Published in final edited form as:

Placenta. 2009 October ; 30(10): 907–913. doi:10.1016/j.placenta.2009.07.003.

Suppression of Protein Phosphatase 2 Differentially Modulates VEGF- and FGF2-Induced Signaling in Ovine Fetoplacental Artery Endothelial Cells

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Abstract

Vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) elicit cellular responses via activation of protein kinases and phosphatases. We have reported that the MEK1/2/ERK1/2 and PI3K/AKT1 pathways are critical for VEGF- and FGF2-stimulated ovine fetoplacental endothelial (OFPAE) cell proliferation. We have also shown that protein phosphatase 3 (PPP3) differentially modulates VEGF- and FGF2-stimulated cell proliferation and activation of ERK1/2 and AKT1 in OFPAE cells. Herein, we investigated if protein phosphatase 2 (PPP2) modulated VEGF- and FGF2-induced ERK1/2, AKT1, and p38 MAPK activation and VEGF- and FGF2-stimulated cell proliferation in OFPAE cells. Small interfering RNA (siRNA) specifically targeting human PPP2 catalytic subunit α (PPP2CA) was used to suppress PPP2CA expression in OFPAE cells. When compared with scrambled siRNA, PPP2CA siRNA decreased ($p < 0.05$) PPP2CA protein levels ($\sim 70\%$) and activity ($\sim 50\%$) without altering protein levels of PPP3 catalytic subunit α (PPP3CA), nitric oxide (NO) synthase 3 (NOS3), ERK1/2, AKT1, and p38 MAPK. FGF2, but not VEGF rapidly (≤ 5 min) induced p38 MAPK phosphorylation. Suppression of PPP2CA enhanced ($p < 0.05$) VEGF-induced AKT1, but not ERK1/2 phosphorylation, whereas inhibited ($p < 0.05$) FGF2-induced ERK1/2 and p38 MAPK and slightly attenuated FGF2-induced AKT1 phosphorylation. Suppression of PPP2CA did not significantly affect VEGF- and FGF2-stimulated OFPAE cell proliferation. Thus, suppression of PPP2CA alone differentially modulated VEGF- and FGF2-induced ERK1/2, AKT1, and p38 MAPK activation, without altering VEGF- and FGF2-stimulated cell proliferation in OFPAE cells. These data also suggest that signaling molecules other than ERK1/2, AKT1, and p38 MAPK are important mediators for VEGF- and FGF2-stimulated OFPAE cell proliferation after PPP2CA suppression.

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Conflict of interest: None

Keywords

Endothelial cell; signaling transduction; placenta

INTRODUCTION

Normal pregnancy is associated with dramatic increases in placental blood flow, which is directly correlated with fetal growth and survival as well as neonatal birth weights and survivability [1-3]. These increases in placental blood flows result from both angiogenesis and vasodilatation [3-5]. Both VEGF and FGF2 are key regulators of placental vascular growth and vasodilatation [6-7]. Several lines of evidence have shown that VEGF and FGF2 promote angiogenesis and production of the potent vasodilator nitric oxide (NO) in placentas, implicating critical roles of VEGF and FGF2 in placental angiogenesis and vasodilatation [5, 8-12].

Cellular responses to VEGF and FGF2 are initiated by binding to their corresponding specific receptors, thereby activating the cytoplasmic tyrosine kinase domains of the receptors. Upon activation, these receptor tyrosine kinases subsequently activate a cascade of downstream protein kinases, including mitogen-activated protein kinase 1/2 (MAPK1/2, also termed as ERK1/2), phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT1), and p38 MAPK [13-18]. ERK1/2, a threonine/tyrosine kinase, is one major target of MAPK kinase 1/2 (MEK1/2), while AKT1 and p38 MAPK, two serine/threonine kinases, are primarily activated by PI3K and MAPK kinase 3/6 (MKK_{3/6}), respectively. p38 MAPK comprises at least four isoforms, α , β , δ , and γ , in which α , β , and δ are ubiquitously expressed, whereas γ appears to be specially expressed in skeletal muscle [16,17]. All three kinases ERK1/2, AKT1, and p38 MAPK are actively involved in regulating endothelial cell proliferation and migration [13-18]. After activation, these protein kinases must be inactivated, returning to a status ready for the next stimulation. Dephosphorylation is the primary mechanism for such inactivation [19-21]. For example, dephosphorylation of either threonine or tyrosine residue of ERK1/2 results in complete inactivation of ERK1/2 [20].

Dephosphorylation of protein kinases is catalyzed by multiple families of protein phosphatases, including serine/threonine phosphoprotein phosphatases (PPP) [19,21]. PPP2 (also termed as PP2A), one of the best-studied members of this family, accounts for the majority of total serine/threonine phosphatase activity in most tissues and cells [22-24]. The PPP2 core enzyme is composed of catalytic (C) and scaffolding (A) subunits, which can further interact with at least eighteen regulatory (B) subunits to form a trimeric holoenzyme [22-24]. The expression of these variable B subunits is developmentally regulated in a tissue specific manner, which controls the activity, specificity, and intracellular locations of the holoenzyme [22-24]. In mammals, the C subunit of PPP2 is encoded by two genes, C α (PPP2CA) and C β (PPP2CB), whose products share 97% homology in amino acid sequences; nonetheless, PPP2CA mRNA expression is about 10 fold more abundant than PPP2CB in most tissues [22,24]. The specific function of these two isoforms is currently unclear. However, mouse embryos null of PPP2CA gene die around embryonic day 6.5, largely due to absence of mesoderm formation, indicating that PPP2CB cannot completely compensate for the absence of PPP2CA in early embryonic development [25].

PPP2 participates in many other cellular processes including cell cycle progression, DNA replication, transcription, protein synthesis, and metabolism [23]. Given the fact that PPP2 is able to dephosphorylate and inactivate both ERK1/2 [26-29], AKT1 [29,30], and p38 MAPK [31], PPP2 mediated cellular responses could be modulated through ERK1/2 and AKT1. Indeed, it has been reported that inhibition of PPP2 activity increases endothelial motility [32,33] and permeability [34]. In addition, inhibition of PPP2 activity promotes stretch-induced

cellular proliferation in bovine aortic endothelial cells [35], suggesting that PPP2 acts as a growth suppressor.

We have reported that the MEK1/2/ERK1/2 and PI3K/AKT1 pathways are critical for VEGF- and FGF2-stimulated OFPAE cell proliferation [36-38]. We have recently demonstrated that PPP3 differentially modulates the VEGF- and FGF2-stimulated cell proliferation as well as the VEGF- and FGF2-induced ERK1/2 activation in OFPAE cells [39]. However, it is still unclear if PPP2 modulates VEGF- and FGF2-induced activation of ERK1/2, AKT1, and p38 MAPK, as well as placental angiogenesis. In this study, we examined if suppression of PPP2CA modulates VEGF- and FGF2-induced ERK1/2, AKT1, and p38 MAPK activation and VEGF- and FGF2-stimulated placental endothelial cell proliferation using OFPAE cells as a model.

METHODS

OFPAE Cell Preparation

Primary ovine fetoplacental artery endothelial (OFPAE) cells were established in our laboratory [40]. All OFPAE cells used in this study were at passages 8-10. Protocols for endothelial isolation and experimental procedures were approved by the Research Animal Care Committees of both the Medical and Public Health School and the College of Agriculture and Life Sciences, and by the Institutional Review Board, University of Wisconsin-Madison.

Design and Transfection of siRNA

The design and transfection of siRNA were carried out as described previously [39]. The siRNA duplex against human PPP2CA was designed based on the protein coding sequence (GenBank # NM_002715) using an online siRNA design program (Dharmacon Inc., Chicago, IL) and synthesized with a 3'-overhanging thymidine dimer (IDT, Coralville, IA). In the preliminary study, we found that out of three pairs of double-strained PPP2CA siRNA, only one pair (Sense: 5'-AGAGGCGAGCCACAUGUUATT-3'; Antisense: 3'-TTUCUCCGCUCGGUGUACAAU-5') at 20-80 nM significantly suppressed PPP2CA protein expression as compared with a pair of scrambled siRNA (Sense: 5'-AGUUUGACCUGCUCUCCAUTT-3'; Antisense: 3'-TTUCAAACUGGACGAGAGGUA-5'). The blast search analysis revealed that the sequence of PPP2CA siRNA did not share homology with any other human genes including PPP2CB (GenBank # NM_004156). This scrambled siRNA was conjugated with Cy3 at 5' end, which was used to monitor the transfection efficiency [39].

Cells were cultured in 60 mm culture dishes in Dulbecco Modified Eagle Medium (DMEM) containing 5% fetal bovine serum (FBS), 5% calf serum (CS), and 1% penicillin-streptomycin (P/S) (all from Gibco, Grand Island, NY). After reaching 50-60% of confluence, cells were washed and cultured in 1 ml of DMEM. PPP2CA siRNA was mixed with siLentFect Lipid Reagent (BioRad, Hercules, CA), diluted in DMEM, and incubated for 20 min at room temperature. This siRNA transfection complex (200 µl) was added to cell cultures to give a final siRNA concentration at 20 nM. After 5 hr of transfection, 1 ml of medium supplemented with 10% FBS, 10% CS, and 2% P/S was added to each well, followed by another 48 hr of culture. Cells were harvested and proteins were prepared for Western blot analysis as described below. Cells transfected with scrambled siRNA at 20 nM were run in parallel, served as controls.

Western Blot Analysis for PPP2CA, PPP3CA, and NOS3 Proteins

Western blot analysis was performed as described [18,36-39]. Cells were washed with cold PBS, harvested by scraping and further lysed by sonication in buffer (20 mM Imidazole-HCl, 2mM EGTA, 2mM EDTA, pH7.0, 0.1 mM phenylethyl sulfony fluoride, 0.01% Triton

X-100, 5 µg/ml leupeptin, 5 µg/ml aprotinin). The lysates were centrifuged (16,000 x g, 4° C for 10 min, and protein concentrations of the supernatant were determined. Proteins (10-15 µg/sample) were separated on 10% SDS-PAGE gels, electroblotted onto Immobilon-P membrane (Millipore, Bedford, MA). For each set of samples, multiple gels were run simultaneously. One membrane was probed with a rabbit polyclonal antibody against human PPP2C (1: 2000; catalog number: sc10402; Santa Cruz Biotechnology, Santa Cruz, CA). Since PPP2CA and PPP2CB in mammals have 97% identity in amino acid sequences, this antibody raised against full length PPP2CA of human origin should also recognize PPP2CB. As far as we were aware, no antibody specifically detecting PPP2CA or PPP2CB is commercially available. The bound antibody was detected using enhanced chemiluminescence detection systems (ECL; Amersham Biosciences, Piscataway, NJ). The membranes were reprobed with a mouse antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:4000; catalog number: RDI-TRK5G4-6C5; Research Diagnostic, Flanders, NJ). Additional membranes were probed with a rabbit antibody against PPP3CA (1: 2000; catalog number: sc9070; Santa Cruz Biotechnology) or NO synthase 3 (NOS3; 1:2500; catalog number: 610296; BD Biosciences, Franklin Lakes, NJ), followed by reprobing for GAPDH. Changes in PPP2CA, PPP3CA, NOS3, and GAPDH protein levels were quantified by a scanning densitometry. Data from four independent experiments on PPP2CA, PPP3CA, and NOS3 protein levels were normalized to GAPDH. To determine if VEGF (recombinant human VEGF₁₆₅, PeproTech, Inc, Rocky Hill, NJ) and FGF2 (bovine FGF2, R and D Systems, Minneapolis, MN) affected PPP2CA protein expression, after 48 hr of siRNA transfection and 16 hr of serum starvation, cells were treated with 10 ng/ml VEGF and FGF2 for additional one or three days, followed by Western blot analysis.

Immunoprecipitation Phosphatase Activity Assay for PPP2CA

Activities of PPP2CA in OFPAE cells were measured using a PPP2 immunoprecipitation phosphatase assay kit (Upstate, Lake Placid, NY) according to the manufacturer's instructions. This method detected the amount of free phosphate generated in a dephosphorylation reaction by measuring the absorbance of malachite green: phosphate complex. Cell extracts (100 µg/sample) obtained as described above were immunoprecipitated using a mouse monoclonal antibody against human PPP2CA. This antibody also recognizes PPP2CB according to the manufacturer. After washing with Tris buffer and the serine/threonine assay buffer, the immunoprecipitates were incubated with a phosphopeptide (a substrate for PPP2; the final concentration was 750 µM), followed by 20 min incubation at 30° C in a shaking incubator. The reaction mix was centrifuged briefly. An aliquot of the supernatant (25 µl) was transferred into a microtiter plate and mixed with freshly made malachite green phosphate solution (100 µl). After 10 min of color development at room temperature, absorbance was detected at 620 nm using a Synergy microplate fluorescence reader (Bio-TEK Instrument, Winooski, VT). In the preliminary study, we detected only PPP2CA, but not PPP3CA and NOS3 in the immunoprecipitates using Western blot analysis (data not shown). To further verify specificity of PPP2CA activity, additional immunoprecipitates were run in the presence of okadaic acid (OA, a widely used PPP2CA inhibitor; Ki = 0.2 nM; Millipore, Billerica, MA) at 1 or 10 nM, at which OA is considered to be a highly selective inhibitor for PPP2. All determinations were performed in duplicates. Controls included the samples immunoprecipitated with preimmune mouse IgG (Vector Laboratory, Burlingame, CA) at the same concentration as the PPP2CA antibody used. Data were corrected by subtracting basal absorbance of the reactions without the phosphopeptide substrate. The amount of phosphate released was calculated from a standard curve. Four independent experiments were run for the PPP2A activity assay.

Western Blot Analysis for ERK1/2, AKT1, and p38 MAPK

After 48 hr of transfection and 16 hr of serum starvation, cells were treated with 10 ng/ml VEGF or FGF2 for 0, 5, 10, 30, or 60 min. Cells were washed with cold PBS, and then harvested

and lysed by sonication in buffer (4 mM sodium pyrophosphate, 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 10 mM sodium fluoride, 2 mM sodium orthovanadate [Na₃VO₄], 1 mM PMSF, 1% Triton X-100, 5 µg/ml leupeptin, 5 µg/ml aprotinin). The lysates were centrifuged and protein concentrations of the supernatant were determined. Proteins (10-15 µg/lane) were separated on 10% SDS-PAGE gels and electroblotted to Immobilon-P membranes. For each set of samples, multiple gels were run simultaneously and each membrane was generally probed with no more than two antibodies. The membrane was probed with antibody against total or phospho-ERK1/2 (1: 2000), total or phospho-AKT1 (1:1000), or total (1:1000) or phospho-p38 MAPK (1:500). The phospho-p38 MAPK antibody was purchased from Promega (Madison, WI) and all other antibodies were from Cell Signaling Technology (Beverly, MA). The membranes were reprobed for a housekeeping gene GAPDH as described above as a loading control. The immunoreactive proteins were detected with the ECL detection system (Amersham Biosciences). Changes in total and phospho-ERK1/2, AKT1, p38 MAPK protein levels were quantified. Data on phospho-ERK1/2 and AKT1 were normalized to total ERK1/2 and AKT1, respectively. Since the total p38 MAPK antibody used only recognized α isoform of p38 MAPK, data on each isoform of phospho-p38 MAPK were normalized to total p38 α . Data on total ERK1/2, AKT1, and p38 MAPK were normalized to GAPDH. At least four independent experiments were run for ERK1/2 and AKT1 and two were run for p38 MAPK.

Cell Proliferation

Cell proliferation was assayed as described previously [18,36,37,39]. Cells after 48 hr of transfection with the PPP2CA or scrambled siRNA were cultured in 96-well plates (4000-6000 cells/well), followed by 16 hr of serum deprivation. Cells were treated without (control) or with VEGF or FGF2 at 0.01, 0.1, 1, 10, and 100 ng/ml (6 wells/dose). After another 48 hr of culture, the number of cells was determined. Briefly, wells were rinsed with PBS, fixed in methanol, air-dried, and stained with 0.1% (w/v) crystal violet. Wells were rinsed with distilled water, and air-dried again. Once dried, cells were lysed with 2% (w/v) sodium deoxycholate solution with gentle agitation. Absorbance was measured at 570 nm on a microplate reader (BioTek Instrument, Winooski, VT). Wells containing known cell numbers (0, 1000, 2000, 5000, 10000, 20000 or 40000 cells/well; 6 wells/cell density) were treated in the similar fashion to establish standard curves. Cell proliferation studies were run in four independent experiments.

Statistics Procedures

Data were analyzed using one-way ANOVA (SigmaStat; Jandel Co., San Rafael, CA). When an F-test was significant, data were compared with their respective control by the Bonferroni's multiple comparison tests or Student's t-test.

RESULTS

Effects of PPP2CA siRNA on PPP2CA Protein Expression and Activity

At 48 hr post-transfection with scrambled siRNA labeled with Cy3, the majority of cells (~ 100%) were positive for Cy3 as we reported recently [39] and exhibited similar morphology to the nontransfected cells [18,36,37,39]. PPP2CA siRNA decreased ($p < 0.05$) PPP2CA protein levels by ~ 70%, but did not alter PPP3CA, NOS3, and GAPDH protein levels (Figure 1). This suppressive effect on PPP2CA protein was maintained for at least 6 days post-transfection (data not shown). As compared with scrambled siRNA, PPP2CA siRNA also decreased ($p < 0.05$) PPP2CA activity by ~ 50% (Figure 2). As a positive assay control, OA at 1 and 10 nM greatly inhibited ($p < 0.05$) the PPP2CA activity (Figure 2).

Effects of PPP2CA siRNA on VEGF- and FGF2-Induced ERK1/2, AKT1, and p38 MAPK Phosphorylation

The basal levels of total and phospho-ERK1/2 and AKT1 in cells transfected with scrambled siRNA were similar to those in untransfected OFPAE cells as previously reported [39,40]. As compared with scrambled siRNA, PPP2CA siRNA did not significantly change basal levels (at time 0) of total ERK1/2 (total ERK1/GAPDH: 3.58 ± 0.98 vs. 3.38 ± 1.05 ; total ERK2/GAPDH: 2.41 ± 0.69 vs. 2.58 ± 0.85 for PPP2CA siRNA: scrambled siRNA) and total AKT1 (3.66 ± 0.99 vs. 3.57 ± 0.95). PPP2CA siRNA did not significantly change basal phospho-ERK1/2 (phospho/total ERK1: 0.12 ± 0.05 vs. 0.15 ± 0.04 ; phospho/total ERK2: 0.34 ± 0.13 vs. 0.47 ± 0.15) and AKT1 (phospho/total AKT1: 0.39 ± 0.09 vs. 0.42 ± 0.12). PPP2CA siRNA also did not alter total ERK1/2 (Figure 3) and AKT1 (Figure 4) levels at any time point of growth factor treatments studied.

In PPP2CA and scrambled siRNA transfected cells, both VEGF and FGF2 time-dependently elevated ($p < 0.05$) ERK1/2 phosphorylation (Figures 3). As compared with scrambled siRNA, PPP2CA siRNA did not affect VEGF-induced ERK1/2 phosphorylation, whereas inhibited ($p < 0.05$) FGF2-induced phosphorylation at 5, 10, 30, and 60 min (Figures 3). In scrambled siRNA transfected cells, FGF2, but not VEGF time-dependently induced ($p < 0.05$) AKT1 phosphorylation (Figure 4). As compared with scrambled siRNA, PPP2CA siRNA enhanced ($p < 0.05$) VEGF-induced AKT1 phosphorylation at 5 and 10 min, whereas decreased FGF2-induced AKT1 phosphorylation; however, this inhibition did not reach statistic significance (Figure 4).

For total p38 MAPK, we detected a single band at ~ 40 kD, which was corresponding to its β isoform (Figure 5;16,17). For phospho-p38 MAPK, one major band, corresponding to β isoform was detected in VEGF-stimulated cells, whereas three bands corresponding to α , β , and δ isoforms were detected in FGF2-stimulated cells (Figure 5). In both scrambled and PPP2CA siRNA treatment groups, VEGF and FGF2 did not significantly change p38 β phosphorylation levels; however, FGF2 time dependently induced p38 α and δ phosphorylation, occurred at 5 and 10 min and then declined at 30 and 60 min (Figure 5). After 30 and 60 min of FGF2 stimulation, levels of phospho-p38 α in scrambled siRNA treatment group, and levels of phospho-p38 δ in both scrambled and PPP2CA siRNA treatment groups were undetectable. As compared with scrambled siRNA, PPP2CA siRNA decreased ($p < 0.05$) FGF2-induced p38 α , but not β and δ phosphorylation, whereas did not altered levels of p38 β phosphorylation in VEGF-treated cells (Figure 5). PPP2CA siRNA did not significantly change basal levels (at time 0) of total p38 β (p38 β /GAPDH: 0.94 ± 0.05 vs. 0.87 ± 0.19 for PPP2CA siRNA: scrambled siRNA) and phospho-p38 β (phospho/total p38 β : 0.74 ± 0.08 vs. 0.50 ± 0.15). PPP2CA siRNA also did not significantly change total p38 β levels at any time point of growth factor treatments studied (Figure 5).

Under serum free culture conditions, both VEGF and FGF2 did not significantly alter PPP2CA protein levels for at least three days after 48 hr of PPP2CA siRNA transfection (data not shown).

Effects of PPP2CA siRNA on VEGF- and FGF2-Stimulated Cell Proliferation

Both VEGF and FGF2 dose-dependently stimulated proliferation of OFPAE cells transfected with PPP2CA or scrambled siRNA (Figure 6), similar to those in untransfected OFPAE cells as previously reported [40]. As compared with scrambled siRNA, PPP2CA siRNA did not significantly alter the VEGF- and FGF2-stimulated cell proliferation.

DISCUSSION

It is well established that VEGF- and FGF2-induced cellular responses are tightly mediated by a complex signaling network involving multiple protein kinases and phosphatases. However, such signaling network mediating VEGF- and FGF2-regulated placental angiogenesis, particularly for the role of protein phosphatases, is poorly defined. In the present study, we have successfully used a double stranded PPP2CA siRNA to specifically suppress PPP2CA protein expression and activity in OFPAE cells. We demonstrated that suppression of PPP2CA did not modulate VEGF-induced ERK1/2 phosphorylation, whereas it slightly enhance VEGF-induced AKT1 phosphorylation and decreased FGF2-induced ERK1/2, AKT1, and p38 MAPK phosphorylation in OFPAE cells. This suppression also did not alter VEGF- and FGF2-stimulated OFPAE cell proliferation. These data indicate that specific suppression of PPP2CA alone differentially modulates VEGF- and FGF2-induced ERK1/2, AKT1, and p38 MAPK activation; however, these modifications on signaling do not suffice to affect VEGF- and FGF2-stimulated OFPAE cell proliferation.

We showed that transfection with specific siRNA caused ~ 70% inhibition of PPP2CA protein levels but only ~ 50% decrease in its activity (Figures 1 and 2). What causes such difference is not clear. Given that the antibody used to immunoprecipitate PPP2CA also recognized PPP2CB according to the manufacturer, it was possible that the immunoprecipitates might contain PPP2CB, potentially contributing a portion of phosphatase activity we detected. More importantly, this 50% reduction in PPP2CA activity was sufficient to significantly suppress FGF2-induced ERK1/2 and p38 MAPK phosphorylation levels, which were much higher than that induced by VEGF. These data suggest that there is differential regulation of PPP2CA on VEGF- and FGF2-induced ERK1/2, AKT1, and p38 MAPK activation in OFPAE cells. Such differential modulation in OFPAE cells is not surprising because we have observed a similar phenomenon after PPP3CA protein expression was specifically knockdowned [39]. Thus, the roles of PPP2CA (current study) or PPP3CA [39] in the modulation of OFPAE cell signaling and proliferation are much more complicated than what we originally thought. It is plausible that once PPP2 or PPP3 is down-regulated, additional signaling pathways other than ERK1/2, AKT1, and p38 MAPK could emerge as major mediators for the VEGF- and FGF2-stimulated cell proliferation.

Inhibition of PPP2 by its pharmacologic inhibitors (OA or cyclosporin A) and its specific siRNA has been shown to enhance growth factor-induced ERK1/2 [26,28], AKT1 [29,30], and p38 MAPK [41] activation in many *in vitro* studies. Conversely, it has also been postulated that acute (hours by OA) and chronic (days by the siRNA) inhibition of PPP2 could lead to distinct modulations of ERK1/2 and AKT1 activation. For example, as opposed to the OA's stimulatory effect, PPP2 siRNA blunts ERK1/2 and AKT1 activation [29]. This is believed to be resulted from PPP2 siRNA-mediated chronic ERK1/2 and AKT1 hyperphosphorylation, leading to downregulation of signaling molecules upstream of Ras in response to growth factors including FGF2 [29]. In the present study, the basal levels (at time 0) of ERK1/2, AKT1, and p38 MAPK phosphorylation were similar between PPP2CA and scrambled siRNA transfected cells. Moreover, PPP2CA inhibition did not attenuate VEGF-induced ERK1/2, AKT1, and p38 MAPK activation in OFPAE cells. Thus, a negative feedback mechanism is unlikely involved in PPP2CA modulation of ERK1/2, AKT1, and p38 MAPK activation in OFPAE cells. It is more likely that differential regulation of other protein phosphatases such as MAPK phosphatase and Phosphatase and TENSin homolog (PTEN) might contribute to the differential activation of ERK1/2, AKT1, and p38 MAPK by VEGF and FGF2 in OFPAE cells transfected with PPP2CA siRNA. An alternative mechanism is that growth factors might induce different modifications (e.g. phosphorylation [42,43]) of different B units, leading to distinct interactions of the specific B subunits with PPP2A/C core enzymes in OFPAE cells. This notion is

supported by the observation that the variable B subunits differentially modulate PPP2 mediated ERK1/2 and AKT1 activation induced by growth factor in the neural cells [29].

Our previous report has shown that VEGF, but not FGF2 induces a slight increase in p38 β phosphorylation in OFPAE cells only at 60 min of VEGF stimulation [44]. In the current study, we observed that both VEGF and FGF2 did not significantly alter p38 β phosphorylation. However, we found that FGF2, but not VEGF induced p38 α and δ phosphorylation. This discrepancy might be explained by the fact that the phospho-p38 MAPK antibody used in the previous study was purchased from a different vendor (Cell Signaling) and recognized only a single isoform of p38 MAPK in OFPAE cells [44]. These data also suggest that p38 MAPK may play an important role in mediating FGF2-, but not VEGF-stimulated cell responses in OFPAE cells.

Our current findings that suppression of PPP2 failed to affect VEGF- and FGF2-stimulated OFPAE cell proliferation are in contrast with the previous reports showing that inhibition of PPP2 activity promotes endothelial cell motility [32,33], permeability [34], and proliferation [35]. It is noteworthy that in all of these previous reports, pharmacological PPP2 inhibitor OA or cantharidin was used to suppress PPP2 activity [32-35]. Being extensively used as inhibitors of PPP2 activity, the specificity of these pharmacological inhibitors, however, is largely dependent on the doses used. For example, it is well known that OA and cantharidin selectively inhibit the activity of purified PPP2 at relatively low doses (OA: IC₅₀ = 0.1 nM; cantharidin: IC₅₀ = 40 nM), while attenuating activity of other protein phosphatases including PPP1 at relatively high doses (OA: IC₅₀ = 10 nM; cantharidin: IC₅₀ = 473 nM; [34,45]). Thus, the stimulatory effects of PPP2 inhibition on endothelial cell motility and permeability observed in those previous studies might result from suppression of multiple protein phosphatases because these studies used relatively high doses of OA (10-100 nM; [32,33] or cantharidin (5-100 μ M; [34]). Conversely, a small increase in stretch-induced cell proliferation caused by a low dose of OA (0.1 nM) implies a less important role of PPP2 in cell proliferation as suggested by Murate et al. [35], which falls in line with the current observation. Moreover, it is possible that such inhibition does not reach a threshold sufficient to alter cellular proliferative responses to VEGF and FGF2 as PPP2CA siRNA decreased only 50% of PPP2CA activity in OFPAE cells. Additionally, siRNA used in the present study was designed to specifically target PPP2CA, whereas OA or cantharidin inhibits total PPP2 activity derived from all catalytic subunits of PPP2. Thus, we also cannot exclude the possibility that PPP2CB might compensate loss of phosphatase activity after PPP2CA was suppressed. Moreover, to rule out that possibility of compensation, the lack of a specific commercial antibody to PPP2CB will likely hinder completion and interpretation of studies on simultaneous knockout of both PPP2 catalytic subunits.

Together with our previous data showing critical roles of PPP3 in mediating VEGF-stimulated OFPAE cell proliferation [39], our present study suggest that PPP2 alone is not a key regulator of VEGF- and FGF2-stimulated OFPAE cell proliferation. Moreover, these data imply that after suppression of PPP2CA alone, signaling pathways other than MEK1/2/ERK1/2, PI3K/AKT1, and p38 MAPK might merge as major signaling in mediating the FGF2-stimulated OFPAE cell proliferation, as we have suggested in OFPAE cells with knockdown of PPP3 [39]. Thus, our current findings further advance our understanding of the complex signaling mechanism controlling placental endothelial function. Future studies are needed to dissect these signaling networks, which might provide fundamental information for modulating placental vasculature and blood flows by altering activation of signaling cascades by angiogenic factors.

Acknowledgments

This work was supported in part by the National Institutes of Health grants HL64703 and HD38843 (RRM/JZ), HL74947 and HL70562 (DBC), HL49210 and HL87144 (RRM).

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Figure 1.

Figure 2.

A.

B.

Figure 3.

A.

B.

Figure 4.

Figure 5.

Figure 6.