様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Molecular regulation of blood vessel size by endothelial cells-the role of Marcksl1.

研究課題名（英文）Molecular regulation of blood vessel size by endothelial cells-the role of Marcksl1.

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研究成果の概要（和文）：血管組織の形成および維持機構は、胚の成長や組織の恒常性を理解するために重要である。本研究で我々は血管の力学的特性を調節する分子として、アクチン結合タンパク質であるMarcksl1を同定した。Marcksl1のノックアウト系統では血管内皮細胞（EC）の細胞サイズや毛細血管の直径が変化した。一方、EC細胞においてMarcksl1を過剰発現させた際にはapical側およびbasal側の細胞膜にblebと呼ばれる突起構造が形成された。このblebの形成は血圧の減少によって抑制されることから、EC細胞におけるMarcksl1の発現量調節は血圧に拮抗するために必須であることが示された。

研究成果の学術的意義や社会的意義
Vascular diseases such as cerebral carvenous malformation and aneurysms are characterized by abnormal vessel structure that are prone to rupture and can lead to death. Findings from our study contribute to the understanding of how vascular malformations arise and lead to the development of therapy.

研究成果の概要（英文）：The efficient distribution gases and solutes to tissues is facilitated by blood flow pumped through blood vessels. The formation and maintenance of vascular tubes are therefore critical for growth and tissue physiology. In this study, we identified a role of the actin-binding protein, Marcksl1, in modulating the mechanical properties of endothelial cell (EC) to regulate cell shape and vessel structure. Increasing and depleting Marcksl1 expression level results in an increase and decrease, respectively, in EC size and vessel diameter. Furthermore, overexpression of Marcksl1 induces ectopic membrane blebbing that is suppressed by reduced blood flow. Detailed analysis reveals that Marcksl1 promotes the formation of linear actin bundles and decreases actin density at the EC cortex. Our findings reveal that a balanced network of linear and branched actin at the EC cortex is essential for conferring resistance to the deforming forces of blood flow and pressure to regulate vessel structure.

研究分野：Vascular development

キーワード：Endothelial cell　Blood vessels　Morphogenesis　Lumen　Mechanobiology　Actin　Marcksl1　Bleb

1. 研究開始当初の背景

The establishment and maintenance of a network of blood vessels is crucial throughout the lifetime of a vertebrate. During development, blood vessels supply oxygen and nutrients to meet the demands of growing tissues such that inadequate blood vessel formation can lead to embryonic lethality. In the adult, the circulatory network of blood vessels caters for the metabolic needs of tissues and organs; serve as conduits through which immune cells travel to sites of infection; and, importantly, new blood vessel formation is necessary for tissue repair. The formation of new blood vessels frequently occurs through sprouting angiogenesis, where new vessels are generated from pre-existing ones. Sprouting angiogenesis is a multicellular process that is tightly regulated in time and space beginning with the formation of new vascular sprouts composed of endothelial cells (ECs). Together, endothelial tip and stalk cells migrate in a collective manner to invade hypoxic tissues. The polarized migration of ECs collectives requires the coordinated behaviour of individual ECs that is mediated through cell-cell junctions, which act as sites of mechanocoupling to transmit force from one cell to another. ECs also undergo extensive changes in cell shape that is adapted to function. Live imaging has revealed that local and transient cell shape changes underlie migration, cell rearrangements, anastomosis and lumen formation, which are cellular behaviours that are critical for building a multicellular tubular vascular network.

ECs become exposed to haemodynamic forces such as shear stress and pressure as soon as they generate lumens. During transcellular lumen formation of angiogenic vessels, blood pressure deforms EC membrane to induce apical membrane blebbing to propel lumen expansion. However, we have observed that membrane blebbing activity decreases in fully perfused vessels, suggesting that an active mechanism exists at the EC cortex to regulate membrane and cell shape.

2. 研究の目的

In this project, we investigated how EC shape is regulated in the presence of blood flow and pressure. To achieve this, we examined the function of the actin bundling protein, Marcksl1, a member of the myristoylated alanine-rich C kinase substrate family proteins, in ECs during the period of lumen formation using the zebrafish embryo as a model system.

3. 研究の方法

(1) Mosaic expression of transgenes in zebrafish

Tol2-based expression constructs (5-10 pg) were co-injected into one-cell stage zebrafish embryos with 50 pg of *Tol2* transposase mRNA. For mosaic expression of transgenes, embryos were analysed at 1 or 2 dpf after injections.

(2) Generation of *marcksl1a* and *marcksl1b* zebrafish mutants

marcksl1a mutants were generated by CRISPR/Cas9-mediated mutagenesis. *marcksl1b* mutants were generated by TALEN-mediated mutagenesis. F_0 founders were identified by outcrossing TALEN or CRISPR/Cas9-injected fish with wildtype fish and screening the offspring for mutations at 2 dpf using T7 endonuclease I (NEB) assay (for TALEN-injected fish) or Sanger sequencing (for CRISPR/Cas9-injected fish).

(3) Live imaging

Embryos were mounted in 0.8% low-melt agarose in E3 medium containing 0.16 mg/mL Tricaine and 0.003% phenylthiourea. Confocal z-stacks were acquired using an inverted Olympus IX83/Yokogawa CSU-W1 spinning disc confocal microscope equipped with a Zyla 4.2 CMOS camera (Andor). Bright-field images were acquired on Leica M205FA microscope.

(4) Microangiography
2 and 3 dpf embryos were injected with 1–2 nL dextran tetramethyl rhodamine or dextran fluorescein (MW= 2,000 kDa, Invitrogen) at 10 mg/mL and imaged immediately. Confocal z-stacks were acquired with an Olympus UPLSAPO 10x/NA 0.4 objective.

(5) Chemical treatments
Latrunculin B (Merck Millipore) was dissolved in DMSO to 1 mg/ml and stored at −20°C. CK666 (SIGMA) was dissolved in DMSO to 50mM and stored at 4°C. All compounds were diluted to the desired concentration in E3 Buffer.

(6) Cell transfections
HUVECs (Lonza, C-2519A) were cultured in EGM medium (Lonza) and used until passage 4. For plasmid transfections, cells were seeded in Opti-MEM medium (Gibco) on poly-L-Lysine and gelatin-coated coverslips and transfected with 2 μg of plasmid using Lipofectamine 3000 (ThermoFisher Scientific). For siRNA transfection, HUVECs were transfected with 20nM siRNA using DharmaFECT1 reagent (Dharmacon). Transfection was repeated after 24 hours. ON-TARGETplus human MARCKSL1 siRNA-SMARTpool (Dharmacon) was used to knockdown *MARCKSL1*. ON-TARGETplus Non-target pool (Dharmacon) was used as control siRNA transfection.

4. 研究成果

(1) <u>An intact cell cortex preserves membrane and cell shape in perfused vessels</u>

In perfused blood vessels, laser ablation of both apical and basal cortices of ECs leads to an immediate deformation at the site of ablation while adjacent membranes remain unperturbed. The deformation resembles a membrane bleb and expands outwards from the vessel wall into the surrounding tissue, indicating that an intact EC cortex is required to resist the deforming forces of blood flow to preserve membrane and cell shape. Analysis of actin cytoskeleton and myosin II localization in ECs using fluorescent probes and live imaging revealed a gradient of actomyosin cytoskeleton along the apical cortex during lumen expansion in 1-day post-fertilization (dpf) embryos. While very little or no F-actin and myosin II were observed at the anterior front of the lumen, a higher level was detected at the posterior segment of the lumen. Analysis of older embryos at 2 and 3 dpf, when ISVs are perfused, showed prominent assembly and localization of F-actin and myosin II at both the apical and basal cortices of ECs. These observations suggest the existence of a temporal switch in actomyosin assembly at the apical cortex that allows apical membrane expansion at low levels (during lumen formation) but confers cortical stiffness to the ECs at higher levels (after lumen is formed). Indeed, short-term inhibition of actin polymerization using a low dose of Latrunculin B, which inhibits actin polymerization, in 2 dpf embryos resulted in a significant increase in the number of blebs that form in ECs. As with laser ablation, these blebs protrude from the basal membrane and into the surrounding tissue membrane. These results suggest that cortical actin cytoskeleton confers a protective mechanism against haemodynamic forces.

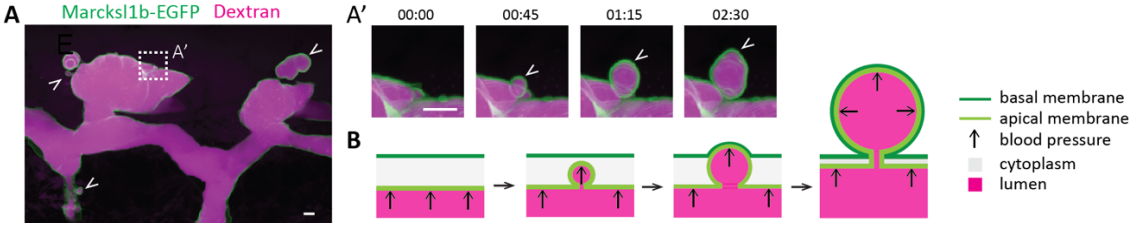

Figure 1. The overexpression of Marcksl1 in ECs induces ectopic basal blebs. (A) Marcks1-induced blebs are comprised of apical and basal membranes (green). Newly formed basal blebs (arrowheads) at the dorsal longitudinal anastomotic vessel (DLAV) are filled with Dextran (magenta). 00:00, minutes:seconds. (B) Schematic representation of how basal blebs are formed. Scale bars, 5 μm.

(2) Marcksl1 regulates endothelial membrane behaviour

To understand how actin cytoskeleton formation and organization are molecularly regulated in ECs, we investigated the role of the actin binding protein, Marcksl1. In zebrafish, two paralogues exist, *marcksl1a* and *marcks1b*, that are expressed in ECs. By tagging the proteins with EGFP, we discovered that both proteins localize to the plasma membrane but become enriched in the apical membrane during lumen formation. To investigate their function in ECs, we performed gain- and loss-of function experiments by mosaic overexpression of the proteins in ECs using an endothelial Gal4-UAS system and by generating *marcksl1a* and *marcksl1b* zebrafish mutants using CRISPR/Cas9 genome editing, respectively. Analyses of membrane behaviour revealed a significant increase and decrease in filopodia formation in ECs with increased expression of Marcksl1a/b and in ECs of *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ zebrafish, respectively. Interestingly, the overexpression in ECs also resulted in ectopic basal membrane blebbing upon exposure to blood flow (Fig. 1). Most of these Marcksl1-induced blebs are transient as many retract after the assembly of actomyosin network in the bleb cortex. Reducing blood flow by decreasing heart contractions suppressed Marcksl1-induced membrane blebbing, indicating that increased Marcksl1 expression level weakens ECs to become more deformable in the presence of haemodynamic forces.

(3) Marcksl1 regulates blood vessel diameter

During clonal analysis of ECs overexpressing either Marcksl1a or Marcksl1b, we frequently observed that segments of intersegmental vessels (ISVs) and dorsal longitudinal anastomotic vessel (DLAV) composed of ECs with increased Marcksl1 levels are wider or bulbous in appearance compared with neighbouring wildtype ECs (Fig. 2A). Quantification confirmed that wildtype (full length) Marcksl1a and Marcksl1b proteins increased vessel diameter when compared to controls (Fig. 2B). This increase in diameter was abrogated when Marcksl1aΔED or Marcksl1bΔED, which lacks the F-actin binding Effector Domain (ED), was expressed, suggesting that the actin-binding domain of Marcksl1 is required for Marcksl1-induced increase in vessel diameter. Analysis of trunk vessels at 2 dpf revealed that the diameter of blood capillaries is decreased in *marcksl1b*$^{-/-}$ and *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ mutants when compared to wildtype embryos (Fig. 2C and D).

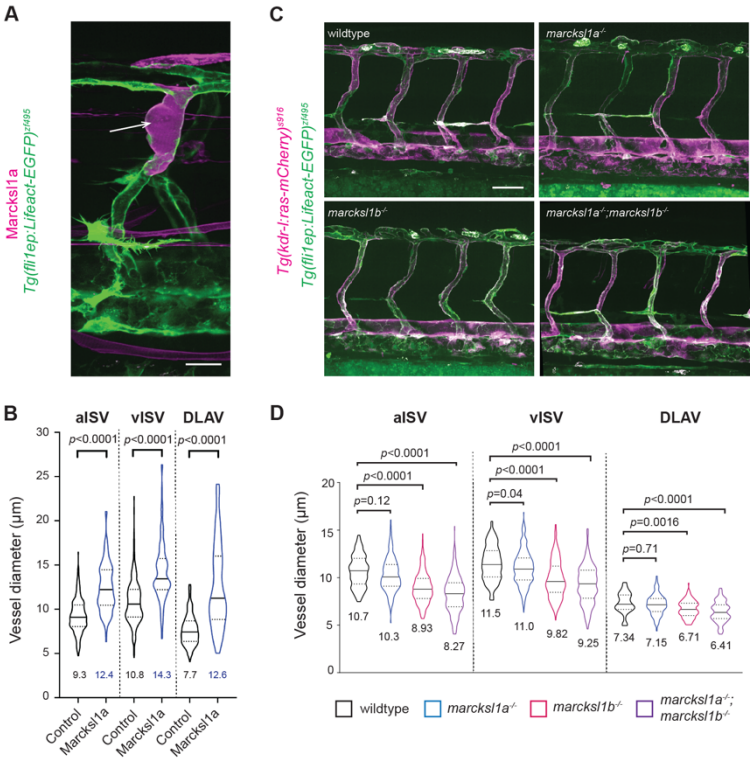

Figure 2. Marcksl1 expression level regulates blood vessel diameter. (A) A Marcks1-overexpressing EC (magenta) is dilated (arrow) compared to surrounding wildtype ECs (green) in an intersegmental vessel (ISV) of a 2 dpf embryo. (B) Measurement of arterial ISV (aISV), venous ISV (vISV) and DLAV diameter shows significantly increased vessel diameter in Marcksl1a-overexpressing ECs when compared to controls. (C) Maximum intensity projection of confocal z-stacks of zebrafish trunk vessels of 2 dpf *marcksl1a*$^{-/-}$, *marcksl1b*$^{-/-}$ and *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ embryos. (D) aISVs, vISVs and DLAV of 2 dpf *marcksl1b*$^{-/-}$ and *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ embryos are significantly narrower than wildtype embryos. Scale bars, 20 µm (A) and 50 µm (C).

(4) Marcksl1 regulates EC number and size

Analysis of EC proliferation during ISV formation revealed a decrease in EC division and EC number in *marcksl1a*$^{-/-}$, *marcksl1b*$^{-/-}$ and *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ zebrafish embryos when compared to wildtype embryos. Although the decrease in EC number can offer an explanation for the decreased vessel diameter in *marcksl1b*$^{-/-}$ and *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ mutants, mosaic experiments where single ECs with ectopic Marcksl1a or Marcksl1b expression can induce vessel dilation and membrane blebbing suggest that Marcksl1 can also regulate vessel diameter independent of cell number by regulating cell shape. Indeed, quantitative cell shape analyses of single ECs of vascular tubes and in human umbilical vein ECs (HUVECs) show that increased or loss of Marcksl1 expression increased or decreased, respectively, the size of ECs when compared to control ECs. Elevated Marcksl1 expression also increased cell aspect ratio (*in vivo* and *in vitro*) and cell protrusions (*in vitro*). Importantly, regions of ISVs composed of single *marcksl1a*$^{-/-}$;*marcksl1b*$^{-/-}$ ECs in wildtype embryos have significantly reduced diameter compared to neighbouring segments composed of wildtype ECs. These experiments indicate that the level of Marcksl1 expression in ECs control EC size and shape to regulate vessel diameter.

(5) Marcksl1 regulates actin organization in the endothelial cell cortex

We next sought to uncover the mechanism by which Marcksl1 regulates EC shape. As Marcksl1a/b localizes at the plasma membrane, we investigated actin organization in the cortex of HUVECs using superresolution microscopy. In confluent cell cultures, we detected a meshwork of actin cytoskeleton decorated with actin nodes at the apical cell cortex. Upon transfection of Marcksl1-EGFP in HUVECs, we detected the co-localization of Marcksl1 protein with actin bundles (Fig. 3A). Additionally, we observed the formation of longer bundles of actin and a decrease in actin density in their vicinity in HUVECs with elevated Marcksl1 expression (Fig. 3B). Combined with the observation that ectopic expression of Marcksl1 increases filopodia formation in ECs in zebrafish, our analyses demonstrate that Marcksl1 favours the generation of linear actin bundles in the cell cortex. The increase in linear actin bundle formation may concomitantly reorganize the surrounding actin network, creating regions with sparse actin within the cortex that may become susceptible to deformations.

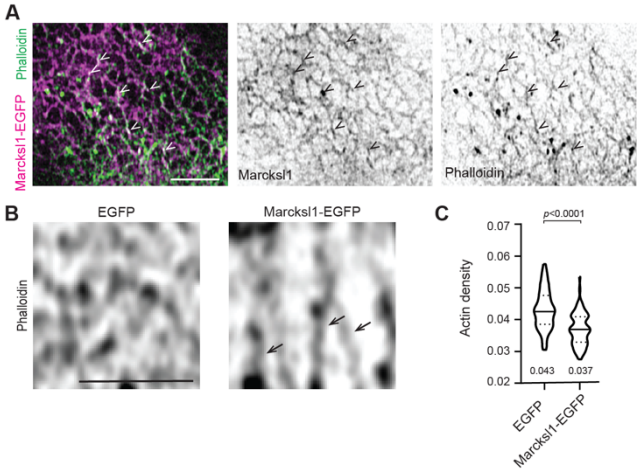

Figure 3. (A) Marcksl1-EGFP co-localizes with actin bundles (arrowheads) in the cortex of HUVECs. (B and C) Overexpression of Marcksl1 in HUVECs results in decreased actin density surrounding linear actin bundles (arrows). Scale bars, 2 µm.

(6) Excessive formation of linear actin bundles weakens EC resistance to haemodynamic forces

We subsequently tested the hypothesis that a balanced network of branched and linear actin bundles is essential for maintaining cortical integrity in perfused vessels exposed to haemodynamic forces. In one approach, we overexpressed the actin bundling protein, Fascin1a, specifically in ECs during lumen formation to increase linear actin bundle formation. In a second approach, we decreased branched actin formation by inhibiting the activity of Arp2/3 using CK666 in 2 dpf embryos. Both experimental manipulations resulted in ectopic basal blebbing, therefore indicating that a tight balance of linear and branched actin bundles is necessary to resist haemodynamic forces, thereby preserving EC membrane and shape in perfused vessels.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

１．著者名 Igor Kondrychyn, Douglas J. Kelly, Nuria Taberner Carretero, Akane Nomori, Kagayaki Kato, Jeronica Chong, Hiroyuki Nakajima, Satoru Okuda, Naoki Mochizuki and Li-Kun Phng	４．巻 11
２．論文標題 Marcksl1 modulates endothelial cell mechanoresponse to haemodynamic forces to control blood vessel shape and size	５．発行年 2020年
３．雑誌名 Nature Communications	６．最初と最後の頁 5476-5493
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41467-020-19308-5	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計8件（うち招待講演　7件／うち国際学会　3件）

１．発表者名 Li-Kun Phng
２．発表標題 Marcksl1 modulates endothelial cell mechanoresponse to haemodynamic forces to regulate cell and vessel shape
３．学会等名 Annual Scientific Meeting of the Japanese Circulation Society, 2021. “Vascular biology”（招待講演）
４．発表年 2021年

１．発表者名 Li-Kun Phng
２．発表標題 Marcksl1 modulates endothelial cell mechanoresponse to haemodynamic forces to regulate cell and vessel shape
３．学会等名 Annual Meeting of the Molecular Biology Society of Japan, 2020. “Decoding and Engineering Cell Shape”（招待講演）
４．発表年 2020年

１．発表者名 Li-Kun Phng
２．発表標題 Endothelial cell mechanoresponse to haemodynamic forces during vascular tubulogenesis.
３．学会等名 Mechanobiology Institute Conference, NUS, Singapore.（招待講演）
４．発表年 2020年

１．発表者名
Li-Kun Phng

２．発表標題
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３．学会等名
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４．発表年
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Li-Kun Phng

２．発表標題
Endothellial cell response to hemodynamic forces in developing blood vessels

３．学会等名
52nd Japan Society for Developmental Biology（招待講演）

４．発表年
2019年

１．発表者名
Li-Kun Phng

２．発表標題
Marcksl1 modulates endothelial cell response to haemodynamic forces to control blood vessel shape and size

３．学会等名
Angiogenesis Gordon Research Conference（国際学会）

４．発表年
2019年

１．発表者名
Li-Kun Phng

２．発表標題
Shaping the vasculature - a balance of endothelial cell biomechanics and haemodynamic forces

３．学会等名
3rd Asia-Australia Vascular Biology Meeting（招待講演）（国際学会）

４．発表年
2019年

１．発表者名 Li-Kun Phng
２．発表標題 Shaping the vasculature - a balance of endothelial cell biomechanics and haemodynamic forces
３．学会等名 42nd Annual Meeting of the Molecular BIology Society of Japan.（招待講演）（国際学会）
４．発表年 2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関