Tspan18 is a novel regulator of the Ca^{2+} channel Orai1 and von Willebrand factor release in endothelial cells

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Supplemental Methods

Cell culture

The DT40 chicken B-cell line was cultured in RPMI medium (Sigma) containing 10% fetal calf serum (Gibco), 1% chicken serum (Sigma), 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. DT40 cells deficient in IP3 receptors[1] were supplied by T. Kurosaki (Osaka University, Japan). The Jurkat human T-cell line was cultured in RPMI medium (Sigma) containing 10% bovine calf serum, 4 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The human embryonic kidney (HEK)-293T (HEK-293 cells expressing the large T-antigen of simian virus 40) cell line and the human HeLa epithelial cell line were cultured in DMEM medium (Sigma) containing 10% fetal calf serum (Gibco), 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from Birmingham Women's Health Care NHS Trust with informed consent[2]. HUVECs were cultured in M199 medium (Sigma) containing 10% fetal calf serum, 0.2% bovine brain extract, 90 μg/ml heparin and 4 mM L-glutamine, on plates coated with 0.1% type 1 gelatin from porcine skin (Sigma).

Quantitative real-time polymerase chain reaction (PCR)

RNA was extracted using a RNeasy Mini Kit with QIAshredder columns (Qiagen). cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using TaqMan assays for Tspan18, Orai1, Orai2, Orai3, 18S and GAPDH. All quantitative PCR data were normalized to 18S or GAPDH, as internal loading controls, and relative data was calculated as described[3].

Lentiviral transduction

HUVECs were lentivirally transduced with Orai1-Myc using our previously described method[4]. In brief, lentivirus were produced in HEK-293T cells by transfection with the psPAX2 lentiviral packaging and pMD2.G envelope constructs, which were gifts from Didier Trono (Addgene), and pLVX-EF1α-IRES-Puro (Clontech) into which human Myc-tagged Orai1[5] was subcloned. Transduced HUVECs were selected in 1 μg/ml puromycin (Invitrogen).

NFAT/AP-1-luciferase transcriptional reporter assay

The NFAT/AP-1-luciferase assay, and β-galactosidase assay to normalize for transfection efficiency, were completed as described[6]. Expression of FLAG-tetraspanin constructs was confirmed by SDS-PAGE of cell lysates in 1% Triton X-100, followed by western blotting using Western Lightning chemiluminescence reagents (Perkin Elmer) and Hyperfilm (Amersham Biosciences).

Co-immunoprecipitation

Transfected HEK-293T cells were lyzed in 1% digitonin (Merck) followed by co-immunoprecipitation using anti-FLAG M2-coupled protein G sepharose (Life Technologies)[7]. SDS-PAGE and western blots with anti-Myc and anti-FLAG antibodies, followed by specific IRDye fluorescent secondary antibodies, were imaged and quantified on the Odyssey Infrared Imaging System (LI-COR Biosciences)[7].

Immunofluorescence microscopy

HeLa cells were transfected with FLAG-Tspan18, Orai1-Myc or both expression constructs, and stained with CellTracker Deep Red Dye (Invitrogen). Cells were fixed,

washed and blocked as described[2], and stained with rabbit anti-Myc and mouse anti-FLAG tag antibodies, followed by Alexa488-conjugated anti-rabbit and Alexa568-conjugated anti-mouse secondary antibodies. Images were captured on a Zeiss LSM 710 confocal microscope using a 40x objective. The Manders coefficients (M1 and M2) were used as the colocalization measure[8].

For imaging of HUVECs transfected with Tspan18 siRNA and lentivirally-transduced with Myc-tagged Orai1, cells were fixed, washed and blocked as described[2], and stained with rabbit anti-Myc and mouse anti-calnexin antibodies. Secondary antibodies and confocal microscopy was as described above. To quantify Myc-Orai1 subcellular localization, the percentage expression of Orai1 and calnexin across a line spanning the width of each cell was determined using ImageJ. The percentage of Orai1 that localized to a calnexin endoplasmic reticulum mask was then quantified.

For imaging of Weibel-Palade bodies, HUVECs were transfected with Tspan18 siRNA and 48 hours later were stained with CellTracker Deep Red Dye (Invitrogen). Cells were then stimulated with 1 U/ml thrombin for 5 minutes. Cells were fixed, washed, blocked and stained with anti-VWF antibody, followed by Alexa488-conjugated anti-rabbit secondary antibody. Z-stack images (0.4 μm slices) were analyzed using Fiji. Images were de-noised and the background was subtracted to remove the VWF signal in the ER. Weibel-Palade bodies were then counted from 3D reconstructions.

For imaging of mouse ear vasculature, anterior ear tissue was prepared and stained as previously described[9], using biotinylated isolectin GS-IB_4 glycoprotein and Alexa647-conjugated streptavidin (ThermoFisher Scientific). Images of whole mounted ear tissue were taken with a Leica DM IRE2 confocal microscope and a 10x objective.

Immunohistochemistry

Formalin-fixed paraffin-embedded 5 μm tissue sections were stained and analyzed as previously described[10], using 0.5 μg/ml MECA32 rat anti-mouse panendothelial cell antibody or rat IgG2a κ isotype control.

ELISA

For the HUVEC VWF release studies, complete M199 media was replaced with OptiMEM for 16 hours. Half of the OptiMEM was taken for a baseline reading, and the remaining OptiMEM was treated with thrombin (1 U/ml) or histamine (20 μM) for 5 minutes before the supernatant was removed. The media was assayed for VWF release using the human VWF ELISA Kit (RayBio).

Platelet aggregation and adhesion to HUVECs

Mouse washed platelets were isolated from whole blood for lumi-aggregometry[11]. For platelet-HUVEC adhesion assays, human washed platelets were labelled with 5 μg/ml calcein-AM (Cambridge, Bioscience) and assays performed as described[12]; HUVECs were first transfected with Tspan18 siRNA and 48 hours later were stimulated with 1 U/ml thrombin for 5 minutes. Five random fields of the endothelial monolayer were imaged using phase-contrast and fluorescence microscopy, and adherent platelets counted using ImageJ.

Coagulation assays

Mouse plasma samples were used in a prothrombin time test with human placental thromboplastin (Thromborel S, Siemens Healthcare Diagnostics Products) and in an activated partial thromboplastin time test with purified soy phosphatides with ellagic acid (Actin FS, Siemens Healthcare Diagnostics Products). All tests were performed on a Sysmex CS-5100 (Sysmex Corporation).

In vivo assays

Mouse models used litter-matched 8-10 week-old mice. For measurement of VWF release, mice were intra-peritoneally injected with 200 µl 1 mM histamine per 15 g body weight. Blood samples were taken 2 days prior and 30 minutes after injection. Plasma was assayed using the mouse VWF ELISA Kit (Generon). Arterial thrombosis models (mechanical-induced injury of the abdominal aorta and $FeCl_3$-induced injury of mesenteric arterioles)[13] and the inferior vena cava deep vein thrombosis model[14] were completed as described. For the tail bleeding hemostasis assay, 3 mm of the tail tip was removed and blood loss monitored for 20 minutes, or until a maximum blood loss of 15% as estimated by weight (assuming a total blood volume of 70 ml/kg)[11]. Myocardial ischemia-reperfusion injury was induced in the left ventricle of the beating heart using a well-established model that involved occluding the left anterior descending artery for 45 minutes using a silk suture[15]. Reperfusion was instigated for 2 hours by removal of the ligature, after which the organ was harvested. Isolated hearts were covered in optimum cutting temperature compound (Tissue-Tek), before being snap frozen in liquid nitrogen. Frozen sections of 10 µm were cut and fixed in acetone for 10 minutes. For immunofluorescence staining, sections were rehydrated in phosphate-buffered saline for 10 minutes, followed by incubation with 10 µg/ml anti-CD16/32 antibody to block Fc receptors. Sections were incubated with 10 µg/ml allophycocyanin-conjugated anti-CD41 antibody to stain platelets, and viewed using an EVOS microscope (ThermoFisher Scientific). Five fields of view were taken for each heart section, and three sections were examined for each mouse, and the images were analyzed using ImageJ. Each field of view was changed to 8-bit and a triangle threshold was added before quantitation. The integrated density value was calculated as a representation of the total number of platelets, and the average aggregate size was also calculated.

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