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Shear-mediated ALK5 expression regulates endothelial activation

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ABSTRACT

Calcific aortic valve disease affects the aortic side of the valve, exposed to low magnitude multidirectional ("disturbed) blood flow, more than it affects the ventricular side, exposed to high magnitude uniaxial flow. Overt disease is preceded by endothelial dysfunction and inflammation. Here we investigate the potential role of the transforming growth factor-β (TGF-β) receptor ALK5 in this process. Although ECs are always subject to shear stress due to blood flow, and their responses to shear stress are important in healthy valve development and homeostasis, low magnitude multidirectional flow can induce pathophysiological changes. Previous work has shown ALK5 to be an important mechanosensor. ALK5 transduces mechanically sensed signals via the activation of the SMAD2/3 transcriptional modulators. However, it is currently unclear precisely how ALK5-mediated shear stress responses translate into pathological changes under conditions of chronically disturbed flow. Here, we demonstrate that ALK5 mechanosensory signalling influences flow-induced endothelial leukocyte adhesion and paracellular permeability. Low magnitude multidirectional flow resulted in downregulation of the receptor, accompanied by increased SMAD2 phosphorylation, in human umbilical vein endothelial cell (HUVEC) monolayers. These changes correlated with elevated monocyte adhesion and significantly increased transendothelial transport of an albumin-sized tracer. These effects were abolished by inhibition of ALK5 kinase activity. Analysis of ALK5 expression patterns in porcine aortic valve tissue corroborated the findings from cell-based experiments. Together, these results suggest that ALK5 has a role in shear stress-associated cardiovascular disease pathology, emphasising the importance of further mechanistic investigations and supporting it as a potential therapeutic target.

1. Introduction

Calcific aortic valve disease (CAVD), largely associated with ageing, is the major reason for surgical aortic valve replacement. The condition develops slowly, beginning with mild thickening of the valve (sclerosis) that can progress to cause significant impairment of valvular function through aortic valve stenosis (AVS) [1]. According to one estimate, AVS affects up to 2–3% of North Americans over the age of 65, and is increasing in prevalence [2].

The early stages of the disease involve endothelial cell (EC) dysfunction characterised by a pro-calcific transcriptional profile that is apparently associated with disturbed flow – or low magnitude multidirectional flow (LMMF) [3]. LMMF has been established as a contributing factor in endothelial activation, which is an important hallmark of vascular atherosclerosis as well as CAVD [4,5]. Endothelial cells respond to LMMF through mechanosensory pathways that control such cellular responses as immune cell recruitment [5], increased endothelial permeability [6], and endothelial-to-mesenchymal transition (EndMT) [7], all of which are hallmarks of CAVD.

The transforming growth factor-β (TGF-β) family of cytokines plays critical roles in normal tissue homeostasis, acting through their cellular receptors. In particular, TGF-β1 interacts either with ALK5 and TGF-β type II receptor (TβRII), or ALK1, endoglin, and TβRII to form heteromeric complexes in ECs. ALK5 induces SMAD2 and 3 phosphorylation to maintain EC quiescence; on the other hand, ALK1 induces SMAD 1, 5, and 8 phosphorylation to activate ECs [8]. The ALK5–SMAD2/3 axis normally functions as a negative regulator of angiogenesis to limit proliferation and apoptosis, and

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Abbreviation

CAVD	Calcific aortic valve disease
AVS	Aortic valve stenosis
EC	Endothelial cell
LMMF	Low magnitude multidirectional flow
EndMT	Endothelial-to-mesenchymal transition
TGF-β	Transforming growth factor-β
TβRII	TGF-β type II receptor
NO	Nitric oxide
HUVEC	Human umbilical vein endothelial cell
EGM-2	Endothelial Cell Growth Medium
THP-1	Human acute monocytic leukaemia suspension line
FBS	Fetal Bovine Serum
HMUF	High magnitude uniaxial flow
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
DMSO	Dimethyl sulfoxide
FITC	Fluorescein isothiocyanate
BSA	Bovine serum albumin

maintain endothelial quiescence under flow [8,9].

Recent studies have also shown that ALK5 is an important receptor in shear-stress mediated endothelial activation [7,9,10]. It is involved in shear stress-mediated alignment of ECs and their release of nitric oxide (NO), both of which are known to promote EC quiescence [9]. Even though TGF-β signalling through the ALK5-SMAD2/3 axis is generally perceived as anti-inflammatory, there are reports suggesting that ALK5 is involved in shear stress-induced endothelial activation. For example, ALK5 has a critical role in shear stress-induced EndMT, arterial inward remodelling and atherogenesis via the SMAD 2/3 pathway [7,11]. These results are consistent with an in-vivo study in which inhibition of TGF-β signalling in ECs abrogated inflammatory responses and atherosclerotic plaque formation in mice [12].

TGF-β signalling relies on receptor internalisation [13]. Activation of SMAD2/3 by ALK5-mediated phosphorylation takes place in early endosomes and is followed by binding of phosphorylated SMAD2/3 to SMAD4 to effect nuclear translocation and downstream transcriptional responses [14]. Interestingly, TGF-β receptors have been shown to undergo constitutive cycles of internalisation and recycling [15]. Internalisation can take place by either clathrin-dependent or -independent pathways, with the former favouring SMAD2/3 signal transduction. Endocytosed receptors are then either returned to the cell surface in a RAB11A-dependent manner [15], or segregated into a degradation pathway (initiated by ubiquitination) that is also the destination of receptors internalised via the clathrin-independent route [14]. Thus, TGF-β receptor activation may result in downstream signalling that is accompanied by apparent downregulation of the receptor [15].

Although ALK5 is known to be an important receptor in EC mechanotransduction, the specific mechanisms leading to endothelial inflammation, vascular calcification and clinical disease are not yet fully understood. In particular, changes in ALK5 expression and its associated pathways in response to LMMF have not been explored in detail. In this study, we investigated ALK5-mediated responses to normal and pro-inflammatory flow conditions in vitro, as a first step in delineating the specifics of pathogenic signalling. Expression of ALK5 was also evaluated in porcine aortic valve tissue, to support the findings based on cell-based experiments.

2. Materials and methods

2.1. Cell isolation and culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from donors with uncomplicated labour at the Hammersmith Hospital, UK. Isolation of HUVECs was approved by the Hammersmith Hospital Research Ethics Committee (ref. 06/Q0406/21) for research purposes. The procedure was conducted as described by Jaffe et al. [16] with minor changes. HUVECs were cultured on 0.1% gelatin-coated flasks. Endothelial Cell Growth Medium (EGM-2) supplemented with the EGM-2 supplement kit (Lonza, Switzerland) was used. Cells between passages 3–5 were used. Human acute monocytic leukaemia suspension line (THP-1) cells (ATCC, USA) were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Sigma-Aldrich, UK). Passages 6–20 were used. All cells were maintained in a humidified incubator at 37 °C under 95% air/5% CO_2.

2.2. Application of shear stress

HUVECs were grown only at the centre or the edge of a 6-well plate; this was achieved through coating the region of interest with fibronectin (Sigma-Aldrich, UK), followed by passivation of the other region with Pluronic F-127 (Sigma-Aldrich, UK) [5,17]. HUVECs were allowed to grow to confluence after seeding before being placed on the orbital shaker (POS-300, Grant Instruments) inside the incubator for another 3 days. The platform orbited in the horizontal plane with an orbital radius of 5 mm and angular velocity of 150 rpm. The orbital motion of the orbital shaker resulted in the swirling motion of the medium, which in turn expose HUVECs to low magnitude multidirectional flow (LMMF, putatively atherogenic) and high magnitude uniaxial flow (HMUF, putatively atheroprotective) at the centre and the edge of the plate, respectively [5].

2.3. SDS-PAGE and western blotting

HUVECs were lysed using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, UK) supplemented with Halt protease and phosphatase inhibitor (Thermo-Fisher Scientific, USA). Lysed proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, USA). Blots were incubated with rabbit anti-ALK5 (dilution of 1:2000, ab31013, Abcam, UK), rabbit anti-phospho-SMAD2 (dilution of 1:1000, 3108s, Cell Signalling Technology, USA), rabbit anti-SMAD2 (dilution 1:2000, 5339s, Cell Signalling Technology, USA), or mouse anti-calnexin (dilution of 1:2000, LS-C179860, Source BioScience, UK) antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated mouse anti-rabbit or goat anti-mouse secondary antibodies (dilution of 1:5000, sc-2357 or sc-2005, respectively, Santa Cruz Biotechnology, USA) for 1 h at room temperature. Calnexin was used as a loading control. Blots were incubated with Clarity ECL substrate (Bio-Rad, USA) before being imaged using a Biospectrum imaging system (UVP, UK). Densitometry was performed using Image Studio Lite software (LI-COR, USA).

2.4. Inhibitor treatment

To investigate the role of ALK5 in HUVECs, cells were pre-treated with an inhibitor of ALK5 (SB431542, 10 μM; Stratech Scientific, UK) for an hour prior to shear stress application, followed by 72 h treatment together with shear stress application. Dimethyl sulfoxide (DMSO) was used as vehicle control.

2.5. THP-1 adhesion assay

THP-1 cell suspension at 1million cells/mL in RPMI 1640 medium was labelled with calcein-AM solution (1 mg/mL, Life Technologies, USA) at a dilution of 1:1000 for 30 min in a humidified incubator. Labelled THP-1 cells were then centrifuged at 200×*g*. Cell pellet resuspended in EGM-2 medium. One million THP-1 cells were applied to a monolayer of HUVECs (cultured in a swirling well or in static) seeded in a 6-well plate for 1 h in the incubator under static condition, followed by three washes with pre-warmed RPMI medium to remove unbound THP-1. Adhered THP-1 were fixed with 4% PFA for 15 min. Ten random fields were imaged using an inverted fluorescence microscope (SP105F, Brunel Microscopes) with a 20 × objective, 470/40 nm excitation filter, 495 nm dichroic mirror and 525/50 nm emission filter. The number of adhered THP-1 cells was quantified using a custom MATLAB script and normalised by the number of HUVECs [5].

2.6. Permeability assay

The permeability assay was derived from Dubrovskyi et al. as reported in our previous studies [6,18,19].Cells were grown on biotinylated gelatin. 24 h before tracer application, serum in the medium was reduced to 2.5%. 0.38 μM fluorescein isothiocyanate (FITC)-labelled avidin in EGM-2 medium was applied for 3 min to the monolayers. Tracer solution was then removed, and any remaining tracer that had not crossed the endothelium and bound to the biotinylated substrate was removed by rinsing with PBS three times. Cells and bound tracer were then fixed with 4% paraformaldehyde. Tracer was measured using a scanning fluorimetric plate reader (SpectraMax M5, Molecular Devices). HUVEC nuclei were visualised by staining with DRAQ5 (dilution of 1:1000) for 15 min.

2.7. Immunohistochemical staining of porcine aortic valve sections

Five μm thick paraffin sections of porcine aortic valve were dewaxed and rehydrated with water. Antigen retrieval was carried out by microwaving in 0.1 M citrate buffer for 10 min and left for a further 20 min in the same buffer then washed twice in PBS. Endogenous peroxidase activity was blocked by incubating the sections with 0.3% hydrogen peroxide for 5 min, washed twice in PBS, followed by incubating with 3% bovine serum albumin (w/v) (BSA) in PBS containing 1% v/v Tween-20. Sections were incubated for 2 h at room temperature with primary antibody against ALK5 (dilution of 1:50, sc398, Santa Cruz Biotechnology, USA). Negative control consisted of 3% BSA in PBS containing 1% v/v Tween 20. Primary antibody was then removed by washing the sections 3 times in PBS followed by a second layer of biotinylated goat anti-rabbit antibodies (BA-1000, Vector laboratories, USA) in PBS. Sections were then washed 3 times in PBS before 1 h incubation with Avidin-Biotin Complex (pk-6100, Vector laboratories, USA). Reactivity was detected using diaminobenzidine tetrahydrochloride (25 mg/mL, Sigma Aldrich, USA) and hydrogen peroxide (0.01% W/V). Sections were then counter stained with Mayers haematoxylin and viewed on a nanozoomer slide scanner microscope (Hamamatsu, Japan).

2.8. Statistical analyses

Data are presented as mean ± standard error of the mean. Statistical analyses were performed by two-way ANOVA with Tukey's post hoc test or Student's t-test using GraphPad Prism 8 (GraphPAD Software Inc., United States). The criterion for significance was $p < 0.05$ (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

3. Results

3.1. Pro-inflammatory flow reduces ALK5 expression and increases SMAD2 phosphorylation

Initial experiments subjected HUVECs to different types of fluidic shear stress – LMMF or HMUF – for 72 h using the orbital shaker. With LMMF (putatively pro-inflammatory flow), ALK5 protein expression was significantly lower and SMAD2 phosphorylation was induced, compared with HMUF (putatively anti-inflammatory flow; Fig. 1, $p < 0.05$).

3.2. ALK5 inhibition abrogates pro-inflammatory flow-induced monocyte adhesion

One important hallmark of endothelial activation is monocyte adhesion, which is known to be induced by pro-inflammatory flow [4]. Here, LMMF induced 18-fold higher adhesion of THP-1 monocytes onto vehicle (DMSO)-treated control HUVECs, compared with HMUF (Fig. 2, $p < 0.0001$). This effect of LMMF was largely abrogated upon treatment with SB431542 (an ALK5 inhibitor), as evidenced by the 83% reduction in monocyte adhesion to SB431542-treated control HUVECs (Fig. 2, $p < 0.0001$). Furthermore, in the presence of SB431542, there was no significant difference in monocyte adhesion between LMMF- and HMUF-sheared HUVECs, suggesting that the ALK5 receptor is required for LMMF-induced THP-1 adhesion to HUVECs.

3.3. ALK5 inhibition also attenuates pro-inflammatory flow-induced permeability

The paracellular permeability of HUVEC monolayers was quantified using an albumin-sized tracer (FITC–avidin). If junctions between cells are compromised due to endothelial activation, more FITC–avidin passes through the monolayer, and will then bind to the biotinylated gelatin substrate beneath the cells. The permeability of LMMF- and HMUF-sheared HUVEC monolayers was measured separately by taking readings at the centre or edge, respectively, of swirling wells. HUVECs were grown only in the centre or the edge of a swirling well to ensure that they only experience specific shear stress. The green dots in Fig. 3 correspond to FITC–avidin captured by the biotinylated gelatin.

Transport of FITC–avidin across vehicle-treated, LMMF-sheared HUVEC monolayers was 7.9-fold higher than for the HMUF-sheared case (Fig. 3, $p < 0.01$). This effect of LMMF shearing was reduced by 85% relative to control upon SB431542 treatment, and with this ALK5 inhibitor, there was also no significant difference in FITC–avidin transport across LMMF- and HMUF-sheared monolayers. These results demonstrate the importance of the ALK5 receptor in LMMF-enhanced paracellular permeability of HUVEC monolayers.

3.4. ALK5 is differentially expressed on the endothelium of ventricularis and fibrosa layers

The in vivo relevance of shear-regulated ALK5 expression was examined by assessing expression of the receptor on ECs of porcine

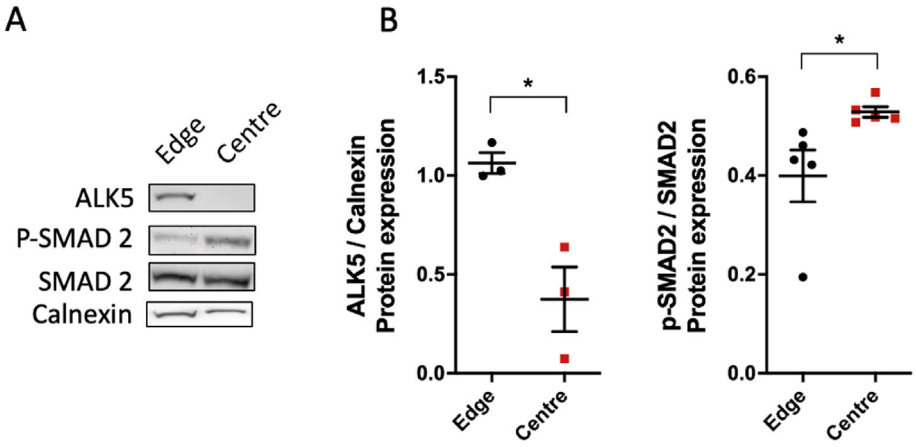

Fig. 1. (A) Representative blots and (B) quantified values of ALK5 expression and SMAD2 activation in sheared HUVECs. Application of LMMF (centre of the well, putatively pro-inflammatory) for 72 h significantly diminished the expression of ALK5 and activated SMAD2 phosphorylation, compared with HUVECs sheared using HMUF (edge of the well, putatively anti-inflammatory). (Student's t-test; $n \geq 3$; *$p < 0.05$).

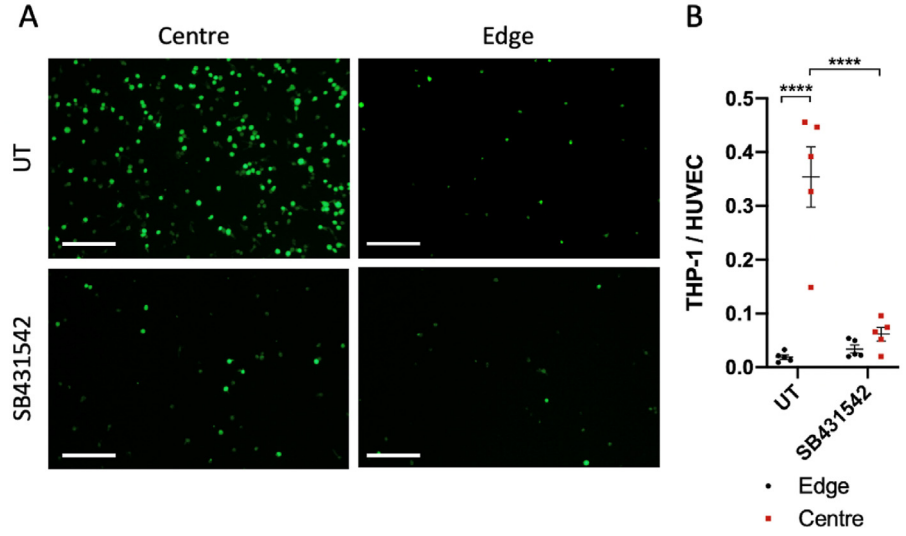

Fig. 2. (A) Representative images and (B) quantification of calcein-AM-stained THP-1 adhesion to HUVECs sheared by LMMF (centre of the well) or HMUF (edge of the well). THP-1 adhesion to HUVECs was increased by LMMF compared with HMUF, but this effect was significantly suppressed by an ALK5 inhibitor (SB431542). Scale bar = 200 μm. Two-way ANOVA followed by Tukey's post hoc test; $n = 5$. ****$p < 0.0001$).

valves. Staining of paraffin-embedded sections with anti-ALK5 antibody (Fig. 4) revealed that there were 31% fewer ALK5-expressing ECs on the fibrosa layer (aortic side) than on the ventricularis layer (ventricular side) (86.4 ± 4.9 versus 54.4 ± 6.2, $p < 0.01$), which are known to experience different flow characteristics in vivo.

4. Discussion

In this study, the orbital shaker ("swirling well") model was used to study the effect of shear stress on ALK5 protein expression. The use of this model is becoming more common in endothelial mechanobiology because of its straightforward setup, ability to apply shear stress for prolonged periods with minimal risk of contamination, small volume of medium (which allows analysis of secreted mediators and the use of costly reagents), and the fact that the swirling motion induces complex and heterogenous flow. Specifically, ECs grown at the centre of a swirling well experience LMMF whereas those at the edge experience HMUF, and these flow types are putatively pro-inflammatory and anti-inflammatory, respectively [20]. In this study, ECs were cultured only in the centre or around the edge of swirling wells—using a differential coating and passivation method described previously [17]—followed by 72 h of shear stress application. We observed significantly lower ALK5 protein expression in the ECs exposed to LMMF, which was additionally found to induce SMAD2 phosphorylation.

Previously, oscillatory shear stress (±4 dyn/cm^2) and low shear stress (5 dyn/cm^2) generated using a flow chamber have also been shown to induce SMAD2 phosphorylation [7,10,11], thus corroborating our findings. However, it should be noted that the earlier studies did not expose ECs to multidirectional flow and only sheared cells for 1–48 h. Another study reported results that contradict our data, showing that high shear stress (10 dyn/cm^2) generated using an orbital shaker induced SMAD2 phosphorylation [21]. This is likely because the authors did not restrict EC growth to only the centre or the edge of each well. In support of this, we have previously documented that soluble mediators released from cells in response to different flow characteristics may be uniformly

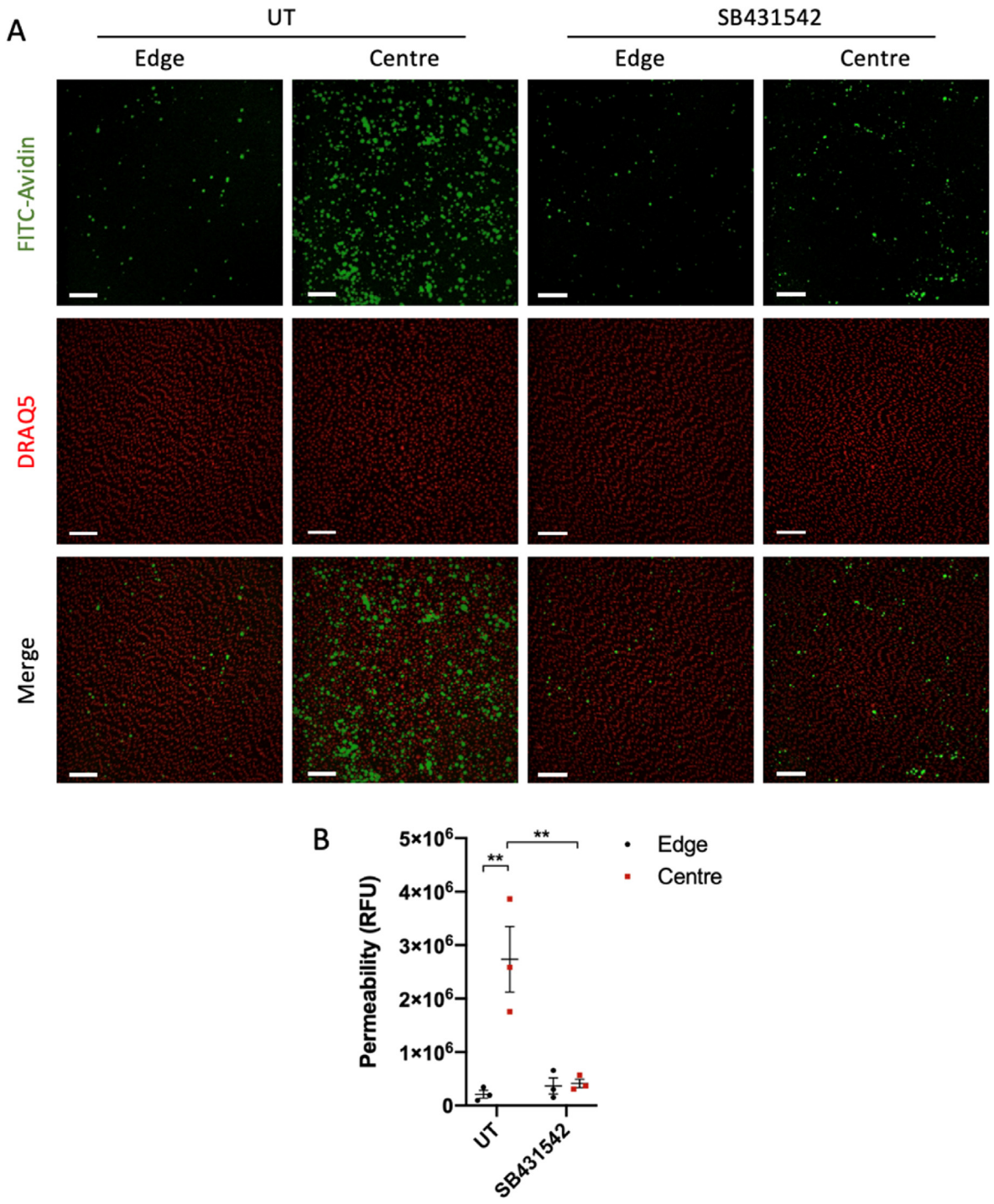

Fig. 3. (A) Representative confocal images of biotin-bound FITC–avidin (green) underneath HUVEC monolayers, and DRAQ5-stained nuclei (red). A higher intensity of FITC–avidin corresponds to increased paracellular permeability. (B) Graph quantifying relative fluorescence units (RFU) for the FITC channel shows that permeability of monolayers was increased by LMMF (centre of the well) compared to HMUF (edge of the well). The effect of LMMF on permeability was abrogated by an ALK5 inhibitor (SB431542). Scale bar = 200 μm. Two-way ANOVA followed by Tukey's post hoc test; n = 3. **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.)

mixed into the overlying medium, and that this can corrupt the apparent relation between shear and EC properties [5,6]. Hence, it is important to grow ECs only in the region of interest when using the orbital shaker model in order to properly understand the effect of shear stress on SMAD2 phosphorylation.

Intriguingly, suppression of ALK5 expression by LMMF might be expected to decrease SMAD2 activation by phosphorylation, but an increase was observed. A possible mechanism to reconcile these observations is endocytosis of the ALK5 receptor; endocytosis of plasma membrane receptors is known to regulate many cellular signalling pathways [22]. ALK5 is internalised through clathrin-dependent endocytosis, and this has already been linked with SMAD2/3 activation and receptor recycling [13–15]. Hence, we infer from the present results that LMMF activates SMAD2

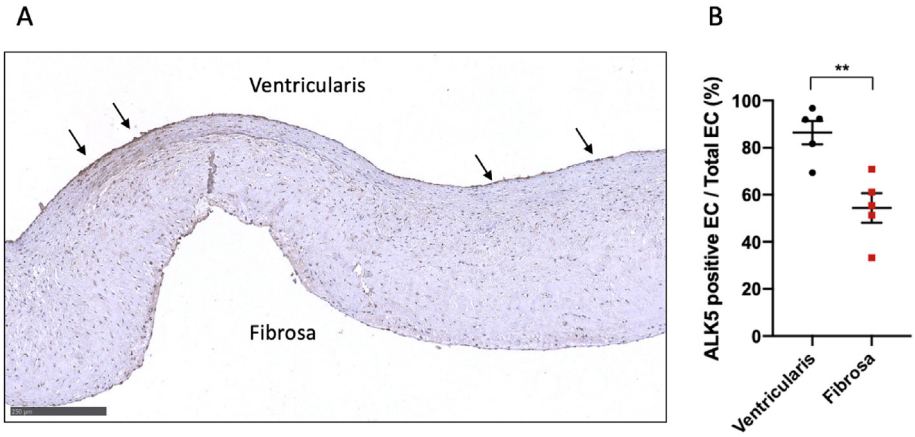

Fig. 4. (A) Representative image from immunohistochemical staining of ALK5 in sections of porcine aortic valve. (B) Graph quantifying ALK5-positive ECs, showing that ALK5 expression was significantly higher in ECs of the ventricularis (ventricular side) than the fibrosa (aortic side) of porcine aortic valve. Scale bar = 250 μm. Student's t-test; n = 5; **p < 0.01.

signalling pathways via ALK5 internalisation and degradation.

Pro-inflammatory flow-induced endothelial activation has been linked to many cardiovascular disorders, including atherosclerosis and CAVD [3,23]. Structurally intact ECs respond to pro-inflammatory flow by upregulating cell adhesion molecules, recruiting leukocytes and increasing permeability. To address these aspects, we measured monocyte adhesion and employed permeability assays based on the binding of FITC–avidin tracer to a biotinylated substrate [5,6,18] under LMMF and HMUF. To understand the role of ALK5, we treated ECs with an ALK5 inhibitor prior to and during shear stress exposure. Consistent with our previous report, LMMF triggered elevated monocyte adhesion and increased paracellular permeability [5,6]; a novel observation was that both of these actions were essentially abrogated by ALK5 inhibition.

The role of the TGF-β signalling pathway in endothelial activation is complex. The current consensus is that activation of the ALK5–SMAD2 signalling axis leads to endothelial quiescence, a state characterised by low expression of cellular adhesion molecules and an intact endothelial cell barrier [8]. However, there is increasing evidence that suggests otherwise. In a knockout mouse model, loss of ALK5 reduced endothelial activation and resulted in a lesser atherosclerotic burden [12]. The present study also produced results suggesting that ALK5 may have an essential role in flow-induced endothelial activation.

To examine the in vivo relevance of the results from our cell based assays, we investigated the ALK5 expression pattern in aortic valve samples. Porcine aortic valve tissue was chosen because ECs on the ventricularis and fibrosa layers are known to experience very different shear stress profiles: ECs of the ventricularis layer are exposed to high magnitude shear stress with minimal direction changes, whereas their counterparts in the fibrosa layer experience low magnitude and multidirectional shear stress (commonly termed “disturbed flow”) [24]. Our analysis revealed lower endothelial ALK5 expression in the fibrosa layer than the ventricularis layer. This finding is consistent with the observed suppression of ALK5 expression in HUVECs sheared with LMMF.

In conclusion, our findings suggest that ALK5 expression in ECs is suppressed by LMMF, potentially via receptor internalisation and recycling. Such a mechanism appears necessary for LMMF-induced endothelial activation, since ALK5 inhibition abrogated LMMF-induced monocyte adhesion and increased paracellular permeability. The role of ALK5 as a mechanoreceptor initiating pro-inflammatory mechanosensitive signalling might offer a new avenue for therapeutic discovery in cardiovascular diseases.

Author contributions

Conceptualization, K.T.P, P.D.W.; methodology, K.T.P., M.G., P.S., and C.M.W.; validation, K.T.P., M.G., P.S., C.M.W.;A.H.C., and P.D.W.; formal analysis, K.T.P. and M.G.; investigation, K.T.P., M.G., P.S., and A.H.C.; resources, A.H.C. and P.D.W.; data curation, K.T.P., M.G., and P.S.; writing—original draft preparation, K.T.P., A.H.C., and P.D.W.; writing—review and editing, K.T.P., M.G., P.S., C.M.W., M.H.Y., A.H.C., and P.D.W.; visualization, K.T.P. and M.G.; supervision, P.D.W.; project administration, K.T.P. and P.D.W.; All authors have read and agreed to the published version of the manuscript.

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Data availability statement

Data supporting the findings of this study are available from the corresponding author on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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