The Ca^{2+} Sensor Stromal Interaction Molecule 1 (STIM1) Is Necessary and Sufficient for the Store-Operated Ca^{2+} Entry Function of Transient Receptor Potential Canonical (TRPC) 1 and 4 Channels in Endothelial Cells[§]

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ABSTRACT

We addressed the requirement for stromal interaction molecule 1 (STIM1), the endoplasmic reticulum (ER) Ca^{2+} -sensor, and Orai1, a Ca^{2+} selective channel, in regulating Ca^{2+} entry through the store-operated channels mouse transient receptor potential canonical (TRPC) 4 or human TRPC1. Studies were made using murine and human lung endothelial cells (ECs) challenged with thrombin known to induce Ca^{2+} entry via TRPC1/4. Deletion or knockdown of TRPC4 abolished Ca^{2+} entry secondary to depletion of ER Ca^{2+} stores, preventing the disruption of the endothelial barrier. Knockdown of STIM1 (but not of Orai1 or Orai3) or expression of the dominant-negative STIM1^{K684E-K685E} mutant in ECs also suppressed Ca^{2+} entry secondary to store depletion. Ectopic expression of WT-STIM1 or WT-Orai1 in *TRPC4*(–/–)-ECs failed to rescue Ca^{2+} entry; however, WT-TRPC4 expression in *TRPC4*(–/–)-ECs restored Ca^{2+} entry indicating the requirement for TRPC4 in mediating

store-operated Ca^{2+} entry. Moreover, expression of the dominant-negative Orai1^{R91W} mutant or Orai3^{E81W} mutant in WT-ECs failed to prevent thrombin-induced Ca^{2+} entry. In contrast, expression of the dominant-negative TRPC4^{EE647-648KK} mutant in WT-ECs markedly reduced thrombin-induced Ca^{2+} entry. In ECs expressing YFP-STIM1, ER-store Ca^{2+} depletion induced formation of fluorescent membrane puncta in WT but not in *TRPC4*(–/–) cells, indicating that mobilization of STIM1 and engagement of its Ca^{2+} sensing function required TRPC4 expression. Coimmunoprecipitation studies showed coupling of TRPC1 and TRPC4 with STIM1 on depletion of ER Ca^{2+} stores. Thus, TRPC1 and TRPC4 can interact with STIM1 to form functional store-operated Ca^{2+} -entry channels, which are essential for mediating Ca^{2+} entry-dependent disruption of the endothelial barrier.

Introduction

Ca^{2+} entry through store-operated channels (SOCs) in endothelial cells (ECs) mediates increased vascular permeabil-

ity (Tiruppathi et al., 2006) and increases inflammatory gene expression (Bair et al., 2009). The transient receptor potential canonical (TRPC) family of channels are components of SOCs (Nilius and Droogmans 2001; Birnbaumer et al., 1996). We showed that human vascular ECs predominantly express isoform TRPC1 (Tiruppathi et al., 2006; Thippogowda et al., 2010). In addition, we showed that inflammatory mediators can induce the expression of TRPC1 in human vascular ECs via the nuclear factor- κB pathway (Paria et al., 2003, 2004, 2006). Increased TRPC1 expression was associated with augmented thrombin-induced Ca^{2+} entry and permeability in-

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ABBREVIATIONS: SOC, store-operated channel; EC, endothelial cell; TRPC, transient receptor potential canonical; STIM1, stromal interaction molecule-1; CRAC, Ca^{2+} release-activated Ca^{2+} ; siRNA, small interfering RNA; sc-siRNA, scrambled siRNA; HUVEC, human umbilical vein endothelial cell; EGM, endothelial growth medium; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; PAR-1, protease-activated receptor-1; *m*, mouse; mAb, monoclonal antibody; pAb, polyclonal antibody; YFP, yellow fluorescent protein; *h*, human; HLMVEC, human lung microvascular endothelial cell; RT, reverse transcription; PCR, polymerase chain reaction; AM, acetoxymethyl ester; TER, transendothelial electrical resistance; HEK, human embryonic kidney; CFP, cyan fluorescent protein; GFP, green fluorescent protein; SOCE, store-operated Ca^{2+} entry; WT, wild type; Tg, thapsigargin.

crease in human ECs (Paria et al., 2004). Deletion of TRPC4 in mice inhibited SOC activity in aortic (Freichel et al., 2001) and lung vascular ECs (Tiruppathi et al., 2002). Thrombin-induced increase in lung microvascular permeability was impaired in TRPC4 knockout [*TRPC4*(-/-)] mice (Tiruppathi et al., 2002). These findings show that TRPC1 and TRPC4 mediate SOC activity in ECs; however, the regulation of SOC by ER-store Ca^{2+} release remains poorly understood. An understanding of their regulation is required for developing drugs targeting the Ca^{2+} entry pathways mediating inflammatory vascular diseases.

A number of studies have shown that Ca^{2+} entry through SOC is mediated by interaction between TRPCs and stromal interaction molecule-1 (STIM1) (Huang et al., 2006; Worley et al., 2007; Zeng et al., 2008; Lee et al., 2010), an ER-resident Ca^{2+} sensing protein (Roos et al., 2005; Zhang et al., 2005). Most TRPCs interact with the C terminus of STIM1 (Yuan et al., 2007; Liao et al., 2009). TRPC1-STIM1 interaction in particular has been studied to address the underlying basis of SOC activity (Huang et al., 2006; Ong et al., 2007; Worley et al., 2007; Yuan et al., 2007). Zeng et al. (2008) showed the electrostatic interaction between TRPC1 acidic residues (Asp639, Asp640) and STIM1 basic residues (Lys684, Lys685) regulating TRPC1 gating. ER store Ca^{2+} release mediated assembly of STIM1 into puncta at ER/plasma membrane interface (Huang et al., 2006; Worley et al., 2007; Zeng et al., 2008; Lee et al., 2010) and in turn STIM1 puncta interacted with TRPCs to activate SOC (Worley et al., 2007; Zeng et al., 2008).

Orai gene family members encode for channels mediating the Ca^{2+} release-activated Ca^{2+} (CRAC) current I_{CRAC} (Prakriya et al., 2006). *Orai* family members *Orai1*, *Orai2*, and *Orai3* are tetraspanning membrane proteins (Feske et al., 2006). In human T cells, a point mutation in *Orai1* caused severe combined immunodeficiency in patients with low I_{CRAC} activity (Feske et al., 2006). Expression of wild-type human *Orai1* in patients with severe combined immunodeficiency in patients' T cells rescued store-operated Ca^{2+} entry and I_{CRAC} activity (Feske et al., 2006). Ong et al. (2007) showed that in the human salivary gland cell line, HSG, knockdown of *Orai1* suppressed SOC-mediated Ca^{2+} entry by >90%, whereas knockdown of TRPC1 reduced Ca^{2+} entry by 50%. In human embryonic kidney (HEK) 293 cells, knockdown of either *Orai1* or TRPC1 reduced SOC-mediated Ca^{2+} entry (Kim et al., 2009). Furthermore, coexpression of TRPC1, *Orai1*, and STIM1 in HEK293 cells showed greater SOC-mediated Ca^{2+} entry compared with coexpression of STIM1 with TRPC1 or *Orai1* channels (Kim et al., 2009). In contrast, in Jurkat cells, the SOC-mediated Ca^{2+} entry and CRAC current were inhibited by knockdown of *Orai1* but not altered by knockdown of TRPC1 or TRPC3 (Kim et al., 2009). These results suggest that molecular makeup of the SOC varies depending on the cell type. In the present study, we focused on the question whether STIM1 and *Orai1* proteins are both required and sufficient for the SOC function in ECs.

We (Freichel et al., 2001; Tiruppathi et al., 2002; Paria et al., 2004; Sundivakkam et al., 2009) have identified the role of TRPC1- and TRPC4-mediated SOC function in ECs; however, regulation of TRPC function in these cells seems to be more complex. A siRNA knockdown study in human umbilical vein endothelial cells (HUVECs) showed that STIM1 or *Orai1* knockdown abolished both SOC and I_{CRAC} currents,

whereas knockdown of TRPC1 or TRPC4 had no effect (Abdullaev et al., 2008). Although the role of *Orai*'s in mediating I_{CRAC} -dependent Ca^{2+} entry has been well described in immune cells (Prakriya et al., 2006), their function in ECs is less clear. Thus, we addressed the role of STIM1 and *Orai1* in activating SOC in ECs. Our results show that TRPC1/4 interaction with STIM1 without involvement of *Orai1* is required and sufficient for functional SOC in ECs.

Materials and Methods

Reagents. Endothelial growth medium (EGM-2) was obtained from Lonza Walkersville, Inc. (Walkersville, MD). Hanks' balanced salt solution (HBSS) and trypsin were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Human α -thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Protease-activated receptor-1 (PAR-1) activating peptide (TFFLRNPNNDK-NH₂) was synthesized as C-terminal amide (Tiruppathi et al., 2002). Fura-2AM was purchased from Invitrogen (Carlsbad, CA). Anti-TRPC1 pAb, anti-TRPC4 pAb, *hOrai1*-siRNA, and mouse (m) specific siRNAs for TRPC1, TRPC4, *Orai1*, *Orai2*, and *Orai3* were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-STIM1 monoclonal antibody (mAb) was from BD Biosciences Transduction Laboratories (Lexington, KY). Control scrambled siRNA (sc-siRNA), *mSTIM1*-siRNA, and *hSTIM1*-siRNA were from Dharmacon (Lafayette, CO). Antibodies specifically react with *Orai1*, *Orai2*, and *Orai3* were from Santa Cruz Biotechnology (Santa Cruz, CA). Yellow fluorescent protein (YFP)-WT-STIM1, YFP-STIM1^{K684E-K685E}

Fig. 1. TRPC, STIM, and *Orai* expression in ECs from WT and *TRPC4*(-/-) mice. **A**, RT-PCR analysis of mRNA expression. Total RNA from ECs of WT and *TRPC4*(-/-) mice was isolated, and RT-PCR was performed to determine the expression of transcripts for TRPCs (TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, TRPC7) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (left). RT-PCR was performed to determine the expression of transcripts for STIMs (STIM1 and STIM2), *Orais* (*Orai1*, -2, -3) and GAPDH using total RNA from WT-ECs (right). Experiment was repeated three times. The results from representative experiments are shown. **B**, Western blot analysis. ECs from WT and *TRPC4*(-/-) mice were lysed and immunoblotted with antibodies specific to TRPC1, TRPC4, STIM1, *Orai1*, or β -actin. Representative blot is shown at left. Immunoreactive protein bands were quantified by densitometry relative to β -actin. Results shown are mean of 3 experiments (right).

mutant, *mCherry-hOrai1* and *mCherry-Orai1^{R91W}* mutant, dominant-negative *TRPC4^{EE647-648KK}* mutant, and *TRPC1^{F562A}* pore mutant constructs were prepared as described previously (Zeng et al., 2008; Kim et al., 2009). WT-*mTRPC4 β* cDNA in pcAGGS-IRS-GFP vector was prepared as described previously (Tang et al., 2000). CFP-Orai3^{E81W} mutant construct was obtained from Dr. Mohammed Trebak's laboratory (Albany Medical College, Albany, NY).

Targeted Disruption of *TRPC4* in Mice. *TRPC4* gene-disrupted mice were generated as described previously (Freichel et al., 2001). Age-matched *TRPC4*(+/+) (wild-type) and *TRPC4*(-/-) littermate mice were used for experiments.

Endothelial Cells. Lung endothelial cells (ECs) from wild-type (WT) and *TRPC4*(-/-) mice were isolated as described by us using antiplatelet EC adhesion molecule-1 mAb (Tiruppathi et al., 2002). After isolation the cells were placed in culture and were immortalized by infection with a retrovirus expressing the polyoma middle T antigen (Primo et al., 2000; Sundivakkam et al., 2009). At 24 h from the time of infection, cells were again purified with antiplatelet EC adhesion molecule-1 mAb (Tiruppathi et al., 2002). The affinity purified cells were grown in MCDB131 medium (Sigma-Aldrich) supplemented with 10% FBS, 10 ng/ml human epidermal growth factor, and 1 μ g/ml hydrocortisone. ECs were characterized by their cobble-

stone morphology, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein uptake, and vascular endothelial cadherin expression at the junctions (Tiruppathi et al., 2002; Sundivakkam et al., 2009). ECs passaged between 4 and 8 were used for experiments. Human lung microvascular ECs (HLMVECs) and HUVECs were obtained from Lonza Walkersville Inc. HLMVECs were grown in EGM-2MV supplemented with 15% FBS, and HUVECs were grown in EGM-2 supplemented with 10% FBS (growth media from Lonza Walkersville Inc.). Both cell types were used between passages 4 and 6.

Reverse Transcription-PCR. Total RNA from *m/hECs* was isolated using TRIzol reagent. RT was performed using oligo (dT) primers and superscript RT (Invitrogen) following the manufacturer's instructions. Species specific primers were used to amplify *TRPCs*, *STIMs* and *Orais* (see details in *Supplemental Methods*).

Cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) Measurement. The cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in ECs was measured using the Ca^{2+} -sensitive fluorescent dye Fura-2/AM (Tiruppathi et al., 2002; Sundivakkam et al., 2009). Cells were grown to confluence on gelatin-coated glass coverslips and then washed two times with serum-free medium and incubated for 2 h at 37°C in culture medium containing 1% FBS. Cells were washed once and loaded with 3 μ M Fura-2/AM

Fig. 2. Impaired store-operated Ca^{2+} entry (SOCE) in *TRPC4*(-/-) ECs. A, left, basal Fura-2 340/380 ratio was measured in the presence of nominal extracellular Ca^{2+} (1.5 mM) in WT and *TRPC4*(-/-) mice ECs. Results shown are mean $\pm$ S.E. of five experiments. A, right, Fura-2 loaded ECs from WT and *TRPC4*(-/-) mice were used to measure thrombin (50 nM)-induced increase in $[\text{Ca}^{2+}]_i$ in presence of extracellular Ca^{2+} . NS, not significant. B, left, basal Fura-2 340/380 ratio was measured in the absence of extracellular Ca^{2+} in WT and *TRPC4*(-/-) mice ECs. Results are mean $\pm$ S.E. of five experiments. B, right, Fura-2 loaded cells placed in Ca^{2+} - and Mg^{2+} -free HBSS were stimulated with thrombin (50 nM). After return of $[\text{Ca}^{2+}]_i$ to baseline levels, CaCl_2 (1.5 mM) was added to extracellular medium to induce Ca^{2+} entry. Arrow indicates time at which cells were stimulated with thrombin (Thr). Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated. Results shown are mean $\pm$ S.E. of four experiments carried out in duplicate (bottom). *, significant difference in Ca^{2+} entry in *TRPC4*(-/-)-ECs compared with WT-ECs. C, thapsigargin (1 μ M)-induced Ca^{2+} entry was measured as in B. Arrow indicates time at which cells were stimulated with Tg. *, significant difference in Ca^{2+} entry in *TRPC4*(-/-)-ECs compared with WT-ECs.

for 30 min. After loading, cells were washed with HBSS and the coverslips were transferred on a perfusion chamber at 37°C and imaged using a semimotorized microscope (Axio Observer D1; Carl Zeiss GmbH, Jena, Germany) equipped with an AxioCam HSM camera (Carl Zeiss) and a Fluor 40× oil immersion objective. Light was provided by the DG-4 wavelength switcher (Princeton Scientific Instruments, Monmouth Junction, NJ). A dual excitation at 340 and 380 nm was used, and emission was collected at 520 nm. The Axio-Vision physiology software module was used to acquire the images at 1-s intervals, and the data were analyzed off-line. In each experiment, 20 to 30 cells were selected to measure change in $[\text{Ca}^{2+}]_i$.

Transendothelial Electrical Resistance. The real-time change in endothelial monolayer resistance (TER) was measured to assess endothelial barrier function (Tiruppathi et al., 1992). Before the experiment, confluent endothelial monolayer was kept in 1% FBS containing medium for 2 h and then thrombin- or PAR-1 agonist peptide-induced change in TER was measured. Data are presented in resistance normalized to its value at time 0 as in Tiruppathi et al. (1992).

siRNA Transfection. ECs grown to ~70% confluence on gelatin-coated culture dishes were transfected with target siRNAs or sc-siRNA using DharmaFECT transfection reagent as per the manufacturer's instructions. At 72 h after transfection, cells were used for Ca^{2+} measurements or harvested for Western blot analysis.

cDNA Transfection. ECs or HEK293 cells transfected with YFP-WT-STIM1, YFP-STIM1^{K684E-K685E} mutant, mCherry-hOrai1, mCherry-Orai1^{R91W}, CFP-Orai3^{E81W}, WT-mTRPC4 β , dominant-negative TRPC4^{EE647-647KK} mutant, or TRPC1^{F562A} pore mutant expression con-

structs were used to measure change in $[\text{Ca}^{2+}]_i$. ECs or HEK293 cells grown to 80% confluence were transfected with indicated plasmid DNA using Superfect reagent (QIAGEN, Valencia, CA) in serum-free medium as in Sundivakkam et al. (2009). ECs were used for experiments 72 h after transfection, whereas HEK293 cells were used for experiments 48 h after transfection. Cells expressing YFP, mCherry, CFP, or GFP were selected to measure change in $[\text{Ca}^{2+}]_i$ as in Zeng et al. (2008) and Lee et al. (2009).

Immunoprecipitation. ECs grown to confluence were challenged with agonists were washed three times with phosphate-buffered saline at 4°C and lysed in lysis buffer as in (Sundivakkam et al., 2009). Lysate protein (300 μg) was subjected to immunoprecipitation. Insoluble material was removed by centrifugation (13,000g for 15 min) before overnight immunoprecipitation with 1 $\mu\text{g}/\text{ml}$ antibody at 4°C. Protein A/G-agarose beads were added to each sample and incubated for 1 h at 4°C. Immunoprecipitates were gently washed three times with wash buffer (Tris-buffered saline containing 0.05% Triton X-100, 1 mM Na_3VO_4 , 1 mM NaF, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 44 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride). Immunoprecipitated proteins were resolved on SDS-polyacrylamide gel electrophoresis and immunoblotted with appropriate antibodies.

Immunoblotting. EC lysates or immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis on a 10% separating gel under reducing conditions and transferred to Duralose membrane. Membranes were blocked with (5% dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 1 h. Membranes were incubated with indicated primary antibody (diluted in blocking buffer) overnight. After three washes, membranes

Fig. 3. TRPC4 expression is required for thrombin- or Tg-induced Ca^{2+} entry in mECs. **A**, WT-ECs transfected with control siRNA (sc-siRNA) or mouse (m) TRPC4-siRNA (see details under *Materials and Methods*) were lysed and immunoblotted with anti-TRPC4 pAb to determine TRPC4 expression (top). The membrane was stripped and probed with anti-β actin mAb as loading control (bottom). **B** and **C**, WT-ECs transfected with sc-siRNA or mTRPC4-siRNA were used to measure thrombin (Thr)- or Tg-induced Ca^{2+} store release-mediated Ca^{2+} entry as described in Fig. 2B. Arrows indicate time of addition of thrombin or Tg. A representative profile is shown in left. Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated. Results shown are mean $\pm$ S.E. of four experiments carried out in duplicate. NS, not significant; *, significant difference in the Ca^{2+} entry in WT-ECs transfected with sc-siRNA compared with TRPC4-siRNA.

Fig. 3. Continued. D, *mTRPC4* cDNA in pcAGGS-IRES-GFP vector was transfected into *TRPC4*(-/-)-ECs (see details under *Materials and Methods*). After transfection, total cell lysates were used to determine TRPC4 expression by Western blot, or GFP-positive cells were used to determine thrombin-induced Ca²⁺ entry. *, significant difference in the Ca²⁺ entry compared with control cells. *n* = 15 in each transfection conditions. E, HA-*hTRPC4*^{EE647-648KK} mutant (2 µg) was transfected in WT-mECs. Transfection efficiency was ~40% assessed by GFP expression plasmid transfection. After transfection, cells were used to determine TRPC4 expression by Western blot (top) or used to measure thrombin-induced Ca²⁺ entry (middle). Change in peak fluorescence ratio (340/380) for Ca²⁺ entry over basal was calculated. Results shown are mean ± S.E. of four experiments carried out in duplicate. *, significant difference in the Ca²⁺ entry in HA-*hTRPC4*^{EE647-648KK} mutant expressing cells compared with control cells.

were incubated with horseradish peroxidase-conjugated secondary antibody. Protein bands were detected by enhanced chemiluminescence method.

Confocal Imaging. ECs were transfected with YFP-WT-STIM1 (2 µg/ml) construct as described above. At 48 h after transfection, cells washed, placed in HBSS, and then confocal live cell images of the YFP-tagged fluorescent protein were acquired near the surface of the cell using 514 nm laser excitation/530 nm LP emission filter with the pinhole set to achieve 1 Airy unit (~0.5-µm optical sections). Image configurations acquired before and after thrombin stimulation were not changed, and the cells were maintained at 37°C.

Statistical Analysis. Comparisons were made with a two-tailed Student's *t* test. Experimental values were reported as mean ± S.E. Differences in mean values between two or more groups were determined by one-way analysis of variance. A *p* value <0.05 was considered statistically significant.

Results

Impaired Store-Operated Ca²⁺ Entry in TRPC4 Knockout Mouse Endothelial Cells. We first determined the expression of TRPCs, STIMs, and Orais in ECs from wild-type (WT) and *TRPC4*(-/-) mice by RT-PCR (Fig. 1A) and Western blot (Fig. 1B). We observed expression of transcripts for TRPC1, TRPC4, and TRPC6 in WT-ECs (Fig. 1A, left); TRPC1 and TRPC6 expression occurred at a low level compared with the TRPC4 transcript. In *TRPC4*(-/-)-ECs, the TRPC4 transcript was not detectable, whereas TRPC1 and TRPC6 expression was similar to WT-ECs (Fig. 1A, left), in agreement with our previous results (Tiruppathi et al., 2002). We also observed the expression of STIM1, STIM2, and Orai1 mRNA in WT-ECs (Fig. 1A, right). In *TRPC4*(-/-)-

ECs, the expression of STIM1, STIM2, and Orai1 mRNA was similar to WT-ECs (data not shown). We also performed Western blot analysis to determine the expression levels of TRPCs, STIM1, and Orai1 proteins in ECs from WT and *TRPC4*($-/-$) mice. TRPC4 protein expression was predominant in WT-ECs, whereas TRPC4 protein expression was not observed in *TRPC4*($-/-$)-ECs (Fig. 1B). In WT and *TRPC4*($-/-$) ECs, we observed expression of TRPC1, STIM1, and Orai1 proteins at similar levels (Fig. 1B).

To determine whether loss of TRPC4 in ECs influences basal intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), we measured fura-2 340/380 ratio in the presence and absence of extracellular Ca^{2+} in ECs from WT and *TRPC4*($-/-$) mice. We observed no significant differences in basal $[\text{Ca}^{2+}]_i$ between WT and *TRPC4*($-/-$) ECs (Fig. 2, A and B, left). Next, we measured thrombin-induced rise in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) using ECs from WT and *TRPC4*($-/-$) mice. Thrombin-induced increase in $[\text{Ca}^{2+}]_i$ was reduced by 80% in *TRPC4*($-/-$)-ECs compared with WT-ECs (Fig. 2A, right). We also measured thrombin-induced Ca^{2+} entry in ECs from WT and *TRPC4*($-/-$) mice. In these experiments, we first depleted the ER Ca^{2+} stores with thrombin in the absence of extracellular Ca^{2+} to open channels and then reapplied Ca^{2+} to the extracellular medium to assess Ca^{2+} entry (i.e., SOCE) through the open channels. This protocol allowed separation

of Ca^{2+} release from Ca^{2+} entry. Thrombin produced a similar initial peak increase in $[\text{Ca}^{2+}]_i$ in WT and *TRPC4*($-/-$) ECs (Fig. 2B, right). In WT-ECs, restoring Ca^{2+} in the extracellular medium after thrombin-induced store-depletion caused an increase in Ca^{2+} entry (Fig. 2B, right). In contrast to WT-ECs, re-addition of Ca^{2+} to the extracellular medium after store depletion caused no significant Ca^{2+} entry in *TRPC4*($-/-$)-ECs (Fig. 2B, right and bottom). We also observed that thapsigargin (Tg)-induced Ca^{2+} entry was blocked in *TRPC4*($-/-$)-ECs (Fig. 2C).

Because thrombin activates the dominant PAR-1 in ECs, we measured PAR-1 agonist peptide (TFLLRNPNKD)-induced Ca^{2+} entry in WT and *TRPC4*($-/-$) mice ECs. We observed the peptide-induced Ca^{2+} entry in WT-ECs was similar to thrombin (Supplemental Fig. 1, A and B), whereas the response was reduced by 80% in *TRPC4*($-/-$)-ECs (Supplemental Fig. 1, A and B). We also investigated the effects of gadolinium (Gd^{3+}) and 2-aminoethoxydiphenyl borate on thrombin-induced Ca^{2+} entry in WT-ECs. Gd^{3+} (10 μM) abolished thrombin-induced Ca^{2+} entry in WT-ECs (Supplemental Fig. 2A). In addition, 2-aminoethoxydiphenyl borate (30 μM) markedly reduced thrombin-induced Ca^{2+} entry in WT-ECs (Supplemental Fig. 2B). These findings demonstrate the importance of TRPC4 expression in SOCE in *mECs*.

To address the functional relevance of the TRPC4 in *mECs*,

Fig. 4. TRPC1 knockdown or TRPC1^{F562A} pore mutant expression failed to inhibit SOCE in *mECs*. A, WT-*mECs* transfected with sc-siRNA or *mTRPC1*-siRNA were lysed and immunoblotted with anti-TRPC1 pAb to determine TRPC1 expression (top). The membrane was stripped and probed with anti-β actin mAb as loading control (bottom). B and C, WT-*mECs* transfected with sc-siRNA or *mTRPC1*-siRNA were used to measure thrombin (Thr)- or Tg-induced Ca^{2+} store release-mediated Ca^{2+} entry as described above. Arrows indicate time of addition of thrombin or Tg. A representative profile is shown at left. Change in peak ratio (340/380) for Ca^{2+} entry over basal was calculated (right). Results are given the mean $\pm$ S.E. of three experiments carried out in duplicate. NS, not significant.

Fig. 4. Continued. D, HA-tagged TRPC1^{F562A} pore mutant was transfected into WT-mECs. After transfection, cells were used to measure TRPC1 expression by Western blot (top), thrombin-induced Ca^{2+} entry (middle), or Tg-induced Ca^{2+} entry (bottom). Results are given the mean $\pm$ S.E. of three experiments carried out in duplicate. NS, not significant.

we measured PAR-1 activation-mediated changes in TER to assess EC retraction (i.e., cell shape change), a prerequisite for the increase in endothelial permeability (Wysolmerski and Lagunoff, 1990; Tiruppathi et al., 1992). Exposure of WT-ECs with either thrombin or PAR-1 agonist peptide produced a $\sim 40\%$ maximum decrease in TER whereas this effect was significantly reduced in *TRPC4*($-/-$)-ECs (Supplemental Fig. 3) indicating that TRPC4 expression in ECs contributes significantly to the thrombin-induced increase in endothelial permeability.

TRPC4 Knockdown Suppresses SOCE in mECs. We addressed whether TRPC4 gene knockdown suppresses SOCE in mECs. In these experiments, we transfected control siRNA (sc-siRNA) or *mTRPC4*-specific siRNA in WT-ECs and determined TRPC4 protein expression in these cells. In TRPC4-siRNA transfected cells, TRPC4 expression was markedly suppressed compared with control or sc-siRNA transfected cells (Fig. 3A). We determined Ca^{2+} entry after thrombin-induced ER store Ca^{2+} depletion in control, sc-siRNA and TRPC4-siRNA transfected cells. We observed thrombin-induced Ca^{2+} entry in control and sc-siRNA transfected cells (Fig. 3B) whereas in TRPC4-siRNA transfected cells, thrombin-induced Ca^{2+} entry was abolished (Fig. 3B). We also observed that Tg-induced Ca^{2+} entry was blocked $>80\%$ in TRPC4-siRNA transfected WT-ECs (Fig. 3C). Next we transiently expressed the WT-*mTRPC4* β isoform in *TRPC4*($-/-$)-ECs. TRPC4 re-expression rescued the throm-

bin-induced Ca^{2+} entry in *TRPC4*($-/-$)-ECs (Fig. 3D). We also expressed the dominant-negative TRPC4^{EE6470648KK} mutant in WT-ECs and observed that thrombin-induced Ca^{2+} entry was markedly reduced in TRPC4^{EE6470648KK} mutant expressing cells compared with control cells (Fig. 3E). These results collectively support the concept that TRPC4 expression contributes to SOCE in mECs.

Because TRPC1 is expressed in mECs and hetero-oligomerizes with TRPC4 to form a functional SOCE complex (Hofmann et al., 2002), we knocked down TRPC1 by transfecting *mTRPC1*-specific siRNA in WT-ECs and determined Ca^{2+} entry after ER store Ca^{2+} depletion with either thrombin or Tg. In TRPC1-siRNA transfected cells, TRPC1 protein expression was inhibited by 80% compared with control (Fig. 4A), but TRPC1 knockdown resulted in $\sim 15\%$ inhibition of Ca^{2+} entry induced by either thrombin or Tg (Fig. 4, B and C). To further address the role of TRPC1, we transfected TRPC1^{F562A}, a pore-mutant expression construct, in WT-ECs and measured thrombin- or Tg-induced Ca^{2+} entry. Expression of the mutant construct in WT-ECs had no significant effect on SOCE (Fig. 4D). Next we determined the effect of stored Ca^{2+} release on the interaction between TRPC1 and TRPC4. We observed that thrombin- or Tg-induced store depletion increased the association between TRPC1 and TRPC4 (Fig. 4, E–G). These results suggest that TRPC1 is not essential for pore forming subunit channel complex but may provide scaffold for TRPC4 in mECs.

Fig. 4. Continued. E, WT-*mECs* were challenged with thrombin for different time intervals. After thrombin stimulation and cells were lysed. Top, total cell lysates were immunoprecipitated with goat anti-TRPC4 pAb and immunoblotted with rabbit anti-TRPC1 pAb. Middle, total cell lysates were immunoprecipitated with rabbit anti-TRPC1 pAb and immunoblotted with goat anti-TRPC4 pAb. Bottom, total cell lysates were immunoblotted with anti- β actin mAb. A representative blot is shown from four different experiments. F, immunoprecipitated protein bands were quantified by densitometry and are expressed in arbitrary units. *, significant difference compared with control cells (not stimulated with thrombin). G, WT-*mECs* were challenged with Tg (1 μ M) for 2.5 min and then used for immunoprecipitation as described in E. Immunoprecipitated protein bands were quantified by densitometry and are expressed in arbitrary units (right). *, significant difference compared with control cells (not stimulated with Tg).

STIM1 Signaling Is Required for SOCE in *mECs*. To address the role of STIM1 in the mechanism of SOCE in *mECs*, we transfected *mSTIM1*-specific siRNA into WT-*ECs* and determined the expression of STIM1. In STIM1-siRNA-transfected cells, STIM1 protein expression was reduced >90% (Fig. 5A) whereas in sc-siRNA-transfected cells, STIM1 expression was not altered (Fig. 5A). To determine whether STIM1 knockdown influences basal intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), we measured Fura-2 340/380 ratio in the presence and absence of extracellular Ca^{2+} in control, sc-siRNA, and STIM1-siRNA transfected *ECs*. We did not observe significant differences in basal $[\text{Ca}^{2+}]_i$ in STIM1-siRNA transfected *ECs* compared with control or sc-siRNA transfected cells (Fig. 5B). Next we measured Ca^{2+} entry in control, sc-siRNA-, or STIM1-siRNA-transfected cells after depleting ER-store Ca^{2+} with thrombin. Thrombin-induced Ca^{2+} entry was markedly reduced in STIM1-siRNA-transfected WT-*ECs* (Fig. 5C) whereas in sc-siRNA-transfected WT-*ECs*, the response was similar to control WT-*ECs*. Tg-induced Ca^{2+} entry was also inhibited in STIM1-siRNA-transfected cells but not in sc-siRNA-transfected or in control WT-*ECs* (Fig. 5D). These findings show that STIM1 expression is required for SOCE in *mECs*.

Next we ectopically expressed YFP-WT-STIM1 or YFP-mutant-STIM1^{K684E-K685E} (dominant negative mutant) in *ECs* from WT and *TRPC4*(-/-) mice. Previous studies have shown that YFP-tagged STIM1 interacts with and gates

TRPC channels (Zeng et al., 2008; Yuan et al., 2009). YFP fluorescence allowed identification of transfected cells for Ca^{2+} imaging. In WT-*ECs* expressing YFP-WT-STIM1, thrombin-induced Ca^{2+} entry was enhanced compared with control cells (Fig. 5E). By contrast, in WT-*ECs* expressing YFP-STIM1^{K684E-K685E}, thrombin-induced Ca^{2+} entry did not occur (Fig. 5E). We also expressed YFP-WT-STIM1 or YFP-mutant-STIM1^{K684E-K685E} in *TRPC4*(-/-)-*ECs*. Expression of either YFP-WT-STIM1 or YFP-STIM1^{K684E-K685E} failed to rescue thrombin-induced Ca^{2+} entry in *TRPC4*(-/-)-*ECs* (Fig. 5F). Thus, STIM1-TRPC4 cosignaling is required for SOCE in *mECs*.

To further delineate the role of TRPC4 in SOCE, we expressed YFP-WT-STIM1 in *ECs* of WT and *TRPC4*(-/-) mice and used confocal imaging to observe STIM1 mobilization as the result of ER-store Ca^{2+} release. In WT-*ECs* expressing YFP-WT-STIM1, we noted that thrombin or thapsigargin stimulation caused formation of STIM1 puncta in a time-dependent manner (Fig. 6, A-F). STIM1 puncta appeared within 10 s of thrombin or Tg addition to cells and persisted over a period of 350 s. In *TRPC4*(-/-)-*ECs*, thrombin- or Tg-induced formation of STIM1 puncta was markedly reduced compared with WT-*ECs* (Fig. 6, A-F). We also coexpressed YFP-STIM1 and TRPC4 in HEK293 cells and measured STIM1 puncta in response to Tg. In this experiment, we observed that TRPC4 and STIM1 coexpression enhanced Tg-induced STIM1 puncta and Ca^{2+} entry (Supplemental

Fig. 5. STIM1 is essential for SOCE in *mECs*. A, suppression of STIM1 by silencing RNA. WT-*mECs* transfected with indicated concentrations of sc-siRNA or *mSTIM1*-siRNA were lysed and immunoblotted to determine STIM1 expression. B, basal Fura-2 340/380 ratio was measured in the presence and absence of nominal extracellular Ca²⁺ (1.5 mM) in control, sc-siRNA-, or STIM1-siRNA-transfected WT-*mECs*. Results shown are mean $\pm$ S.E. of five experiments. C and D, left, suppression of Ca²⁺ entry with RNA silencing of STIM1 expression. WT-*mECs* were transfected with indicated concentrations of sc-siRNA or *mSTIM1*-siRNA were used to measure thrombin- or Tg-induced Ca²⁺ entry. Arrows indicate time of addition of thrombin or Tg. The results from representative experiments are shown. Change in peak fluorescence ratio (340/380), after Ca²⁺ restoration represents Ca²⁺ entry. Results are given as mean $\pm$ S.E. of four experiments carried out in duplicate (right). NS, not significant; *, significant difference in the Ca²⁺ entry between sc-siRNA-transfected cells and STIM1-siRNA-transfected cells.

Fig. 4, A and B). These results indicate that TRPC4 expression is essential for the STIM1 mobilization, an obligatory step in the activation of SOCE in *mECs*.

Orai Family Channels Are Not Required for SOCE in *mECs*. We determined the expression of Orai family channels in *mECs*. We observed that Orai1 was dominantly expressed in *mECs* (Fig. 1, A and B; Fig. 7A). Orai2 expression was not detectable, whereas Orai3 was expressed at low levels compared with Orai1 in *mECs* (Fig. 7A). Because Orai1 is predominantly expressed in *mECs*, we suppressed Orai1 expression by transfecting WT-*ECs* with *mOrai1*-specific siRNA. In Orai1-siRNA-transfected cells, Orai1 protein expression was reduced by more than 80% compared with control or sc-siRNA-transfected WT-*ECs* (Fig. 7A). We did not observe any significant change in thrombin- or Tg-induced Ca²⁺ entry in Orai1 depleted WT-*ECs* compared with

control WT-*ECs* (Fig. 7, B and C). Furthermore, we observed that Gd³⁺ blocked thrombin-induced Ca²⁺ entry in Orai1-depleted WT-*ECs* (Supplemental Fig. 5). Although, Orai2 was not detectable in *mECs*, we transfected *mOrai2*-siRNA and measured thrombin-induced Ca²⁺ entry in WT-*ECs*. Thrombin-induced Ca²⁺ entry was not significantly altered in Orai2-siRNA transfected cells compared with sc-siRNA or control cells (Fig. 7D). Next, we suppressed Orai3 in WT-*ECs* with *mOrai3*-siRNA and used these cells to measure thrombin-induced Ca²⁺ entry. Orai3 suppression also had no significant effect on thrombin-induced Ca²⁺ entry (Fig. 7E).

To further test the role of Orai proteins in *mECs*, we ectopically expressed *mCherry*-tagged Orai1 in *ECs* from WT and *TRPC4*(-/-) mice and measured thrombin-induced Ca²⁺ entry. In this experiment, we selected only *mCherry*-expressing cells for Fura-2 imaging to assess Ca²⁺ entry. We

Fig. 5. Continued. E, WT-mECs were transfected with YFP-WT-STIM1 (2 μg) or YFP-STIM1^{K684E-K685E} mutant (2 μg) construct. YFP-expressing cells (10–15 cells/experiment) were imaged for thrombin-induced Ca^{2+} entry as described above. The results from representative experiments are shown (left). Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated. E, right, results shown are mean $\pm$ S.E. of four experiments. *, difference in the Ca^{2+} entry between control and YFP-WT-STIM1 transfected cells or YFP-WT-STIM1 transfected cells and YFP-STIM1^{K684E-K685E} mutant transfected cells as indicated. F, TRPC4^{-/-}-ECs were transfected with YFP-WT-STIM1 (2 μg) or YFP-STIM1^{K684E-K685E} mutant (2 μg) construct. YFP-expressing cells were imaged for thrombin-induced Ca^{2+} entry as described above. Experiments were repeated three times. The results from representative experiments are shown.

observed no significant effect on thrombin-induced Ca^{2+} entry in WT-ECs expressing *mCherry*-tagged Orai1 (Fig. 7F). In addition, we observed that the expression of *mCherry*-tagged Orai1 in TRPC4^{-/-}-ECs failed to restore thrombin-induced Ca^{2+} entry in these cells (Fig. 7F). We used HEK293 cells as positive control cells to test Orai1 function. HEK293 cells expressed STIM1 and Orai1 proteins, and silencing of Orai1 markedly reduced Tg-induced Ca^{2+} entry (Supplemental Fig. 6). Tg-induced Ca^{2+} entry was significantly increased in *mCherry*-tagged Orai1 expressing HEK293 cells compared with control cells (Supplemental Fig. 6) and coexpression of *mCherry*-tagged Orai1 with YFP-STIM1 in HEK293 cells further increased Tg-induced Ca^{2+} entry compared with the cells expressing only *mCherry*-tagged Orai1 (Supplemental Fig. 6). Next, we expressed dominant-negative Orai1^{R91W} and Orai3^{E81W} mutants in WT-mECs. Expression of either mutant Orai1^{R91W} or Orai3^{E81W} in WT-mECs failed to inhibit SOCE (Fig. 7G). These results collectively support the conclusion that Orai family proteins are not involved in the mechanism of SOCE in mECs.

STIM1 Knockdown Prevents SOCE in Human ECs. Because the above studies were in mECs and hECs predominantly express TRPC1 (Tiruppathi et al., 2006), we tested whether Orai1 regulates this channel hECs, and thereby SOCE. Using HLMVECs, we observed expression of the SOC constituents TRPC1, TRPC4, STIM1, Orai1, and Orai3 (Fig. 8A; Supplemental Fig. 7A). We suppressed individually

endogenous expression of TRPC1, TRPC4, STIM1, or Orai1 by transfecting target-specific siRNA in HLMVECs. TRPC1 knockdown in HLMVECs inhibited >80% of thrombin-induced Ca^{2+} entry (Supplemental Fig. 7B), whereas TRPC4 knockdown suppressed ~50% thrombin-induced Ca^{2+} entry (Supplemental Fig. 7B). We also expressed the TRPC1^{F562A} pore mutant and dominant-negative TRPC4^{EE647-648KK} mutant in HLMVECs. TRPC1^{F562A} pore-mutant expression blocked ~70% of the thrombin-induced Ca^{2+} entry (Supplemental Fig. 7C) whereas expression of the TRPC4^{EE647-648KK} mutant inhibited ~25% of the thrombin-induced Ca^{2+} entry (Supplemental Fig. 7C). These results indicate that TRPC1 is the dominant channel in HLMVECs SOCs. Similar to results in mECs above, STIM1 knockdown markedly reduced thrombin-induced Ca^{2+} entry in HLMVECs (Fig. 8, B and C). Tg-induced Ca^{2+} entry was also suppressed in STIM1 knockdown HLMVECs (data not shown). However, Orai1 knockdown had no significant effect on thrombin-induced Ca^{2+} entry (Fig. 8, D and E) in HLMVECs. These results demonstrate that TRPC1-STIM1 cosignaling is essential for SOCE in HLMVECs.

This observation is different from the recent report indicating that knockdown of Orai1 but not TRPC1 or TRPC4 suppressed thrombin-induced Ca^{2+} entry in HUVECs (Abdullaev et al., 2008). To reconcile these differences, we performed knockdown experiments using HUVECs. Knockdown of TRPC1, Orai1, or STIM1 markedly reduced thrombin-

Fig. 6. TRPC4 expression is essential for ER-store Ca^{2+} release-induced STIM1 puncta formation in *mECs*. A and B, *mECs* from WT and *TRPC4*^{-/-} mice were grown to ~70% confluence on glass-bottomed 35-mm dishes and were transfected with YFP-WT-STIM1 expression construct (2 $\mu\text{g}/\text{dish}$). After transfection, cells were washed and placed in HBSS, and then confocal live cell images of the YFP-tagged fluorescent protein were acquired (see details under *Materials and Methods*). Times at which thrombin (50 nM) was added are indicated. Insets show the magnified areas of interest. Note that within seconds of thrombin addition, STIM1 puncta are seen in WT but not in *TRPC4*^{-/-}-*mECs*. The images acquired from representative experiments are shown in A and B. Scale bar, 10 μm . C, STIM1 puncta formed after thrombin addition at 150 s was quantified and results shown are mean $\pm$ S.E. $n = 6$ from each group; *, significantly different from WT-*mECs*.

induced Ca^{2+} entry in HUVECs (Supplemental Fig. 8). These results show in HUVECs (as distinct from *hEC* and *mEC* results) that Orai1 (Abdullaev et al., 2008) and TRPC1 (Thippegowda et al., 2010) are both essential for SOCE.

ER-Stored Ca^{2+} Depletion Induces STIM1 Binding to TRPCs. To address whether ER store Ca^{2+} release mediates the interaction between STIM1 and TRPC4 and can thereby activate Ca^{2+} entry in ECs, we challenged WT-ECs with Tg or thrombin and used the cells for immunoprecipitation analysis. Depletion of ER-stored Ca^{2+} with either Tg or thrombin induced interaction between STIM1 and TRPC4 in a time-dependent manner (Fig. 9, A and B). It is noteworthy that we observed the presence of Orai1 in STIM1-TRPC4 complexes; however, Orai1 association with the STIM1-TRPC4 complex was not altered in response to ER-stored Ca^{2+} release (Fig. 9C). Furthermore, TRPC1 was also shown to bind to STIM1 upon stimulation of HLMVECs with either thrombin or Tg (data not shown). These results show that depletion of stored Ca^{2+} is required for STIM1 binding to TRPC1/4, which in turn activates SOCE in ECs.

Discussion

We investigated the role of the ER-localized Ca^{2+} -sensing protein STIM1 and the CRAC channel protein Orai1 in activating the SOCE function of TRPCs in ECs. We observed expression of STIM1 and Orai1 in ECs from humans and mice. Knockdown of STIM1 suppressed SOCE activity of TRPC1 and TRPC4, indicating that STIM1 was essential for SOCE in ECs expressing these channels. Orai1 expression was not required for SOCE in primary human and mouse ECs. However, Orai1 was involved in regulating SOCE in HUVECs, consistent with evidence on the importance of Orai1 in mediating Ca^{2+} entry in these cells (Abdullaev et al., 2008). The difference with HUVECs may reflect the specialized nature and uniqueness of these cells, which are often, and perhaps incorrectly, used as a surrogate for ECs (Watson et al., 1995).

TRPC family members TRPC1, TRPC4, and TRPC5 form SOCs (Hofmann et al., 2002), whereas TRPC3, TRPC6, and TRPC7 form receptor-operated channels (Nilius and Droog-

Fig. 6. Continued. D and E, *mECs* from WT and *TRPC4*(^{-/-}) mice were transfected with YFP-WT-STIM1 expression construct used for confocal imaging to assess Tg-induced STIM1 puncta formation. F, STIM1 puncta formed after Tg addition at 150 s was quantified, and results shown are mean ± S.E. *n* = 6 from each group; *, significantly different from WT- *mECs*.

mans, 2001). We have shown that TRPC1 and TRPC4 are essential for the SOC function in ECs (Tiruppathi et al., 2002; Paria et al., 2004, 2006). Freichel et al. (2001) showed, using *TRPC4*(^{-/-}) mice that TRPC4 is essential for SOCE in ECs. We also observed markedly reduced Ca^{2+} entry in *TRPC4*(^{-/-}) mouse ECs in response to PAR-1 activation, resulting in a reduced lung vascular permeability response (Tiruppathi et al., 2002). In addition, TRPC4 mediates Ca^{2+} entry during transition from a proliferating to quiescent phenotype in the human dermal microvascular EC line (HMEC-1) (Graziani et al., 2010). These findings collectively indicate a key role for TRPC1 and TRPC4 in mediating Ca^{2+} entry in response to Ca^{2+} store depletion in ECs.

Recent studies have demonstrated that Ca^{2+} entry through TRPC channels is influenced by their association with Orai1 and STIM1 (Liao et al., 2007; Yuan et al., 2007; Alicia et al., 2008; Jardin et al., 2008, 2009; Yuan et al., 2009). To address the role of STIM1 and Orai1 proteins in

regulating SOCE mediated by TRPCs in ECs, we isolated cells from WT and *TRPC4*(^{-/-}) mice and determined the expression of TRPCs, STIMs, and Orais. There was essentially similar expression of these proteins in WT-ECs and *TRPC4*(^{-/-})-ECs. Using Fura-2 to determine PAR-1-mediated Ca^{2+} entry, we observed the loss of Ca^{2+} entry in *TRPC4*(^{-/-})-ECs in response to PAR-1 activation or thapsigargin. On silencing TRPC4 expression using siRNA in WT-ECs, we also showed the loss of thrombin- or Tg-induced Ca^{2+} entry. However, silencing TRPC1 expression in WT-ECs inhibited ~15% of thrombin-induced Ca^{2+} entry. It is noteworthy that re-expression of TRPC4 in *TRPC4*(^{-/-})-ECs restored PAR-1-induced Ca^{2+} entry, whereas the expression of the dominant-negative TRPC4^{EE647-648KK} mutant in WT-ECs markedly reduced PAR-induced Ca^{2+} entry. These key findings suggest that TRPC4 is responsible for SOCE in *mECs*, consistent with a previous report (Tiruppathi et al., 2002).

Fig. 7. Orai proteins function is not obligatory for SOCE in ECs. **A**, left, Western blot analysis of WT- *m*ECs using specific antibodies that reacts with Orai1, Orai2, and Orai3. Orai1 expression was dominant, Orai2 expression was not detectable, and Orai3 expression was relatively low compared with Orai1. **A**, right, WT- *m*ECs transfected with indicated concentrations of sc-siRNA or *m*Orai1-siRNA transfection were used for Western blot to determine Orai1 expression. **B** and **C**, cells were used to measure thrombin- or Tg-induced Ca²⁺ entry. Arrows indicate time of addition of thrombin or Tg. **A** representative profile is shown in **B** and **C**, left. Change in peak fluorescence ratio (340/380) for Ca²⁺ entry over basal was calculated and plotted in right. Results shown are the mean $\pm$ S.E. of four experiments carried out in duplicates. NS, not significant.

Because there was similar expression of STIM1 in WT and *TRPC4*($-/-$) ECs, we next addressed the function of STIM1 in mediating SOCE. STIM1 knockdown by greater than 80% abolished SOCE activated by either thrombin or Tg, indicating that STIM1 signaling is required for TRPC4-mediated Ca²⁺ entry in ECs. STIM1 activation of Ca²⁺ entry through *h*TRPC1 involves electrostatic interactions between STIM1 Lys-rich domain (Lys684 and Lys685) and TRPC1 C-terminal Asp639 and Asp640 (Zeng et al., 2008). Ca²⁺ entry through TRPC1 channels can be blocked by expression of STIM1 Lys-rich domain mutant, STIM1^{K684E-K685E} (Zeng et al., 2008). Like *h*TRPC1, *m*TRPC4 C-terminal contains negatively charged (Glu648 and Glu649) residues, indicating that charge interaction between STIM1 and TRPC4 activates Ca²⁺ entry through TRPC4 in *m*ECs. To test this concept, we ectopically expressed YFP-dominant negative-STIM1^{K684E-K685E} mutant (Zeng et al., 2008) in WT-ECs and found an unusually low

Ca²⁺ entry in response to thrombin compared with control cells expressing WT construct (YFP-WT-STIM1). In contrast to our findings in WT-ECs, we did not observe thrombin-induced Ca²⁺ entry in *TRPC4*($-/-$)-ECs expressing either YFP-WT-STIM1 or YFP-dominant negative-STIM1, indicating that TRPC4 is required for SOCE in *m*ECs. These results show that TRPC4 interacts with STIM1 to activate SOCE in ECs.

To further define the role of STIM1 in the mechanism of TRPC4-mediated SOCE in ECs, we visualized STIM1 puncta formation secondary to ER stored-Ca²⁺ release after transducing YFP-STIM1. In WT-ECs, STIM1 puncta were clearly seen in response to thrombin- or Tg-induced ER stored-Ca²⁺ release, whereas the response was greatly suppressed in *TRPC4*($-/-$)-ECs. Thus, formation of STIM1 puncta requires TRPC4 and formation of these at the ER-plasma membrane interface is required for Ca²⁺ entry in ECs.

Recent studies have shown that STIM1 associates with the

Fig. 7. Continued. D, WT-mECs transfected with sc-siRNA or *mOrai2*-siRNA were used to measure thrombin-induced Ca^{2+} entry. Experiment was repeated three times. The results from representative experiments are shown. E, WT-mECs transfected with sc-siRNA or *mOrai3*-siRNA were used to measure thrombin-induced Ca^{2+} entry (bottom) or Western blot to determine Orai3 expression [top; ECL, longer exposure time (30 min) used to detect]. Experiments were repeated three times. The results from representative experiments are shown. F, mECs from WT and *TRPC4* $^{-/-}$ mice were transfected with indicated concentrations of *mCherry*-tagged WT-*hOrai1* construct. After transfection, 10 to 15 *mCherry*-expressing cells were used to measure thrombin-induced Ca^{2+} entry. Arrows indicate time of addition of thrombin. A representative profile is shown at top. Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated. Results shown are the mean $\pm$ S.E. of four experiments (bottom). G, WT-mECs transfected with WT-*mCherry*-Orai1, *mCherry*-Orai1 $^{\text{R91W}}$, or CFP-Orai3 $^{\text{E81W}}$ expression construct were used to measure thrombin-induced Ca^{2+} entry as above (top). Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated. Results shown are the mean $\pm$ S.E. of four experiments (bottom).

*I*_{CRAC}-channel protein Orai1 to form a functional SOC in different cell types (Ong et al., 2007; Cheng et al., 2008; Kim et al., 2009; Covington et al., 2010). In addition, TRPCs may

interact with STIM1 and Orai1 to form a functional SOC (Ong et al., 2007; Cheng et al., 2008; Kim et al., 2009; Liao et al., 2009; Lee et al., 2010). However, we observed that Orai1

Fig. 8. Effects of TRPC1, TRPC4, STIM1, or Orai1 knockdown on thrombin-induced Ca^{2+} entry in human ECs. **A**, RT-PCR was used to determine the mRNA expression profile for STIMs and OraIs in HLMVECs and HUVECs. **B**, HLMVECs transfected with sc-siRNA or *h*STIM1-siRNA were lysed and immunoblotted with anti-STIM1 mAb (top). The blots were stripped and probed with anti-β actin mAb as loading control (bottom). **C**, after transfection, cells were used to measure thrombin-induced Ca^{2+} entry (left). Arrows indicate time of addition of thrombin. Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated (right). Results shown are mean $\pm$ S.E. of four experiments carried out in duplicate. *, significantly different from cells transfected with sc-siRNA. **D**, HLMVECs transfected with sc-siRNA or *h*Orai1-siRNA were immunoblotted with anti-Orai1 pAb (top). The blots were stripped and probed with anti-β actin mAb as loading control (bottom). **E**, cells were used to measure thrombin-induced Ca^{2+} entry (left). Arrow indicates time at which cells were stimulated with thrombin. Change in peak fluorescence ratio (340/380) for Ca^{2+} entry over basal was calculated (right). Results shown are mean $\pm$ S.E. of four experiments carried out in duplicate. NS, not significantly different from control or sc-siRNA transfected cells.

knockdown had no effect on SOCE induced by either thrombin or Tg. Ectopic expression of Orai1 in *m*ECs of WT and *TRPC4*($-/-$) mice also had no effect on thrombin-induced Ca^{2+} entry. Moreover, Orai1 expression failed to restore SOCE in *TRPC4*($-/-$)-ECs. We also suppressed the expression of the other CRAC channel proteins Orai2 and Orai3 with siRNA in *m*ECs. Suppression of either Orai2 or Orai3 had no effect on thrombin-induced Ca^{2+} entry in *m*ECs. In addition, we expressed Orai1^{R91W} and Orai3^{E81W} mutants in WT-ECs and observed that expression of these mutants had no effect on SOCE. Because this lack of involvement of Orai1 could be species-specific, we investigated the contribution of TRPCs, STIMs, and OraIs in the mechanism of SOCE in *h*ECs, HLMVECs. We observed expression of TRPCs (TRPC1, TRPC4, and TRPC6), STIM1, Orai1, and Orai3 in

HLMVECs. TRPC1 knockdown prevented >80% thrombin-induced Ca^{2+} entry in HLMVECs, whereas TRPC4 knockdown prevented ~50% of thrombin-induced Ca^{2+} entry, indicating the role of both TRPCs in activating SOCE in HLMVECs. We also suppressed expression of STIM1 and the dominant Orai protein Orai1 in HLMVECs and measured thrombin-induced Ca^{2+} entry after RNA silencing. Thrombin-induced Ca^{2+} entry was blocked in STIM1-depleted HLMVECs but not in the Orai1-depleted cells, consistent with the *m*EC results. Thus, Orai1 in contrast to STIM1 does not play a role in mediating SOCE in *h*/*m*ECs.

That Orai1 does not regulate SOCE in ECs is different from the findings of Abdullaev et al. (2008) showing an Orai1-dependent I_{CRAC} channel mediated Ca^{2+} entry in HUVECs. We also showed quite a similar role of Orai1 in

Fig. 9. ER Ca^{2+} store depletion induces interaction between TRPC4 and STIM1 in ECs. **A**, WT-mECs were stimulated with Tg (1 μM) for different time intervals at 37°C. After Tg stimulation, cells were lysed and immunoprecipitated (IP) with anti-TRPC4 pAb. The precipitated proteins were immunoblotted (IB) with anti-STIM1 mAb (first) or Orai1 pAb (second). Total cell lysates were immunoblotted with anti-STIM1 mAb (third) or anti-Orai1 pAb (fourth). **B**, WT-mECs treated with or without thrombin (50 nM) for 5 min at 37°C were lysed and immunoprecipitated with either anti-TRPC4 pAb or anti-STIM1 mAb. The precipitated proteins were immunoblotted with anti-STIM1 mAb (first) and anti-TRPC4 pAb (second). Total cell lysates were immunoblotted with anti-TRPC4 pAb (third) and anti-STIM1 mAb (bottom). **C**, WT-mECs treated with or without thrombin (50 nM) for 5 min at 37°C were lysed and immunoprecipitated with anti-TRPC4 pAb. The precipitated proteins were immunoblotted with anti-Orai1 pAb (first). Total cell lysates were immunoblotted with anti-Orai1 pAb (second) or anti-TRPC4 pAb (third). The experiments described in A to C were repeated three times with similar results. Results shown are from representative experiments.

regulating SOCE in HUVECs, suggesting that the discrepancy is related to the use of HUVECs, a specialized cell that does not reflect the complex characteristics of most ECs (Watson et al., 1995). Orai proteins are highly Ca^{2+} selective (Salido et al., 2009), whereas TRPC proteins are nonselective cation channels permeable to Ca^{2+} , Na^{+} , and Cs^{+} (Salido et al., 2009). We observed expression of Orai1, Orai2, and Orai3 proteins in HUVECs; thus, Orai proteins may contribute to Ca^{2+} -selective SOC activity in HUVECs that may be required for specialized function of human umbilical veins. We observed that lung *h*mECs dominantly express Orai1 isoform, and its knockdown had no significant effect on thrombin-induced Ca^{2+} entry, indicating that TRPCs function as a nonselective cation channel in ECs.

In summary, we showed that STIM1 activates TRPC1/4-mediated SOCE in ECs. Association between TRPC and

STIM1 was required for Ca^{2+} entry in ECs secondary to store depletion. These findings demonstrate the importance of the interaction between the Ca^{2+} -sensing STIM1 with SOCE channel TRPC1 or TRPC4 in ECs in mediating Ca^{2+} entry and disrupting the endothelial barrier. However, Orai1 was not required for activation of SOCE in ECs, although Orai1 protein was expressed, suggesting that it has another, as-yet-unrecognized function in ECs.

Authorship Contributions

Participated in research design: Sundivakkam, Freichel, Yuan, Flockerzi, and Tiruppathi.

Conducted experiments: Sundivakkam, Freichel, Singh, and Yuan.

Wrote or contributed to the writing manuscript: Sundivakkam, Vogel, Malik, and Tiruppathi.

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