

# Effects of FSS on the expression and localization of the core proteins in two Wnt signaling pathways, and their association with ciliogenesis

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**Abstract.** Fluid shear stress (FSS) may alter ciliary structures and ciliogenesis, and it has been reported that the Wnt signaling pathway may regulate cilia assembly and disassembly. The present study aimed to investigate the effects of FSS on primary cilia, the Wnt/ $\beta$ -catenin and Wnt/PCP signaling pathways, and the association among them. In the present study, human umbilical vein endothelial cells were subjected to FSS of differing velocities for various periods of time using a shear stress device. Subsequently, immunofluorescence and quantitative polymerase chain reaction were used to detect the expression and localization of the following core proteins:  $\beta$ -catenin in the Wnt/ $\beta$ -catenin signaling pathway; and dishevelled segment polarity protein 2 (Dvl2), fuzzy planar cell polarity protein (Fuz) and VANGL planar cell polarity protein 2 (Vangl2) in the Wnt/planar cell polarity (PCP) signaling pathway. Furthermore, the colocalization of Dvl2 with the basal body was analyzed under low FSS and laminar FSS. The results demonstrated that low FSS promoted the expression of *Dvl2* and its colocalization with the basal body. Although *Fuz* expression was decreased with increasing duration of FSS, no visible alterations were detected in its localization, it was ubiquitously localized in the ciliated region. Conversely, the expression of *Vangl2* was increased by laminar FSS, and  $\beta$ -catenin was translocated into the nucleus at the early stage of low FSS. These findings suggested that Dvl2 may participate in low FSS-induced ciliogenesis and  $\beta$ -catenin may participate at the early stage, whereas *Vangl2* may be associated with laminar FSS-induced cilia disassembly.

## Introduction

Fluid shear stress (FSS) refers to friction that is generated by blood flow, which acts on the vascular wall. Endothelial cells (ECs) are directly exposed to FSS, and are able to respond to differential stress induced by constantly varying flow patterns and velocities by altering their shape, polarity and patterns of gene activity (1). Primary cilia are considered one of the major stress sensors in vascular endothelium (2). The primary cilium is a non-motile microtubule-based structure present at the surface of almost every mammalian cell, which extends from the basal body. The basal body functions as the template for ciliogenesis, and regulates the entry and exit of proteins into and out of the cilia that are required for cilia assembly (3).  $\gamma$ -tubulin, which is a basal body marker, is associated with initiation of cilia assembly. Intraflagellar transport 88 (IFT88) is a transport protein for tubulin and is also a marker for ciliogenesis. In recent years, it has been reported that primary cilia are key coordinators of signaling pathways during development and tissue homeostasis; therefore, cilia-associated disorders, known as ciliopathies, can affect numerous organ systems, and include autosomal recessive polycystic kidney disease and nephronophthisis (4). The mechanosensing function of primary cilia depends on the following mechanoproteins: Polycystins 1 and 2 (5). An abrupt alteration in FSS can be detected by these sensory proteins localized in the cilia, and these alterations are transduced and translated via a complex pathway of intracellular signaling. Notably, polycystin can become functionally inactive following exposure to high FSS (6). Previous studies have suggested that alterations in FSS may also alter ciliary structure by shortening the cilia or leading to depolymerization (7,8). Furthermore, not all parts of the vasculature possess cilia. Scanning electron microscopy indicated that primary cilia are distributed in embryonic endocardium and participate in cardiac differentiation (9), and are found in the branches and bends of vessels, which is associated with atherosclerosis (10). In addition, the distribution pattern of monocilia is associated with the pattern of shear stress, and primary cilia disassemble in ECs under laminar FSS (7). In the past few decades, it has been reported that shear flow is an influencing factor in ciliogenesis (11). Although some studies have reported that oscillatory fluid flow may stimulate the

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assembly of primary cilia via an increase in the surrounding number of microtubules (12), little is currently known about the mechanism underlying flow-induced primary cilia assembly and disassembly in ECs (11).

Recently, the Wnt signaling pathway has been revealed to be associated with the primary cilium and FSS. The Wnt proteins are a family of 19 highly conserved secreted glycoproteins that act via frizzled (Frz) receptors, or via a complex that is composed of Frz and low-density lipoprotein-receptor-related proteins 5 and 6. At present, three distinct intracellular Wnt signaling cascades are well established: The Wnt/ $\beta$ -catenin pathway, which is the canonical Wnt signaling pathway; the Wnt/planar cell polarity (PCP) signaling pathway; and the Wnt/ $\text{Ca}^{2+}$  pathway. The Wnt/ $\beta$ -catenin pathway is mediated by  $\beta$ -catenin, which accumulates in the cytoplasm in the presence of Wnt, and can then be translocated to the nucleus (12). The Wnt/PCP signaling pathway, which was discovered in *Drosophila*, serves a role in establishing cell polarity during development of the organism (13). In mammals, Wnt/PCP is essential for neural tube closure, fur patterning, hair bundle orientation in the inner ear and axonal guidance. The core PCP genes include dishevelled segment polarity proteins 1-3 (*Dvl1-3*), VANGL planar cell polarity proteins 1-2 (*Vangl1-2*), *Frz*, cadherin, and Prickle-like proteins 1-4. Another group of PCP proteins comprises the 'effector' molecules, including fuzzy planar cell polarity protein (Fuz), inturned and Fritz (14). The Wnt signaling pathway has been reported to be associated with the mechanical force stimulation. In numerous tissue types, particularly in the endothelium, PCP develops in response to shear flow (15-17). To the best of our knowledge, the mechanisms that differentially trigger and control these signaling pathways remain to be fully understood. It has recently been proposed that hemodynamic shear stress causes the polarization of ECs in the direction of flow, and the noncanonical Wnt signaling pathway may reduce endothelial shear sensitivity by regulating the cell polarity (18). Notably, the Wnt signaling pathway is essential for ciliogenesis. Numerous proteins in the Wnt signaling pathway have been confirmed to colocalize with the basal body, and mutations in these proteins induce ciliary mislocalization or deficiency (19). Furthermore, the cilium functions as a regulatory switch to control the balance between canonical and noncanonical Wnt pathways (20). However, the association between ciliogenesis, Wnt/ $\beta$ -catenin and Wnt/PCP signaling pathways has yet to be elucidated. In addition, very little is known about whether these two signaling pathways are involved in primary ciliogenesis induced by FSS in ECs.

In the present study, to determine the role of Wnt/ $\beta$ -catenin and Wnt/PCP signaling pathways in FSS-induced ciliogenesis, the cells were subjected to differing velocities of FSS for various durations using a shear stress device. Subsequently, immunofluorescence and quantitative polymerase chain reaction (qPCR) were used to assess activation of the Wnt/ $\beta$ -catenin and Wnt/PCP signaling pathways, in order to determine the effects of FSS on the two pathways and primary cilia in ECs. Furthermore, the colocalization of Dvl2 and the basal body under low and laminar FSS was analyzed. The results indicated that under low FSS for 12 h, ECs could induce the localization of Dvl2 to the basal body, whereas laminar FSS led to the mislocalization of Dvl2, thus suggesting that

low FSS may induce primary ciliogenesis via the Wnt/PCP signaling pathway.

## Materials and methods

**Cell culture.** Primary human umbilical vein ECs (HUVECs) were cultured in a humidified incubator at 37°C in vascular cell basal medium in the presence of vascular endothelial growth factor supplemented with 1% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from American Type Culture Collection, Manassas, VA, USA).

Cells grown on matrix-treated culture slides (75x25x1.0 mm; Flexcell International Corporation, Burlington, NC, USA) were subjected to FSS using a shear stress device (Streamer®; Flexcell International Corporation) for 0, 6, 12 and 18 h at 37°C. As the inside diameter of the hose, the length and width of the slide are fixed, the magnitude of FSS is controlled by the flow rate of the liquid, which is controlled by the injection pump, based on the following FSS formula:  $\text{FSS} = 6\mu Q/a2b$ , where  $\mu$  refers to the apparent viscosity of the media;  $a$  refers to height;  $b$  to width; and  $Q$  to flow rate. Shear stress was regulated independently at 0, 1 and 15 dynes/cm<sup>2</sup> using a computer-controlled peristaltic pump, and cells that underwent FSS for 0 h were considered negative control samples. Subsequently, cells were harvested for RNA and protein extraction.

**Immunofluorescence.** Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 3 min at 4°C in PBS. Subsequently, the cells were blocked with 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) three times (5 min per time) at room temperature in PBS. Protein expression was detected by immunofluorescence using the following primary antibodies at 4°C overnight: Mouse anti-Dvl2 (cat. no. sc-8026, 1:1,000) and mouse anti- $\beta$ -catenin (cat. no. sc-59737, 1:2,000) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), goat anti-Vangl2 (cat. no. SAB2501092, 1:2,000), rabbit anti- $\gamma$ -tubulin (cat. no. T5192, 1:1,000), rabbit anti-Fuz (cat. no. HPA041779, 1:1,000) and rabbit anti-IFT88 (cat. no. SAB1302866, 1:1,000) (Sigma-Aldrich; Merck KGaA). Subsequently, cells were incubated with corresponding secondary antibodies for 30 min at room temperature: Donkey Alexa Fluor 488 or Alexa Fluor 594 anti-mouse, -rabbit or -goat (cat. no. A-21202, 1:1,000; cat. no. A-21203, 1:1,000; cat. no. A-210207, 1:1,000; cat. no. A-11058, 1:1,000; Molecular Probes; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Nuclei were counterstained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.), and images were captured using a fluorescence microscope (Olympus IX83; Olympus Corporation, Tokyo, Japan) at room temperature.

**Reverse transcription (RT)-qPCR.** RNA was isolated using the RNeasy kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. cDNA was synthesized using the Superscript III reverse transcriptase and random hexamer primers according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using TB Green™ Premix Ex Taq™ II (Tli RnaseH Plus) (Takara Bio, Inc.) on samples from three independent experiments, according to the manufacturer's protocol. The thermocycling

conditions were as follows: Predenaturation at 95°C for 3 min; followed by 40 cycles of denaturation, at 95°C for 10 sec, and annealing and extension at 60°C for 30 sec. All experiments were conducted in duplicate, and *GADPH* was used as the housekeeping gene for normalization and quantification. The relative fold-change in expression was calculated using the  $2^{-\Delta\Delta C_q}$  method ( $C_q$  values <30) (21). Analysis was performed according to the manufacturer's protocol. Primer sequences are listed in Table I.

**Western blot analysis.** Cells were harvested from each group, and radioimmunoprecipitation assay lysis and extraction buffer (Thermo Fisher Scientific, Inc.) containing 1% phenylmethylsulfonyl fluoride was used to obtain cell lysates for protein expression detection by western blotting. Protein concentration was determined using the bicinchoninic acid method; protein samples (25  $\mu$ g) were then subjected to electrophoresis (5% stacking gel and 8% separation gel). Samples were transferred to polyvinylidene fluoride membranes in electric transfer buffer (0.58% Tris base, 0.29% glycine, 0.037% SDS, 1% methanol) at 4°C for 120 min. Subsequently, the membranes were blocked in Tris-buffered saline-0.5% Tween with 1% skimmed milk at room temperature for 2 h, and were probed with rabbit anti-human Dvl2 antibody (cat. no. SAB2100634, 1:1,000; Sigma-Aldrich; Merck KGaA) and mouse anti-human GAPDH antibody (cat. no. ab9484, 1:10,000; Abcam) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated sheep anti-rabbit and rabbit anti-mouse secondary antibodies (cat. nos. A16172 and 61-6520, 1:3,000; Thermo Fisher Scientific, Inc.) at room temperature for 2 h, respectively. The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagent (Clarity™ Western ECL Substrate; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and semi-quantified using Quantity One software 4.6.7 (Bio-Rad Laboratories, Inc.). The ratio of the optical density of the target protein to that of GAPDH was estimated as the relative expression level of the target protein.

**Statistical analysis.** All experiments were repeated three times. All data are presented as the means  $\pm$  standard error of the mean. Differences between means were determined using unpaired two-tailed Student's t-tests.  $P < 0.05$  was considered to indicate a statistically significant difference using SPSS 15.01 (SPSS, Inc., Chicago, IL, USA).

## Results

**mRNA expression levels of the core proteins in two Wnt signaling pathways under low and laminar FSS.** To assess the effects of various types of FSS on the expression of core proteins in the Wnt/ $\beta$ -catenin and Wnt/PCP pathways, the mRNA expression levels were measured and quantified using qPCR (Fig. 1). Cells loaded with FSS at 0 h were considered the negative control group. The results demonstrated that the expression levels of *Dvl2* were increased with time and maximal expression was detected at 18 h. Furthermore, the expression levels of *Dvl2* under low FSS (1 dynes/cm<sup>2</sup>) were significantly higher than those under laminar FSS (15 dynes/cm<sup>2</sup>) ( $P < 0.05$ ); expression exhibited a 1.9-fold increase in response to low FSS compared with laminar FSS at 18 h (Fig. 1A). Conversely,

Table I. Primer sequences of the genes detected by quantitative polymerase chain reaction.

Gene	Primer sequences (5'-3')
<i><math>\beta</math>-catenin</i>	F: 5'-CTGGCCATATCCACCAGAGT-3' R: 5'-GAAACGGCTTTTCAGTTGAGC-3'
<i>Dvl2</i>	F: 5'-TTCAACGGAAGGGTGGTATC-3' R: 5'-TGGCAAAGGAGGTAAAGGTG-3'
<i>Vangl2</i>	F: 5'-ATCGGACTCTCCGGAACCTT-3' R: 5'-TCAGCAGATACTGCCCTGTG-3'
<i>Fuz</i>	F: 5'-ACTGAGGAACCAGGCACAG-3' R: 5'-TCAAAGAAGTGGGGTGAGG-3'
<i>IFT88</i>	F: 5'-GAGAGGCTCTGCATTGACC-3' R: 5'-CCTGCACTTTTGCCTTTTC-3'
<i><math>\gamma</math>-tubulin</i>	F: 5'-AGAACGGCTGAATGACAGGT-3' R: 5'-TTGATCTGGGAGAAGGATGG-3'
<i>GAPDH</i>	F: 5'-CAGGAGGCATTGCTGATGAT-3' R: 5'-GAAGGCTGGGGCTCATTT-3'

*Dvl2*, dishevelled segment polarity protein 2; F, forward; *Fuz*, fuzzy planar cell polarity protein; *IFT88*, intraflagellar transport 88; R, reverse; *Vangl2*, VANGL planar cell polarity protein 2.

the expression levels of *Fuz* were decreased with time, and the effects of low FSS were less than those of laminar FSS (Fig. 1B). Notably, the mRNA expression levels of the core protein of the PCP signaling pathway, *Vangl2*, were very low prior to 18 h; however, the expression increased sharply under laminar FSS for 18 h, ~100-fold (Fig. 1C). Although  *$\beta$ -catenin*, which is the core protein of the Wnt/ $\beta$ -catenin pathway, was also influenced by laminar FSS, no significant alterations were noted with time under low FSS, which was mostly equivalent to that of the control group. At the early stage of laminar FSS, the expression levels of  *$\beta$ -catenin* were significantly increased; expression was 2-fold that of the control group. However, the expression was decreased to basal levels with increasing time (Fig. 1D). Therefore, this protein may be considered an effector at the early stage under the influence of laminar FSS. The expression levels of *Dvl2* protein were detected, and were significantly increased in response to low FSS, by ~2-fold ( $P < 0.05$ ). Furthermore, *Dvl2* protein expression was suppressed under laminar FSS ( $P < 0.05$ ) as compared with cells at 0 h (Fig. 1E and F).

**mRNA expression levels of the basal body protein  $\gamma$ -tubulin and the primary cilia protein IFT88 under low and laminar FSS.** The mRNA expression levels of the basal body protein  *$\gamma$ -tubulin* were assessed under low FSS (1 dynes/cm<sup>2</sup>) and laminar FSS (15 dynes/cm<sup>2</sup>) for 6, 12, and 18 h. Cells loaded with FSS at 0 h were considered a negative control group. The results indicated that the expression levels of  *$\gamma$ -tubulin* were higher under laminar FSS than under low FSS before 12 h, with a considerable difference at 12 h ( $P < 0.05$ ); however, this phenomenon was reversed after 18 h ( $P < 0.05$ ) (Fig. 2A). Similar to  *$\gamma$ -tubulin*, the mRNA expression levels of the primary cilia protein, *IFT88*, were decreased before 12 h in

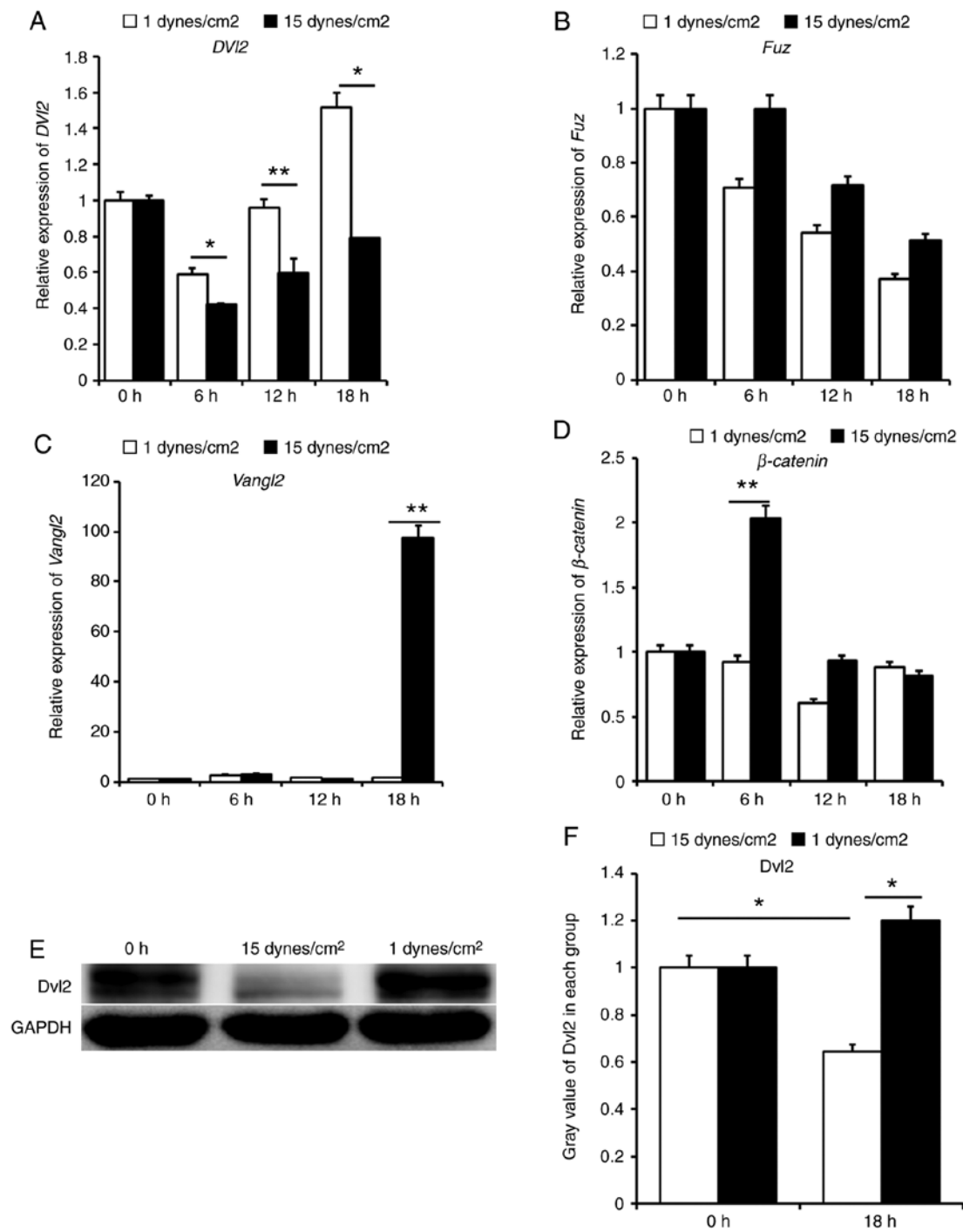


Figure 1. Relative expression of core proteins in the Wnt signaling pathway and the protein expression levels of Dvl2. (A) Low and laminar FSS promote the expression of *Dvl2* with increasing time; the highest expression was observed after low FSS loading for 18 h, which exhibited a 1.9-fold increase compared with under laminar FSS. (B) Expression of *Fuz* was highest during the early stage, and declined with increasing time; however, no statistical difference was observed between each group. (C) Expression levels of *Vangl2* were low in almost all groups, with the exception of the 18 h laminar FSS group. (D)  $\beta$ -catenin exhibited a temporary increase under laminar FSS at 6 h, ~2-fold of that under low FSS. (E) Expression levels of Dvl2 were higher in the low FSS group compared with in the laminar FSS group. (F) Gray value was estimated by Quantity One software; relative expression in the low FSS group was ~2-fold that in the laminar FSS group. Data are presented as the means  $\pm$  standard error of the mean, n=5. \*\*P<0.01, \*P<0.05 vs. laminar FSS. FSS, fluid shear stress; *Dvl2*, dishevelled segment polarity protein 2; *Fuz*, fuzzy planar cell polarity protein; *Vangl2*, VANGL planar cell polarity protein 2.

each group, and no significance was presented between these groups at 6 or 12 h. After 18 h, *IFT88* expression was increased under low FSS compared to that under laminar FSS (P<0.01) (Fig. 2B). These results indicated that low FSS may promote membrane localization of the basal body of primary cilia and cilia assembly.

*Localization of the core proteins in the two Wnt signaling pathways under low and laminar FSS.* In order to obtain the activation status of the canonical and noncanonical Wnt signaling pathways under various types of FSS, the localization of  $\beta$ -catenin (the core protein of canonical Wnt signaling) and Dvl2, Vangl2 and Fuz (the core proteins of

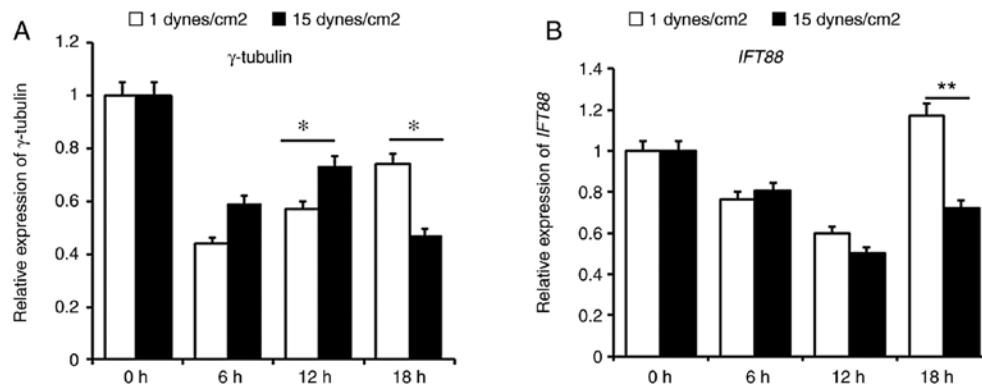


Figure 2. Relative expression of core proteins of primary cilia. (A) Expression levels of  $\gamma$ -tubulin were promoted under low FSS 18 h. (B) Expression levels of IFT88 were markedly increased after 18 h under low FSS (1 dyne/cm<sup>2</sup>, low FSS; 15 dynes/cm<sup>2</sup>, laminar FSS). \*\*P<0.01, \*P<0.05 vs. laminar FSS. FSS, fluid shear stress; IFT88, intraflagellar transport 88.

Wnt/PCP signaling) was assessed by immunofluorescence (Figs. 3-7).

Under low FSS (1 dyne/cm<sup>2</sup>) for 6, 12 and 18 h, the localization of these proteins altered in a time-dependent manner, with the exception of Fuz, which was permanently localized at a specific point in the cytoplasm during the whole process (Fig. 3A-C). The levels of  $\beta$ -catenin were increased in the cytoplasm under low FSS, and it entered the nucleus after 6 h, after which, it exited and accumulated around the nucleus. Notably, after 18 h,  $\beta$ -catenin was clustered in the cytoplasm (Fig. 4A-C). Similarly, the core protein of the PCP signaling pathway, Vangl2, was scattered around the nucleus after 12 h under low FSS, and some was aggregated in the cytoplasm at 18 h (Fig. 5A-C). In addition, under low FSS, Dvl2 was mainly localized in the cytoplasm, with some aggregated expression near the nucleus after 12 h (Figs. 6Bb-Db and 7Bb-Db).

Under laminar FSS (15 dynes/cm<sup>2</sup>) at 6 h, no distinct localization of  $\beta$ -catenin was observed in the cytoplasm; however, it clustered at 18 h (Fig. 4D-F). Conversely, under laminar FSS, Vangl2 was always localized in the cytoplasm and dispersed into the nucleus at 18 h (Fig. 5F). Under laminar FSS, Dvl2 was dispersed in the cytoplasm before 18 h, and its expression was markedly reduced at 18 h (Figs. 6Eb-Gb and 7Eb-Gb).

**Localization of the basal body protein  $\gamma$ -tubulin and the primary cilia protein IFT88.** In most negative control cells loaded with FSS at 0 h,  $\gamma$ -tubulin was primarily localized in the nucleus, and only a small number of cells exhibited cytoplasmic localization of this protein (Fig. 6Aa). Notably, localization in the cytoplasm was not fixed; therefore, some protein could be localized in the proximity of the nucleus, whereas some might be distally localized (Fig. 7Aa). When cells were loaded with low FSS for 6 h,  $\gamma$ -tubulin was not expressed in all cells (Fig. 6Ba), whereas in some cells IFT88 began to localize to the edge of the nucleus (Fig. 7Ba).  $\gamma$ -tubulin was observed in the majority of cells after 12 h (Fig. 6Ca-Da). Notably,  $\gamma$ -tubulin and IFT88 were localized to the edge of the nucleus, at the same area, after 12-18 h under low FSS (Fig. 7Ca-Da).

At the early stage of laminar FSS, the aggregation and localization of  $\gamma$ -tubulin near the nucleus was most marked at 6 h (Fig. 6Ea). However, when it was loaded with laminar FSS

for 12 h, only a few cells were observed harboring  $\gamma$ -tubulin (Fig. 6Fa), and identification of cytoplasmic expression after 18 h was difficult (Fig. 6Ga). In addition, localization of IFT88 was rarely observed in the cytoplasm during the whole process of laminar FSS loading (Fig. 7Ea-Ga).

**Colocalization of Dvl2 with  $\gamma$ -tubulin and IFT88 under low and laminar FSS.** The colocalization of Dvl2 with  $\gamma$ -tubulin and IFT88 was observed under low and laminar FSS. The results did not show any localization of Dvl2 and IFT88 in the ciliated area in static cells, which was considered as a negative control sample, and  $\gamma$ -tubulin and IFT88 could only be observed in only some cells (Figs. 6Ac and 7Ac). However, 12 h after loading with low FSS (1 dyne/cm<sup>2</sup>), not only did the maximum number of cells exhibit  $\gamma$ -tubulin localization, but they also exhibited Dvl2 at the same position (Fig. 6Cc), and there colocalization of IFT88 and Dvl2 was detected (Fig. 7Cc). IFT88 was mainly localized at the primary cilia, whereas Dvl2 was detected at the base, thus indicating that they were not on the same focal plane; however, these were observed to be positioned in the same area. Similarly, after 18 h, obvious colocalization of Dvl2 with  $\gamma$ -tubulin and IFT88 was noted (Figs. 6Dc and 7Dc). Conversely, 12 h after loading with laminar FSS (15 dynes/cm<sup>2</sup>), Dvl2 colocalized with  $\gamma$ -tubulin in some cells (Fig. 6Fc); however, at 18 h, the localization of  $\gamma$ -tubulin and IFT88 with Dvl2 was reduced. These findings suggested that Dvl2 did not localize in a specific area despite its abundant appearance in the cytoplasm, which indicates that Dvl2 did not colocalize with  $\gamma$ -tubulin and IFT88 under laminar FSS at 18 h (Figs. 6Gc and 7Gc).

## Discussion

Under static conditions, little free  $\beta$ -catenin is observed; however, it accumulates and translocates to the nucleus when stimulated by specific signaling. Previous *in vivo* and *in vitro* experiments indicated that activation of the Wnt/ $\beta$ -catenin signaling pathway is the normal response to mechanical stimulation (22). Wnt/ $\beta$ -catenin increases the sensitivity of osteocytes to FSS (23). A recent study demonstrated that blood FSS increases the concentration of  $\beta$ -catenin in the cytoplasm (24). In the present study, the expression levels

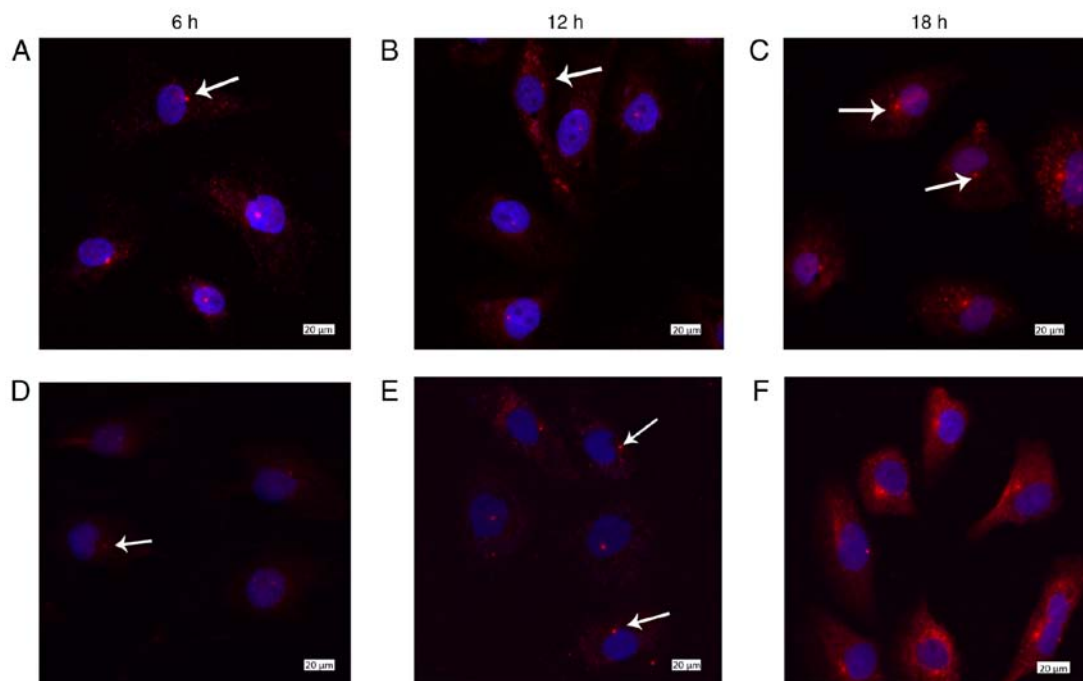


Figure 3. Immunofluorescence staining reveals that Fuz is localized at the ciliary area in the cytoplasm throughout the process of FSS. (A-C) Cells were loaded with low FFS for 6-18 h and localization of Fuz did not change (arrows). (D-F) Cells were loaded with laminar FFS for 6-18 h; Fuz was always localized in the ciliated region, which is similar to that in the case of low FFS (arrows). Scale bars=20  $\mu$ m. FFS, fluid shear stress; Fuz, fuzzy planar cell polarity protein.

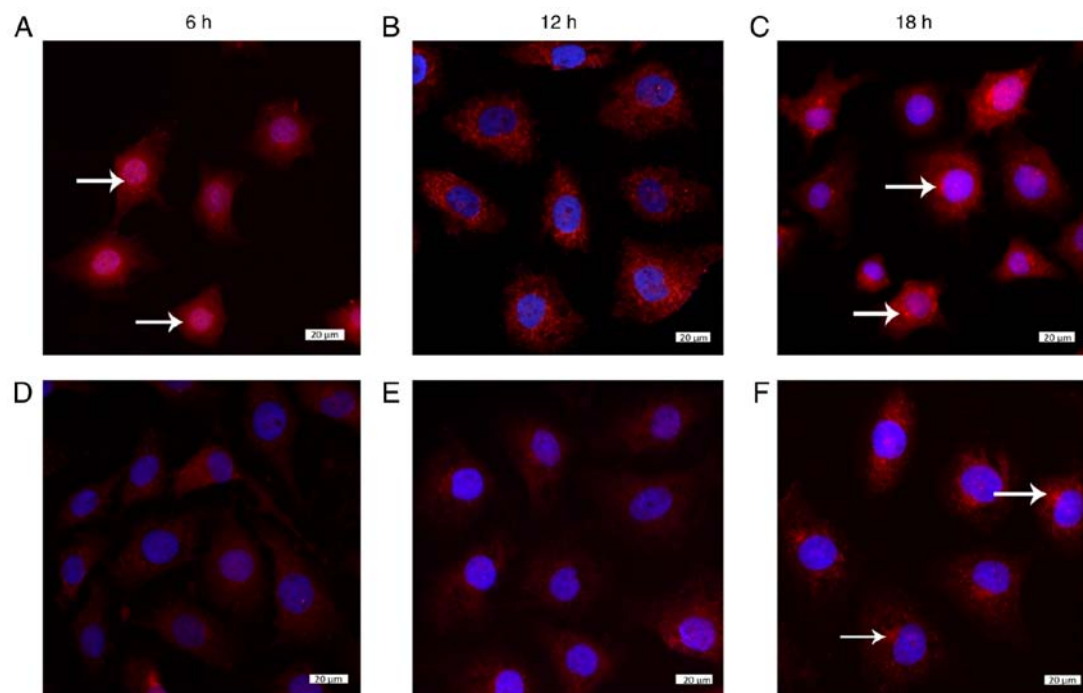


Figure 4. Immunofluorescence staining reveals that  $\beta$ -catenin responds differently to various types of FSS. (A-C) Cells were loaded with low FSS for 6-18 h;  $\beta$ -catenin was translocated to the nucleus at 6 h (arrows in 6 h image), and was then gradually transferred to a specific localization in the cytoplasm (arrows in 18 h image). (D-F) Cells were loaded with laminar FSS for 6-18 h, no translocation to the nucleus occurred during the whole process, and cytoplasmic expression was reduced. Some accumulation around the nucleus could be observed only at 18 h (arrow in 18 h image). Scale bars=20  $\mu$ m. FSS, fluid shear stress.

of  $\beta$ -catenin were upregulated at the early stage of laminar FSS, and translocation to the nucleus was observed at the early stage of low FSS, thus suggesting that FSS could activate Wnt/ $\beta$ -catenin for a short duration. Notably, although an upregulation of the protein was observed under the action of

laminar FSS, translocation to the nucleus was not observed; therefore, further studies on the activation of the downstream genes are required.

Dvl2 is a key scaffolding protein, and a branching point at the Wnt/ $\beta$ -catenin and Wnt/PCP pathways, which has a pivotal

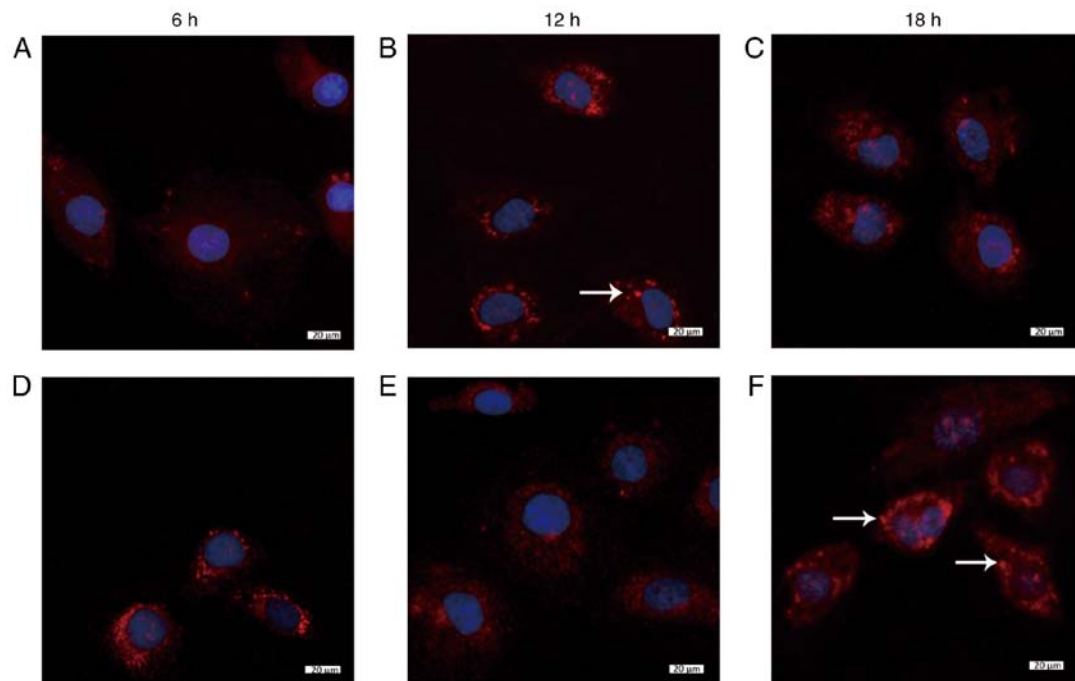


Figure 5. Immunofluorescence staining reveals that laminar FSS promotes the expression and localization of Vangl2 in the cytoplasm. (A-C) Cells were loaded with low FSS for 6-18 h, and cytoplasmic localization was difficult to observe, with the exception of a small quantity of the protein at 12 h (arrow in 12 h image). (D-F) Cells were loaded with laminar FSS for 6-18 h, and a large amount of Vangl2 was localized in the cytoplasm at 18 h (arrow in 18 h image). Scale bars=20  $\mu$ m. FSS, fluid shear stress; Vangl2, VANGL planar cell polarity protein 2.

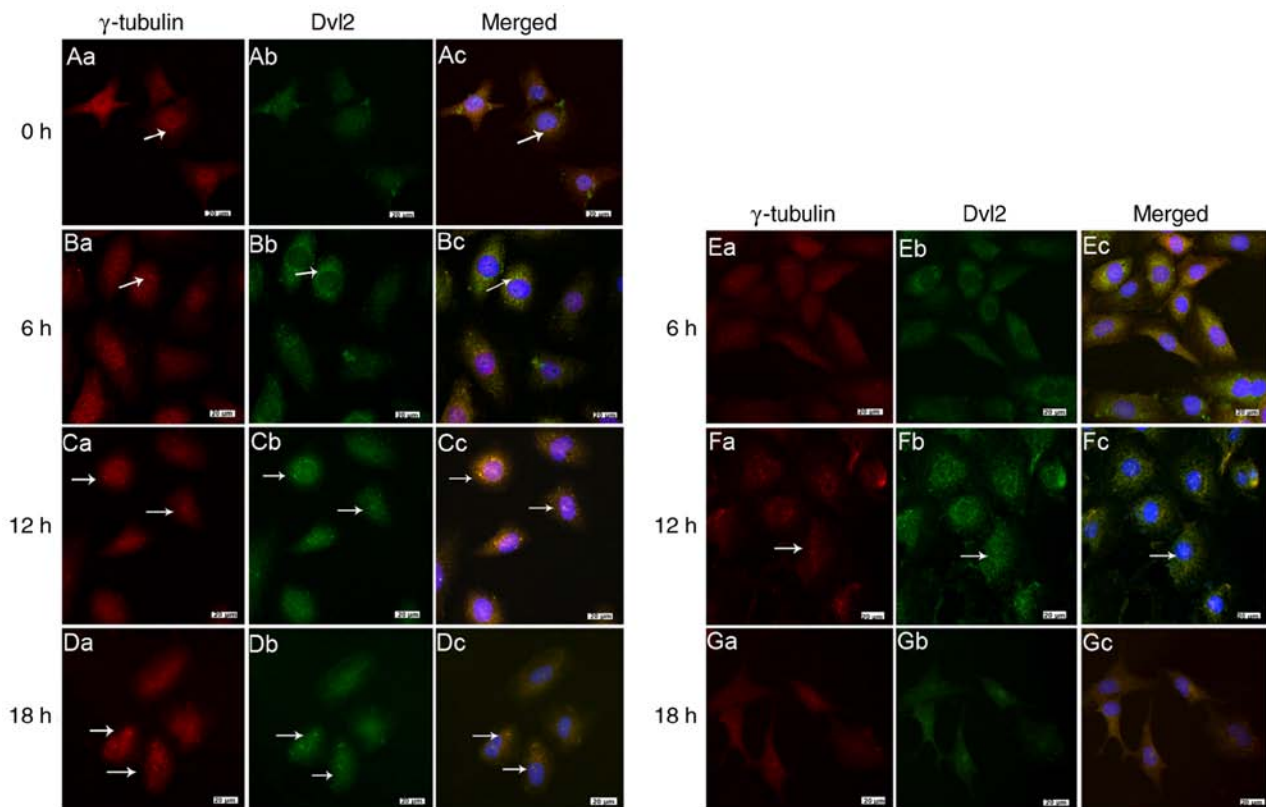


Figure 6. Immunofluorescence staining reveals that low FSS improves Dvl2 localization to the basal body, which is inhibited by laminar FSS. (Aa-Ac) Stationary cultured cells. (Ba-Dc) Cells were loaded with low FSS. (Ea-Ge) Cells were loaded with laminar FSS.  $\gamma$ -tubulin was labeled with red fluorescence and Dvl2 with green. (Aa-Da) Low FSS increased the number of cells labeled with  $\gamma$ -tubulin at the position of the basal body (arrows). (Ea-Ga) No localization of this protein was observed under laminar FSS except for individual cells at 12 h (arrow). (Ab-Db) Similar to  $\gamma$ -tubulin, fluorescent labeling of Dvl2 increased with time under low FSS and accumulated at one point between 12 and 18 h (arrows). (Eb-Gb) Labeling of Dvl2 was similar to that of  $\gamma$ -tubulin under laminar FSS (arrow). (Ac-Gc) Yellow fluorescence showed colocalization of these two proteins, only little could be observed in the stationary cultured cells; however, colocalization increased with low FSS (arrows), yet it was dispersed in cells under laminar FSS at 18 h (arrow). Scale bars=20  $\mu$ m. Dvl2, dishevelled segment polarity protein 2; FSS, fluid shear stress.



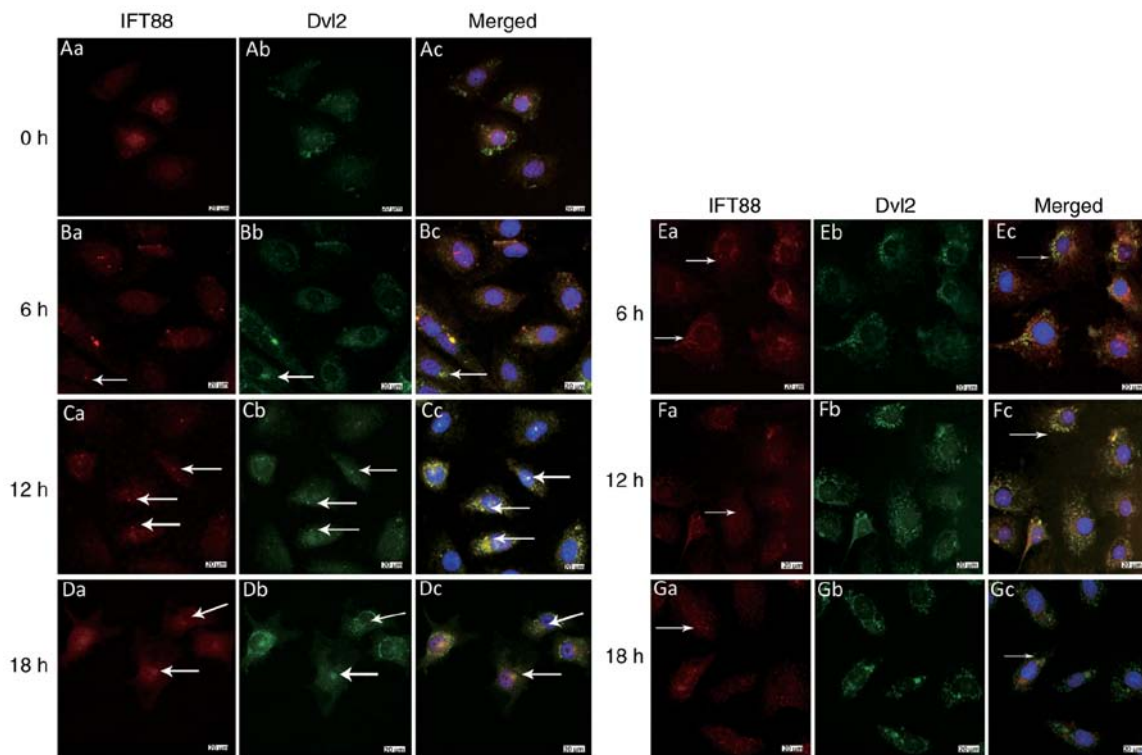


Figure 7. Immunofluorescence staining reveals that low FSS enhances the accumulation of IFT88 at the basal body, and this protein may participate in cilia assembly. (Aa-Ac) Stationary cultured cells. (Ba-Dc) Cells were loaded with low FSS. (Ea-Gc) Cells were loaded with laminar FSS. IFT88 was labeled with red fluorescence and Dvl2 with green. (Aa-Da) Loading of low FSS increased the expression of IFT88 (arrows). (Ea-Ga) Loading of laminar FSS led to only a few cells expressing the protein (arrows). (Ac-Gc) Yellow fluorescence indicated the colocalization of these two proteins, which could be observed in nearly all cells at 12 h (arrows), and after 18 h in response to low FSS, these two proteins were separated, rendering it difficult to capture them in the same focal plane (arrow); however, the proteins were dispersed in some cells under the action of laminar FSS at 18 h (arrows). Scale bars=20  $\mu$ m. Dvl2, dishevelled segment polarity protein 2; FSS, fluid shear stress; IFT88, intraflagellar transport 88.

role in Wnt signaling. It acts as a molecular switch in the activation of Wnt/PCP and the suppression of Wnt/ $\beta$ -catenin pathways. In vascular ECs, Wnt signaling has been reported to contribute towards the development of atherosclerosis; the risk factor, hypercholesterolemia, selectively activates canonical Wnt signaling over the noncanonical pathway by specifically facilitating membrane recruitment of Dvl and its interaction with other proteins (25). However, the effects of low FSS, another risk factor for atherosclerosis, on Dvl2 are not yet fully understood. In osteoblast cells, the gene expression levels of *Dvl2* and  $\beta$ -catenin are affected by mechanical stress (26,27). In the present study, detection of the gene and protein expression levels demonstrated the laminar FSS could inhibit the expression of Dvl2 and its localization to the basal body, whereas low FSS led to enhanced localization when loaded for 12-18 h. Therefore, in vascular ECs, external FSS could influence the expression and cellular localization of Dvl2.

Another core protein, Vangl2, is also affected by FSS. Recently, Curtis-Whitchurch *et al* reported that laminar flow (~15 dynes/cm<sup>2</sup> for 2-48 h) was able to induce phosphorylation of Vangl2 in cultured human ECs compared with under static conditions; however, the disrupted flow led to uniform distribution throughout the cells (28). In the current study, HUVECs were exposed to laminar FSS (15 dynes/cm<sup>2</sup>) and low FSS (1 dynes/cm<sup>2</sup>) for 6, 12 and 18 h. qPCR demonstrated that although Vangl2 was insensitive to low FSS, it could be substantially upregulated by laminar flow. Similarly, fluorescence labeling indicated that laminar FSS increased the

expression of this protein in the cytoplasm and it was also dispersed in the nucleus at 18 h. Therefore, Vangl2 may not participate in cellular activity induced by low FSS, which is essential for ECs under physiological mechanical conditions.

Unlike Dvl2 and Vangl2, Fuz is an effector of PCP signaling. However, studies regarding the effects of mechanical forces on Fuz are absent. In the present study, it was demonstrated that, although expression was decreased with increasing FSS time, this was not statistically significant; therefore, it was suggested that Fuz was not affected by FSS with increasing loading time. These findings suggested that it may be a stable protein that is not susceptible to external mechanical signals.

Ciliogenesis is considered to be associated with cell-cycle dependent cellular progression. However, it has been reported that it may also be influenced by external factors, including osmotic stress (29). Iomini *et al* reported that primary cilia of human ECs disassemble under laminar FSS (8). Previous reports have also demonstrated that ciliogenesis in ECs relies on the type of FSS during the initial stages of cardiac development and atherosclerosis (9,30). It was previously revealed that low FSS could enhance ciliogenesis, whereas laminar FSS could suppress ciliogenesis (31,32). However, the mechanism underlying flow-induced ciliogenesis remains to be elucidated (31).

Both Wnt/ $\beta$ -catenin and Wnt/PCP signaling pathways are associated with ciliogenesis. Several proteins, such as inversin, Dvl, Vangl2 and turned are enriched at the basal body (20). Stabilization of Dvl2 at the basal body is essential for the func-



tion of primary cilia. Dvl2 functions with inturned and Rho in the docking of basal bodies at the apical plasma membrane (33). Notably, it has been reported that Dvl2 may participate in the disassembly of cilia. Lee *et al* reported that Dvl2 participates in primary cilia disassembly, which is regulated throughout the cell cycle (33). However, the possibility of tissue-, time- and environment-dependent differences in ciliary association with Wnt signaling cannot be excluded. Furthermore, whether this protein is also required for the primary cilia assembly induced by FSS has not yet been reported. In the present study, it was demonstrated that the variation trends of the basal body protein  $\gamma$ -tubulin and the cilia assembly protein IFT88 were the same as the core protein Dvl2 in the Wnt signaling pathway. In addition, Dvl2 was gradually localized to the basal body during low FSS-induced ciliogenesis. However, this phenomenon was not observed under laminar FSS. Therefore, it may be hypothesized that in vascular ECs, Dvl2 is a critical signal molecule for the assembly, instead of disassembly, of primary cilia induced by mechanical stress.

Vangl2 is also known to be associated with basal body orientation. In zebrafish embryos, although Vangl2 is not required for ciliogenesis, it controls the posterior tilting of the primary cilia and is required for asymmetric localization (34). It has previously been demonstrated that Vangl2 participates in two PCP signals, while regulating the apical docking and polarity of cilia in ependymal cells (14). Notably, the dock of ependymal cilia basal bodies relies on the coupling between hydrodynamic forces and the PCP protein, Vangl2, within a limited duration (35). This feature indicates that Vangl2 may have a role in ciliogenesis regulated by the hydrodynamic force. In the present study, it was demonstrated that the altered gene expression and cellular localization of Vangl2 was in agreement with ciliogenesis under low FSS, as well as, with cilia disassembly under laminar FSS. Furthermore, due to the relationship of this protein with cilia disassembly (36), it was speculated that Vangl2 might serve a dual role in ciliary fate influenced by FSS. Under low FSS, although the expression of the protein did not change, its cytoplasmic localization could guide basal body orientation. In addition, under laminar FSS, the strong expression and cellular accumulation of the protein might be associated with the primary cilia disassembly via another pathway.

Fuz also localizes to the basal body. In *Xenopus* and mice, the loss of Fuz protein disrupts ciliogenesis, which in turn, might impair the formation of primary cilia in the skin (37-39). Fuz controls cilia assembly and signaling by recruiting Rab8 and Dvl to the primary cilium (40). Furthermore, Fuz is required for normal IFT dynamics in vertebrate cilia, and has a specific role in trafficking of retrograde, but not anterograde, IFT-B proteins (41). The present study indicated that, although Fuz was not markedly influenced by various types of FSS with regards to expression and localization, it was located at the base of primary cilia in the cytoplasm during the whole process. Considering that it appears to control the subcellular localization of the core PCP protein Dvl2, it may be inferred that, although Fuz was not regulated by an external mechanical signal, its stationary cellular localization may propitiously orient Dvl2 to pitch at the basal body.

Although there is no direct evidence indicating the localization of  $\beta$ -catenin to the basal body, its translocation to the

nucleus has been associated with mutation-induced ciliopathies (42), thereby suggesting an indirect relationship between  $\beta$ -catenin and ciliogenesis. Immunofluorescence demonstrated that both low and laminar FSS loading for a prolonged period promoted the clustering of this protein at a specific point in the cytoplasm, in the region of the basal body, thus suggesting that mechanical stress is crucial in stabilizing  $\beta$ -catenin in the cytoplasm. Although the alterations in  $\beta$ -catenin cellular location are not synchronous with that of the basal body protein  $\gamma$ -tubulin, and Vangl2 and Fuz in the PCP signaling pathway under various types of FSS, its localization to the ciliary region indicated its potential connection to primary cilia. Since the trigger of these two signaling pathways is diverse, and the correlation between cilia and Wnt/ $\beta$ -catenin is currently unclear, an in-depth investigation into whether  $\beta$ -catenin is essential for FSS-induced cilia assembly or disassembly is required.

In conclusion, the results demonstrated that low FSS promoted the expression of *Dvl2* and its colocalization with the basal body. Furthermore the expression of *Vangl2* was increased by laminar FSS, and  $\beta$ -catenin was translocated into the nucleus at the early stage of low FSS. These findings suggested that Dvl2 may participate in low FSS-induced ciliogenesis and  $\beta$ -catenin may participate at the early stage, whereas Vangl2 may be associated with laminar FSS-induced cilia disassembly. The results have important clinical significance for exploring the relationship between shear stress and the inflammatory response of endothelial cells.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XS and YS conceived and designed the study. YL, XL and BS performed the experiments. DL processed data. XS and YS wrote the paper. XS, YS, YL and XL reviewed and edited the manuscript. All authors read and approved the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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