

Phosphatidylinositol 3-Kinase Class II α -Isoform PI3K-C2 α Is Required for Transforming Growth Factor β -induced Smad Signaling in Endothelial Cells^{*[S]}

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Sho Aki[‡], Kazuaki Yoshioka[‡], Yasuo Okamoto[‡], Noriko Takuwa^{‡§}, and Yoh Takuwa^{‡1}

From the [‡]Department of Physiology, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920-8640, Japan and the

[§]Department of Health and Medical Sciences, Ishikawa Prefectural Nursing University, Kahoku, Ishikawa 929-1210, Japan

Background: TGF β receptor signals through Smad phosphorylation, which is dependent on endocytosis of TGF β receptors and the Smad anchor protein SARA localized on endosomes.

Results: Class II PI3K-C2 α is necessary for TGF β receptor endocytosis into SARA-containing endosomes, SARA-Smad complex formation, and Smad phosphorylation.

Conclusion: PI3K-C2 α serves endosomal TGF β receptor signaling.

Significance: PI3K-C2 α is a key molecule that is generally engaged in endosomal receptor signaling.

We have recently demonstrated that the PI3K class II- α isoform (PI3K-C2 α), which generates phosphatidylinositol 3-phosphate and phosphatidylinositol 3,4-bisphosphates, plays crucial roles in angiogenesis, by analyzing PI3K-C2 α knock-out mice. The PI3K-C2 α actions are mediated at least in part through its participation in the internalization of VEGF receptor-2 and sphingosine-1-phosphate receptor S1P₁ and thereby their signaling on endosomes. TGF β , which is also an essential angiogenic factor, signals via the serine/threonine kinase receptor complex to induce phosphorylation of Smad2 and Smad3 (Smad2/3). SARA (Smad anchor for receptor activation) protein, which is localized in early endosomes through its FYVE domain, is required for Smad2/3 signaling. In the present study, we showed that PI3K-C2 α knockdown nearly completely abolished TGF β 1-induced phosphorylation and nuclear translocation of Smad2/3 in vascular endothelial cells (ECs). PI3K-C2 α was necessary for TGF β -induced increase in phosphatidylinositol 3,4-bisphosphates in the plasma membrane and TGF β receptor internalization into the SARA-containing early endosomes, but not for phosphatidylinositol 3-phosphate enrichment or localization of SARA in the early endosomes. PI3K-C2 α was also required for TGF β receptor-mediated formation of SARA-Smad2/3 complex. Inhibition of dynamin, which is required for the clathrin-dependent receptor endocytosis, suppressed both TGF β receptor internalization and Smad2/3 phosphorylation. TGF β 1 stimulated Smad-dependent VEGF-A expression, VEGF receptor-mediated EC migration, and capillary-like tube formation, which were all abolished by either PI3K-C2 α knockdown or a dynamin inhibitor. Finally, TGF β 1-induced microvessel formation in Matrigel plugs was greatly

attenuated in EC-specific PI3K-C2 α -deleted mice. These observations indicate that PI3K-C2 α plays the pivotal role in TGF β receptor endocytosis and thereby Smad2/3 signaling, participating in angiogenic actions of TGF β .

PI3Ks are a family of enzymes that phosphorylate membrane inositol phospholipids at the 3' position of the inositol ring and comprise three classes (classes I–III) (1, 2). Class I PI3Ks, which mainly generate phosphatidylinositol 3,4,5-bisphosphates, are activated by receptor tyrosine kinases and G protein-coupled receptors to mediate activation of Akt and Rac, stimulating cell proliferation and migration. In contrast to the well characterized class I PI3Ks, physiological roles of class II PI3Ks, which have three members: PI3K-C2 α , PI3K-C2 β , and PI3K-C2 γ , were not well understood (3–7). We have recently revealed that PI3K-C2 α plays a crucial role in angiogenesis and maintenance of the endothelial barrier integrity in an endothelial cell (EC)² autonomous manner (8). PI3K-C2 α is localized in clathrin-coated pits and endocytic vesicles, early endosomes, and the *trans*-Golgi network (8–11) and is thought to predominantly generate phosphatidylinositol 3-phosphate (PtdIns(3)P) and/or phosphatidylinositol 3,4-bisphosphates (PtdIns(3,4)P₂) *in vivo* differently from class I PI3K (3–5, 7, 12–14). Our data showed that PI3K-C2 α regulates vesicular trafficking in EC and thereby is indispensable for vesicular transport-mediated delivery of cargos including the endothelial adhesion molecule VE-cadherin and ligand binding-induced endocytosis of the receptor tyrosine kinase VEGF receptor-2 (VEGFR2) and the G protein-

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[S] This article contains supplemental Movies S1 and S2.

¹ To whom correspondence should be addressed: Dept. of Physiology, Kanazawa University School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640 Japan. Tel.: 81-76-265-2165; Fax: 81-76-234-4223; E-mail: ytakuwa@med.kanazawa-u.ac.jp.

² The abbreviations used are: EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; HMVEC, human microvascular endothelial cell; C2 α , PI3K-C2 α ; C2 β , PI3K-C2 β ; C2 α' , C2 α -siRNA-resistant C2 α ; kdC2 α' , kinase-deficient mutant of C2 α ; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphates; ALK, activin receptor-like kinase; wtALK5, wild-type ALK5; caALK5, constitutively active ALK5 mutant; wt, wild-type SARA; S1P, sphingosine-1-phosphate; Δ SBD-SARA, Smad-binding domain-deleted mutant; SMC, smooth muscle cell; VEGFR, VEGF receptor; MLEC, mouse lung endothelial cell; MASM, mouse aortic smooth muscle; vWF, von Willebrand factor; PLA, proximity ligation assay; p-, phosphorylated.

coupled receptor S1P₁ (8, 15, 16). Signaling of VEGFR2 and S1P₁ was defective in PI3K-C2 α -depleted EC: the receptor endocytosis was inhibited, and the signaling on endosomes, particularly Rho GTPase activation, was impaired. These defects result in impaired migration, proliferation, and intercellular junction formation in EC. It is unknown whether and how PI3K-C2 α regulates signaling of other angiogenic receptors. In addition to our studies, a general regulatory role for PI3K-C2 α in endocytosis through the generation of PtdIns(3,4)P₂ in the plasma membrane was recently reported (14).

TGF β is involved in the regulation of migration and proliferation of EC, production of basement membrane, and differentiation and recruitment of mural cells, thus being essential for normal vascular formation (17–20). TGF β signals through type I and type II TGF β receptors, which are both serine/threonine transmembrane kinases (21–23). TGF β binds to type II receptor, which phosphorylates and activates type I receptors, activin receptor-like kinase (ALK) 1, and ALK5. ALK1 and ALK5 in turn phosphorylate the receptor-regulated Smads, Smad1 and Smad5 (Smad1/5) and Smad2 and Smad3 (Smad2/3), respectively. Phosphorylated receptor-regulated Smads form complexes with the common mediator Smad4 and the Smad complexes translocate into the nucleus to regulate gene transcription. It was proposed that TGF β signaling pathways via ALK1 and ALK5 in EC may play a balancing role for controlling proliferation and migration of EC during angiogenesis (24, 25). Of the two TGF β signaling pathways, EC-specific gene ablation of either ALK5 or Smad2/3 resulted in the similar vascular abnormalities, indicating a pivotal role of endothelial ALK5-Smad2/3 pathway in the angiogenic effect of TGF β (19, 20, 26, 27). SARA (Smad anchor for receptor activation) protein contains the binding domains for both Smad2/3 and the TGF β receptor complex and is localized in the early endosomes through its FYVE domain, which specifically recognizes and binds to PtdIns(3)P (28). Previous studies (28–31) demonstrated that upon TGF β stimulation, the TGF β receptor complex undergoes clathrin-dependent endocytosis into the early endosomes containing SARA and that the proper localization of SARA in the early endosomes and the TGF β receptor internalization into the SARA-containing endosomes are the events necessary for TGF β -induced phosphorylation of Smad2/3 and the following nuclear translocation of the Smad complexes. It is likely that PI3Ks are involved in TGF β receptor internalization, the endosomal localization of SARA, and thus TGF β signaling. However, it is unknown which isoform of PI3K is engaged in the processes of TGF β signaling.

In the present study, we studied a role for PI3K-C2 α in TGF β -induced Smad2/3 signaling in EC. We found that TGF β -induced Smad2/3 phosphorylation, Smad2/3-dependent gene expression, and angiogenic responses were strongly dependent on PI3K-C2 α . PI3K-C2 α was required for TGF β receptor internalization but not the endosomal localization of SARA. These observations suggest that PI3K-C2 α plays an indispensable role in endosomal TGF β receptor signaling.

EXPERIMENTAL PROCEDURES

Cells—Human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland), the human microvascular endothe-

lial cells (HMVECs), and mouse lung vascular endothelial cells (MLECs) were plated onto type I collagen (Nitta Gelatin, Osaka, Japan)-coated dishes and flasks and allowed to grow under 5% CO₂ at 37 °C in complete endothelial growth medium containing 2% FBS and growth factor supplements (EGM-2 (catalog no. CC3156; Lonza) for HUVECs and EBM-2-MV (catalog no. CC4147; Lonza) for HMVECs and MLECs). HUVECs between passages 4 and 6 were used for all experiments. MLECs and mouse aortic smooth muscle (MASM) cells were isolated from *Pik3c2a^{flox/flox}* mice and used for *in vitro* assays as described previously (8, 32, 33). HEK293T cells, MASM cells, and Caco2 cells were cultured in 10% FBS (Invitrogen Gibco)-supplemented DMEM for HEK293T and Caco2 cells (catalog no. 041-29775; Invitrogen Gibco) and advanced DMEM for MASM cells (catalog no. 12491; Invitrogen Gibco).

Small Interfering RNA, Plasmids, and Transfection—Knockdown of endogenous PI3K isoforms and Smad4 were performed with the siRNAs that were synthesized using a Silencers siRNA construction kit (catalog no. AM1620; Ambion, Austin, TX) according to the manufacturer's instructions. The target sequences were: 5'-AAGGTTGGCACTTACAAGAAT-3' and 5'-AAGTAAGCCTAAGGTGGATAA-3' for human PI3K-C2 α #1 and #2, respectively; 5'-AAGCCGGAAGCTTCTGGGTTT-3' for PI3K-C2 β ; 5'-GGACAACCTGTTTCATATAG-3' for class I PI3K p110 α ; 5'-AAACTCAACACTGGCTAATTA-3' for Vps34; 5'-AATACATTCCTCACTGCACACCC-3' for Smad4; and 5'-GGGGGAAATACGACTTAGTAGG-3' for ALK5. HUVECs were transfected by incubating with the siRNAs in the presence of Lipofectamine 2000 (catalog no. 11668-019; Invitrogen) for 48–72 h before the experiments. MASM cells isolated from *Pik3c2a^{flox/flox}* mice were grown to 70% confluency and then infected with the adenovirus encoding Cre recombinase (Ad-Cre) in the absence of serum. Adenovirus encoding LacZ (Ad-LacZ) was used as control. After 1 h of adenovirus infection, the growth medium containing 10% FBS was added, and the cells were allowed to recover for the next 48 h. The expression vector for GFP-PI3K-C2 α was described previously (8). The expression vectors for the PI3K-C2 α -specific siRNA-resistant form of PI3K-C2 α (C2 α ^r) and the kinase-deficient mutant (D1268A) of GFP-PI3K-C2 α (GFP-kdPI3K-C2 α) were generated using a standard PCR-based method (34). In C2 α ^r, the codons AAG-GTT-GGC-ACT-TAC for the amino acids Lys⁷²⁸-Val⁷²⁹-Gly⁷³⁰-Thr⁷³¹-Tyr⁷³² were replaced by the nucleotides AAA-GTC-GGT-ACC-TAT, which encodes the same amino acids. The changes in these nucleotides rendered C2 α ^r resistant to the PI3K-C2 α -specific siRNA. The expression vectors for FLAG-tagged wild-type SARA (wtSARA), FLAG-tagged Smad-binding domain (the amino acids 665–704)-deleted SARA mutant (Δ SBD-SARA), FLAG-tagged wild-type ALK5 (wtALK5), FLAG-tagged constitutively activated mutant (T202D) of ALK5 (caALK5), and GFP-PH^{TAPP1} were purchased from Addgene (Cambridge, MA). The expression vector for GFP-FYVE^{SARA} was provided by Dr. S. Itoh (Showa Pharmaceutical University) (26). The expression vectors for FLAG-tagged Smad2 and FLAG-tagged Smad3 were provided by Dr. K. Miyazono (University of Tokyo) (35).

Immunoblotting and Immunoprecipitation Analysis—At 48 h after siRNA transfection, the cells were serum-starved

Crucial Role of PI3K-C2 α in TGF β Signaling

with M199 (Invitrogen Gibco) containing 0.5% fatty acid-free BSA (catalog no. A6003; Sigma-Aldrich) for 4 h and then stimulated with 5 ng/ml TGF β 1 (catalog no. 240-B; R&D Systems, Minneapolis, MN). The cells were washed in PBS and lysed in the cell lysis buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM CaCl₂, 0.5% Triton X-100, 100 mM NaF, 1 mM Na₃VO₄) supplemented with Complete Protease inhibitor mixture (Roche Applied Science) by scraping, followed by centrifugation for 15 min at 16,000 \times g at 4 °C. The resultant supernatants were taken, electrophoresed on 8% SDS-PAGE, and transferred onto PVDF membrane (Millipore, Billerica, MA). The membranes were blocked in PBS containing 5% BSA and incubated with respective antibodies overnight. The antibodies used are PI3K-C2 α (catalog no. 611046; BD Biosciences, San Jose, CA), PI3K-C2 β (catalog no. 611342; BD Biosciences), p110 α (catalog no. 4249; Cell Signaling, Danvers, MA), Vps34 (catalog no. 4263; Cell Signaling), total Smad2/3 (catalog no. 610842; BD Biosciences), phosphorylated Smad2 (p-Smad2) (catalog no. 3101; Cell Signaling), phosphorylated Smad3 (p-Smad3) (catalog no. 9520; Cell Signaling), total ERK1/2 (catalog no. 9102; Cell Signaling), phosphorylated ERK1/2 (catalog no. 4370; Cell Signaling), total Smad4 (catalog no. 9515; Cell Signaling); SARA (catalog no. sc-9135; Santa Cruz, Dallas, TX), phosphoserine (catalog no. 618100; Invitrogen), and β -actin (catalog no. A1978; Sigma-Aldrich). The membranes were incubated with alkaline phosphatase-conjugated secondary antibodies (anti-mouse IgG antibody, catalog no. 7056; anti-rabbit IgG antibody, catalog no. 7054) (Cell Signaling) and visualized by color reaction using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Wako, Osaka, Japan). The band intensities were determined using Image Gauge (Fuji Film, Tokyo, Japan). The values were normalized for the value of β -actin as a loading control and expressed as multiples over the normalized values of untreated controls.

For immunoprecipitation assay, HEK293T cells were co-transfected with the expression vectors for FLAG-Smad3, either FLAG-SARA or FLAG- Δ SBD-SARA, and either FLAG-wtALK5 or FLAG-constitutively activated ALK5 (caALK5) and 72 h later were lysed in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) supplemented with Complete Protease inhibitor cocktails. The lysates were incubated with anti-SARA antibody for 1 h at 4 °C with rocking, followed by the incubation with protein G-agarose beads (catalog no. 1-719-416; Roche) for 1 h at 4 °C. After the beads were washed five times, they were mixed with 2 \times Laemmli's SDS sample buffer and boiled. The resultant samples were analyzed with immunoblotting using respective antibodies.

Immunohistochemistry and Immunofluorescence Staining—HUVECs were plated onto type I collagen-coated glass bottom dishes (MatTek Corporation, Ashland, MA) and allowed to adhere to dishes in EGM-2 growth medium overnight. The cells were rinsed with prewarmed PBS once and fixed in prewarmed 4% fresh paraformaldehyde in PBS for 10 min, washed with PBS, and then permeabilized in 0.2% Triton X-100 in PBS for 15 min when necessary. After the cells were incubated with 5% normal goat serum for 60 min to inhibit nonspecific protein binding, the cells were incubated with rabbit polyclonal anti-p-

Smad2 antibody (catalog no. AB3849; Millipore), mouse monoclonal anti-Smad2/3 antibody (catalog no. 610842; BD Biosciences), rabbit polyclonal anti-SARA antibody, or mouse monoclonal anti-EEA1 antibody (catalog no. 610456; BD Biosciences) for 2 h at room temperature or overnight at 4 °C. The cells were incubated for 60 min at room temperature with Alexa Fluor 488-conjugated goat anti-mouse (catalog no. A31620; Molecular Probes), Alexa Fluor 488-conjugated goat anti-rabbit (catalog no. A11034), Alexa Fluor 594-conjugated goat anti-mouse (catalog no. A31624), Alexa Fluor 594-conjugated goat anti-rabbit (catalog no. A31620), and secondary antibodies diluted at 1:1000 in PBS. Where appropriate, the cells were counterstained with DAPI (catalog no. D1306; Molecular Probes) for 5 min. The cells were mounted on Fluoromount (catalog no. K024; Diagnostic BioSystems, Pleasanton, CA) and observed under a custom confocal microscope unit as described in detail previously (8). For immunohistochemistry of the sections of Matrigel plugs, the sections of paraformaldehyde-fixed, paraffin-embedded Matrigel plug were deparaffinized and processed in heat-induced target retrieval to unmask the antigen using with target retrieval solution (Dako, Carpinteria, CA) (30). The sections were incubated with Dako blocking solution (catalog no. X0909; Dako) for 10 min to inhibit nonspecific protein binding. After blocking, the sections were stained with rabbit polyclonal anti-von Willebrand factor (vWF) (catalog no. A0082; Dako) for 60 min at room temperature. The sections were incubated with the secondary antibody of an EnVision kit (catalog no. K4002; Dako) for 60 min, and the color reaction was developed. Where appropriate, the sections were counterstained with hematoxylin. The sections were examined using a BX41 inverted microscope (Olympus, Tokyo, Japan), and vWF-positive microvessel numbers were determined with ImageJ software.

Proximity Ligation Assay (PLA) Staining—The cells were fixed in prewarmed 4% fresh paraformaldehyde in PBS for 10 min and permeabilized in 0.2% Triton X-100 in PBS for 15 min when necessary. After the cells were incubated with rabbit polyclonal anti-ALK5 antibody (catalog no. sc-398; Santa Cruz), mouse monoclonal anti-Smad2/3 antibody, and mouse monoclonal anti-SARA (catalog no. sc-133071; Santa Cruz) antibody overnight at 4 °C, *in situ* protein interactions were detected using the Duolink proximity ligation assay kit according to the manufacturer's instructions (Olink Bioscience, Uppsala, Sweden). The cells were stained with anti-EEA1-Alexa Fluor 594 (M176 A59; MBL, Nagoya, Japan).

RNA Isolation and Quantitative PCR Analysis—Total RNA in HUVECs was isolated using TRIzol reagent (Invitrogen). One μ g of total RNA was reverse-transcribed into the first strand cDNA using QuantiTect RT Kit (catalog no. 205311; Qiagen). Quantitative real time PCRs were performed using FAM-conjugated TaqMan inventoried assay from Applied Biosystems for human PI3K-C2 α (Hs0090461_m1) and human VEGF-A (Hs00900055_m1). 18 S rRNA (Hs99999901_s1) probe was used as an internal control. The mRNA expression levels were normalized for the expression of 18 S rRNA mRNA, and the results were expressed as multiples over control values. Comparative quantitative analysis was performed using the GeneAmp 7300 system (Applied Biosystems, Foster City, CA) based on the $\Delta\Delta$ Ct method.

Wound Healing/Scratch and Tube Formation Assay—For wound healing/scratch assay (32), confluent HUVEC monolayers were scratched with a standard 20- μ l pipette tip and incubated in M199 containing 1% FBS in the presence of recombinant human VEGF-A (50 ng/ml) (catalog no. 100-20; PeproTech, Rocky Hill, NJ), TGF β 1 (5 ng/ml), dynasore (80 μ M) (catalog no. D7693; Sigma-Aldrich), ALK5 inhibitor II (2-(3-(6-methylpyridin-2-yl)-1-*H*-pyrazol-4-yl)-1,5-naphthyridine) (5 μ M) (catalog no. 616452; Merck-CalbioChem), and VEGFR2 inhibitor SU1498 (10 μ M) (catalog no. 572888; Merck-Calbiochem) for 8 h. The microphotographs were taken at 0 and 8 h, and the wound width was determined with ImageJ software. For tube formation assays, siRNA-transfected HUVECs (2.0×10^4 cells) in M199 containing 1% FBS were seeded onto 200 μ l of growth factor-reduced Matrigel (BD Biosciences) in a 24-well plate in the absence and presence of VEGF-A (50 ng/ml), TGF β 1 (5 ng/ml), dynasore (80 μ M), ALK5 inhibitor (5 μ M), and VEGFR2 inhibitor (10 μ M) and were incubated for 12 h. Tube formation was quantified by measuring cumulative tube length in five random microscopic fields/well using ImageJ software under a BIOREVO microscope (Keyence, Osaka, Japan).

Matrigel Plug in Vivo Angiogenesis Assay—All of the mice used in this study were bred and maintained at the Institute for Experimental Animals, Advanced Science Research Center, Kanazawa University under specific pathogen-free conditions. All procedures were conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan and approved by the Committee on Animal Experimentation of Kanazawa University. *Pik3c2a*^{ΔEC} (*C2α*^{ΔEC}) (*Pik3c2a*^{flox/flox}; *Cdh5*(PAC)-*CreER*^{T2}) and *Pik3c2a*^{ΔSMC} (*C2α*^{ΔSMC}) (*Pik3c2a*^{flox/flox}; *SM22α*-*Cre*) mice were described previously (8). Cre-negative littermates were used as controls. To verify the efficiency of Cre recombination, Cre mice were mated with mice from the Cre reporter transgenic line *ROSA26-LacZ* (B6.129S4-*Gt(ROSA)26Sortm1Sor*/J, Jackson Lab). All mice had a C57BL/6J genetic background. For *Pik3c2a* gene inactivation in adult mice, tamoxifen (10 mg/ml corn oil) (catalog no. T5648; Sigma-Aldrich) was administered seven times by intraperitoneal injection of 100 μ l of tamoxifen solution. For Matrigel plug assay (32, 36), recombinant VEGF-A (200 ng/ml), FGF2 (400 ng/ml) (catalog no. AF-100-18B; PeproTech), and heparin (100 mg/ml) (Sigma-Aldrich) were mixed with growth factor-reduced Matrigel. The Matrigel solutions (300 μ l each) were injected subcutaneously into the groin area close to the dorsal midline (most angiogenic portion) of anesthetized mice. Matrigel plugs were harvested on day 10 and fixed overnight in 4% paraformaldehyde for paraffin embedding and the following immunohistochemistry.

VEGF-A ELISA Assay—Human VEGF-A protein levels in the conditioned medium of HUVEC cultures were determined using human VEGF-A ELISA immunoassay (catalog no. DVE00; R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Optical density was measured at 450 nm using a 540-nm correction with a Multiskan GO (Thermo Fisher Scientific, Waltham, MA).

Statistical Analysis—The data are presented as means \pm S.E. and expressed as the percentages or multiples relative to the

values in control cells. Statistical significance was analyzed using Prism 5 software (GraphPad Software Inc., San Diego, CA). Statistical significance was analyzed either by one-way or two-way analysis of variance followed by Bonferroni test as appropriate. Results with $p < 0.05$ were considered statistically significant.

RESULTS

TGF β 1-induced Phosphorylation and Nuclear Translocation of Smad2/3 Are Dependent on Class II PI3K-C2 α —We studied roles of PI3K isoforms in TGF β 1-induced Smad2/3 stimulation in vascular EC. HUVECs were transfected with either of the specific siRNAs against class I PI3K p110 α , class II PI3K-C2 α and PI3K-C2 β , and class III Vps34, or scrambled siRNA (sc-siRNA) as control. Each siRNA effectively inhibited the expression of respective PI3K proteins but not other PI3K isoform proteins (Fig. 1, A and B). In sc-siRNA-transfected control HUVECs, TGF β 1 induced a gradual increase in Smad3 phosphorylation, which reached the plateau level of an 8-fold increase over the basal at 30 min (Fig. 1B). Transfection of PI3K-C2 α -siRNAs (*C2α*-siRNAs #1 and #2) markedly inhibited TGF β 1-induced stimulation of Smad3 phosphorylation throughout the observation time period. TGF β 1 also induced Smad2 phosphorylation, which was greatly suppressed by PI3K-C2 α knockdown (Fig. 1C). In contrast, knockdown of either p110 α , PI3K-C2 β , or Vps34 failed to inhibit TGF β 1-induced phosphorylation of Smad2/3. TGF β 1 did not stimulate phosphorylation of Smad1, Smad5, or Smad8 (Smad1/5/8) in HUVECs (Fig. 1D). In contrast, BMP9 induced phosphorylation of Smad1/5/8 (Fig. 1E) but not Smad2/3 (data not shown). Differently from the case of TGF β 1, PI3K-C2 α knockdown did not suppress BMP9-induced Smad1/5/8 phosphorylation. In addition, PI3K-C2 α knockdown did not alter TGF β 1-induced activation of the noncanonical pathway ERK, which is known to be independent of Smad2/3 (37), in HUVECs. Similar to HUVECs, TGF β 1 stimulated phosphorylation of Smad2/3 in HMVECs and MLECs in a PI3K-C2 α -dependent manner (Fig. 2, A and B). TGF β 1 also stimulated phosphorylation of Smad1/5/8 in HMVECs, which was also dependent on PI3K-C2 α (Fig. 2A). Unlike vascular endothelial cells, deletion of PI3K-C2 α in MASM cells and human colonic epithelial carcinoma Caco2 cells did not inhibit TGF β 1-induced phosphorylation of Smad2/3 (Fig. 2, C and D). Thus, PI3K-C2 α was necessary for TGF β -induced Smad2/3 phosphorylation in vascular EC but not in vascular smooth muscle.

Stimulation with TGF β 1 induced robust nuclear translocation of Smad2/3 in sc-siRNA-transfected HUVECs as evaluated with anti-Smad2/3 immunofluorescent staining (Fig. 3A). We also observed that TGF β 1 stimulated nuclear accumulation of p-Smad2, which was also markedly suppressed by PI3K-C2 α knockdown (Fig. 3B). In *C2α*-siRNA-transfected HUVECs, the exogenous expression of the *C2α*-siRNA-resistant form of PI3K-C2 α (*C2α'*) but not wild-type PI3K-C2 α (wtC2 α) restored TGF β -induced Smad activation (Fig. 3, C and D). In sc-siRNA-transfected HUVECs, the overexpression of wild-type PI3K-C2 α by itself did not change TGF β -induced Smad translocation into the nucleus. Moreover, we determined the effect of the expression of the kinase-deficient mutant of *C2α'* (kdC2 α') on Smad2/3

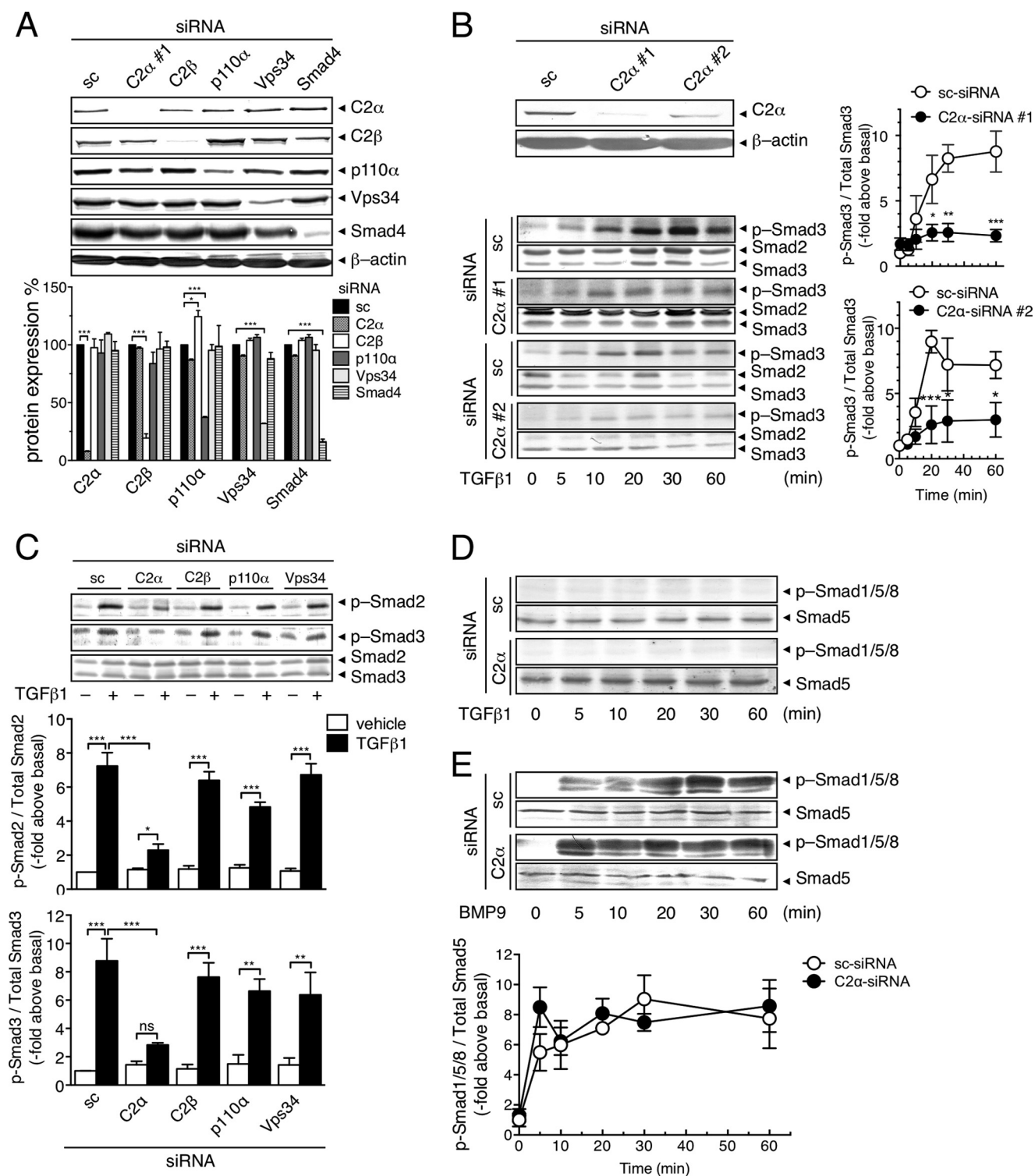


FIGURE 1. PI3K-C2 α is required for TGF β 1-induced Smad2/3 phosphorylation in EC. A, siRNA-mediated knockdown of PI3K isoforms, HUVECs were transfected with PI3K-C2 α #1 (C2 α #1), PI3K-C2 β (C2 β), p110 α , Vps34, and Smad4-specific siRNA or scrambled (sc)-siRNA, and the expression of the PI3K proteins, Smad4, and β -actin as a loading control were analyzed with immunoblotting. Upper panel, representative blots. Lower panel, quantified data. B, time-dependent phosphorylation of Smad3 in response to TGF β 1 in C2 α #1, C2 α #2, or sc-siRNA transfected HUVECs. Serum-starved cells were stimulated with TGF β 1 (5 ng/ml) for the indicated time periods. The cell lysates were subjected to immunoblot analysis for p-Smad3 and total Smad2 and Smad3. Upper left panel, effects of siRNA-mediated knockdown of PI3K-C2 α . Lower left panel, representative blots of Smads. Right panel, quantified data. C, effects of knockdown of PI3K isoforms on TGF β 1-induced Smad2 and Smad3 phosphorylation. HUVECs that had been transfected with either of C2 α #1-, C2 β -, p110 α -, and Vps34-specific siRNAs or sc-siRNA were stimulated with TGF β 1 (5 ng/ml) for 30 min, followed by the immunoblot analysis for p-Smad2, p-Smad3, and total Smad2 and Smad3. Upper panel, representative blots. Lower panel, quantified data. D, time-dependent phosphorylation of Smad1/5/8 in response to TGF β 1 in HUVECs. The cells were treated as in B. The cell lysates were subjected to immunoblot analysis for p-Smad1/5/8 and total Smad5. E, time-dependent phosphorylation of Smad1/5/8 in response to BMP9 in HUVECs. Serum-starved cells were stimulated with BMP9 (10 ng/ml) for the indicated time periods. The cell lysates were subject to immunoblot analysis for p-Smad1/5/8 and total Smad5. In A–E, the data are means \pm S.E. of three or four determinations ($n = 3$ or 4). In all figures, the asterisks indicate statistical significance between the indicated groups at the levels of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). ns, statistically not significant.

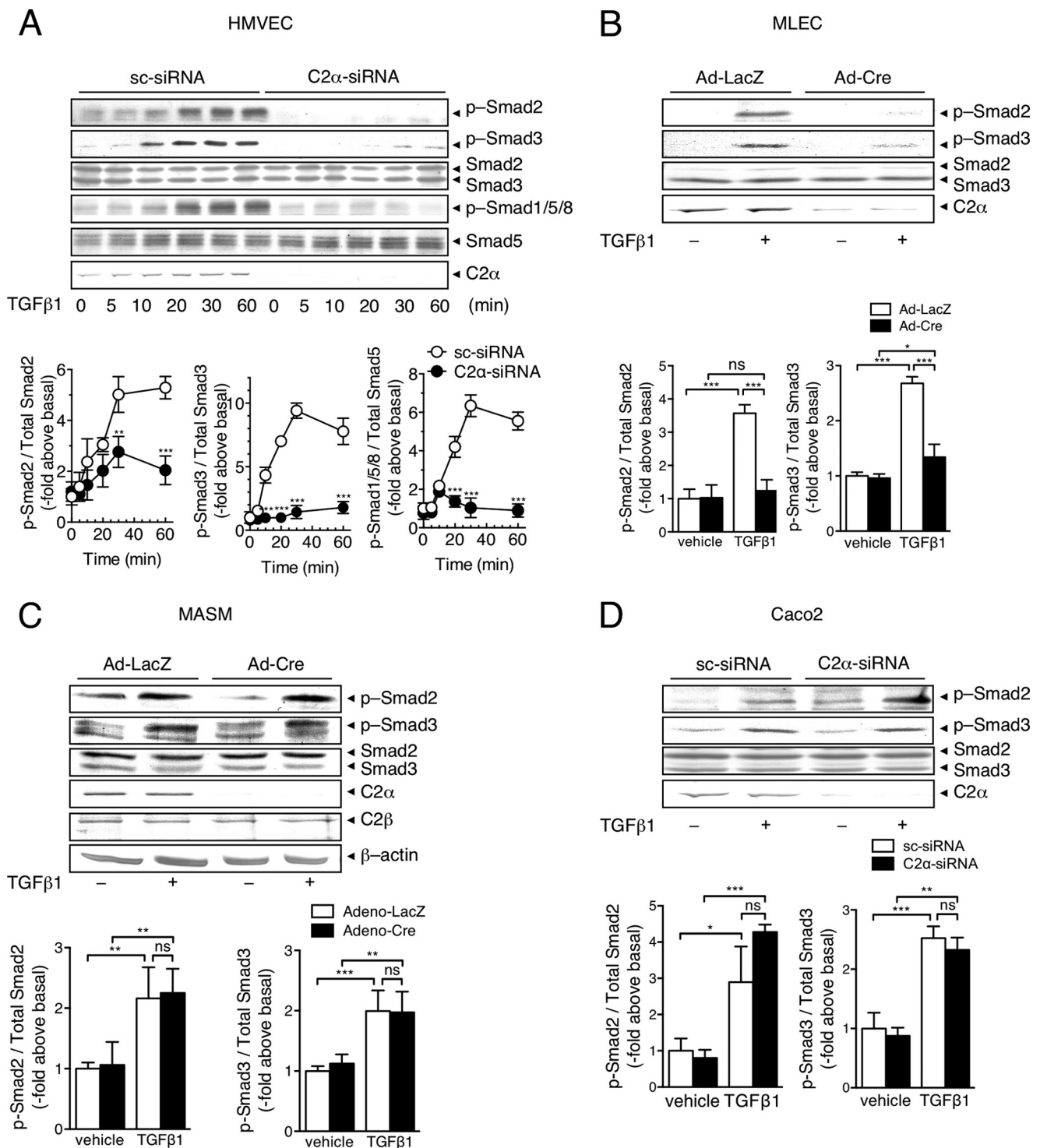


FIGURE 2. PI3K-C2 α is required for TGF β -induced Smad2/3 phosphorylation in EC but not smooth muscle cells or epithelial cells. A, TGF β 1-induced time-dependent phosphorylation of Smad2/3 and Smad1/5/8 in HUVECs. Serum-starved cells were stimulated with TGF β 1 (5 ng/ml) for the indicated time periods. The cell lysates were subjected to immunoblot analysis for p-Smad1/5/8 and p-Smad2/3. B, effects of Cre-mediated deletion of C2 α on TGF β 1-induced Smad3 phosphorylation in MLECs. The cells were infected with adenoviruses encoding LacZ (Ad-LacZ) or Cre recombinase (Ad-Cre), stimulated as in A, and analyzed for p-Smad3. C, effects of Cre-mediated deletion of C2 α on TGF β 1-induced Smad3 phosphorylation in MASM cells. The cells were treated and analyzed as in A. D, effects of knockdown of PI3K isoforms on TGF β 1-induced phosphorylation of Smad2/3. Caco2 were treated and analyzed as in A. In A–D, upper panels indicate representative blots, and lower panels indicate quantified data ($n = 3$ –5). The data are expressed as multiples relative to the values in TGF β 1-nonstimulated sc-siRNA transfected cells or Ad-Cre-transfected cells.

nuclear translocation. In sc-siRNA-transfected control HUVECs, the expression of kdC2 α inhibited TGF β 1-induced Smad2/3 nuclear accumulation, differently from wtC2 α expression (Fig. 3D). In C2 α -siRNA-transfected HUVECs, the expression of

kdC2 α did not restore TGF β 1-induced Smad2/3 nuclear accumulation, differently from wtC2 α . These observations together suggest that PI3K-C2 α fulfills an indispensable role for TGF β 1-induced Smad2/3 activation through its kinase activity in EC.

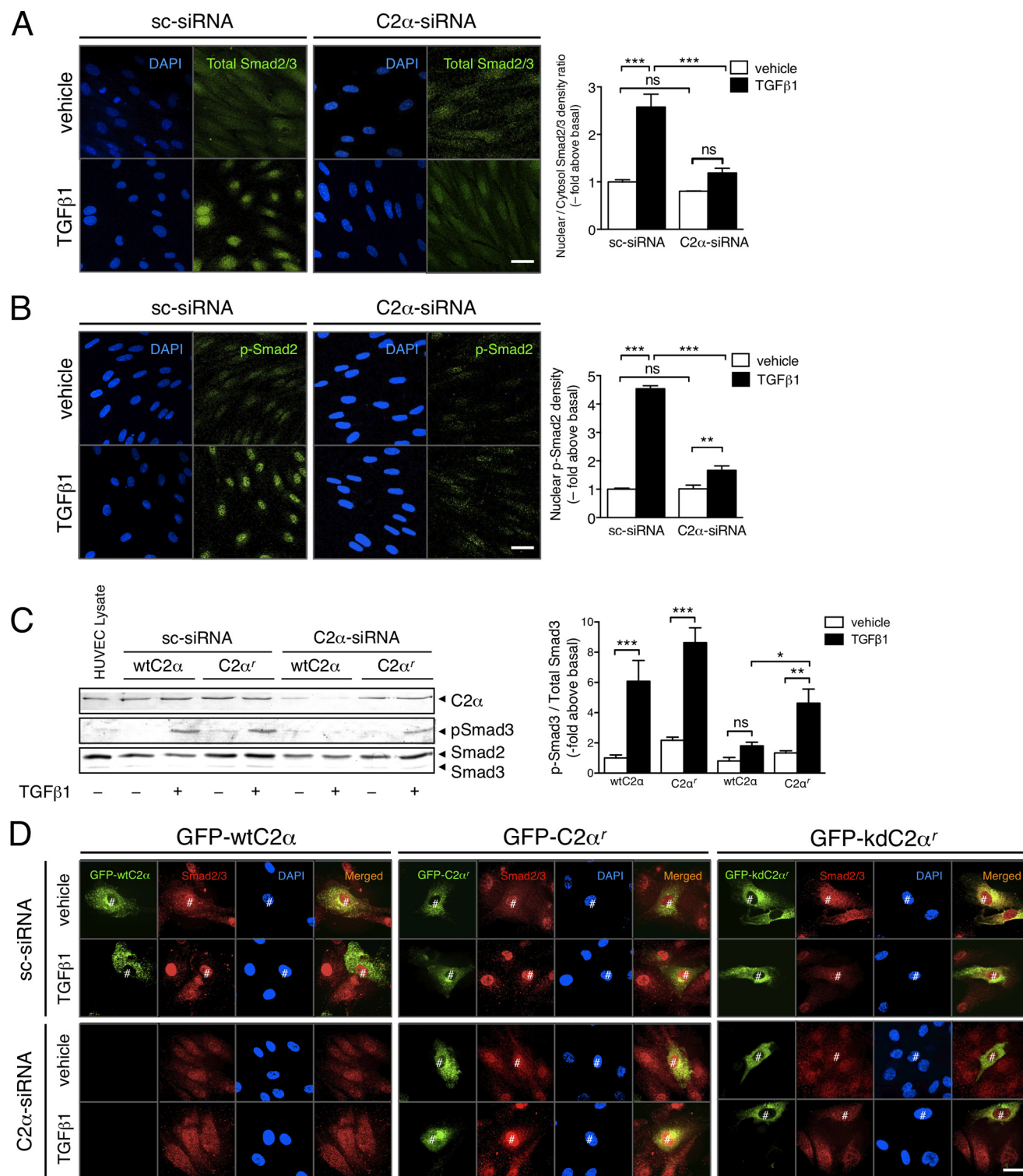


FIGURE 3. TGF β 1-induced nuclear translocation of Smad2/3 depends on PI3K-C2 α in EC. *A* and *B*, immunofluorescent staining of Smad2/3 (*A*) and p-Smad3 (*B*) in TGF β 1-stimulated HUVECs. The cells were transfected with C2 α #1 siRNA or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min, followed by anti-Smad2/3 antibody or anti-p-Smad3 antibody staining. Nuclei were stained by DAPI. *Left panels*, representative confocal images of the stained cells. *Right panels*, quantified data. The data were obtained from 48 cells per group. *Scale bar*, 20 μ m. *C*, HUVECs were transfected with either wtC2 α or C2 α -siRNA-resistant C2 α (C2 α^r) and either C2 α #1 siRNA or sc-siRNA. The cells were stimulated with TGF β 1 (5 ng/ml) for 30 min, followed by immunoblot analysis for p-Smad3. *Left panel*, representative blots. *Right panel*, quantified data ($n = 3$). *D*, HUVECs were transfected with either GFP-wtC2 α , GFP-C2 α^r , or GFP-tagged kinase deficient mutant of C2 α^r (GFP-kdC2 α^r) and either C2 α -specific siRNA or sc-siRNA. The cells were stimulated with TGF β 1 (5 ng/ml) for 30 min, followed by immunofluorescent staining with anti-Smad2/3. Nuclei were stained by DAPI. *Scale bar*, 20 μ m. #, transfected cells.

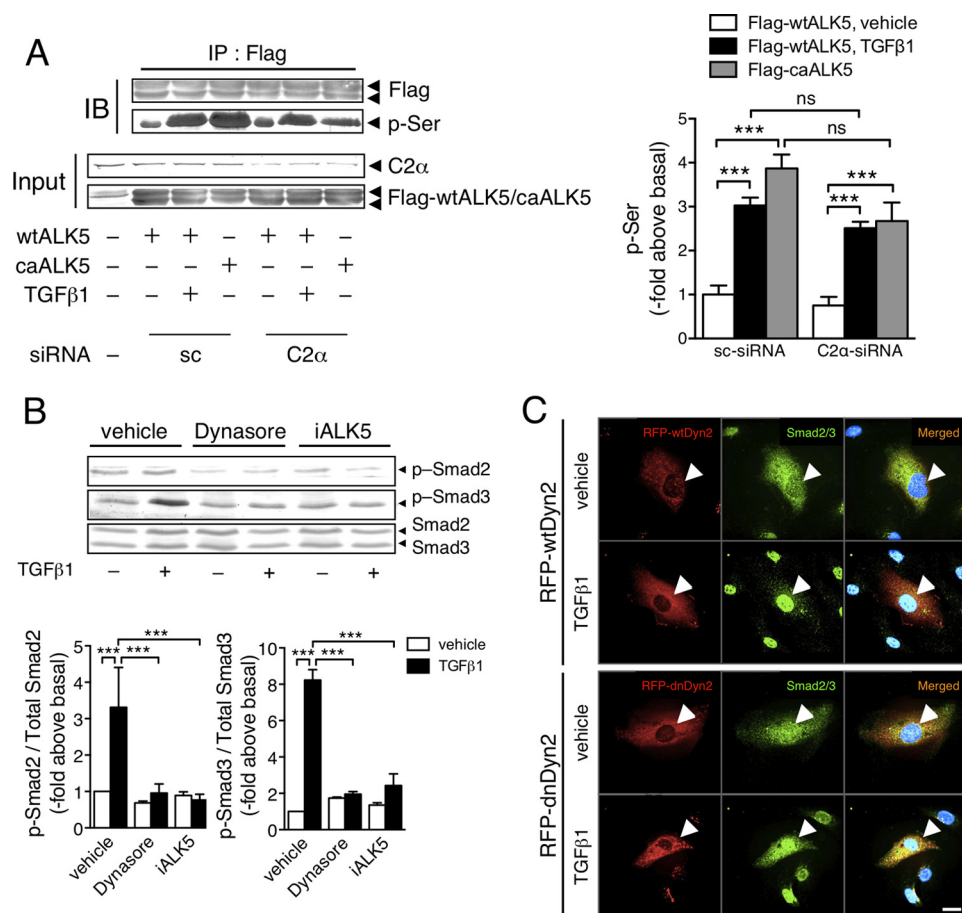


FIGURE 4. TGF β signaling requires PI3K-C2 α distally to TGF β 1-induced ALK5 phosphorylation and endocytosis. *A*, effects of C2 α depletion on TGF β 1-induced ALK5 phosphorylation. *Left panel*, representative blots. *Right panel*, quantified data. HEK293T cells were transfected with either FLAG-wtALK5 or FLAG-caALK5 and either C2 α #1 siRNA or sc-siRNA. The cell lysates were immunoprecipitated with anti-Flag antibody, followed by immunoblotting (IB) using anti-Flag and anti-phosphoserine (p-Ser) antibodies. A portion of the cell lysates was analyzed for the expression of the indicated proteins with IB. *IP*, immunoprecipitation. The data are from three independent experiments, which yielded comparable results, and are expressed as multiples over the values in wtALK5 expressed in sc-siRNA-transfected cells. *B*, effects of the endocytosis inhibitor dynasore and the ALK5 inhibitor (iALK5) on TGF β 1-induced Smad3 phosphorylation. The cells were pretreated or not with dynasore (80 μ M) or ALK5 inhibitor (5 μ M) for 30 min and stimulated with TGF β 1 (5 ng/ml) for 30 min ($n = 5$). *C*, effects of the expression of RFP wild-type dynamin2 (RFP-wtDyn2) or RFP dominant negative dynamin2 (RFP-dnDyn2) on TGF β 1-induced nuclear translocation of Smad2/3. The cells were transfected with the RFP-wtDyn2 or RFP-dnDyn2 expression vectors and stimulated with TGF β 1 (5 ng/ml) for 30 min, followed by anti-Smad2/3 immunostaining. Nuclei were stained by DAPI. The arrowheads denote the transfected cells. Scale bar, 20 μ m.

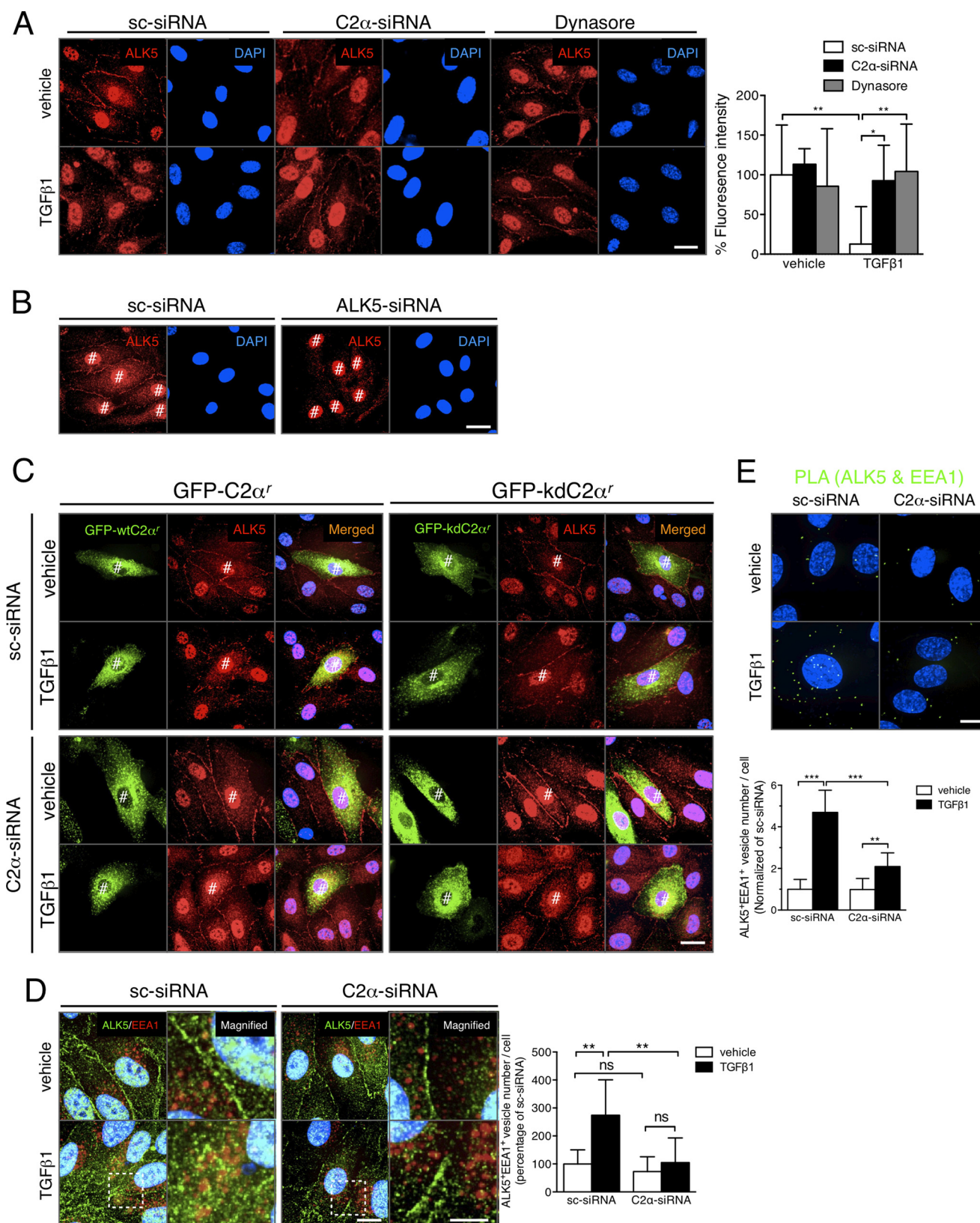
TGF β 1-induced TGF β Receptor Internalization into SARA-containing Endosomes Is Dependent on PI3K-C2 α —TGF β 1 stimulation induced an increase in serine phosphorylation of ALK5 in HEK293T cells transfected with wtALK5 (Fig. 4A). In HEK293T cells, we observed PI3K-C2 α dependence of TGF β 1-induced Smad2/3 phosphorylation (data not shown). PI3K-C2 α knockdown did not inhibit TGF β -induced serine phosphorylation of wtALK5, implying that PI3K-C2 α is necessary for the TGF β receptor signaling step, which is distal to phosphorylation of type I TGF β receptor. Previous studies (28–31) showed that TGF β 1 stimulation triggered clathrin-dependent endocytosis of TGF β receptor and that TGF β receptor endocytosis was required for TGF β activation of Smad2/3 signaling. We tested the effect of dynasore, an inhibitor of dynamin that is necessary for clathrin-dependent endocytosis, in EC. Treatment of HUVECs with dynasore abolished TGF β 1-induced phosphorylation of Smad2/3, like an ALK inhibitor (Fig. 4B). Likewise, the expression of the dominant negative dynamin2 mutant but not wild-type dynamin2 inhibited nuclear translocation of Smad2/3 (Fig. 4C). These observations suggested that

TGF β receptor endocytosis was required for Smad2/3 signaling. TGF β 1 stimulation promoted the internalization of type I TGF β receptor ALK5 into the intracellular compartment, which was prevented by PI3K-C2 α knockdown (Fig. 5A). Likewise, dynasore suppressed TGF β 1-induced ALK5 internalization. In these immunostainings, the anti-ALK5 antibody stained nuclei. ALK5 knockdown did not abolish or reduce the nuclear staining in anti-ALK5 immunostaining (Fig. 5B), suggesting that the nuclear staining was nonspecific. In sc-siRNA-transfected HUVECs, the expression of kdC2 α partially inhibited TGF β 1-induced ALK5 internalization, differently from that of wtC2 α expression (Fig. 5C). In C2 α -siRNA-transfected HUVECs, the expression of kdC2 α did not restore TGF β 1-induced ALK5 internalization. Double immunofluorescent staining of ALK5 and the early endosome marker EEA1 showed that TGF β 1 induced the internalization of ALK5 into the EEA1-positive early endosomes (Fig. 5D). PLA staining to detect interaction or close co-localization of two molecules showed that TGF β 1 stimulation induced the close co-localization of ALK5 and EEA1 (Fig. 5E, green dots), which was nearly abolished by PI3K-C2 α knockdown.

Crucial Role of PI3K-C2 α in TGF β Signaling

Because SARA is located in the early endosomes and acts as a scaffold for Smad2/3 phosphorylation by ALK5 (28–30), we studied the requirement of SARA for TGF β /ALK5 signaling, the possible co-localization of TGF β 1 receptors and SARA, and

the effect of PI3K-C2 α knockdown on the co-localization. Knockdown of SARA nearly completely suppressed TGF β -induced Smad2/3 phosphorylation (Fig. 6A), indicating that SARA is essential for TGF β /ALK5 signaling in HUVECs. Dou-



ble immunofluorescent staining using anti-SARA and anti-EEA1 showed that SARA was localized mainly in the EEA1-positive early endosome compartment in sc-siRNA-transfected HUVECs (Fig. 6B). TGF β 1 increased the SARA- and EEA1-double positive endosomes. PI3K-C2 α knockdown did not affect the numbers of either SARA-positive or EEA1-positive vesicles in nonstimulated cells but abolished TGF β 1-induced increase in SARA- and EEA1-double positive early endosomes. PLA staining for ALK5 and SARA, combined with anti-EEA1 immunostaining, showed that TGF β 1 stimulation induced the close co-localization of ALK5 and SARA and that a portion of the closely co-localized ALK5 and SARA existed in the EEA1-positive early endosomes (Fig. 6C). Knockdown of PI3K-C2 α inhibited the close co-localization of ALK5 and SARA in EEA1-positive early endosomes. These observations indicate that PI3K-C2 α is involved in TGF β 1-induced ALK5 internalization into the SARA-containing endosomes through its kinase activity.

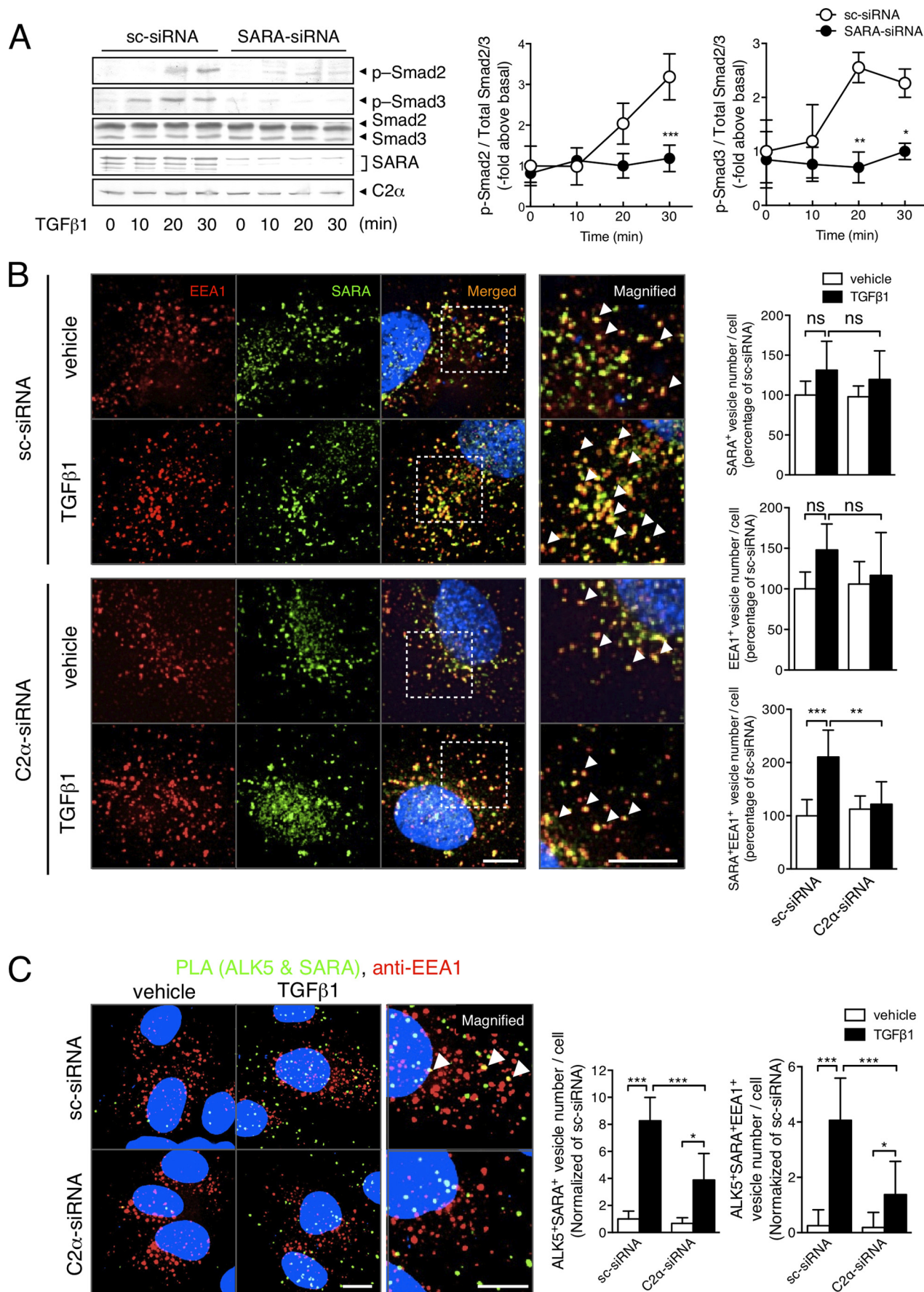
Endosomal Enrichment of PtdIns(3)P and Localization of SARA Are Not Dependent on PI3K-C2 α —PI3K-C2 α was previously reported to generate PtdIns(3)P and PtdIns(3,4)P₂ *in vivo* (8, 12–14). Because SARA is localized in early endosomes by binding PtdIns(3)P through its FYVE domain, we studied the effects of knockdown of PI3K-C2 α and other PtdIns(3)P-producing PI3K on the cellular level and localization of PtdIns(3)P and PtdIns(3,4)P₂ by observing GFP fluorescence of HUVECs that had been transfected with the expression vectors for the PtdIns(3)P-specific probe GFP-FYVE^{SARA} or the PtdIns(3,4)P₂-specific probe GFP-PH^{TAPP1}. In sc-siRNA-transfected nonstimulated HUVECs, the GFP-FYVE^{SARA} signal was a punctate pattern as reported previously in nonendothelial cells (29) (Fig. 7A), suggesting that PtdIns(3)P was enriched in endosomes. TGF β 1 stimulation did not change GFP-FYVE^{SARA} signal. Among three PI3Ks, knockdown of PI3K-C2 α did not affect GFP-FYVE^{SARA} signal. In contrast, that of either PI3K-C2 β or Vps34 obviously reduced the density of vesicular GFP-FYVE^{SARA} signals. Consistent with these observations, knockdown of either PI3K-C2 β or Vps34 reduced anti-SARA-positive endosomes unlike PI3K-C2 α knockdown and augmented the diffuse cytosolic anti-SARA staining (Fig. 7B), suggesting that PI3K-C2 β and Vps34 but not PI3K-C2 α generate PtdIns(3)P in SARA-containing endosomes. Thus, PI3K-C2 α seems to participate in TGF β -induced signaling, without altering the endosomal distribution of SARA.

In contrast to the effects on PtdIns(3)P level, TGF β 1 induced a rapid increase in GFP-PH^{TAPP1} signal in lamellipodia (Fig. 7C and supplemental Movies S1 and S2), suggesting a localized increase in the membrane PtdIns(3,4)P₂ level in TGF β 1-stimulated cells. Notably, PI3K-C2 α knockdown abolished TGF β 1-induced increase in GFP-PH^{TAPP1} signals, indicating that PI3K-C2 α is involved in the generation of PtdIns(3,4)P₂ in lamellipodia. The recent study implicated PI3K-C2 α -generated PtdIns(3,4)P₂ in endocytosis (14). Therefore, it is an interesting possibility that PI3K-C2 α is involved in TGF β 1-induced endocytosis of TGF β receptor through forming PtdIns(3,4)P₂ in the plasma membrane.

PI3K-C2 α Is Required for SARA-Smad2/3 Complex Formation—Because SARA is associated with Smad2/3 and acts as a scaffold for Smad2/3 phosphorylation by type I TGF β receptor ALK5, we studied the PI3K-C2 α dependence of SARA-Smad2/3 complex formation, using anti-SARA immunoprecipitation and the following anti-Smad2/3 immunoblotting. For the experiments, we employed HEK293T cells for efficiency of gene transduction. We co-transfected HEK293T cells with the expression vectors for either wtSARA or the Δ SBD-SARA and either wtALK5 or caALK5, with or without Smad2/3 expression vectors. We detected co-immunoprecipitation of Smad2/3 in the anti-SARA immunoprecipitates in the cells transfected with Smad2/3, wtSARA, and wtALK5. However, without Smad2/3 transfection, we did not detect Smad2/3 in the anti-SARA immunoprecipitates (Fig. 8A). In sc-siRNA-treated control cells that had been transfected with wtSARA and wtALK5, we detected the association of Smad3 and Smad2 with SARA (Fig. 8, B and C). The expression of caALK5 substantially stimulated the association of Smad3 and Smad2 with SARA and resultant phosphorylation of Smad3 and Smad2, which were both markedly inhibited by the expression of Δ SBD-SARA. In contrast, in PI3K-C2 α -depleted cells, caALK5 expression barely stimulated the association of Smad3 and Smad2 with SARA and phosphorylation of Smad3 and Smad2. Thus, PI3K-C2 α is required for ALK5-mediated formation of the SARA and Smad2/3 complex and phosphorylation of Smad2/3.

We also studied the interaction of endogenous SARA and Smad2/3 in HUVECs using PLA staining. TGF β 1 promoted SARA-smad2/3 interaction in the endosomes in sc-siRNA-transfected control HUVECs. A portion of the PLA signal was co-localized with EEA1, indicating that SARA and Smad2/3 complex were located in the early endosomes. PI3K-C2 α knockdown inhibited TGF β 1-stimulated SARA-smad2/3 interaction (Fig. 8D). The observations indicate that TGF β 1 stimulation of the

FIGURE 5. PI3K-C2 α is required for TGF β 1-induced internalization of TGF β receptor into the early endosomes in EC. A, effects of C2 α depletion or dynasore on TGF β 1-induced internalization of endogenous ALK5. The cells were either transfected with C2 α #1 siRNA or sc-siRNA or pretreated with dynasore (80 μ M) for 30 min and stimulated with TGF β 1 (5 ng/ml) for 30 min. *Left panel*, representative confocal images of the stained cells. *Right panel*, quantified data of fluorescence intensity per cell that were obtained from 24 cells per group. Nuclei were stained by DAPI. Scale bar, 20 μ m. B, effects of ALK5 knockdown on the nuclear staining in anti-ALK5 immunostaining. HUVECs were transfected with ALK5-specific siRNA (ALK5-siRNA) or sc-siRNA, followed by anti-ALK5 immunofluorescent staining. Nuclei were stained by DAPI. Scale bar, 20 μ m. #, nonspecific nuclear signals. C, effects of the expression of a kinase-deficient C2 α mutant on TGF β 1-induced ALK5 internalization. HUVECs were transfected with either GFP-C2 α ' or GFP-kdC2 α ' and either C2 α #1 siRNA or sc-siRNA. The cells were stimulated with TGF β 1 (5 ng/ml) for 30 min, followed by immunofluorescent staining with anti-ALK5 antibody. Nuclei were stained by DAPI. #, transfected cells. D, double immunofluorescent staining of ALK5 (green) and EEA1 (red) in TGF β 1-stimulated HUVECs. The cells were transfected with C2 α #1 siRNA or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min. *Left panel*, representative confocal images of the stained cells. Magnified views of the boxed areas are also shown. Nuclei were stained by DAPI. Scale bar, 20 μ m. *Right panel*, quantified data of the ALK5/EEA1-double positive vesicle numbers per cell that were obtained from 24 cells per group. E, PLA staining of ALK5 and EEA1 (green) in TGF β 1-stimulated HUVECs. The cells were transfected with C2 α #1 siRNA or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min. Nuclei were stained by DAPI. *Upper panel*, representative confocal images of the stained cells. Scale bar, 20 μ m. *Lower panel*, quantified data of the numbers of ALK5/EEA1 interactions per cell that were obtained from 24 cells per group.



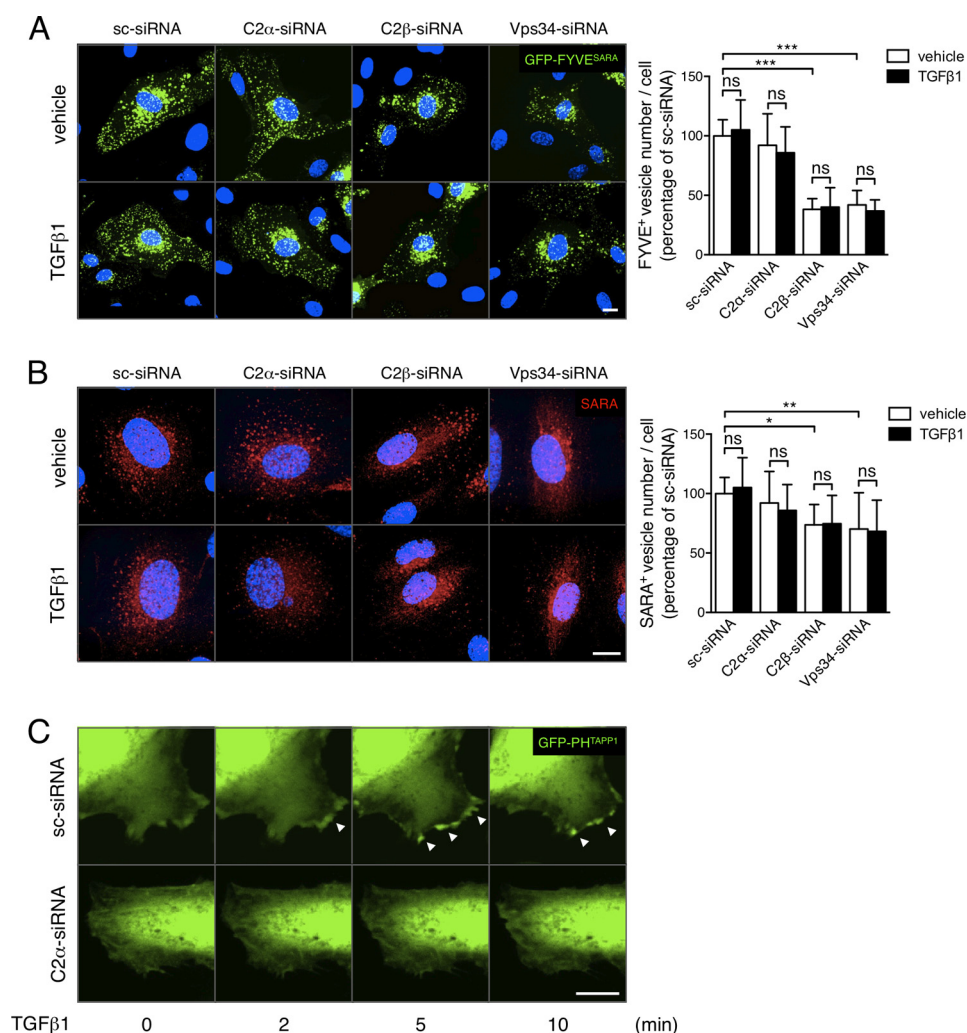


FIGURE 7. PI3K-C2 α is not required for PtdIns(3)P enrichment or the localization of SARA in the endosomes but for TGF β -induced increase in PtdIns(3,4)P₂ in lamellipodia. A, GFP-FYVE^{SARA} fluorescence. The HUVECs were co-transfected with the GFP-FYVE^{SARA} expression vector and either C2 α #1 siRNAs or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min or left untreated. Nuclei were stained by DAPI. Left panel, representative confocal images. Right panel, quantified data of the numbers of GFP-FYVE fluorescence-positive vesicles per cell that were obtained from 48 cells per group. B, anti-SARA immunofluorescent staining. The HUVECs were transfected with either C2 α -siRNAs or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min or left untreated. Nuclei were stained by DAPI. Left panel, representative confocal images. Right panel, quantified data of anti-SARA-positive vesicles per cell that were obtained from 48 cells per group. Scale bar, 20 μ m. C, GFP-PH^{TAPP1} fluorescence. The HUVECs were co-transfected with the GFP-PH^{TAPP1} expression vector and either C2 α -siRNAs or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min or left untreated. Nuclei were stained by DAPI. Confocal images at 2, 5, and 10 min after the additions of TGF β 1 or vehicle are shown.

interaction of endogenous SARA and Smad2/3 in the endosomes requires PI3K-C2 α in HUVECs.

PI3K-C2 α Is Required for TGF β 1-induced VEGF-A Expression in EC—Consistent with previous studies (38–41), TGF β 1 increased the expression of mRNA and protein of VEGF-A in control HUVECs (Fig. 9, A–C). The stimulatory effects of TGF β 1 were inhibited by knockdown of the common Smad Smad4, suggesting the involvement of Smad2/3. Furthermore,

TGF β 1-induced VEGF-A expression was abolished by the pharmacological blockade of ALK5 (Fig. 9D). These observations together suggested that TGF β 1-induced VEGF-A up-regulation was dependent on the ALK5-Smad pathway. In agreement with the involvement of the canonical Smad pathway in TGF β 1-induced VEGF-A expression, knockdown of PI3K-C2 α , but not PI3K-C2 β , p110 α , or Vps34, inhibited TGF β 1-induced VEGF-A expression (Fig. 9, A–C). Moreover, treat-

FIGURE 6. PI3K-C2 α is required for TGF β receptor internalization into SARA-containing endosomes in EC. A, effects of SARA depletion on TGF β 1-induced Smad2/3 phosphorylation in HUVECs. The cells were transfected with SARA-specific siRNA or sc-siRNA. Serum-starved cells were stimulated with TGF β 1 (5 ng/ml) for the indicated time periods and subjected to immunoblot analysis for p-Smad2, p-Smad3, and total Smad2 and Smad3. Left panel, representative blots. Right panel, quantified data ($n = 3$). B, double immunofluorescent staining of EEA1 (red) and SARA (green) in TGF β 1-stimulated HUVECs. The cells were transfected with C2 α #1 siRNA or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min. Nuclei were stained by DAPI. Right panel, quantified data of the numbers of SARA (upper panel), EEA1 (middle panel), and SARA/EEA1-double (lower panel) positive vesicle numbers per cell that were obtained from 48 cells per group. Scale bar, 20 μ m. C, PLA staining (green) of ALK5 and SARA and anti-EEA1 immunostaining (red) in TGF β 1-stimulated HUVECs. The cells were transfected with C2 α #1 siRNA or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min. Left panel, representative confocal images of the stained cells. Green and red denote PLA signals and immunostaining signals, respectively. Nuclei were stained by DAPI. Scale bar, 20 μ m. Right panel, quantified data of the numbers of PLA signals (left graph) and PLA signal/anti-EEA1-double positive vesicle numbers per cell (right graph) that were obtained from 24 cells per group.

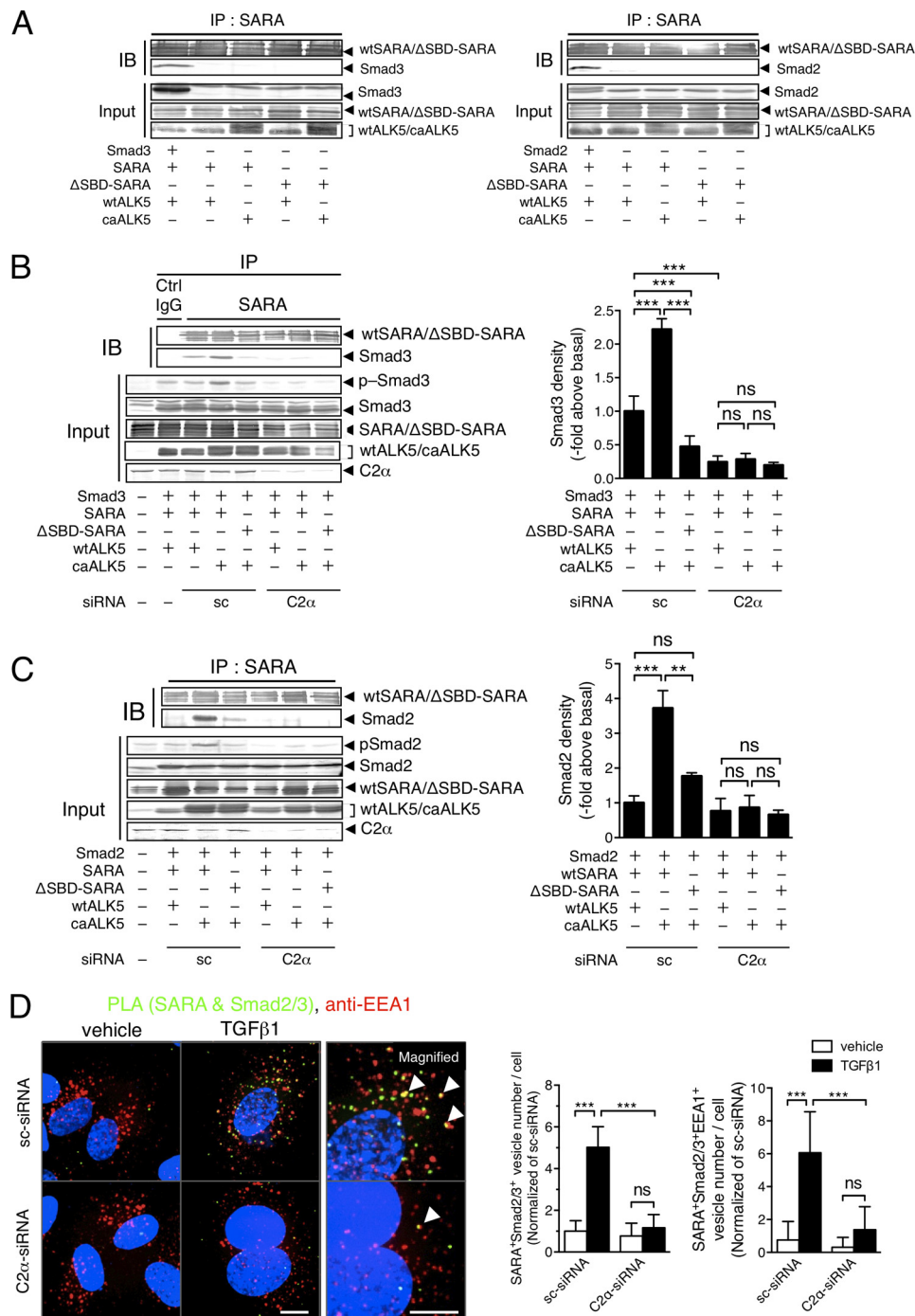


FIGURE 8. SARA-Smad3 complex formation is dependent on PI3K-C2 α . A–C, analyses of complex formation between SARA and Smad2/3 by co-immunoprecipitation-immunoblotting in HEK293T cells. A, the cells were transfected with the expression vectors for either wtSARA or Δ SBD-SARA and either wtALK5 or caALK5, with or without Smad2/3 expression vectors. The cell lysates were immunoprecipitated with anti-SARA antibody, followed by immunoblotting (IB) using anti-SARA, anti-Smad3, or anti-Smad2 antibody. B and C, the cells were co-transfected with the expression vectors for either Smad2 or Smad3, either wtSARA or Δ SBD-SARA, either wtALK5 or caALK5, and either C2 α #1 siRNA or sc-siRNA. The cell lysates were immunoprecipitated with anti-SARA antibody, followed by immunoblotting using anti-SARA, anti-Smad3 antibody in B, and anti-Smad2 antibody in C. Portions of the cell lysates were analyzed for the expression of the indicated proteins with immunoblotting (Input). IP, immunoprecipitation; Ctrl, control. Left panel, representative blots. Right panel, quantified data of the amounts of Smad3 and Smad2 in immunoprecipitates from the cells transfected as indicated. The data are means \pm S.E. from four independent experiments, which yielded comparable results, and expressed as multiples over the values in wtALK5- and sc-siRNA-transfected cells. D, PLA staining of SARA and Smad2/3 (green) and anti-EEA1 immunostaining (red) in TGF β 1-stimulated HUVECs. The cells were transfected with C2 α #1 siRNA or sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 30 min. Left panel, representative confocal images of the stained cells. Green and red denote PLA signals and immunostaining signals, respectively. Nuclei were stained by DAPI. Scale bar, 20 μ m. Right panel, quantified data of the numbers of PLA signals (left graph) and PLA signal/anti-EEA1 staining-double positive vesicle numbers per cell (right graph) that were obtained from 24 cells per group.

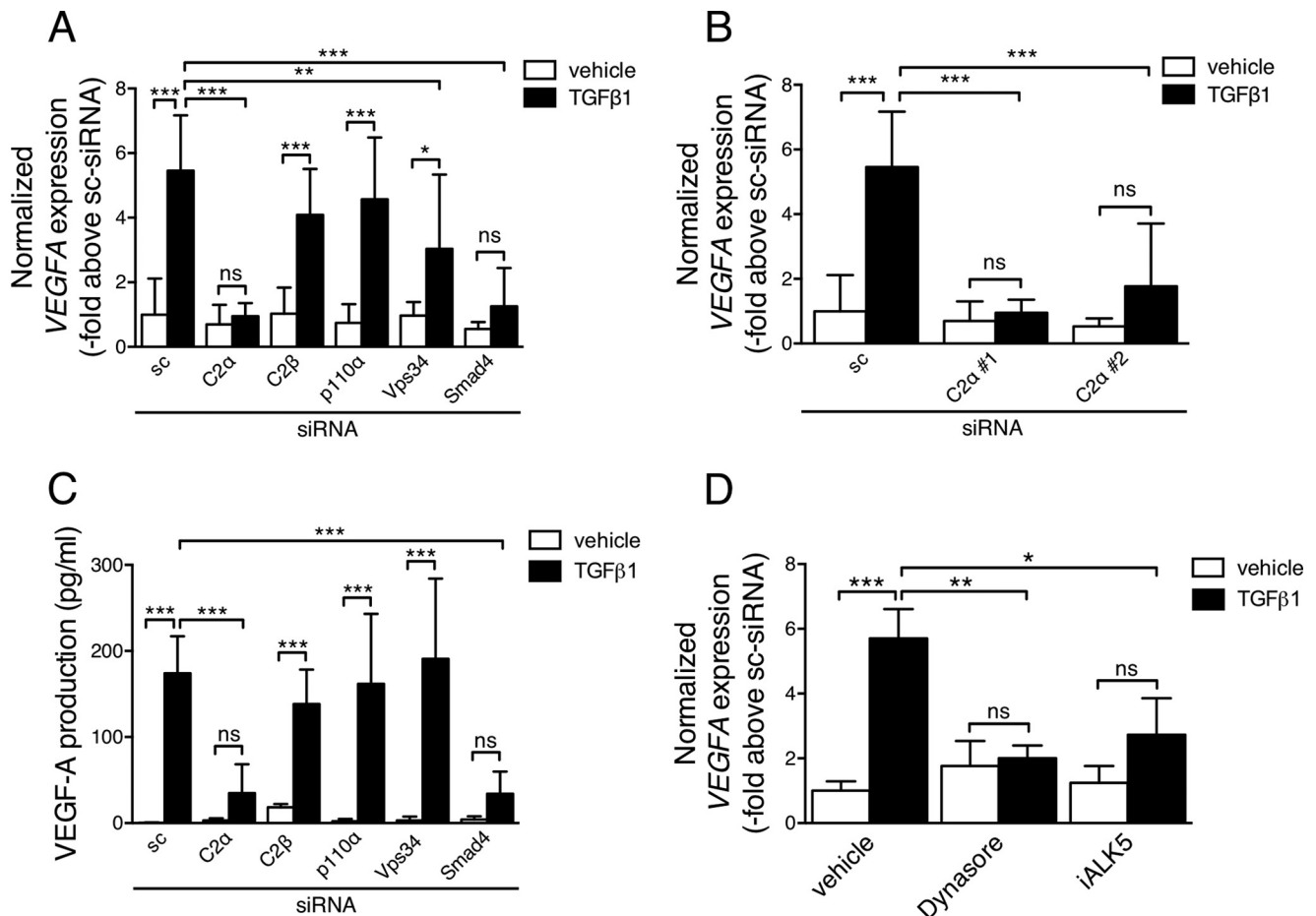


FIGURE 9. PI3K-C2 α is required for TGF β 1-induced, Smad-dependent VEGF-A production in EC. HUVECs were transfected with either of C2 α (#1), C2 β , p110 α , Vps34, and Smad4-specific siRNA or sc-siRNA and stimulated with TGF β 1 in the presence and absence of the indicated inhibitors. **A**, VEGF-A (VEGFA) mRNA expression in the cells stimulated with TGF β 1 (5 ng/ml) for 6 h. The VEGFA mRNA expression levels were determined with real time PCR and were corrected for 18 S rRNA level ($n = 6$). **B**, VEGFA mRNA expression in the cells were transfected with either of C2 α #1, C2 α #2, and sc-siRNA and stimulated with TGF β 1 (5 ng/ml) for 6 h. The VEGFA mRNA expression levels were determined with real time PCR and were corrected for 18 S rRNA level ($n = 3$). **C**, VEGF-A peptide concentrations in the media of the cells stimulated with TGF β 1 (5 ng/ml) for 12 h ($n = 3$). **D**, effects of dynasore and ALK5 inhibitor on VEGFA mRNA expression. The cells were pretreated or not with dynasore (80 μ M) or iALK5 (5 μ M) for 30 min and stimulated with TGF β 1 (5 ng/ml) for 6 h ($n = 5$). In **A–D**, the data are expressed as multiples over the values in TGF β 1-nonstimulated sc-siRNA-transfected or vehicle-treated control cells.

ment of HUVECs with dynasore abolished TGF β 1-induced VEGF-A mRNA expression with suppression of Smad3 phosphorylation (Figs. 4B and 9C). These findings indicate that PI3K-C2 α - and endocytosis-dependent Smad2/3 signaling mediates TGF β -induced VEGF-A expression in EC.

PI3K-C2 α Is Required for TGF β 1-induced Endothelial Cell Migration, Tube Formation, and in Vivo Angiogenesis—In a wound healing assay, PI3K-C2 α knockdown inhibited migration of HUVECs induced by either TGF β 1 or VEGF-A (Fig. 10, A and B). The ALK5 inhibitor suppressed TGF β 1-induced cell migration. Interestingly, the inhibitor of VEGFR2 suppressed not only VEGF-induced but also TGF β 1-induced cell migration, indicating that TGF β 1-induced cell migration is dependent on VEGFR2. Likewise, PI3K-C2 α knockdown inhibited tube formation induced by either TGF β 1 or VEGF-A (Fig. 10, C and D). The inhibition of VEGF-A-induced tube formation by PI3K-C2 α knockdown is most likely because VEGFR2 signaling is dependent on PI3K-C2 α as we demonstrated previously (8). The ALK5 inhibitor and the VEGFR2 inhibitor blocked TGF β 1-induced tube formation (Fig. 10, E and F). In addition, dynasore suppressed TGF β 1-induced tube formation. These

observations suggest that TGF β 1-induced, ALK5-mediated stimulation of endothelial migration and morphogenesis is dependent on stimulation of VEGF-A expression and VEGFR2 signaling, in which PI3K-C2 α and the endocytic process are involved.

We finally investigated a role for PI3K-C2 α in *in vivo* angiogenesis, using a Matrigel plug assay in conditional PI3K-C2 α knock-out mice. We subcutaneously injected Matrigel plug with or without TGF β 1 in mice with endothelial specific deletion of PI3K-C2 α (C2 α ^{ΔEC}) or smooth muscle-specific PI3K-C2 α deletion (C2 α ^{ΔSMC}) and compared angiogenesis in both mutant mice with that in control mice (C2 α ^{flax/flax}). The inclusion of TGF β 1 in Matrigel increased the formation of anti-vWF-positive microvessels in Matrigel plugs in control mice, compared with vehicle (Fig. 11, A–F). In contrast, in C2 α ^{ΔEC} mice TGF β 1 failed to stimulate microvessel formation. In C2 α ^{ΔSMC} mice, however, TGF β 1 stimulated microvessel formation in Matrigel plugs to the similar extent as in control mice (Fig. 11, D–F). We performed double immunofluorescent staining of p-Smad2 and EC marker CD31 in Matrigels containing either TGF β or vehicle that had been implanted in C2 α ^{ΔEC}

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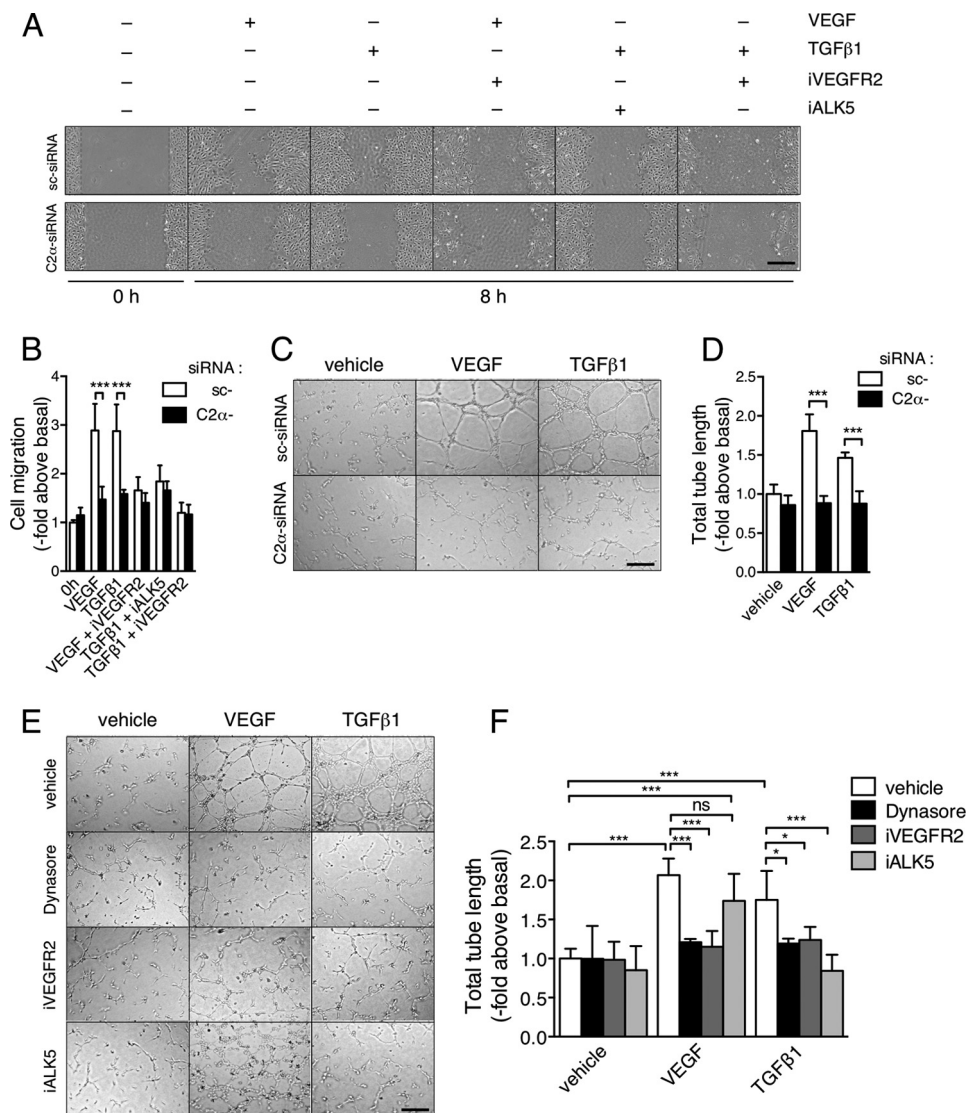


FIGURE 10. TGF β 1-induced endothelial cell migration and tube formation are VEGFR2-mediated and PI3K-C2 α -dependent. A and B, effects of inhibitors of VEGFR2 and ALK5 on TGF β 1- and VEGF-induced cell migration. HUVECs were stimulated with TGF β 1 (5 ng/ml) or VEGF (50 ng/ml) in the presence and absence of iALK5 (5 μ M) and the VEGFR2 inhibitor (iVEGFR2) (10 μ M). Cell migration was determined with scratch wounding healing assay. A, representative microscopic views. B, quantified data ($n = 4$). C and D, effects of C2 α -knockdown on TGF β 1- and VEGF-induced tube formation. siRNA-transfected cells were stimulated with VEGF-A (50 ng/ml) or TGF β 1 (5 ng/ml) for 12 h. C, representative microscopic views; D, quantified data ($n = 4$). E and F, effects of dynasore (80 μ M), iVEGFR2 (10 μ M), and iALK5 (5 μ M) on TGF β 1- and VEGF-induced tube formation. E, representative microscopic views. F, quantified data ($n = 4$).

and C2a^{DSMC} mice. C2a^{DEC} mice showed much reduced p-Smad2- and CD31-double positive cells compared with C2a^{DSMC} (Fig. 11G), suggesting that Smad2 activation in EC was attenuated in C2a^{DEC} mice. These observations suggest that TGF β 1-induced microvessel formation in Matrigel plugs is dependent on PI3K-C2 α that is expressed in EC but not smooth muscle.

DISCUSSION

Accumulated evidence indicates that TGF β receptor-Smad2/3 signaling is dependent on the endocytosis of the TGF β receptor complex (28–31). Upon TGF β binding, TGF β receptors are internalized into early endosomes, where the Smad anchor SARA is enriched through its FYVE domain. SARA interacts with Smad2/3, facilitating Smad2/3 phosphorylation and thereby their nuclear translocation. PI3K may be involved in at least two steps of

these TGF β receptor signaling processes: TGF β -induced TGF β receptor internalization and the endosomal localization of SARA. In the present study, we identified class II PI3K-C2 α as PI3K isoform that is engaged in TGF β -induced activation of Smad2/3 signaling. Our data indicate that PI3K-C2 α is required for the endocytosis of TGF β receptor but not for endosomal localization of SARA.

The present observation in EC that TGF β -induced Smad2/3 phosphorylation is dependent on TGF β receptor internalization into the EEA1-positive, SARA-containing early endosomes (Figs. 4–6) is similar to the previous observations in other types of cells including HeLa cells, HepG2 cells, and Mv1Lu cells (28, 30, 31), although some discrepant results on the necessity of SARA for ALK5/Smad2/3 signaling were reported (42, 43). Either PI3K-C2 α depletion (~80–90%) or the expression of the kinase-deficient C α mutant strongly sup-

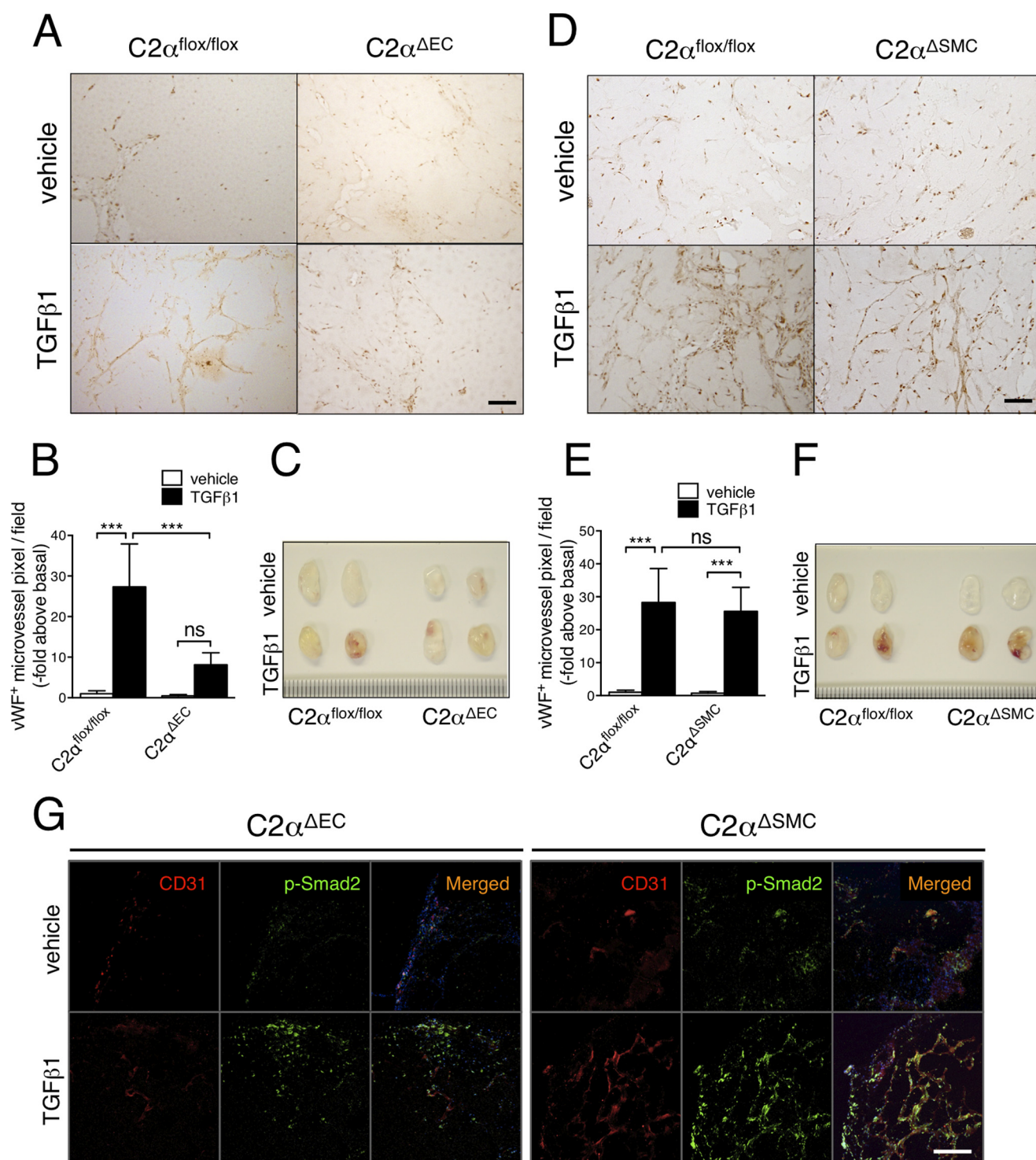


FIGURE 11. Endothelial PI3K-C2 α is necessary for TGF β 1-induced angiogenesis in vivo. Matrigels containing PBS (vehicle) or TGF β 1 were injected into the subcutaneous tissues on the back of EC-specific C2 α -deleted ($C2\alpha^{\Delta EC}$), SMC-specific C2 α -deleted ($C2\alpha^{\Delta SMC}$), and control ($C2\alpha^{flox/flox}$) mice. Matrigel plugs were removed 10 days later and analyzed for microvascular formation by immunohistochemistry using anti-vWF antibody. *A* and *D*, representative views of anti-vWF immunostained sections of Matrigel plugs in $C2\alpha^{\Delta EC}$ (*A*) and $C2\alpha^{\Delta SMC}$ (*D*) mice. *B* and *E*, quantified data of neovessel formation in Matrigel plugs (seven mice per group). *C* and *F*, representative gross views of Matrigel plugs resected from mice. *G*, double immunofluorescent staining of CD31 (red) and p-Smad2 (green) in Matrigel plugs in $C2\alpha^{\Delta EC}$ (left panel) and $C2\alpha^{\Delta SMC}$ (right panel) mice. Nuclei were stained by DAPI. Scale bar, 50 μ m.

pressed the internalization of TGF β receptor, like the dynamin inhibitor dynasore (Fig. 5). Because either PI3K-C2 α depletion or dynasore markedly inhibited TGF β -induced Smad2/3 phosphorylation (Figs. 1, *B* and *C*, and 4*B*), there is a good correlation between TGF β -induced TGF β receptor internalization

and Smad2/3 phosphorylation. As discussed in detail below, PI3K-C2 α depletion did not compromise the endosomal distribution of the Smad anchor SARA. Based on these findings, it is reasonable to suggest that PI3K-C2 α is involved in TGF β receptor-activated Smad2/3 signaling largely through regulat-

ing TGF β receptor internalization. In addition, the overexpression of wtC2 α did not affect TGF β -induced Smad2/3 activation or ALK5 internalization, suggesting that the endogenous level of wtC2 α was sufficient for full activation of TGF β receptor-induced Smad2/3 signaling.

We recently demonstrated in EC that ligand binding-triggered endocytosis of two different classes of cell surface receptors, VEGFR2 and S1P₁, was dependent on PI3K-C2 α (8, 15). Interestingly, PI3K-C2 α depletion inhibited only a part of multiple signaling pathways activated by VEGF and S1P: Rho activation in VEGF signaling and Rac activation in S1P signaling. We observed using FRET imaging technique that both VEGF-induced Rho activation and S1P-induced Rac activation occurred in PtdIns(3)P-enriched endosomes, as well as the plasma membrane. The present study together with those previous observations indicate that PI3K-C2 α participates in signaling on the endosomes, upon the activation of different classes of receptors including receptor tyrosine kinases, G protein-coupled receptors, and receptor serine/threonine kinases. Thus, the ability of PI3K-C2 α to regulate the endocytosis of different classes of cell surface receptors controls endosomal signaling.

A recent study (14) suggested a novel mechanism about a general role of PI3K-C2 α in clathrin-dependent endocytosis in non-vascular cells; the formation of PtdIns(3,4)P₂ by PI3K-C2 α at clathrin-coated pits and late endocytic intermediates before dynamin-mediated fission recruited the PtdIns(3,4)P₂-effector protein SNX9, promoting maturation of clathrin-coated pits toward endocytic vesicles. They suggested that PI3K-C2 α formed PtdIns(3,4)P₂ from PtdIns(4)P, which was generated through 5'-dephosphorylation of PtdIns(4,5)P₂ enriched in the clathrin-coated pits. We observed that TGF β 1 induced the rapid and sustained formation of lamellipodia with a local lamellipodial increase in PtdIns(3,4)P₂ (Fig. 7C). In the lamellipodial region of the plasma membrane, endocytosis carries membrane-anchored Rho GTPases and integrins to the cell interior, and these molecules are recycled to the specific regions of the plasma membrane, which promotes lamellipodial protrusion (44). Considering the rapid onset of TGF β 1-induced lamellipodial formation and an increase in PtdIns(3,4)P₂ level, these responses very likely represent non-genomic effects of TGF β 1. It remains to be clarified how TGF β induces a rapid increase in the level of PtdIns(3,4)P₂ through a mechanism involving PI3K-C2 α in EC.

The interaction of the FYVE domain in SARA with PtdIns(3)P, a predominant phosphoinositide in the endosomes, serves the endosomal localization of SARA (28, 29, 45). Previous studies (12, 46) showed that PI3K-C2 α formed PtdIns(3)P in cells. These observations together with the finding of the endosomal localization of PI3K-C2 α (8) suggested that PI3K-C2 α might be responsible for enrichment of PtdIns(3)P in the endosomes. However, the present study showed that PI3K-C2 α depletion did not reduce PtdIns(3)-enriched endosomes (Fig. 7A). Instead, knockdown of either PI3K-C2 β or Vps34 reduced PtdIns(3)-enriched endosomes. In agreement with these findings, knockdown of PI3K-C2 β or Vps34 but not PI3K-C2 α reduced SARA-containing vesicles (Fig. 7B). Our observations of the effects of PI3K-C2 β and Vps34 depletion on SARA distribution are consistent with the previous reports showing that

the nonselective PI3K inhibitor wortmannin totally changed the endosomal localization of SARA to a diffuse cytosolic pattern with inhibition of Smad signaling at the relatively low concentrations of 50–100 nM (29, 45). This range of concentration of wortmannin does not effectively inhibit PI3K-C2 α because PI3K-C2 α is relatively resistant to wortmannin compared with the other PI3K (5, 7, 47). Thus, PI3K-C2 α is not a major enzyme to be responsible for the accumulation of PtdIns(3)P in the SARA-localized vesicular compartment. Our data indicate that the other PI3K including class II PI3K-C2 β and class III Vps34 are involved in PtdIns(3)P accumulation in this compartment. Another point of interest is that partial reductions (25–30%) of SARA association with the endosomes by depletion of either PI3K-C2 β or Vps34 (Fig. 7B) did not inhibit Smad2/3 phosphorylation (Fig. 1C), which suggests that such partial reductions of the SARA association with the endosomes do not compromise Smad signaling.

We observed that PI3K-C2 α depletion suppressed TGF β -induced Smad2/3 signaling in several different vascular EC of human and mouse origins, but not vascular smooth muscle or epithelial cells (Figs. 1 and 2). In addition, Smad2/3 phosphorylation mediated by caALK5 in HEK293 cells was also PI3K-C2 α -dependent (Fig. 8). Therefore, there appears to be some cell type specificity concerning the PI3K-C2 α dependence. A few explanations for this may be possible. Another class II PI3K member, PI3K-C2 β , possesses the similarities to PI3K-C2 α , in the distribution of the expression, the structure, the clathrin binding capacity, and the substrate specificity (7). Functionally, PI3K-C2 β is necessary for growth factor signaling (48) and cell migration with its lamellipodial distribution (49). Therefore, in vascular smooth muscle cells and epithelial cells, PI3K-C2 β may be able to compensate for PI3K-C2 α depletion. Alternatively, it might be possible that TGF β -induced Smad2/3 signaling could be cell type-specific, because it was reported that the dependence of Smad2/3 signaling on endocytosis differed, depending on cell type (50).

In EC, TGF β -induced activation of Smad2/3 signaling pathway is linked to the up-regulation of VEGF-A gene expression (39, 41). Consistent with the essential role of PI3K-C2 α in TGF β -induced Smad signaling activation, TGF β stimulation of VEGF-A expression was completely and specifically dependent on PI3K-C2 α (Fig. 9). Interestingly, TGF β -induced endothelial cell migration and tube formation were both dependent on VEGFR2 (Fig. 10). It is reasonable to conceive that VEGF-A-stimulated cell migration and tube formation requires PI3K-C2 α because VEGFR2 signaling and transport of VE-cadherin and other molecules are dependent on vesicular trafficking (8, 51). Hence, TGF β -induced stimulation of these cellular responses requires PI3K-C2 α for at least at two steps, *i.e.* TGF β -induced Smad2/3 signaling-dependent VEGF expression and VEGF activation of VEGFR2 signaling. PI3K-C2 α has a significant *in vivo* functional role in TGF β -induced neovessel formation at an organismal level as demonstrated by the observations in EC-specific PI3K-C2 α -deleted mice (Fig. 12).

Previous studies showed that TGF β stimulated proliferation and migration of ECs via ALK1, whereas TGF β inhibited proliferation and migration of ECs via ALK5 (24, 52), although some studies (41, 53) showed that ALK5-mediated stimulation of cell migra-

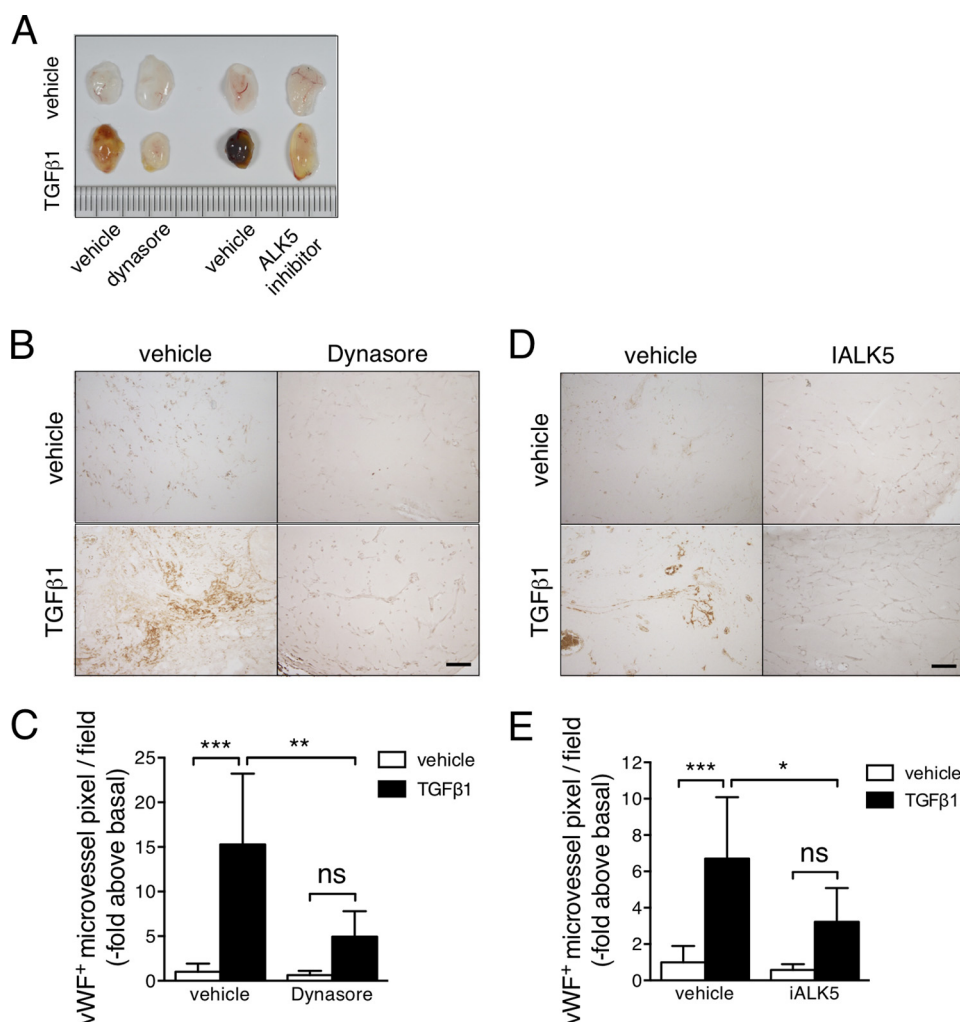


FIGURE 12. Receptor endocytosis and ALK5 are necessary for TGF β 1-induced angiogenesis *in vivo*. Matrigels containing of PBS (*vehicle*) or TGF β 1 and dynasore (200 μ M) or IALK5 (20 μ M) were injected into the subcutaneous tissues on the back. Matrigel plugs were removed 10 days later and analyzed for microvascular formation by immunohistochemistry using anti-vWF antibody. *A*, representative gross views of Matrigel plugs resected from mice. *B* and *D*, representative views of anti-vWF immunostained sections of Matrigel plugs containing dynasore (*left panel*) and IALK5 (*right panel*). *C* and *E*, quantified data of the effects of dynasore and IALK5 on neovessel formation in Matrigel plugs (six mice per group).

tion. The present observations suggest that ALK5-mediated stimulation of EC migration involves an indirect mechanism, *i.e.* stimulation by autocrine/paracrine VEGF. TGF β -induced phosphorylation of Smad1/5/8 was dependent on PI3K-C2 α , as well as Smad2/3 phosphorylation (Fig. 2A). It is intriguing to see how ALK1-mediated Smad1/5/8 phosphorylation is dependent on PI3K-C2 α in a future study.

In summary, the present study indicates that PI3K-C2 α is indispensable for TGF β -induced Smad signaling through being engaged in the internalization of TGF β receptors into the Smad anchor SARA-containing early endosomes. Thus, our study suggests that PI3K-C2 α is essential for endosomal signaling of TGF β receptors. The elucidation of the role for PI3K-C2 α in TGF β receptor signaling opens the new avenue for understanding in more depth normal TGF β actions and their derangements in diseases.

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Signal Transduction:
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Sho Aki, Kazuaki Yoshioka, Yasuo Okamoto,
Noriko Takuwa and Yoh Takuwa
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