Matrix stiffness regulates the tight junction phenotypes and local barrier properties in tricellular regions in an iPSC-derived BBB model

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Abstract

The blood-brain barrier (BBB) can respond to various mechanical cues such as shear stress and substrate stiffness. In the human brain, the compromised barrier function of the BBB is closely associated with a series of neurological disorders that are often also accompanied by the alteration of brain stiffness. In many types of peripheral vasculature, higher matrix stiffness decreases barrier function of endothelial cells through mechanotransduction pathways that alter cell-cell junction integrity. However, human brain endothelial cells are specialized endothelial cells that largely resist changes in cell morphology and key BBB markers. Therefore, it has remained an open question how matrix stiffness affects barrier integrity in the human BBB. To gain insight into the effects of matrix stiffness on BBB permeability, we differentiated brain microvascular endothelial-like cells from human induced pluripotent stem cells (iBMEC-like cells) and cultured the cells on extracellular matrix-coated hydrogels of varying stiffness. We first detected and quantified the junction presentation of key tight junction (TJ) proteins. Our results show matrix-dependent junction phenotypes in iBMEC-like cells, where cells on softer gels (1 kPa) have significantly lower continuous and total TJ coverages. We also determined that these softer gels also lead to decreased barrier function in a local permeability assay. Furthermore, we found that matrix stiffness regulates the local permeability of iBMEC-like cells through the balance of continuous ZO-1 TJs and no junction regions ZO-1 in tricellular regions. Together, these findings provide valuable insights into the effects of matrix stiffness on TJ phenotypes and local permeability of iBMEC-like cells.

Key words: iPSC-derived BBB; matrix stiffness; tight junctions; junction presentation

Introduction:

The brain is one of the softest organs in the human body. Brain mechanical properties, including stiffness, are particularly susceptible indicators for pathophysiological changes in neural tissue[1]. Previous studies have shown that brain stiffness is altered in several neurological disorders such as Alzheimer's disease[2, 3] and multiple sclerosis[4, 5]. In addition, increased tissue stiffness is a hallmark of various brain tumors[6]. Physiological and anatomical information suggests that the stiffness of brain tissue is defined by various factors such as cellular density, extracellular matrix (ECM) composition, spatial cellular interactions and cell-ECM interactions[7]. The blood-brain barrier (BBB) is a complex and dynamic vascular interface that is embedded within the vascular basement membrane, and emerging evidence suggests that it may sense and modify its behavior in response to changes in the stiffness of the cerebral vascular bed. Dysfunction of BBB properties has been implicated in the progression of neurological diseases and tumors and is considered to be an important pathological feature [8, 9]. An open question has remained about whether BBB dysfunction is a cause or consequence of these devastating diseases. In addition, the impact of brain or vascular bed stiffness on BBB dysfunction has not been comprehensively studied. Therefore, gaining a deep understanding of the correlation between the alteration of brain stiffness and BBB properties would shed light on the potential contribution of brain stiffness to aspects of BBB dysfunction, or vice versa. Furthermore, a general understanding of how matrix stiffness impacts BBB properties is quite useful as the field considers design parameters such as stiffness in engineered BBB models.

The BBB is comprised of a highly specialized monolayer of brain microvascular endothelial cells (BMECs) that selectively transport nutrients and exclude neurotoxic substances from peripheral circulation to the brain[10]. The tight junction (TJ) complexes joining adjacent BMECs restrict BBB paracellular permeability by sealing the paracellular space, resulting in high trans-endothelial electrical resistance (TEER) values[11, 12]. The morphology, expression, location, and distribution of cell-cell junction complexes are directly influenced by mechanical cues such as shear stress and stiffness in peripheral tissues[13]. Endothelial cells (ECs) on stiff substrate exert larger traction forces and prominent stress fibers extending across the cells [14, 15]. Previous work has shown that the expression of junction proteins in peripheral endothelial cells such as human umbilical vein ECs (HUVECs) and aortic ECs decreases on stiffer substrates[16]. Matrix stiffness regulates the TJ complex through mechanical tension and the assembly of TJ proteins[17], and we have found that conditions associated with decreasing cell contractility (e.g., soft substrates, biochemical inhibition of myosin II, or biochemical activation of cyclic adenosine monophosphate signaling) lead to increases in zonula occudens-1 (ZO-1) coverage in primary human BMECs[18]. Furthermore, we have shown that stiffer substrates promote neutrophil transmigration through

HUVECs stimulated with tumor necrosis factor alpha (TNF-α)[15] or oxidized low-density lipoprotein[19]. Interestingly, another study showed that peripheral blood lymphocytes and natural killer cells prefer transmigrating through the endothelium via the paracellular route in dermal microvasculature ECs on stiffer substrates [20].

Although the primary BMECs provided valuable cell sources for *in vitro* modeling of the BBB, it has been acknowledged that these cells may have compromised properties in predicting BBB function. Recent advancements have shown that BMEC-like cells derived from human induced pluripotent stem cells (iPSCs) (iBMEC-like cells) can recapitulate several critical properties of human BMECs, including the expression of tight junctions, efflux pumps, and nutrient transporters[21-28]. However, an open question has remained about whether substrate stiffness has similar impacts on other BMEC models such as iBMEC-like cells. One recent study showed non-significant changes in TJ morphology of iBMEC-like cells as a function of substrate stiffness, although the TEER values in iBMEC-like cells increased with increased substrate stiffness. These results led us to the questions of whether substrate stiffness influences the permeability of iBMEC-like monolayers and, more generally, whether there are correlations between the junction phenotypes of TJ proteins and BBB permeability.

To answer these questions, we have recently developed a Junction Analyzer Program (JAnaP) that can efficiently quantify cell morphology and junction phenotypes[18]. Using this program, we have evaluated the junction architecture of primary BMECs in response to matrix composition, substrate stiffness, tumor cell-secreted factors, and various drug treatments[18, 29-32]. Moreover, our lab has used a local permeability assay (modified from Dubrovskyi *et al*. study[33]) that quantitatively correlates cell-cell junction phenotype with local permeability[31]. These techniques provide new tools for reevaluating the correlation between TJ proteins and barrier integrity in response to matrix stiffness. To build upon the Bosworth *et al*. study[28], here we investigated how matrix stiffness impacts TJ phenotypes and local permeability of iBMEC-like cells. We show that increased matrix stiffness promotes cell-cell junction protein coverages in iBMEC-like cells. Furthermore, we found that the presence of continuous junctions and non-junctions regulate the local permeability of iBMEC-like cells, particularly in tricellular regions. Our findings provide previously unidentified insights into the complex role of TJ proteins in mechanosensing and the correlation of the junction phenotypes to the permeability of BBB function.

Methods

Cell culture

Human iPSCs (IMR90-1 and DF19-9-11 T.H; both from WiCell) were maintained on Matrigel (Corning) in E8 medium (Thermo Fisher) as previously described[34]. Approval for our use of human iPSCs was granted by the University of Maryland at Baltimore ESCRO committee. Differentiation of BMECs was conducted as previously described[24]. Briefly, iPSCs were singularized with Accutase and seeded on Matrigel-coated plates at a density of 1-1.25×10^5 cells/cm^2 in E8 medium containing 10 μM Y27632 (R&D System). The following day, the medium was changed to E6 medium (Thermo Fisher), initiating differentiation. E6 medium was changed every day thereafter. On day 4, the medium was changed to endothelial cell (EC) culture medium. EC culture medium was comprised of human endothelial serum-free medium (Thermo Fisher) supplemented with 1% platelet-poor plasma-derived serum (PDS) (Thermo Fisher catalog #50-443-029), 20 ng/mL basic fibroblast growth factor (bFGF) (Peprotech), and 10 mM ascorbic acid (RA) (Sigma). On day 6, cells were dissociated with Accutase (Thermo Fisher) and subcultured onto PA gels, glass coverslips, or glass-bottom plates coated with human placenta-derived Collagen type IV (400 μg/ml) (Sigma) and human plasma-derived Fibronectin (100 μg/ml) (Sigma). On day 7 (1 day after subculture), the medium was changed to EC medium without RA and bFGF for maintenance. Cells on day 8 (Day 2 after subculture) were collected for future analysis.

Polyacrylamide (PA) hydrogel preparation

PA gels were polymerized on glass coverslips (22 × 22 mm and 12 mm round, VWR) and mechanically tested via atomic force microscopy as described thoroughly in our previous publication[18]; here we summarize the protocol. To create substrates of various stiffness, acrylamide and bisacrylamide (bis) (Biorad) were combined at the following concentrations: 3% acrylamide + 0.2% bis (1 kPa), 7.5% acrylamide + 0.075% bis (2.5 kPa), 8% acrylamide + 0.2% (15 kPa), and 15% acrylamide + 1.2% bis (194 kPa) [18, 35]. After polymerization, gels were activated with sulfo-SANPAH (Thermo Fisher) and exposed to 365 nm ultraviolet light for 10 mins; this step was repeated twice with a phosphate buffered saline (PBS) wash in between UV treatments. Gels were then washed with PBS three times and coated with Collagen type IV (400 μg/ml) and Fibronectin (100 μg/ml) at 4°C overnight. For experiments on glass, cells were plated on coverslips or 24-well glass bottom plates coated with Collagen type IV (400 μg/ml) and Fibronectin (100 μg/ml). Before cell seeding, PA substrates were washed twice with PBS and incubated with cell culture medium for 10 mins. A total of 2×10^6 cells were seeded on the glass or PA hydrogels for the following experiments.

Hyaluronic acid (HA) and Gelatin hydrogel preparation

HA/gelatin hydrogels were formed using the HyStem-C kit (Sigma). The kit included four components: Glycosil (thio-modified hyaluronic acid), Gelin-S (thio-modified gelatin), Extralink

(polyethylene glycol diacrylate), and degassed, deionized (DG) water. Hyaluronic acid (HA)/gelatin hydrogels were prepared as we described previously in Pranda *et al*.[32]. Briefly, all components were thawed for 60 min at room temperature. Next, Glycosil and Gelin-S were reconstituted with DG water and rocked at room temperature until the solution turned clear and slightly viscous. Extralink was dissolved in DG water to 15%, then diluted into aliquots of 10%, and 5%. Glycosil and Gelin-S were mixed in a 1:1 ratio, and then the Glycosil/Gelin-S solution was combined with the appropriate concentration of Extralink to make the final concentration of Extralink within the hydrogel as 1, 2, and 3%, respectively. Gel solutions were added in 24-well inserts with polyester (PET) membrane (Falcon), spread out on the insert, and allowed to gelate for 20 mins. Once crosslinked, hydrogels were coated with Collagen type IV (400 μg/ml) and Fibronectin (100 μg/ml) overnight. Transwells directly coated with Collagen type IV/Fibronectin overnight served as a hydrogel-free control transwell. Before the cell seeding, transwells with or without HA/gelatin hydrogels were washed twice with PBS and incubated in medium for 10 mins. Then, 5×10^5 cells were seeded onto the HA/gelatin hydrogels, followed by measurement of the TEER value at various timepoints.

Atomic force microscopy

A NanoWizard 4a BioScience AFM (JPK Instruments AG) and commercially available cantilevers (SAA-SPH-1UM, Bruker) with cylindrical tip (tip radius, 1μm) were used for measuring the Young's modulus of HA/gelatin hydrogels. The spring constant of the cantilever was determined by the thermal tune calibration method before each experiment and was of similar order of magnitude as the manufacturer's specifications (0.25 N/m). For the measurement of HA/gelatin hydrogels, mechanical data were obtained using AFM in contact mode. Three distinct 10 x 10 μm areas across three hydrogels for each condition were measured. The resulting force curves were recorded with the AFM working software (JPK Instruments) and processed with JPK image processing software (JPK Instruments AG).

Tanswell and TEER measurements

Each day, TEER of iBMEC-like cells on HA/gelatin hydrogels in the transwell was measured using an EVOM2 voltohmmeter with STX3 chopstick electrodes (World Precision Instruments). TEER values were also recorded from an empty transwell and were subtracted from sample measurements, and the resulting values were multiplied by the surface area of the transwell inserts.

Immunochemistry

iBMEC-like cells were rinsed with phosphate-buffered saline (PBS) (Thermo Fisher) twice and fixed in 4% paraformaldehyde (PFA) (Thermo Fisher) for 10 min. Cells were then treated with 0.25%

Triton X-100 (Sigma) for 10 min. Cells were washed twice with PBS and blocked with 2% goat serum (Abcam) in PBS for 1 hour at room temperature. Primary antibodies were diluted in 2% goat serum, and cells were incubated in the primary antibodies at 4°C overnight. The next day, cells were washed three times with PBS and incubated in secondary antibodies diluted in 2% goat serum for 1 hour at room temperature. Cells were then washed with PBS three times, followed by treatment with Hoechst (Thermo Fisher) for 5 min. Cells were rinsed three times with PBS and then mounted. Cells were visualized using an inverted IX83 microscope (Olympus). A detailed list of antibodies is shown in Table S1.

Junction analysis

To quantify various cellular parameters, the fluorescent images were analyzed using our lab's Junction Analyzer Program (JAnaP, available through https://github.com/StrokaLab/JAnaP)[18], which semi-automates cell edge analysis and defines junction phenotype based on image thresholds. To define cell edges of interest, a random selection of cells with visible cell perimeters from each ZO-1 image was manually waypointed through the program. The variants feature was used to project the ZO-1 image waypoints to corresponding occludin images. The junctions were isolated from background noise using intensity thresholds that were determined through the Python Jupyter Notebook. Appropriate threshold values were manually selected to best isolate junction pieces consistently through each data set. The JAnaP automatically calculated cell morphology parameters such as area, perimeter, circularity, and solidity. Then, the identified junction pieces were classified as continuous, punctate, or perpendicular, and were represented as a percent of the total cell edge. Each presentation type was defined by coinciding junction length and aspect ratio based on the cell edge. Any junction that coincided with the cell edge for more than 15 pixels was characterized as a continuous junction. Any non-continuous junction that had an aspect ratio of more than 1.2 was characterized as a perpendicular junction. Any junction piece that did not fit in to either definition was characterized as a punctate junction.

XperT permeability assay

To visualize leaky areas of iBMEC-like cells on PA hydrogels, PA hydrogels of different stiffness were polymerized on 10 mm round coverslips as described above. Fibronectin was biotinylated using EZ-Link NHS-LC-LC-Biotin (Thermo Fisher) according to the manufacturer's instructions. Coverslips with PA hydrogels were transferred to 24-well plates. Biotinylated Fibronectin (100 ng/ml) and Collagen type IV (400 ng/ml) were added to the 24-well plate wells. For the samples on glass, 10 mm coverslips were exposed to UV for 20 min and coated in biotinylated Fibronectin and Collagen type IV. The biotinylated Fibronectin adsorption was performed overnight at 4 °C. The next day, plates were washed with PBS twice, and iBMEC-like cells were plated at 2.5×10^5 cells/cm^2. Cells were cultured in EC medium for 1 day and

then changed to EC medium without bFGF and RA for another day. At day 8 (2 days after subculture), 50 μg/ml of FITC-avidin was added to 24-well plates for 3 min. and this enabled binding reaction of biotinylated fibronectin at permeable sites of iBMEC monolayers. After 2 washing steps with PBS, cells were fixed with 4% PFA. Three biological replicates were performed for each group. Cells were immunostained for ZO-1 using the method described above.

Fluorescence intensity measurements

To measure the fluorescence intensity of iBMEC-like cells in the local permeability assay, the coverslips of iBMEC-like cells were transferred to 24-well black plates, and 200 μl PBS was added to each well. A standard curve was made with serial dilution of FITC-avidin. The fluorescence of matrix-bound FITC-avidin was measured on a Spark Multimode Microplate Reader. The concentration of bound FITC-avidin in leaky areas of iBMEC-like cells was quantified by comparing the fluorescence intensity to the standard curve.

Local permeability assay analysis

Two steps of analysis were performed as elucidated in our previous study[18] to analyze the results of the XperT permeability assay and correlate the permeable region to junction phenotypes. Briefly, the first step was to process the green channel of FITC-avidin in ImageJ. The images of the green channel were converted to 8-bit, and an appropriate threshold intensity value was manually applied (and kept constant across all samples) to create a binary image showing the permeable region (PR). To quantify PR area, the Wand tracing tool and freehand selection tool were used on the PR threshold images in ImageJ. PRs were categorized as bicellular (Bi), tricellular (Tri), and multicellular (Multi) regions depending on the number of cells they contacted. “Bi” was defined as the PRs located at the areas where two cells were in contact. “Tri” was defined as the PRs in the area where three or more cells met. Multi was defined as the PRs in the area containing more than two tricellular regions or covering more than one whole cells. The second step was to analyze the junction phenotypes of the cells in the PRs using the JAnaP. Every single cell border in the PRs was waypointed in each image. The JAnaP-associated Jupyter Notebook was then used to generate the categorized junctions. Junction images were then overlaid onto the PR threshold images for the measurements. To calculate the percentage of junctions along the cell perimeters with PRs, overlaid images were manually traced in ImageJ using the segmented line tool. Ten images from each of the 3 trials were measured, and the average count of each category per image was calculated. The PR area measurements were averaged over all PRs within the respective category.

Statistical analysis

GraphPad Prism 8 was used for all statistical analysis and graph generation. For statistical analysis, a D'Agostino-Pearson normality test was performed to identify the normality of the data. If the data was normal, a one-way ANOVA with Tukey's multiple comparison post hoc testing was used for analysis. The non-parametric Kruskal–Wallis ANOVA test with Dunn's multiple comparison post hoc testing was used for the data sets that were not normally distributed. Linear regression was used for the junction analysis from the sample image to compare the junction presentation with global permeability. Statistical significance was indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Errors bars represent the standard deviation or standard Error of the mean as noted in the figure caption. All data represent pooled values from three independent trials.

Results

Substate stiffness changes the morphometric shape of iBMEC-like cells

We used two hiPSCs lines: the iPSC DF19-9-11T.H (DF19) line and the IMR90-1 line. The iPSCs expressed typical pluripotent stem cell markers Nanog, TRA-1-60, SSEA, and Oct3/4 (Fig. S1). iBMEC-like cells derived from the above iPSC lines expressed GLUT-1, occludin, ZO-1, claudin-5, and VE-Cadherin, indicating the typical BBB phenotypes (Fig. S2). To examine the influence of matrix stiffness on iBMEC-like cells, we used PA hydrogels to mimic a wide range of stiffness conditions (Fig. 1A) that represent healthy and pathological brain tissue. After 6 days of differentiation, iBMEC-like cells were detached and seeded on PA hydrogels with various stiffnesses (1 kPa, 2.5 kPa, 15 kPa, 194 kPa). iBMEC-like cells were also seeded onto glass coverslips as controls. After subculturing the iBMEC-like cells on different substrates for 2 days (Day 8), we performed immunostaining of TJ markers (ZO-1 and occludin) (Fig. 1B and C) and analyzed the junction phenotypes and morphological parameters of TJ proteins with the JAnaP (Fig. 1A). Our results showed that the solidity, circularity, perimeter, and area of DF19 iBMEC-like cells on softer PA hydrogels (1 kPa) were significantly lower than the iBMEC-like cells on stiffer gels (Fig. S3A and B). Likewise, the solidity and circularity in IMR90 iBMEC-like cells also decreased in the 1 kPa group (Fig. S3A and B). However, unlike in the DF19 iBMEC-like cells, we observed no significant difference in the perimeter and area in IMR-90-1 iBMEC-like cells (Fig. S4A and B) with various substrate stiffnesses, which may be attributed to the cell line difference. These results indicate that substrate stiffness modestly affects the morphometric shape descriptions in iBMEC-like cells.

Substrate stiffness alters the junction phenotypes in iBMEC-like cells

To assess whether substrate stiffness affects the junction coverage patterns, we used the JAnaP to categorize the junctions into three types: continuous, punctate, and perpendicular junctions. We found that continuous junctions of ZO-1 in the 15 kPa and 194 kPa groups were significantly increased compared to

the 1 kPa, 2.5 kPa, and glass groups in both DF19 and IMR90-1 iBMEC-like cells (Fig. 2A and S5A). Although the punctate and perpendicular junctions of ZO-1 showed varying junction pattens across different stiffnesses (Fig. 2A and S5A), the total junction coverage pattern of ZO-1 (Fig. 2B and S5B) was similar to that of the continuous junctions, since the continuous junctions made up the majority of the cell perimeter. The continuous junctions of occludin were significantly higher in DF19 iBMEC-like cells seeded on glass but lower on 194 kPa gels compared to other groups (Fig 2C). Furthermore, the corresponding punctate junctions of occludin were significantly lower in the glass group compared to groups of softer substrates in both DF19 and IMR90-1 iBMEC-like cells (Fig. 2C and S5C). Interestingly, although TJ proteins ZO-1 and occludin showed different junction phenotypes, the total coverage of these two proteins showed a similar pattern in their continuous junctions (Fig. 2D and S5D). Claudin-5, a crucial tight junction protein that maintain the integrity of the BBB, was examined for its expression and junction presentation of iBMEC-like cells. Our findings demonstrated that Claudin-5 was expressed in iBMEC-like cells on various substrate stiffness (Fig. 3A). Notably, increased matrix stiffness was associated with an elevation in continuous Claudin-5 junctions and a reduction in the punctate and perpendicular Claudin-5 junctions (Fig. 3B). Furthermore, the overall coverage of Claudin-5 is consistent with the pattern of continuous junctions which are increased with substrate stiffness (Fig. 3C). Therefore, our results indicate that the substrate stiffness alters the TJ phenotypes in iBMEC-like cells. Moreover, the continuous junctions are crucial in determining the patterns of total junction coverage in iBMEC-like cells.

Junctions at the tricellular regions contribute to the local permeability in iBMEC-like cells

To determine if the junction phenotype correlated with the global permeability of the iBMEC-like cells, we attempted to graft the PA hydrogel onto a transwell to test the TEER value. However, forming an intact PA hydrogel layer on the PET Transwell membrane was challenging. Hence, we generated HA/gelatin gels with stiffnesses ranging from 0.9 kPa to 4.5 kPa (Fig. S6A), which is in the range of the softer PA gels. We seeded the IMR90-1 iBMEC-like cells on the HA/gelation hydrogels for 1 week and measured the TEER values daily. We found no significant differences in the TEER values in the iBMEC-like cells on HA/gelation hydrogels in this range of stiffness when compared to the “no gel” control on day 2 after subculture (Fig. S6B), suggesting that the barrier integrity weakly correlates with altered junction phenotypes in response to different substrate stiffnesses. Our previous study reported that the barrier permeability identified by the local permeability assay somewhat correlates with junction coverage in primary BMECs[31]. To determine if the substrate stiffness-dependent alterations of junction phenotypes we observed in iBMEC-like cells were related to the local permeability of iBMEC-like cells, we performed a local permeability assay on the iBMEC-like cells plated on PA hydrogels of varying stiffnesses (Fig. 1A). The barrier permeability of iBMEC-like cells was detected and visualized *in situ* via FITC-avidin-binding

to biotinylated substrates (Fig. 4A). In parallel with the JAnaP analysis, we quantified the site-specific barrier permeability and corresponding junction phenotypes within the permeable region (Fig. 4B). Measurements of overall intensity using a plate reader showed that iBMEC-like cells on softer gels (1 kPa) had higher levels of FITC-avidin to the substrate (Fig. 4C), indicating that the cells are more permeable on 1 kPa than on stiffer gels. Detailed analysis of microscopy images revealed that the permeable regions in the iBMEC-like cell monolayers tended to be located at tricellular regions (Fig. 4A and D). The number of tricellular PRs was significantly higher than the bicellular and multicellular PRs in all the stiffness groups (Fig. 4D). By contrast, in our previous study of primary BMECs, the permeable regions were more often located between adjacent cells (bicellular regions) [31]. These results suggest that the junction proteins at tricellular regions may be responsible for controlling the local permeability of iBMEC-like cells.

Substrate stiffness affects the local permeability of iBMEC-like cells in the continuous junction and no junction regions

To understand how junction phenotype contributes to the local permeability of iBMEC-like cells in tricellular regions, we quantified the cell-cell junctions by imaging the phenotypes of ZO-1 in the permeable regions of iBMEC-like cells on varying substrate stiffnesses (1 kPa to 194 kPa). Using the previously described method[31], we traced and measured the different junction phenotypes (continuous, perpendicular, and punctate) in the permeable regions of iBMEC-like cells (Fig. 4B). Our results showed that the iBMEC-like cells on softer gels (1 kPa) had a significantly larger PR area than other groups (Fig. 5A and G). The percentage of continuous junctions along the PR length on the 1 kPa gel was significantly lower than that on the stiffer gel groups for IMR90-1 and DF19 derived iBMEC-like cells (Fig. 5C and I). Along with its continuous junctions, the total coverage of ZO-1 showed a similar pattern in PR of IMR90-1 iBMEC-like cells (5B and C). Furthermore, the percentage of no-junction cell perimeter regions along the PR length was increased in the iBMEC-like cells on the 1 kPa gel (Fig. 5D and J). Meanwhile, no apparent differences were observed in perpendicular and punctate junctions along the PR length across the stiffness groups (Fig. 5E, F and K, L). Together, these data suggest that substrate stiffness regulates barrier permeability, at least in part, through junction protein phenotypes. In general, there was higher permeability in regions of no junction, or less continuous junctions, at the cell perimeter, and these regions were more abundant on softer (1 kPa) gels.

The junction phenotype in the PR is correlated with the substrate stiffness but not PR area

Having found that substrate stiffness regulates local permeability of iBMEC-like cells through continuous and no junction regions, we were curious about how the specific junction phenotype correlated with PR area. Therefore, we categorized the extent of permeability as the PR area and performed a linear

regression analysis to investigate the correlation between PR area and junction presentation. We found no statistically significant correlation between TJ protein ZO-1 percent junction (of any type) and PR area (Fig. 6A and B). Although decreased junction coverage led to increased local permeability, preliminary evidence showed that the relationship between the PR areas and any specific junction phenotype is nonlinear and non-monotonic. Therefore, local permeability may be determined by the dynamic changes of different junction phenotypes, especially continuous junctions and no junctions, instead of specific junction phenotypes.

Discussion

The degree to which brain stiffness contributes to BBB dysfunction during the progression of neurological disease has not been well studied. Here, we demonstrated that substrate stiffness affects morphometric shapes and junction presentation of iBMEC-like cells. Our findings show new insights into cell-cell junctions, where TJ proteins in iBMEC-like cells respond subtly and sensitively to mechanical cues, and this occurs via a trend that differs from peripheral endothelial cells and from primary HBMECs. The junction phenotypes in iBMEC-like cells on soft substrates showed subtle decreases in continuous and total junction coverage, along with a significant increase in permeability to FITC-avidin, especially at tri-cellular regions. These findings suggest that soft substrates (1 kPa) lead to a functionally-relevant breakdown in iBMEC-like cell monolayer integrity. Intriguingly, in our previous work, we observed similar subtle changes in junction presentation primary BMECs, but with the opposite trend, where softer (1 kPa) substrates led to increased continuous junction coverage[18]. Furthermore, we and others have previously found that soft substrates promote improved barrier function of HUVECs to neutrophils in TNF-α-stimulated[15] or oxLDL-stimulated[19] endothelium.

We chose to focus on tissue stiffness because this factor can offer a sensitive and generic readout for chemical changes in various tissues. Alteration of brain stiffness can accompany pathological changes in neurological disorders, and it is possible that these mechanical changes can be detected before histological changes are visible. For instance, brain tissue becomes significantly softer in Multiple Sclerosis[36] and Alzheimer's disease[2], while increasing myelin content[37] and intermediate filaments[38] stiffen brain tissue. In addition, increasing brain stiffness has been identified as a hallmark of both low-grade and metastatic tumors[39]. The *in vitro* modeling of brain stiffness, in terms of the mechanical stiffness of brain tissue, has been largely motivated by matrix structure[40]. The brain ECM is a complex network of proteins and carbohydrates that provides matrix structural support to brain. The composition and organization of the ECM can influence the mechanical properties, including the stiffness,

of brain tissue [13]. BBB cells adhered to the vascular basement membrane have specialized adhesion proteins on their surface that mediate the cell-cell interactions and cell-matrix interactions[41]. *In vitro* models of brain stiffness often involve culturing cells on substrates with different ECM components, such as collagen or fibronectin, which can affect cell-matrix interactions and influence tissue stiffness[40]. However, to our knowledge, no studies have directly measured the basement membrane stiffness (i.e., Young's modulus) of brain capillaries, as stiffness measurements are technically challenging in such small vessels. Meanwhile, AFM has been used to quantify the stiffness of de-endothelialized *ex vivo* peripheral blood macrovessels. Healthy peripheral vascular stiffness is usually in the single digit kPa range, and diseased vessels (e.g., from ApoE-null mice, a model of atherosclerosis) or injured vessels can be at least an order of magnitude stiffer[42-45]. Furthermore, one could hypothesize that brain endothelial cells lining those capillaries can mechanosense the stiffness of the basement membrane (which may or may not be elevated in cases where brain tissue stiffness is also elevated) and/or the stiffness of the surrounding brain tissue. *In vitro* studies have suggested that cells can mechanosense through up to 10-20 μm of matrix[46] and hence it is possible that brain endothelial cells may mechanosense changes in extravascular tissue stiffness. Due to these challenges, most studies are conducted on bulk samples such as hydrogels. To address this limitation, we have taken into consideration the wide range of reported measurement of Young's modulus of human brain tissue from a series of studies, spanning the stiffness of a healthy human brain (1 - 8 kPa) to at least one order of magnitude larger than that in different disease conditions.

The TJ protein complexes are comprised of multiple interacting proteins that form a network and serve as principal hubs in regulating the physical barrier properties of the BBB. ZO-1 binds to the actin cytoskeleton, acting as a bridge that connects the transmembrane proteins and cytoskeletal proteins. Occludin and Claudin-5, key components of the TJ strand in brain ECs, are crucial for TJ formation and regulation of BBB permeability. Here, we observed distinct differences in junction phenotypes in iBMEC-like cells, where the increasing matrix stiffness elevates the continuous junction and total coverage of ZO-1, Occludin, and Claudin-5, albeit to different degrees across the three proteins. Moreover, we found that substrate stiffness modestly affects the morphometric shape descriptions in iBMEC-like cells. Recent studies have shown non-significant changes in TJ morphology of iPSC-derived iBMEC-like cells as a function of substrate stiffness [28]. However, our previous research has shown that the TJ phenotype in primary human brain microvascular endothelial cells (HBMECs) roughly correlates with BBB permeability[31]. Therefore, we wondered if these changes affect the endothelial monolayer integrity, and hence permeability, on stiffer substrates.

In vitro experiments demonstrated that lung microvascular ECs exhibit decreased TEER and discontinuous junctions on stiffer substrates[47].TEER values are strong indicators of the integrity of the passive barrier function of BBB in *in vitro* models[48]. So, we first measured the TEER to assess whether the TEER-related barrier integrity correlated with the changes in TJ phenotypes. We found non-significant differences in the TEER value of iBMEC-like cells on HA/gelatin hydrogels with stiffnesses in the narrow but physiological range from 0.9 kPa to 4.5 kPa and PET membrane (Young's modulus ≈ 2-3 GPa) on day 2 after subculture. Bosworth *et al*. also found a minor but non-significant difference in the TEER from day 0 to day 11 after subculture on gels, although in their work iBMEC-like cells exhibited a significantly higher TEER value after the subculture at day 12[28]. Notably, Bosworth *et al*., similarly to us, used different hydrogels for engrafting the iBMEC-like cells in the transwell system, where substrates and the range of gel stiffnesses differ from PA hydrogels[28]. The consistent results in the two studies may rule out bias caused by different materials. Despite the weak influences of substrate stiffness on global barrier integrity, as measured via TEER, the transcriptome analysis of their study found a bulk of genes influenced by the substrate stiffness[28], which indicates the global TEER value may not be sensitive enough to reflect the subtle changes of the passive barrier function of the BBB. Thus, more sensitive techniques are necessary to reveal the correlation between the subtle changes in junction phenotypes and BBB barrier function.

To probe the possible correlation between junction phenotype and BBB permeability, we used a modified local permeability assay described in our prior study[31] to visualize the BBB's permeable region along with the local junction phenotype presentation *in situ*. We quantified the junction phenotype in conjunction with the local permeability of iBMEC-like cells on the PA hydrogels. Surprisingly, the iBMEC-like cells on the softer gels had more permeable regions than on the stiffer gels. Interestingly, most of these permeable regions were in tricellular regions, unlike in our previous study, where permeable regions in primary BMECs were found to be located in the bicellular regions[31]. Such contrast may indicate a different regulation pattern of barrier permeability in iBMEC-like cells through junction proteins at tricellular regions.

TJs at tricellular regions are increasingly recognized as necessary for mechanical sensing and restricting barrier function[49-51]. In our study, strong signals of the TJ proteins in the iBMEC-like cells were generally always spotted in the tricellular regions. Furthermore, a recent study identified the tricellular junctional proteins as targeting sites for T-cell diapedesis across the BBB[52]. Thus, we raised the question: do TJs in tricellular regions regulate the barrier permeability in iBMEC-like cells? To investigate if TJ presentation correlated with the local permeability in tricellular regions, we measured the permeable area and quantified the corresponding junction presentation of ZO-1 in that area. The results showed that the

substrate stiffness dramatically changed the coverage of continuous and no junctions of ZO-1 in the PR area, but not the coverage of perpendicular and punctate junctions. These results suggest that substrate stiffness influences the barrier permeability in iBMEC-like cells through the ZO-1 located at the apical side of the tricellular regions. Consistent with this notion, it has been reported that ZO-1 distributes along the apicobasal axis at tricellular regions in epithelial cells. ZO-1 interacts with Angulin-1 to recruit claudins along the apicobasal axis, which is responsible for the obliteration of the paracellular gap in tricellular regions[53, 54].

We found that the continuous junctions determine the total junction coverage in iBMEC-like cells. The continuous junction is generally considered a more mature and stable phenotype[55]. Decreasing the continuous junction leads to more permeable regions that strongly correlate with the PR area in primary BMECs[31]. In our current study, we found that continuous and no junctions mainly drove the changes in the TJ protein ZO-1. We then speculated if the continuous junction of ZO-1 plays a central role in determining the PR area. Our results demonstrate that the PR area does not correlate with any subcellular junction phenotypes of ZO-1, which suggest the PR area may be determined by the dynamic changes of the continuous and no junctions.

As described above, our finding suggests a strong correlation between the junction presentation and local permeability in the tricellular region in iBMEC-like cells. It has been shown that tricellular regions are the sites of large molecule permeation and immune cell diapedesis across the BBB[52]. In addition, the loss of tricellular TJ proteins promotes the cell invasion and migration in pancreatic cancer cells[56]. Thus, more permeable regions around the tricellular region induced by a softer microenvironment may lead to the open windows on the brain endothelial cells and make the BBB more vulnerable for immune cell penetration or cancer cell invasion. Our results are in line with previous experimental studies that have shown increasing stiffness can decrease the gap generation at tricellular regions in HUVECs. Meanwhile, more stable cell-cell junctions result in fewer transmigrated cells. In contrast, the increased gaps opening at tricellular regions on the soft substrate facilitated transmigration of cancer cells[57]. Furthermore, earlier research has demonstrated that high junction integrity was associated with predominantly transcellular migration, while decreased integrity of junctions resulted in a switch to paracellular diapedesis [58] Another study further revealed that decreasing substrate stiffness promoted the paracellular-route of transendothelial migration of melanoma cancer cells[20]. Thus, our findings raise the possibility that substrate stiffness regulates the paracellular route through the TJ presentation in the tricellular regions, thereby influencing the transmigration process of cancer cells. Moreover, it is worth noting that decreased brain stiffness is linked to increased disease severity in several degenerative diseases, such as Alzheimer's disease[2, 3].

How the underlying mechanisms by which softer microenvironments in the brain affect the disease progression remain elusive. Our study provides valuable insights into the mechanism that TJ protein/ZO-1 regulates the local barrier permeability in the soft environment. Future studies may introduce the immune cells or cancer cells in more complex BBB models to demonstrate the mechanisms of their trans-endothelial migration under disease conditions.

Despite the significant changes in local permeability and junction presentation of ZO-1, we observed that the global/bulk permeability assay (TEER) has no significant differences in iBMEC-like cells in response to different substrate stiffness. These results indicated that the TEER could not reflect these subtle and local changes. Tight junctions can interact with adherens junctions and actin to regulate barrier permeability[59]. Recent evidence showed that the association between ZO-1 and actin is extremely weak in epithelial cells, which is necessary for establishing a robust barrier function[60]. Echoed by our results, the changes in junction presentation of ZO-1 did not abolish the barrier integrity in iBMEC-like cells. In this study, we only focus on the junction presentation of ZO-1 in the permeable regions. Future studies will explore more on the junction presentation of the other junction proteins. Moreover, the dynamic changes of junctions in response to different substrate stiffness will provide more evidence for the junction presentation and interactions.

Overall, our results provide new evidence that matrix stiffness regulates the local permeability in iBMEC-like cells in tricellular regions through the presence or absence of continuous sections of the TJ protein ZO-1. Our findings provide valuable insights into the changes in junction architecture and barrier permeability in response to different matrix stiffnesses. Since BBB dysfunction has been linked to many diseases, it is necessary to understand the regulation of local permeability and other physiologic responses in iBMEC-like cells derived from iPSCs from a healthy patient; these could serve as a baseline comparison to pathologic iBMEC-like cells, which in the future could be used to model diseases using iPSCs from patients. Understanding the influence of matrix stiffness on junction presentations and barrier permeability could lead to the development of new treatments for diseases associated with BBB dysfunction or drug delivery across BBB systems. Furthermore, the human brain continues to undergo considerable architectural changes which are reflected in the regional changes of brain mechanical properties during brain development[61]. Understanding how brain stiffness changes alter the cell-cell junction properties is critical towards our understanding of the junction regulation in normal brain function and development.

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Authors' contributions

LY and KMS designed the research and wrote the manuscript. LY performed cell culture, immunostaining, microscopy, and all other experiments. LY, JWJ, CD, and UG performed JAnaP analysis. RM and KB aided in cell culture and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The JAnaP is available for download at https ://github.com/Stroka Lab/JAnaP.

Ethics approval and consent to participate

Ethics approval for all studies were obtained from the University of Maryland Institutional Biosafety Committee and University of Maryland, Baltimore, Embryonic Stem Cell Research Oversight (ESCRO) Committee.

Competing interests

The authors declare no competing interests.

Figure legends

Fig. 1. Immunocytochemical analysis of BBB markers of iBMEC-like cells on PA hydrogels.
(A) Schematic workflow for the experiments. iPSCs were differentiated to iBMEC-like cells. On day 6, iBMEC-like cells were dissociated and subcultured on the Collagen type IV and fibronectin-coated PA hydrogels and plates. A local permeability assay and JAnaP analysis were performed on the iBMEC-like cell two days after subculture (day 8). (B) Expression of ZO-1 and occludin in DF19 and IMR90-1 iBMEC-like cells on PA hydrogels at Day 8. Scale bars represent 20 μm.

Fig. 2. Junction phenotypes of iBMEC-like cells on PA hydrogels of varying stiffness.
DF19 iBMEC-like cells on PA hydrogels at day 8 were stained for ZO-1 and occludin. (A) The presentation of continuous, punctate, and perpendicular junctions for ZO-1 are shown respectively. (B) The total junction coverage of ZO-1 in DF19 iBMEC-like cells. (C) The presentation of continuous, punctate, and perpendicular junctions for occludin are shown respectively. (D) The total junction coverage of occludin in DF19 iBMEC-like cells. $402 \leq N \leq 453$, where N is the number of cells pooled from three trials. A one-way ANOVA with Tukey's multiple comparison post hoc testing was used for statistical analysis. Errors bars represent the standard deviation of the mean. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Fig. 3. IMR90-1 iBMEC-like cells on PA hydrogels at day 8 were stained for Claudin-5. (A) Expression of Claudin-5 in IMR90-1 iBMEC-like cells on PA hydrogels at day 8. Scale bars represent 20 μm. (B) (A) The presentation of continuous, punctate, and perpendicular junctions for Claudin-5 are shown respectively. (B) The total junction coverage of Claudin-5 in IMR90-1 iBMEC-like cells. $102 \leq N \leq 126$, where N is the number of cells pooled from three trials. A one-way ANOVA with Tukey's multiple comparison post hoc testing was used for statistical analysis. Errors bars represent the standard deviation of the mean. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Fig. 4. Local permeability of iBMEC-like cells on PA hydrogels of varying stiffness.
(A) Representative immunofluorescence images of ZO-1 and FITC-Avidin in iBMEC-like cells cultured for 2 days on PA hydrogels. (B) Image processing. Composite image of ZO-1 (red) and FITC-avidin (green), labeled to identify examples of the permeable region (PR) categories. Images of bound FITC-avidin are processed in ImageJ to generate 8-bit binary images of PRs. The raw junctional protein images are processed in the JAnaP to generate images of categorized junctions (Orange: continuous junction, Yellow: perpendicular junction, Purple: punctate junction), which can be overlaid onto the PR images. (C) Plate reader detected fluorescence intensity of FITC-Avidin bound in permeable regions. (D) Percentage of PR

types in total PRs from three trials. A one-way ANOVA with Tukey's multiple comparison post hoc testing was used for statistical analysis. Errors bars represent the standard deviation of the mean. All scale bars are 20 μm. **p<0.01; ***p<0.001; ****p<0.0001.

Fig. 5. Permeated region and junction presentation analysis.

(A-F) Local permeability assays were performed in IMR90 iBMEC-like cells on PA hydrogels. (A) PR areas were analyzed per image. 22≤N≤28, where N is the number of images pooled from three trials. (B-F) Percentage of ZO-1 junctions (total, continuous, perpendicular, and punctate junctions) and no junction along the cell edges colocalized with PRs. 20≤N≤40, where N is the number of PRs from three trials. (G-L) Local permeability assays were performed in DF19 iBMEC-like cells on PA hydrogels. (G) PR areas were analyzed based on per image. 24≤N≤31, where N is the number of images from three trials. (H-L) Local permeability assays were performed in DF19 iBMEC-like cells on PA hydrogels. PR areas and percentage of ZO-1 junctions (total, continuous, perpendicular, and punctate junctions) and no junction along the cell edges colocalized with PRs were analyzed. 82≤N≤202, where N is the number of PRs from three trials. The non-parametric Kruskal–Wallis ANOVA test with Dunn's multiple comparison post hoc testing was used for the data sets that were not normally distributed (C, D, E, and F). A one-way ANOVA with Tukey's multiple comparison post hoc testing was used for other statistical analysis. Errors bars represent the standard error of the mean. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Fig. 6. Junction presentation versus permeated region area.

The correlation between PR area and the percent of each ZO-1 continuous, discontinuous, and no junction regions at the cell edge co-localized with a PR for in (A) IMR90-1 or (B) DF19 iBMEC-like cells. Each data point represents one image, and results were fit using linear regression.

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