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Intermedin restricts vessel sprouting by inhibiting the loosening of endothelial junction

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ABSTRACT

Vessel sprouting from pre-existing vasculature is a key step for the formation of a functional vasculature. The low level of vascular endothelial growth factor (VEGF) induces normal and stable angiogenesis, whereas high level of VEGF causes irregular and over sprouted vasculature. Intermedin (IMD) is a novel member of calcitonin family, and was found to be able to restrict the excessive vessel sprouting. However, the underlying mechanism had not been elucidated. In this study, using in vitro and in vivo angiogenic models, we found that the loosening of endothelial junction could significantly increase the ability of low-dose VEGF to induce vessel sprouting. IMD inhibited the junction dissociation-induced vessel sprouting by re-establishing the complex of vascular endothelial cadherin on the cell–cell contact. Our findings suggested a novel mechanism through which IMD could restrict the excessive vessel sprouting by preventing the endothelial junction from dissociation, and provide new insight into the understanding of the regulation of sprouting angiogenesis.

1. Introduction

Vessel sprouting and branching from pre-existing vasculature is a key step for the establishment of a hierarchical vasculature and proper vascular function. Vascular endothelial growth factor (VEGF) is the most important pro-angiogenic protein and promotes vessel sprouting predominantly [1]. In cancers, the blood vessels are disorganized and tortuous compared to the normal vasculature, mainly because of the vascular overgrowth and excessive sprouting induced by the overexpressed VEGF [2–4]. Intermedin (IMD) is a novel member of the calcitonin gene related peptides [5,6] and was reported to play a role in the regulation of angiogenesis [7]. We previously reported that IMD was able to restrict the VEGF-induced excessive vessel sprouting for the normalization of tumor vasculature [8]. However, the underlying mechanism is not yet elucidated.

Vascular endothelial cadherin (VE-cad) is an endothelial-specific trans-membrane component of the adherens junction (AJ), which has emerged as a master regulator of endothelial cell–cell adhesive properties [9,10]. The process of vessel sprouting was suggested to undergo multiple steps: the endothelial cell loosening its junction, tip cell selection, and the subsequent stalk elongation [1]. VEGF can cause a rapid and reversible increase in vessel permeability by inducing VE-cad dissociation and loosening the endothelial junction [1,9]. On the other hand, IMD could stabilize the AJ by maintaining VE-cad complex [11,12]. Based on the facts that VEGF promotes but IMD inhibits vessel sprouting, and VEGF disrupts but IMD maintains the endothelial barrier function, we hypothesized that IMD may restrict the excessive vessel sprouting by the prevention of endothelial junction loosening. In

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this study, we used in vitro and in vivo angiogenic models to test this hypothesis.

2. Materials and methods

2.1. Cells and reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cords and grown in EGM-2 (Lonza, Cat. CC-3162). Human skin fibroblasts (HSFs) for co-culturing with HUVECs were isolated from surgical specimens and routinely grown in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2.

The VEGF human and mouse recombinant protein (Cat. PHC9391 & PMG0113) were purchased from Invitrogen. The peptides of human and mouse IMD were synthesized and purchased from Shinegene. The antibody for VE-cadherin (Cat. #2500) was from Cell Signaling Technology. Alexa Fluor®488 goat-anti-rabbit secondary antibody (Cat. A11008) and Isolectin B4 (Cat. 121412) were from Invitrogen. The low molecular FITC-dextran (Cat. FD70s) was from Sigma–Aldrich.

2.2. Fibrin bead assay

A detailed protocol has been described [13]. In brief, dextran-coated Cytodex 3 microcarrier (GE, Cat. 17-0485-01) were coated with HUVECs at a concentration of 400 HUVECs per bead. At the following day, beads were washed and re-suspended at a concentration of 200 beads/ml in 2.5 mg/ml of fibrinogen (Sigma, Cat. F8630), with 0.15 U/ml of aprotinin (Sigma, Cat. A1153). 500 μl of fibrinogen/bead solution was added to 0.625 IU of thrombin (Sigma, Cat. T7009) in 1 well of a 24-well culture plate. The fibrinogen/bead solution was allowed to clot, and 2×10^4 HSFs were plated on top of the gel clot. The medium was replaced every other day until desired growth was achieved.

2.3. siRNA constructs and delivery

Small interfering RNA (siRNA) for VE-cadherin was purchased from Dharmacon (Chicago, IL). A scrambled non-silencing siRNA that doesn't share sequence homology with any known human mRNA from a BLAST search was used as control for target siRNA. The control siRNA conjugated with Cy5 was used for determining

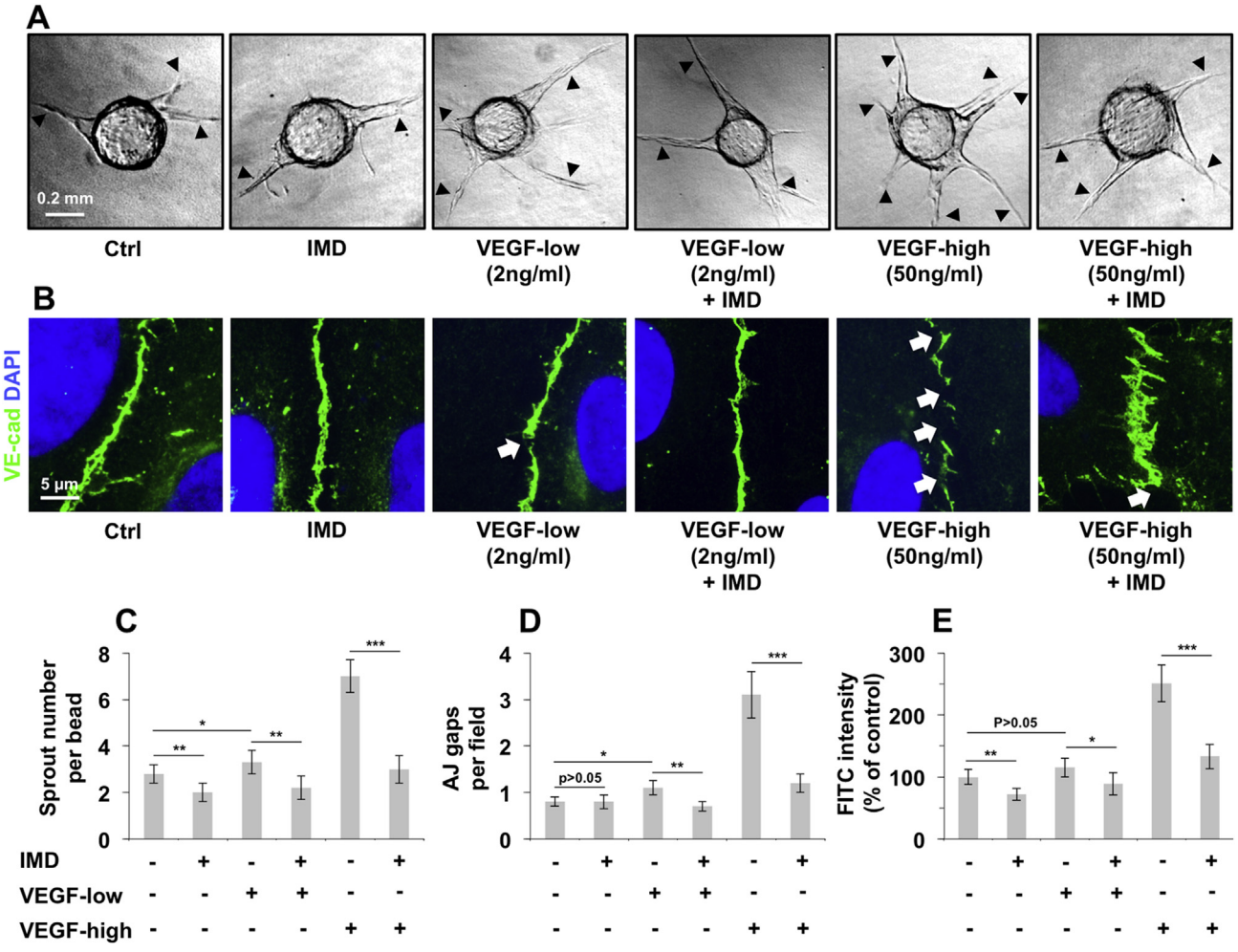

Fig. 1. IMD decreased vessel sprouting accompanied by the prevention of endothelial junction loosening. (A) The fibrin beads assay was performed as described in the Materials and Methods. VEGF-$_{low}$ (2 ng/ml) and VEGF-$_{high}$ (50 ng/ml) with or without IMD (1 μM) were added at day 1 and supplemented every other day when the medium was replaced. Representative images showed the vessel sprouting from the surface of beads at day 7. The arrowheads indicated the vessel sprouts. (B) The HUVECs were grown as monolayers and treated with VEGF-$_{low}$ or VEGF-$_{high}$ for 1 h, followed by the addition of IMD for another 1 h, and stained for VE-cad (green) and DAPI (blue). The images were acquired at 1000 × magnification using oil immersion lens. The white arrows indicated the AJ gaps between the adjacent cells. (C) The number of vessel sprouts per bead was quantified in 10 randomly chosen beads per well. (D) The number of AJ gaps per field was quantified in 10 randomly chosen fields per well. (E) The fluorescence intensity was measured and expressed relative to the control. Data was presented as mean ± S.D. of 3 independent experiments performed in duplicate wells. *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed t-test), similarly hereinafter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

uptake and distribution in the transfected HUVECs. In vitro transient transfection was performed according to the manufacturer's instruction using X-tremeGENE siRNA transfection reagent (Roche, CO).

2.4. Immunofluorescence analysis of cells

The HUVECs were plated on the top of cell slides and cultured in 6-well plates. VEGF at different concentration (2 ng/ml & 50 ng/ml), thrombin (0.2 IU/ml), and IMD (1 μM/ml) were added as indicated in Fig. 1 and Fig. 2. At the end of the experiments, the slides were fixed with cold acetone, washed with PBS, and incubated with the primary antibody (anti-VE-cad, 1:100) over night at 4 °C. The slides were then incubated with AlexaFluor488-secondary antibody (1:200) for 30 min at room temperature, washed with PBS, followed by DAPI (20 μg/ml) staining for 5 min. Each slide was covered with 50 μl ProLong® Gold Antifade Mountant (Invitrogen) and sealed with nail polish.

2.5. Endothelial cell monolayer hyperpermeability

HUVECs were grown as monolayers on fibronectin coated Transwell plates. VEGF at different concentration (2 ng/ml & 50 ng/ml), thrombin (0.2 IU/ml), and IMD (1 μM/ml) were added as indicated in Figs. 1 and 2. The FITC-labeled albumin (5 mg/ml) was added to the upper or luminal chamber of the Transwell plate and was allowed to equilibrate for 30 min 100 μl of the media samples were collected from the upper and lower chambers and the fluorescent intensity of FITC using a fluorometric plate reader at excitation/emission 494 nm/520 nm. The data were calculated as percentage of the control values.

2.6. Retina study

All animal experiments were approved by the Animal Ethics Committee of Sichuan University and performed according to institutional and international guidelines. The neonatal C57BL/6

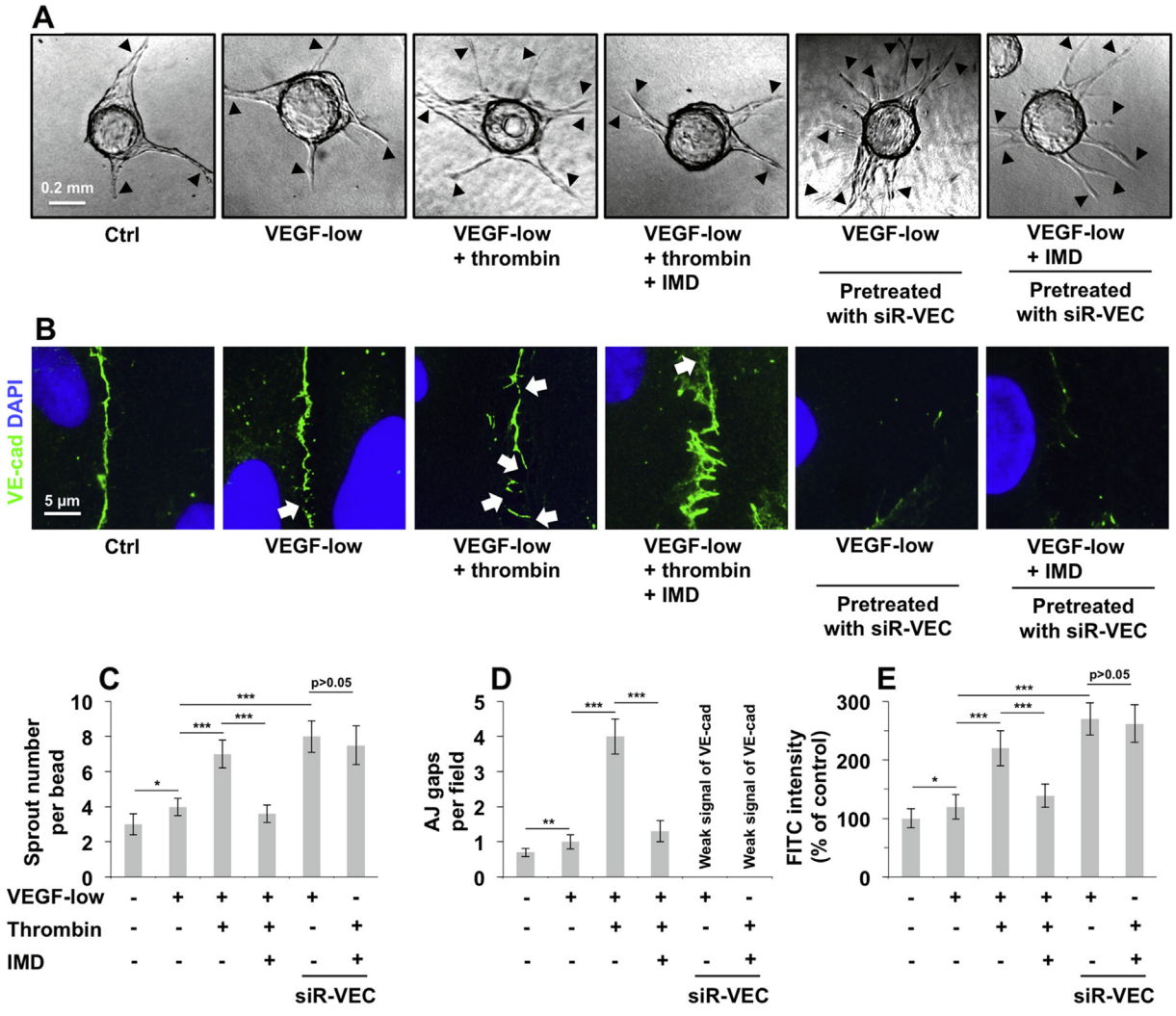

Fig. 2. The loosening of endothelial junctions facilitated low-dose VEGF to induce vessel sprouting, which could be antagonized by IMD in a VE-cad dependent way. (A) The fibrin beads assay was performed, VEGF-$_{low}$ (2 ng/ml), thrombin (0.2 IU/ml), and IMD (1 μM) were added at day 1. In the VE-cad knockdown groups, siR-VEC was transfected into the HUVECs before the cells coated on the surface of the beads at day 0. The arrowheads indicated the vessel sprouts. (B) The HUVEC monolayer was treated with VEGF-$_{low}$ with or without thrombin for 1 h, followed by the addition of IMD for another 1 h. In the VE-cad knockdown groups, siR-VEC was pre-transfected into the cells before they were plated onto the cell slides. The cell slides were stained for VE-cad (green) and DAPI (blue), and images were acquired at 1000 × magnification. The white arrows indicated the AJ gaps between the adjacent cells. (C) The number of vessel sprouts per bead was quantified in 10 randomly chosen beads per well. (D) The number of AJ gaps per field was quantified in 10 randomly chosen fields per well. Note that in the VE-cad knockdown groups, the fluorescent signal from VE-cad complex was too weak to observe. (E) The fluorescence intensity was measured and expressed relative to the control. Data was presented as mean ± S.D. of 3 independent experiments performed in duplicate wells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mice were received intraocular injection of VEGF (50 ng), thrombin (0.2 IU) with or without IMD (1 μg; the reagents in a total volume of 1 μl saline) at postnatal day 2.5 (P2.5) and P5.5, 1 μl saline was injected into the other eye as a control. At P5.5, 30 min after the intraocular injection, the mice were anesthetized and injected with FITC-dextran (2 mg in 50 μl saline) through jugular vein and allowed to circulate for 15 min. Eyes were then collected and fixed with 2% paraformaldehyde (PFA) in PBS for 2 h. The retinas were carefully dissected, washed with PBS, and blocked with 10% goat serum in PBST for 3 h, then incubated overnight with Isolectin B4 to label the whole retinal vasculature.

2.7. Image analysis and statistics

The beads in fibrin gel were examined using Nikon TE2000 inverted microscope and photographed using SPOT color camera system (Diagnostic Instruments). Images of the cell slides and flat mounted retinas were acquired using Zeiss Z2 fluorescence microscope. All images were analyzed using Image-Pro Plus analysis software (version 5.0.9.2). Vessel sprouting was analyzed by quantifying the number of branch points per field. All values are expressed as mean ± SD. Statistical differences among groups were examined using the 2-tailed Student *t* test, and a P value < 0.05 was considered statistically significant.

3. Results

We first investigated the vessel sprouting using the fibrin beads assay, in which the human umbilical vascular endothelial cells (HUVECs) can sprout from the surface of dextran-coated beads embedded in fibrin gels [13]. We found that low-dose VEGF (VEGF-$_{low}$, 2 ng/ml) only slightly promoted the vessel sprouting, whereas high-dose VEGF (VEGF-$_{high}$, 50 ng/ml) markedly increased the number of vessel sprouts (Fig. 1A,C). On the contrary, the treatment of IMD significantly inhibited the vessel sprouting in the VEGF-$_{low}$, VEGF-$_{high}$ and control groups (Fig. 1A,C).

We then examined how VEGF and IMD affect the endothelial junctions. The immunofluorescence assay using anti-VE-cad showed that low-dose VEGF only slightly affected the integrity of the adherens junction (AJ), whereas high-dose VEGF caused AJ gaps between adjacent ECs (Fig. 1B,D). IMD repaired the high-dose VEGF-induced AJ gaps by promoting a zipper-like VE-cad complex formation (Fig. 1B,D). To test whether the endothelial junction is effectively re-established and functional, we performed the monolayer permeability assay, and found that IMD decreased the permeability in all three groups, suggesting that the endothelial junctions were functionally re-established by IMD (Fig. 1E).

We next designed an experiment to investigate whether loosening of endothelial junction can improved the ability of low-dose VEGF to induce vessel sprouting. Thrombin is a known factor that can potently dissociate the endothelial barrier and increase the permeability [14,15]. We found that the combination of thrombin potently induced the AJ dissociation (Fig. 2B,D) and endothelial hyperpermeability (Fig. 2E). In addition, the combination with thrombin significantly increased the ability of low-dose VEGF to induce vessel sprouting (Fig. 2A,C), similar to the effect of high-dose VEGF. The treatment of IMD markedly reduced the junction loosening, hyperpermeability and vessel sprouting induced by the combination of thrombin and low-dose VEGF (Fig. 2A–E). These results suggested that the loosening of endothelial junction may play an important role in the process of vessel sprouting, and IMD may restrict the excessive vessel sprouting by preventing the endothelial junction from dissociation.

VE-cad is an endothelial-specific trans-membrane protein and regulates the endothelial junction predominantly. In addition, the VE-cad complex is the major component to directly form the endothelial AJ [9,10]. Knockdown the expression of VE-cad using siRNA (siR-VEC) significantly weakened the AJ and disrupted the junctions between adjacent ECs (Fig. 2B). The disruption of EC junction markedly increased the ability of low-dose VEGF to increase endothelial permeability (Fig. 2B,E) and induce vessel sprouting (Fig. 2A,C). However, in this circumstance, IMD could not decrease the permeability and inhibit vessel sprouting (Fig. 2A–E). The result is consistent with previous reports that IMD stabilizes the endothelial barrier function by maintaining the integrity of VE-cad complex [8,11]. Without the expression of VE-cad, IMD could not promote the formation of VE-cad complex, therefore could not prevent the AJ dissociation and subsequent vessel sprouting. The result indicated that the ability of IMD to control vessel sprouting is VE-cad dependent.

We finally tested this hypothesis in vivo using the developmental retinal vasculature during the first post-natal week of mice. High-dose VEGF and thrombin with or without IMD were directly injected into one eye at postnatal day 2.5 (P2.5) and P5.5, and PBS was injected at the same time into another eye as a control. (Note that we didn't use low-dose VEGF as a separate group in the in vivo study for there has been a relatively low level of VEGF existing throughout the body, which is necessary for the physiological angiogenesis.) At P5.5, 30 min after the final intraocular injection, the mice were anesthetized and perfused with FITC-conjugated low molecular dextran (70kD) to show the vessel perfusion (overlapped area, yellow) and leakage (green). The retinas were then removed and stained with Isolectin B4 (IB4) to show the whole vasculature (red). We found that high-dose VEGF induced a highly branched vessel network compared to the control, indicating that VEGF significantly promoted the vessel sprouting (Fig. 3A,D; the branch points were marked with *). Thrombin had a similar but weaker effect on the promotion of vessel sprouting (Fig. 3A,D). Both VEGF and thrombin caused severe leakage in the retina (Fig. 3C,E). The co-treatment of IMD significantly reduced the vessel leakage induced by VEGF or thrombin (Fig. 3C,E), indicating that IMD could strengthen the EC junction in vivo. Importantly, IMD inhibited the excessive vessel sprouting concurrently (Fig. 3C,E). Taken together with the in vitro and in vivo findings, we may conclude that IMD restrict the vessel sprouting by preventing the dissociation of endothelial junction in a VE-cad dependent way.

4. Discussion

We previously reported that IMD, a calcitonin family member, was capable to inhibit the excessive vessel sprouting [8]. However, the underlying mechanism had not been elucidated. VEGF is considered as the most prominent factor to induce vessel sprouting [2–4]. Interestingly, VEGF was originally identified as a vascular permeability factor in 1983 [16]. VEGF increases vessel permeability by inducing VE-cad dissociation and loosening the endothelial junction [1,9]. It was suggested that the dissociation of endothelial junction may be involved in the process of vessel sprouting [1]. Based on the facts that VEGF promotes but IMD inhibits vessel sprouting [2–4,8], and VEGF disrupts but IMD maintains the endothelial barrier [1,9,11,12], we hypothesized that the prevention of endothelial junction loosening may be the mechanism through which IMD could restrict the excessive vessel sprouting.

In this study, we found that low concentration of VEGF (2 ng/ml) only slightly affected the vessel sprouting and endothelial barrier function, and showed no significant effect on the endothelial permeability, whereas high concentration of VEGF (50 ng/ml) markedly induced the vessel sprouting, endothelial junction loosening, and endothelial hyperpermeability. The results are

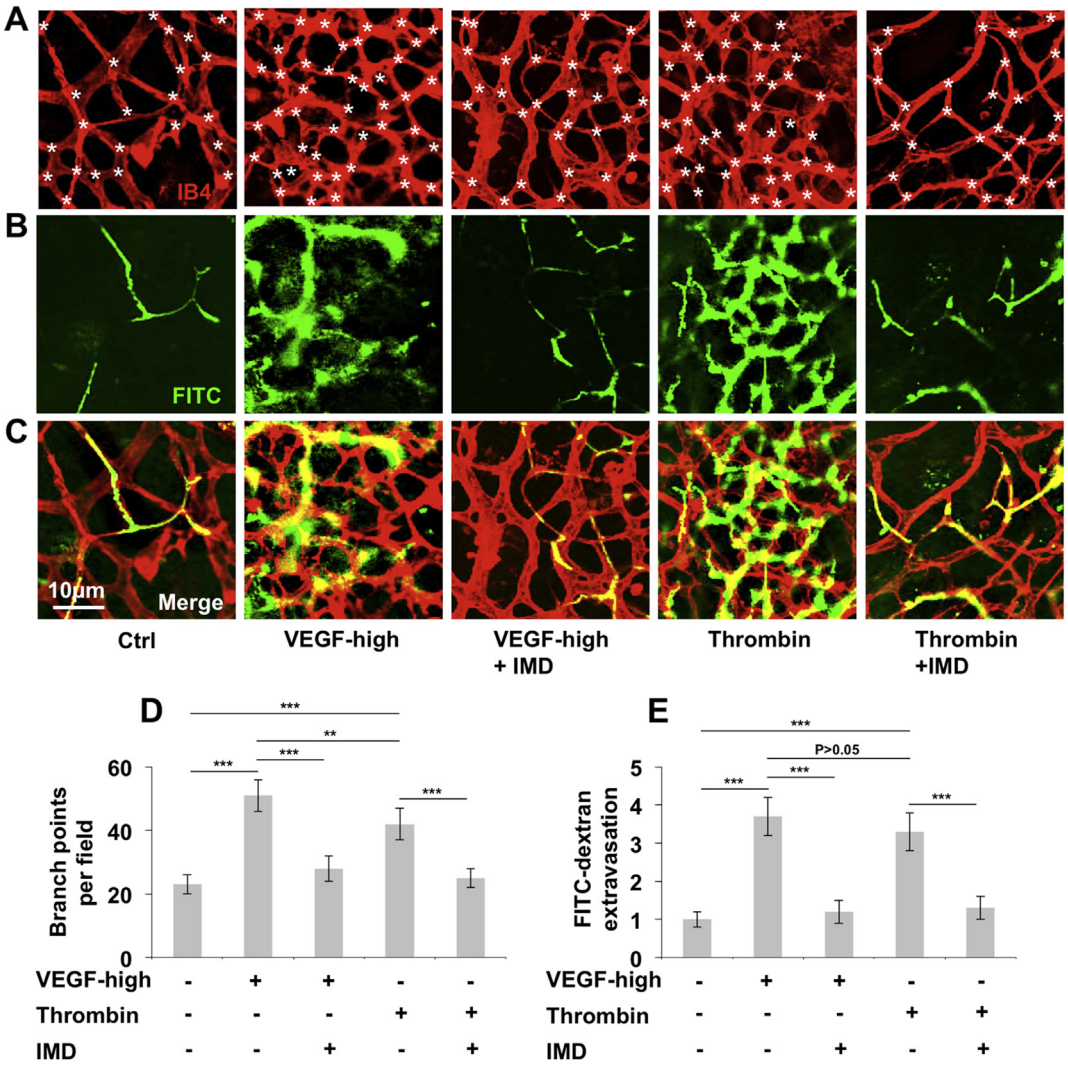

Fig. 3. IMD inhibited the junction loosening-induced vessel sprouting in vivo. (A,B) VEGF (50 ng), thrombin (0.2 IU) with or without IMD (1 μg) in a total volume of 1 μl saline was injected into one eye of the neonatal mice (C56BL/6) at P2.5. and P5.5 1 μl saline was injected into another eye as a control. The retinas were perfused with low molecular FITC-dextran (green) and stained with IB4 (red) as described in the Materials and Methods. The representative image showed the retinal vasculature. * indicated the branch points of the vessels. (C) The merged images showed the vessel leakage. The red fluorescence indicated the retinal vasculature, yellow indicated the dextran left in the vessels, and green indicated the leaked dextran outside the vessels. (D) The number of branch points of the vessels was quantified in 10 randomly chosen fields. (E) The dextran extravasation from the vessels was determined by the area of green signal relative to that of yellow signal. Data was presented as mean ± S.D. (n = 10 per group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

consistent with past reports that low level of VEGF in the micro-environment induces normal and stable angiogenesis, whereas high level of VEGF causes irregular and over branched vasculature [17,18]. Thrombin is a known factor that can potently disrupt the endothelial junction and increase the permeability [14,15]. Interestingly, thrombin significantly improved the ability of low-dose VEGF to induce vessel sprouting. The administration of IMD inhibited the hyperpermeability-induced junction loosening, and reduced the vessel sprouting concurrently. Another prominent molecule to regulate endothelial cell-to-cell junction is VE-cad, which is specifically expressed in ECs and the major component of AJ [9,10]. The knockdown of VE-cad could also increase the ability of low-dose VEGF to induce more vessel sprouts. IMD could not inhibit the VE-cad knockdown-induced hyperpermeability or vessel sprouting. This is consistent with previous observations that IMD stabilizes the endothelial barrier function by maintaining the integrity of VE-cad complex [8,11]. Without the expression of VE-cad, IMD would not be able to promote the formation of endothelial adherens junction, therefore could not prevent the dissociation of EC junction and vessel sprouting.

Vessel sprouting is a crucial step for the establishment of a functional vasculature. This process was suggested to be initiated by the loosening of EC junction and the selection of tip cell [1]. Previous studies mainly focused on the tip cell selection, and found that VEGF and Notch/DLL4 signaling are the prominent pathways to control the tip/stalk cell switch and vessel sprouting [1,19]. However, whether the junction loosening is also an important step to initiate the vessel sprouting, or just a subsequent event after tip cell selection, is still unknown. There have been hundreds of papers in the study of tip cell selection and vessel sprouting, but the study that directly investigates the relationship between junction loosening and vessel sprouting is rare. In this study, we provide in vitro and in vivo evidences showing that there may be a casual relationship between the loosening of endothelial junction and vessel sprouting. The dissociation of EC junction may increase the chance for the tip cell selection, or help the tip cell to migrate from the

streamlined surface of existing vessels, or increase the exchange of pro-angiogenic factors between the basal and luminal sides. Which mechanism is underlying this process need to be determined in the future studies.

Taken together, our data provide in vitro and in vivo evidences to show that the loosening of endothelial junction may promote vessel sprouting, and IMD could restrict the excessive vessel sprouting by preventing the EC junction from dissociation. These findings provide new insight into the understanding of the regulation of sprouting angiogenesis.

Conflict of interest

None declared.

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