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Elucidating the pathogenesis underlying bicuspid aortic valve disease using new disease models
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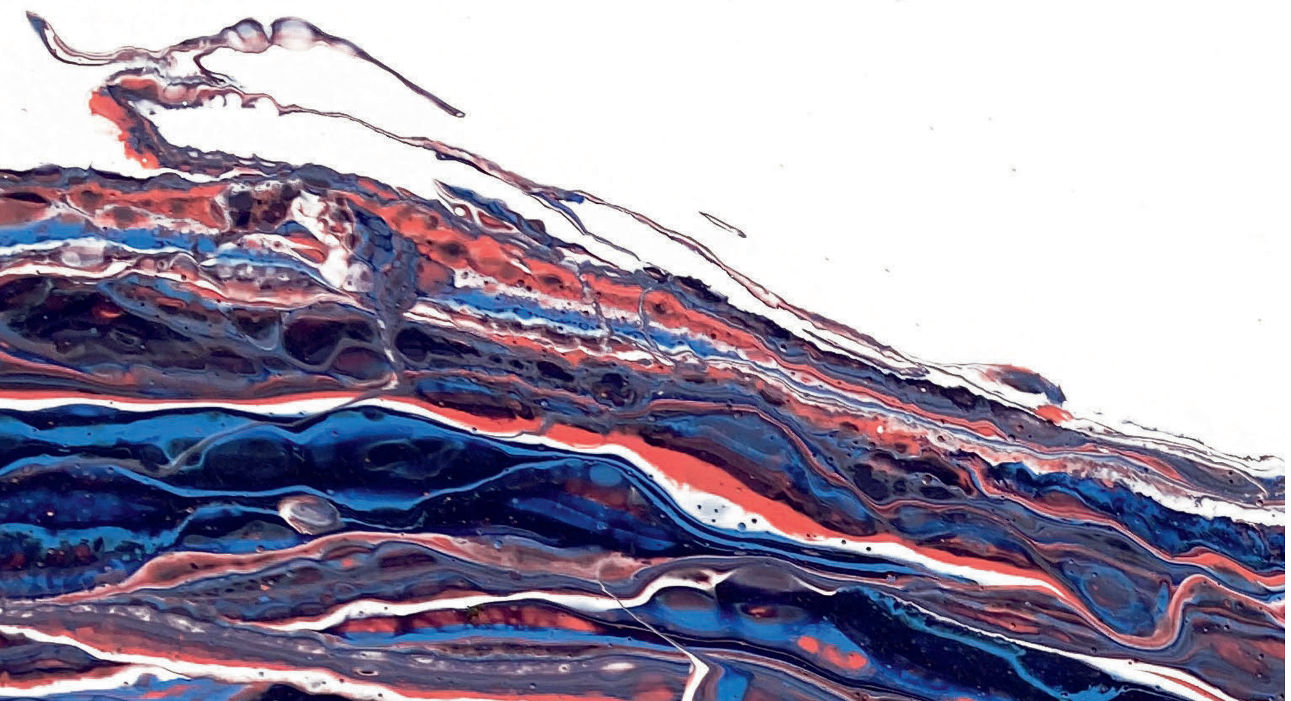

Chapter 7

A role for Four-and-a-Half LIM-domain 2 in aortic valve calcification

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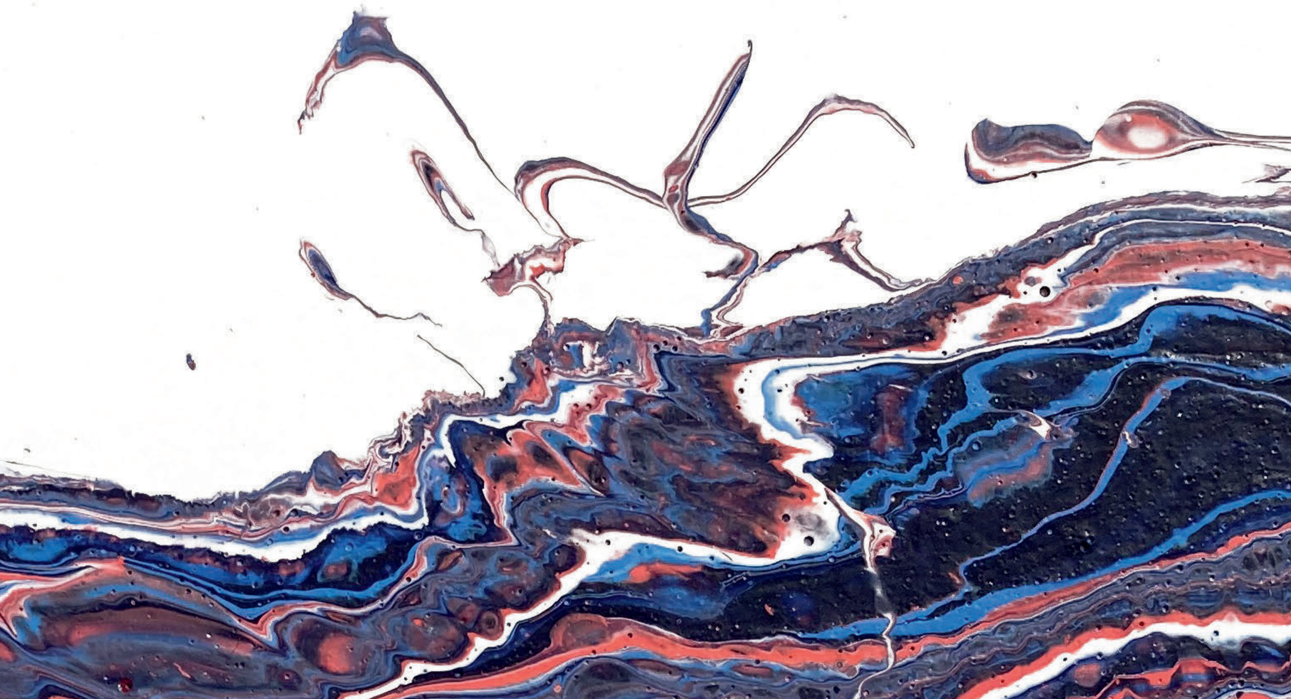

Abstract

Calcific aortic valve disease (CAVD) is a cardiovascular pathology involving fibrosis, matrix degradation, cell death and mineral accumulation in the aortic valves. CAVD ultimately leads to severely calcified leaflets with impaired motion, requiring valve replacement surgery. Although the protein four-and-a-Half LIM-domain 2 (FHL2) is involved in both osteogenic differentiation processes and cellular responses to alterations in the cardiovascular homeostasis, the role of FHL2 in CAVD has not yet been explored. Therefore, we stained sections of calcified human aortic valves and demonstrated FHL2 expression in valvular interstitial cells (VICs) throughout the leaflets. Interestingly, *ex vivo* tissue culture of whole mouse hearts under calcifying conditions revealed significant differences in the localization of calcification in the aortic valves of FHL2$^{-/-}$ mice compared to valves from WT mice. The calcified areas observed in the FHL2$^{-/-}$ aortic valves were mainly located at the aortic side of the valve whereas in WT hearts the calcification was mostly located at the ventricular side. Our results demonstrate a role for FHL2 in aortic valve calcification and show that the effect of FHL2 on cellular calcification is location dependent.

Introduction

Calcific aortic valve disease (CAVD) is a common, progressive disease of the aortic valves occurring in approximately 1% of the population by the age of 70 [1]. CAVD progresses from mild valvular thickening without obstruction of blood flow to the final stage of CAVD termed aortic stenosis characterized by severe calcification with impaired leaflet motion. Currently the only treatment option is surgical replacement of the dysfunctional valve. Although the initiation is not known, inflammation and extracellular matrix (ECM) remodeling are related to the valvular fibrosis in CAVD [2-4]. Calcified nodules can arise via osteogenic differentiation of the valvular interstitial cells (VICs), a process which is characterized by the expression of proteins such as osteopontin, osteocalcin and RUNX2 [5-7]. Another process that can cause valvular calcification is dystrophic calcification. Dystrophic calcification involves apoptosis of the VICs and the deposition of minerals in the valve [8].

Four-and-a-Half LIM-domain 2 (FHL2) is a scaffold protein able to bind numerous different proteins, thereby affecting many cellular processes such as proliferation and differentiation. Amongst others, FHL2 has been shown to play a role in the cardiovascular stress response. For example, in the absence of FHL2, β-adrenergic stimulation was reported to cause exaggerated hypertrophy and carotid artery ligation cause increased smooth muscle cell (SMC) proliferation in FHL2$^{-/-}$ mice, resulting in artery occlusion [9,10]. With regard to calcification, FHL2 has been shown to alter transcriptional activity of RUNX2 and increase the expression and activity of alkaline phosphatase [11]. In addition, FHL2 has been reported to stimulate the differentiation of mesenchymal stem cells towards osteoblasts and to reduce osteoclasts activity [12]. As a consequence, mice develop osteopenia when they have a deficiency for FHL2 [13].

Despite the known role of FHL2 in calcification and cardiovascular pathologies, a function for FHL2 in valvular stenosis has not yet been explored. To increase understanding of the process of valvular stenosis and investigate if FHL2 impacts on these processes, we investigated the role of FHL2 in calcification of murine aortic valves in *ex vivo* cultured hearts.

Results

FHL2 is present in pre-stenotic and stenotic human aortic valves

We first analyzed the expression of FHL2 and RUNX2 in human aortic valves. Therefore, we collected valves from patients with differing severities of aortic valve stenosis. In total 7 valvular samples were studied (including 3 bicuspid aortic valves). Of these samples, there were 3 samples with an ascending aortic

aneurysm and 2 samples with an aortic root aneurysm. The alizarin red (AR) staining demonstrated that 3 out of 7 samples had calcified nodules. FHL2 and RUNX2 were detected in all samples, independent of the presence of calcified nodules (Figure 1). RUNX123$^+$ and FHL2$^+$ cells were quantified but there was no significant difference in the percentage of FHL2$^+$ or RUNX123$^+$ cells between AR$^+$ and AR$^-$ samples (Figure 1I,J) (AR$^+$ mean FHL2=63.63%, SEM:13.62%; AR$^-$ mean FHL2=42.40%, SEM=16.45%; p=0.340 | AR$^+$ mean RUNX123=77.53%, SEM:5.56%; AR$^-$ RUNX123=60.03%, SEM=7.38%; p=0.137). There was no significant correlation between FHL2 and RUNX123 expression (data not shown).

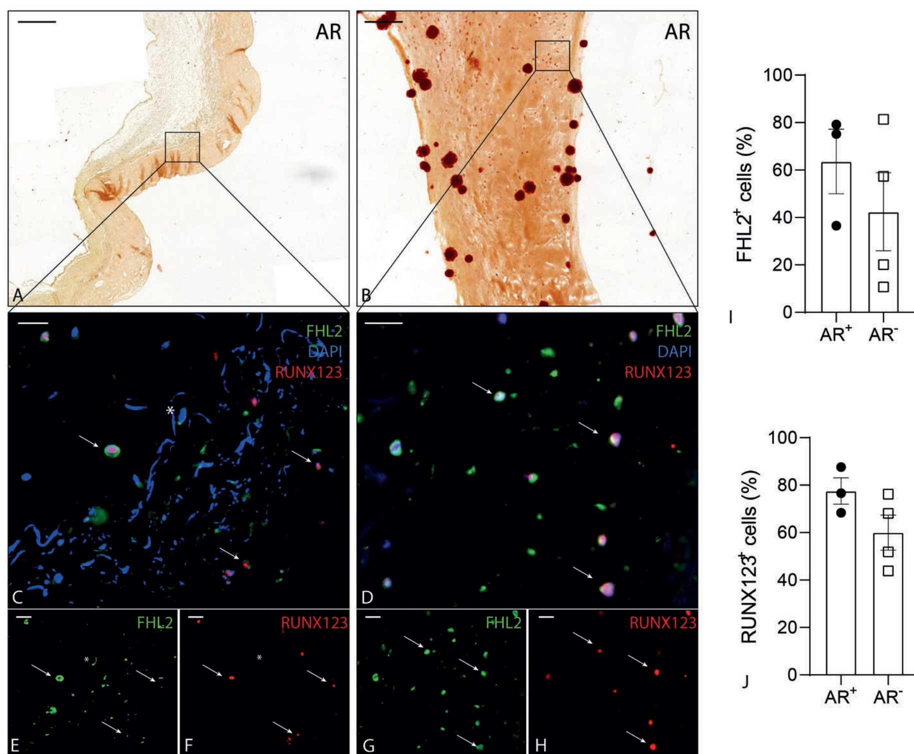

Figure 1. Images of FHL2 and RUNX123 in aortic valve tissue. A,B. Overview images of a non-calcified (A) and calcified (B) aortic valve leaflet stained with AR. C,D. Magnifications of locations indicated in images A (C) and B (D) stained with FHL2, DAPI and RUNX123. E-H. Single channel images of image C (E,F) and D (G,H) for FHL2 (E,G) and RUNX2 (F,H). I,J. Graphs of the quantification of FHL2 and RUNX123 in aortic valve tissue with (AR+) or without (AR-) calcifications. Arrows indicate cells positive for DAPI, FHL2 and RUNX123. * indicates FHL2-DAPI positive cells negative for RUNX123. Scalebar in images A+B indicates 200 μm and in images C-H indicates 20 μm.

Altered location of calcification in FHL2$^{-/-}$ *ex vivo* cultured murine valves

Since human valves in the end-stage of CAVD do not provide mechanistic insights of the role of FHL2 in valvular calcification, we used a miniature tissue culture system (MTCS) to study the role of FHL2 in valvular calcification. Ex vivo culture using the MTCS allows the study of valvular calcification in a natural environment, accurately modeling important factors for CAVD development such as tissue stiffness, strain and cellular and ECM contact [14-20]. Therefore, we cultured WT and FHL2$^{-/-}$ murine hearts *ex vivo* for 7 days in medium supplemented with PI. AR staining showed a diverse range in calcification of the aortic valves of both WT (n=10) and FHL2$^{-/-}$ hearts (n=10), ranging from no calcification to severe calcification (Figure 2A).

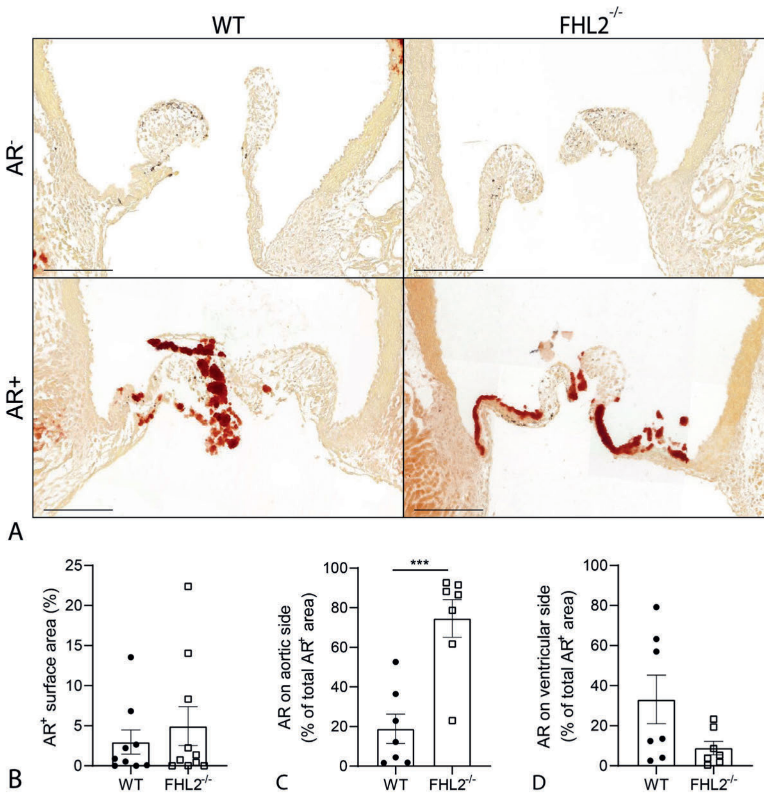

Figure 2. Calcification assay performed on *ex vivo* cultured WT and FHL2-/- aortic valves. A. Representative images of valves stained with AR staining after *ex vivo* culture with PI. A range of phenotypes was observed: valves without calcification (AR-) up to high amounts of calcification (AR+) B. Quantification of calcification in aortic valves cultured *ex vivo* with calcifying medium (WT n=9, FHL2-/- n=10). C. Calcification at the aortic side in WT and FHL2-/- valves (N=7). D. Calcification at the ventricular side in WT and FHL2-/- valves (n=7). Scalebar is 200μm, ***=p<0.001.

When analyzing the total amount of AR^+ area per valve, there was no significant difference between WT and $FHL2^{-/-}$ valves (WT mean=2.97%, SEM:1.51%; FHL2 mean=4.96%, SEM=2.43%; p=0.508) (Figure 2B). Interestingly, when analyzing the localization of the calcified nodules, $FHL2^{-/-}$ valves showed a significant increase of AR^+ surface area at the aortic side of the valves compared to WT aortic valves (WT mean=18.85%, SEM:7.41%; FHL2 mean=74.62%, SEM=9.48%; p=0.0006) (Figure 2C). Complementing this difference is a trend towards a smaller area of calcification at the ventricular side of the valves of $FHL2^{-/-}$ valves compared to WT (WT mean=33.15%, SEM:12.15%; FHL2 mean=8.95%, SEM=3.38%; p=0.079) (Figure 2D). Calcification in the center of the leaflet did not reveal any differences between WT and $FHL2^{-/-}$ valves (data not shown).

Discussion

Since FHL2 is reported to be involved in calcification and cardiovascular pathology, this study aimed to investigate the role of FHL2 in aortic valve calcification. Our results show that human aortic valves with different types and stages of aortic valve disease express FHL2 independent of the degree of calcification of the valve and the expression of the osteogenic transcription factor RUNX2. In addition, absence of FHL2 did not significantly alter the amount of calcification in the aortic valves after 7 days of ex vivo culture in medium supplemented with PI. However, the location of the calcification nodules was significantly different between WT and FHL2-/- ex vivo cultured aortic valves. The calcification nodules in the FHL2-/- valves were mainly located towards the aortic side whereas in the WT valves they were more located at the ventricular side of the valve.

Despite the relation between FHL2 and RUNX2 that has been described previously, to the best of our knowledge, this is the first study investigating the expression of FHL2 and RUNX2 in human aortic valves [11]. We observed FHL2 and RUNX123 expression in the human valve, but were unable to find a relation between FHL2 and RUNX123 expression in the human aortic valve. This appears to be in contrast with several studies indicating that FHL2 expression increases RUNX2 transcriptional activity and thereby calcification [11,13,21]. This apparent contrast could be due to the limitation of tissue samples in demonstrating cause and consequence and/or a temporal difference between FHL2 and RUNX2 expression. In addition, we were unable to relate FHL2 and runx123 to the presence or absence of calcified nodules in the aortic valve.

Although human tissue is the most accurate study sample when investigating protein expression in relation to a disease, it provides limited opportunities for manipulating and investigating the underlying molecular processes. Therefore, we studied the valves from $FHL2^{-/-}$ mice using the MTCS to culture the valves

ex vivo. This allowed us to gain more insight in the role for FHL2 is in valvular calcification whilst keeping the valve in its natural environment [22,23]. Several studies have indicated the importance of the local microenvironment in the calcification process, underscoring the value of maintaining the *in vivo* tissue structure when using the MTCS [14-20]. The results from the *ex vivo* culture experiments demonstrated that not the amount of calcification but the localization is affected by FHL2. The aortic valves of FHL2$^{-/-}$ mice cultured for 7 days in PI showed an increased localization of the calcified area at the aortic side, whereas in the WT valves there is a higher amount of calcification at the ventricular side. The flow on the aortic valves *in vivo* and during *ex vivo* culture is different between the aortic and the ventricular side of the aorta. Similar to diastolic conditions *in vivo*, in *ex vivo* culture the aortic side of the leaflet experiences pressure, the ventricular side is exposed to stretch [23,24]. Interestingly, FHL2 has been shown to translocate to the nucleus upon stretch where FHL2-β-catenin/RUNX2 interactions take place [11,25]. This could explain the differences we observed in the location of calcified nodules between WT and FHL2$^{-/-}$ valves. The FHL2-β-catenin/RUNX2 interactions may be stimulated by the stretch to translocate to the nucleus in WT valves whereas the absence of this translocation and stimulation in FHL2$^{-/-}$ hearts may cause the altered localization of the calcification.

With these experiments we show that FHL2 has the potential to impact on to valvular calcification. To further understand the role FHL2 plays in valvular calcification, it would be interesting to map FHL2 in CAVD in a large patient cohort. Moreover, our data demonstrate an intriguing role for flow and/or strain on FHL2 in the aortic valves requiring research models that accurately mimic the valvular environment.

Materials and methods

Staining of the human aortic valves

Seven human aortic valves were obtained from patients undergoing valvular replacement surgery after consent. The aortic valves were fixated in 4%PFA and embedded in paraffin before sectioning (5μm). To stain for calcification, the sections were deparaffinized and hydrated before the sections were incubated with 2% Alizarin Red. For immunostainings sections were deparaffinized, rehydrated and antigen retrieval was performed by heating the sections to 97°C in sodium citrate buffer (pH 6.0) for 11 minutes. Subsequently, sections were incubated o/n at rt with the primary antibodies (Monoclonal mouse-FHL2 antibody, F4B2-B11 ThermoFisher, 1:250; monoclonal rabbit-RUNX123 antibody; ab92336 Abcam; 1:100) diluted in 1%BSA-PBST before incubating the slides with the secondary

antibodies diluted in 1%BSA-PBST for 40 minutes and 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, D3571 Life Technologies 1:1000) for 5 minutes to stain for nuclei. Sections were mounted using Prolong Gold (Invitrogen, Carlsbad, CA, USA; P36934). Between the staining steps, all sections were rinsed in PBS (2x) and PBST (1x). All slides were scanned with the Panoramic 250 slide scanner (version1.23, 3DHISTECH Ltd.) and visualized using Caseviewer (version2.3, 3DHISTECH Ltd.). FHL2 and RUNX2 positive and negative cells were manually quantified in at least three images from different locations per valve. These quantifications were correlated to the presence or absence of local calcification.

Ex vivo culture of mouse aortic valves

All animal experiments were performed in 2-6 months old mice with a mixed genetic background (B6;129) according to protocols approved by the animal welfare committee of the Leiden University Medical Center and conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mouse hearts were cultured in the MTCS as previously described [22,23]. In short and with the following modifications; mice were anesthetized with 4% isoflurane and the heart was removed and transferred to the perfusion chamber. The inflow needle of the perfusion chamber was inserted into the aorta and ligated with a suture. Flow (1000 ml/min) was introduced using a pump and PI medium (growth medium supplemented with 3mM sodium phosphate (Sigma)) was directed through the aorta towards the closed aortic valve into the coronary circulation. The medium exited the heart via the right atrium and recirculated to the reservoir. The medium was replaced twice a week. After culture for 1 week the hearts were isolated and fixed overnight with 4% PFA/PBS. Fixed mouse hearts were dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin, and sectioned at 6mm. The sections were deparaffinized and hydrated before subsequent staining. For visualization of the calcification, the sections were incubated with 2% Alizarin Red.

Quantification calcification of the *ex vivo* cultured valves

The surface area of the murine *ex vivo* cultured valves positive for AR was measured manually. To calculate the percentage of AR-positive area, the AR positive surface area was divided by the total surface area of the valve leaflets. At least 6 sections with 96 mm interval were used per heart and the measurements were averaged. The location of the calcification was determined by measuring the area of calcification bordering the leaflet edge at the ventricular side of the leaflet, the aortic side of the leaflet or neither (center calcification).

Statistics

Statistical analysis was performed using Graphpad Prism (version 8). Data was tested for significance using Students t-test for unpaired data. Data are reported as mean±SEM. P-value ≤ 0.05 was considered significant.

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