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An endogenous inhibitor of angiogenesis downregulated by hypoxia in human aortic valve stenosis promotes disease pathogenesis

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ABSTRACT

Aortic valve stenosis is the most common valve disease in the western world. Central to the pathogenesis of this disease is the growth of new blood vessels (angiogenesis) within the aortic valve allowing infiltration of immune cells and development of intra-valve inflammation. Identifying the cellular mediators involved in this angiogenesis is important as this may reveal new therapeutic targets which could ultimately prevent the progression of aortic valve stenosis. Aortic valves from patients undergoing surgery for aortic valve replacement or dilation of the aortic arch were examined both ex vivo and in vitro. We now demonstrate that the anti-angiogenic protein, soluble fms-like tyrosine kinase 1 (sFlt1), a non-signalling soluble receptor for vascular endothelial growth factor, is constitutively expressed in non-diseased valves. sFlt-1 expression was, however, significantly reduced in aortic valve tissue from patients with aortic valve stenosis while protein markers of hypoxia were simultaneously increased. Exposure of primary-cultured valve interstitial cells to hypoxia resulted in a decrease in the expression of sFlt-1. We further reveal using a bioassay that siRNA knock-down of sFlt1 in valve interstitial cells directly results in a pro-angiogenic environment. Finally, incubation of aortic valves with sphingosine 1-phosphate, a bioactive lipid-mediator, increased sFlt-1 expression and inhibited angiogenesis within valve tissue. In conclusion, this study demonstrates that sFlt1 expression is directly correlated with angiogenesis in aortic valves and the observed decrease in sFlt-1 expression in aortic valve stenosis could increase valve inflammation, promoting disease progression. This could be a viable therapeutic target in treating this disease.

1. Introduction

Aortic Valve Stenosis (AVS) is the world's most common disease of the heart valves, affecting 3% of the population over the age of 65 in the developed world [1]. The prevalence of AVS increases with age and with longer life-expectancies, the significant burden that it carries is likely to increase [2]. There is currently no pharmaceutical treatment for AVS, with surgical insertion of a new prosthetic valve the only option once disease progression has reached a critical stage [3,4]. Thus, the requirement for non-invasive, medical treatments which could slow or halt the progression of AVS remains an important unmet clinical need.

Healthy aortic valves consist of thin valve leaflets and are almost completely avascular. An important early process in the pathogenesis of AVS is valve thickening, known as aortic sclerosis, promoted by inflammatory signals and cellular proliferation [5,6]. As aortic sclerosis progresses, there is a significant increase in blood vessels within the valve leaflet [5,6]. Although the development of new blood vessels (angiogenesis) is typically driven by hypoxia, there are no previous mechanistic studies of hypoxia and the associated angiogenesis in AVS [6,7]. Inhibition of angiogenesis is, however, a potentially attractive target in the treatment of AVS and has previously been targeted in other diseases, including cancer [8,9].

Hypoxia drives angiogenesis through hypoxia-inducible factor 1a (HIF-1α). Under normoxic conditions, HIF1α is rapidly degraded [10]. However, with the onset of hypoxia, this degradation is prevented and HIF1α forms a heterodimer with hypoxia inducible factor 1b (HIF-1β)

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[11]. This protein complex will then translocate to the nucleus where it acts as a transcription factor. Several genes regulated by HIF1α are important for angiogenesis, including vascular endothelial growth factor (VEGF) family [12,13]. The VEGF family includes five growth factors, all of which carry roles in the development of new blood or lymphatic vessels [14,15]. The VEGF family bind to specific tyrosine kinase receptors of the VEGF receptor family [16]. One of these receptors is soluble fms-like tyrosine kinase 1 (sFlt1), a soluble, alternatively spliced, isoform of the membrane bound VEGF receptor 1 (VEGFR1/Flt1) [17,18]. sFlt1 differs from VEGFR1 as it lacks a transmembrane and intracellular kinase domain, thus having a unique C-terminus [19]. sFlt1 does, however, carry an extracellular Ig-like domain identical to VEGFR1. This means that sFlt1 can bind and block the signalling of VEGF isoforms, thus preventing angiogenesis [20]. sFlt1 has previously been implicated as an important factor in some diseases, including pre-eclampsia [21–23]. As sFlt-1 is a soluble receptor, it is also present in plasma [25]. It is produced in several different cell types and released via unknown mechanisms into the extracellular space, where it can bind VEGF [24].

This study has examined the effect of hypoxia on the aortic valve tissue during the pathogenesis of AVS, investigating its role as a driver of angiogenesis via VEGF-dependent cellular signalling. The results demonstrate that hypoxia is key to the progression of angiogenesis in AVS, in part, via regulation of the soluble VEGF receptor sFlt1 expression. We also delineate sFlt1 as an angio-static factor critical in the maintenance of normal aortic valve avascularity. Furthermore, we show that upregulation of sFlt1 expression and the subsequent inhibition of angiogenesis in the aortic valve can be achieved via sphingosine 1-phosphate, thus highlighting sphingosine 1-phosphate receptors as novel drug targets to slow the progression of AVS, subsequently inhibiting angiogenesis in the aortic valve.

2. Materials and methods

2.1. Aortic valve tissue

Predominately, aortic valves were collected from patients undergoing aortic valve replacement surgery within the cardiothoracic surgery department at Aberdeen Royal Infirmary. Non-stenotic aortic valves were obtained from patients undergoing valve replacement surgery due to developmental conditions that cause dilation of the aortic arch. The aortic valve tissue taken from patients with aortic arch dilation displayed no sign of hypertrophy or stenosis [26]. All tissue was collected after informed written consent from participants. These procedures were approved by the local ethics committee (National Health Service Grampian Research & Development, project number 10/S0802/53). Unless otherwise stated, this valve tissue was used for all experiments except immunohistochemistry. For immunohistochemistry, tissue was obtained from the NHS Grampian Biorepository. Valves were not age or sex matched.

2.2. Cell culture

Human aortic valvular interstitial cells (VICs) were cultured using an explant method. Human aortic valve tissue was dissected in a laminar-flow hood under sterile conditions. The endothelial layer was removed by mechanical dissociation. Sections of aortic valve tissue (1 mm^3) were plated onto 6-well culture plates in Dulbecco's Modified Eagle Media (Sigma-Aldrich, UK) supplemented with 20% foetal bovine serum (Biowest, USA), 1% L-Glutamine (Sigma-Aldrich, UK) and 1% penicillin/streptomycin (Sigma-Aldrich, UK). VICs were grown onto 6-well plates from aortic valve tissue for 7–14 days before being transferred into cell culture flasks. VICs were maintained and used until passage 7. Cells were incubated at 37 °C, 5% CO2.

Human umbilical vein endothelial cells (HUVECs) were maintained in Dulbecco's Modified Eagle Media with 10% foetal bovine serum, 1% L-glutamine and 1% Penicillin/Streptomycin and passaged upon reaching 80–90% confluency.

In order to create a hypoxic environment for cells in vitro, a ProPx C21 hypoxic incubation chamber (BioSpherix) was used. The hypoxic chamber was placed inside a regular incubator and set to 1% O2 and 5% CO2 with dH2O to humidify the chamber. This O2 level was selected to replicate the hypoxic environment seen in AVS and cells were incubated for 24 h.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed on both diseased aortic valves (from patients undergoing valve replacement surgery and healthy control aortic valves (from patients undergoing surgery for aortic regurgitation). Sections (10 μm thick) were cut from formalin-fixed paraffin-embedded tissue. After removal of the paraffin the samples underwent heat-induced epitope retrieval in 20 mM citrate buffer (pH 6.1) (Aligent, UK). All samples were stained using the Dako Autostainer (Aligent, UK). Post-staining samples underwent an H&E counterstain.

Slides were imaged on Zeiss AxioscanZ1 slide scanner (Carl Zeiss AG, Deu) and processed using the Zen Blue software (Carl Zeiss AG, Deu). Analysis was performed either by quantifying macro structures (neovessels). Alternatively, when quantifying the expression level of sFlt1 an immunoreactivity score (IRS) was used. IRS is calculated by 'Positive cells score' x 'Staining Intensity' = IRS score, on a scale of 1–12 (For % of positive cells 1 $\leq$ 10% cells, 2 = 10–50% positive cells, 3 = 51–80% positive cells, 4 $\geq$ 80% positive cells. For staining intensity 1 = mild reaction, 2 = moderate reaction, 3 = intense reaction).

The following antibodies were used; rabbit anti-VEGF Receptor 1 (soluble) (1:200, #36–1100 Thermo Fisher, USA), rabbit anti-VEGF-A (1:1000, ab46154, Abcam, UK), mouse anti-HIF1α (1:500, ab1, Abcam, UK). Control tissue was included for each sample. Human placental tissue was used as a control for anti-VEGF Receptor 1 (soluble), human lung was used for anti-VEGF-A and human lung carcinoma was used for anti-HIF1α. Optimisation runs were performed to determine the optimal concentration for each antibody on each control tissue. Negative controls with only secondary antibody added were used on each run with control tissue.

2.4. Immunofluorescence microscopy

Aortic valve tissue was fixed in 4% paraformaldehyde, incubated with 15% sucrose at 4 °C for 60–90 min and rapidly frozen in super-cooled Freon-22. The frozen was transferred to liquid nitrogen for storage. Tissue was embedded in Tissue-Tek 4583 OCT compound (Fischer Scientific) and sections were cut at 10 μm thickness. Frozen aortic valve tissue was cryosectioned using a Lecia CM1900 cryostat (Lecia, Milton Keynes, UK). Sections were transferred to poly L-lysine-coated slides and incubated with 3% BSA/PBS and primary antibodies: mouse anti-smooth muscle a-actin (1:1000 dilution, MAB1420 R&D Systems USA) and rabbit anti-VEGF Receptor 1 (soluble) (1:200, #36–1100 Thermo Fisher, USA) overnight at 4 °C. Control sections had either no primary antibody added and species-specific serum (to the same dilution as primary antibodies). Sections were washed 3 × 3% BSA/PBS and incubated with secondary antibody for 1 h at room temperature. F(ab')2 fragment of IgG Alexa Fluor 488- and IgG Alexa Fluor 546-conjugated secondary antibodies were used (1:500 dilution). Sections were washed 3 × 3% BSA and 1 x PBS. The nuclear stain DAPI was added. Vectashield mounting medium was applied to prevent photobleaching, coverslip added and slides sealed. Slides were visualised under confocal microscopy (Zeiss) using Zen software. Immunofluorescence was quantified using Image J.

2.5. Immunoblotting

Proteins from primary cultured VICs and from ex vivo aortic explants

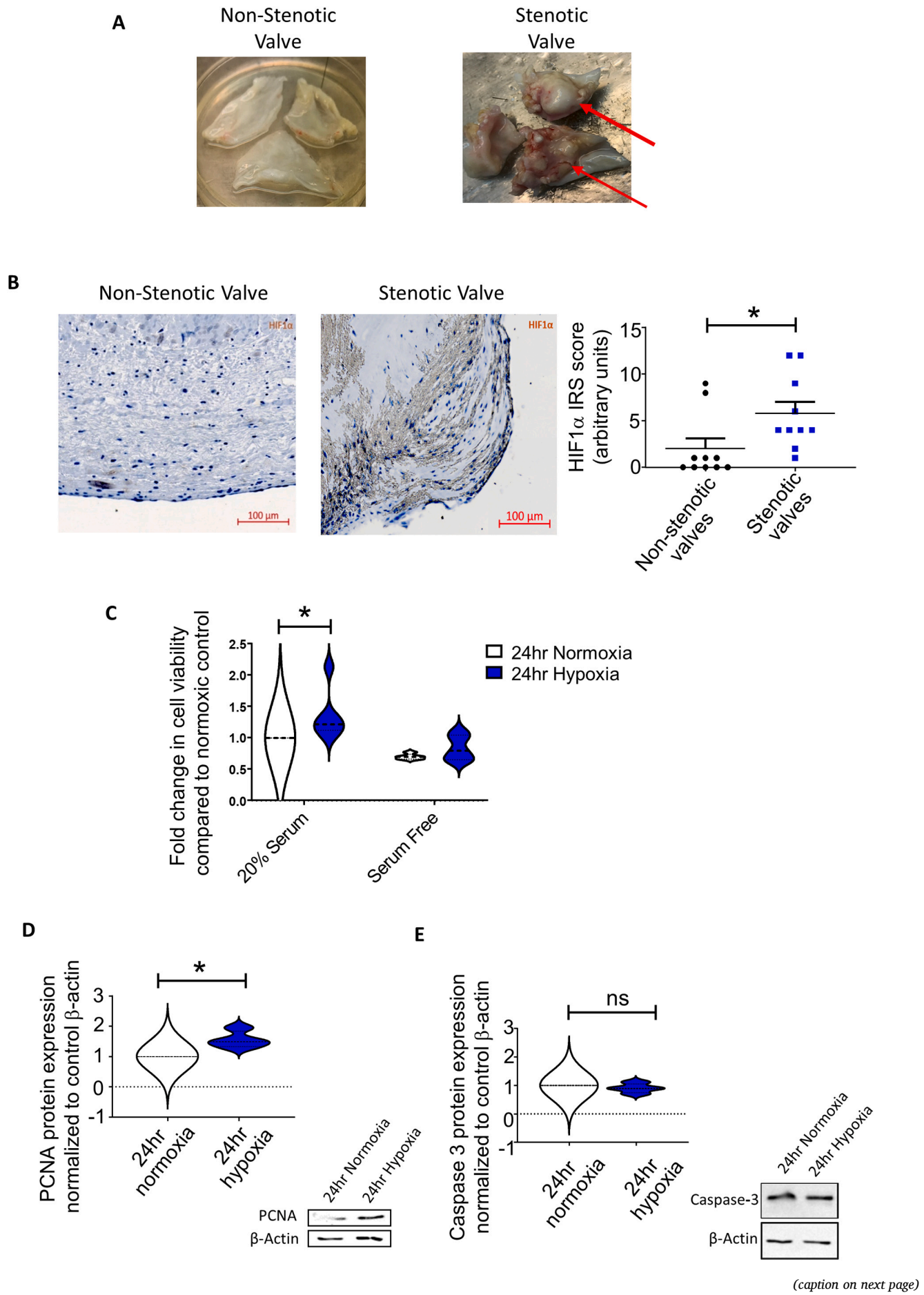

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Fig. 1. Hypoxia drives the proliferation of human aortic valve interstitial cells, contributing to early disease progress. (A) Representative images of both non-stenotic and stenotic aortic valves which were obtained from patients undergoing surgery. Red arrows represent calcified nodules in the stenotic aortic valve. (B) Immunohistochemistry of HIF1α on non-stenotic (healthy) human aortic valves and stenotic (diseased) aortic valves. Quantification of HIF1α was performed using immunoreactivity score ($n = 10$). (C) MTT assay analysing cell proliferation of human valvular interstitial cells (VICs) isolated from stenotic aortic valves incubated for 24 h in normoxia or hypoxia in either 20% or 0% serum ($n = 6$). (D) Immunoblot analysis of cell proliferation marker PCNA in human VICs incubated for 24 h in normoxia or hypoxia, both in 20% serum, with representative immunoblot images shown, normalised to β-actin loading control ($n = 4$). (E) Immunoblot analysis of cell apoptosis marker caspase-3 in human VICs isolated from stenotic aortic valves incubated for 24 h in normoxia or hypoxia, both in 20% serum, with representative immunoblot images shown, normalised to β-actin loading control (n = 4). Results are presented as the mean ± SEM. *P* values in panels B, D, E were determined by students *t*-test or non-parametric equivalent. p valve in panel C was determined by 2-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$. Each 'n' number in panels C, D and E represents cells derived from valves of a different patient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were extracted using RIPA lysis buffer containing cOmplete™ Protease Inhibitor Cocktail (Roche, Sus). Protein samples (10 μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane sheets (GE Healthcare Life Sciences, USA) using standard protocols. Proteins immunodetected were visualised using an enhanced chemiluminescence (ECL) kit (Bio-Rad Laboratories, USA) and quantified by densitometry scanning using ImageJ software (National Institutes of Health, USA). The following antibodies were used: rabbit anti-VEGF-A (1:1000, ab46154 Abcam, UK), goat anti-VEGFR1/Flt-1 (1:200, AF321 R&D Systems, USA), mouse anti-PCNA (1:1000, 2586, Cell Signalling Technology, USA), rabbit anti-Caspase-3 (1:1000, 9662, Cell Signalling Technology, USA). Rabbit anti-GAPDH (1:1000, #2118 Cell Signalling Technology, USA) and rabbit anti-beta Actin (1:1000, #4970 Cell Signalling Technology, USA) were used as loading controls. The following secondary antibodies conjugated to peroxidase were used: goat anti-rabbit (1:5000, ab97080 Abcam, UK), goat anti-mouse (1:5000, ab97040 Abcam, UK), donkey anti-goat (1:5000 Abcam, UK).

2.6. siRNA knockdown

VICs were transfected with siRNA directed to human sFlt1 (Santa Cruz Biotechnology, sc-270,075, USA) or Control siRNA-A (sc-37,007). sFLT-1 siRNA targets the unique c-terminus of sFLT-1 and does not therefore target mFLT-1. siRNA transfections were performed using Lipofectamine® 2000 (Thermo Fisher, USA) and Opti-MEM I Reduced Serum Medium (Thermo Fisher, USA). siRNA was used at a final concentration of 30 nM siRNA. Cells were then incubated for 24 h before being transfected at the same concentration for a second time to increase knock-down efficiency. Four hours after the second transfection the cell media was changed to Endothelial Cell Media (PromoCell, Deu). 24 h after this media changed the media was collected and stored at −70 °C for future use in tubule formation assays. At this point protein from the transfected, VICs were harvested to assess the knockdown efficiency of sFlt1 by Western blotting.

2.7. Tubule formation assay

Endothelial cell tubule formation (angiogenesis) assays were performed using HUVECs with conditioned Endothelial Cell Media, collected from VICs exposed to hypoxia or siRNA transfection. Wells from 96-well plates were coated with 50 μL of Matrigel® basement membrane matrix LDEV-free (Corning, USA). HUVECs are then plated into coated wells at a density of 1500 cells/well in 100 μL of the appropriate media and incubated at 37 °C, 5% CO2 for six hours. Each well is then imaged on the EVOS XL microscope (Thermo Fisher, USA) and analysed using the Angiogenesis Analyzer for ImageJ (Giles Carpentier) plug-in within the ImageJ software.

2.8. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for human VEGF (Invitrogen, USA) was performed on supernatant collected from VICs exposed to hypoxia. The assay was performed according to manufacturer's instructions. Range of measurable values were 15.6–1000 pg/mL.

2.9. Quantitative polymerase chain reaction

RNA was extracted from VICs exposed to hypoxia using the RNeasy kit (QIAGEN, Deu) according to manufacturer's instructions. cDNA was made from the extracted RNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Sus). Quantitative real-time polymerase chain reaction was performed using SYBR green chemistry with Takyon ROX 2× MasterMix dTTP Blue (Eurogentec, Bel). Samples were run using the StepOne Plus Real-Time PCR System (Applied BioSystems, USA). qPCR was analysed using the StepOne Plus software (Applied Biosystems, USA). VEGF-A and β-Actin primers were used as previously described [27,28]. S1PR receptor primers were used as follows, S1PR1 Forward: 3′ – CTCCGTGTTCAGTCTCCTCG – 5′, S1PR1 Reverse: 3′ - ATTGCTC CCGTTGTGGAGT – 5′. S1PR2 Forward: 3′ – TCGGCCTTCATCGT-CATCCTCT – 5′, S1PR2 Reverse: 3′ – CCTCCCGGGCAAACCACTG -5′. S1PR3 Forward: 3′ – CTTGGTCATCTGCAGCTTCATC – 5′, S1PR3 Reverse: 3′ - TCATTGTCAAGTGCCGCTCGAT – 5′. S1PR4 Forward: 3′ – GACGCTGGGTCTACTATTGCC – 5′, S1PR4 Reverse: 3′ - CTCCCGTAG-GAACCACTG – 5′. S1PR5 Forward: 3′ - GCGCACCTGTCCTGTACTC – 5′, S1PR5 Reverse: 3′ – GTTGGTGAGCGTGTAGATGATG – 5′. 28S primers were also used as a housekeeping gene as follows, 28S Forward: 3′ – GCCTAGCAGCCGACTTAGAA – 5′, 28S Reverse: 3′ – AAATCA-CATCGCGTCAACAC – 5′. All primers were manufactured by Sigma-Aldrich, UK.

2.10. MTT cell viability assay

VICs were plated at 10,000 cells/well in 100 μL of defined media, all conditions were performed in triplicate. MTT assay was performed as manufacturer's instructions using the Invitrogen MTT Cell Viability kit (Invitrogen, US). Serum starvation was used as a control experiment to determine whether hypoxia could alter cell viability in the absence of serum. The absorbance level of each well was then read on a BMG Labtech Plate Reader at a wavelength of 590 nm. Data was analysed using the Fluostar Omega software.

2.11. Preparation of sphingosine 1-phosphate

Lyophilised sphingosine 1-phosphate (S1P) was solublised in methanol at a stock concentration of 1 mM. Once solubilised, S1P was stored in 50 μL aliquots at −20 °C. To prepare S1P for use it was first incubated in a 37 °C water bath for 20 min and then vortexed. The solvent was then flushed from the aliquots under nitrogen gas, which leaves the compound in the Eppendorf tube. The S1P was then reconstituted in 10% DMSO prepared within 4 mg/mL bovine serum albumin (BSA), vortexed thoroughly again for 2 min and then placed back in the water bath at 37 °C for 30 min prior to use. S1P was used at an experimental range between 10 nM and 10 μM. As a vehicle control 10% DMSO in a 4 mg/mL BSA was used and diluted appropriately.

2.12. Aortic valve ex vivo angiogenesis assay

Fresh aortic valve leaflets from patients undergoing valve replacement surgery were dissected under sterile conditions into 1 mm3

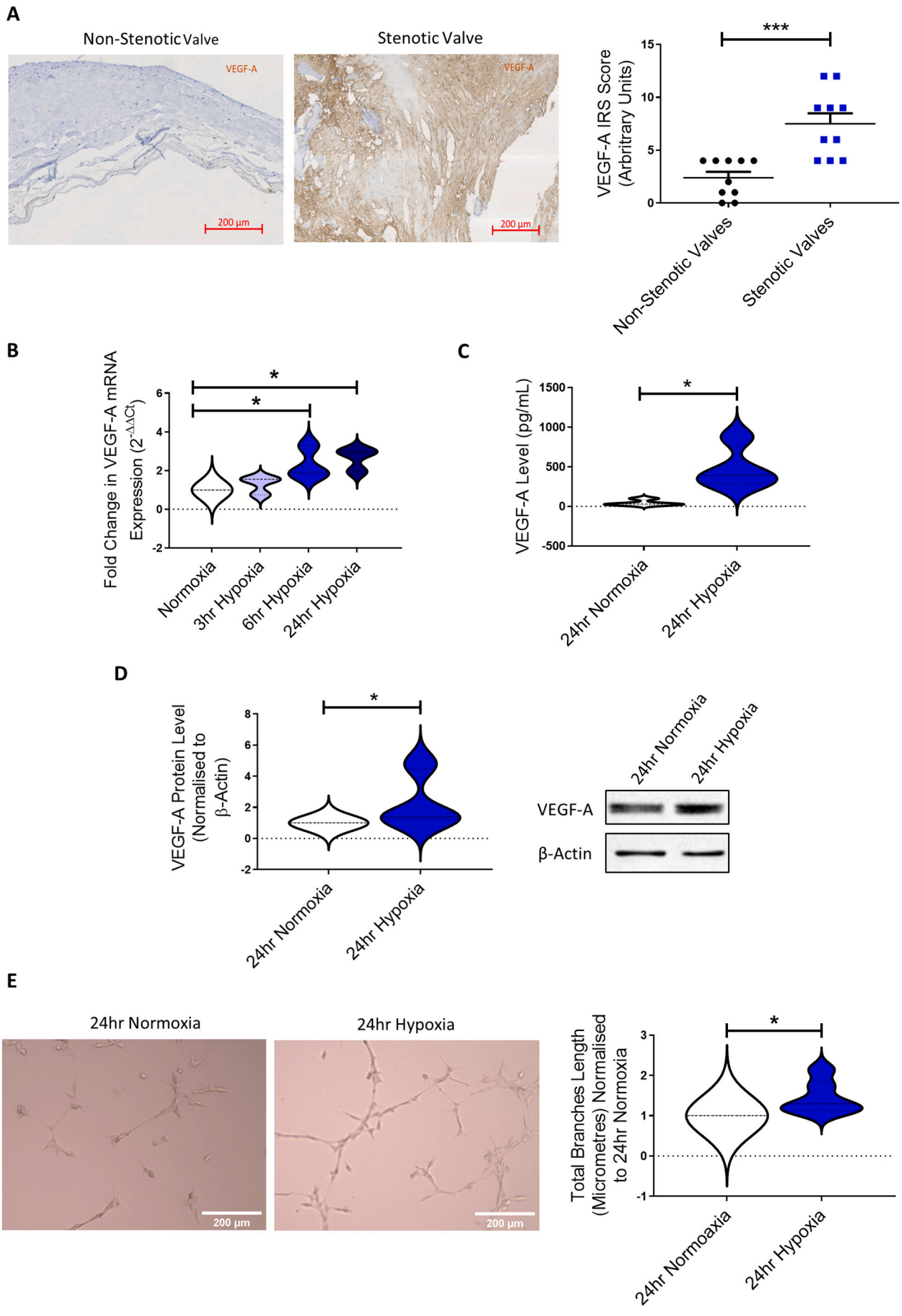

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Fig. 2. Hypoxia produces a pro-angiogenic environment in aortic valves and increases the expression of pro-angiogenic factor VEGF-A. (A) Immunohistochemistry of VEGF-A on non-stenotic aortic valves and stenotic human aortic valves obtained from the Grampian Biorepository. Quantification of VEGF-A was performed using immunoreactivity score ($n = 10$). (B) qPCR measuring VEGF-A mRNA levels in human VICs isolated from stenotic aortic valves incubated in normoxia or 3, 6 or 24 h in hypoxia. Data is expressed as 2-ΔΔCt relative to the normoxic control ($n = 3$). (C) ELISA measuring the supernatant collected from human VICs isolated from stenotic aortic valves incubated in 24 h normoxia or hypoxia (n = 4). (D) Immunoblot analysis of VEGF-A in human VICs isolated from stenotic aortic valves incubated for 24 h in normoxia or hypoxia with representative immunoblot images shown, normalised to β-actin loading control ($n = 6$). (E) Conditioned media experiment on human umbilical vascular endothelial cells (HUVECs) from supernatant collected from VICs incubated for 24 h in normoxia or hypoxia. Human VICs were cultured after isolation from stenotic aortic valves obtained from AVS patients following surgery. Representative light-microscopy images of HUVECs tubule formation are shown. Quantification of the total pixels from the branch length of HUVEC tubule formation ($n = 6$). Results are presented as the mean ± SEM. p values in panels A, C, D, E, were determined by students t-test or non-parametric equivalent. p valve in panel B was determined by 1-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Each 'n' number in panels B-E represents cells derived from valves of a different patient.

sections with appropriate care taken not to damage the outer endothelial layer of the tissue. Fresh tissue was embedded in 100 μL of Matrigel in a 24-well plate and allowed to set at 37 °C for 15 min. 500 μL of Endothelial Cell Media 2 was added. Valve sections were treated with S1P at various concentrations as stated or 10% DMSO in a 4 mg/mL BSA control. Media and treatment changes are made every 2 days. Branching neovessels from aortic valve samples are imaged on days 5, 8, 10 and 12 on an EVOS XL light microscope. Angiogenesis was quantified by counting the number of endothelial microvessels sprouting from explants using phase contrast microscopy. Treatments were performed in triplicate.

2.13. Statistical analysis

Data is expressed as mean ± SEM. Statistical analysis was performed using Prism software (GraphPad Software, USA). For single comparisons a student's t-test or non-parametric equivalent was applied. For multiple comparisons a one-way ANOVA was applied with a Bonferroni post hoc test. For correlation analysis, linear regression was used. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Hypoxia drives the proliferation of human aortic valve interstitial cells

The thickening and morphological changes which occur in the aortic valve during AVS was apparent in valves from patients undergoing valve replacement surgery, compared to those valves from patients with aortic regurgitation (Fig. 1A). Immunohistochemistry revealed that stenotic aortic valve tissue had a significant increase in the protein expression of HIF1α, the canonical marker of tissue hypoxia (Fig. 1B). This was present throughout the valve tissue in the interstitial cells. The effects of hypoxia on primary cultured human VICs from stenotic aortic valves was examined. Surprisingly, VICs underwent significant proliferation following 24 h of incubation in a hypoxic incubator in the presence of 20% serum (1% O2, 5% CO2) (Fig. 1C). In the absence of serum, VIC proliferation was not significantly changed in hypoxic compared to normoxic conditions (Fig. 1C).

Human VICs from stenotic valves grown in hypoxia also demonstrated a significant increase in proliferating cellular nuclear antigen (PCNA), a DNA clamp and marker of cellular proliferation (Fig. 1D). As hypoxia leads to the initiation of apoptosis in many cell types, the protein expression of caspase-3, an apoptotic marker was investigated under hypoxic conditions. In VICs cultured in hypoxia, there was no change in capase-3 expression compared to cells in normoxic conditions (Fig. 1E).

3.2. Hypoxia drives aortic valve neovascularisation and the expression of pro-angiogenic VEGF-A whilst decreasing the expression of angio-static factor sFlt1

As hypoxia is a canonical stimulant of angiogenesis, we investigated the ability of hypoxia in the aortic valve to stimulate the growth of new blood vessels. Angiogenesis is a feature of the early stages of AVS development [27]. Expression levels of the angiogenic growth factor VEGF-A expression were significantly increased in stenotic aortic valves when compared to non-stenotic aortic valves (Fig. 2A). This increased VEGF-A protein expression was observed throughout the aortic valve interstitial tissue. To determine whether hypoxia directly regulates VEGF-A expression, we examined VICs under hypoxic conditions. qPCR revealed an increase in the mRNA expression of VEGF-A in VICs after both 6 h and 24 h of hypoxia compared to human VICs in normoxic conditions (Fig. 2B). VEGF-A protein levels were also significantly increased in the supernatant from VICs collected following 24 h of incubation in hypoxic compared to normoxic supernatant (Fig. 2C). Immunoblotting demonstrated that the intracellular protein levels of VEGF-A was also significantly increased in VICs that had been incubated in hypoxia, compared to VICs in normoxia (Fig. 2D). To assess if the increased VEGF expression could result in a pro-angiogenic environment, in vitro tubule formation assays were used. Firstly, supernatant was collected from stenotic human VICs which were incubated in hypoxic conditions for 24 h. Human umbilical vein endothelial cells (HUVECs) were subsequently incubated in this supernatant and plated onto Matrigel within a 96-well plate (Fig. 2E). HUVECs incubated with supernatant from VICs under hypoxia for 24 h displayed significantly more in vitro tubule formation compared to HUVECS incubated with medium from normoxic human VICs (Fig. 2E). Together these data demonstrate that VICs are an important source of VEGF in aortic valves and can produce a pro-angiogenic environment via this mechanism.

As VEGF was significantly increased in valves from AVS and increased in hypoxia, a hallmark of AVS development, we investigated the possibility that angiostatic factors may be conversely decreased. One such factor, the soluble VEGR receptor sFlt1, has not previously been examined in aortic valves but is an important angiostatic mediator in the cornea where its expression maintains avascularity [17]. We first assessed whether sFlt-1 is expressed in non-stenotic aortic valves. Valve leaflets obtained directly from patients were immuno-labelled and examined using confocal microscopy. This revealed that sFlt-1 was expressed throughout the valve sections in a discrete cell-like pattern (Fig. 3A). In non-stenotic valve leaflets, the predominant cell type is interstitial cells with valve endothelial cells covering the outer leaflet. To determine if sFlt-1 was contained within interstitial cells, sections were double-labelled with anti-smooth-muscle α-actin antibody (a marker for VICs). The merged labelling demonstrates that most sFlt-1 is within the same structures, although not entirely overlapping (Fig. 3A). All cells observed expressed smooth-muscle α-actin, indicating almost exclusive presence of VICs in these valves. This provides strong evidence that sFlt-1 is normally expressed in non-diseased aortic valves within VICs. This indicates that VICs express sFlt1 which could be released into the surrounding aortic valve tissue, thereby inhibiting angiogenesis.

We next determined whether sFlt1 was altered in valves from patients with AVS compared to (non-stenotic) valves from patients with aortic regurgitation. Immunohistochemistry on fixed valve leaflet sections obtained from the Grampian biorepository demonstrated that there was a significant decrease in the expression of sFlt1 in stenotic aortic valves versus the non-stenotic aortic valves (Fig. 3B). As stenotic valves result in intra-valve hypoxia in vivo, the effects of hypoxia on

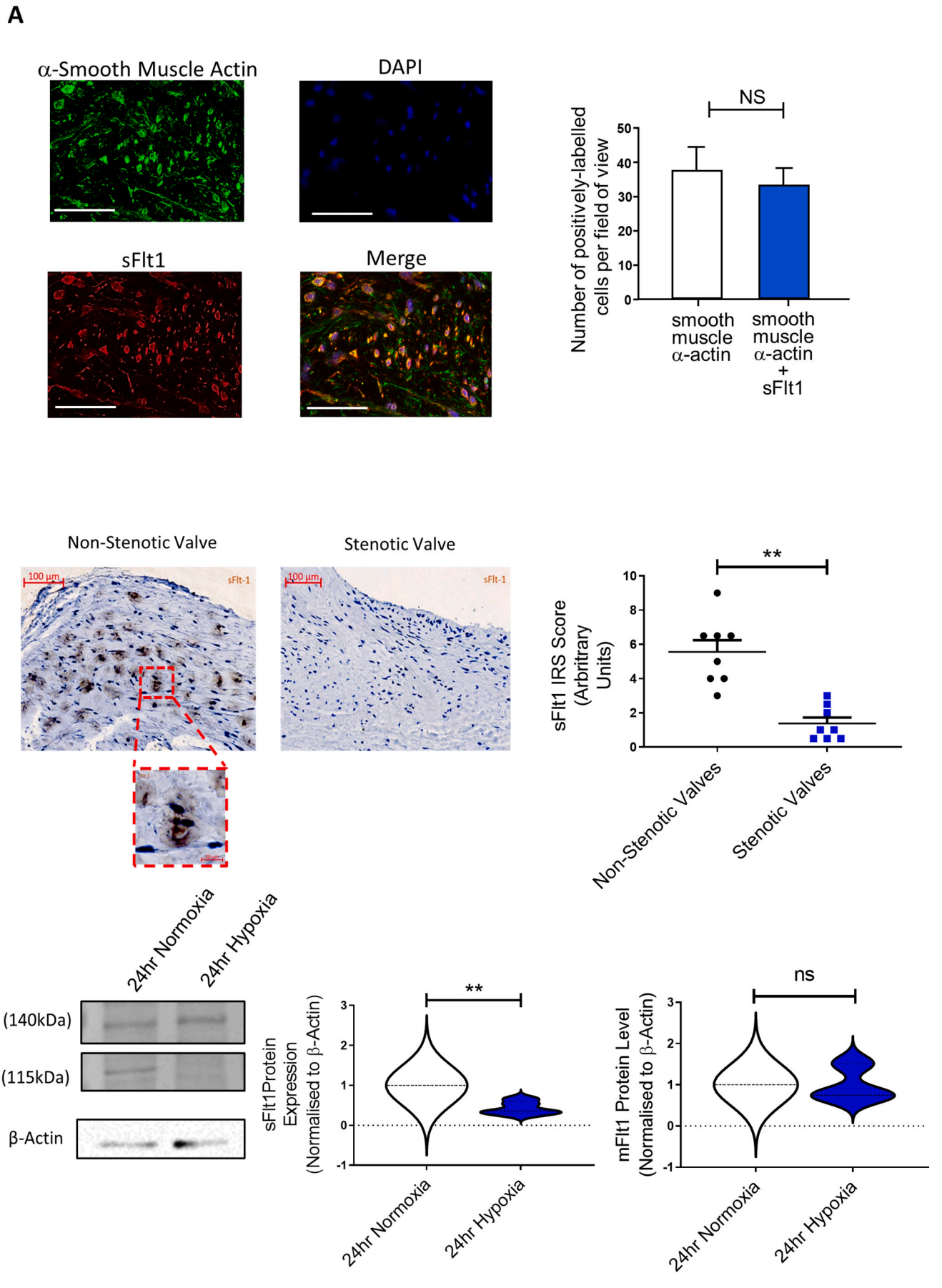

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Fig. 3. sFlt1 is reduced in AVS and regulated by hypoxia. (A) Co-localization of VIC protein marker (smooth muscle α-actin) and sFlt1 using immunofluorescent confocal microscopy in non-stenotic human aortic valves obtained from patients undergoing surgery for aortic regurgitation. Images show typical field of view with single fluorophores and merged image, scale bar represents 50 μm. Co-localization of smooth muscle α-actin and sFlt-1 was determined by comparing the number of cells which displayed labelling for smooth muscle α-actin alone with labelling for both proteins (n = 3 individual valves averaged from 2 fields of view per valve). NS = not significant, Student's *t*-test (B) Immunohistochemistry of sFlt1 on non-stenotic aortic valves and stenotic human aortic valves. Quantification of sFlt1 was performed using immunoreactivity score (n = 10). Inset is a higher magnification view revealing cellular detail. (C) Immunoblot analysis of mFlt-1 (n = 3) and sFlt1 ($n = 5$) in human VICs isolated from stenotic aortic valves incubated for 24 h in normoxia or hypoxia with representative immunoblot images shown, normalised to β-actin loading control. (Results are presented as the mean ± SEM. p values were determined by students t-test. * = $p < 0.05$, ** = $p < 0.01$. Each 'n' number represents cells derived from valves of a different patient.

cultured VICs derived from AVS valves was determined.

Despite sFlt-1 expression being significantly decreased in stenotic valve tissue (Fig. 3B), sFlt1 protein was still detected using immunoblotting in VICs derived from AVS patients (Fig. 3C). There was, however, significantly expression decreased of sFlt-1 in VICs exposed to hypoxic conditions compared to VICs incubated in normoxic conditions. This indicates that sFlt-1 expression is decreased by hypoxia in aortic valves (Fig. 3C). Interestingly, under the same hypoxic conditions there was no change in the intracellular protein expression of the membrane bound isoform of this receptor, mFlt1 (VEGFR1) (Fig. 3C).

3.3. sFlt1 maintains aortic valve avascularity and an angiostatic environment in the human aortic valve

As sFlt1 expression is altered in VICs from stenotic valves under hypoxia conditions (while mVEGFR1 is unchanged), we next investigated the function of sFlt1 in the regulation of angiogenesis. As we demonstrated that VICs are a source of sFlt-1 in aortic valves, we determined whether modulating sFlt-1 expression in VICs could potentially regulate angiogenesis. sFlt1 expression was knocked down using siRNA in primary cultured human VICs from the valves of AVS patients. Immunoblotting for sFlt-1 demonstrated that approximately 50% knockdown was achieved by siRNA targeted to sFlt1 compared to VICs treated with the control siRNA (Fig. 4A). 24 h following siRNA knockdown, the supernatant was collected from the siRNA-treated VICs and used to perform an in vitro tubule formation assay with HUVECs. HUVECs incubated with supernatant from sFlt1 siRNA-treated VICs revealed a significant increase in tubule formation compared to HUVECs incubated with supernatant from control siRNA-treated cells (Fig. 4B). In further in vitro tubule formation assays, HUVECs were incubated with two different concentrations of recombinant human sFlt1 (10 ng/mL and 100 ng/mL) and a vehicle control. Recombinant sFlt1 significantly inhibited in vitro tubule formation when compared to the vehicle control (Fig. 4C).

3.4. S1P can upregulate sFlt1 and produce an angiostatic environment in human aortic valves

Our data suggest that increasing the expression of sFlt-1 could potentially inhibit the vascularisation of the aortic valve. During a preliminary screen of G-coupled protein receptors (GPCRs) in human VICs, the sphinogosine 1-phosphate (S1P) receptors (S1PR) were demonstrated to be highly expressed in both stenotic and non-stenotic VICs (data not shown). S1PR bind bioactive lipid-mediator S1P28and S1P receptors 1–5, have previously been shown to modulate angiogenesis [29,30]. S1P is naturally-occurring in plasma and produced and stored in several cell types. We therefore targeted S1P receptors and tested the ability of S1P to modulate angiogenesis in aortic valves from AVS patients. Human aortic valve tissue was embedded in Matrigel and cultured for 12 days treated with either 1 μM S1P or vehicle control (Fig. 5A). Sprouting neovessels grow into the Matrigel and can be quantified by light microscopy. In the S1P-treated tissue, there was a significant decrease in the number of sprouting neovessels after 8 days when compared to the vehicle control. This was maintained at days 10, 12 and 14 days (Fig. 5A). As we have demonstrated the role of sFlt1 in regulating aortic valve vascularisation, we further investigated whether S1P could regulate the expression of sFlt1. Aortic valve tissue from the above experiments in Fig. 5A was incubated with S1P for 14 days and sFlt1 protein expression assessed by immunoblotting. Aortic valve tissue incubated with S1P (1 μM) significantly increased sFlt1 protein expression compared to control (Fig. 5B). As S1P is able to inhibit angiogenesis in aortic valves, S1P receptor levels were quantified in cultured human VICs using qPCR to determine expression patterns of different isoforms (Fig. 5C). The S1P receptor 3 had the highest mRNA expression levels in cultured human VICs.

4. Discussion

In this study, we demonstrate for the first time that hypoxia induces angiogenesis in human aortic valves via dual actions; 1) by increasing production of the pro-angiogenic growth factor VEGF-A and, 2) by simultaneously decreasing expression of the angiostatic VEGF receptor sFlt1. These effects are involved in the progression of AVS. Finally, activation of S1P receptors increased sFlt-1 expression and inhibit angiogenesis in the human aortic valves. As inhibiting angiogenesis is a potential therapeutic target in AVS, increasing the expression of sFlt1 via S1P receptor activation may be a future strategy.

Hypoxia is an established canonical driver of angiogenesis within the context of cancer [9], wound-healing [31] and other cardiovascular diseases, such as myocardial infarction [32]. However, the study of angiogenesis in AVS has, until now, been limited. In the early stages of AVS, the aortic valve tissue undergoes a significant level of thickening, a process known as aortic sclerosis [33]. This thickening of this avascular human aortic valve results in insufficient O_2 delivery to the valve tissue, creating a hypoxic environment. This was reflected in the observed increase in HIF1α in the human stenotic aortic valve leaflets. As these valves are removed from patients with moderate to severe AVS, it is worth noting that the hypoxic environment probably remains throughout the development of AVS as previously observed [34]. We did not directly assess the cell type that was responsible for this increase, however, other studies have demonstrated that human VICs can produce HIF1α [5,34]. In our results, one unexpected but novel observation was the proliferation and increased expression of markers of proliferation in isolated human VICs upon hypoxia incubation. The vast majority of cells in the systemic circulation cease to proliferate in hypoxia and undergo programmed cell death [35,36]. This proliferative phenotype in hypoxic conditions is observed in other cell types including pulmonary vascular smooth muscle cells, neural stem cells and cancer cells where studies have shown this increase in proliferation despite hypoxic conditions occurs via several different metabolic changes [37–40]. These include upregulation of both cellular and mitochondrial glutamine uptake which offsets reduced mitochondrial pyruvate availability for adenosine triphosphate production [40]. In pulmonary artery smooth muscle cells, hypoxia increases proliferation, the molecular pathways for this are not

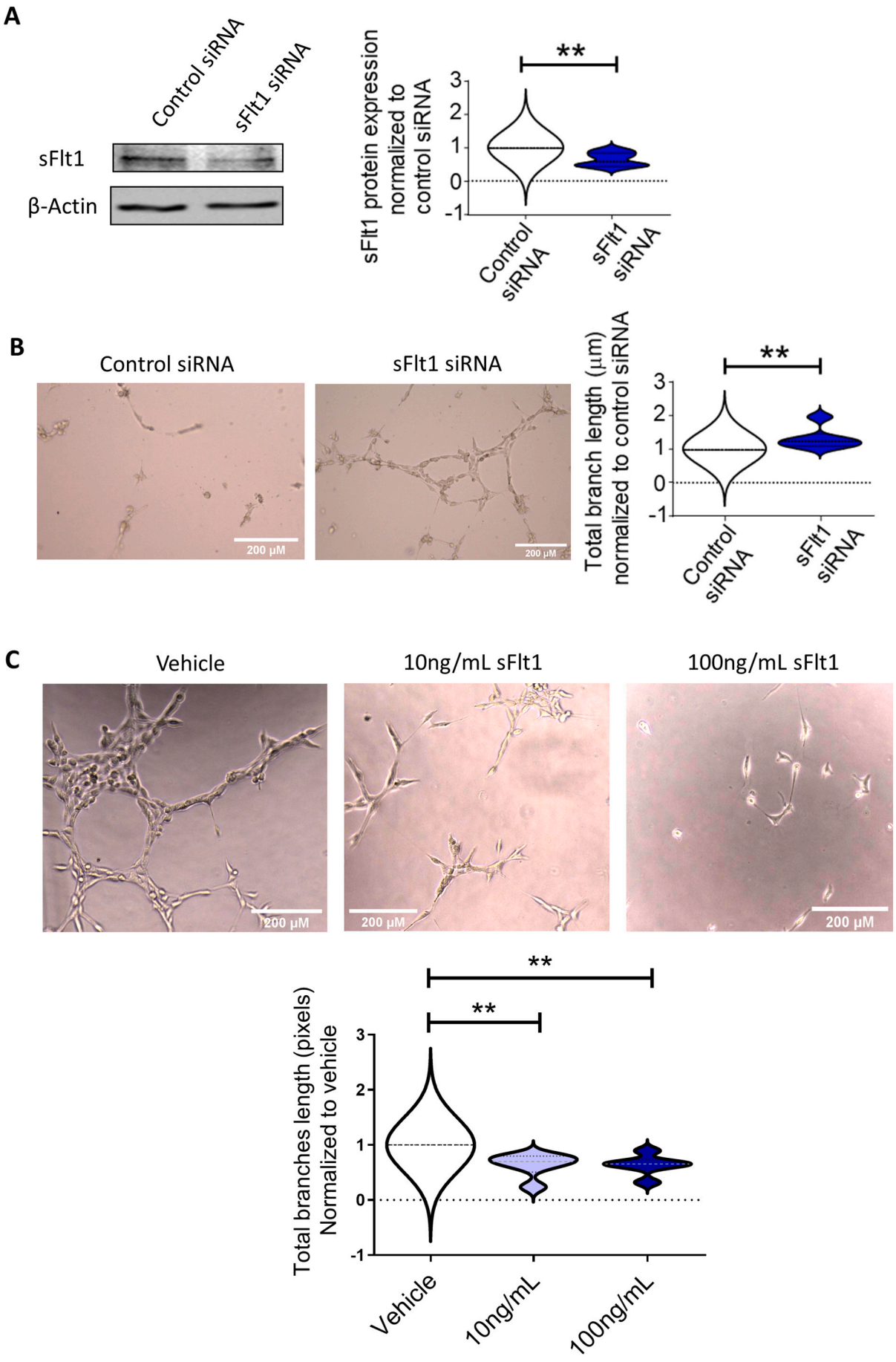

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Fig. 4. sFlt1 maintains aortic valve avascularity and addition of sFlt1 can inhibit angiogenesis. (A) Immunoblot analysis of sFlt1 siRNA knockdown in human valvular interstitial cells (VICs) isolated from stenotic aortic valves treated with either control siRNA or sFlt1 siRNA as indicated. Representative immunoblot images shown, normalised to β-actin loading control (n = 6). (B) Conditioned media experiment on human umbilical vascular endothelial cells (HUVECs) from supernatant collected from human VICs isolated from stenotic aortic valves treated with control siRNA or sFlt1 siRNA as indicated. Representative light-microscopy images of HUVECs tubule formation are shown. Quantification of the total pixels from the branch length of HUVEC tubule formation (n = 6). (C) HUVEC tubule formation assay with added recombinant sFlt1 at concentrations of 10 ng/mL and 100 ng/mL. phospho-buffered saline was used as vehicle. Representative light-microscopy images of HUVEC tubule formation under each condition are shown (n = 6). Results are presented as the mean ± SEM. p values in panels A, B were determined by students t-test or non-parametric equivalent. p valve in panel C was determined 1-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$. Each 'n' number represents cells derived from valves of a different patient.

delineated but may be related to metabolic changes similar to those observed in cancer cells [40]. Whilst we have not investigated this finding further, the unique phenotype of VICs observed in our study may play a critically important role in the early stages of AVS.

Growing evidence has now demonstrated the importance of angiogenesis as a process important in the pathogenesis of AVS [5,6]. Our observation that hypoxia can stimulate the expression of VEGF-A in human VICs from AVS patients is consistent with previous studies in this cell type although we did not directly measure the ability of hypoxia in non-stenotic VICs to increase VEGF expression [5]. Furthermore, we demonstrate the increased presence of VEGF-A in tissue from human stenotic aortic valves when compared to non-stenotic human aortic valves. Although this highlights the ability for human VICs to produce this pro-angiogenic factor, angiogenesis is a tightly controlled process which involves a balance of both pro-angiogenic and angiostatic factors, with inter- and counter-acting mechanisms [12,14,16] Therefore, our observation of the ability of conditioned media from human VICs incubated in hypoxia to functionally stimulate the formation of in vitro tubules is an important finding. We propose that the balance of pro-angiogenic and angiostatic proteins is altered and that hypoxia induces a milieu of factors released from VIC promoting the growth of neovessels. Alongside the increase in pro-angiogenic VEGF-A, we revealed that there was a significant decrease in the angiostatic factor sFlt1 in stenotic human aortic valve tissue. As we demonstrate here, sFLT-1 is typically expressed in VICs in vivo. This soluble receptor binds to VEGF-A and thus prevents VEGF-A from binding to pro-angiogenic transmembrane VEGF receptors present on endothelial cells [17,24]. The pathological relevance of this novel finding was further indicated by a reduction in sFlt1 expression in human VICs incubated under hypoxic conditions. Simultaneously, there was no change in the levels of mFlt1 (VEGFR1), the membrane-bound isoform of sFlt1 indicating a process of hypoxia-mediated alternative splicing of this gene, which contains an HRE and could be partly responsible for tipping the overall effect towards a pro-angiogenic environment [41]. The mechanisms involved in the differential effect of hypoxia on sFlt1 and mFlt1 are currently unclear. sFlt1 remains is known to be under the control of hypoxia in a cell-type specific manner. Consistent with our results, it has also been seen that hypoxia can downregulate sFlt1 in microvascular endothelial and cancer cells [42,43]. The expression of sFlt1 could therefore be an important switch between pro-and anti-angiogenic molecular cues in AVS.

Consistent with our data for a role of sFlt-1 in AVS, we observed that conditioned media from stenotic human VICs in which sFlt1 expression had been knocked down resulted in increased in vitro tubule formation. By contrast, physiologically relevant levels of recombinant sFlt1 protein reduced in vitro tubule formation in endothelial cells [44]. sFlt1 expression is essential in the maintenance of corneal avascularity suggesting that sFlt-1 may work in synergy to inhibit angiogenesis in various avascular areas of the body [17]. sFlt1 overexpression is also a canonical marker in the pregnancy disorder pre-eclampsia, where it inhibits pro-angiogenic factors and prevent proper blood vessel formation in the placenta [21,45–47]. Although this is the first time sFlt1 has been suggested to be important in the progression of angiogenesis during AVS, previous literature has pointed to several other factors involved in the same process. Chondromodulin-1 is an anti-angiogenic glycoprotein that that was found to be also expressed in healthy cardiac valves yet was downregulated during the onset of AVS [6]. Functionally chondromodulin-1, secreted by VICs, was able to suppress angiogenesis in vitro. Chondromodulin-1 is also highly expressed and important in maintain the avascularity of the eye further indicating similarities between the avascular aortic valve leaflet and the cornea [48]. Pro-angiogenic factors have also been identified to be expressed in stenotic aortic valves. Periostin was found to be upregulated in the degenerated aortic valve, specifically in the presence of neovessels, and able to promote endothelial cell migration and tubule formation [49]. Endothelial cells from aortic valves are phenotypically distinct from aortic endothelial cells although whether these differences are involved in AVS is still not clear [35]. Our study has used HUVECs to act as a read-out for our bioassay with conditioned medium (which provide a stable and consistent cell phenotype), though ideally endothelial cells cultured from aortic valves would have provided a more accurate phenotype. Further research is required to fully understand any phenotypic changes which occur in aortic valve endothelial cells as a result of the pro-angiogenic stimuli in AVS. Regardless, our data suggest that sFlt1 is an integral part of a larger network of factors which maintain valvular avascularity in healthy aortic valves and whose balance is altered in AVS. We demonstrate here for the first time that hypoxia is, at least in part, one of the driving forces regulating this balance.

Inhibition of sFlt1 expression as an integral part of the hypoxic response in AVS, raising the possibility of maintaining expression of sFlt-1 as a therapeutic approach in AVS. This could prevent hypoxia-induced angiogenesis and therefore slow disease pathogenesis. S1P has an established role in the vascular system as well as the regulation of angiogenesis [29,30]. In the current study, S1P inhibited neovessel growth in ex vivo human aortic valve tissue from stenotic aortic valves. Furthermore, we observed that ex vivo stenotic aortic valve tissue incubated with S1P increased sFlt1 protein expression. Such cellular effects would be beneficial. The targeting of S1P receptors has also recently proven to be successful with the development of FTY720-P for the treatment of relapsing-remitting multiple sclerosis [50]. S1P has had limited previous attention in the context of AVS, however, it was previously demonstrated to be pro-inflammatory in VICs but only in synergy with lipopolysaccharide [51]. S1PR3 was observed to have by far the highest mRNA expression of any of the S1P receptors in human stenotic VICs, and this is a strong indication that it would also have the highest protein expression. S1PR3 has a wide array of cellular functions in the vascular system and could prove to be a promising drug target, particularly through ligands which are S1PR3 specific [52].

In summary, we have demonstrated a novel link between hypoxia and a pro-angiogenic environment in human aortic valves via an inhibition of sFlt1. We also demonstrate that treatment with S1P is able to increase sFlt1 expression in VICs and inhibit neovessel growth in ex vivo aortic valve tissue, highlighting a potential therapeutic approach in AVS. This knowledge will further allow for the development of drugs that are able to target sFlt1 in the treatment of AVS.

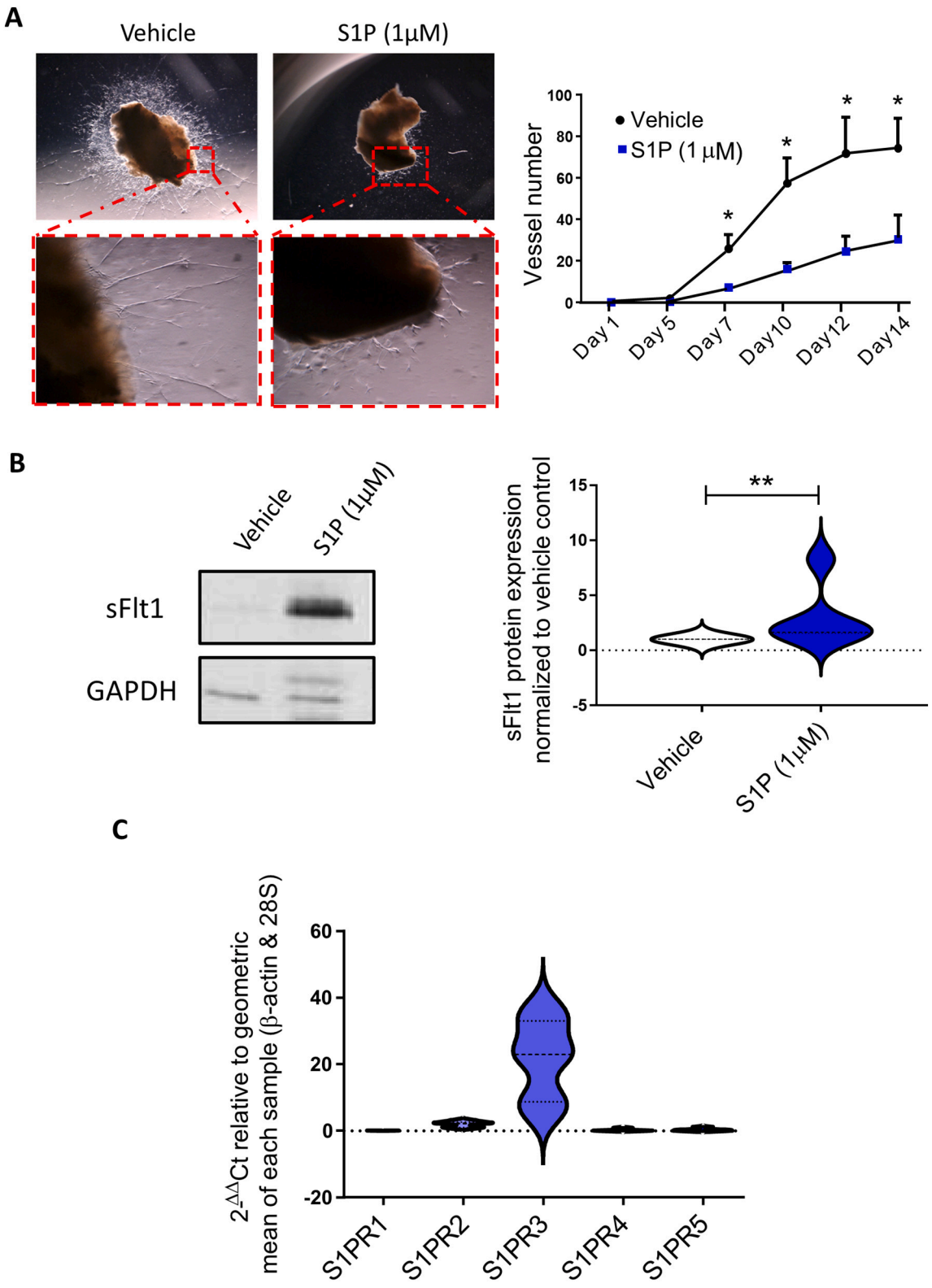

Fig. 5. S1P treatment can inhibit angiogenesis in the human aortic valve and upregulate sFlt1. (A) Representative images of ex vivo human aortic valve tissue from patients with AVS, embedded in Matrigel, treated with S1P (1 μM) or vehicle control. Red inset displays increased magnification of neovessel growth. Neovessels were quantified on days 1, 5, 8, 10, 12 and 14 after embedding in Matrigel ($n = 4$). (B) Immunoblot analysis of sFlt1 in human aortic valve tissue treated with 1 μM S1P or vehicle control as indicated in methods section. Representative immunoblot images shown, normalised to GAPDH loading control (n = 5). (C) qPCR analysis of S1PR1–5 levels in human stenotic VICs incubated using SYBR green technology. 2-ΔΔCt levels were compared relative to the geometric mean of two housekeeping genes: β-actin and 28S. This provides a quantitative measure of S1PR expression of each receptor (n = 4), each condition was performed in triplicate. Results are presented as the mean ± SEM. p values were determined by students t-test or non-parametric equivalent * = $p < 0.05$, ** = $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

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Glossary

sFlt1: soluble fms-like tyrosine kinase 1
AVS: Aortic Valve Stenosis
HIF-1α: hypoxia-inducible factor 1α
HIF-1β: hypoxia inducible factor 1β
VEGF: vascular endothelial growth factor
VEGFR1/Flt1: VEGF receptor 1
VICs: valvular interstitial cells
HUVECs: Human umbilical vein endothelial cells
IHC: Immunohistochemistry
ELISA: Enzyme-linked immunosorbent assay
PBS: phosphate-buffered saline
S1P: sphingosine 1-phosphate
BSA: bovine serum albumin
PCNA: proliferating cellular nuclear antigen
GPCRs: G-coupled protein receptors