Canine mitral valve disease: investigating the role of miR-143 in valvular interstitial cell activation

Ellen Finch B.S., Dawn Meola, B.S., Aaron Williams, Ellie Kudej, Vicky Yang PhD DVM DACVIM (Cardiology)

Cummings School of Veterinary Medicine at Tufts University

ellen.finch@tufts.edu

Mentor:

Vicky Yang

Abstract

Objective: To investigate the role of miR-143 in the activation of canine valvular interstitial cells (VICs) in myxomatous mitral valve disease (MMVD).

Methods: VICs were isolated from two deceased donor canines from Foster Hospital for Small Animals at Tufts University and transfected with either a miR-143 mimic or inhibitor using Lipofectamine, and vehicle (Lipofectamine) only treated was used as control. Following stimulation, RNA and protein were extracted from the VICs. Western blot analysis was used to assess changes in the protein expression of alpha-smooth muscle actin (α-SMA), and qPCR was used to evaluate the gene expressions of α-SMA and extracellular matrix (ECM)-associated genes involved in MMVD pathogenesis.

Results: Transfection with miR-143 inhibitor showed consistent downregulation of α-SMA protein and reduced expression of ECM-associated genes. miR-143 mimic transfection resulted in a mild decrease in α-SMA with no consistent gene expression changes compared to control.

Conclusions: miR-143 inhibitor treatment reduced α-SMA expression in VICs, suggesting that miR-143 may inhibit fibroblast-to-myofibroblast transition. These findings support the need for future research into uncovering miR-143's role in modulating VIC activation in MMVD.

Clinical Relevance: Understanding the effects of molecular regulators such as miR-143 provides insight into MMVD pathogenesis and could reveal molecular targets for therapeutic intervention to slow or prevent the progression of MMVD in dogs, and potentially in human mitral valve disease.

Introduction

Myxomatous mitral valve disease (MMVD) is the leading acquired cardiac disease in dogs and is characterized by progressive degeneration of and myxoid formation in the mitral valve [3]. These changes can lead to mitral regurgitation and, in severe cases, congestive heart failure or death [1]. Despite its high prevalence, the molecular mechanisms underlying MMVD remain incompletely understood [1]. The structure and functional consequences of canine MMVD closely resemble human mitral valve prolapse (MVP), a progressive and degenerative valve disease affecting 2-3% of the human population [3]. Therapy for the disease is limited, partly due to limits of rodent models to adequately demonstrate the natural pathogenesis of this polygenic heritable disease [3]. Due to their similarities, canine MMVD serves as a more robust naturally occurring disease model for human MVP [3].

The extracellular matrix (ECM) of diseased valves transition from thin and uniformly organized to thick and disorganized [4]. Increased glycosaminoglycan accumulation and excessive deposition of collagen fibers result in impaired valve coaptation and subsequent regurgitation [3, 4]. A hallmark of MMVD is the transition of valvular interstitial cells (VICs) from quiescent fibroblasts to activated myofibroblasts, which produce abnormal ECM and mediate remodeling [2,4]. This phenotypic switch is marked by increased expression of alpha-smooth muscle actin (α-SMA), a cytoskeletal protein involved in contractile function and extracellular matrix remodeling [3]. Recent work has suggested that inflammation, particularly the activation and infiltration of macrophages, may be important in MMVD-development [5].

Equally important in MMVD is the dysregulation of microRNAs (miRNAs)—small, non-coding RNAs that regulate gene expression at the post-transcriptional level – particularly miR-145 [3]. Not only is miRNA dysregulation in VICs important in disease development, but other cell types within the valve tissue can affect VIC behavior by sharing their dysregulated miRNA. Given the increase in activated macrophage in disease valves, miRNA produced by these macrophages will likely influence VIC phenotype. Preliminary sequencing data from our lab revealed that activated macrophages (M1/M2) produce extracellular vesicles containing elevated levels of several miRNAs, including miR-143 (unpublished data). Interestingly, miR-143 is often co-expressed with miR-145, a miRNA found to be overexpressed in VICs from MMVD-affected valve tissue [3].

We therefore hypothesized that the miR-143 produced by activated macrophages will contribute to the fibroblast-to-myofibroblast transition of canine VICs. The goal of this study is to investigate whether miR-143 can induce α-SMA expression in VICs, a marker of VIC activation, confirming its potential role in MMVD pathogenesis and provide a new therapeutic target for both dogs and humans.

Materials and Methods

VIC isolation

Canine hearts were obtained from donor dogs at the Tufts University Veterinary Teaching Hospital under the tissue donation program, and VICs were isolated based on a previously established protocol [2,3]. First, the mitral valves were finely sliced with a scalpel before further

digestion in a solution containing 7 U/mL collagenase I (Sigma-Aldrich), 7 U/ml collagenase XI (Sigma-Aldrich), 4 U/ml DNase (Sigma-Aldrich), and 4 U/ml hyaluronidase (Sigma-Aldrich) at 37 °C with agitation for 1 hr. The digested cells were filtered through a 70μm filter and cultured in αMEM media (Lonza) with 10% FBS (Hyclone), 0.3mg/ml L-glutamine (Corning), and 100U/ml penicillin and 100μg/ml streptomycin (Hyclone) onto untreated plastic culture plates. After 24 hours, the culture media was replaced to remove the unattached valvular endothelial cells, and remaining cells were passaged once (P1) and cryopreserved until later use.

VIC culture

VICs harvested from normal canine mitral valves were used. Once thawed, VICs (P1) were plated at ~1–7 × 10^4 cells/well in 6-well plates with 1 mL αMEM + 10% FBS. Once reaching 60-70% confluency, cells were washed twice with phosphate buffer saline (PBS), and the media was replaced by serum-free defined chemical media composed of Dulbecco's Modified Eagle Medium (DMEM) with 25 mM HEPES (Life Technologies, Carlsbad, CA, USA), 1× penicillin–streptomycin, 1× l-glutamine, 1× Insulin-Transferrin-Selenium (Gibco, Waltham, MA, USA), 5 ng/mL recombinant human fibroblast growth factor 2 (Invitrogen, Waltham, MA, USA), and 5 ng/mL recombinant human platelet-derived growth factor AB (Invitrogen). All cultures were kept incubated at 37 °C in a humidified atmosphere with 5% CO_2.

VIC stimulation and transfection

At 80–95% confluency (2 to 4 days post-plating; 24 h after change to serum-free defined media), cells were assigned to one of four conditions: control, lipofectamine (vehicle only)

control, miR-143 mimic (Thermo Fisher, Waltham, MA, USA), or miR-143 inhibitor (Thermo Fisher). Transfections were performed using Lipofectamine RNAiMAX (Thermo Fisher) for 2 to 72 hr, depending on the experimental condition. Cells were then trypsinized and harvested for protein and RNA isolation.

RNA isolation

Total RNA was isolated from VICs using mirVana miRNA isolation kit (Invitrogen) according to the manufacturer's instructions. Sample RNA concentration was then determined using the Agilent RNA 6000 Nano Kit with a 2100 Bioanalyzer according to the manufacturer's instructions.

cDNA synthesis and qPCR

cDNA was synthesized from isolated VIC RNA with the HT First Strand Kit (Qiagen) according to manufacturer's instructions. RNA input varied due to low concentration in some samples. RT-qPCR was performed using RT^2 SYBR Green qPCR Mastermix (Qiagen) with a QuantStudio 3 analyzer. RPS19 was used as a housekeeping gene, and primers were chosen based on previous reports as listed in Table 1.

Table 1: mRNA primers used in PCR analysis

Abbr.	Full name	Function
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	Housekeeping gene
ACTA	Alpha-Smooth Muscle Actin	Myofibroblast activation marker
CTGF	Connective Tissue Growth Factor	Fibrosis/ECM production
Fibro	Fibronectin	ECM glycoprotein

DCN	Decorin	ECM proteoglycan
BGN	Biglycan	ECM proteoglycan
Coll1A1	Collagen Type I Alpha 1 chain	Fibrillar collagen
Coll3A1	Collagen Type III Alpha 1 chain	Fibrillar collagen
LUM	Lumican	ECM proteoglycan
ELN	Elastin	Elastic fiber protein
Smemb	Non-muscle Myosin Heavy Chain-B (MYH10)	Cell motility marker
TGFB1	Transforming Growth Factor Beta 1	Pro-fibrotic cytokine
TGFB2	Transforming Growth Factor Beta 2	Pro-fibrotic cytokine
TGFB3	Transforming Growth Factor Beta 3	Pro-fibrotic cytokine
TGFBR	TGF Beta Receptor 1	TGF-β signaling receptor
TGFBR	TGF Beta Receptor 1	TGF-β signaling receptor
VCAN	Versican	ECM proteoglycan

Protein Isolation and immunoblotting

Protein was isolated from VICs using M-PER reagent (Thermo Fisher) according to the manufacturer's instructions, and concentrations were determined with the BCA Protein Assay Kit (Thermo Fisher). Two western blot protocols were used depending on protein signal strength. For low protein signals, an ECL-based detection method instead of a fluorescence-based method of detection was used given the higher sensitivity of this approach.

For both protocols, 3 μg of protein was separated by electrophoresis. For the fluorescent detection method, Bolt 4-12% Bis-Tris gels (Invitrogen, NW04120) were used, followed by protein transfer using an iBlot 2 Dry Blotting System (Invitrogen) with PVDF membrane. Membranes were incubated with primary antibodies overnight at 4°C (α-SMA: Sigma–Aldrich A5228, mouse monoclonal, 1:500; β-actin (housekeeping protein): Cell Signaling Technology 3700, mouse monoclonal, 1:1000), followed by incubation with the secondary antibody for 1 hr

at room temperature (Horse anti-mouse: Invitrogen, 31806). The membrane was further treated with ABC Vectastain (Vector Labs, PK6100) and DAB Substrate Kit, Peroxidase (Vector Labs, SK-4100) for signal detection. For the ECL protocol, Mini-Protean TGX gels (BioRad, 4561094) were used, followed by protein transfer onto a PVDF membrane. Primary antibody incubation was similarly performed overnight at 4^{o}C (α-SMA: Sigma–Aldrich A5228, mouse monoclonal, 1:1000–1:5000; vinculin (housekeeping protein): Cell Signaling, 4650S, rabbit polyclonal, 1:1000), followed by secondary antibody treatment for 1 hr at room temperature (Goat anti-rabbit: Invitrogen, 31460; Goat anti-mouse: BioRad, 170-6516). Detection was performed using ECL substrate (Biorad Clarity Western ECL), and membranes were imaged on a LI-COR imaging system. Protein expression intensity analysis was performed using ImageJ v.1.53e.

Statistics

Due to insufficient biological replicates, no statistical analysis was performed. Graphical representation of the data was created using GraphPad Prism v.10.

Results

miR-143 inhibition may control activation of VICs

Protein and gene expression analysis of one of the VIC samples with robust Western blot and qPCR signals (Dog 78, 24 hr post-transfection) demonstrated coordinated downregulation of α-SMA protein and several ECM–associated genes (CTGF, Smemb, VCAN, BGN, TGFB2, etc.) compared to controls. These findings are consistent with reduced VIC activation.

miR-143 overexpression may not have an important role in VIC activation

Contrary to the original hypothesis, western blot analysis of miR-145 mimic-treated cells did not reveal upregulation of α-SMA protein expression. Instead, a modest decrease was observed. qPCR analysis for mimic-treated samples was inconclusive due to low RNA yield and inconsistent amplification.

	ACTA	CTGF	Fibro	DCN	BGN	C1A1	C3A1	LUM	ELN	Smemb	TGFB2	TGFB3	TGFBR1	VCAN
miR-143 Mimic	1.1	-1.3	1.1	1.1	-1.2	1.1	1.2	-1.0	1.5	-1.3	-1.1	1.1	1.0	-1.2
miR-143 Inhibitor	-1.8	-1.8	-1.9	-1.7	-2.3	-1.7	-2.3	-1.6	-1.8	-1.6	-1.8	-2.1	-2.3	-1.9

Figure 1: Gene expression fold change compared to vehicle-only control for α-SMA and several extracellular matrix-related genes for Dog 78 VICs, transfected with miR-143 mimic or inhibitor for 24 hours.

Methodology Development and Optimization

High Sensitivity Western Blot

A significant portion of project time was devoted to developing and optimizing a higher sensitivity ECL western blot detection. This process required multiple trials with different housekeeping proteins and antibody dilutions to achieve a reproducible, high-quality signal. The optimized ECL protocol is now in place for future laboratory use.

Effects of Culture Duration and Cell Density

Because the effects of miR-143 on VICs has not been previously explored, we want to understand the effects of culture duration post-transfection and to optimize culture condition for transfection studies. We tested several culture durations (ranging from 2 to 72 hrs) and varying initial plating densities to maintain cell viability. Previous reports had suggested culturing for 72 hrs was required to detect expression changes [6]. Our results showed that maximum increase in α-SMA protein expression after 4 hrs of culture after transfection with miR-143 mimic, while a small decrease in α-SMA expression was noted with miR-143 inhibitor transfection at the same timepoint (Fig 2). Furthermore, longer culture durations required lower initial cell plating density to prevent overgrowth of cells at time of harvest.

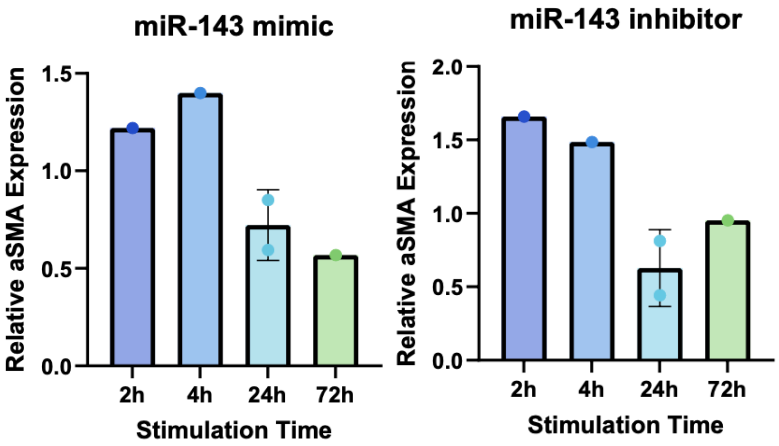

Figure 2: Relative α-SMA protein expression compared to vehicle-only control for two VIC cell lines transfected with miR-143 mimic or inhibitor at various time points. Data suggests peak upregulation of α-SMA protein at hour 4 post-transfection followed by downregulation over longer culture time periods post transfection. miR-143 inhibitor transfection resulted in a gradual decrease in α-SMA protein expression with time.

Discussion

The data from the most robust miR-143 inhibitor-transfected VICs provide preliminary support for the hypothesis that miR-143 contributes to VIC activation. Inhibition of miR-143 resulted in downregulation of α-SMA and multiple ECM-related genes, preventing fibroblast-to-myofibroblast transition in VICs. However, the limited number of high-quality replicates, resulting from inconsistent cell growth, low RNA and protein yield, and limited time for protocol optimization prevented definitive conclusions.

The unexpected reduction in α-SMA protein expression in mimic-treated samples after 24 hrs of culture may reflect a combination of biological and technical factors. miRNA effects can vary depending on cell type and their transcriptional state. As our data suggests, the effects of miR-134 may not be long lasting as peak α-SMA protein expression was seen at 4 hrs post transfection, but the expression quickly diminished with time. Our results further suggest a temporal relation between gene and protein expressions after miR-143 transfection. Additionally, miR-143 targets numerous genes for suppression and therefore regulates multiple pathways, some of which may indirectly suppress α-SMA in VICs. High baseline expression of miR-143 could also result in functional saturation, where increasing the concentration of miR-143 would have minimal effects on target gene and protein expression. Technical limitations may have also influenced results. Efficiency of Lipofectamine transfection of miR-143 was not verified.

Conclusion

This study provides preliminary evidence that miR-143 inhibition may suppress VIC activation, as indicated by reduced α-SMA and ECM gene expression. The lack of expected upregulation with miR-143 overexpression and the variability across replicates highlight the need for additional experiments to optimize culture conditions to better understand the effects of miR-143 in VICs.

Acknowledgments

The author thanks the pet owners who have contributed to the tissue donation program.

Disclosures

The authors are not aware of any conflicts of interest. No AI-assisted technologies were used in the writing of this manuscript.

Funding

This research was funded by the Cummings School of Veterinary Medicine Summer Research Program, as well as existing funds of the Yang Lab.

References

1. Keene B. W., Atkins C. E., Bonagura J. D., Fox P. R., Haggstrom J., Fuentes V. L., Oyama M. A., Rush J. E., Stepien R., Uechi M. (2019). ACVIM consensus guidelines for the diagnosis and treatment of myxomatous mitral valve disease in dogs. J Vet Intern Med, 33(3), 1127-1140. https://doi.org/10.1111/jvim.15488

2. Yang, V. K., Tai, A. K., Huh, T. P., Meola, D. M., Juhr, C. M., Robinson, N. A., & Hoffman, A. M. (2018). Dysregulation of valvular interstitial cell let-7c, miR-17, miR-20a, and miR-30d in naturally occurring canine myxomatous mitral valve disease. PLOS ONE, 13(1), e0188617. https://doi.org/10.1371/journal.pone.0188617

3. Yang, V. K., Moyer, N., Zhou, R., Carnevale, S. Z., Meola, D. M., Robinson, S. R., Li, G., & Das, S. (2024). Defining the role of the miR-145—KLF4—αSMA axis in mitral valvular interstitial cell activation in myxomatous mitral valve prolapse using the canine model. International Journal of Molecular Sciences, 25(3), 1468–1468. https://doi.org/10.3390/ijms25031468

4. Hulin, A., Anstine, L. J., Kim, A. J., Potter, S. J., DeFalco, T., Lincoln, J., & Yutzey, K. E. (2018). Macrophage transitions in heart valve development and myxomatous valve disease. Arteriosclerosis, Thrombosis, and Vascular Biology, 38(3), 636–644. https://doi.org/10.1161/atvbaha.117.310667

5. LeRoy, E.N., Williams, A.A., Skor, M.O., Lyons, C.E., Martinot, A.J., Yang, V.K. (2025). *Distribution of ionized calcium-binding adaptor molecule 1-positive macrophages and*

papillary muscle fibrosis in dogs with myxomatous mitral valve disease. Journal of Veterinary Cardiology, (in press). https://doi.org/10.1016/j.jvc.2025.10.007

6. Chen, Y., Wang, Z., Chen, Y., Zhang, Y., & Zhang, S. (2022). *MicroRNA-143-3p regulates proliferation and apoptosis of synovial fibroblasts via targeting HMGB1 in rheumatoid arthritis*. Experimental and Therapeutic Medicine, 24(1), 1–10. https://doi.org/10.3892/etm.2022.11225