Mechanical factors influence $\beta$-catenin localization and barrier properties

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Abstract

Mechanical forces are of major importance in regulating vascular homeostasis by influencing endothelial cell behavior and functions. Adherens junctions are critical sites for mechanotransduction in endothelial cells. $\beta$-catenin, a component of adherens junctions and the canonical Wnt signaling pathway, plays a role in mechanoactivation. Evidence suggests that $\beta$-catenin is involved in flow sensing and responds to tensional forces, impacting junction dynamics. The mechanoregulation of $\beta$-catenin signaling is context-dependent, influenced by the type and duration of mechanical loads. In endothelial cells, $\beta$-catenin’s nuclear translocation and signaling are influenced by shear stress and strain, affecting endothelial permeability. The study investigates how shear stress, strain, and surface topography impact adherens junction dynamics, regulate $\beta$-catenin localization, and influence endothelial barrier properties.

Insight box

Mechanical loads are potent regulators of endothelial functions through not completely elucidated mechanisms. Surface topography, wall shear stress and cyclic wall deformation contribute overlapping mechanical stimuli to which endothelial monolayer respond to adapt and maintain barrier functions. The use of custom developed flow chamber and bioreactor allows quantifying the response of mature human endothelial to well-defined wall shear stress and gradients of strain. Here, the mechanoregulation of $\beta$-catenin by substrate topography, wall shear stress, and cyclic stretch is analyzed and linked to the monolayer control of endothelial permeability.

Keywords: vascular mechanobiology; $\beta$-catenin signalling; mechanotransduction; barrier function

Introduction

Mechanical forces, key regulators of vascular homeostasis, exert effects on endothelial cells influencing their behavior and functions. Critical sites for mechanotransduction in endothelial cells are the adherens junctions (AJs), that are characterized by a dynamic configuration and adapt to specific tissue requirements triggered by hemodynamic forces stimuli. All the mechanical force-induced adjustments of the AJ structure are important for the dynamic control of endothelial barrier functions.

The AJ core component is a complex consisting of the transmembrane protein Vascular Endothelial (VE)-cadherin that binds cytoplasmic partners $^1$ among which the transcription co-factor $\beta$-catenin $^2$. Besides being a component of the AJs, $\beta$-catenin is one of the key elements of the canonical Wnt signaling $^3$. When it is released from the junctions or when it is stabilized by Wnt pathway activation, it can translocate to the nucleus and regulate gene expression $^2, 4$.

A growing body of evidence reports about the mechanoactivation of AJ components. VE-cadherin together with VEGFR2 and PECAM-1 form the flow sensor (mechanosensitive complex) that is required for the cell alignment in the direction of flow $^5$. Moreover, VE-cadherin-based AJs are responsive to tensional forces from the cytoskeletal components increasing the size of the junctions $^6$, and Vinculin is recruited to the AJs during force-dependent remodeling $^7$.

Mechanoregulation of $\beta$-catenin signaling pathway has been reported to be cell context dependent, and specifically related to the type and duration of the mechanical load applied $^8$. Tzima and co-workers suggested that $\beta$-catenin assists in the formation of the flow sensor $^5$, but its role in endothelial shear stress-mediated mechanotransduction remains elusive. $\beta$-catenin increases nuclear translocation and signaling in endothelial cells exposed to both pulsatile and oscillatory shear stress in vitro $^9$, through the activation of a Wnt-dependent pathway. In vivo, nuclear accumulation of $\beta$-catenin is detected in the atheroprone region of the aortic arch, as well as in vitro under atherogenic flow stimulation $^10$, via a Wnt-independent mechanism. Of interest, $\beta$-catenin mechanosignaling has been extensively studied in bone development and metabolisms, where it has been shown that in osteoblasts laminar shear stress induces the dissociation of $\beta$-catenin from N-cadherin, with the first that accumulates into the nucleus and signals $^11$. Increased $\beta$-catenin nuclear localization in endothelial cells can be triggered by nanopatterned topography $^12$ as well as by high strain cyclic stretch $^13$, with no evident detection of its transcriptional activity. It has
also been shown that mechanical strain induced cell cycle re-entry of quiescent epithelial monolayers through the nuclear localization and transcriptional activity of a group transcription factors among which β-catenin [14].

Being mechanotransduction at cell–cell contacts fundamental for the homeostasis of the endothelium, in the present work we studied how physiological values of shear stress and strain, and topography affect AJ dynamic and regulate the fraction of β-catenin that is bound to VE-cadherin or localized in the nucleus, controlling its downstream signaling pathway and endothelial permeability. We show here that these stimuli differently affect β-catenin subcellular localization and signaling, thus tuning the barrier properties of endothelial monolayers.

Materials and methods
Surface topography
Surface patterning was realized through so called “breath figures” (BF), generated as previously reported [15]. Briefly, membrane of 400 μm thickness and 14 mm diameter were fabricated using an RTV (room temperature vulcanizing) silicone. For flat substrates, the applied silicone was left to fully cure, producing a flat membrane of thickness. For the BF patterned substrates, a cycle of temperature and humidity was applied as described in [15] to induce condensation imprints on the curing elastomer surface. Specifically, the process parameters room temperature curing time tRTcure = 20 min and high temperature curing time tHTcure = 22 min were used before the freezer step and humidity step. The irregular arranged BF patterns feature a diameter and a center-to-center distance of the wells with a relatively broad distribution (average values of 52.9 ± 1.5 μm and 93.3 ± 7.2 μm, respectively), while the well depth is about 6.3 ± 0.6 μm. The resulting surface topography supports the in vitro generation of mature human endothelia [15].

Cell culture, passaging and seeding
Primary human umbilical vein endothelial cells (HUVEC; 3 different batches with n=4 pooled donors each, Promocell) were grown in Endothelial Cell Growth Medium (C-22010, Promocell) and were maintained at 37°C and 5% CO2. In all the experiments endothelial cells were seeded at high initial density (450 cell/mm²), immediately confluent and grown in static conditions for 72 h to generate mature monolayers as previously reported [16], unless otherwise specified. All reported experiments were performed using cells with less than six passages in vitro.

Gelatin coating
Gelatin coating was performed similarly as previously described [16]. Briefly, all surfaces were coated with glutaraldehyde (Sigma-Aldrich)-crosslinked gelatin (#104070, Merck Millipore, Germany) as follows: samples were incubated for 1 h at 37°C with 1.5% gelatin followed by a crosslinking with 2% glutaraldehyde solution for 15 min at room temperature (RT). The glutaraldehyde was then replaced by 70% ethanol. After 1 h at RT, 5 washes with PBS (Thermo Fisher Scientific) followed by overnight (O/N) incubation with 2 mM glycine in PBS were performed. Before cell seeding, slides were washed with PBS.

Paracellular permeability assay
The assay was carried out as previously reported [17]. Briefly, endothelial monolayers were cultured on biotin-conjugated 1.5% gelatin-coated substrates. At selected time points (end of experiments) Oregon green 488-conjugated avidin (Thermo Fisher Scientific) was added to the culture medium at a final concentration of 25 μg/ml for 3 min. The medium was then removed, and the cells were fixed with 37°C warm 4% paraformaldehyde (PFA) for 15 min. Residual unbound avidin was removed by washing samples with PBS after fixation.

DKK1 treatment
Recombinant human DKK1 protein powder (R&D systems) was reconstituted in PBS at stock concentration of 100 μg/ml. Samples were incubated with DKK1 at a final concentration of 100 ng/ml for 24 h. For control untreated samples, the same amount of PBS was added to the cells. After 24 h stimulations cells were fixed with 4% PFA.

Immunofluorescence and image acquisition
Fixed samples were permeabilized for 10 min with PBS 0.5% Triton X-100 and incubated for 1 h at RT in a blocking solution 2% Bovine Serum Albumin (BSA) in PBS. Samples were then incubated with primary antibodies diluted in blocking solution O/N at 4°C, washed with PBS, followed by appropriate secondary antibody incubation for 1 h at RT. DAPI was used to counterstain nuclei.

Confocal microscopy was performed at RT using an automated Nikon Ti spinning disk confocal microscope (Nikon) equipped with an Andor DU-888 camera (Oxford Instruments) and a pE100 LED illumination system (CoolLED Ltd) with violet (405-nm laser diode), blue (488 nm; Argon), yellow (561 nm; solid state) and red (633 nm; HeNe) excitation laser lines. For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings. Image acquisition was performed using a 63X/1.4 NA and 40X oil immersion objective (Plan Fluor, Nikon). Fluorescent Z-stacks of the signals emitted were acquired selecting the optical filters based on the respective emission. Only adjustments of brightness and contrast were used in the preparation of the figures.

Antibodies
The following primary antibodies were used: mouse anti β-catenin (IF: 1:100, BD Transduction Laboratories, 610154); rabbit anti p-S552-β-catenin(IF: 1:100, Cell Signaling, 56515); mouse anti GSK-3β (3D10) (WB: 1:2000, Cell Signaling 98325); rabbit anti phospho-GSK-3β (Ser9) (5B3) (WB: 1:2000, Cell Signaling, 9323S); mouse anti-GAPDH (WB: 1:1000, Santa Cruz, #sc-47724).

The following secondary antibody were used: donkey anti-mouse Alexa fluor 488 (1:200, #A21202) conjugated; and donkey anti-rabbit Alexa Fluor 555 (IF: 1:200, #A32794) conjugated. Cell nuclei were counterstained by DAPI (1:1000, 62248) from Thermo Fisher Scientific. The secondary antibodies for western blot were anti-mouse IgG, HRP-linked (WB: 1:2000, #7076) and anti-rabbit IgG, HRP-linked (WB: 1:2000, #7074) All secondary antibodies were from Thermo Fisher Scientific.

RT-qPCR
Real time quantitative polymerase chain reaction (RT-qPCR) was used to evaluate expression of different genes. Total RNA was extracted and purified using Qiagen RNA mini kit (Qiagen) following the manufacturer’s instructions. The RNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific). 600 ng of total RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer’s instructions. cDNAs amplification was performed using Power
track SYBR green gene expression assay (Thermo Fisher Scientific) and an ABI/Prism 7900 HT thermocycler. Specific primers for the target genes were:

AXIN2 forward 5′-TACACTCCTTATGGGGCCTA-3′ and reverse 5′-TTGGTACACTGAAATTGTTG-3′ [18], CLAUDIN1 forward 5′-GTCTTGGACTCCTGGTACTG-3′ and reverse 5′-CACCCTCATC GTCTTCAAGCA-3′ [19], CLAUDIN5 forward 5′-ATGTCGAGGTGCG CGGCTCT-3′ and reverse 5′-GTCTTTGACTCCTGGTACATTG-3′ (from OriGene); PLVP forward 5′-CAATCAAGAGGATCATCTGGTCG C-3′ and reverse 5′-CTATCTCACCTCAGGCTTCTT-3′ (from OriGene); LECF1 forward 5′-CTACCCATCCTCACTGTGCT-3′ and reverse 5′-GGATGTTCTGGTCTTACGTGAGG-3′ (from OriGene); GAPDH forward 5′-AAGTGGGAATCGACCGAT-3′ and reverse 5′-CTCTGTGAAATGTTGAGC-3′ [20].

For gene expression quantification, the comparative Ct (ΔΔCt) method was used. Briefly, the average Ct values of the housekeeping gene GAPDH was calculated and used as reference (Ct-ref). For each gene of interest, the ΔCt was calculated as follows: ΔCt = Ct gene – Ct-ref. The gene expression level was expressed as 2−ΔΔCt for each sample. Finally, for each experiment, the 2−ΔΔCt value of each sample was normalized on the corresponding control group, to yield the relative expression level (2−ΔΔCt). The resulting values were used for the plot and the statistical analysis.

Image analysis

To analyze the nuclear and junctional localization of β-catenin, images of total β-catenin, p-S552-β-catenin and DAPI are used. Nuclei and cell–cell junction location were segmented from the DAPI and total the β-catenin signals, respectively. The segmented nuclei and cell–cell junction images were then binarized to generate masks. By masking the p-S552-β-catenin image with the junctional total β-catenin mask, the mean fluorescent intensity of p-S552-β-catenin at the cell–cell junctions was calculated as I-junc. For each individual nucleus, the mean intensity of p-S552-β-catenin (I-nuc) was calculated from nuclei-masked p-S552-β-catenin images. Each individual nuclear p-S552-β-catenin average intensity I-nuc was compared to the averaged junctional intensity (I-junc). If I-nuc was larger than I-junc, this nucleus was marked as p-S552-β-catenin positive. Finally, the ratio between N-positive, the number of positive nuclei, and N-total, the number of total nuclei, was calculated and reported.

To evaluate p-S552-β-catenin cell–cell junctional localization, images of total β-catenin and p-S552-β-catenin were used. All confocal images were first projected with maximum intensity into 2D images and corrected for background illumination. Calculated threshold values were imposed for both the β-catenin and p-S552-β-catenin channels to segment the cell–cell junctions. Pearson’s correlation coefficient, an indicator of the colocalization, was determined using the colocalization plug-in of Imaris (Bitplane Scientific Software). Basically, the colocalization plug-in calculates the correlation of pixel intensities across both images.

To measure the junctional leakage in the paracellular permeability assay, images of 488-conjugated avidin were used. The permeability was determined by calculating the ratio between A-avidin, the area where avidin signal was present, and A-total, the total image area. A custom-made Matlab script was used to read the image, dynamically threshold and segment the biotin-avidin-bound area and calculate the ratio A-avidin/A-total.

Statistical analysis

Statistical comparisons were performed using a nonparametric Mann–Whitney test. All quantitative measurements reported as bar plots are expressed as average values ± standard deviation (SD). The number of biological independent experiments (N) and the number of technical replicates (N) is reported in the corresponding figure legends. All statistical analysis was performed using Origin (OriginLab, USA) and p less than 0.05 was considered significant (*P < 0.05, **P < 0.01, ***P < 0.001).

Western blotting

Cells were lysed in boiling Laemmli sample buffer [2 % SDS, 20 % glycerol, and 125 mM Tris–HCl (pH 6.8)]. Protein concentration was determined using a BCA Protein Assay kit (Thermo Fisher Scientific). Equal amounts of proteins were loaded on gel, separated by SDS-PAGE and transferred to a nitrocellulose membrane (Thermo Fisher Scientific). After blocking and incubation with primary and horseradish peroxidase-linked secondary antibodies, specific bindings were detected using a chemiluminescence system (Thermo Fisher Scientific). Western blot bands were quantified using optic densitometry software (Image Lab 6.0.1, Biorad).

Flow experiments

Flow experiments were performed in a custom-designed parallel plate flow chamber as previously described [15, 21, 22]. The flow rate was selected to reach the target WSS values, in accordance with the following equation: \( \tau = 6 \mu / W^2 H \), where \( \tau \) refers to the WSS, \( Q \) the volumetric flow rate, \( \mu \) dynamic viscosity of water at 37°C, \( W \) the width of the chamber, and \( H \) the height of the chamber. Specifically, cells were conditioned for 18 h with a flow yielding a nominal physiological WSS of 1.4 Pa [23].

Cyclic stretch experiments

An inflation-based custom-made bioreactor [24, 25] was used to apply 150 mbar internal pressure leading to an equibiaxial tension state in the elastomeric substrate. The corresponding deformation of the endothelial monolayers was ~13% (apex principal strain), which is within the physiological range [23]. The strain was applied at a frequency of 1 Hz for 18 h.

Results

Surface topology influences β-catenin nuclear localization

Surface topology affects (Wnt/β-catenin signaling in many cell model systems [26–28]. We previously developed a surface modification that is characterized by the generation of microscale wells (breath figures, BF) [15] and we showed that it affected VE-cadherin phosphorylation on Tyrosine residue responsible for the dynamic changes of AJs stability [16]. Since the phosphorylation of VE-cadherin has direct effects on the stability of β-catenin at AJs [29], we investigated the intracellular distribution of β-catenin within endothelial monolayers in static culture on BF vs flat conditions. In particular, we determined the level of phosphorylation of β-catenin at Serine 552 (SS52), also known as active β-catenin, which is a crucial post-translational modification that affects its stability, subcellular localization, and activity [30, 31].

When compared to monolayers cultured on flat substrates, endothelial cells on BF topography showed an increase in nuclear localization of β-catenin (Fig. 1A and B left panel), although no differences in its junctional localization were detected (Fig. 1A and B right panel). We then tested if the higher nuclear localization of β-catenin detected in the BF samples was correlated with an increase in its transcriptional activity. Therefore, by RT-qPCR we analyzed the expression of the known target genes of β-catenin AXIN2 and LEF1, and of a subset of endothelial β-catenin target genes encoding for protein.
regulating monolayer barrier functions CLDN1, CLDNS5, and PLVAP, respectively. As shown in Fig. 1C none of the target genes showed significant variations. Finally, we evaluated barrier integrity using a vascular permeability assay imaging-based (Fig 1D) [17]. Endothelial monolayers cultured on both substrates were characterized by a continuous and pronounced staining of β-catenin at AJs, and rare small paracellular focal sites of avidin leakage (Fig 1E). Quantitative analysis showed that paracellular avidin leak was decreased in cells cultured on BF substrates (Fig. 1F), although the measured values for both tested conditions are in the range of that reported for stable and mature monolayers [17, 32].

The results indicate that surface topography increases nuclear localization of β-catenin, with implications for barrier properties. No associated changes in β-catenin transcriptional activity could be identified.

**Wnt signalling is involved in BF-mediated β-catenin nuclear localization in static culture**

In the absence of Wnt signaling cytoplasmic β-catenin is rapidly degraded by the destruction complex, while when Wnt signaling pathway is activated, it initiates a cascade of events that ultimately leads to the stabilization and accumulation of β-catenin in the cytoplasm. The increased concentration of cytoplasmic β-catenin promotes its shuttling into the nucleus, where it binds to transcription co-factors and regulates gene transcription [33]. To determine if Wnt signaling was involved in BF-mediated nuclear relocalization of β-catenin, we inhibited the pathway treating endothelial monolayers with Dickkopf-related protein 1 protein (DKK1). As shown in Fig 2, DKK1 treatment strongly limited β-catenin nuclear accumulation in BF cultured monolayers and reduced it for the flat substrate. As a result, no significant difference between BF and flat substrate is observed after treatment. Importantly, DKK1 did not affect the amount of p-S552-β-catenin stabilized at cell–cell contacts. Overall, these data suggest that Wnt signaling takes part in the nuclear localization of p-S552-β-catenin in monolayers grown on both flat and BF patterned surfaces, and that the different nuclear localization is not due to a shuttling of β-catenin from the AJs.

**Flow strongly triggers β-catenin signaling from the AJs**

Endothelial cells undergo extensively morphological remodeling in response to altered (physiological) fluid flow, including cell elongation and reorientation [34]. These dynamic processes require the constant remodeling of AJs, which is mediated in part by the phosphorylation of VE-cadherin [16] that was shown to trigger the β-catenin-cytoskeletal release in static condition [29]. Based on this evidence, we analyzed the intracellular localization of p-S552-β-catenin in endothelial monolayers seeded on flat and BF substrates exposed to physiological values of flow [15]. As shown in Fig 3A and B, in cells exposed to flow the nuclear level of p-S552-β-catenin strongly increased compared to the relative static controls. Of interest, a concomitant reduction of the junctional fraction of p-S552-β-catenin was observed, which was stronger for the case with flat substrate. Based on the observed involvement of Wnt in the regulation of the subcellular localization of β-catenin (Fig 2), we checked the activation of the glycogen synthase kinase-3β protein (GSK3β), a negative regulator of the Wnt signaling pathway that is part of β-catenin destruction complex [35]. We did not detect any difference in the phosphorylation level of GSK3β (Fig. S1) suggesting that Wnt signaling was not differently active and that its contribution to β-catenin nuclear localization is the same in these experimental conditions.

We then evaluated β-catenin transcriptional activity by analyzing by RT-qPCR some specific target genes. Conversely with what was observed for static culturing (Fig 1C), the increased nuclear localization of p-S552-β-catenin induced by flow correlated with increased transcriptional activity (Fig 3C), similarly for flat and BF substrates. While no difference in AXIN2 expression was detected, in line with the same activation of the destruction complex, all the other targets were modulated by the flow according to the known activation or inhibition activity of β-catenin on their promoters [4, 36–39]. The expression of the transcription factor lymphoid enhancer binding factor 1 (LEFI) a key mediator of β-catenin signaling [40] was significantly increased by flow, as well as that of plasma-lemma vesicle-associated protein (PLVAP) an endothelial cell-specific protein central regulator of fenestrae formation and increase of permeability [41]. The expression of two members of the claudin family of proteins of the tight junction complex [42], encoded by the genes CLDN1 and CLDNS5, respectively, were instead significantly downregulated by flow. Both PLVAP and claudins are key regulators of barrier functions, and it has been previously reported that their expression patterns are opposite during development [43].

We then moved to assess the monolayer permeability under flow. Given the similar effect of flow on nuclear p-S552-β-catenin localization for flat and BF patterned surfaces, the paracellular permeability assays were carried out only on the flat surface condition. As shown in Fig 3D, we detected an increase in permeability of endothelial monolayers under physiological value of flow compared to the static control.

Overall, these data suggest that flow-induced mechanical forces can regulate β-catenin signaling and that this regulation is mediated by the mechanosensing and mechanotransduction properties of the AJs. The lack of Wnt signaling modulation suggests the signaling from the AJs as primary contributor to the observed effects.

**Cyclic stretch does not influence β-catenin signaling**

Endothelial monolayers coherently remodel under stretch [23], and we previously reported that this adaptation is strictly dependent on the AJ dynamics [24]. Cyclic stretch of endothelial monolayers induces AJ remodeling and reinforcement [44].

We evaluated the subcellular localization of p-S552-β-catenin under cyclic stretch conditions. Using a custom-made bioreactor [24], we cyclically stretched endothelial monolayers for 18 h at a frequency of 1 Hz to reach an equibiaxial strain of 13% in the fully inflated state. We observed that cyclic stretch reduced the nuclear amount of p-S552-β-catenin and alongside increased its junctional localization, suggesting a p-S552-β-catenin shuttling from the nucleus to the junctions. Of interest, these changes were observed for both the flat and the BF patterned surface (Fig 4A and B). Despite the differences in localization, the expression of β-catenin target genes was unchanged by cyclic stretch (Fig 4C), suggesting that this condition of stretch did not modify β-catenin signaling activity.

Upon inflation of the circular membrane, radial and circumferential gradients of strains are generated [24]. As shown in Fig S2, strains in both directions are equal in the center, and their values reduce moving towards the edges of the membrane, with stronger decrease for the radial component. As a result, in the center region of the membrane (referred to as R1 in Fig S2) an equibiaxial strain is produced, while in the edge of the membrane (referred to as R4
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Figure 1. β-catenin subcellular localization, signaling and barrier functions in static culturing condition. (A) Representative images of p-S552-β-catenin localization in endothelial monolayers seeded on flat surface (top) and BF surface (bottom). Left panel: Total β-catenin, right: p-S552-β-catenin. Scale bar: 100 μm. (B) Quantification of nuclear and junctional localization of p-S552-β-catenin. Results are presented as percentage of p-S552-β-catenin positive nuclei and as Pearson’s coefficient. N = 3 biological independent experiments, N’ = 3 technical replicates; **P < 0.005. (C) RT-qPCR analysis of CLDN1, CLDN5, PLVAP, LEF1 and AXIN2. Results are normalized to the flat control group. N = 3 biological independent experiments, N’ = 3 technical replicates. (D) Schematic diagram of the paracellular permeability assay (created with BIORENDER.com). (E) Representative immunofluorescence images of endothelial paracellular permeability, junctions are stained with β-catenin and leakages are highlighted by avidin (asterisks). Scale bar: 100 μm. (F) Quantification of paracellular permeability as percentage of biotin-avidin bound area. N = 3 biological independent experiments, N’ = 3 technical replicates; **P < 0.005.
in Fig. S2), the strain is uniaxial, with a transition situation in R2 and R3. Therefore, we performed additional analysis by separating and comparing the edge (R4) and the center (R1) regions on the flat surface. As shown in Fig. 5A, the analysis confirmed a reduction of the nuclear amount of p-S552-β-catenin both at the edge and at the center compared to the static control, with the edge region displaying the lowest level. We then evaluated the barrier properties of the monolayer among the different regions R1 and R4, taking into consideration their different loadings. Despite the reduction of nuclear p-S552-β-catenin level induced by the cyclic stretch, the monolayer permeability increased on both the region R1 and the region R4 compared to the static control, with the highest value measured for the edge region. These results indicate that, differently from what observed for the flow, the stretch-induced increase of endothelial permeability is not positively regulated by β-catenin signaling pathway, suggesting that other mechanisms could be involved.

Discussion and conclusion

Endothelial cells are continuously stimulated by a range of contact-derived or flow-derived mechanical loads, including substrate topography, shear stress and substrate strain [23], which influence cellular responses through mechanotransduction mechanisms [45]. In this context, the identification and characterization of the pathways translating mechanical forces into biological functions is of high interest. Several studies have demonstrated that mechanical forces can influence the integrity of the endothelial barrier, thus modulating vascular permeability [46]. These mechanical stimuli can lead to changes in the cell–cell junction structural and functional organization, to induce reorientation of endothelial cells with corresponding alterations in cytoskeletal organization and cell polarity [34, 47], ultimately affecting the transport of macromolecules across the endothelium. These latter aspects were not investigated in the present work. Maladaptation to mechanical stimuli or...
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Figure 3. β-catenin subcellular localization, signaling and barrier functions in monolayers under 1.4 Pa of wall shear stress. (A) Representative images of p-S552-β-catenin localization in endothelial monolayers seeded on flat surface (top) and BF surface (bottom) in static (left) and flow (right) cell culture conditions. Left panels: Total β-catenin, right panels: p-S552-β-catenin. Scale bar: 100 μm. (B) Quantification of nuclear and junctional localization of p-S552-β-catenin. Results are presented as percentage of (p-S552-β-catenin) positive nuclei and as Pearson’s coefficient. N = 3 biological independent experiments, N’ = 3 technical replicates; **P < 0.005, ***P < 0.001. (C) RT-qPCR analysis of CLDN1, CLDN5, PLVAP, LEF1 and AXIN2. Results are normalized to the flat control group. N = 3 biological independent experiments, N’ = 3 technical replicates; *P < 0.05. (D) Representative immunofluorescence images of endothelial paracellular permeability, junctions are stained with β-catenin and leakages are highlighted by avidin (asterisks) and quantification of paracellular permeability as percentage of biotin-avidin bound area. N = 3 biological independent experiments, N’ = 3 technical replicates; **P < 0.005. Scale bar: 100 μm. Arrows indicate the direction of the flow.
Figure 4. β-catenin subcellular localization, signaling and barrier functions in monolayers under cyclic stretch. (A) Representative images of p-S552-β-catenin localization in endothelial monolayers seeded on flat surface (top) and BF surface (bottom) in static (left) and cyclic stretch (right) cell culture conditions. Left panels: Total β-catenin, right panels: p-S552-β-catenin. Scale bar: 100 μm. Arrows indicate the direction of the stretch. (B) Quantification of nuclear and junctional localization of p-S552-β-catenin. Results are presented as percentage of (p-S552-β-catenin) positive nuclei and as Pearson’s coefficient. N = 4 biological independent experiments, N’ = 3 technical replicates; *P < 0.05. (C) RT-qPCR analysis of CLDN1, CLDN5, PLVAP, LEF1 and AXIN2. Results are normalized to the flat control group. N = 3 biological independent experiments, N’ = 3 technical replicates.

pathological (supraphysiological) levels of mechanical loads ultimately lead to the disruption of endothelial barrier function, a common feature associated with various cardiovascular diseases [48].

The present work analyzes the mechanostimulation of β-catenin pathways following physiological stimuli and its impact on the fine tuning of endothelial paracellular permeability, assessed by the small molecule avidin interaction with biotin at coating surface. Flow, cyclic strain and substrate BF were considered as it was previously reported that shear stress [49–51] and strain [52–54] influence endothelial permeability, and indirect evidence suggested that surface topography could do it as well [55]. Using HUVEC cells as an in vitro model of endothelial monolayer, we analyzed β-catenin subcellular localization and signaling upon the different mechanical stimuli.
β-catenin belongs to the armadillo family of proteins and has dual functionality in the cells depending on its intracellular localization. At the cellular membrane, it contributes to establishing adherens junctions by connecting classical cadherins to the actin cytoskeleton [56]. When transported to the nucleus, it combines with co-transcription factors creating the β-catenin transcriptional complex. This complex oversees the transcription of numerous target genes that are crucial for controlling endothelial homeostasis [57] and barrier functions [4]. This study revealed that single mechanical stimuli differently affected β-catenin intracellular localization. Our results showed that cells in contact with the BF topography presented a higher level of nuclear p-S552-β-catenin (active β-catenin), compared to the control, and no modification of the amount that is localized at cell-cell contacts. This latter suggests that the active β-catenin that relocalised into the nucleus does not shuttle from the junctions. However, this increased nuclear localization of β-catenin did not trigger changes in the expression of its target genes, and the analysis of the barrier properties revealed instead a slight decrease of permeability induced by the surface topography.

It has been previously reported that the nuclear localization of transcription factors does not necessarily imply an increase in their transcriptional activity [58] due to multiple reasons, such as the nuclear availability of β-catenin co-factors [59], post-translational modifications and protein–protein interactions [60], and the spatial organization of transcription factors within the nucleus, such as their co-localization in transcription factories, and the accessibility of chromatin domains [61, 62]. Of interest, substrate micro-topography, known to inhibit gene expression by decreasing histone acetylation and reducing the accessibility of chromatin, could potentially counteract the increased nuclear localization of β-catenin [63].

Interestingly, endothelial monolayer exposed to physiological levels of flow exhibited a release of active β-catenin from the AJs and a concomitant strong re-localization into the nucleus, on both types of surfaces. This triggered the mechanical activation of β-catenin transcriptional activity, as we observed increased expression of level of expression of PLVAP and LEF1 genes for which β-catenin is an activator [64, 65] and the decreased level of expression of CLDN5 and CLDN1 genes for which β-catenin acts as repressor [4, 64, 66, 67]. Both Claudins and PLVAP are involved, with opposite effects, in the control of barrier properties, and their expression during development is inversely correlated [43]: CLDN5 expression increases over time while that of PLVAP decreases, as well as the β-catenin transcriptional activity [36, 64], thus allowing the proper formation of the barrier. In our experimental conditions, the modulation of expression of PLVAP and Claudins by physiological levels of flow suggested an overall impairment of endothelial barrier function, which was further confirmed by permeability assay. A tuning of permeability was detected as endothelial monolayers were more permeable to avidin under flow compared to the static control across all surface conditions.

The literature on β-catenin signaling under mechanical stretch is mainly restricted to osteoblast and mesenchymal stem cell
model systems [8] where opposite results have been reported. The effect of stretch on endothelial permeability is not well understood, as it was reported that it can either increase or decrease endothelial permeability, depending on the specific experimental conditions and cell types used, and the mechanisms underlying these effects are not fully elucidated [53, 54].

Here we showed that cyclic stretch triggered a reduction of nuclear active β-catenin and a parallel increase of the junctional fraction, which did not impact on the transcriptional regulation of the target genes. This is not surprising since, as it was reported by previous literature [68, 69], in confluent monolayers of cells the majority of β-catenin is sequestered by VE-cadherin at AJs and the levels of cytoplasmic free β-catenin are kept low. This implies that β-catenin signaling activity is already impaired in static conditions and the reduction of nuclear active β-catenin induced by stretch does not further impact on it. Moreover, in addition to the already mentioned possible reasons why different levels of transcription factor nuclear localization do not always coincide with changes in their transcriptional activity, one possible further explanation could be the influence of cyclic stretch, which has been shown to promote chromatin accessibility [70], and this could counteract the reduced nuclear localization of β-catenin.

Despite no difference in the transcription of the genes contributing to the regulation of the barrier functions, we found that cyclic stretch slightly increased the permeability of endothelial monolayers in a way that depended on the magnitude and orientation of the stretch applied. This suggests that remodeling and weakening of the junctions is a direct consequence of the strain applied and the associated mechanical loading of cell-cell contacts. In line with this hypothesis, we detected an increase in junctional localization of active β-catenin, which could reflect the cells’ attempt to counteract the stretch-induced dismantling. Increased β-catenin at the junctions could be the consequence of its shuttling from the nucleus or it could be a de novo phosphorylation of junctional β-catenin on S552 by AKT pathway activation. The latter has been reported to occur in other cell types in response to mechanical stimulation to regulate junctional remodeling [71].

The variations of barrier functions detected in this work show that mechanical stimulation can significantly contribute to physiological processes. Indeed, small changes in endothelial permeability are relevant in the dynamic regulation of tissue fluid balance, controlling the homeostasis and the exchange of nutrients and waste [72]. Moreover, they are involved in vascular tone and blood pressure regulation, in the response to hypoxia [73], and in the inflammatory response, where variations in permeability modulate leukocyte extravasation and the transport of cytokines and chemokines [74]. Lastly, a tuning of endothelial permeability is essential during the formation of new blood vessels during physiological angiogenesis, supplying nutrients and oxygen to growing tissues [75]. While controlled variations in endothelial permeability are crucial for normal physiology, permeability dysregulation can contribute to various diseases such as atherosclerosis, diabetes, and cancer [76].

In conclusion, our work highlights the role of β-catenin for the mechanotransduction of different individual mechanical cues and its contribution to the regulation of endothelial barrier functions by tuning the expression of junctional proteins. Future work is needed to better understand the effect of multiple simultaneous mechanical stimuli and to elucidate the mechanisms by which they are integrated by endothelial cells.

**Supplementary data**

Supplementary data is available at INTBIO Journal online.

Conflict of interest: The authors declare no conflict of interest.

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**Data availability**

The data underlying this article will be shared on reasonable request to the corresponding author (C.G.).

**References**


Mechanical factors influence \(\beta\)-catenin localization


