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The endothelial E3 ligase HECW2 promotes endothelial cell junctions by increasing AMOTL1 protein stability via K63-linked ubiquitination

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ABSTRACT

Cell-to-cell junctions are critical for the formation of endothelial barriers, and its disorganization is required for sprouting angiogenesis. Members of the angiomotin (AMOT) family have emerged as key regulators in the control of endothelial cell (EC) junction stability and permeability. However, the underlying mechanism by which the AMOT family is regulated in ECs remains unclear. Here we report that HECW2, a novel EC ubiquitin E3 ligase, plays a critical role in stabilizing endothelial cell-to-cell junctions by regulating AMOT-like 1 (AMOTL1) stability. HECW2 physically interacts with AMOTL1 and enhances its stability via lysine 63-linked ubiquitination. HECW2 depletion in human ECs decreases AMOTL1 stability, loosening the cell-to-cell junctions and altering subcellular localization of yes-associated protein (YAP) from cytoplasm into the nucleus. Knockdown of HECW2 also results in increased angiogenic sprouting, and this effect is blocked by depletion of ANG-2, a potential target of YAP. These results demonstrate that HECW2 is a novel regulator of angiogenesis and provide new insights into the mechanisms coordinating junction stability and angiogenic activation in ECs.

1. Introduction

Angiogenesis is a process that forms new blood vessels and is coordinated by the dynamics of quiescent and activated endothelial cells (ECs). The quiescent state of ECs is maintained until they respond to pro-angiogenic factors, such as VEGF and ANG-2, which promote the liberation of ECs by loosening cell-to-cell junctions [1,2]. Once the junctions become disorganized, subsequent intracellular signaling induces cells to actively proliferate and migrate to form new vessels. Conversely, when ECs come into close contact with each other, actively proliferating ECs form stable intercellular junctions [3,4]. Regulation of endothelial cell-to-cell junctions is critically important in angiogenesis, and incorrect junctional permeability is the primary contributing factor for morbidity and mortality in vascular diseases [5,6].

The angiomotin (AMOT) family is characterized as a group of tight junction proteins that are expressed in ECs and regulate vascular permeability [7–10]. For example, AMOT co-localizes with ZO-1 by cell-to-cell contacts in ECs. In addition, ectopic expression of AMOT results in decreased permeability compared with that in control cells [7], and loss of AMOT-like 1 (AMOTL1) in ECs results in increased vascular permeability in vitro. Further, AMOTL1-transfected morphant zebrafish showed decreased junction stability of stalk cells in the dorsal aorta in vivo [8]. Moreover, AMOT-like 2 (AMOTL2) associated with VE-cadherin for transmission of mechanical force during aortic vessel lumen expansion [11]. A recent study showed that the protein stability of AMOTL1 was required for the formation of the tight junctions [12]. Nevertheless, the mechanism by which AMOTL1 stability is regulated in ECs remains largely unknown.

HECW2, also known as Nedd4-like E3 ubiquitin-protein ligase (NEDL) 2, belongs to the NEDD4 family, which includes nine other members: NEDD4, NEDD4-2, ITCH, SMAD-specific E3 ubiquitin protein ligase (SMURF) 1, SMURF2, WW domain-containing E3 ubiquitin protein ligase (WWP) 1, WWP2, NEDL1, and NEDL2 [13–18]. This family of proteins is characterized by distinct domains: a Ca^{2+}/lipid-binding domain (C2 domain) involved in membrane targeting, 2–4 WW domains that interact with a PPXY motif, and homologous with E6-associated protein C-terminus (HECT)-type ligase domain required for

Abbreviations: AMOT, angiomotin; AMOTL1, angiomotin-like 1; AMOTL2, angiomotin-like 2; YAP, yes-associated protein; ANG-2, angiopoietin-2; VE-cadherin, vascular endothelial cadherin; EPC, endothelial progenitor cell; OEC, outgrowing endothelial cell; HUVEC, human umbilical vein endothelial cell; EC, endothelial cell.

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catalytic activities. This family of E3 ligases regulates diverse cellular processes, such as proliferation, migration, differentiation, invasion, and neuronal cell apoptosis via regulation of target protein interaction with the WW-PPXY domains [19–22].

We recently proposed a molecular mechanism involving the YAP in vascular ECs. YAP is a transcriptional co-activator that plays a role in maintaining cellular homeostasis [23]. YAP was found to be expressed in the angiogenic front region where vessels were sprouting and regulated by the VE-cadherin-mediated PI3K/AKT pathway. Once YAP was activated by disruption of junctions, YAP translocated into the nucleus and induced ANG-2 expression, thereby promoting angiogenic sprouting in vitro [24].

Here we investigate the role of HECW2 in ECs. We demonstrate that HECW2 enhances endothelial cell-to-cell junctions through the regulation of AMOTL1 protein stability via lysine 63-linked polyubiquitination. Furthermore, depletion of HECW2 stimulates YAP translocation into the nucleus, thereby promoting EC sprouting via increased ANG-2 expression.

2. Materials and methods

2.1. Cell culture and antibodies

Human umbilical vein ECs (HUVECs) were isolated from human umbilical cord veins using collagenase, as previously described [25], and cells (between passage 2 and 7) were cultured on 2% gelatin-coated dishes using M199 medium (Invitrogen, Carlsbad, CA), containing 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor (R&D systems, Minneapolis, MN, USA), and 5 U/ml heparin. HUVECs were grown at 37 °C in a humidified 95%/5% (vol/vol) mixture of air and CO_2. The following antibodies were used: HECW2 and AMOTL1 (Atlas Antibodies, Stockholm, Sweden); ANG-2 (R&D systems, Minneapolis, MN, USA); YAP, LAMIN A/C, GFP, GAPDH, and β-ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-YAP (S127; Cell Signaling, Danvers, MA, USA); and angiopoetin-2 (R&D systems, Minneapolis, MN, USA).

2.2. Plasmids

The HECW2 and AMOTL1 open-reading frame (ORF)-containing plasmids Flag-HECW2 WT, FLAG-AMOTL1 and 2, and HA-HECW2 WT were generated in the Vascular genomics laboratory, Yonsei University. The AMOT130 ORF-containing plasmid HA-AMOT130 was purchased from Addgene (Cambridge, MA, USA), and AMOT130 ORF was subcloned into a FLAG vector. The HECW2 C1540A mutant was generated by site-directed mutagenesis. HECW2 deletion mutants were generated by polymerase chain reaction (PCR). V5-Ub was kindly provided by Dae-Won Kim (Yonsei University). pHM6-HA-Ub and mutants were kindly provided by Jaewhan Song (Yonsei University). pEGFP-C2 was used as a transfection control (Clontech, San Diego, CA).

2.3. Transfection of siRNA

HUVECs were transfected with scrambled and HECW2 siRNAs using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) for 3 h. Efficiency of siRNA was assessed by RT-PCR and Western blot 48 h after transfection. siRNA targeting HECW2 was purchased from Dharmacon Inc. (siGENOME HUMAN (MQ-007192-00-0005)) (Lafayette, CO, USA).

2.4. Immunoprecipitation

HUVECs were plated on 100-mm-diameter dishes. The cells were lysed with lysis buffer [150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.1% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture]. After centrifugation for 10 min at 13,000 rpm, the supernatant was removed, and the cell lysate was incubated with indicated antibodies for 12 h at 4 °C. The cells were incubated with protein G-Sepharose beads (GE Healthcare, Buckinghamshire, United Kingdom) for 2 h at 4 °C. The samples were then centrifuged and washed three times with lysis buffer and added to 2× sample buffer and then boiled.

2.5. Ubiquitination assay

HEK293T cells were transfected with plasmids expressing FLAG-AMOTL1, HA-HECW2, and HA-tagged Ub. After 24 h of transfection, the cells were lysed and harvested in phosphate-buffered saline (PBS) containing 10 nM NEM to prevent deubiquitination. Cells were lysed in 1% SDS by boiling for 10 min, followed by dilution to 0.1% SDS by the addition of lysis buffer, protease inhibitors, and NEM. Lysed samples were immunoprecipitated with a FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA), followed by Western blotting.

2.6. Fibrin gel beads sprouting assay

HUVECs were mixed with dextran-coated Cytodex 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a concentration of 400 HUVECs per bead in 1 ml of EGM-2 medium (Clonetics, Walkersville, MD, USA). Beads with cells were shaken gently every 20 min for 4 h at 37 °C and 5% CO_2. After incubation, mixtures of beads and cells were transferred to a 25-cm^2 tissue culture flask (BD Biosciences, Bedford, MA, USA) and incubated in 12–16 h in 5 ml of EGM-2 at 37 °C and 5% CO_2. The following day, mixtures of beads and cells were washed three times with 1 ml EGM-2 and were resuspended at a concentration of 250 cell-coated beads/ml in 2 mg/ml fibrinogen (Sigma) with 0.15 U/ml of aprotinin (Sigma) at a pH of 7.4. Five hundred microliters of fibrinogen/bead solution was added to 1 unit of thrombin (Sigma) in 1 well of a 24-well tissue culture plate. The fibrinogen/bead solution was allowed to clot for 5 min at room temperature and for 20 min at 37 °C and 5% CO_2. One milliliter of EGM-2 (which contains 10% FBS) was added to each well and was equilibrated with the fibrin clot for 30 min at 37 °C and 5% CO_2. The medium was removed from the well and replaced with 1 ml of fresh medium with or without additional growth factors. Twenty thousand skin fibroblasts were plated on the clot and the medium was changed every other day. Bead assays were monitored for 10 days.

2.7. Immunofluorescence staining

Scramble or HECW2 siRNA-transfected HUVECs cultured on coverslips in a 12-well plate were fixed with 4% paraformaldehyde ((PFA)-PBS) at room temperature for 20 min and were permeabilized with 0.1% Triton X-100 in PBS with Tween-20 for 15 min. After blocking by addition of 1% bovine serum albumin in PBS with Tween 20 for 1 h, cells were incubated with primary antibodies in blocking buffer at 4 °C overnight. After washing with PBS, cells were incubated with Alexa Fluor 488 goat *anti*-mouse for 30 min at room temperature. Nuclei were stained with 4, 6 diamidino-2-phenylindole (DAPI; Molecular Probes, Carlsbad, California, USA). Cells were mounted, and images were acquired by a laser scanning confocal microscope (LSM 700 META, Carl Zeiss). For quantification, at least 500 cells were counted in each sample.

2.8. Lentivirus production

Human TRC HECW2 shRNA constructs were purchased from Dharmacon (RHS4533-EG57520). To produce the lentivirus, pLenti-shHECW2 and 2nd generation packaging DNA were transfected into HEK293T cells. After 48 h, the supernatant was harvested and filtered using a 0.45 μm filter to remove cellular debris. Lentivirus was titrated to 10^7 TU per ml.