

Aortic valve disease augments vesicular microRNA-145-5p to regulate the calcification of valvular interstitial cells via cellular crosstalk

Goody, PR^{1*}, Christmann, D^{1*}, Goody, D⁶, Nehl, D¹, Becker, K², Wilhelm-Jüngling, K², Uchida³, S, Moore IV, JB⁴, S., Zimmer, S¹, Bakhtiary, F⁵, Pfeifer, A⁶, Latz, E⁷, Nickenig, G¹, Jansen, F¹, Hosen, MR,¹

* Co-first authors

Affiliations:

1. Heart Center Bonn, Molecular Cardiology, Department of Internal Medicine II, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany.
2. Endothelial Signaling and Metabolism, Institute for Cardiovascular Sciences, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany.
3. Center for RNA Medicine, Department of Clinical Medicine, Aalborg University, Frederikskaj 10B, 2., DK-2450 Copenhagen SV, Denmark.
4. The Christina Lee Brown Environment Institute, Department of Medicine, University of Louisville, 302 E Muhammad Ali Blvd, Louisville, KY 40202, USA.
5. Department of Cardiac Surgery, Heart Center Bonn, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany.
6. Institute of Pharmacology and Toxicology, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany.
7. Institute of Innate Immunity, University Hospital Bonn, University of Bonn, Venusberg-Campus 1, 53127 Bonn, Germany.

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Corresponding author:

Mohammed Rabiul Hosen, PhD

Molecular Cardiology, Heart Center Bonn

Department of Internal Medicine II

Rheinische Friedrich-Wilhelms University Bonn

Venusberg-Campus 1, 53127 Bonn

Phone: +49(0) 228-287 51487

Fax: +49(0) 228-287 51482

E-mail: hosenmr@uni-bonn.de

55 **Abstract**

56 **Rationale:** Aortic valve stenosis (AVS) is a major contributor to cardiovascular death in the elderly population
57 worldwide. MicroRNAs (miRNAs) are highly dysregulated in patients with AVS undergoing surgical aortic valve
58 replacement (SAVR). However, miRNA-dependent mechanisms regulating inflammation and calcification or
59 miRNA-mediated cell-cell crosstalk during the pathogenesis of AVS are still poorly understood. Here, we
60 explored the role of extracellular vesicles (EV)-associated *miR-145-5p*, which we showed to be highly
61 upregulated upon valvular calcification in AVS in mice and humans.

62 **Methods:** Human TaqMan miRNA arrays identified dysregulated miRNAs in aortic valve tissue explants from
63 AVS patients compared to non-calcified valvular tissue explants of patients undergoing SAVR.
64 Echocardiographic parameters were measured in association with the quantification of dysregulated miRNAs
65 in a murine AVS model. *In vitro* calcification experiments were performed to explore the effects of EV-*miR-*
66 *145-5p* on calcification and crosstalk in valvular cells. To dissect molecular miRNA signatures and their effect
67 on signaling pathways, integrated OMICS analyses were performed. RNA sequencing (RNA-seq), high-
68 throughput transcription factor (TF) and proteome arrays showed that a number of genes, miRNAs, TFs, and
69 proteins are crucial for calcification and apoptosis, which are involved in the pathogenesis of AVS.

70 **Results:** Among several miRNAs dysregulated in valve explants of AVS patients, *miR-145-5p* was the most
71 highly gender-independently dysregulated miRNA (AUC, 0.780, p-value, 0.01). MiRNA arrays utilizing patient-
72 derived- and murine aortic-stenosis samples demonstrated that the expression of *miR-145-5p* is significantly
73 upregulated and correlates positively with cardiac function based on echocardiography. *In vitro* experiments
74 confirmed that *miR-145-5p* is encapsulated into EVs and shuttled into valvular interstitial cells. Based on the
75 integrated OMICs results, *miR-145-5p* interrelates with markers of inflammation, calcification, and apoptosis.
76 *In vitro* calcification experiments demonstrated that *miR-145-5p* regulates the *ALPL* gene, a hallmark of
77 calcification in vascular and valvular cells. EV-mediated shuttling of *miR-145-5p* suppressed the expression of
78 *ZEB2*, a negative regulator of the *ALPL* gene, by binding to its 3' untranslated region to inhibit its translation,
79 thereby diminishing the calcification of target valvular interstitial cells.

80 **Conclusion:** Elevated levels of pro-calcific and pro-apoptotic EV-associated *miR-145-5p* contribute to the
81 progression of AVS via the *ZEB2-ALPL* axis, which could potentially be therapeutically targeted to minimize
82 the burden of AVS.

83 **Keywords:** aortic valve stenosis, microRNA, extracellular vesicles, cellular crosstalk, valvular calcification

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87 **Nonstandard Abbreviations and Acronyms**

88	AVS	aortic valve stenosis
89	CVD	cardiovascular disease
90	ECs	endothelial cells
91	EVs	extracellular vesicles
92	VICs	vulvular interstitial cells
93	VECs	vulvular endothelial cells
94	HCAEC	human coronary artery endothelial cell
95	LV	left ventricle
96	LVEF	left ventricular ejection fraction
97	miR	microRNA
98	miRNA	microRNA
99	NGS	next generation sequencing
100	RBP	RNA-binding protein
101	RIP	RNA immunoprecipitation
102	SAVR	surgical aortic valve replacement
103	TAVR	transcatheter aortic valve replacement
104	TEM	transmission electron microscopy

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122 **Clinical Significance**

123 **What is known?**

- 124 1. Aortic valve stenosis (AVS) is the most prevalent structural heart valve disease requiring surgical or
125 interventional valve replacement. Currently, no medical treatment option is available to slow, halt, or
126 reverse the progression of the disease.
- 127 2. AVS induces pressure overload on the left ventricle (LV), resulting in concentric hypertrophy and LV
128 dysfunction.
- 129 3. AVS is not an exclusively degenerative disease that leads to fibrosis and calcification of the valve
130 cusps but rather a chronic inflammatory disease, in which mechanical strain and shear stress lead to
131 endothelial dysfunction and immune cell infiltration, which induces chronic inflammation, apoptosis
132 and differentiation of valvular interstitial cells into osteoblast-like cells.
- 133 4. Increasing osteoblastic differentiation and the formation of macrocalcifications are hallmarks of the
134 later stages of AVS.

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136 **What is the new information we provide?**

- 137 1. During aortic valve stenosis, expression pattern of vesicle-associated regulatory miRNAs is altered.
- 138 2. Patient-derived aortic valve tissue demonstrated an increased expression of *miR-145-5p* in humans,
139 as well as in aortic valve explants from an experimental murine AVS model.
- 140 3. *MiR145-5p* contributes to calcification of the aortic valve through ZEB2, a transcriptional repressor of
141 ALPL, in valvular interstitial cells.
- 142 4. Extracellular vesicular shuttling of *miR-145-5p* contributes to valvular cell-cell crosstalk and plays a
143 role in the pathogenesis of AVS.

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148 **Introduction**

149 Aortic valve (AV) disease is a significant contributor to cardiovascular death worldwide and shows a
150 prevalence of over 2% in cardiovascular patients over 60 years of age¹. The 2-year mortality rate is greater
151 than 50% when symptoms of severe aortic valve stenosis (AVS) are manifest, including dyspnoea, angina
152 pectoris, or cardiac syncope¹⁻². While AVS is in part a degenerative disease, with “wear and tear” driving
153 pathological fibrosis and calcification of the valve cusps, chronic inflammation is also a significant contributor
154 to disease pathogenesis¹⁻². AVS can be divided into distinct phases. Initial endothelial damage, due to
155 mechanical and shear stress, leads to infiltration of phospholipids (PL) and low-density lipoprotein particles
156 (LDL). PL and LDL can be oxidized in the valve cusps, creating a pro-inflammatory milieu. Infiltration by
157 monocytes and T-cells characterizes the initiation phase of the disease. Pro-inflammatory cytokines, secreted
158 by classically activated macrophages and CD-8⁺ T-cells can induce apoptosis and differentiation of valvular
159 interstitial cells (VIC) into osteoblast-like cells³⁻⁴. Arising cell debris acts as a further promotor of inflammation
160 and can function as an initiator for microcalcification. Increasing osteoblastic differentiation and the formation
161 of macrocalcification are indicative of the next phase of AVS, termed the propagation phase. Currently, no
162 pharmacological treatments targeting either phase of the disease are available, and the only treatment option
163 is surgical or interventional valve replacement (SAVR or TAVR)⁵⁻⁷.

164 MicroRNAs (miRNAs) are small noncoding RNAs that are involved in cardiovascular diseases⁷⁻⁸. When
165 compared to healthy valves, miRNAs have been shown to be differentially expressed in aortic valve tissues from
166 patients undergoing valve replacement surgery due to AVS^{Ref}. MiRNAs can also be packaged into extracellular
167 vesicles (EV) to be transferred between different cells⁸⁻¹⁰. This phenomenon has been investigated in many
168 diseases, including different types of cancer and in atherosclerosis¹¹⁻¹³. We and others have demonstrated
169 miRNA transfer between endothelial cells (EC), cardiomyocytes (CM), and smooth muscle cells (SMC), leading
170 to direct genetic and phenotypic effects on target cells^{8,14}. Recently, we showed that an increase in EV-*miR-*
171 *122-5p* in patients with AVS represents a novel mechanism for the deterioration of cardiac function in patients
172 following TAVR⁸. EVs harbor *miR-122-5p* and facilitate its shuttling into CM by direct interaction with a
173 multifunctional RNA-binding protein (RBP), heterogeneous nuclear ribonucleoprotein U (hnRNPU), to regulate
174 the viability of CM⁸. Furthermore, vesicular shuttling of *miR-30c-5p* is regulated by hnRNPU in a sequence-
175 specific manner, which controls EC function and is augmented in CAD¹⁴. EVs have also been shown to play a
176 role during AVS, with released EVs from VICs and macrophages acting as crystallization sites for
177 microcalcification¹⁵⁻¹⁸. However, the horizontal transfer of vesicle-bound contents (e.g. miRNAs, proteins) and its
178 effect on valvular calcification have not been well investigated.

179 The aim of our study was to explore whether tissue-resident miRNA content differ between patients with and

180 without AVS. Further, we examined whether vesicular RNA contents are distinctive and how such differences
181 impact disease progression through cell-cell communication via EVs. We demonstrated for the first time that
182 vesicular *miR-145-5p* is upregulated in clinical and experimental settings of valvular calcification (i.e in AVS
183 patients, our murine AVS model, and during *in vitro* calcification). Further functional and mechanistic studies
184 revealed that EV-*miR-145-5p* is an important regulator of valvular calcification and osteoblastic differentiation of
185 intestinal cells via vesicular shuttling.

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187 **Methods**

188 A detailed methods section is provided in the online Data Supplement.

189 **Study approval and Human specimen**

190 All clinical samples and measurements were obtained after informed consent from patients following ethical
191 approval by the ethics committee of the University of Bonn (approval number: AZ78/17). Aortic valve specimens
192 were collected from patients undergoing SAVR for either severe aortic stenosis or aortic regurgitation in
193 cooperation with the Department of Heart Surgery, University Hospital Bonn, Germany.

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195 **Data availability**

196 MiRNA array data are available from the Gene Expression Omnibus (GEO) under the accession number:
197 GSE1905689. RNA-seq data are available from the GEO under the accession number GSE190539. The raw
198 data of proteome and transcription factor arrays are provided as online data supplements. All further data that
199 support the findings of this manuscript are available upon request from the corresponding author.

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201 **Statistical analysis**

202 Normally distributed continuous variables were presented as the mean \pm standard deviation (SD). Continuous
203 variables were tested for normal distribution using the Kolmogorov–Smirnov test. Categorical variables are
204 given as frequencies and percentages. For continuous variables, the two-tail, unpaired Student t-test or Mann–
205 Whitney U test were used for the comparison between the two groups. For the comparison of >2 groups, the
206 one-way ANOVA with Bonferroni correction for multiple comparisons test was used. All tests were two-sided.
207 Statistical significance was assumed when the null hypothesis could be rejected at $p < 0.05$. Statistical analysis
208 was performed with IBM SPSS Statistics version 20 (IBM Incorporation, USA) and GraphPad Prism 9
209 (GraphPad Inc, USA).

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212 Results

213 Baseline characteristics and identification of differentially regulated miRNAs in valve tissue from AVS 214 patients post-SAVR

215 To identify differentially expressed miRNAs in valve tissues, the AVS patients were characterized. Baseline
216 characteristics indicated that comorbidities influencing the prognosis after SAVR [e.g., pulmonary disease and
217 coronary artery disease (CAD)] were similar between the groups (AVS vs. AI). Of note, other cardiovascular risk
218 factors such as type-II diabetes (Type II DM), body mass index (BMI), dyslipidemia, smoking history, as well as
219 creatinine levels, were noted to be elevated in patients with AVS (Table 1). To identify differentially expressed
220 miRNAs in valve tissue explants from AVS patients, we performed an unbiased RT-qPCR-based human miRNA
221 array in the screening cohort (Figure 1A-B, Figure S1A). Explanted valves from patients with aortic insufficiency
222 (AI, no AVS) due to dilatation of the aortic root or ascending aorta served as controls when valve cusps did not
223 exhibit signs of calcification. Explanted valves from AVS patients displayed calcifications, increased collagen
224 deposition, elastin, and fibrin in aortic valve tissue sections stained with alizarin red staining followed by light-
225 microscopy imaging (Figure 1A). Interestingly, our array data revealed several miRNAs (*miR-145-5p*, *miR-let-7b*,
226 *miR-1201*, *miR-145-3p*, *miR-29b*, *miR-126-3p*, *miR-29c*, *miR-126-5p*, *miR-133b*, *miR-518d*, *miR-127-5p*, and
227 *miR-143-5p*) (Figure 1B-C) to be differentially expressed between AVS patients and controls after SAVR
228 [Threshold values: |fold change| >1.5 and p-value <0.05 (FDR-adjusted)]. Of these differentially expressed
229 miRNAs, *miRNA-145-5p* exhibited a more than 3-fold (p=0.001) increase in AVS tissues compared to those
230 sourced from control patients (no-AVS). Said findings were recapitulated in a validation cohort via RT-qPCR,
231 wherein *miR-145-5p* was significantly upregulated in AVS patients relative to controls (n=25 and n=10,
232 respectively; p=0.01) (Figure 1D). The increased expression of *miR-145-5p* was independent of sex, with no
233 significant difference being observed between male and female AVS patients in this validation cohort (Figure
234 1E).

235 Among the differentially expressed miRNAs, only *miR-145-5p* showed a significant diagnostic value in AVS
236 patients when a ROC analysis was performed. This analysis suggested that *miR-145-5p* [AVS vs. controls] was
237 a reliable predictor of the pathogenesis of disease (AUC=0.780, p=0.01; Figure 1F). We found that a
238 significantly higher percentage of AVS patients demonstrated increased *miR-145-5p* expression. In addition, a
239 correlation analysis of *miR-145-5p* expression with the level of calcification from AVS to controls suggests that
240 *miR-145-5p* is a critical regulator of the calcification state of AVS patients (with a Spearman coefficient of r=
241 0.25344, p=0.0156) (Figure 1G).

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244 ***MiR-145-5p* is highly upregulated in both human stenotic aortic valves and the murine AVS model**

245 Most miRNAs are evolutionarily conserved, which helps in understanding their functions using model animals
246 such as mice. To screen for species-conserved miRNAs in diseased aortic valve tissues, RT-qPCR-based
247 miRNA arrays were performed using human (calcified tissue vs. control) and murine aortic valve tissue (wire
248 injury vs. sham). Among eleven miRNAs that were shown to be differentially regulated (Figure 1H), *miR-145-5p*
249 was expressed highly in both humans and mice. Based on the above-mentioned data, we sought to investigate
250 the functional importance and molecular mechanism of *miR-145-5p* in AVS.

251 To further investigate the role of *miR-145-5p* in AVS, we employed a graded wire-injury model of AVS in mice
252 (Figure 2A). A wire-induced aortic valve injury led to the development of severe stenosis, as demonstrated by
253 elevated blood flow velocities four weeks after the operation (Figure 2A-B). The ejection fraction (EF) and
254 cardiac output were inversely regulated in these mice, suggesting that there is an AVS-induced decrease of EF
255 (Figure 2C-D) in comparison to baseline (when compared to sham-operated mice). RT-qPCR analysis revealed
256 that *miR-145-5p* was highly upregulated in mice with AVS (Figure 2E).

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258 ***MiR-145-5p* expression in interstitial cells is higher than endothelial cells in valve tissues**

259 To elucidate the mechanism of action of *miR-145-5p*, we established several *in vitro* culture models. We
260 established an improved isolation protocol for valvular endothelial cells (VECs) and valvular interstitial cells
261 (VICs) from human calcified and non-calcified AV tissues explanted during SAVR (Figure 3A). In brief, human
262 VICs and VECs (patVIC and patVEC) were isolated from explanted AVs using multiple steps of collagenase
263 digestion and CD105 (Endoglin) Magnetic Activated Cell Sorting (MACS) for EC purity (Figure 2B). We further
264 obtained human VICs and VECs (hVIC and hVEC) from a healthy young donor who died from a non-
265 cardiovascular-related event. Patient-derived and commercially available cells were characterized via the
266 expression of different endothelial and interstitial cell markers via qRT-PCR. A comparison of characteristic
267 marker expression in these cells was performed (Figure S2A-D) to confirm cellular identity of isolated and
268 commercially available valve cells. Immunofluorescence staining for prototypical endothelial and interstitial cell
269 markers and gene expression were performed by qRT-PCR (Figure 3B-E, Figure S2A-D). PatVICs and hVICs
270 stained positively for α -SMA, which was also further confirmed by qRT-PCR of these cells (Figure 3B-E). In
271 contrast, patVECs and hVECs showed high expression levels of endothelial markers, such as vWF, PECAM1,
272 and CDH5 (Figure S2A-D), thus further confirming that our MACS-based isolation was valid and reproducible.

273 To investigate the cell-specific expression of *miR-145-5p* isolated from AV-tissues after SAVR, we quantified
274 *miR-145-5p* expression in VECs and VICs. Our qRT-PCR quantification revealed that VICs express a
275 significantly higher level of *miR-145-5p* in comparison to VECs (Figure 3F) suggesting that *miR-145-5p* may

276 play a role in the pathogenesis of AVS in mice and humans through its expression in specific valve cells.

277

278 **AVS increases the level of *miR-145-5p* in aortic valve tissue and EVs derived from plasma of patients**

279 Recent studies have shown that different extracellular vesicle (EV) populations (e.g. exosomes or small EVs,
280 large EVs, apoptotic bodies) act as vehicles to transfer short or long RNAs into nearby or distant recipient cells
281 and play a role in cellular crosstalk^{8,14}. Encapsulation of miRNAs into small or large EVs provide dramatic
282 resistance to blood- or tissue-resident exonucleases. MiRNAs can also be secreted bound to LDLs (low-
283 density lipoproteins, HDL (high-density lipoproteins), and RBPs (e.g., argonaute, hnRNPU, hnRNP A2B1,
284 hnRNPK, HuR)¹⁴⁻¹⁵.

285 The characterization of EVs derived from AV tissue or plasma was performed according to the current
286 guidelines of the International Society of Extracellular Vesicles (ISEV) using nanoparticle tracking analysis
287 (NTA), transmission electron microscopy (TEM), and immunoblotting for vesicular markers, including
288 tetraspanins (Flotillin, CD63, CD81) (Figure 4B-D).

289 To identify the mode of transport of *miR-145-5p*, we determined the plasma component of AVS-patients in
290 which *miR-145-5p* may be detected. After isolation of large EVs, small vesicles (also known as exosomes),
291 and vesicle-free plasma (Figure S3A)^{8, 14, 25-26}, the large EV population (170-800 nm) isolated from aortic valve
292 tissues explanted from patients with AVS after SAVR demonstrated a significantly higher level of *miR-145-5p*
293 expression in comparison to control (vesicle-free plasma) (Figure 4A), suggesting that *miR-145-5p* is
294 contained in large EVs. To verify *miR-145-5p* secretion and transport in large EVs, a vesicle-RNA degradation
295 assay was performed (Figure 4E-F). We found that proteinase K digestion before treatment with RNase did
296 not affect *miR-145-5p* levels. In contrast, treatment with Triton X-100, which acts as a detergent to disrupt the
297 phospholipid membrane of vesicles, before treatment with RNase led to near-complete degradation of *miR-*
298 *145-5p*. These findings indicate that extracellular *miR-145-5p* may be incorporated into large EVs, protecting
299 the RNA from resident or circulating nucleases. Altogether, these data suggest that extracellular *miR-145-5p* is
300 predominantly encapsulated and secreted in large EVs.

301

302 **Vesicular shuttling augments the expression level of *miR-145-5p* in recipient valvular interstitial cells**

303 As dysfunction of VICs is crucial to the development of AVS in the murine AVS model and human, we explored
304 whether VICs can transfer *miR-145-5p* into target VICs (other neighboring VICs) and established a co-culture
305 model of donor and recipient VICs to investigate intercellular communication via EVs. After transfecting VICs
306 with *miR-145-5p* mimic, large EVs were isolated and incubated with target VICs, revealing that *miR-145-5p* is
307 upregulated in target VICs in both control and mimic-transfected cells (Figure 4G). This result suggests that

308 horizontal EV-mediated cell-to-cell transfer of *miR-145-5p* increases the levels of *miR-145-5p* in target VICs.
309 To examine whether the intercellular transfer of *miR-145-5p* occurs via shuttling by large EVs in a paracrine
310 manner, fluorescently labeled large EVs were isolated from VICs and incubated with acceptor VICs. The results
311 show that the fluorescently labeled EVs can be internalized into recipient VICs (Figure S3B). Furthermore, large
312 EVs isolated from donor VICs transfected with fluorescently labeled *miR-145-5p* confirmed the uptake of EV-
313 *miR-145-5p* into recipient VICs (Figure 4H), indicating that EV-mediated shuttling of *miR-145-5p* could act as an
314 intercellular mediator of calcification within the aortic valve during stenosis progression.

315 316 ***MiR-145-5p* regulates pro-calcification marker genes in VICs**

317 Recent studies have reported that miRNAs can influence the calcification process in vascular and valvular
318 cells via regulation of pro-calcific genes or proteins³¹⁻³². To examine whether the calcification of VICs is
319 mediated by *miR-145-5p*, we performed *in vitro* calcification experiments. The level of calcification was
320 assessed by utilizing alizarin red staining to quantify matrix calcium on deposition (Figure 5A). To further
321 elucidate any direct involvement of *miR-145-5p* during calcification of VICs, we proceeded to assess the
322 expression of calcification marker genes via qRT-PCR in cells treated with either a *miR-145-5p* mimic or
323 inhibitor (Figure 5B, Figure S4A-B). Interestingly, upon silencing of *miR-145-5p*, downregulation of calcific
324 genes, including alkaline phosphatase, biomineralization associated (*ALPL*), zinc finger E-box binding
325 homeobox 2 (*ZEB2*), RUNX family transcription factor 2 (*RUNX2*), secreted phosphoprotein 1 (*SPP1*), matrix
326 Gla protein (*MGP*), KLF transcription factor 4 (*KLF4*), and SMAD Family Member 5 (*SMAD5*), was observed.
327 These data suggest that *miR-145-5p* could regulate key genes in calcification processes that can be partly
328 abrogated by overexpression of *miR-145-5p* (Figure S4A-B).

329 Among the dysregulated genes involved in calcification processes, *ALPL* was observed to be upregulated
330 upon induction of calcification in *miR-145-5p* overexpressed VICs (Figure S4B-C). The inverse correlation
331 between *miR-145-5p* and *ALPL* was further confirmed when VICs were incubated with osteogenic medium
332 (OM) to induce calcification (Figure 5C), indicating that *miR-145-5p* regulates the *ALPL* expression in a dose-
333 dependent manner (Figure S4C). To examine whether *miR-145-5p* regulates the transcription of the *ALPL* or
334 ALP activity itself, we quantified alkaline phosphatase activity upon transfection of VICs with mimic and
335 inhibitors followed by induction of calcification *in vitro*. Upon knockdown of *miR-145-5p* in VICs, we observed
336 reduced enzymatic activity of ALP (Figure 5D), suggesting that *miR-145-5p* may modulate the expression of
337 *ALPL*, thus diminishing ALP activity.

338

339

340 ***MiR-145-5p* may regulate calcification of VICs through ZEB2 and tissue nonspecific alkaline**
341 **phosphatase (*ALPL*)**

342 Several studies revealed that *ALPL* transcription can be regulated by numerous transcription factors (TFs). For
343 example, *ZEB2* is a known repressor of the *ALPL* and was reported to be increased in healthy heart tissues,
344 including AV tissue³⁰⁻³². Given that *miR-145-5p* was reported to bind to *ZEB2* to regulate cellular gene
345 expression during epithelial-mesenchymal transition (EMT) in metastasis⁴², we sought to further characterize its
346 regulation by *miR-145-5p*. To study whether *ZEB2* has binding sites for *miR-145-5p*, we analyzed the genomic
347 architecture of the *ZEB2*. Interestingly, genomic analysis and target prediction of *miR-145-5p* binding
348 demonstrated that the *ZEB2* contains potential binding sites for numerous miRNAs, including *miR-145-5p*, 3290
349 bp upstream of the first two exons in its 3'-UTR (untranslated region). This suggests that *miR-145-5p* may bind
350 to *ZEB2* (Figure 5E), consequently regulating the expression of its cognate *ALPL* mRNA and thus protein
351 synthesis.

352 Given that *ALPL* is a central player in calcification²⁹⁻³², we sought to further characterize its regulation by *miR-*
353 *145-5p*. We reciprocally quantified the expression of *ALPL* and its transcriptional repressor, *ZEB2*, in the same
354 experiments upon knockdown of *miR-145-5p*. The induction of calcification by osteogenic medium showed that
355 the expressions of *ZEB2* and *ALPL* are inversely correlated, an effect also seen upon induction of *ZEB2* by
356 BMP (bone morphogenic proteins) (Figure 5F), suggesting that *miR-145-5p* regulates the transcription of the
357 *ALPL* gene via its transcriptional repressor *ZEB2* by binding to the 3'-UTR of *ZEB2*. To examine whether this
358 regulation has any effect on the cellular function of VICs, we performed experiments focusing on cellular viability
359 and functions; namely, the activation of caspase 3/7 for apoptosis, cell viability via MTT reduction, and cell
360 migration via scratch-wound healing assays upon silencing or overexpressing *miR-145-5p*. The results revealed
361 that *miR-145-5p* can regulate apoptosis and viability of VICs as a pro-apoptotic miRNA (Figure 5G-I). Taken
362 together, our data suggest that *miR-145-5p* exerts a pro-apoptotic effect that is a prerequisite of the initiation of
363 valvular calcification in AVS.

364

365 **RNA sequencing reveals that *in vitro* calcification alters calcific gene and miRNA expression profiles in**
366 **valvular interstitial cells**

367 To assess calcification-induced gene expression signatures in the VICs, we performed bulk RNA sequencing of
368 calcified VICs upon incubation with OM for 7 days and controls (without OM), resulting in a robust differential
369 expression of pro-calcific markers, e.g., *ADAMS18*, *RUNX2*, *ALPL*, *COL11A1*, and *MMP13* (Figure 6A). We
370 further analyzed the miRNA expression profile of these VICs and found *miR-145-5p* among the dysregulated
371 miRs (Figure 6B), providing further confirmation of our *in vitro* and *in vivo* data, which identify *miR-145-5p* to be

372 involved in the calcification process of VICs. Further bioinformatics analysis via DAVID (Database for
373 Annotation, Visualization and Integrated Discovery) and KEGG (Kyoto Encyclopedia of Genes and Genomes)
374 revealed that induced pathways involve osteogenesis and apoptosis among the top-regulated pathways,
375 providing further evidence of the calcification potential of VICs (Figure S5A).

376

377 ***MiR-145-5p* may exert its function via the *miR-145-5p*-ZEB2-ALPL axis in calcific valvular interstitial cells**

378 To gain further insight into cellular pathways related to calcification regulated by *miR-145-5p*, an unbiased high
379 throughput pathway reporter array for transcription factor activity was performed. VICs were either transfected
380 with mimic or inhibitor of *miR145-5p* and control. The cells were then transfected with two plasmids, one
381 carrying a firefly luciferase downstream of a transcription factor binding site and a renilla luciferase under the
382 control of a CMV promoter as transfection control. After 24 h, transcription factor activity was calculated by
383 luminescence measurements and normalized with control (Figure 6C, Figure S5B). Among the highly active
384 transcription factors, *NFkB* (*Nuclear Factor Kappa B Subunit 1*), *STAT1* (*Signal Transducer And Activator Of*
385 *Transcription 1*), *STAT3* (*Signal Transducer And Activator Of Transcription 3*), *P53* (*cellular tumor antigen p53*)
386 were dysregulated in the reporter array analysis (Figure 6C, Figure S5B, Table S1).

387 To examine how *miR-145-5p* regulates apoptosis in a more unbiased manner, we performed a proteome
388 profiler assay with antibodies against 35 apoptosis-related proteins with VIC lysates after knockdown of *miR*-
389 *145-5p* and or control (Figure 6D, Figure S5C, Table S2). Interestingly, several pro-apoptotic proteins (pro-
390 caspase-3, cleaved caspase-3, fas/TNFRSF6, p53, etc.) were increased, whereas antiapoptotic proteins
391 (hsp27, catalase, HIF-1 α , etc.) were decreased. The dysregulation of these apoptotic proteins upon *miR-145*-
392 *5p* knockdown suggests that miR-145-5p regulates viability and apoptosis of VICs during calcification (Figure
393 6D). These data are in line with our pathway analysis and reporter arrays, as these proteins are known to
394 modulate different stages of calcification. Finally, we assessed whether overexpression or knockdown of *miR*-
395 *145-5p* supports our data and whether overexpression of *miR-145-5p* rescues the pro-apoptotic/calcific effect
396 of ZEB2 and ALPL. We repeated calcification in VICs upon overexpression and knockdown of *miR-145-5p*
397 followed by alizarin red staining and found that overexpression of *miR-145-5p* directly increases calcification
398 (Figure 6E). As expected, when the expression of ZEB2 protein was quantified via immunoblotting with *miR*-
399 *145-5p* mimic and siRNA against ZEB2, we found reduced expression of ZEB2 in mimic- and siZEB2-treated
400 VICs, whereas the expression was increased in inhibitor-treated VICs, suggesting that *miR-145-5p* directly
401 regulates ZEB2, which acts on *ALPL* expression as a transcriptional repressor (Figure 6F). To determine the
402 involvement of miR-145-5p-ZEB2-ALPL as a mediator of the calcification process in VICs, we further quantified
403 mRNA expression, which supports our finding that overexpression of *miR-145-5p* suppresses ZEB2,

404 augmenting the expression of *ALPL* in VICs (Figure 6G).

405 Taken together, the above-mentioned data demonstrate that regulation of *ZEB2-ALPL* by *miR-145-5p* triggers
406 apoptosis and calcification of VICs and that the level of expression of *miR-145-5p* is important for this
407 mechanism, which acts in a *miR-145-5p-ZEB2-ALPL*-dependent manner (Figure 7). Overexpression of *miR-*
408 *145-5p* in the AV induces inflammation, calcification, and apoptosis leading to stiffening of the aortic valve
409 cusps and narrowing of the aortic valve orifice, ultimately inducing pressure overload of the LV and valvular
410 heart failure.

411

412 **Discussion**

413 The data from the present study demonstrate a newly identified role for EV-miRNAs that are differentially
414 expressed and associated with calcification in AVS patients. *Mir-145-5p* expression is correlated with
415 calcification of AV tissues explanted from patients undergoing SAVR due to severe AVS and was found to
416 have predictive value for calcification in AVS. Based on the clinical findings of our study, we have further
417 described and explored the functional role of *miR-145-5p* in valvular cells in clinical and in pathological murine
418 models. Of note, the level of *miR-145-5p* was found to be higher in VICs isolated from aortic valve tissue
419 explants from aortic stenosis patients/AVS mice compared to control patients/sham-operated mice and was
420 released into the circulation in a vesicle-associated form. Mechanistically, *miR-145-5p* interferes with the
421 translation of *ZEB2* mRNA by binding to its 3'-UTR. Since it is a negative regulator of the *ALPL* gene, reduction
422 of *ZEB2* upregulates *ALPL* expression, resulting in calcification of VICs. The effects triggered by *miR-145-5p* *in*
423 *vitro*, such as decreasing viability and migration, increasing inflammation, calcification and simultaneously
424 inducing apoptosis are regarded as prerequisites for the initiation of AVS pathogenesis.

425 EV-mediated cell-cell crosstalk is gaining more interest in the scientific community due to its implications for
426 non-invasive diagnostics and therapeutic potential. Circulating EVs can be used as biomarkers of disease, as
427 has been shown in various types of cancer and also in cardiovascular diseases, such as atherosclerosis and
428 vascular calcification¹⁹⁻²⁴. EVs can be secreted by a variety of cells relevant to cardiovascular diseases,
429 including ECs, platelets, and immune cells. Higher levels of circulating EVs can be observed in patients with
430 classical cardiovascular risk factors such as smoking, hypertension, *diabetes mellitus* and dyslipidemia, and in
431 patients with coronary artery disease (CAD)²⁵. Increased levels of circulating endothelium-derived EVs
432 correlate with higher rates of major adverse cardiovascular and cerebral events (MACCE) in patients with
433 stable CAD²⁵⁻²⁸. Not only EV levels, but also more importantly, their cargo composition can predict and
434 influence cardiovascular events. One of the first studies to demonstrate this showed that higher levels of EV-
435 bound miR-126 and miR-199a inversely correlate with MACCE and revascularization-free survival in a patient

436 cohort with stable CAD²⁸. These observations gave rise to several mechanistic studies, demonstrating the
437 beneficial and detrimental effects of EVs and their cargo on EC and smooth muscle cells (SMC) in the course
438 of atherosclerosis. Transferred EV-incorporated miRNAs are involved in endothelial regeneration, SMC
439 phenotype switching, osteoblastic differentiation, and vascular calcification²⁹. Under calcifying conditions, EVs
440 can be packaged with miRNAs targeting mRNAs for proteins actively involved in osteoblastic differentiation
441 and calcification by a variety of cell types, including SMCs, ECs, and macrophages, demonstrating their
442 important role in vascular calcification processes²⁹⁻³³. Recently, we demonstrated that EV-incorporated *miR-*
443 *122-5p* post-transcriptionally represses *BCL2*, an anti-apoptotic gene, which is central to cell viability and
444 apoptosis⁸. The levels of circulating EV-bound *miR-122-5p* were found to be an indicator of heart function in
445 patients with low or no LVEF improvement after TAVR⁸. EV-incorporated miRNAs have the potential to bind
446 mRNAs that encode for master regulators of osteoblastic differentiation and calcification such as
447 *RUNX2 (Runt-related transcription factor 2)* and *ALPL (Alkaline-phosphatase)*. However, the involvement of
448 EV-bound miRNA cargoes in valvular calcification remains unknown.

449 *MiR-145-5p* belongs to the miR-143/145 cluster of miRNAs, which was reported to be dysregulated (either as
450 a cluster, or one of its members) during essential hypertension, atherosclerosis, CAD, and pulmonary arterial
451 hypertension (PAH)³⁴⁻³⁶. Patients with stable CAD show lower levels of circulating *miR-145-5p* than healthy
452 controls, but patients with unstable angina display elevated levels and *miR-145-5p* levels correlate with infarct
453 size during myocardial infarction. Furthermore, *miR-145-5p* can be transferred between endothelial and
454 vascular SMCs via EVs³⁴. Mechanistically, physiological laminar flow induces *miR-145-5p* expression in ECs
455 in a KLF2-dependent manner, which leads to packaging into sEVs and transfers to vascular SMCs, where
456 *miR-145-5p* induces an atheroprotective vascular SMC phenotype³⁴. During myocardial infarction, *miR-145-5p*
457 also seems to exert a protective effect by inhibiting apoptosis via the Akt3/mTOR signaling pathway³⁷⁻³⁹. In
458 contrast, the miR-143/145 cluster was found to be upregulated in symptomatic atherosclerotic carotid plaques,
459 when compared to asymptomatic controls, suggesting a role in plaque destabilization³⁹. On the other hand,
460 *miR-145-5p* overexpression in ApoE^{-/-} mice via lentiviral vectors was able to reduce aortic atherosclerotic
461 plaque size. These stabilized plaques displayed an increased fibrous cap, more collagen content, and fewer
462 pro-inflammatory macrophages than the plaques from untreated littermates³⁹. In patients with PAH, miR-143
463 and miR-145 levels were found to be higher in pulmonary arterial SMCs (PASMCs) than from healthy
464 controls. *In vitro* experiments demonstrated that *miR-145-5p* influenced PASMC migration and apoptosis,
465 while *miR-145-5p* knockdown ameliorated the development of PAH in a hypoxia induced PAH mouse model *in*
466 vivo⁴⁰⁻⁴¹. Recently, miR-143 was shown to promote valvular calcification by inhibiting matrix Gla protein,
467 (MGP), which itself inhibits calcification and is necessary for valve homeostasis²⁰. Thus, the miR-143/145

468 cluster appears to have multiple roles in the cardiovascular system, ranging from protective to harmful.

469 *MiR-145-5p* had not previously been investigated in the pathology of AVS. To further validate the involvement

470 of this miRNA in AVS, we examined the AV of mice that had undergone wire-induced injury of the AVs and

471 developed severe AVS. In line with the patient data, we observed an upregulation of *miR-145-5p* in the AV

472 tissue of diseased mice. Since our initial screenings were performed on whole human tissue samples, we

473 sought to identify the cell type that might be responsible for this increase. MiRNA analysis of primary cells from

474 human tissue explants demonstrated a significantly higher expression of *miR-145-5p* in VICs than VECs,

475 suggesting a role during calcification and osteoblastic differentiation. To investigate the function of *miR-145-5p*

476 in VICs, we analyzed its expression *in vitro* under calcifying conditions. A so-called osteogenic medium induces

477 calcification and osteoblastic differentiation of VICs in an ALPL-dependent manner. In our *in vitro* experiments,

478 *miR-145-5p* as well as ALPL levels were significantly upregulated under calcifying stimuli. *In silico* target

479 prediction revealed a specific *miR-145-5p* binding site in the 3'UTR region of ZEB2/SIP1. Of note, the binding

480 of *miR-145-5p* to this binding site in ZEB2 has already been confirmed by a luciferase promoter assay in

481 another experimental setting⁴². Importantly, ZEB2 has been shown to be a transcriptional repressor of ALPL⁴².

482 The qRT-PCR analysis confirmed that ZEB2 expression is decreased under calcifying conditions and

483 negatively correlates with *miR-145-5p* and ALPL mRNA levels *in vitro*. To further elucidate the *miR-145-*

484 *5p/ZEB2/ALPL* axis, we overexpressed *miR-145-5p* in VICs, which led to a significant downregulation of ZEB2

485 and a consecutive upregulation of ALPL. Furthermore, cells that were simultaneously treated with OM and

486 *miR-145-5p* mimic showed an increased ALPL expression, while *miR-145-5p* ablation diminished ALPL in OM

487 incubated cells, thus indicating an important role of *miR-145-5p* in the modulation of ALPL-dependent

488 calcification.

489 Since we observed higher levels of *miR-145-5p* in AV tissue- and blood-derived large EVs (IEVs) from AVS

490 patients, we sought to further investigate the transfer of *miR-145-5p* between VICs via EVs. Fluorescence

491 microscopy and copy number experiments confirmed the uptake of PKH26-labelled EVs (a lipophilic

492 membrane dye), fluorescently labeled-*miR-145-5p*, and unlabeled *miR-145-5p* containing EVs by target cells,

493 demonstrating intercellular communication between VICs via EV-bound *miR-145-5p*. The transfer of miRNA

494 cargo between valvular cells might therefore play an important role in AVS initiation and progression. The

495 herein identified regulatory *miR-145-5p-ZEB2-ALPL* axis presents a potential target for the development of

496 new, RNA-based therapies. Furthermore, *in vivo* experiments utilizing EV-incorporated *miR-145-5p* mimics

497 and inhibitors will lead to a better understanding of the involved mechanisms and suggest possible therapeutic

498 strategies. Taken altogether, in this study, using unbiased miRNA profiling, we have identified significantly

499 dysregulated miRNAs in AV tissue from patients with AVS. Among several miRNAs that are commonly

500 dysregulated in mice and humans under AVS, *miR-145-5p* levels were most significantly upregulated and
501 selected for further validation in a larger patient cohort, which subsequently confirmed its expression signature
502 in AVS patients. Interestingly, *miR-145-5p* levels in tissue-derived and circulating large EVs isolated from AVS
503 patients were also higher when compared to control (no AVS) patients. By RNA-sequencing, high-throughput
504 TF array, and proteome arrays utilizing our *in vitro* calcification model of VICs, we revealed that *miR-145-5p*
505 regulates a key process in calcification, i.e. inhibition of ZEB2, a DNA-binding transcription factor that
506 regulates transcription and translation of the ALPL protein, regarded as a hallmark of calcification in valvular
507 and vascular calcification. However, these conclusions from *in vitro* experiments are based on transient
508 overexpression or inhibition of *miR-145-5p* and therefore do not allow us to firmly conclude that *miR-145-5p* is
509 an indispensable factor to promote direct calcification processes in the AV. Importantly, *miR-145-5p* is highly
510 overexpressed in mice and human calcified AV tissues and silencing of *miR-145-5p* reduces apoptosis of VICs
511 and promotes migration and viability of VICs, suggesting that the potential therapeutic effects of
512 pharmacological *miR-145-5p* inhibition for AVS treatment may be transferable into human AVS patients.

513

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516

517 **Author Contributions**

518 MRH and PRG conceptualized the study. MRH and PRG prepared the manuscript. MRH, PRG, DC, DG, DN,
519 and KB performed experiments. KWJ, JBM, and SU provided scientific input and provided materials. SZ, FB,
520 provided facilities for the murine model and patient-tissue samples. AP, FJ, EL, and GN contributed to the
521 funding of the project, and provided input on the project. All authors have read and approved the final
522 manuscript.

523

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528

529 **Disclosures**

530 None.

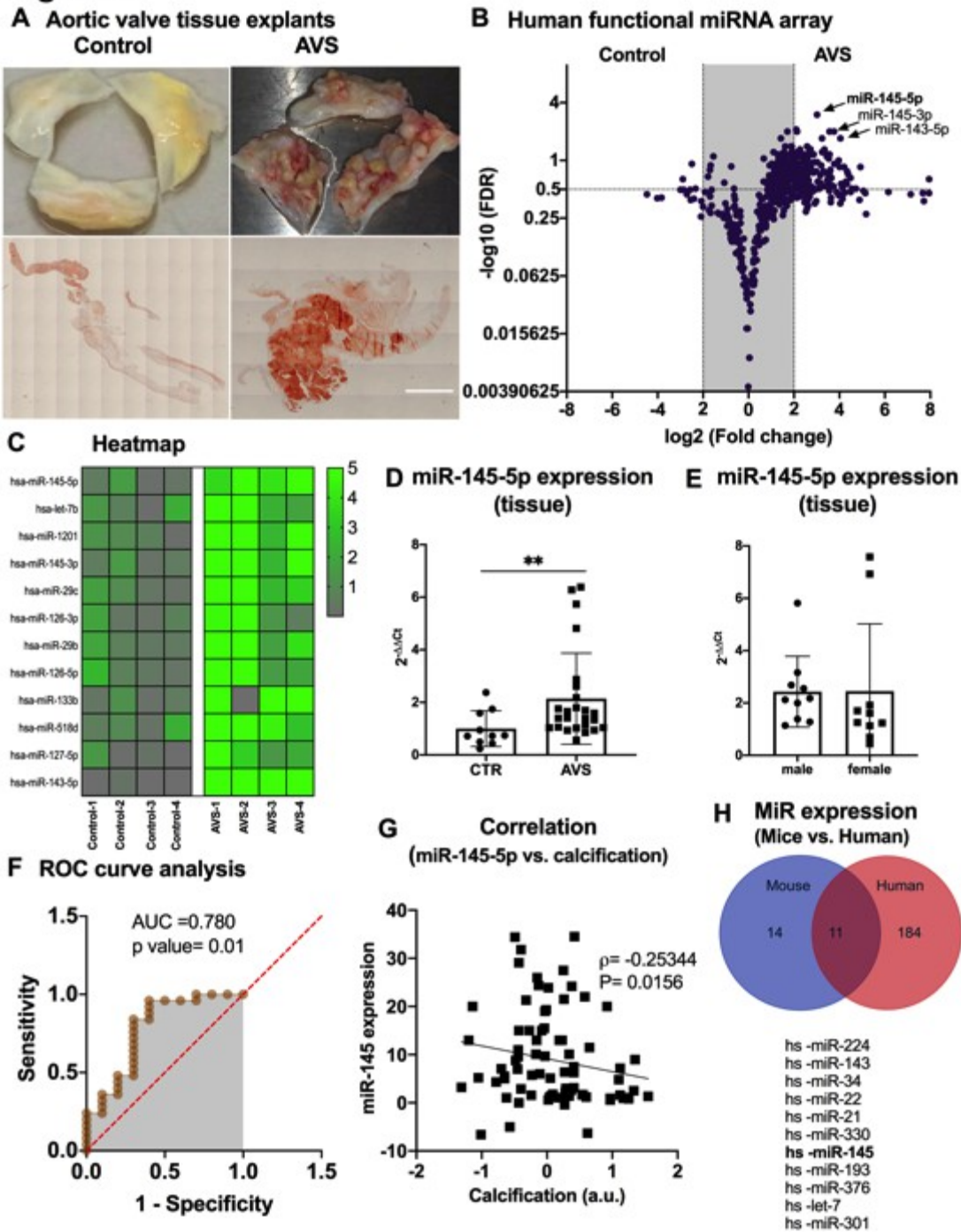
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Figure 1



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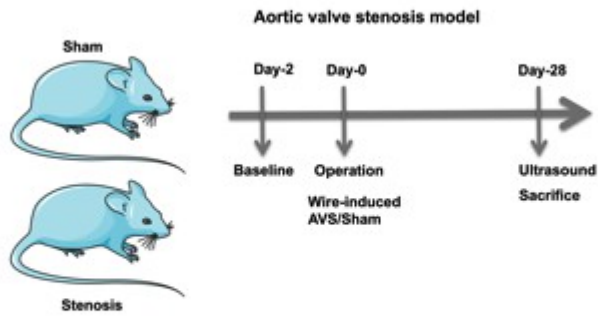
Figure 1. Investigation and profiling of miRNAs in stenotic aortic valves from patients who underwent surgical valve replacement with aortic valve stenosis.

(A) Representative images of human calcified (AVS) and non-calcified (no AVS=AI) aortic valves explanted from patients undergoing aortic valve replacement surgery (upper part) and stained with alizarin red staining (lower part). (B) Volcano plot showing differentially regulated human miRNAs in explanted valve tissues derived from patients who underwent SAVR. Thresholds (black dotted lines) of a two-fold change and p -values (FDR-adjusted) < 0.05 were used to distinguish the miRNAs of interest. $n=4$ for control (aortic insufficiency), $n=4$ for aortic valve disease (AVS). (C) Heatmap showing top-regulated miR expression of tissue-resident miRs were analyzed in explanted aortic valve tissues derived from controls ($n=4$) and AVS patients ($n=4$). (D) Expression of tissue-associated *miR-145-5p* was analyzed in control and AVS patients by qRT-PCRs. Data represent the mean \pm SEM (** $p < 0.01$, CTR, $n=10$, AVS $n=30$, by Student t-test, two-tail, unpaired). (E) Expression of tissue-associated *miR-145-5p* was analyzed in male and female patients by qRT-PCRs. Data represent the mean \pm SEM (ns, $p < 0.06$, Male, $n=12$, AVS $n=12$, by Student t-test, two-tail, unpaired). (F) ROC curve analysis for the prediction of expression of *miR-145-5p* in-patients with AVS and controls. (AUC, 0.780, ** p -value 0.01). (G) Correlation analysis of *miR-145-5p* expression with the level of calcification. (Spearman coefficient of $r = -0.25344$, $p = 0.0156$). (H) Venn

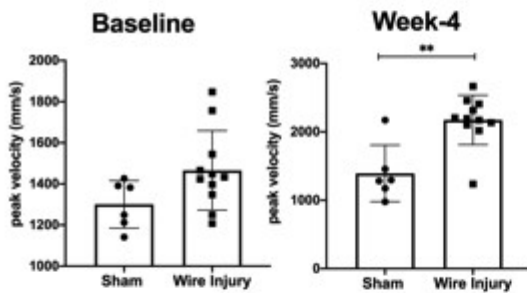
682 diagram representing the dysregulated and common miRNAs in aortic valve samples from mice (sham, n=5, AVS, n=5)
683 and patients with AVS (control, n=4, AVS, n=4). Mice were subjected to a wire injury to induce aortic valve stenosis *in vivo*.
684 Aortic valves from 5 mice were pooled and analyzed via miRNA array, and 5 aortic valves from sham-operated mice were
685 used as controls. *MIR-145-5p* was found to be amongst the upregulated miRNAs in both human and murine stenotic
686 valves. The common miRNAs are shown in the list with a cut-off >2 fold, p-value < 0.05 (FDR-adjusted). SAVR, surgical
687 aortic valve replacement; AVS, aortic valve stenosis; miRNA, microRNA; ROC, receiver-operating characteristic curve.
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Figure 2

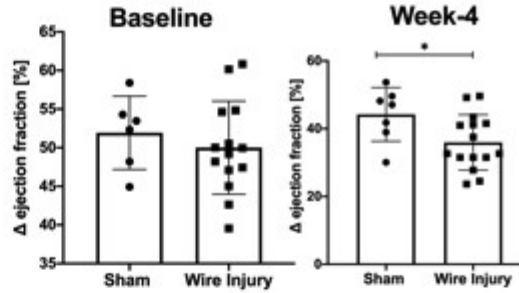
A Workflow of AVS model



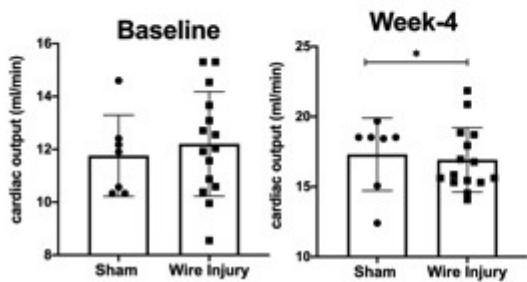
B Peak velocity



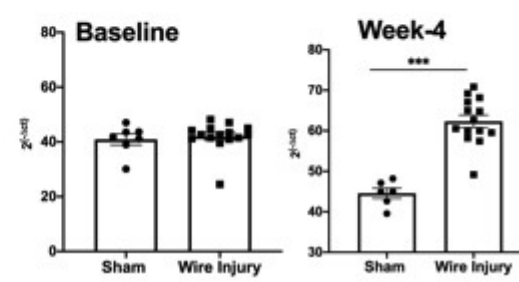
C Ejection fraction



D Cardiac output



E MiR-145-5p expression



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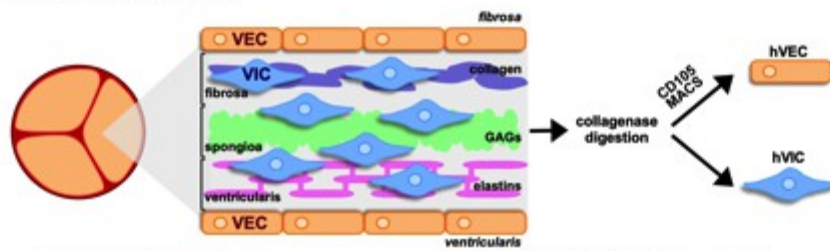
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Figure 2. *MiR-145-5p* is differentially regulated in a murine model of aortic valve stenosis.

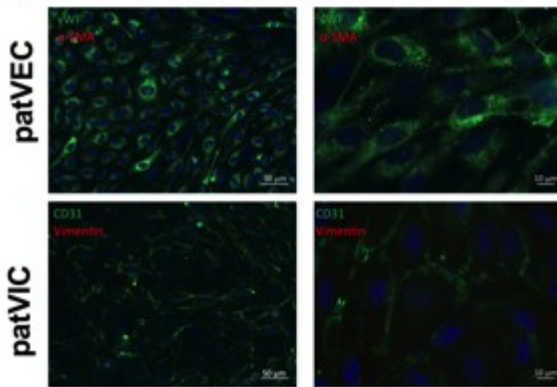
(A) Timeline of the operation used to induce aortic valve stenosis in a murine model. The induction of aortic valve stenosis in mice is achieved by inserting a coronary wire into the left ventricle and rotating it. (B) Peak velocities over the aortic valve after sham operation or wire injury show induction of stenosis, as visualized with peak velocity, 4 weeks after surgery. Statistical significance was shown between, Sham-day-1 vs. Sham-week-4 and Stenosis-day-1 vs. Stenosis-week-4 (** $p < 0.01$, sham, $n = 13$; stenosis, $n = 13$; SEM; One-way ANOVA with Bonferroni multiple comparisons test). (C) Change in the ejection fraction after sham operation or operation creating severe stenosis on day-1 and week-4, as compared to before the surgery. Statistical significance was shown between, Sham-day-1 vs. Sham-week-4 and Stenosis-day-1 vs. Stenosis-week-4 (* $p < 0.05$, sham, $n = 13$; stenosis, $n = 13$; SEM; One-way ANOVA with Bonferroni multiple comparisons test). (D) Cardiac output after sham operation or operation creating severe stenosis on day 1 and week 4 (* $p < 0.05$, sham, $n = 13$; stenosis, $n = 13$; SEM; One-way ANOVA with Bonferroni multiple comparisons test). (H) *MiR-145-5p* expression, as determined by RT-qPCR, in valvular tissue after sham operation or operation creating severe stenosis on day 1 and week-4 (* $p < 0.05$, sham, $n = 13$; stenosis, $n = 13$; SEM; One-way ANOVA with Bonferroni multiple comparisons test). AVS, aortic valve stenosis.

Figure 3

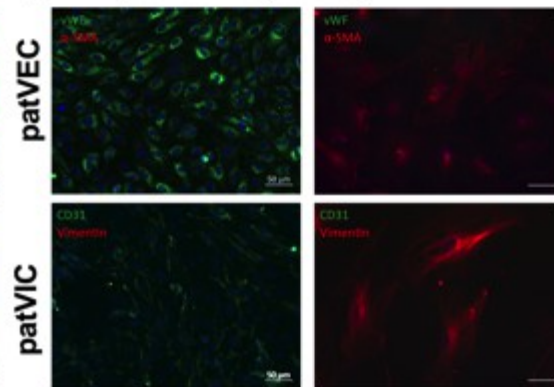
A Aortic valvular cell isolation



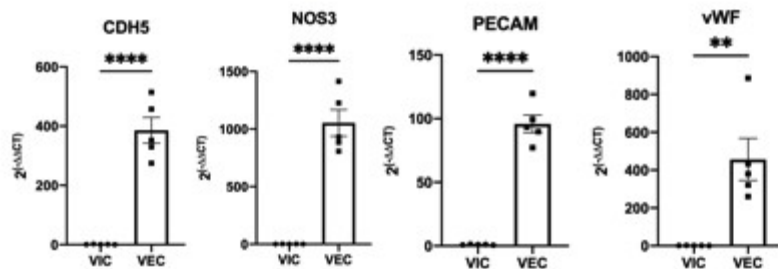
B patVEC/VIC (AVS)



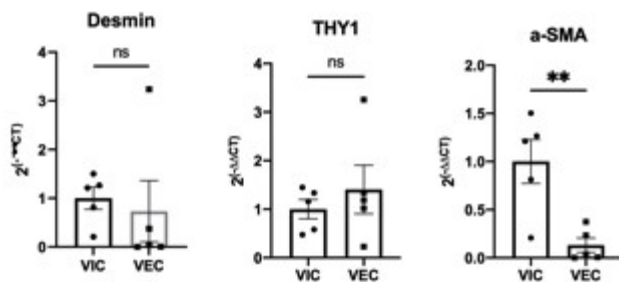
C patVEC/VIC (Control)



D Marker expression (patVEC from AVS)



E Marker expression (patVIC from AVS)



F miR-145-5p expression

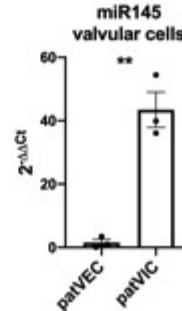


Figure 3. Isolation and characterization of patient-derived valvular cells.

(A) Schematic representation of the structure of valvular matrix and isolation process of valvular cells from aortic valve tissue explant after SAVR. Minced aortic valve tissues have been subjected to collagenase digestions overnight at optimal cell culture conditions under gentle rotations. MACS-affinity-based selection was performed to isolate VECs, whereas; a preplating step is required for VICs to separate from the remaining cell mixture. (B-C) Characterization of isolated aortic valve cells by using different surface markers, corresponding to VECs- and VICs-derived from AVS patients that underwent SAVR and compared with the corresponding controls. Representative immunofluorescence images of isolated VECs (patVECs) and VICs (patVICs) by using their characteristic markers. (D-E) Expression of characteristic markers of isolated valvular cells, (VECs, and VICs), isolated from patients with AVS post SAVR. Data are depicted as Student t-tests. (****p<0.0001, **p<0.01, n=5, two-tailed, unpaired). Expression of *miR-145-5p* was normalized to the internal control gene, *RNU6*. (F) Expression of *miR-145-5p* was measured in VECs and VICs isolated from patients with AVS after SAVR. Data are depicted as Student t-test. (**p <0.01, n=3, two-tailed, unpaired). Expression of *miR-145-5p* was normalized to the internal control gene, *RNU6*. VICs, valvular interstitial cells; VECs, valvular endothelial cells; SAVR, surgical valve replacement; vWF, von-Willebrand factor; a-SMA, alpha-smooth muscle actin; DAPI, 4',6-Diamidin-2-phenylindol; PECAM1, platelet endothelial cell

721 adhesion molecule; CDH5, cadherin 5/VE-Cadherin; NOS3, nitric oxide synthase 3/eNOS; DES, desmin; THY1, Thy-1 Cell
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Figure 4

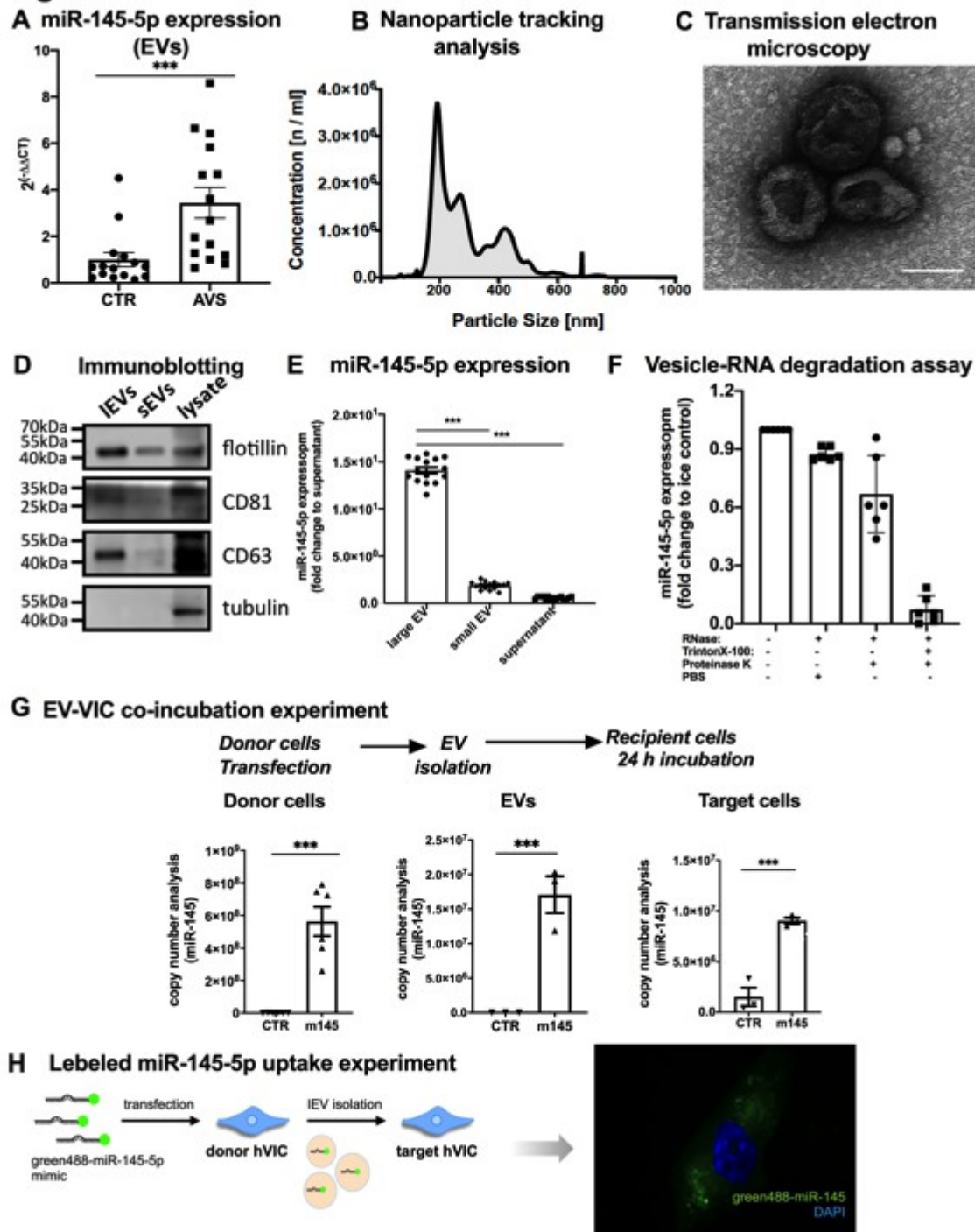


Figure 4. *MiR-145-5p* may regulate the calcification of recipient VICs via vesicular transfer.

(A) Expression of *miR-145-5p* was measured in large EVs isolated from aortic valve tissues derived from patients with AVS and corresponding controls. Taqman-based expression analysis was performed to determine *miR-145-5p* in large EVs. Data is depicted with Student t-tests. (***) $p < 0.001$, $n = 15$, two-tailed, unpaired). Expression of *miR-145-5p* was normalized to the internal control gene, *RNU6*. Large EVs were isolated by using centrifugation at 20,000 g, according to the protocol published by our group and others (20-21, 29-31). (B) NTA was used to determine the diameter (~200–700 nm) and concentration of large EVs isolated from valve tissue from patients undergoing SAVR (C) TEM image (85,000 x magnification) of pelleted large EVs (diameter ~300–700 nm) derived from the valve tissue of patients with AVS. (D) Western blot analysis of the expression of small and large EV-markers. (E) *MiR-145-5p* expression was assessed in different EV populations isolated from VIC cultures by qRT-PCR (***) $p < 0.001$, $n = 15$, by 1-way ANOVA with Bonferroni correction for multiple comparisons test). Cel-miR-39 was used for normalization. EVs and exosomes were isolated by centrifugation at 20,000 g and 100,000 g. (F) Vesicle-RNA degradation assays. VIC-derived EVs were treated in parallel using different conditions followed by RNase A digestion. *MiR-145-5p* was quantified by qRT-PCR (* $p < 0.05$, compared with the untreated group; ns: not significant, $n = 3$, by 1-way ANOVA with Bonferroni correction for multiple comparisons test). (G) Co-incubation experiments with large EV isolated from *miR-145* overexpressed VIC and control. VICs were transfected with control, *miR-145-5p* mimic and large EVs were isolated and co-incubated with VICs under similar

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788 treatment conditions to quantify the differential *miR-145-5p* transfer via large EVs to the target cells. *MiR-145-5p*
789 expression was assessed in donor VICs, isolated large EVs, and target VICs that were treated with large EVs, using copy-
790 number analysis (** $p < 0.001$, $n=6$, by 1-way ANOVA with Bonferroni multiple comparisons test). (H) EV-incorporated *miR-*
791 *145-5p* uptake experiments in VICs. PKH26-labeled large EV and green fluorescent 488-labeled *miR-145-5p* were co-
792 incubated for 24 hours to allow incorporation of EV-associated *miR145-5p* into recipient VICs. EVs, extracellular vesicles;
793 AVS, aortic valve stenosis; TEM, transmission electron microscopy; NTA, nanoparticle-tracking analysis; VECs, valvular
794 endothelial cells; VICs, valvular interstitial cells.

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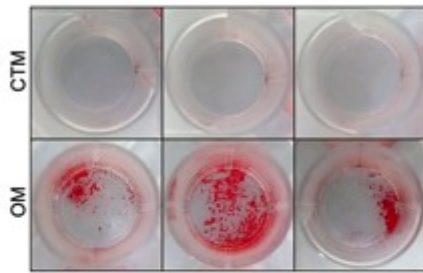
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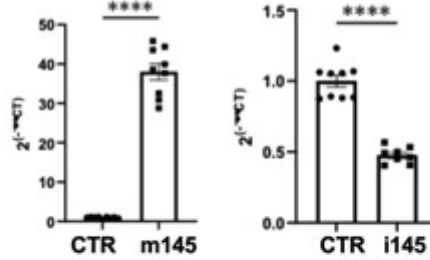
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Figure 5

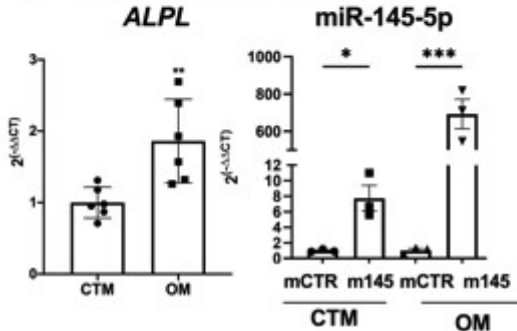
A Alizarin red staining



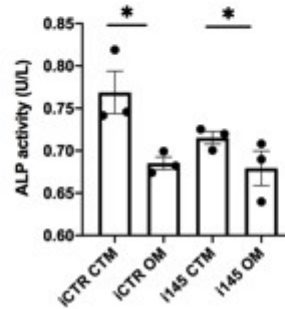
B MiR-145-5p expression



C Expression of transcript



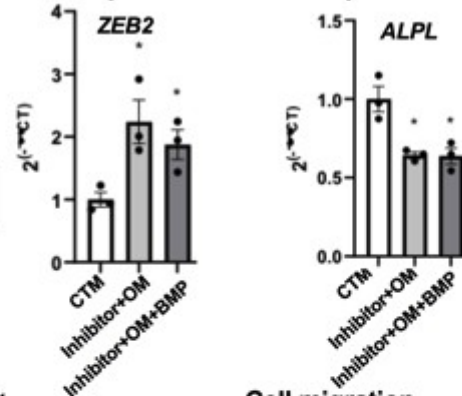
D ALP activity (U/ml)



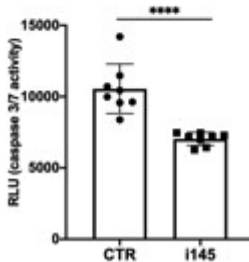
E Genomic map of human ZEB2 gene



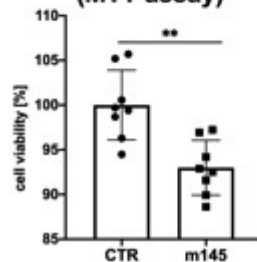
F Expression of transcript



G Caspase 3/7 activity



H Cell viability (MTT assay)



I Cell migration (Scratch assay)

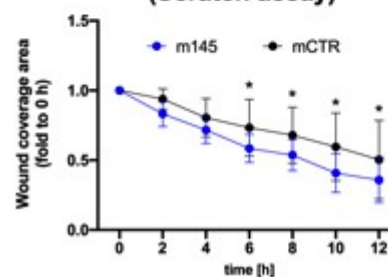


Figure 5. *MiR-145-5p* is a crucial regulator of valvular calcification and cellular function.

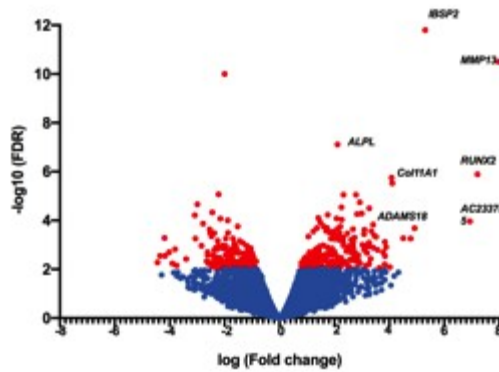
(A) *In vitro* calcification experiment in VICs followed by quantification via Alizarin red staining on day 7. The deposition of calcium in the cellular matrix was stained and quantified with alizarin red after induction of *in vitro* calcification in VICs purchased from a commercial vendor (representative images of n=3). (B) Overexpression and knockdown quantification of *miR-145-5p* by small oligonucleotides in VICs. Expression data for *miR-145-5p* in VICs have been compared with control and depicted as ****p<0.001, n=9, by 1-way ANOVA with Bonferroni multiple comparisons test. (C) Taqman-based quantification of gene expression of ALPL as a hallmark of calcification of VICs and *miR-145-5p* after induction of *in vitro* calcification by using osteogenic medium (OM) for 7 days. Expression data for ALPL mRNA in VICs and *miR-145-5p* has been compared with control medium and control *miR-145-5p* mimic and depicted as *p<0.05, **p<0.01, ***p<0.001, n=3-6, by 1-way ANOVA with Bonferroni multiple comparisons test. (D) Quantification of ALP activity in VICs after transient *miR-145-5p* overexpression or inhibition and simultaneous incubation with OM (n=3, 2-way ANOVA with Bonferroni multiple comparisons test). (E) Genomic map of the ZEB2 gene, a known regulator of ALPL, with predicted binding sites for hsa-*miR-145-5p*, in the 3'-UTR. ZEB2 is a known repressor of ALPL and contains multiple binding sites for numerous miRs, including, miR-145-5p. (F) Expression of ZEB2 and ALPL mRNA in VICs after induction of *in vitro* calcification for 7 days. Data is depicted as *p<0.05, n=3, by 1-way ANOVA with Bonferroni multiple comparisons test. (G) The level of

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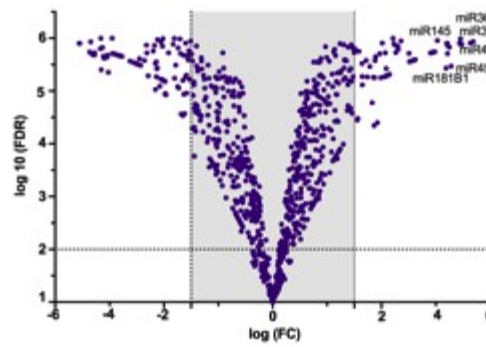
821 apoptosis was assessed via caspase 3/7 activity after incubation with H₂O₂ (100 μM) for 24h. The data was analyzed by 1-
822 way ANOVA with Bonferroni's multiple comparison test. (****p<0.001, n=8). (H) Cell viability was determined by using MTT
823 assays (incubation with H₂O₂ (100 μM)). Data was analyzed by 1-way ANOVA with Bonferroni's multiple comparison test.
824 (**p<0.01, n=8). (I) Cell migration via scratch-wound was determined by using bright-field microscopy after overexpression
825 of miR-145-5p and control at different time points. Cell-free areas were measured to assess the migration capacity of VICs
826 at 0h, 2h, 4h, 6h, 8h, 10h, and 12h time points after scratch-wound. The data was analyzed by 1-way ANOVA with
827 Bonferroni's multiple comparison test. (*p<0.05, n=3). ZEB2, Zinc Finger E-Box Binding Homeobox 2; ALPL, Alkaline
828 Phosphatase; CTM, control media, OM, osteogenic medium; UTR, untranslated region; VICs, valvular interstitial cells;
829 miRs, microRNAs; H₂O₂, hydrogen peroxide; AVS, aortic valve stenosis.

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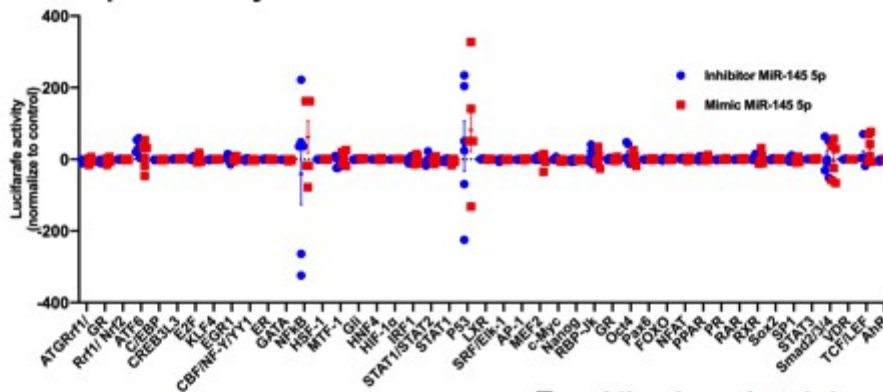
Figure 6 A RNA sequencing



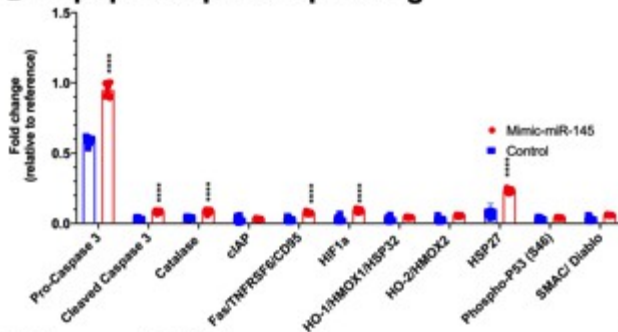
B miR profiling



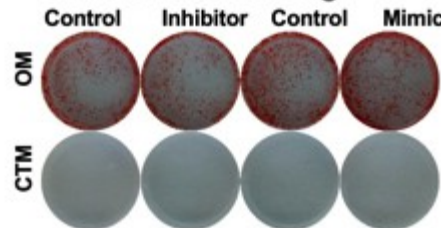
C Signal 45 reporter array



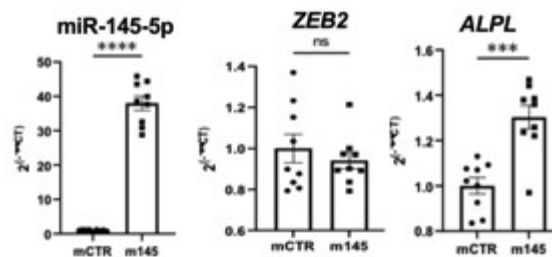
D Apoptosis protein profiling



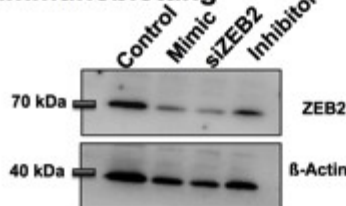
E Alizarin red staining



G Expression of transcripts



F Immunoblotting



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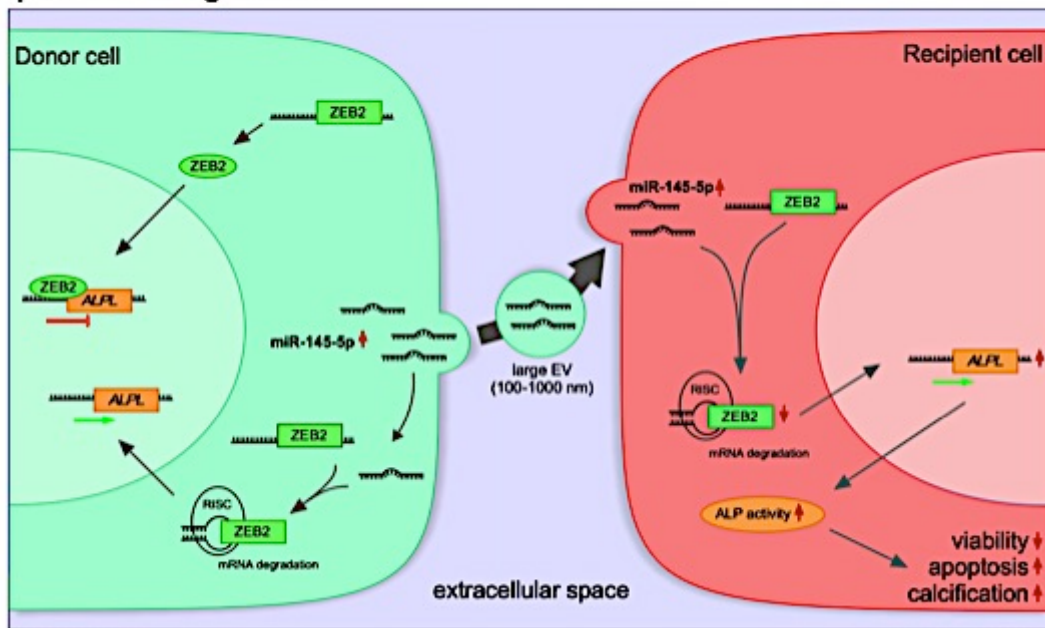
Figure 6. Transcriptomic profiling of calcified VICs confirms that miR-145-5p regulates calcification and apoptosis by regulating *ALPL2* and other calcific genes.

(A) Volcano plot of RNA sequencing data of calcified human VICs (7 days) vs. controls that demonstrated an upregulation of genes that are central to calcification, such as *ALPL*, *RUNX2*, *MMP13*, *ADAMTS18*, *COL11A1*, and *AC233755*, etc. Furthermore, an upregulation of known inflammation-, and apoptosis-regulated genes upon induction of calcification for 7 days was observed (n=4; SEM; FDR-adjusted). (B) Volcano plot with profiling of dysregulated miRNAs in calcified VICs (7 days) in comparison to control VICs with a cut off 1.5 fold (n=4; SEM; FDR-adjusted). The top and statistically significantly upregulated miRNAs are represented. (C) A high throughput transcription factor activity reporter luciferase assay upon oligonucleotide-mediated depletion or overexpression of miR-145 vs. control in VICs was performed 48h after transfection. Depicted are the activities of 45 transcription factors in inhibitor, *miR-145-5p* treated cells and control cells (control-treated) 48h after transfection with the reporter constructs. Pro-Caspase-3, Caspase-3, HSP27, HIF1a, and other apoptosis-related pathways are significantly upregulated. Data represent mean \pm SEM (****p<0.0001, n \geq 6). (E) Representative bright-field images of *in vitro* calcification experiment in VICs followed by quantification via Alizarin red staining on day 7. The deposition of calcium in the cellular matrix quantified and stained with alizarin red after induction of

864 *in vitro* calcification in VICs purchased from a commercial vendor (representative images of n=3). (F) Western blotting for
865 ZEB2 protein after transfection with control, *miR-145-5p* inhibitor (Inhibitor), siRNA against ZEB2 (siZEB2) and *miR-145-5p*
866 mimic (Mimic). (G) Overexpression data of *miR-145-5p*, ZEB2 mRNA, and ALPL in VICs upon transfection and
867 subsequent induction of calcification for 7 days to assess the expression of the transcripts (^{ns}p>0.05, ***p<0.001,
868 ****p<0.0001, n=9, by 1-way ANOVA with Bonferroni multiple comparisons test). ZEB2, Zinc Finger E-Box Binding
869 Homeobox 2; RUNX2, Runt-related transcription factor 2; ALPL, Alkaline Phosphatase; HSP27, Heat shock protein 27;
870 HIF1a, Hypoxia-inducible factor 1-alpha; CTM, control media, OM, osteogenic medium; UTR, untranslated region; VICs,
871 valvular interstitial cells; miRs, microRNAs; AVS, aortic valve stenosis.
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Figure 7

Proposed working model



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Figure 7. Large extracellular vesicular *miR-145-5p* regulates calcification and apoptosis of valvular interstitial cells by regulating *BCL2* and other apoptotic genes.

The model proposed for *miR-145-5p* action, in which it is shuttled to the recipient cells via ZEB2–*miR-145-5p* conjugates to regulate calcific gene networks by binding to 3'-UTR, such as ALPL, to control the inflammation, calcification, and apoptosis of VICs. ICs, valvular interstitial cells; miRs, microRNAs; RISC, RNA-induced silencing complex; UTR, untranslated region; ZEB2, Zinc Finger E-Box Binding Homeobox 2; ALPL, Alkaline Phosphatase.

911 **Table and legends:**

Table 1. Baseline characteristics of the study population

	Control	AVS
Total population	10	30
Clinical parameters		
Age (mean)	68.6	69.7
NYHA level	3.3±0.5	3.1±0.5
BMI	24.3±6.7	25.2±3.7
CAD	5 (50)	13 (43.3)
Hypertension	8 (80)	26 (86.6)
AVA in cm ²	0.81	>2.0
Vmax in m/s	4.45	<1.0
Pmean in mmHg	N.D.	50.7
LV function (mean)	50.4	58.3
eGFR (ml/min)	54.2±15.8	51.6±20.0
Mild-Severe mitral regurgitation	7 (70)	17 (70)
Cardiovascular risk factors		
Type II DM	0 (0)	11 (36.7)
BMI (mean)	24.2	29.288
Dyslipidemia	4 (40)	23 (76.7)
Smoker	4 (40)	12 (40)
Creatinine in mg/dl (mean)	1.14	1.04

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Baseline demographic, laboratory and echocardiographic parameters of the validation study population. P values reflect the comparison between two groups. NYHA, New York Heart Association; eGFR, estimated glomerular filtration rate; CAD, Coronary artery disease; Vmax, Velocity maximum, Pmean, mean pressure; BMI, Body mass index; AVA, Aortic valve area