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

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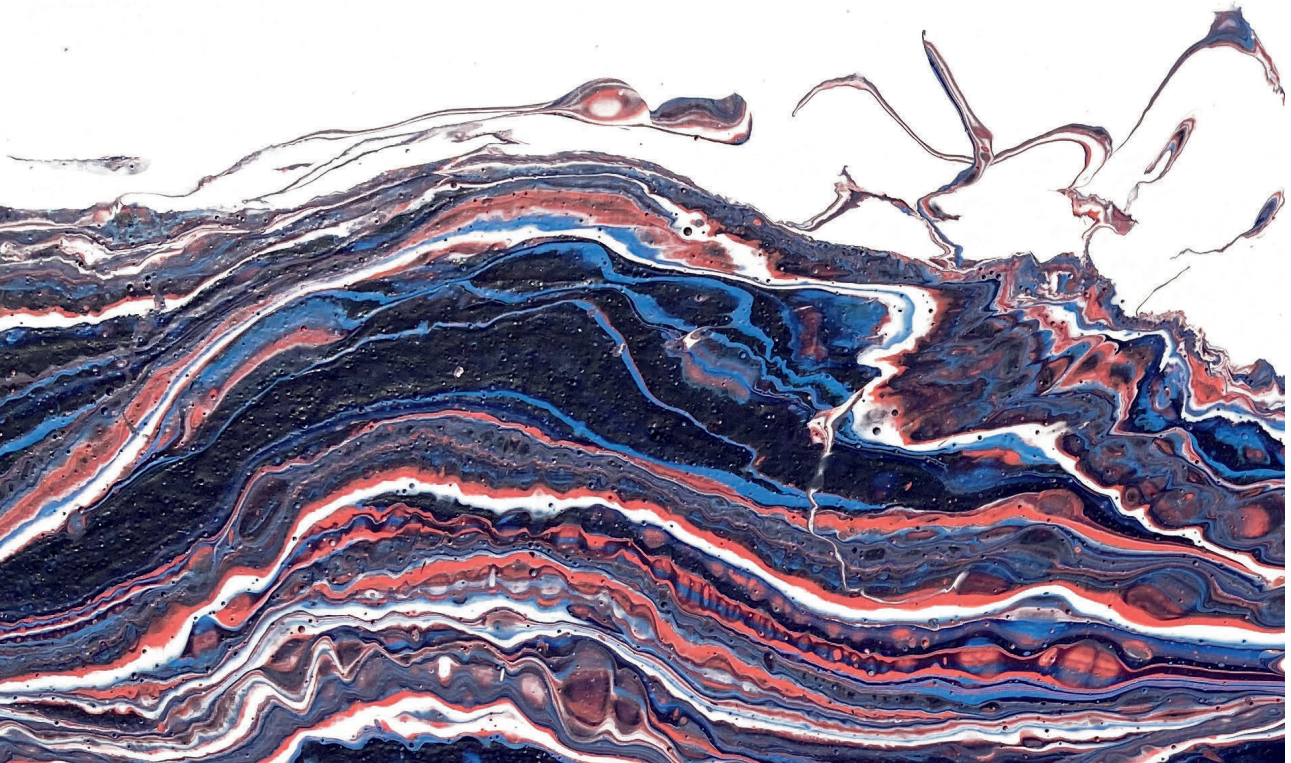

Chapter 8

A role for FHL2 in aortic dilation in BAV patients

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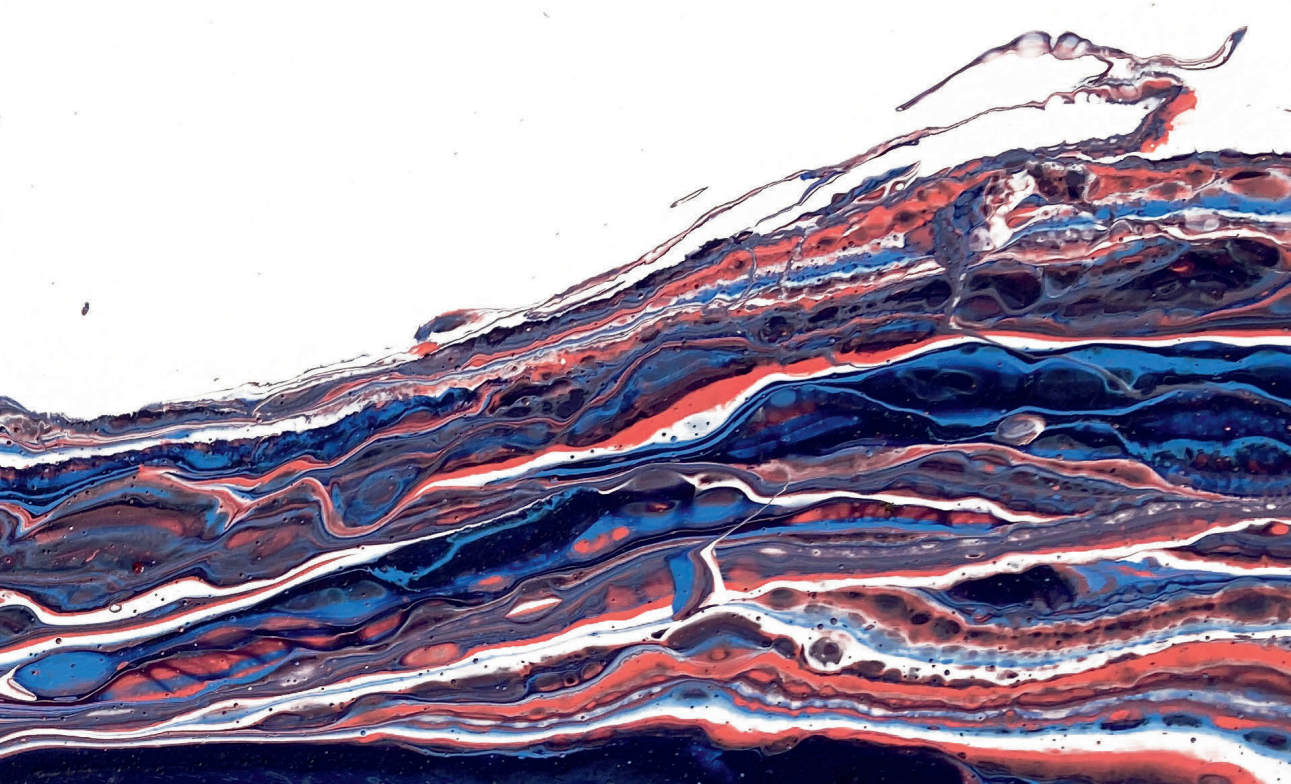
To be submitted



Abstract

Bicuspid aortic valve is the most common congenital heart defect and is associated with increased prevalence of thoracic aortic aneurysm and rupture. During aortic dilation, the structure of the vessel wall is weakened by degeneration of the extracellular matrix and phenotypic switching of the vascular smooth muscle cells (vSMCs) from a contractile to synthetic phenotype. Four-and-a-Half LIM-domain 2 (FHL2) is involved in vSMC phenotypic switching in carotid artery lesions. To investigate whether FHL2 also plays a role in the phenotypic switching of vSMCs in thoracic aortic dilation, we studied the expression of FHL2 in the non-dilated and dilated thoracic aorta of patients with a tricuspid (TAV) and bicuspid aortic valve (BAV). Moreover, we studied the behaviour of aortic vSMCs isolated from FHL2$^{-/-}$ mice and the levels of FHL2 in the conditioned medium of these cells. Finally, FHL2 levels were investigated in plasma from BAV patients with and without dilation.

Immunofluorescent analysis revealed different expression patterns of FHL2 protein in the aortic wall of BAV and TAV patients. While FHL2 was expressed in the vSMCs in the intima, inner media and adventitia, a different pattern was observed in the middle of the media where FHL2 was mainly present in the extracellular space. Areas lacking nuclei and α-smooth muscle actin (αSMA) expression, contained high amounts of extracellular FHL2. Furthermore, our *in vitro* studies showed that FHL2$^{-/-}$ vSMCs lose their contractile phenotype faster than WT vSMCs and that vSMCs secrete extracellular vesicles containing FHL2. Moreover, we showed that FHL2 is present in human plasma. However, because FHL2 plasma levels were highly variable between patients, no relation between FHL2 levels and aortic dilation or other patient characteristics was observed. In summary, we identified an inverse relationship between FHL2 and αSMA as a new and interesting role for FHL2 in vSMC contractility in aortic dilation. Moreover, the presence of FHL2 in extracellular vesicles and peripheral blood suggests a potential extracellular signaling role for this protein.

Introduction

The most common congenital heart defect is bicuspid aortic valve (BAV) in which, instead of the usual 3 leaflets, the aortic valve consists of 2 leaflets [1]. Although a BAV can function normally throughout life, people with BAV have an increased risk of developing an ascending aortic aneurysm, potentially leading to rupture of the aorta [2]. Aortic dilation is caused by degeneration of the extracellular matrix (ECM), the elastic lamellae and phenotypic switching of vascular smooth muscle cells (vSMCs) from a contractile to a synthetic phenotype [3-5].

In a healthy aortic wall, vSMCs have a contractile phenotype; a state in which they express high levels of contractile proteins such as α-smooth muscle actin (α SMA) and SM22α [6,7]. When homeostasis is altered, e.g. after vessel wall damage, vSMCs can undergo a phenotypic switch from a contractile state to a synthetic state [8,9]. Synthetic vSMCs are characterised by a high proliferative and migratory potential and express less contractile proteins than contractile vSMCs [7,10]. Serum response factor (SRF) plays a central and crucial role in vSMC phenotypic switching by regulating both contractile and synthetic gene transcription [7,11-13]. SRF also promotes the expression of the scaffold protein Four-and-a-Half LIM-domain protein 2 (FHL2). Interaction between FHL2 and SRF has been shown to inhibit transcription of SRF-responsive smooth muscle genes and knockdown of FHL2 increased the contractility of vSMCs in a collagen matrix contractile assay [14,15]. FHL2 is reported to modulate the cardiovascular stress response and impact on vSMC contractility in carotid artery lesions [16-19]. However, the role of FHL2 in aortic dilation has not yet been studied. Therefore, this study aims to investigate the role of FHL2 in thoracic aortic dilation in TAV and BAV patients.

Results

FHL2 is expressed throughout the intima, media and adventitia of dilated aorta

To determine the expression of FHL2 in the ascending aortic wall, a staining for αSMA and FHL2 was performed on aortic tissues with a range of different phenotypes from healthy to dilated and severely remodeled. In all samples FHL2 protein was detected, however the pattern and cellular localization differs within the media, adventitia and in the intima depending on the degree of remodeling observed in the aortic wall. In the remodeled intima/inner media, FHL2 was mainly present in vSMCs as indicated by the co-localization of αSMA and FHL2 (Figure 1A). FHL2 and αSMA expression were present at different locations within the cells in the inner media, whereas the expression of FHL2 in αSMA$^+$ cells co-localized with αSMA expression within the vSMCs in severely remodeled intimal tissue (Figure 1A,B). In the media, FHL2 was mainly found extra-

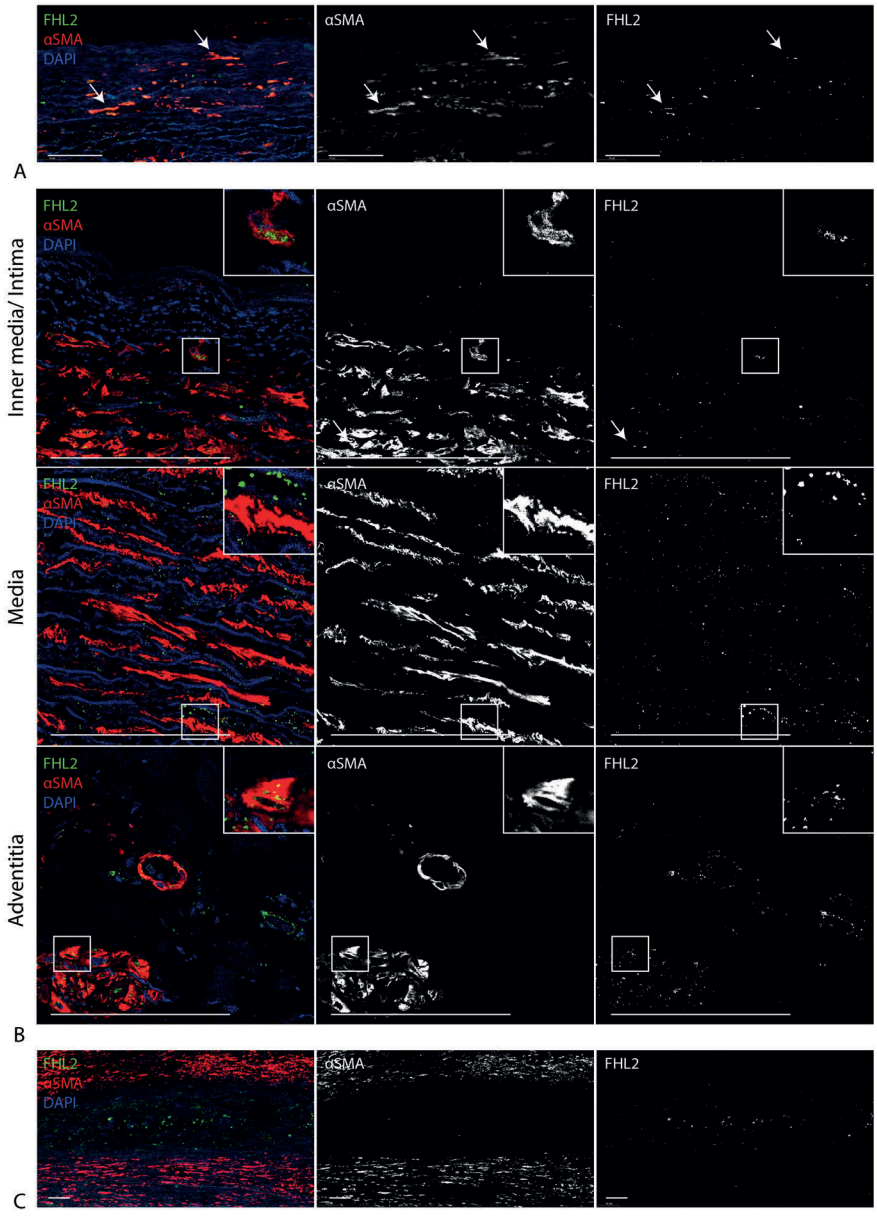


Figure 1. FHL2 expression in the thoracic aortic wall. A. Representative image of a remodeled intima. Arrows indicate areas where αSMA and FHL2 overlap. B. Representative images showing FHL2 and αSMA expression in the inner media, media and adventitia of the vessel. C. Representative image showing a medial area with decreased αSMA and increased FHL2 expression. Bars indicate 100 μm.

cellularly in a speckled pattern. Only very little FHL2 expression was observed within the medial vSMCs. In the adventitia both intracellular and extracellular expression of FHL2 was clearly present. Interestingly, in areas in the middle of the aortic wall with clear medial degeneration (indicated by the absence of αSMA expression, nuclei and elastic lamellae) high expression of FHL2 protein was observed (Figure 1C).

FHL2 is increased in dilated TAV aorta

Large variation in the expression of FHL2 staining could be observed between different aortic samples (Figure 2A). To determine if the amount of FHL2 could be related to specific patient characteristics, we performed a WB analysis on aortic samples from TAV and BAV patients with (D) and without (N) aortic dilation (TAV N n=7, TAV D n=11, BAV N n=8, BAV D n=14). We observed that FHL2 in the aorta of TAV samples with dilation was significantly increased compared to all other groups ($F(3, 36) = 0.355$, $p=0.0155$ ($R^2=0.25$), TAV N $t(16)=2.886$, $p=0.011$, $r=0.34$, BAV N $t(17)=3.486$, $p=0.0028$, $r=0.42$ and BAV D $t(23)=2.362$, $p=0.027$, $r=0.20$).

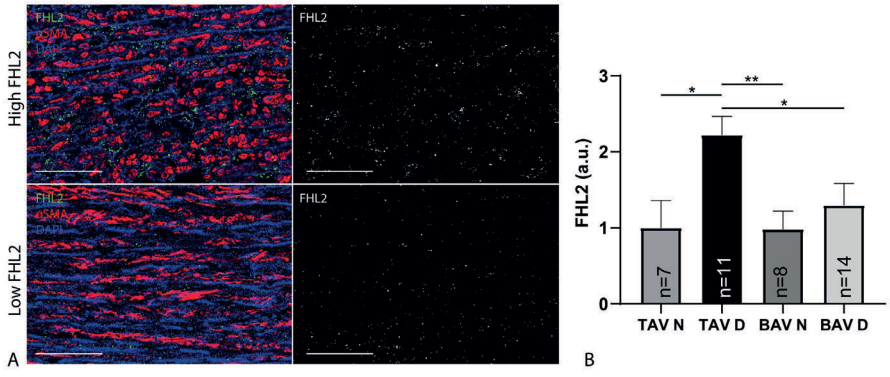


Figure 2. FHL2 is increased in TAV dilated aorta. A. Images showing high and low amount of FHL2 in the media. B. Quantification of FHL2 in aortic patient samples. N= non-dilated, D=dilated. Bars indicate 50 μm. *, P<0.05 **, P<0.01.

FHL2$^{-/-}$ smooth muscle cells lose their contractile phenotype *in vitro*

To explore the relation between FHL2 and the contractility of vSMCs, we cultured aortic vSMCs isolated from FHL2$^{-/-}$ and WT mice. After the first passage, vSMCs from both WT and FHL2$^{-/-}$ mice showed the same elongated morphology (Figure 3A) and expressed αSMA (data not shown). During culture the FHL2$^{-/-}$ vSMCs lost their contractile morphology and became cobble shaped (Figure

3A). qPCR analysis confirmed a significant decrease in αSMA in the FHL2$^{-/-}$ (M=0.0996, SD=0.095) compared to the WT vSMCs (M=1, SD=0.13) (p<0.0001) (Figure 3B).

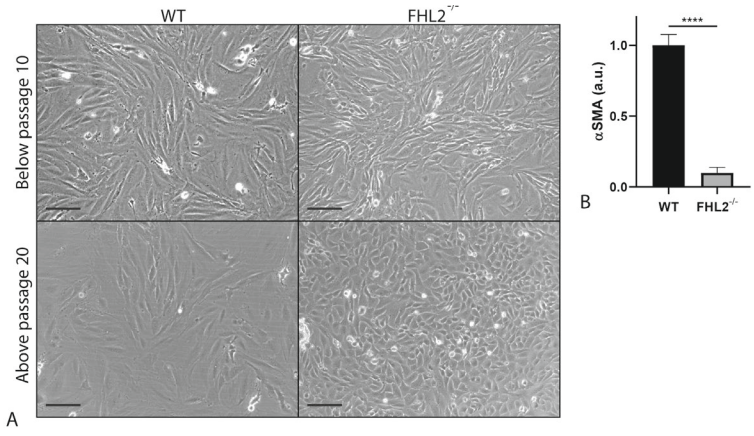


Figure 3. FHL2$^{-/-}$ SMCs lose contractile morphology quicker than WT. A. Representative images of early (below p10) and late passage (above p20) WT and FHL2$^{-/-}$ SMCs. B. qPCR results of αSMA in late passage WT and FHL2$^{-/-}$ SMCs. Bars indicate 200 μm. ****, P<0.001.

FHL2 is secreted in conditioned medium and plasma

As mentioned, FHL2 staining shows a speckled pattern in the aortic media. To determine if FHL2 was secreted extracellularly, WB was performed on extracellular vesicles isolated from vSMC conditioned medium. This confirmed that FHL2 is present in the extracellular fraction (Figure 4A). Moreover, manipulation of the intracellular FHL2 levels by knocking down or overexpressing FHL2 in the vSMCs indicated that the amount of FHL2 in the conditioned medium could be altered (Figure 4B,C).

As FHL2 can be secreted, we determined whether FHL2 was present in plasma from BAV patients and control subjects. Indeed, FHL2 could be detected in human plasma. Moreover, a large variation in the amount of FHL2 between the different samples was observed (Figure 4D). To determine if FHL2 may serve as a biomarker to detect aortic dilation, we determined the levels of FHL2 in plasma of 3 groups: TAV without aortic dilation, BAV patients without aortic dilation and BAV patients with aortic dilation (Table 1). No correlation was observed between the FHL2 plasma levels and aortic dilation or BAV ($H(2) = 0.72$, $p = 0.65$) (Figure 4E). Analysing the data in more detail we observed two distinct groups; one group with a high FHL2 in plasma and another group with a low amount of FHL2 in plasma. However, we were unable to identify which factors, such

as gender, age, height, weight or medication use of the patients, discriminates between the high or low FHL2 plasma levels.

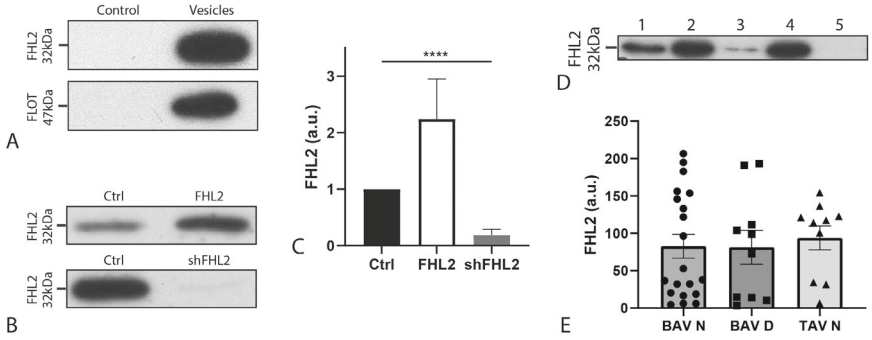


Figure 4. FHL2 in CM and extracellular vesicles. A. WB for FHL2 and FLOT on vesicles and vesicle deprived medium (control) conditioned by haSMCs. B. WB for FHL2 on haSMC conditioned medium with overexpression and knockdown of FHL2. C. Quantification of WB results for FHL2 on medium conditioned by SMCs. D. Representative image of WB for FHL2 on patient plasma samples. E. Quantification of WB for FHL2 on patient plasma samples. N=non-dilated, D=dilated, a.u.= arbitrary units. ****, P<0.001.

Table 1. Patient characteristics

Group (N)	Age (mean,SD)	Height (mean cm,SD)	Weight (mean,SD)	Gender (male%)
TAV N (10)	37.6, 9.6	176.0, 7.1	77.4, 13.6	50
BAV N (20)	35.7, 12.9	171.6 , 13.1	70.0, 10.6	45
BAV D (10)	51.0, 7.0	187.2, 8.1	91.1,16.7	100

Discussion

In the present study, we determined if the scaffold protein FHL2 is involved in aorta dilation by analyzing the expression of FHL2 in vSMCs in relation with αSMA, vSMC contractility and the dilating aorta. We detected FHL2 throughout the aortic wall either in vSMCs or in the extracellular space. Interestingly, especially in areas of severe medial degeneration, the expression level of FHL2 was very high. Although all aortic walls showed FHL2 expression, the amount of FHL2 was significantly higher in the dilated TAV aorta compared to non-dilated TAV aorta, dilated and non-dilated BAV aorta. Furthermore, FHL2 was secreted by the vSMCs in extracellular vesicles and present in human plasma.

We found increased FHL2 protein levels in dilated TAV aortas compared to non-dilated TAV aortas. Interestingly, this increase was not found in BAV dilated aorta compared to non-dilated BAV aorta samples. Since FHL2 is involved in a wide variety of processes, a difference in expression could be related to or caused by one of the multiple differences known between TAV and BAV aortas. For example; FHL2 impacts smooth muscle cell contractility and it is known that BAV aortic tissue contains less αSMA than TAV aortas [14,15,20]. Furthermore, FHL2 can influence the proliferation of cells and, in dilated aortas, the gene expression profile of TAV vSMCs indicate a more proliferative phenotype than BAV vSMCs [21,22]. In line with previous studies on carotid artery vSMCs, our *in vitro* studies suggest that increased levels of FHL2 stimulate a contractile phenotype in aortic vSMCs [17]. Therefore, the increased expression of FHL2 in the dilated TAV aorta could be an attempt of vSMCs to maintain or regain a contractile phenotype. The lack of an increased FHL2 expression in BAV dilated aortas could therefore play a role in the increased prevalence of aortic aneurysms in BAV patients. [15,17].

Not only the amount, but also the expression pattern of FHL2 showed interesting differences. FHL2 in the aorta was expressed intracellularly in vSMCs in the inner media and adventitia while in the middle media and adventitia FHL2 was expressed extracellularly. Our finding that FHL2 can be secreted by vSMCs could explain the speckled FHL2 expression pattern in the media and adventitia of the dilated aorta. Interestingly, the expression of intracellular FHL2 in the intima and adventitia may also be stimulated by different mechanisms and have different effects than the extracellular FHL2 in the media. FHL2 secretion has also been reported for other cell types such as myoblasts and mesenchymal cells [23,24]. Since vSMCs can secrete FHL2 and FHL2 expression is increased in the dilated TAV aorta, we hypothesized that plasmic FHL2 levels could serve as a biomarker for aortic dilation. Therefore, we compared FHL2 plasma levels of TAV controls to BAV patients with and without aortic dilation. Unfortunately, we were unable to correlate the level of FHL2 in plasma to the degree of aortic dilation, which might be due to the number of patient samples analysed. Increasing the number of patients might improve sensitivity. However, the presence of two distinct groups in the data (high and low plasmic FHL2 levels) would imply that plasmic FHL2 levels are related to a common factor. We were unable to relate the plasmic FHL2 levels to factors such as gender, weight, height, age or use of medication. For future research we aim to include TAV patients with a dilating aorta, as we found an increased expression of aortic FHL2 in this group. Unfortunately, we were unable to include plasma samples of these patients into our study.

In conclusion, FHL2 is secreted by vSMCs into the aortic extracellular space and into the circulation In local areas with severe medial degeneration and in TAV aortic dilation, FHL2 expression is increased. These results highlight the intricate interaction FHL2 has with vSMC contractility.

Material and methods

Aortic samples

Ascending aortic wall sample collection and handling was carried out according to the official guidelines of the Medical Ethical Committee of Leiden University Medical Center (LUMC), Leiden, and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (www.FMWV.nl).

In short, material was collected during surgery from TAV and BAV patients with and without aortic dilation (defined by > 44mm). After 24hr fixation in 4% formalin, samples were decalcified in a formic acid buffer (120hr), embedded in paraffin and sectioned transversally (5μm).

Cell culture

Human aortic vSMCs were isolated from aortic tissue as described previously [25]. Informed consent was obtained according to the protocols of the Medical Ethical Committee of the Academic Medical Center (Amsterdam). The cells were cultured in DMEM:F12 supplemented with 10% fetal bovine serum (FBS) and PenStrep (100U/ml, Gibco). Cells were transduced with lentivirus containing FHL2-CMV overexpression construct [26], GFP-CMV control, shFHL2 [27] or PLKO1 control (Mission Library, Sigma) via o/n incubation and refreshing the medium the next day. Cells were refreshed at least 3 times before starting the experiments. To condition medium, cells were incubated with DMEM:F12 for 24 hours after which the medium was collected and used for WB or extracellular vesicle isolation. Mouse aortic vSMCs were isolated from WT and FHL2$^{-/-}$ mice via collagenase digestion and dissociation as described previously and approved by an independent animal ethic committee of the Amsterdam Medical Center (Amsterdam, permit number DBC102226) [17]. Cells were cultured in DMEM supplemented with 20% FBS and PenStrep. Expression of αSMA was confirmed at P1 via qPCR.

Plasma samples

Venous blood samples were collected from patients after informed consent at the Erasmus University Medical Center (Rotterdam). The study has been approved by the local Medical Ethical committee (MEC14-225). We included adult patients

with BAV and with and without aortic dilation defined by sinus of Valsalva ≥40 mm and/or aortic size index ≥2.1 cm/m^2 (ascending aorta). TAV control samples were isolated from people without known cardiovascular diseases. Exclusion criteria were: age <18 years, previous valve or aortic replacement, pregnancy or not capable of understanding or signing informed consent. Peripheral blood was collected in EDTA coated tubes and plasma was isolated by centrifuge for 10 min at 2000G. Afterwards it was stored at -80 °C within two hours after withdrawal.

Extracellular vesicle isolation

Conditioned medium (approximately 80 ml) was spun for 15 min at 2000G, 4°C. The supernatant was carefully isolated without disturbing the pellet and subsequently spun at 10.000G, 4°C for 30 min to further clear debris. To isolate the vesicles the debris-free supernatant was spun 70 min ultracentrifuge at 100.000G, 4°C. The pellet containing the vesicles was washed by adding PBS and isolated by centrifugation for 70 min using the ultracentrifuge at 100.000G, 4°C. Finally, the pellet was resuspended in PBS.

Immunohistochemistry

Sections were deparaffinised, 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 room temperature with the primary antibodies (Monoclonal mouse-FHL2 antibody, F4B2-B11ThermoFisher, 1:250; Rabbit-αSMA, ab5694, Abcam, 1:500) diluted in 1%BSA-PBST before incubating the samples 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).

Protein isolation and Western blot

To isolate protein from the aortic samples and cells, a lysis step was used. Both crushed aortic samples and cells were lysed in cold radio immunoprecipitation (RIPA) lysis buffer supplemented with protease inhibitors (Complete protease inhibitor cocktail, 11697498001, Roche Diagnostics, Basel, Switzerland). Protein concentration was determined using BCA protein assay (23225, ThermoFisher Scientific, Waltham, MA, USA). Equal amounts of protein were diluted using 5x sample buffer. To perform WB on (the extracellular fraction of) conditioned medium and plasma the lysis step was not required. For these samples, equal volumes were supplemented with 5x sample buffer (CM cleared from debris by

centrifugation step, plasma 1:1000 diluted in 1x SB). Ponceau staining was performed to check for equal loading. From this point, all samples follow the same protocol. After 5 minutes at 98°C, the samples were loaded onto and separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (IPVH00010, Merck). Membranes were blocked in Tris-buffered saline, 0.2% Tween-20 (TBST) containing 10% dry milk and incubated overnight with primary antibodies (FHL2: Abcam, ab202584, 1:2000; FLOT: Santa Cruz, SC-25506, 1:1000; αSMA: Sigma, A2547, 1:10.000) diluted in 5% BSA in TBST. Membranes were washed 3 times with TBST and incubated with a horseradish peroxidase-conjugated goat-anti-rabbit antibody (31458, ThermoFisher Scientific) diluted 1:10.000 in 10% dry milk in TBST. Membranes were washed with TBST again after which protein expression was detected using enhanced chemiluminescence (WesternBright Quantum, Advanstra) and visualized on x-ray film (Fuji film).

mRNA isolation and quantitative RT-PCR

RNA was isolated using the ReliaPrep RNA cell miniprep kit (Z6012, Promega) according to the manufacturer's protocol. RevertAid First Strand cDNA Synthesis (K1622, ThermoFisher Scientific) was used to generate cDNA according to the manufacturer's protocol, after which qRT-PCR was performed using GoTaq qPCR Master Mix (A6001, Promega).

Table 2. Primers

αSMA	FW: 5′-TCGGTGGCTCCATCCTGGCT-3′	RV: 5′-TGCTAGAGGCAGAGCAGGGGG-3′
GAPDH	FW: 5′-AGCCACATCGCTCAGACAC-3′	RV: 5′-GCCCAATACGACCAAATCC-3′
ARP	FW: 5′-CACCATTGAAATCCTGAGTGATGT-3′	RV: 5′-TGACCAGCCGAAAGGAGAAG-3′

Statistical analysis

All results were obtained in 3 independent experiments, unless otherwise mentioned. Statistical assays were performed using Graph Pad Prism (version 7). Correlations were tested using One-Way ANOVA (WB aortic FHL2) or Kruskal-Wallis test (patient plasma FHL2) when required. All other statistical tests were performed using Students t-test for unpaired samples. P-values < 0.05 were considered significantly different.

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