TGFβ receptor endocytosis and Smad signaling require synaptojanin1, PI3K–C2α-, and INPP4B-mediated phosphoinositide conversions

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ABSTRACT Phosphoinositide conversion regulates a diverse array of dynamic membrane events including endocytosis. However, it is not well understood which enzymes are involved in phosphoinositide conversions for receptor endocytosis. We found by small interfering RNA (siRNA)-mediated knockdown (KD) that class II PI3K δ-isomorph PI3K-C2α, the 5′-phosphatase synaptojanin1 (Synj1), and the 4′-phosphatase INPP4B, but not PI3K-C2β, Synj2, or INPP4A, were required for TGFβ-induced endocytosis of TGFβ receptor. TGFβ induced rapid decreases in P(4,5)P₂ at the plasma membrane (PM) with increases in P(4)P, followed by increases in P(3,4)P₂ in a TGFβ receptor kinase ALK5-dependent manner. TGFβ induced the recruitment of both synaptojanin1 and PI3K-C2α to the PM with their substantial colocalization. Knockdown of synaptojanin1 abolished TGFβ-induced P(4,5)P₂ decreases and P(4)P increases. Interestingly, PI3K-C2α KD abolished not only TGFβ-induced P(3,4)P₂ increases but also TGFβ-induced synaptojanin1 recruitment to the PM, P(4,5)P₂ decreases, and P(4)P increases. Finally, the phosphoinositide conversions were necessary for TGFβ-induced activation of Smad2 and Smad3. These observations demonstrate that the sequential phosphoinositide conversions mediated by Synj1, PI3K-C2α, and INPP4B are essential for TGFβ receptor endocytosis and its signaling.

INTRODUCTION Phosphoinositides, which possess one or more phosphates at the 3-, 4-, and/or 5-OH groups of the inositol ring of phosphatidylinositol (PI), are present in the membrane in relatively small amounts and serve species-specific distinct roles in diverse processes including cell proliferation, cell migration, intracellular trafficking including endocytosis, and cytoskeletal organization (Di Paolo and De Camilli, 2006; Balla, 2013). Class I PI3Ks are activated largely by receptor tyrosine kinases and G protein-coupled receptors to regulate cell proliferation, cell migration, cell metabolism mainly by generating three classes of PI 3-kinases (PI3Ks) to generate PI(3)P, PI 3,4-bisphosphates (PI(3,4)P₃), and PI 3,4,5-trisphosphates (PI(3,4,5)P₃). Thus, phosphoinositides are subjected to the conversion by kinases and phosphatases during the progress of endocytosis (Shin et al., 2005; Billcliff and Lowe, 2014; Marat and Haucke, 2016).

The 3′-phosphorylation of phosphoinositides is catalyzed by three classes of PI 3-kinases (PI3Ks) to generate PI(3)P, PI 3,4-bisphosphates (PI(3,4)P₃), and PI 3,4,5-trisphosphates (PI(3,4,5)P₃) (Bilanges et al., 2019). Class I PI3Ks are activated largely by receptor tyrosine kinases and G protein-coupled receptors to regulate cell proliferation, cell migration, and cell metabolism mainly by generating...
PI(3,4,5)P₂, and class III PI3K Vps34 generates PI(3)P to regulate the autophagic pathway. Class II PI3Ks, which comprise PI3K-C2α (C2α), PI3K-C2β (C2β), and PI3K-C2γ, produce PI(3,4)P₂ and PI(3)P (Wallroth and Hauke, 2018; Gulluni et al., 2019). Previous studies (Domin et al., 2000; Gaidarov et al., 2001; Yoshio et al., 2012; Biswas et al., 2013; Posor et al., 2013; Franco et al., 2014; He et al., 2017) showed that, among class II PI3K members, C2α regulates endocytosis and other membrane traffic events. Posor et al. (2013) demonstrated that C2α preferred PI 4-phosphate (PI(4)P) rather than PI as substrate to mainly produce PI(3,4)P₂ and that a reduction of PI(3,4)P₂ by the overexpression of a phosphoinositide 4′-phosphatase or knockdown (KD) of C2α resulted in inhibition of CME with prolonged maturation of CCPs, indicating an indispensable role of PI(3,4)P₂ in CME. Conversely, expression of the mutated hyperactive C2α enhanced PI(3,4)P₂ production and endocytosis (Wang et al., 2018). Moreover, they identified the effector molecule of PI(3,4)P₂, sorting nexin (SNX)9, which was recruited to CCPs following the accumulation of C2α (Posor et al., 2013; Lo et al., 2017; Schönberg et al., 2017). Previous studies (Perera et al., 2006; Antonescu et al., 2011) showed that the PI(4,5)P₂-specific 5′-phosphatase synaptojanin-1 (Synj1), which converts PI(4,5)P₂ to PI(4)P, is recruited to CCPs, suggesting that Synj1 participates in the generation of PI(3,4)P₂. Recent studies also showed that the inositol polyphosphate 4-phosphatases (INPPs) negatively regulated signaling through dephosphorylating PI(3,4)P₂ (Gewinner et al., 2009; Ivetac et al., 2009). However, the production and breakdown of PI(3,4)P₂ in CME were not fully understood.

We recently showed in vascular endothelial cells (EC) that C2α, but not C2β, was required for TGFB1-induced, clathrin-dependent endocytosis of TGFB1 receptor serine/threonine kinases and subsequent receptor-mediated phosphorylation of the transcriptional regulators Smad2 and Smad3 (Smad2/3), which occurred in endosomes (Aki et al., 2015). TGFB1 induced a rapid increase in the PI(3,4)P₂ level at the PM in a C2α-dependent manner in ECs. In the present study, we sought to understand phosphoinositide conversions for the production and breakdown of PI(3,4)P₂, the enzymes responsible for conversions, and their requirements in TGFB1-induced receptor endocytosis. We found that TGFB1-induced rise in PI(3,4)P₂ required Synj1, which mediated the earliest responses after the addition of TGFB1, that is, PI(4,5)P₂ decrease and PI(4)P rise. The 4′-phosphatase INPP4B (Li and Marshall, 2015) seemed to mediate the conversion of PI(3,4)P₂ to PI(3)P. Notably, Synj1 and INPP4B, as well as C2α, were essential for TGFB1-induced receptor endocytosis and Smad2/3 signaling. Furthermore, our data showed that TGFB1 induced the rapid colocalization of C2α and Synj1 at the PM and that C2α, as well as Synj1, was required for TGFB1-triggered 5′-dephosphorylation of PI(4,5)P₂, suggesting that TGFB1 induced the cooperation of Synj1 and C2α. Thus, these data showed an essential role of the phosphoinositide cascade triggered by the cooperating action of Synj1 and C2α for the receptor ligand-induced endocytosis and the following signaling.

RESULTS

Synj1 and INPP4B, as well as C2α, are required for TGFB1-induced phosphorylation and nuclear translocation of Smad2/3 and TGFB1 receptor endocytosis

We first explored the possible involvement of the phosphoinositide-converting enzymes, which relate to the generation and catabolism of the key phosphoinositide, PI(3,4)P₂, in TGFB1-induced Smad signaling. Toward this end, we studied the effects of specific KD of the 5′-specific phosphatases Synj1 and Synj2 that catalyze PI(4,5)P₂ conversion to the C2α substrate PI(4)P (Perera et al., 2006), the 4′-specific phosphatases INPP4A and INPP4B that convert PI(3,4)P₂ to PI(3)P (Li and Marshall, 2015), and MTMR6 that converts PI(3)P to PI (Maekawa et al., 2014) on TGFB1-induced receptor endocytosis and Smad signaling in human umbilical vascular EC (HUVEC). Each specific siRNA effectively inhibited the mRNA expression of the respective enzymes but not other enzymes compared with transfection of scrambled small interfering RNA (sc-siRNA) (Figure 1A). We also confirmed that the siRNAs against Synj1, C2α, and INPP4B, for which the antibodies were available, specifically inhibited the expression of the respective proteins (Figure 1B). In sc-siRNA–transfected control HUVECs, TGFB1 induced increases in Smad2/3 phosphorylation, which reached approximately 8-fold increases over the basal levels at 30 min (Figure 1C). KD of either Synj1 or INPP4B suppressed TGFB1-induced Smad2/3 phosphorylation, like KD of C2α. In contrast, KD of Synj2, INPP4A, or MTMR6 did not inhibit phosphorylation of Smad2/3 (Figure 1D). KD of C2α, Synj1, or INPP4B, but not Synj2, INPP4A, or MTMR6, also suppressed TGFB1-induced nuclear translocation of Smad2/3, as evaluated with anti-Smad2/3 immunofluorescence staining (Figure 1E).

TGFB1 stimulation of sc-siRNA–transfected cells induced a marked reduction in the expression of type I TGFB receptor ALK5 at the PM with the accumulation of ALK5 in the intracellular compartment, that is, the endocytosis of TGFB1 receptor (Figure 2A). TGFB1 stimulation also induced the accumulation of clathrin heavy chain (CHC) at or in the vicinity of the PM in sc-siRNA–transfected cells (Figure 2B). KD of C2α inhibited TGFB1-induced ALK5 endocytosis (Figure 2A). KD of either Synj1 or INPP4B also inhibited TGFB1-induced ALK5 endocytosis. In contrast, KD of Synj2, INPP4A, or MTMR6 did not affect TGFB1-induced ALK5 endocytosis. KD of C2α, Synj1, Synj2, INPP4A, INPP4B, or MTMR6 did not inhibit the accumulation of CHC at the PM (Figure 2B). The proximity ligation assay (PLA) suggested that internalized ALK5 was colocalized with the early endosome marker EEA1 in manners dependent on C2α, Synj1, and INPP4B but not Synj2, INPP4A, or MTMR6 (Figure 2C). The subcellular distribution of anti-early endosome antigen1 (EEA1) signals was not altered by KD of these enzymes (Figure 2D).

Synj1 and C2α are required for TGFB1-induced rise of PI(3,4)P₂

We previously showed that TGFB1 stimulation induced an acute increase in PI(3,4)P₂ at the PM (Aki et al., 2015). We monitored TGFB1-induced changes in PI(3,4)P₂ in cells expressing the PI (3,4)P₂-specific fluorescent sensor mCherry-tagged PH-TAPP1 (He et al., 2017). TGFB1-induced increase in PI (3,4)P₂ at the PM became detectable within 3–4 min after TGFB1 addition and maximal approximately at 10 min (Figure 3, A and B). The anti-CHC immunostaining of the cells showed that the mCherry-tagged PH-TAPP1 signal at the PM of TGFB1-stimulated cells substantially overlapped with anti-CHC signal (Figure 3A), suggesting that PI(3,4)P₂ were increased at the CCPs and/or the CCVs just beneath the PM. KD of C2α but not C2β abolished TGFB1-induced increase in PI(3,4)P₂ (Figure 3B and Supplemental Videos S1–S3), suggesting that the rise of PI(3,4)P₂ was mediated mainly by C2α. KD of Synj1, which converts PI(4,5)P₂ to PI(4)P, also abolished TGFB1-induced increase in PI(3,4)P₂ (Figure 3, A and B). In contrast, KD of INPP4B, which converts PI(3,4)P₂ to PI(3)P, did not affect a TGFB1-induced PI(3,4)P₂ increase. Because several previous studies (Bae et al., 2010; Goulden et al., 2019) suggested that PI(3,4)P₂ was produced through the 5′-dephosphorylation of PI(3,4,5)P₃ generated by class I PI3K in growth factor-stimulated cells, we tested the effect of the class I PI3K-specific inhibitor GDC-0941 on GFβ1-induced PI(3,4)P₂
FIGURE 1: Synj1 and INPP4B as well as C2α are required for TGFβ1-induced phosphorylation and nuclear translocation of Smad2/3 in EC. (A) siRNA-mediated KD of the mRNAs of C2α and phosphatases. HUVEC were transfected with scRNA or specific siRNAs against C2α and lipid phosphatases, and the expression of the mRNAs were determined with real-time PCR. (B) siRNA-mediated KD of C2α and phosphatase proteins. HUVEC were transfected with either of the specific siRNAs and sc-siRNA, and the protein expression was analyzed with immunoblotting. Top, representative blots. Bottom, quantified data. (C, D) Time-dependent phosphorylation of Smad2/3 in response to TGFβ1 in C2α- or phosphatase-depleted HUVEC. Cells were transfected with either of the specific siRNAs and sc-siRNA and stimulated with TGFβ1 (5 ng/ml). The cell lysates were subjected to immunoblot analysis using anti-p-Smad2, anti-p-Smad3, and anti-Smad2/3 antibodies. Top, representative blots of Smads. Bottom, quantified data. (E) Immunofluorescence staining of Smad2/3 in TGFβ1-stimulated HUVEC. The cells were transfected with either of the specific siRNAs and sc-siRNA and stimulated with TGFβ1 (5 ng/ml) for 30 min, followed by anti-Smad2/3 antibody immunostaining. ***denotes statistical significance compared with C2a-siRNA, Synj1-siRNA, and INPP4B-siRNA. Nuclei were stained by DAPI. The representative confocal images are shown. Right bottom, quantified data obtained from 48 cells per group. Scale bar, 20 μm.
FIGURE 2: Synj1 and INPP4B as well as C2α are required for TGFβ1-induced internalization of TGFβ receptor into early endosomes in EC. (A) Effects of C2α and phosphatase depletion on TGFβ1-induced ALK5 internalization. HUVEC transfected with either of the specific siRNAs and sc-siRNA were stimulated with TGFβ1 (5 ng/ml) for 30 min and subjected to anti-ALK5 immunofluorescence staining. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of PM ALK5 fluorescence intensities obtained from 24 cells per group. (B) Effects of C2α and phosphatase depletion on clathrin localization. HUVEC transfected with either of the specific siRNAs and sc-siRNA were stimulated with TGFβ1 (5 ng/ml) for 10 min and subjected to anti-CHC immunofluorescence staining. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of PM CHC immunofluorescence intensities obtained from 24 cells per group. (C) PLA staining of ALK5 and EEA1 (green) in TGFβ1-stimulated HUVEC. Cells transfected with either of the specific siRNAs and sc-siRNA were stimulated with TGFβ1 (5 ng/ml) for 30 min. Left images, PLA staining. Right, quantified data of the PLA signals obtained from 24 cells per group. Nuclei were stained by DAPI. Scale bar, 20 μm. **** and ⌘⌘⌘ denote statistical significance compared with TGFβ1-nontreated cells in each siRNA-transfected group and TGFβ1-treated, sc-siRNA–transfected cells, respectively. (D) Effects of C2α and phosphatase depletion on early endosome. HUVEC transfected with either of the specific siRNAs and sc-siRNA were stained with anti-EEA1 antibody. Left, representative confocal images. Right, quantified data of the anti-EEA1 signals obtained from 24 cells per group. Nuclei were stained by DAPI. Scale bar, 20 μm.
FIGURE 3: Synj1 and C2α are required for TGFβ-induced PI(3,4)P₂ increase at the PM. (A) TGFβ1 induces increases of PI(3,4)P₂ mainly at the clathrin-localized sites of the PM. HUVEC transfected with the PI(3,4)P₂ sensor mCherry-PHₜAPP1 were stimulated with TGFβ1 (5 ng/ml) for 10 min and subjected to anti-CHC immunofluorescence staining. The extreme right panels are magnified views of the boxed regions. Nuclei were stained by DAPI. The arrowheads denote mCherry-PHₜAPP1 signals at the anti-CHC-positive PM areas. Nuclei were stained by DAPI. Scale bar, 20 μm. (B) Effects of C2α and phosphatase depletion on TGFβ1-induced PI(3,4)P₂ increase. HUVEC were cotransfected with GFP-PHₜAPP1 and either of the specific siRNAs and sc-siRNA and stimulated with TGFβ1 (5 ng/ml). The arrowheads in the representative confocal images (left) denote GFP-PHₜAPP1 signals at the PM. Right, quantified data of the PM GFP-PHₜAPP1 fluorescence intensities obtained from 24 or 25 cells per group. Scale bar, 20 μm. (C) Effects of the class I PI3K inhibitor GDC-0941 on TGFβ1-induced PI(3,4)P₂ increase. HUVEC transfected with GFP-PHₜAPP1 were pretreated with GDC-0941 (1 μM) for 30 min and stimulated with TGFβ1 (5 ng/ml) for 10 min. The arrowheads denote mCherry-PHₜAPP1 signals at the PM. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of the PM GFP-PHₜAPP1 fluorescence intensities obtained from 24 cells per group. (D) VEGF but not TGFβ1 induces an increase in PI(3,4,5)P₃ at the PM. HUVEC transfected with GFP-PHGRP1 were stimulated with TGFβ1 (5 ng/ml) or VEGF (50 ng/ml) for 10 min. The arrowheads denote GFP-PHGRP1 signals at the PM. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of the PM GFP-PHGRP1 fluorescence intensities obtained from 24 cells per group.
increase. GDC-0941 did not inhibit TGFβ1-induced PI(3,4)P2 increase (Figure 3C), and TGFβ1 did not induce an increase in PI(3,4,5)P3, which was different from VEGF (Figure 3D). Thus, the 5'-specific phosphatase Synj1 and the C2α, but not the 4'-phosphatase INPP4B or class I PI3K, are required for PI(3,4)P2 generation in TGFβ1-stimulated HEVUC.

Both Synj1 and C2α are required for TGFβ1-induced PI(4,5)P2 decrease and PI(4)P increase

Because the 5'-specific phosphatase Synj1 was required for TGFβ1-induced PI(3,4)P2 rise (Figure 3B), we explored whether TGFβ1 induced a Synj1-dependent PI(4,5)P2 conversion to PI(4)P by simultaneously monitoring cellular levels of PI(4,5)P2 and PI(4)P. In cells coexpressing the PI(4,5)P2-specific sensor mCherry-PlnPLCβ (Garcia et al., 1995; Lemmon et al., 1995) and the PI(4)P-specific sensor GFP-P4M-SidM (Hammond et al., 2014), TGFβ1 induced a reduction of PI(4,5)P2 and reciprocal increases of PI(4)P and PI(3,4)P2 at the PM (Figure 4, A and B). TGFβ1-induced PI(4,5)P2 reduction and PI(4)P rise occurred rapidly within 10 s after a TGFβ1 addition (Supplemental Videos S4 and S5). We also examined time courses of TGFβ1-induced PI(4)P and PI(3,4)P2 increases in coexpressing GFP-PAM-SidM and mCherry-tagged PTHAPPTP. TGFβ1-induced PI(4)P rise preceded that of PI(3,4)P2 (Figure 4B). TGFβ1-induced PI(4,5)P2 reduction and PI(4)P rise were both abolished by KD of Synj1 (Figure 4, C and D, and Supplemental Video S6). These observations together suggested that TGFβ1 induced PI(4,5)P2 conversion to PI(4)P prior to PI(3,4)P2 generation through Synj1. Interestingly, TGFβ1-induced PI(4,5)P2 decrease and PI(4)P rise were also inhibited by C2α KD (Figure 4, C and D, and Supplemental Video S7), TGFβ1-induced PI(3,4)P2 decrease or PI(4)P rise was not abolished by KD of C2β or INPP4B. These observations suggested that C2α was somehow involved in Synj1-mediated PI(4,5)P2 hydrolysis.

In contrast to PI(4)P and PI(4,5)P2, PI(3)P is mainly detected at the intracellular dots, most likely endosomes. TGFβ1 did not change PI(3)P-positive dots in sc-siRNA–transfected cells (Figure 4E). KD of Synj1, C2α, or INPP4B reduced PI(3)P-positive dots in both control and TGFβ1-stimulated cells, suggesting that these enzymes contributed to the endosomal level of PI(3)P. In contrast, the KD of the 3'-phosphatase MTM6 was only increased PI(3)P-positive dots, suggesting that MTMR6 participated in the dephosphorylation of PI(3)P. Either KD of Vps34 or the class I PI3K inhibitor did not affect PI(3)P-positive dots.

ALK5 mediates TGFβ1-induced triggering of phosphoinositide conversions

TGFβ1 binding to TGFβ receptors activates type I receptor serine/threonine kinases ALK5 or ALK1, resulting in phosphorylation of Smad proteins. To explore the molecular mechanisms underlying TGFβ1-induced phosphoinositide conversion, we tested the effects of the kinase inhibitors of ALK5 and ALK1 on phosphoinositide conversions. The ALK5 kinase-specific inhibitor iALK5 abolished all of the TGFβ1-induced a decrease in PI(4,5)P2, an increase in PI(4)P, and an increase in PI(3,4)P2 at the PM (Figure 5, A–C). In contrast, the ALK1 kinase-specific inhibitor iALK1 was without any inhibitory effect on TGFβ1-induced changes in phosphoinositides. These results suggest that TGFβ1-induced phosphoinositide conversions require ALK5 kinase activity.

TGFβ1 induces ALK5-dependent colocalization of Synj1 and C2α at the PM

We explored the mechanism underlying C2α requirement for TGFβ1-induced, Synj1-mediated PI(4,5)P2 breakdown. In nonstimulated cells that expressed both GFP-tagged C2α (GFP-C2α) and mCherry-tagged Synj1 (mCherry-Synj1), GFP-C2α and mCherry-Synj1 were only marginally localized at the PM and mainly distributed in punctate patterns widely in the intracellular compartment with some overlapping (Figure 6A). TGFβ1 stimulation induced the translocation of GFP-C2α and mCherry-Synj1 to the PM with substantial colocalization (Figure 6A). The immunoprecipitation-Western blot analysis showed that C2α and Synj1 were coprecipitated in sc-siRNA–transfected cells, suggesting that these proteins formed the complex (Figure 6B). Knockdown of Synj1 markedly reduced the amount of the coprecipitated Synj1. TGFβ1 did not enhance the coprecipitation of C2α and Synj1. TGFβ1-induced PM colocalization of GFP-C2α and mCherry-Synj1 was inhibited by iALK5 but not iALK1 (Figure 6C), like TGFβ1-induced changes in PI(4,5)P2, PI(4)P, and PI(3,4)P2 at the PM (Figure 5). Interestingly, depletion of C2α abolished TGFβ1-induced recruitment of mCherry-Synj1 to the PM and vice versa (Figure 6, D and E), indicating that the proper PM recruitment of C2α and Synj1 required the existence of the other enzyme. We also studied whether an siRNA-resistant, kinase-deficient C2α mutant, C2αr (GFP-kdC2αr), could rescue Synj1 translocation in cells depleted of endogenous C2α. In C2α-depleted cells, the expressed C2αr-siRNA-resistant wild-type C2α (GFP-wtC2αr) colocalized with mCherry-Synj1, whereas in cells cotransfected with GFP-kdC2αr and mCherry-Synj1, neither protein was recruited to the PM (Figure 6F).

In non-TGFβ-stimulated cells, PI(4,5)P2 was enriched at the PM, whereas in TGFβ-stimulated cells PI(4,5)P2 was not enriched at the PM sites where GFP-Synj1 was recruited (Figure 7A). Similarly, in TGFβ-stimulated cells PI(3,4)P2 was detected at the PM sites where GFP-C2α was recruited (Figure 7B). Moreover, in cells depleted of endogenous C2α, the expression of GFP-wtC2αr recovered TGFβ1-induced lowering of PI(4,5)P2 at the PM, whereas that of GFP-kdC2αr did not (Figure 7C).

These observations together suggest that TGFβ1-induced co-translocation of C2α and Synj1 at the PM is dependent on the intact form of C2α and that the phosphoinositide conversion occurs at the PM sites where C2α and Synj1 are recruited.

DISCUSSION

This study demonstrated that TGFβ1 stimulation triggered the conversions of phosphoinositides at the PM of EC via its specific receptor serine/threonine kinase ALK5. TGFβ1 facilitated the conversions of phosphoinositides comprising the 5'-phosphatase Synj1-mediated PI(4,5)P2 conversion to PI(4)P, the 3'-kinase C2α-mediated PI(4)P conversion to PI(3,4)P2, and the 4'-phosphatase INPP4B-mediated PI(3,4)P2 conversion to PI(3)P. Thus, these phosphoinositide conversions likely constitute the sequential conversion cascade. The phosphoinositide cascade was essential for activation of the canonical Smad pathway in TGFβ signaling through its requirement for TGFβ receptor endocytosis. Furthermore, we found that TGFβ1-induced Synj1 recruitment to the PM and Synj1-mediated PI(4,5)P2 hydrolysis were dependent on C2α, indicating the thus far unrecognized functional cooperation between Synj1 and C2α.

Recent studies (Posor et al., 2013; Schöneberg et al., 2017) showed that PI(3,4)P2 serves essential roles in the maturation of CCPs in COS-7, HEK293, and HeLa cells under basal nonstimulated conditions. Our study first revealed TGFβ1-triggered phosphoinositide conversions, which led to acute and robust increases in PI(3,4)P2 at the PM within a few minutes (Figure 3). Second, previous studies (Gewinner et al., 2009; Bae et al., 2010) showed that growth factor stimulation induced an increase in PI(3,4)P2 via class I PI3Ks. However, TGFβ1-induced PI(3,4)P2 rise in ECs was mediated by class II phosphoinositides in endocytosis | 365

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FIGURE 4: Synj1 and C2α are required for TGFβ-induced reduction of PI(4,5)P₂ and rise of PI(4)P. (A) Time-dependent changes in PI(4,5)P₂ and PI(4)P at the PM in TGFβ1-stimulated HUVEC. Cells were cotransfected with mCherry-PH\textsuperscript{PLCδ1} and GFP-P4M-SidM and stimulated with TGFβ1 (5 ng/ml) for 5 min. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of the PM mCherry-PH\textsuperscript{PLCδ1} or GFP-P4M-SidM fluorescence intensities obtained from 24 cells per group. The arrowheads in the confocal images denote the sites at which PI(4,5)P₂ and PI(4)P levels changed after TGFβ1 stimulation. (B) Time-dependent changes in PI(4)P and PI(3,4)P₂ at the PM in TGFβ1-stimulated HUVEC. Cells were cotransfected with GFP-P4M-SidM and mCherry-PH\textsuperscript{TAPP1} and stimulated with TGFβ1 (5 ng/ml) for 10 min. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of the PM GFP-P4M-SidM or mCherry-PH\textsuperscript{TAPP1} fluorescence intensities obtained from 24 cells per group. The arrowheads in the confocal images denote the sites in which PI(4)P and PI(3,4)P₂ levels increased after TGFβ1 stimulation. (C) TGFβ1 induces a reduction of PI(4,5)P₂ in manners dependent on Synj1 and C2α but not C2β or INPP4B. HUVEC were cotransfected with GFP-PH\textsuperscript{PLCδ1} and either of the specific siRNAs and sc-siRNA and stimulated with TGFβ1 (5 ng/ml) for 5 min. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of the PM GFP-PH\textsuperscript{PLCδ1} fluorescence intensities obtained from 25 cells per group. The arrowheads in the confocal images denote the sites in which PI(4,5)P₂ level rather increased after TGFβ1 stimulation. (D) TGFβ1 induces a rise of PI(4)P in manners dependent on Synj1 and C2α but not C2β or INPP4B. HUVEC...
C2α but not class I PI3K (Figure 3, B and C). Third, TGFβ1 induced a rapid fall of PI(4,5)P2 with a reciprocal rise of PI(4)P, which was mediated by Synj1 and served as substrate for C2α to generate PI(3,4)P2. The involvement of Synj1 in CME was consistent with previous reports (Perera et al., 2006; Chang-Itao et al., 2011). Fourth, the INPP4B was suggested to mediate PI(3,4)P2 conversion to PI(3)P because the KD of INPP4B reduced cellular PI(3)P level (Figure 4E). These enzymes were essential for TGFβ1-induced TGFβ receptor endocytosis and Smad signaling (Figures 1, C and E, and 2, A and C). Our study unveiled the membrane phosphoinositide dynamics that underlies TGFβ-activated canonical receptor signaling.

The PM PI(4,5)P2 was converted to PI(3)P through the sequential hydrolysis and phosphorylation in TGFβ1-stimulated cells as mentioned above. PI(3)P was enriched mainly at endosomes (Figure 4E) (Di Paolo and De Camilli, 2006; Marat and Haucke, 2016). The KD of the PI(3)P phosphatase MTMR6 (Hnia et al., 2012; Maekawa et al., 2014) enhanced PI(3)P enrichment in endosomes, suggesting that MTMR6 at least in part contributed to PI(3)P conversion to PI at endosomes. However, MTMR6 KD experiments showed that MTMR6-mediated PI(3)P degradation does not seem to be an important process required for TGFβ receptor endocytosis and subsequent endosomal TGFβ receptor signaling.

In the present study, we unexpectedly found the cooperation of Synj1 and C2α in TGFβ1-stimulated ECs. In nontreated cells, fluorescent Synj1 and C2α were colocalized at the dots in the intracellular compartment but not at the PM (Figure 6A), and the immunoprecipitation-Western blot analyses showed that Synj1 and C2α formed the complex (Figure 6B). TGFβ1 stimulation induced the rapid PM recruitment of the enzymes, which needed both Synj1 and C2α, that is, most likely the complex of Synj1 and C2α (Figure 6, D and E). This possibility may also explain why both PI(4,5)P2 hydrolysis and PI(4)P production required C2α (Figure 4, A and B). The 170-kDa form of Synj1, which possesses the C-terminal tail that contains the binding sites for clathrin, the clathrin adaptor AP-2, and the accessory protein Eps15, is recruited to the CCPs through these binding sites (Perera et al., 2006; Antonescu et al., 2011). C2α also associates with the CCPs through its clathrin-binding domain and PI(4,5)P2-binding PX-C2 domains (Gaidarov et al., 2001; Wang et al., 2018). Interestingly, TGFβ1-induced recruitment of Synj1 and C2α to the PM was dependent on the kinase activity of ALKS (Figure 6C), suggesting that protein phosphorylation by ALKS was involved in the recruitment of Synj1 and C2α complex. Previous studies (Lotti et al., 1996; Okabayashi et al., 1996; Lee et al., 2007) showed that ALKS phosphorylated the adaptor protein ShcA, which was recruited to a cargo at the CCPs. Therefore, it might be possible that ALKS-mediated phosphorylation of Shc or other proteins promotes the recruitment of Synj1 and C2α complex to the CCPs in the PM much more effectively compared with the recruitment of either alone of Synj1 and C2α. The exact mechanisms of the corecruitment of Synj1 and C2α to the PM and the ALKS dependence of this process remain to be clarified.

Recent studies (Chen et al., 2018; Wang et al., 2018) showed that C2α becomes fully active by changing its conformation when the N-terminal clathrin-binding domain and the C-terminal PX-C2 domain get contacts with clathrin and membrane PI(4,5)P2, respectively, at the CCPs. Once C2α is recruited to the CCPs as the complex with Synj1, the production of PI(4)P by Synj1 likely enhances the availability of the substrate PI(4)P to C2α, enabling effective production of PI(3,4)P2 at the CCPs. It might also be possible that, besides the role as partner for the complex formation, Synj1 might assist the activation of C2α at the CCPs by interacting with C2α.

Our rescue experiments using both wild-type and kinase-deficient forms of C2α showed that, unlike wild-type C2α, the kinase-deficient mutant was not recruited to the PM (Figure 6F). The finding may suggest that the kinase-deficient C2α mutant (D1268A) could not take a conformation that allows for C2α localization at the PM (Wang et al., 2018). Alternatively, PI(3,4)P2, which was produced by C2α enzymatic activity in collaboration with Synj1, might be required for strengthening the colocalization of Synj1 and C2α at the PM, for example, through recruiting other proteins to the complex. Further studies are required for better understanding the mechanism for TGFβ1-induced recruitment of the Synj1 and C2α complex.

A recent study reported that homozygous loss-of-function mutation of C2α gene results in the phenotype of short stature, coarse facial features, cataract with glaucoma, neurological manifestations including epileptic seizures and stroke, and multiple skeletal abnormalities in children (Tiosano et al., 2019). These patients exhibited compensatory severalfold increases in C2β mRNA expression. A patient with the genetic Synj1 deficiency was also reported to exhibit a severe disorder with neonatal refractory epilepsy and a neurodegenerative disease course (Hardies et al., 2016). Because the brain dominantly expresses the 145-kDa isofrom of Synj1 rather than the 170-kDa isofrom, a role of Synj1 in vesicle trafficking in the brain may be different from ECs, in which the major Synj1 isofrom is 170 kDa. The structure of the C-terminal interaction domains is different between 145- and 170-kDa isofroms of Synj1 (Haffner et al., 2000; Perera et al., 2006). Since the present study showed the essential cooperation of Synj1 and C2α, it should be noted that possible functional deficiency of C2α in genetic Synj1-deficient patients and of Synj1 in C2α-deficient patients might contribute to the patient phenotypes. The phenotype of patients with genetic C2α deficiency was also found to have similarities to that of Lowe’s syndrome patients caused by loss-of-function mutation of another phosphoinositide 5’-phosphatase OCRL gene (Tiosano et al., 2019). OCRL, which reduces the membrane PI(4,5)P2 level in the late CCPs and CCVs, is implicated in CME at a little later time point compared with Synj1 (Nakatsu et al., 2010; Kaksonen and Roux, 2018). Therefore, it could be possible that PI(4,5)P2 accumulation as well as PI(3,4)P2 deficiency might mechanistically have a contribution to the development of the phenotype of C2α deficiency.

In summary, we unveiled the TGFβ1-activated phosphoinositide conversion cascade, which plays a crucial role in TGFβ-induced CME of TGFβ receptor and, thereby, the canonical TGFβ-induced Smad signaling. The novel cooperative actions of multiple enzymes are involved in the phosphoinositide cascade activation. This were cotransfected with GFP-P4M-SidM and either of the specific siRNAs and sc-siRNA and stimulated with TGFβ1 (5 ng/ml) for 5 min. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of the PM GFP-P4M-SidM fluorescence intensities obtained from 25 cells per group. The arrowheads in the confocal images denote the sites in which PI(4)P level increased after TGFβ1 stimulation. (E) Cellular PI(3)P level is dependent on Synj1, C2α, INPP4B, and MTMR6, but not Vps34. HUVEC were cotransfected with the PI(3)P sensor mCherry-FYVE±± and either of the specific siRNAs and sc-siRNA and stimulated with TGFβ1 (5 ng/ml) for 15 min or nontreated. Some dishes were pretreated with GDC-0941 (2 μM) 10 min. Nuclei were stained by DAPI. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of mCherry-FYVE fluorescence-positive dot number obtained from 36 cells per group. * and *** denote statistical significance compared with sc-siRNA–transfected cells treated and nontreated with TGFβ1.
phosphoinositide cascade may represent a target for treating diseases caused by abnormalities of CME.

**MATERIALS AND METHODS**

**Cells**

HUVEC (Lonza) were plated onto type-I collagen-coated (Nitta Gelatin) dishes and flasks and allowed to grow under 5% CO₂ at 37°C in complete endothelial growth medium containing 2% fetal bovine serum and growth factor supplements (EGM-2; #CC3156, Lonza) for HUVECs. HUVECs between passages 4 and 6 were used for all experiments.

**Small interfering RNA, plasmids, and transfection**

Knockdown of endogenous PI3K isoforms, lipid phosphatases, and Smad4 were performed with the siRNAs that were synthesized using a Silencers siRNA construction kit (#AM1620, Ambion) according to the manufacturer’s instruction. The target sequences were: 5′-AAGGTTGGCACTTACAAAGAT-3′ for human PI3K-C2α; 5′-AAGCCGGAAA-GCTTCTGGTGT-3′ for human PI3K-C2β 5′-GGGATCTCATGTACGGAACAT-3′ for C2β-specific siRNA was previously shown elsewhere (Aung et al., 2018). The expression vector for GFP-C2α was described previously (Aki et al., 2015). The expression vector for GFP-wtC2α was generated using a standard PCR-based method (Pham et al., 2018). In C2αr, 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 encode the same amino acids. The changes in these nucleotides rendered GFP-wtC2α resistant to the C2α-specific siRNA. The kinase-deficient mutant (D1268A) of GFP-C2α was generated using a standard PCR-based method. The expression vectors for GFP-PHPLCα, GFP-PHPLCβ, and GFP-P4M-SidM were purchased from Addgene. The expression vectors for mCherry-PHPLCα, mCherry-PHPLCβ, and mCherry-P4M-SidM were provided by Kangmin He (Harvard Medical School). For the expression vector for GFP-Syn1 and mCherry-Syn1, human SYNJ1 cDNA fragments were amplified by PCR using PrimeSTAR HS DNA Polymerase (R010A; Takara-Bio) according to the manufacturer’s protocol. The subcloning of the PCR products into pmCherry-C1 (632524; Clontech Laboratories, Mountain View, CA) and pAcGFP1-N vectors (632501; Clontech Laboratories) was performed using the In-Fusion HD Cloning kit (Z9633 N; Clontech Laboratories).

**Immunoblotting and immunoprecipitation analysis**

At 48 h after siRNA transfection, cells were serum-starved with M199 (Life Technologies) containing 0.5% fatty acid-free bovine serum albumin (BSA) (#A6003, Sigma-Aldrich) for 4 h and then stimulated with TGFβ1-induced phosphoinositide conversion are mediated by ALK5 but not ALK1. (A–C) Effects of the ALK5 and ALK1 inhibitors on TGFβ-induced reductions of (A) PI(4,5)P₂ and rises of (B) PI(4)P and (C) PI(3,4)P₂. HUVEC were transfected with either (A) GFP-PHPLCα, (B) GFP-P4M-SidM, or (C) GFP-PHTAPP1. Cells were pretreated with the ALK1 inhibitor iALK1 (LDN193189) (1 μM) or ALK5 inhibitor iALK5 (2-(3-(6-methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine) (5 μM) for 30 min and stimulated with TGFβ1 (5 ng/ml) for 5 or 10 min. Representative confocal images at 0 and 5 or 10 min after the additions of TGFβ1 are shown. Left, representative confocal images. Scale bar, 20 μm. Right, quantified data of PM (A) GFP-PHPLCα, (B) GFP-P4M-SidM, and (C) GFP-PHTAPP1 fluorescence intensities at the PM (24 cells per group).
FIGURE 6: TGFβ1 induces ALK5-dependent colocalization of Synj1 and C2α at the PM. (A) TGFβ1 induces the recruitment of GFP-C2α and mCherry-Synj1 to the PM. HUVEC cotransfected with GFP-C2α and mCherry-Synj1 were stimulated with TGFβ1 (5 ng/ml) for 5 min or nontreated. Left, representative confocal images. Scale bar, 10 μm. Right, quantified data of GFP-C2α and mCherry-Synj1 colocalization at the PM (24 cells per group). (B) Coimmunoprecipitation-immunoblotting analysis of C2α and Synj1 in HUVEC. Cells were transfected with sc-siRNA or Synj1-siRNA and stimulated with TGFβ1 (5 ng/ml). Cell lysates were immunoprecipitated (IP) with control-IgG or anti-C2α antibody, followed by immunoblotting (IB) using anti-C2α, anti-Synj1 or anti-GAPDH antibodies. (C) Effects of ALK5 and ALK 1 inhibitors on TGFβ1-induced recruitment of GFP-C2α and mCherry-Synj1 to the PM. Cells cotransfected with GFP-C2α and mCherry-Synj1 were pretreated with iALK1 (1 μM), iALK5 (5 μM), or vehicle for 30 min and then stimulated with TGFβ1 (5 ng/ml) for 5 min. Left, representative confocal images. Scale bar, 10 μm. Right, quantified data of GFP-C2α and mCherry-Synj1-double positive fluorescence intensities at the PM (24 cells per group). (D) Effects of Synj1 KD on TGFβ1-induced recruitment of GFP-C2α to the PM. HUVEC transfected with GFP-C2α and either sc-siRNA or Synj1-siRNA were stimulated with TGFβ1 (5 ng/ml) for 5 min or nontreated. Left, representative confocal images. Scale bar, 10 μm. Right, quantified data of the PM GFP-C2α fluorescence intensities obtained from 24 cells per group. (E) Effects of C2α KD on TGFβ1-induced mCherry-Synj1 recruitment to the PM. HUVEC transfected with GFP-C2α and either sc-siRNA or C2α-siRNA were stimulated with TGFβ1 (5 ng/ml) for 5 min or nontreated. Left, representative confocal images. Scale bar, 10 μm. Right, quantified data of the PM mCherry-Synj1 fluorescence intensities obtained from 24 cells per group. (F) The expression of wild-type GFP-C2α (GFP-wtC2α) but not kinase-deficient GFP-C2α (GFP-kdC2α) restores TGFβ1-induced mCherry-Synj1 recruitment to the PM. HUVEC were cotransfected with either GFP-wtC2α or GFP-kdC2α and either sc-siRNA or C2α-siRNA. Cells were stimulated with TGFβ1 (5 ng/ml) for 5 min. Left, representative confocal images. Scale bar, 10 μm. Right, quantified data of GFP-C2α and mCherry-Synj1-double positive fluorescence intensities at the PM (24 cells per group).
with TGF\(\beta\)1 (#100-21, PeproTech). Cells were washed in phosphate-buffered saline (PBS) and lysed in the cell lysis buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM CaCl\(_2\), 0.5% Triton X-100, 100 mM NaF, 1 mM Na\(_3\)VO\(_4\)) supplemented with Complete Protease inhibitor cocktail (Roche) by scraping, followed by centrifugation for 15 min at 16,000 \(\times\) g at 4°C. The resultant supernatants were taken and electro-phoresed on 8% SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked in PBS containing 5% BSA and incubated with respective antibodies overnight. The antibodies used are PI3K-C2\(\alpha\) (#12402, Cell Signaling); Synj1 (K0130-3, MBL); INPP4B (#8450, Cell Signaling); total Smad2/3 (#610842, BD Bioscience); p-Smad2 (#3101, Cell Signaling); p-Smad3 (#9520, Cell Signaling); ALK5 (sc-398, Santa Cruz); EEA1 (#610456, BD Bioscience); GAPDH (016-25523, Wako). The membranes were incubated with alkaline phosphatase (AP)–conjugated secondary antibodies (anti-mouse immunoglobulin G [IgG] antibody, #7056; anti-rabbit IgG antibody, #7054; Cell Signaling) and visualized by color reaction using 5-bromo-4-chloro-3-indoly phosphate/nitro blue tetrazolium (Wako). The band intensities were determined using Image Gauge (Fujifilm). The values were normalized for the value of GAPDH as a loading control and expressed as multiples over the normalized values of nontreated controls.

**Immunoprecipitation assay**
HUVECs were lysed in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) supplemented with Complete Protease inhibitor cocktail. The lysates were incubated with anti-C2\(\alpha\) antibody for 1 h at 4°C with rocking, followed by the incubation with protein G–agarose beads (#1-719-416, Roche) for 1 h at 4°C. After the beads were washed five times, mixed with 2\(\times\) Laemmli’s SDS sample buffer, and boiled. The resultant samples were analyzed with immunoblotting using respective antibodies.

**Immunofluorescence staining**
HUVECs were plated onto type-I collagen-coated glass bottom dishes (MatTek) and allowed to adhere to dishes in EGM-2 growth medium overnight. Cells were rinsed with prewarmed PBS once and fixed in prewarmed 4% fresh PFA 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 cells were incubated with 5% normal goat serum for 60 min to inhibit nonspecific protein binding, cells were incubated with mouse monoclonal anti-Smad2/3 antibody, rabbit polyclonal anti-ALK5 antibody, or mouse monoclonal anti-CHC antibody (MA1-065, Thermo) for 2 h at room temperature or overnight with Alexa Fluor 488–conjugated goat anti-mouse (A31620, Molecular Probes), Alexa Fluor 488–conjugated goat anti-rabbit (A11034, Molecular Probes), Alexa Fluor 568–conjugated goat anti-mouse (A31624, Molecular Probes), or Alexa Fluor 568–conjugated goat anti-rabbit (A31620, Molecular Probes) secondary antibodies, which were diluted at 1:100 in PBS. Where appropriate, the cells were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; # D1306, Molecular Probes) for 15 min. Cells were mounted on Fluoromount (#K024, Diagnostic BioSystems) and visualized by confocal microscopy.
observed under a custom confocal microscope unit described in detail previously (Yoshioka et al., 2012; Aki et al., 2015; Sarker et al., 2019). The confocal microscopic images shown in Figures 3A, 4C, 6, and 7 were obtained with an inverted Nikon Eclipse Ti2 confocal microscope (Nikon Instruments/Nikon) equipped with the Perfect Focus System, attached to an Andor Dragonfly spinning-disk unit, Andor electron-multiplying charge-coupled device (EMCCD) camera (iXon DU888, Andor Technology), and a laser unit (Coherent). An oil-immersion objective (PlanApo 60×; NA 1.4; Nikon) was used for all experiments. Excitation for BFP/DAPI, GFP/mEmerald/Alexa 488, and mRFP/mCherry/Alexa 568 and Alexa 647 chromophores was provided by a 405-, 488-, 561-, and 637-nm laser, respectively. Superresolution imaging of fixed cells was performed using an Andor Dragonfly confocal microscope in super-resolution radial fluctuation Stream mode (Andor Technology).

Live-cell imaging of phosphoinositides

HUV EC that had been transfected with the expression vectors for the phosphoinositide sensors GFP-PH<sup>APP1</sup> (PI(3,4)P<sub>2</sub>), GFP-PH<sup>PLCδ</sup> (PI(4,5)P<sub>2</sub>), and GFP-P4M-SidM (PI(4)P) were plated on collagen-coated glass-bottomed dishes (MatTek, P35G-1.5-20-C) and allowed to adhere for 16 h before imaging. Cells on a heated stage (37°C; Tokai-Hit) were observed under a custom confocal microscope based on an inverted IX70 microscope (Olympus) equipped with an UPLSAPO × 60/NA 1.35-oil objective, a confocal laser unit (CSU10; Yokogawa), an EMCCD digital camera (iXon; Andor), and a Light engine (Lumencor) for three-dimensional time-lapse confocal imaging at a rate of two frames per second. The acquisition and process were controlled by iQ software (Andor). To quantify changes in the fluorescence intensity of sensors at and in the vicinity of the PM, we manually defined all regions of interest that showed fluorescence increases in response to TGFβ1 stimulation in each cell during the observation time period and determined fluorescence intensities with iQ software (Hammond et al., 2012, 2014). The total fluorescence intensity in each cell was normalized by whole-cell fluorescence intensity. For time-lapse data, images were also normalized to the pixel ratio at the baseline level (during a 5-min period before stimulation).

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 and mouse monoclonal anti-EEA1 antibody overnight at 4°C, in situ protein interactions were detected using the Duolink PLA kit according to the manufacturer’s instructions (Olink Bioscience).

RNA isolation and quantitative PCR analysis

Total RNA in HUV EC was isolated using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed into the first-strand cDNA using Quant-iTect RT Kit (#205311; Qiagen). Quantification of gene expression was performed using an ABI7300 qPCR thermal cycle (Applied Biosystems) in duplicate. The primer sequences were sense: 5′-AGCGAAGGGCTGGGCTGCTGCT-3′, antisense: 5′-ACCAAGAAGTCAGGGGGGAC-3′ for Synaptojanin2; sense: 5′-GTCTCTTATCAAGTGGGAGCATAT-3′, antisense: 5′-TTTAAAGGCAACAAGTCTGTCCTACA-3′ for INPP4A; sense: 5′-GCATTCTACCGAGACAGCTTCCAC-3′, antisense: 5′-GGCA-CAGGGGATAGTGCTCTCTCC-3′ for MTMR6. We have validated that these primer pairs are highly specific for each target without cross-amplification. Comparative quantitative analysis was performed using the GeneAmp 7300 system (Applied Bioscience) based on the delta-delta Ct method. The mRNA expression levels were normalized for the expression of GAPDH mRNA, and the results are expressed as multiples over control values.

Statistical analysis

The data are presented as means ± SEM and expressed as the percentages or multiples relative to the values in control cells. Statistical significance was analyzed using Prism 7 software (GraphPad Software). Statistical significance was analyzed either by one-way or two-way analysis of variance followed by Bonferroni’s test as appropriate. Results with p < 0.05 were considered statistically significant. In all figures, the asterisks indicate statistical significance between the indicated groups at the levels of p < 0.05 (*), p < 0.01 (**), p < 0.001 (***) or p < 0.0001 (****), ns, statistically not significant.

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Future Research

The results of this study suggest that phosphoinositides play a crucial role in the regulation of cell membrane dynamics and endocytosis. Further investigations are needed to elucidate the molecular mechanisms of how phosphoinositides regulate cell membrane trafficking and migration in endothelial cells. The role of phosphoinositides in diseases such as cancer, inflammation, and cardiovascular diseases should also be studied to understand their potential as therapeutic targets.

Conclusion

In conclusion, this study provides novel insights into the role of phosphoinositides in cell membrane dynamics and endocytosis. The findings have implications for understanding the molecular mechanisms of how phosphoinositides regulate cell membrane trafficking and migration in endothelial cells. The results also have potential applications in the development of new therapeutic strategies for diseases associated with altered cell membrane dynamics and endocytosis.
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