Validating KLF4 as a key control for miR-145 initiated α-SMA expression in canine VICs

Runzi Zhou V’26
Cummings School of Veterinary Medicine at Tufts University

Under the supervision of:

Vicky Yang, D.V.M., Ph.D., DACVIM
Associate Professor
Department of Clinical Sciences

Signature of Mentor

This summer research was supported, in part, by:

AVMA/AVMF
Boehringer Ingelheim Scholars Program
Cummings School of Veterinary Medicine at Tufts University Dean’s Office

OBJECTIVE
To validate the role of Krüppel-like factor 4 (KLF4) in regulating α-SMA expression in canine valvular interstitial cells (VICs), thereby elucidating its contribution to the pathogenesis of myxomatous mitral valve disease (MMVD).

SAMPLES
The study included VICs harvested from both diseased and normal canine mitral valves. The diseased samples were used for the gain-of-function experiments and the normal samples were used for the loss-of-function experiments. A total of six samples were analyzed, three for each experiment.

PROCEDURES
The gain-of-function was assessed by stimulating diseased/myofibroblastic VICs with recombinant human KLF4 protein at concentrations of 0.1 and 1.0 ng/ml. The loss-of-function was performed using KLF4-specific siRNA on normal/fibroblastic VICs. Western blot and real-time PCR were used to quantify α-SMA protein and gene expression, respectively.

RESULTS
The gain-of-function experiments revealed that KLF4 stimulation significantly reduced α-SMA protein expression at 0.1 ng/ml ($p = 0.015$), with a fold change of 0.87 ± 0.09 compared to the control. In contrast, the siRNA designed for this study was not effective in changing the α-SMA protein expression, although there was an increase in α-SMA gene expression.

CLINICAL RELEVANCE
The results of this study provide evidence supporting KLF4 as a key regulator of the fibroblast-to-myofibroblast transition in VICs by modulating α-SMA expression, suggesting that the KLF4 – α-SMA pathway could serve as a potential therapeutic target for MMVD treatment. However, further investigation of the downstream KLF4 – α-SMA pathway and its effects on VICs is needed to fully understand the MMVD pathogenesis and to optimize future therapeutic strategies.

Myxomatous mitral valve disease (MMVD) is the most common acquired heart disease in dogs. It is characterized by disruption of the extracellular matrix in the mitral valve leaflets (Figure 1), which leads to regurgitation and congestive heart failure. Despite its prevalence, the etiology of this disease is unknown. Histologically, valvular interstitial cells (VICs) in MMVD undergo a transition from fibroblasts to myofibroblasts (Figure 2), marked by increased alpha-smooth muscle actin (α-SMA) expression. MicroRNAs (miRNAs) are a class of endogenous, small non-coding RNAs that play a pivotal role in post-transcriptional regulation of gene expression by inhibiting mRNA translation, has and we have demonstrated an upregulated expression of miR-145 in VICs derived from diseased canine mitral valves. miR-145 has been shown to direct inhibitory effect on Krüppel-like factor 4 (KLF4) in other cell types such as vascular smooth muscle cells. KLF4, a member of the KLF transcription factor family, is associated with critical cellular processes such as proliferation, differentiation, migration, and apoptosis.

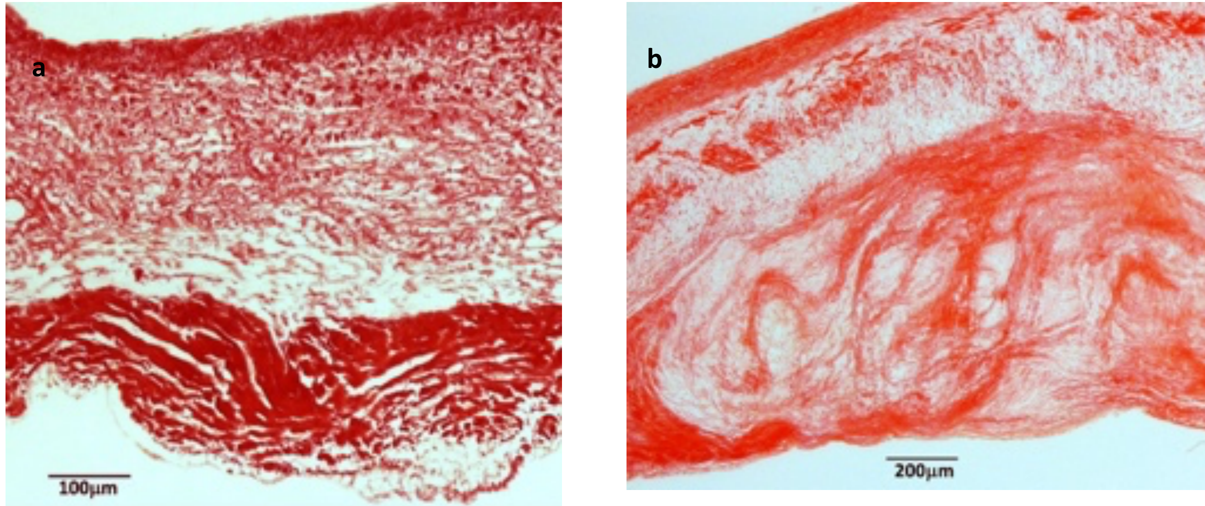


Figure 1- Normal valve structure (a), and a valve leaflet affected by MMVD (b).

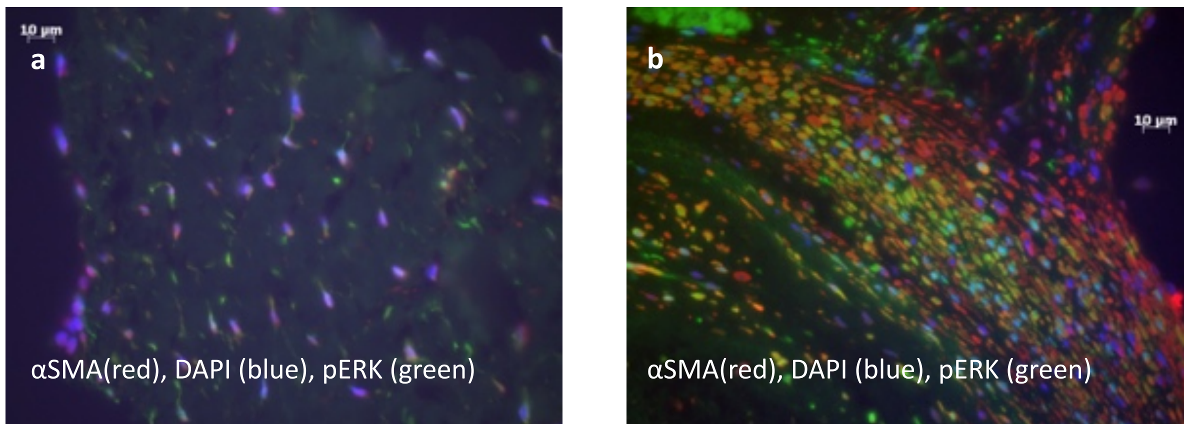
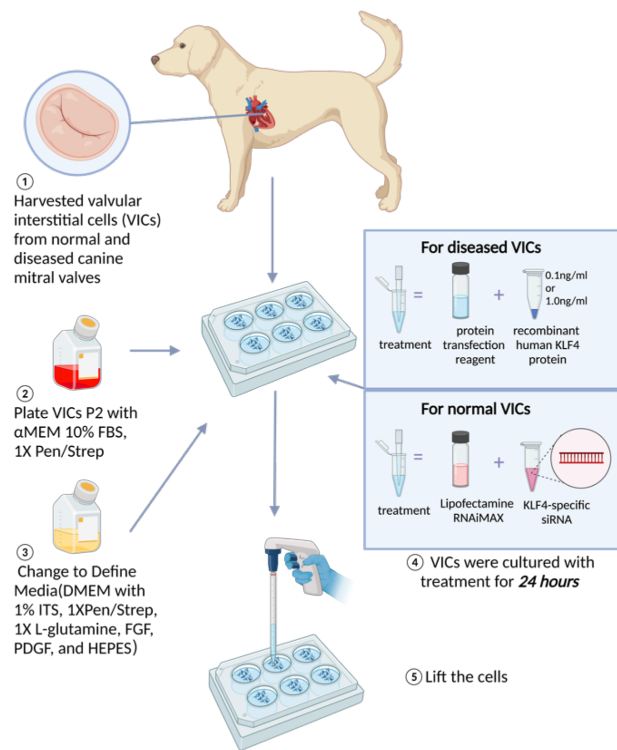


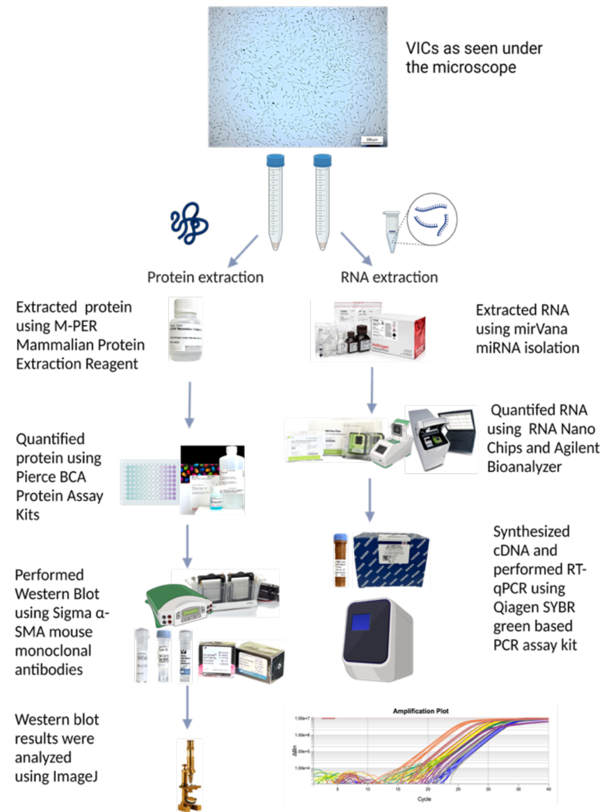
Figure 2- Low α-SMA expression shown in normal valve sections (a), while α-SMA expression increased in affected valve sections (b).

We therefore hypothesized that miR-145 induces the fibroblast-to-myofibroblast transition in VICs through the inhibition of KLF4.

Methods

We conducted a series of KLF4 gain and loss of function experiments. For the gain of function experiment, passage 2 (P2) VICs harvested from diseased canine mitral valves (n=3) were plated in αMEM with 10% FBS and 1× penicillin-streptomycin. Subsequently, VICs were transitioned to serum free media (DMEM with 25 mM HEPES (Life Technologies), 1× penicillin–streptomycin, 1× l-glutamine, 1× Insulin- Transferrin-Selenium (Gibco), 5 ng/mL recombinant human fibroblast growth factor 2 (Invitrogen), and 5 ng/mL recombinant human platelet-derived growth factor AB (Invitrogen).). Recombinant human KLF4 protein (ab169841) was subsequently added to the media at concentrations of 0.1 and 1.0 ng/ml for 24 hours. RNA and protein isolation was then performed with the miRVana RNA isolation kit (Invitrogen) and M-Per extraction reagent (Thermo Fischer), respectively, followed by protein quantification using Pierce BCA Protein





Assay Kits and RNA quantification using the RNA Nano Chips and Agilent Bioanalyzer. Protein expressions were examined via Western blot analysis. 3-20 µg of total protein was loaded for electrophoresis and transferred to the polyvinylidene difluoride membrane. The membrane was processed for immunoblotting against α-SMA (Sigma-Aldrich A5228; clone 1A4, monoclonal mouse antibody; 1:500 dilution, antibody ID: AB_262054), KLF4 (Abcam ab129473, clone rabbit polyclonal, 1:1000 antibody ID: AB_2941800), β-actin (CST 3700, mouse monoclonal, clone: 8H10D10; 1:1000, antibody ID: AB_2242334). Secondary antibodies used were anti-rabbit IgG (Vector BA-1000, goat polyclonal, 1:250, antibody ID: AB_2313606) and anti- mouse IgG (Vector BA-2000, horse polyclonal, 1:250, antibody ID: AB_2313581). Immunoblot images for α-SMA and KLF4 were analyzed using ImageJ and normalized against β-actin signal. cDNA was synthesized using the RT^2 HT First Strand Kit (Qiagen). and the gene expression of α-SMA was assessed through real-time PCR using RT^2 SYBR Green qPCR Mastermix (Qiagen).

In contrast, for the loss of function experiment, we inhibited the expression of KLF4 with the use of KLF4-specific siRNA designed with the siDESIGN program (Horizon Discovery). VICs from normal canine mitral valves (n=3) were transfected with KLF4-specific siRNA in serum free media for 24 hours using Lipofectamine RNAiMAX, (Thermo Fisher). Subsequently, RNA and protein were isolated as described above to analyze α-SMA gene and protein expressions.

Results

In the context of our gain-of-function experiment, Western blotting **(Figure 3)** revealed KLF4 stimulation at 0.1ng/ml significantly reduced α-SMA expression ($p = 0.015$; one way ANOVA with Dunnett's

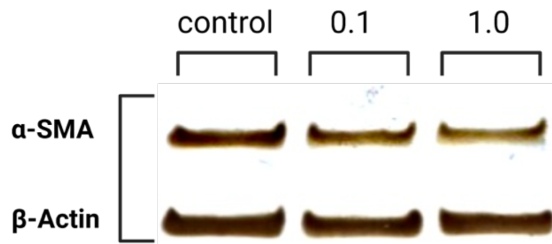


Figure 3- Western Blot analysis of α–SMA normalized with β-actin.

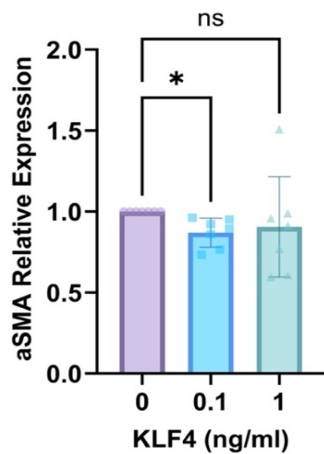


Figure 4- 0.1 ng/ml KLF4 stimulation significantly reduced α-SMA expression, while the average expression of α-SMA was also reduced with 1.0 ng/ml KLF4 stimulation.

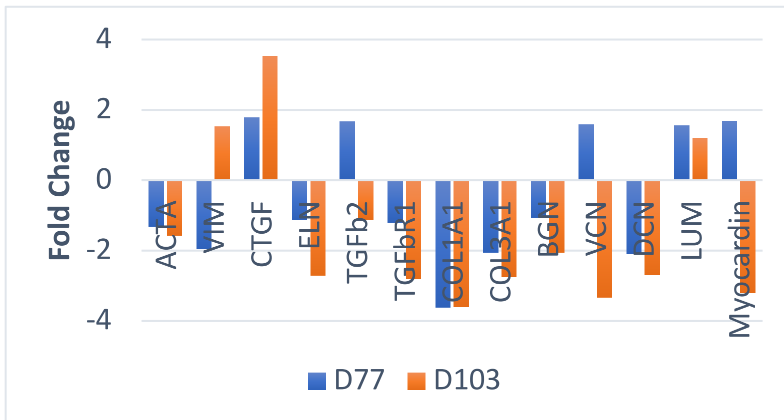


Figure 5- Gene expression for Diseased VIC P2 treated with KLF at 0.1ng/ml.

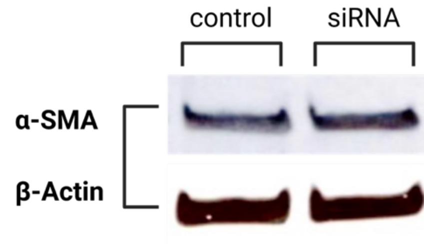


Figure 6- Western Blot analysis of α–SMA after siRNA treatment.

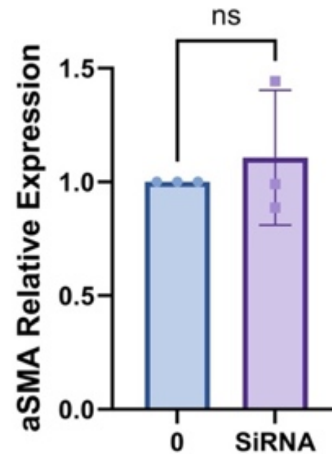


Figure 7- No significant changes in α-SMA protein expression.

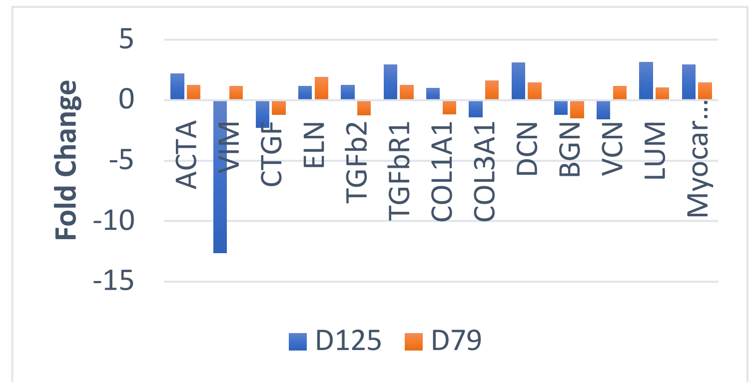


Figure 8- Gene expression for Normal VIC P2 treated with KLF-specific siRNA.

multiple comparison test), with a fold change of 0.87 ± 0.09 compared to the control group **(Figure 4)**. The average expression of α-SMA was also reduced with 1.0 ng/ml KLF4 stimulation, although it did not reach statistical significance. Additionally, gene expression analysis of α-SMA (ACTA), collagen, and glycosaminoglycans following

KLF4 treatment (n=2) showed a decreased in the α-SMA (ACTA) gene expression **(Figure 5).**

In the loss-of-function experiment, we observed no significant changes in α-SMA protein expression after KLF4 siRNA treatment **(Figure 6 & 7).** However, RT-PCR analysis **(Figure 8)** revealed an increase in the gene expression of α-SMA (ACTA) following treatment with KLF4-specific siRNA (n=2).

Discussion

The results of our study suggest that KLF4 may be an important regulator of α-SMA expression in VICs, controlling the fibroblast-to-myofibroblast transition of VICs. As this transition is a hallmark of MMVD development, KLF4 may be a potential target for therapeutic intervention.

In our gain-of-function experiments, the stimulation of diseased canine VICs with recombinant human KLF4 protein resulted in a statistically significant reduction in α-SMA expression. This reveals that KLF4 acts as an inhibitor for α-SMA expression. As our previous work demonstrated that miR-145 is upregulated in MMVD and promotes the fibroblast-to-myofibroblast transition by increasing α-SMA expression in VICs, and it suppresses KLF4 expression concurrently, the result from this study further confirms the likely miR-145 – KLF4– α -SMA pathway.

However, the inhibition of KLF4 using siRNA did not lead to a corresponding increase in α-SMA protein expression, although there was an increase in gene expression. This discrepancy between gene and protein expression of α-SMA following the KLF4-specific siRNA suggests that the KLF4 siRNA design may need to be further optimized, with the demonstration of KLF4 gene expression decreases first, prior to the study or its effect on α-SMA protein and gene expressions.

Conclusion

miR-145↑ →KLF4↓ → α-SMA↑

Stimulation with recombinant human KLF4 protein led to a decrease in both α-SMA protein and gene expressions. Conversely, stimulation with KLF4-specific siRNA resulted in an upregulation of α-SMA gene expression. These findings provide strong evidence supporting the regulatory role of KLF4 in VICs fibroblast-to-myofibroblast transition in MMVD. Consequently, the KLF4 – α-SMA pathway could serve as a potential therapeutic target for MMVD treatment. We will further investigate the KLF4-α-SMA pathway and its downstream effects on VICs to fully understand MMVD pathogenesis.

Acknowledgements

This work was supported by the Boehringer Ingelheim Scholars Program, and NIH (K01OD028205).

We thank Dawn Meola and Vicky Yang for their support.

Reference

1. Aupperle H, Disatian S. Pathology, protein expression and signaling in myxomatous mitral valve degeneration: comparison of dogs and humans. J Vet Cardiol 2012;14:59-71.

2. Borgarelli M, Buchanan JW. Historical review, epidemiology and natural history of degenerative mitral valve disease. J Vet Cardiol 2012;14:93-101.

3. Tien YT, Chang MH, Chu PY, et al. Downregulation of the KLF4 transcription factor inhibits the proliferation and migration of canine mammary tumor cells. Vet J 2015;205:244-253.