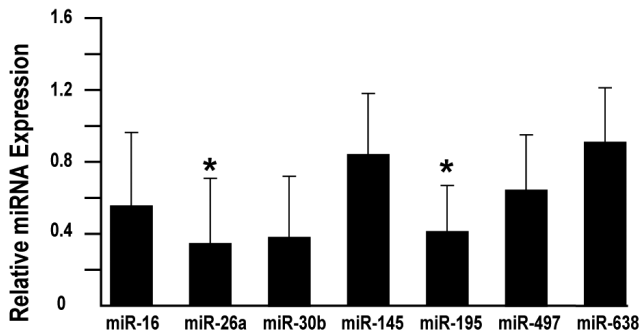


Figure 1: *miR-26a* and *miR-195* were expressed at lower levels in AS valves. QRT-PCR was performed on the AS ($n = 4$) and AI ($n = 5$) valve samples to validate the miRNA microarray results. *miR-26a* was reduced by 65% in the AS valves, while *miR-195* was decreased by 59% (* $p < 0.05$ for both). Notably, *miR-30b* was reduced by 62% ($p < 0.06$).

genes in AVICs, mimics for these miRNAs were transfected into human AVICs and the RNA was harvested for qRT-PCR after four days. A panel of calcification- and valve-related genes was examined via qRT-PCR.

miR-26a repressed several of the calcification-related genes in vitro (Fig. 2). *ALPL* mRNA levels were reduced by 38% ($p < 0.01$), *BMP2* mRNA by 36% ($p < 0.04$), and *SMAD1* by 26% ($p < 0.01$). *MiR-26a* increased the mRNA level of genes that may have roles in inhibiting calcification, including *JAG2* (31%, $p < 0.01$) and *SMAD7* (15%, $p < 0.01$). Of note, there was an increase in two genes that are considered pro-calcific; *RUNX2* was increased by 16% ($p < 0.01$) and *SMAD5* by 21% ($p < 0.01$).

miR-30b also appeared to down-regulate calcification-related gene pathways (Fig 3). *miR-30b* repressed

SMAD1 by 18% ($p < 0.02$) and *SMAD3* by 12% ($p < 0.05$). *BMP2* was repressed by 39%, with a p-value (< 0.07) that approached significance. *JAG2* was increased by 14% ($p < 0.01$) and *SMAD7* by 40% ($p < 0.02$). In contrast, *NOTCH1* expression was decreased by 19% ($p < 0.01$).

miR-195 activated calcification-related genes by *BMP2* (68%, $p < 0.01$), *RUNX2* (11%, $p < 0.01$), *SMAD1* (9%, $p < 0.04$), *SMAD3* (4%, $p < 0.02$), and *SMAD5* (17%, $p < 0.01$) (Fig. 4). Increases were also shown in the expression of *JAG2* and *SMAD7* (by 13%, $p < 0.01$ and 26%, $p < 0.01$, respectively), both of which may repress calcification.

Discussion

To the present authors' knowledge, this is the first report of altered miRNA levels in aortic valve disease, and their possible role in aortic valve calcification. Given that BAV, which is present in 1-2% of the population, is the most common risk factor for aortic valve calcification, efforts were focused on comparing miRNA levels in BAV with AS or AI. The miRNA levels in the fused leaflets were compared by using miRNA arrays and qPCR. The miRNA arrays identified seven miRNAs with differential expression in the fused leaflets of BAV, compared to the unfused leaflet. It was interesting to note that three miRNAs (*miR-16*, *miR-195*, and *miR-497*) from the same family were found to be significant for their differences between the AS and AI phenotypes. miRNA qPCR showed that both *miR-26a* and *miR-195* were decreased in AS patients as compared to AI patients, in a statistically significant manner, while *miR-30b* was down-regulat-

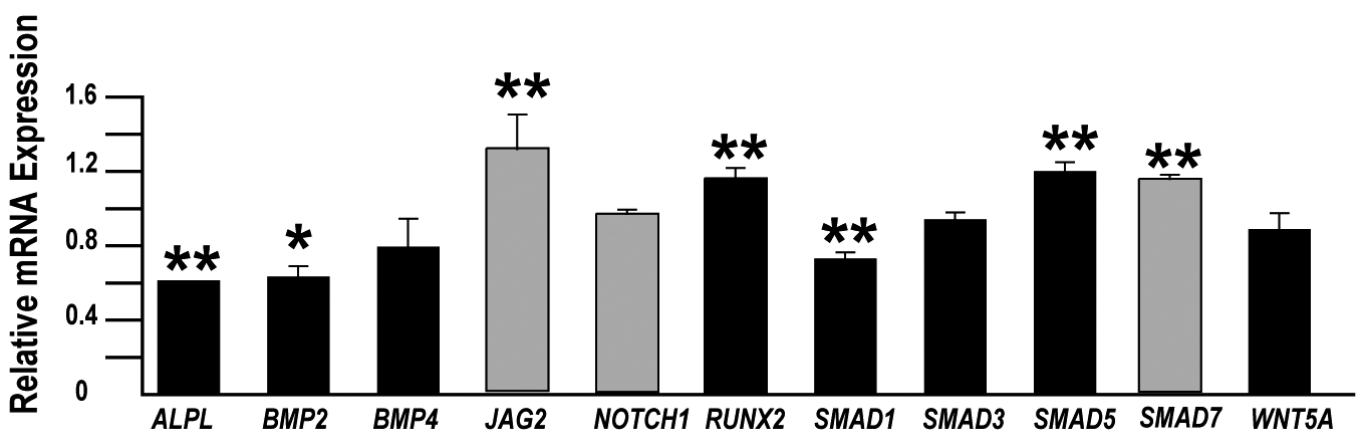


Figure 2: In human AVICs, *MiR-26a* predominantly repressed pro-calcification genes (black bars), while increasing the expression of anti-calcification genes (gray bars). Specifically, *miR-26a* treatment resulted in a decreased expression of *ALPL* (38%), *BMP2* (36%), and *SMAD1* (26%). At the same time, *JAG2* and *SMAD7* were increased by 31% and 15%, respectively, in cells treated with *miR-26a*. However, the increases in the pro-calcification genes *RUNX2* (16%) and *SMAD5* (21%) went against the trend ($n = 3$; * $p < 0.05$; ** $p < 0.01$).

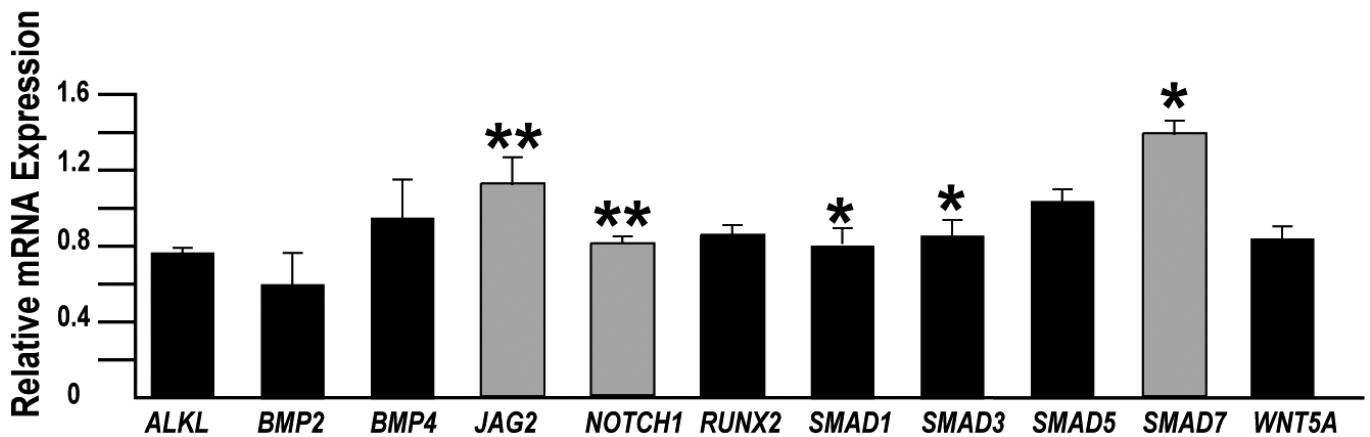


Figure 3: In human AVICs, MiR-30b predominantly repressed pro-calcification genes (black bars), while increasing the expression of anti-calcification genes (gray bars). AVICs treated with miR-30b mimic had decreased SMAD1 (18%) and SMAD3 (12%) mRNA levels. JAG2 and SMAD7, two anti-calcification genes, had 14% and 40% increases in mRNA expression, respectively. The 19% decrease in NOTCH1 expression went against the trend ($n = 3$; $*p < 0.05$; $**p < 0.01$). Of note, BMP2 was reduced by 68% ($p < 0.07$).

ed ($p = 0.06$, which approached statistical significance). It is acknowledged that there is a likelihood of additional miRNAs being altered but not detected by the microarray, since calcification is not a uniform process throughout the leaflet, and the experimental design employed examined the expression patterns in the entire leaflet. Furthermore, miRNAs may have a different role in the early pathogenesis, whereas the valve samples were obtained during the late stages of the disease process.

In order to examine the role of miR-26a, miR-195, and miR-30b on calcification-related genes, cultured human AVICs were transfected with miRNA mimics and qPCR then performed for calcification-related genes. Interestingly, it was found that miR-26a repressed pro-calcification genes such as BMP2,

RUNX2, SMAD1, and SMAD5, whilst at the same time there was an increase in the anti-calcification genes such as SMAD7 and JAG2. Indeed, JAG2, a ligand for the Notch signaling pathway, may repress aortic valve calcification via NOTCH1, which has been shown to repress aortic valve calcification (5,8). SMAD7 has inhibitory roles towards BMP signaling, and also inhibits transforming growth factor-beta (TGF- β) signaling (27,28). miR-30b has a similar pattern of repressing calcification-related genes and activating SMAD7 and JAG2, whereas MiR-195 appears to activate several pro-calcification-related genes.

The study findings indicate that these miRNAs (miR-26a, miR-195, and miR-30b) caused an in-vitro modulation of the mRNA levels of calcification-related genes. It appears that miRNAs may have a key role in

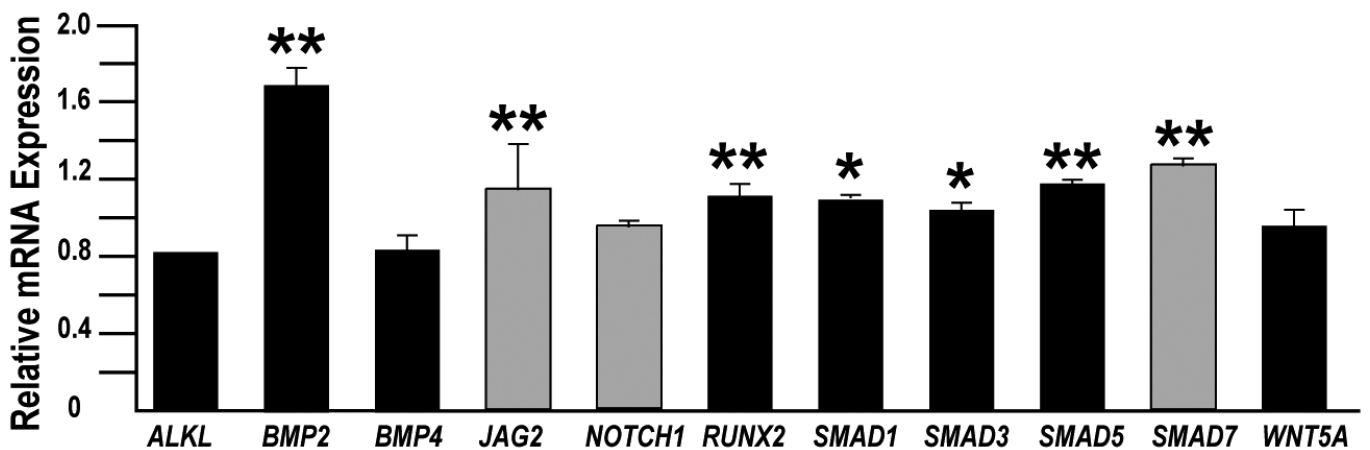


Figure 4: MiR-195 predominantly activated pro-calcification gene expression (black bars), while repressing anti-calcification genes (gray bars). BMP2 (68%), RUNX2 (11%), SMAD1 (9%), SMAD3 (4%), and SMAD5 (17%) were each increased in miR-195 treated AVICs. The increases in JAG2 (13%) and SMAD7 (26%) went against the trend ($n = 3$; $*p < 0.05$; $**p < 0.01$).

the evolving understanding of the genetic pathways involved in aortic valve calcification, and therefore merit investigation as a possible treatment modality.

For the future, studies are being developed to examine these miRNAs in a large cohort of BAVs, with different clinical phenotypes. One aspect of these studies will be to characterize the role of the miRNAs in different cell types within the aortic valve.

Study limitations

The primary limitation was the significant age difference between patients in the AI and AS groups; however, such a difference is typical because AS normally requires surgery later in life. This also raised the possibility that age is a contributing factor to the alterations in miRNA. The decreases in miR-26a and miR-30b may contribute to calcification of the aortic valve, as they modulate the genes related to calcification. Further studies will be required to determine how miRNAs are regulated in the aortic valve leaflets, including the role of aging on miRNA expression. A second limitation was that specific calcification-related genes being targeted by the miRNA could not be identified. Although attempts were made to perform *in-silico* analyses using publically available target prediction programs, none of the genes with altered expression in response to treatment with miRNA mimics had predicted binding sites for the relevant miRNA. However, as the understanding of relationships between miRNAs and their target genes continues to improve, it will most likely become easier to identify the specific calcification-related genes targeted by the miRNAs.

In conclusion, miR-26a, miR-30b, and miR-195 were reduced in the aortic valve leaflets of patients undergoing AVR as a result of calcific stenosis, as compared to those undergoing AVR due to valve insufficiency. *In vitro*, increased levels of miR-26a and miR-30b were seen to repress the calcification pathways by modulating calcification-related mRNAs, whereas miR-195 tended to activate the calcification pathways.

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