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# Compartmentalizing Proximal FGFR1 Signaling in Ovine Placental Artery Endothelial Cell Caveolae<sup>1</sup>

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## ABSTRACT

Caveolae orchestrate the dominant placental angiogenic growth factor fibroblast growth factor 2 (FGF2) signaling primarily via FGF receptor 1 (FGFR1) in placental artery endothelial cells; however, how the proximal FGF2/FGFR1 signaling is organized in the caveolae is obscure. We have shown in the present study that the FGFR substrate 2 $\alpha$  (FRS2 $\alpha$ ) is physically associated with FGFR1, and both are targeted to the caveolae via interaction with caveolin-1 in ovine fetoplacental artery endothelial cells. Treatment with FGF2 rapidly stimulated time- and concentration-dependent FRS2 $\alpha$  tyrosine phosphorylation and recruited the cytosolic growth factor receptor-bound protein 2 (GRB2)-GRB2-associated binding protein 1 (GAB1) complex to the caveolae, where they formed a ternary complex with FRS2 $\alpha$ . Disruption of caveolae by cholesterol depletion with methyl-beta-cyclodextrin inhibited FGF2-induced FRS2 $\alpha$  tyrosine phosphorylation, and it blocked the FGF2-induced recruitment of GRB2 and GAB1 to the caveolae and formation of the FRS2 $\alpha$ -GRB2-GAB1 complex in the caveolae, as well as activation of the PI3K/AKT1 and MAPK1/2 pathways. Thus, these findings have demonstrated that the proximal fibroblast growth factor (FGF2/FGFR1) signaling is compartmentalized in the placental endothelial caveolae via the FGFR substrate 2 $\alpha$  that mediates formation of a FRS2 $\alpha$ -GRB2-GAB1 complex.

*caveolae, caveolin-1, FRS2 $\alpha$ , placental endothelial cells, proximal FGF2/FGFR1 signaling*

## INTRODUCTION

Fibroblast growth factors (FGFs) are a large family of multifunctional peptide growth factors, including 28 distinct members encoded by 22 distinct genes in humans. They play pivotal roles in many different cellular processes, including proliferation, differentiation, migration, and cell survival, during all stages of prenatal and postnatal life [1]. The pleiotropic activities of FGFs are mediated by a family of FGF receptors (FGFRs) that are distinct receptor tyrosine kinases (TRKs; FGFRs 1–4) encoded by distinct genes [2]. The FGF/FGFR signaling is unique in that a specific membrane-linked

docking protein FGFR substrate 2 $\alpha$  (FRS2 $\alpha$ , also called Suc-associated neurotrophic factor-induced tyrosine-phosphorylated target, or SNT) is required [3, 4]. FRS2 $\alpha$  is rapidly tyrosine phosphorylated upon FGF stimulation [5] and functions as a “conning center” for the coordinated assembly of multiple signaling proteins for precisely activating specific intracellular signaling pathways, such as mitogen-activated protein kinases 1/2 (MAPK1/2) and phosphoinositide-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT1) pathways. FRS2 $\alpha$  contains six tyrosine residues in the C-terminus, with four (Tyr<sup>196/306/349/392</sup>) binding to an SH2 domain-containing adaptor protein (growth factor receptor-bound protein 2 [GRB2]) [3, 6] and the others (Tyr<sup>436/471</sup>) binding to an SH2 domain-containing tyrosine phosphatase (SHP2) [7]. FRS2 $\alpha$  bridges ligated FGFR1 and adaptor proteins, such as GRB2, Son of sevenless (Sos), and GRB2-associated binding protein 1 (GAB1) [3, 4, 8], unlike the other RTKs, such as vascular endothelial growth factor and epidermal growth factor receptors, which directly interact with GRB2 [9, 10]. Activated Sos leads to Ras activation on the plasma membrane where active Ras<sup>GTP</sup> recruits Raf-1 to the plasma membrane for mediating MEK1-dependent MAPK1/2 activation [11], representing the most characterized RTK-initiated cell signaling for cell survival, proliferation, metabolism, and gene expression [10]. The Ras/MAPK1/2 pathway can be also activated through FRS2 $\alpha$ -Shp2 complex [7]. Although FGF2 potently activates the PI3K/AKT pathway, presumptively through the formation of the FRS2 $\alpha$ -GRB2-GAB1 complex [8, 12, 13], how this is achieved is much less understood.

Caveolae are the  $\Omega$ -shaped plasma membrane microdomains enriched in sphingolipids and cholesterol [14–16]. Various growth factor receptors and cell signaling molecules have been found to be compartmentalized in the caveolae, suggesting an important role for caveolae in signal transduction [17]. Caveolin-1 (cav-1) is the principal structural protein of caveolae [18] that are abundantly present in terminally differentiated cells [19], including endothelial cells [20]. Cav-1 is essential for the formation of caveolae, as evidenced by the fact that ectopic expression of caveolin-1 leads to caveolae formation [21] and the loss of caveolae in *cav-1*<sup>-/-</sup> mice [22]. *Cav-1*<sup>-/-</sup> mice display impaired nitric oxide signaling, uncontrolled proliferation of pulmonary endothelial cells, and dramatic changes in vascular permeability [22], suggesting a critical role for cav-1/caveolae in angiogenesis. Cav-1 functions as a “scaffolding” protein directly interacting with various signaling molecules, and it integrates specific transmembrane signaling pathways activated by various stimuli in the caveolae [23]. Thus, it has been postulated that caveolae functions as a platform for signaling control of cell activity and reactivity [24, 25].

FGFs are major growth factors of the placenta, with FGF2 as the dominant form [26–28]. FGF2 is expressed by the trophoblast and endothelial cells in the uteri and placentas in ruminants [28–30] and humans [27]. Ovine fetoplacental artery

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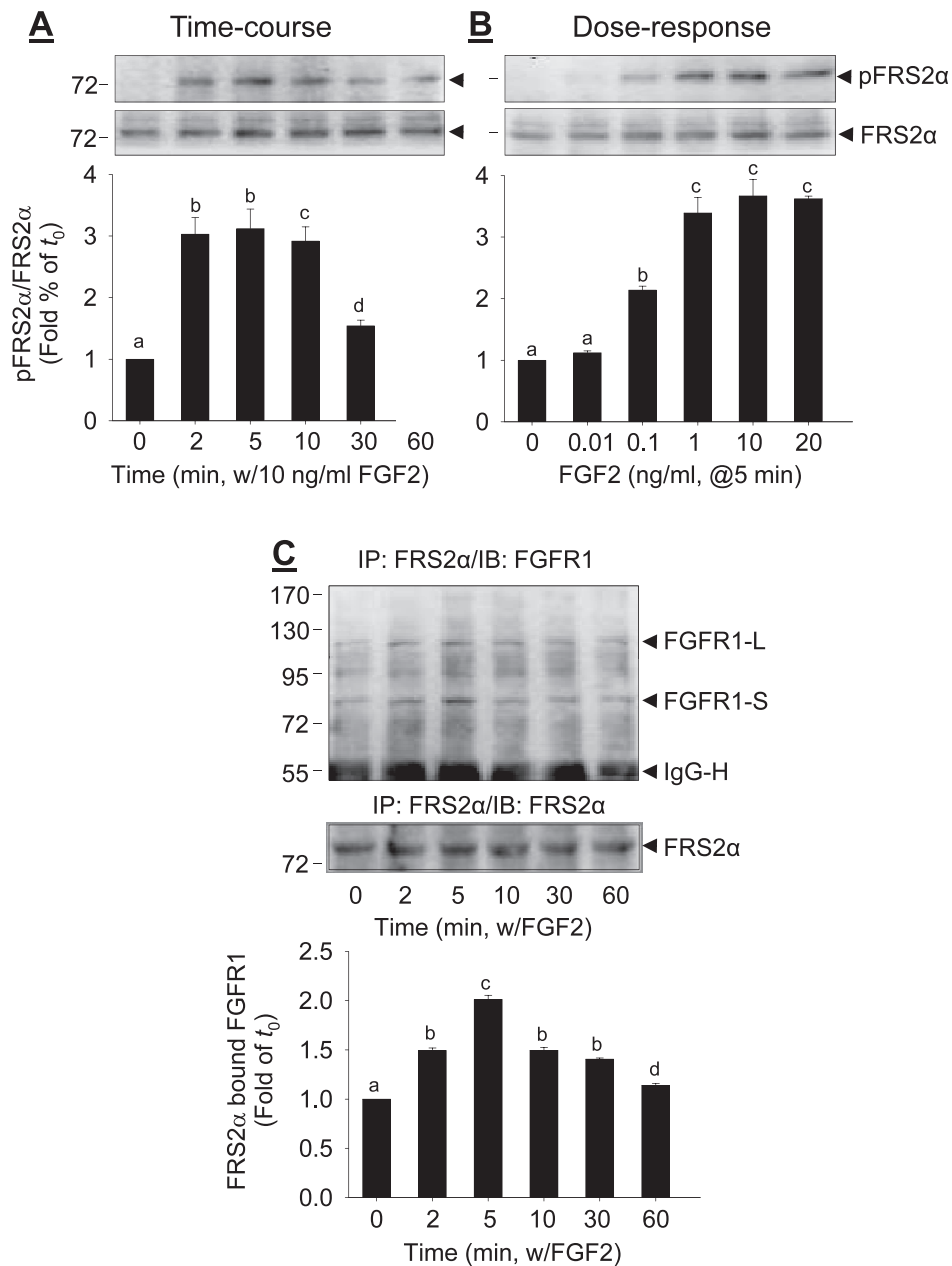


FIG. 1. FGF2-induced FRS2 $\alpha$  phosphorylation and binding to FGFR1 in oPPAE cells. The cells were treated with 10 ng/ml FGF2 up to 60 min (A) or with increasing concentrations of FGF2 for 5 min (B). Total cell lysates were subjected to immunoblotting analysis of FRS2 $\alpha$  phosphorylation. C) Co-IP of FGFR1 and FRS2 $\alpha$ . The oPPAE cells were treated with or without FGF2, and total cell lysates were immunoprecipitated with anti-FRS2 $\alpha$  antibody. The immunoprecipitates were subjected to immunoblotting with anti-FGFR1 and anti-FRS2 $\alpha$  antibodies. Experiments were repeated four times using cells from different pregnant ewes. Band intensities were quantified and expressed as mean  $\pm$  SD. Bars with different superscripts differ significantly ( $P < 0.05$ ). FGFR1-L, FGFR1 long form; FGFR1-S, FGFR1 short form; IgG-H, immunoglobulin heavy chain.

endothelial (oPPAE) cells express FGF2 both in vivo [28, 31] and in vitro [32]. Fetoplacental (cotyledonary) FGF2 mRNA expression in vivo [28] and protein secretion ex vivo are developmentally regulated, greatest at  $\sim$ Days 120–130 of ovine pregnancy [28, 31]. Expression of FGF2 changes little in uteroplacental (caruncular) tissues but increases exponentially in fetoplacental tissues in late ovine gestation, implicating that FGF2 functions as a fetal angiogenic factor for branching angiogenesis that occurs mainly in the fetal cotyledonary tissues [28]. In oPPAE cells, we have recently reported that activation of the MAPK and PI3K pathways by FGF2 is mainly mediated by FGFR1, which is compartmentalized in the caveolae and paradoxically regulated by cav-1 [33]. However,

how the proximal FGFR1 signaling is regulated in these cells has not been reported. FRS2 $\alpha$  protein contains myristyl anchors in their NH<sub>2</sub>-terminus, which is essential for targeting FRS2 $\alpha$  to the plasma membrane and important for FRS2 $\alpha$  phosphorylation and subsequent activation of downstream signaling pathways in response to FGF or nerve growth factor stimulation [3]. Although previous studies have shown that FRS2 $\alpha$  is spatially present in the caveolae/lipid rafts in human neuroblastoma cells [34, 35], whether FRS2 $\alpha$  is compartmentalized in the caveolae in placental endothelial cells remains to be determined. In this study, we hypothesize that the proximal FGF2/FGFR1 signaling via FRS2 $\alpha$  is compartmentalized in placental endothelial caveolae via direct interaction with cav-1.

We found that FRS2 $\alpha$  is stably partitioned in the caveolae via interaction with cav-1. Treatment with FGF2 rapidly recruited GRB2 and GAB1 to the caveolae, where they form a complex with FRS2 $\alpha$ . Disrupting caveolae inhibited the FGF2-induced FRS2 $\alpha$  phosphorylation, blocked the formation of the FRS2 $\alpha$ -GAB1-GRB2 complex, and inhibited downstream PI3K/AKT pathway activation. Thus, these findings suggest that the proximal FGF2/FGFR1 signaling is compartmentalized in the endothelial caveolae.

## MATERIALS AND METHODS

### Antibodies and Chemicals

Recombinant FGF2 (157 amino acids) and mouse monoclonal antibody (mAb) of FRS2 $\alpha$  were from R&D Systems. Rabbit polyclonal antibody (pAb) of FGFR1 was from Zymed. Rabbit pAb against GAB1 and mouse mAb against GRB2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit pAbs against phospho-FRS2 $\alpha$ <sup>Tyr196</sup> (pFRS2 $\alpha$ ), phospho-AKT<sup>ser473</sup> (pAKT1), phospho-MAPK1/2<sup>Thr202/Tyr204</sup> (pMAPK1/2), AKT1, and MAPK1/2 were from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were obtained from Pierce. Caveolin scaffolding domain (Cav-SD; amino acids 82–101) fused with the N-terminus to the antennapedia internalization sequence (amino acids 43–58) and its negative control peptides (Cav-SDX) were from EMD Calbiochem. Cell culture supplies were from Invitrogen/GIBCO. Methyl- $\beta$ -cyclodextrin (M $\beta$ CD) and other reagents were from Sigma-Aldrich unless indicated otherwise.

### Cell Culture and Preparation of Total Cell Extracts

The oPFAE cells were isolated from four late (Days 120–130, gestation  $\approx$ 147 days) pregnant ewes as previously described [32]. The animal (sheep) use protocol was approved by the University of California, San Diego, Animal Subjects Committee. Cells were subcultured in MCDB-131 containing 10% fetal bovine serum and were used at passages 7–11. Prior to each experiment, subconfluent ( $\sim$ 70%–80%) cells were serum starved in M-199 containing 1% fetal bovine serum, 0.1% bovine serum albumin, and 25 mM HEPES overnight. Following stimulation, total cellular proteins were harvested in a nondenaturing lysis buffer [36] and protein concentration was measured.

### Immunofluorescence Microscopy

Cells were fixed with 3.7% paraformaldehyde in PBS on ice for 20 min. After rinsing with ice-cold PBS three times, cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min on ice. After incubation with 5% bovine serum albumin in PBST (PBS with 0.1% Tween 20) for 1 h at room temperature, the cells were incubated with the primary antibody overnight, followed by incubation with Rhodamine Red-X-conjugated goat anti-rabbit immunoglobulin G (IgG) or Alexa<sup>488</sup>-conjugated goat anti-mouse IgG (1:500 dilution) for 2 h at room temperature. After washing three times with PBST, cells were mounted in Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). The cells were examined under an inverted Leica fluorescent microscope, and images were captured with a digital camera. Data were analyzed by SimplePCI software (Hamamatsu Corp., Sewickley, PA).

### Immunoprecipitation, SDS-PAGE, and Western Blot Analysis

Equal amounts of cellular proteins (500  $\mu$ g per sample) were precleared by incubation with 50% protein A/G agarose beads (20  $\mu$ l) for 1 h at 4°C, followed by centrifugation (15 000  $\times$  g, 30 sec). The samples were then incubated with specific antibody (1  $\mu$ g) overnight at 4°C. Protein A/G agarose beads (20  $\mu$ l) were added to pull down the targeted proteins. After washing three times, the immunoprecipitated samples or total protein extracts were run on SDS-PAGE and analyzed by Western blotting as described previously [36]. The relative density of a protein band was calculated by multiplying the absorbance of the surface areas using the National Institutes of Health ImageJ software.

### Competitive Binding Studies

Serum-starved oPFAE cells were treated with or without Cav-SD or Cav-SDX peptides (5  $\mu$ M) for 2 h at 37°C. The cells were then lysed with a nondenaturing buffer [36]. Total cell extracts (500  $\mu$ g per sample) were precleared with protein G agarose beads and then used for immunoprecipitation

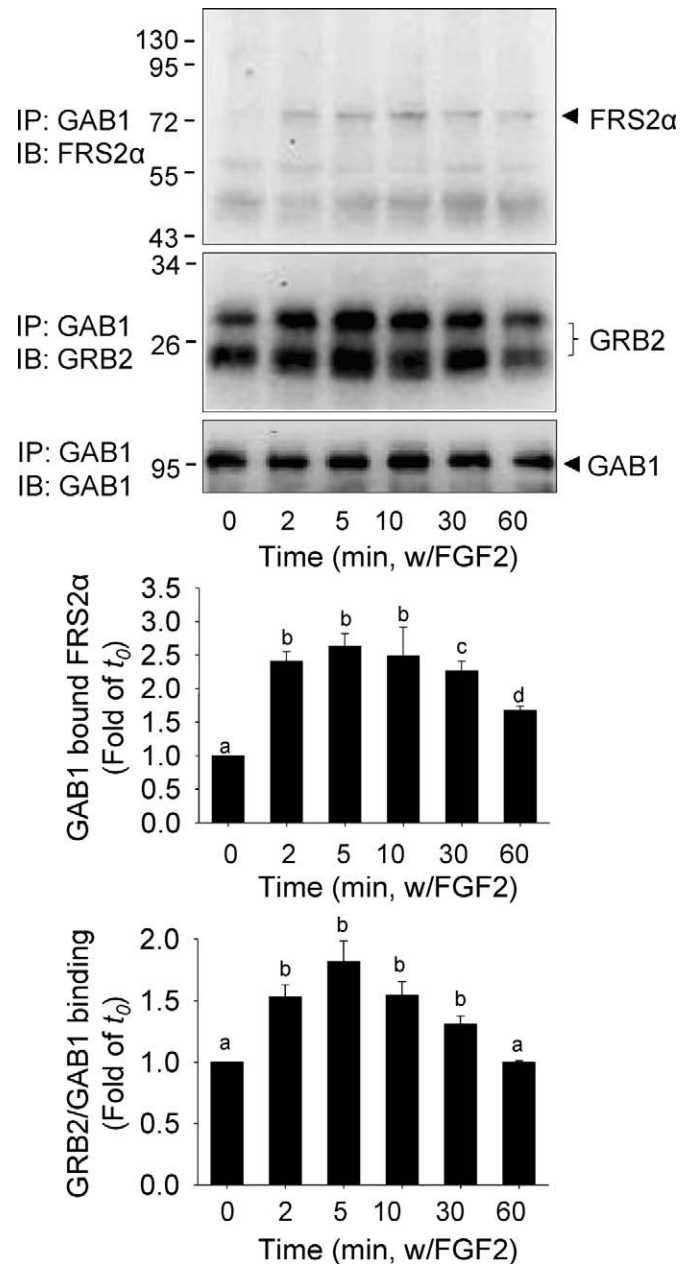


FIG. 2. Effects of FGF2 on the formation of FRS2 $\alpha$ -GAB1-GRB2 complex in oPFAE cells. The cells were treated with 10 ng/ml FGF2 up to 60 min. Total cell lysates were prepared for IP with anti-GAB1 antibody. The immunoprecipitates were subjected to immunoblotting with anti-GAB1, anti-GRB2, and anti-FRS2 $\alpha$  antibodies. Experiments were repeated four times using cells from different pregnant ewes, and band intensities were quantified and expressed as mean  $\pm$  SD. Bars with different superscripts differ significantly ( $P < 0.05$ ).

(IP) with rabbit anti-cav-1 pAb (1  $\mu$ g). The IP samples were analyzed by immunoblotting with the anti-FRS2 $\alpha$  and anti-cav-1 antibodies.

### Caveolae Isolation

Detergent-free isolation of caveolae membranes was performed exactly as previously described [36]. Briefly, the cells ( $\sim$ 2  $\times$  10<sup>7</sup>) were homogenized and sonicated on ice in 2.5 ml of 0.5 M sodium carbonate buffer (pH 11.0) with protease inhibitors. The homogenates ( $\sim$ 5 mg of proteins) were adjusted to 4 ml of MBS buffer (25 mM MES [pH 6.5], 0.15 M NaCl) containing 45% sucrose and were placed at the bottom of 12.5-ml ultracentrifuge tubes. A 5%–35% discontinuous sucrose gradient in MBS was formed above as 4 ml each. Samples were centrifuged at 39 000 rpm ( $\approx$ 260 000  $\times$  g) for 16–20 h in an SW41 rotor (Beckman Instruments). Fractions (1 ml each) were collected from

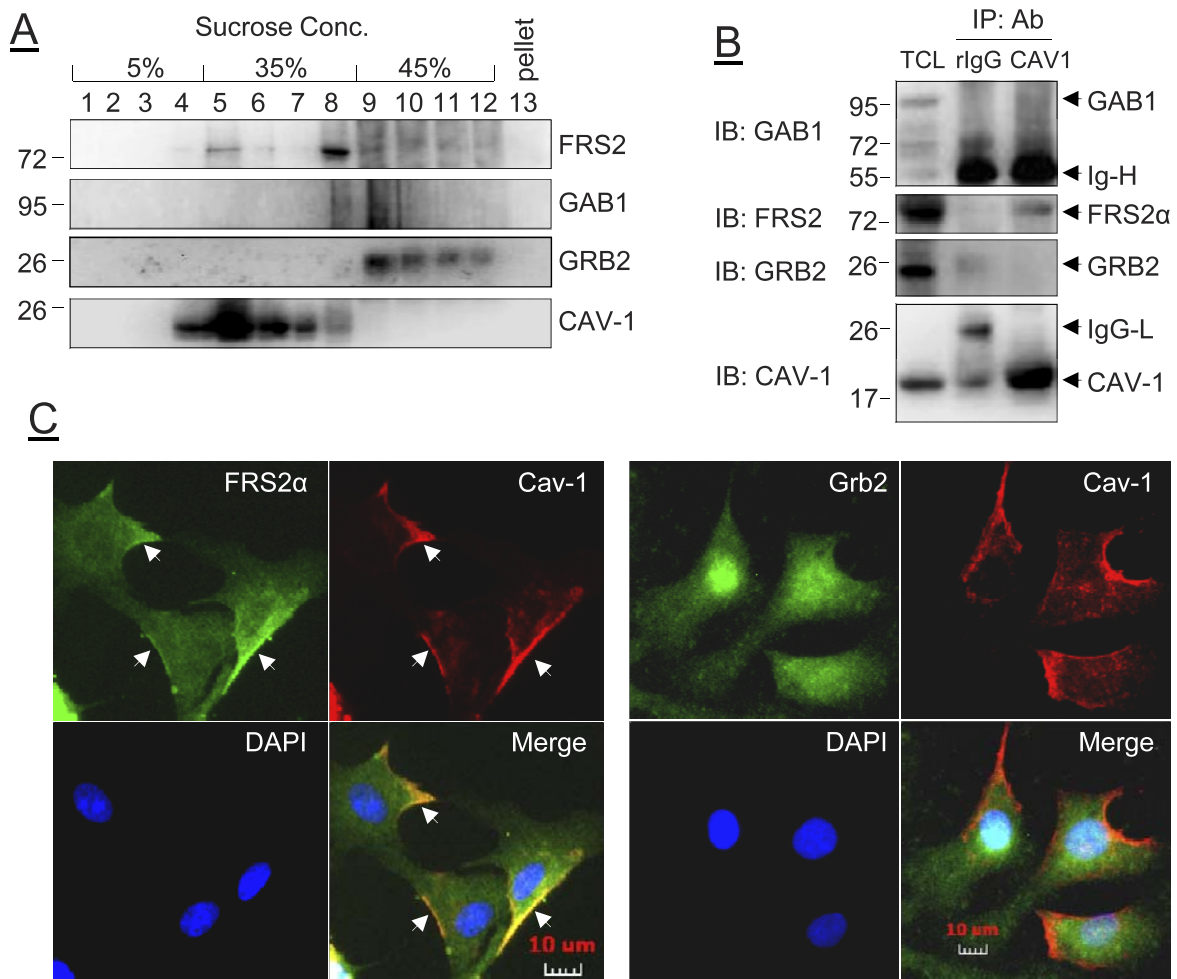


FIG. 3. Effects of FGF2 on the subcellular localization of FRS2 $\alpha$ , GRB2, and GAB1 in oFPAE cells. **A**) The resting cells were homogenized and fractionated by sucrose-gradient ultracentrifugation. Fractions were subjected to immunoblotting with antibodies against FRS2 $\alpha$ , GAB1, GRB2, and cav-1. **B**) Co-IP of FRS2 $\alpha$ , GRB2, and GAB1. The cells were treated with or without FGF2, and total cell lysates were immunoprecipitated with anti-cav-1 antibody. The immunoprecipitates were immunoblotted with antibodies against FRS2 $\alpha$ , GRB2, GAB1, and cav-1. Blots of a typical experiment are shown representing similar data from four experiments using cells from different pregnant ewes. TCL, total cell lysate; Ab, antibody; rIgG, rabbit immunoglobulin; IgG-H, immunoglobulin heavy chain; IgG-L, immunoglobulin light chain. **C**) Cells grown on glass coverslips were fixed and subjected to double-immunofluorescence labeling with anti-FRS2 $\alpha$  or anti-GRB2 paired with anti-cav-1 antibodies, followed by corresponding Rhodamine Red-X-conjugated goat anti-rabbit IgG or Alexa Fluor<sup>488</sup>-conjugated goat anti-mouse IgG secondary antibodies. Cells were mounted in Prolong Gold antifade reagent with DAPI and examined under an inverted Leica fluorescence microscope for image acquisition by a digital camera. Arrows indicate plasma membrane FRS2 $\alpha$  and Cav1 and their colocalization.

the top and mixed with 5 $\times$  SDS sample buffer<sup>5</sup> immediately for immunoblotting analysis.

### Experimental Replication and Statistical Analysis

All experiments were repeated at least four times using cells from different pregnant ewes. Data were presented as means  $\pm$  SD and analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni test for multiple comparisons using SigmaStat 3.5 (Systat Software Inc.). Student paired *t*-test was used for comparison of data between two groups. Significance was defined as  $P < 0.05$ .

## RESULTS

### FGF2 Induced FRS2 $\alpha$ Tyrosine Phosphorylation in a Time- and Concentration-Dependent Manner

Treatment with FGF2 stimulated rapid tyrosine phosphorylation of FRS2 $\alpha$  at Try<sup>196</sup> in a time-dependent (Fig. 1A) and dose-dependent (Fig. 1B) fashion in oFPAE cells. When cells were treated with 10 ng/ml FGF2 up to 60 min, FRS2 $\alpha$

phosphorylation began within 2 min, reaching a peak level (3.2-fold of control;  $P < 0.05$ ) within 5–10 min. Thereafter, it declined gradually but did not return to the baseline at 60 min (Fig. 1A). When the cells were treated with increasing concentrations (0.01–100 ng/ml) of FGF2 for 5 min, as little as 0.1 ng/ml FGF2 was able to induce FRS2 $\alpha$  phosphorylation. Treatment with 10 ng/ml FGF2 dramatically stimulated FRS2 $\alpha$  phosphorylation, consistent with the time-course studies. Higher doses of FGF2 did not further enhance FRS2 $\alpha$  phosphorylation. Thus, FGF2-stimulated FRS2 $\alpha$  phosphorylation maximizes around 5 min after treatment with 10 ng/ml FGF2.

### FGF2 Increases FGFR1-Bound FRS2 $\alpha$

In oFPAE cells, FRS2 $\alpha$  interacted with FGFR1 in the absence and presence of FGF2 (Fig. 1C). However, the amounts of FGFR1-bound FRS2 $\alpha$  were increased by treatment with 10 ng/ml FGF2 in a time-dependent manner, reached their maximum (approximately two times that of time zero;  $P <$

0.05) within 2 min after FGF2 treatment, and then declined gradually, but did not return to the baseline at 60 min.

#### *FGF2 Induced the Formation of an FRS2 $\alpha$ -GRB2-GAB1 Complex*

The proximal FGF/FGFR signaling is mediated by the adaptor protein GRB2 binding to FRS2 $\alpha$  via the SH2 domain and to GAB1 and other signaling molecules via the SH3 domain [3]. In oFPAE cells, very low levels of GAB1 were found to be bound to FRS2 $\alpha$  without FGF2 stimulation (Fig. 2). However, treatment with 10 ng/ml FGF2 rapidly stimulated the amounts of FRS2 $\alpha$ -bound GAB1, maximizing (up to about 2.6 times of that of the time zero;  $P < 0.05$ ) within 2 min and declining after 30 min of FGF2 stimulation. In resting cells, GAB1 was found to be associated with GRB2, and the binding was significantly enhanced by treatment with 10 ng/ml FGF2 (up to about 1.8 times of that of the time zero,  $P < 0.05$ ; Fig. 2). Thus, these results suggest assembly of a ternary complex containing FRS2 $\alpha$ , GRB2, and GAB1 following FGF2 stimulation.

#### *FRS2 $\alpha$ , but Not GRB2 or GAB1, Was Physically Bound to Cav-1 in Resting Endothelial Cell Caveolae*

FRS2 $\alpha$  is targeted to the plasma membrane via myristylation [3]. In human neuroblastoma cell LAN-1 cells, FRS2 is located in the plasma membrane caveolae-like microdomain [34, 35]. By using the well-established discontinuous sucrose gradient isolation procedure [36, 37], FRS2 $\alpha$  was found in the purified caveolae membranes coexisting with cav-1, although most FRS2 $\alpha$  was found in the noncaveolae fractions; neither GAB1 nor GRB2 was detected in the caveolae fractions in the resting cells (Fig. 3A). We used co-IP studies to verify these findings. As shown in Figure 3B, only FRS2 $\alpha$ , but not GRB2 or GAB1, was associated with cav-1 in the resting cells. We also preformed double immunofluorescence labeling experiments to localize FRS2 $\alpha$ , GRB2, and cav-1 in the cells. As shown in Figure 3C, FRS2 $\alpha$  and GRB2 (in green fluorescence) were found to be localized throughout the cells; however, only FRS2 $\alpha$ , but not GRB2, was localized in the plasma membrane. Cav-1 (in red) was mainly present at the leading edges of cells. In the merged micrographs, FRS2 $\alpha$ , but not GRB2, was found to be colocalized with cav-1 along the plasma membrane.

#### *FGF2 Regulates FRS2 $\alpha$ Interaction with Cav-1*

Targeting signaling molecules to caveolae is mainly mediated via the so-called “caveolin binding motif,” defined as  $\phi$ X $\phi$ XXXX $\phi$  or  $\phi$ XXXX $\phi$ XX $\phi$  (where  $\phi$  is one of the aromatic amino acids Trp, Phe, or Tyr [38]), which can directly interact with the caveolin scaffolding domain of caveolin proteins. Amino acid sequence analysis revealed that FRS2 $\alpha$  protein possesses one caveolin-binding motif (<sup>65</sup>YGYDSNLF<sup>72</sup>). We used synthetic Cav-SD peptides to verify whether FRS2 $\alpha$  binds to cav-1 in oFPAE cells. As shown in Figure 4A, pretreatment with the Cav-SD, but not Cav-SDX, peptides reduced the amounts of cav-1-bound FRS2 $\alpha$  to one third of that in resting oFPAE cells ( $P < 0.05$ ). In addition, time-course studies showed that FGF2 dynamically regulated FRS2 $\alpha$  and cav-1 interaction. Treatment with 10 ng/ml FGF2 rapidly decreased the amount of cav-1-bound FRS2 $\alpha$  to less than half of that in the resting cells within 5–10 min; this inhibition was lost after 60 min after FGF2 treatment (Fig. 4B).

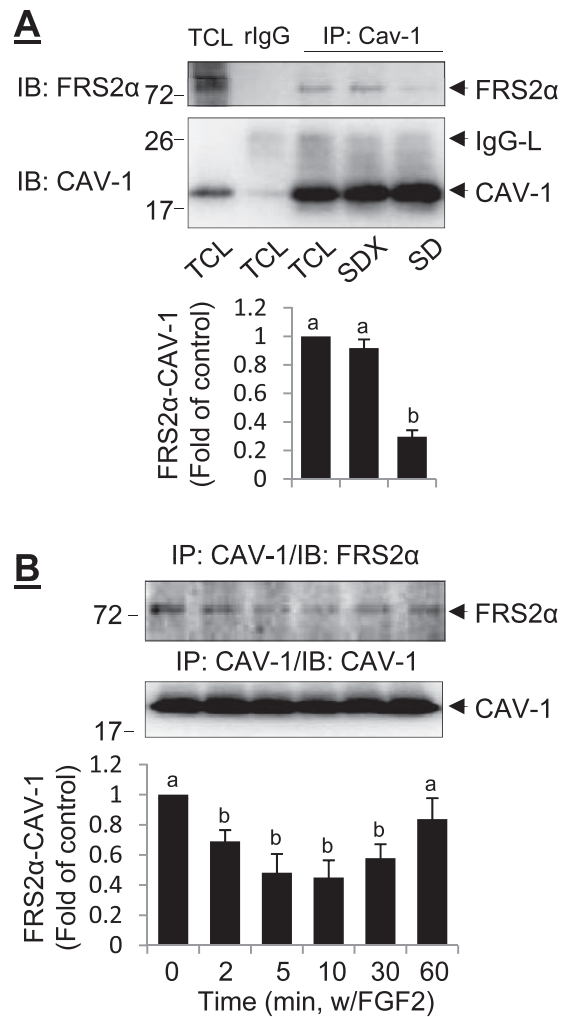


FIG. 4. Effects of FGF2 on FRS2 $\alpha$  interaction with cav-1 in oFPAE cells. **A**) Caveolin scaffolding domain (SD) peptide competes off cav-1 binding to FRS2 $\alpha$ . The cells were pretreated with 5  $\mu$ M Cav-SD or Cav-SDX (control) peptides, and total cell lysates were prepared for immunoprecipitation with anti-cav-1 antibody. The immunoprecipitates were immunoblotted with anti-FRS2 $\alpha$  and anti-cav-1 antibodies. **B** and **C**) The oFPAE cells were treated with or without 10 ng/ml FGF2 up to 60 min. Total cellular proteins were immunoprecipitated with anti-cav-1 antibody followed by immunoblotting with anti-FRS2 $\alpha$  and anti-cav-1 antibodies. Experiments were repeated four times using cells from different pregnant ewes, and band intensities were quantified and expressed as mean  $\pm$  SD. Bars with different superscripts differ significantly ( $P < 0.05$ ). TCL, total cell lysate; rIgG, rabbit immunoglobulin; IgG-H, immunoglobulin heavy chain; IgG-L, immunoglobulin light chain.

#### *Integral Caveolae Is Essential for the Formation of Caveolar FRS2 $\alpha$ -GRB2-GAB1 Complex in Response to FGF2 Stimulation*

Cholesterol depletion with M $\beta$ CD has been widely used as a means for studying the role of integral caveolae in signal transduction [39]. In oFPAE cells, treatment with 10 mM M $\beta$ CD for 1 h resulted in loss of caveolae [36]. Treatment with FGF2 (10 ng/ml, 5 min) resulted in the recruitment of GRB2 and GAB1 to the caveolae in oFPAE cells without M $\beta$ CD pretreatment (Fig. 5). However, when the cells were pretreated with M $\beta$ CD to disrupt integral caveolae, FGF2 was unable to recruit GRB2 and GAB1 to the caveolae (Fig. 5A). Furthermore, without M $\beta$ CD pretreatment FGF2 (10 ng/ml, 5 min) was able to induce the formation of the FRS2 $\alpha$ -GRB2-

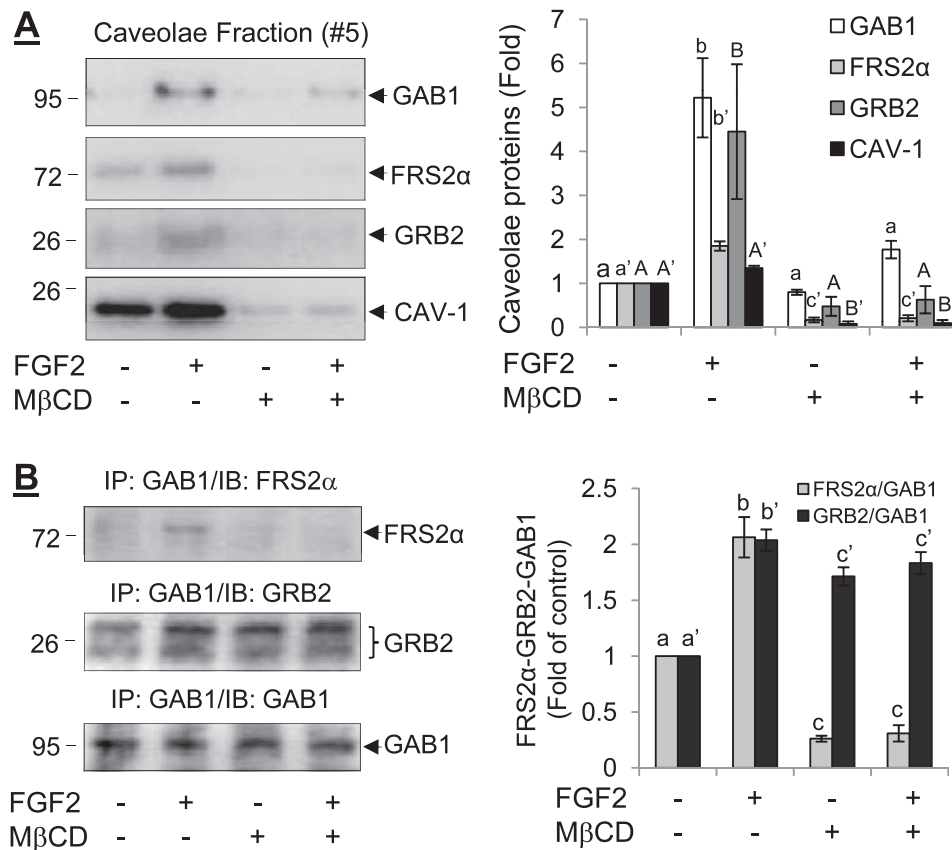


FIG. 5. Effects of caveolae disruption with MβCD on FGF2-induced FRS2α-GRB2-GAB1 formation in oFPAE cells. **A**) The cells were pretreated with or without 10 mM MβCD for 1 h, followed by stimulation with 10 ng/ml FGF2 for 5 min. Cells were lysed to isolate the caveolae membranes by sucrose-gradient fractionation. The caveolae membrane fractions were analyzed by immunoblotting with antibodies for FRS2α, GRB2, GAB1, and cav-1. **B**) The cells were pretreated with or without 10 mM MβCD for 1 h, followed by stimulation with 10 ng/ml FGF2 for 5 min. Total cellular proteins were prepared for IP with anti-GAB1 antibody, followed by immunoblotting with antibodies for FRS2α, GRB2, and GAB1. Experiments were repeated four times using cells from different pregnant ewes, and band intensities were quantified and expressed as mean ± SD. Bars with different superscripts differ significantly ( $P < 0.05$ ).

GAB1 complex in the oFPAE cell caveolae. However, with MβCD pretreatment FGF2 was unable to do so (Fig. 5B).

#### Integral Caveolae Is Required for the Full Activation of FGF2 Signaling

We also investigated the effects of caveolae disruption with MβCD on the FGF2-induced FRS2α phosphorylation and activation of MAPK1/2 and AKT. As summarized in Figure 6, treatment with FGF2 (10 ng/ml, 5 min) potentially increased the levels of phosphorylated FRS2α, MAPK1/2, and AKT1 in oFPAE cells. However, pretreatment with 10 mM MβCD for 1 h inhibited FGF2-induced FRS2α phosphorylation and activation of MAPK1/2 and AKT1.

#### DISCUSSION

We have demonstrated in the present study that the proximal FGF2/FGFR1 signaling is compartmentalized in the caveolae via FRS2α interactions with cav-1 in placental endothelial cells. This conclusion is drawn based on the following findings. First, treatment with FGF2 rapidly stimulates tyrosine phosphorylation of FRS2α that is physically associated with FGFR1 in the caveolae [33]. Second, FRS2α, but neither GAB1 nor GRB2, is found in the caveolae, where it is associated with cav-1 in resting endothelial cells. Third, GRB2 is associated with GAB1 but neither is associated with FRS2α

in resting endothelial cells; however, treatment with FGF2 stimulated the formation of a ternary complex containing FRS2α, GRB2, and GAB1 in the caveolae. Fourth, disruption of integral caveolae with MβCD blocks the formation of the FRS2α-GRB2-GAB1 complex and significantly inhibits the activation of the downstream MAPK1/2 and AKT1. Together with our most recent report demonstrating a paradoxical role for cav-1/caveolae in regulating the FGF2/FGFR1 signaling control of angiogenic responses via the MEK/ERK2/1 and PI3K/AKT pathways in oFPAE cells [33], our current findings have further strengthened the concept that caveolae functions as a focal center for orchestrating FGF2/FGFR1 signaling control of placental angiogenesis.

A major finding in our present study is that of caveolar targeting of the proximal FGF2/FGFR1 signaling via the formation of the FRS2α-GRB2-GAB1 complex. This is because although GAB1 has been considered a major player for mediating the activation of the PI3K/AKT1 pathway by FGF via FGFRs [8, 12, 13], little is known regarding the proximal FGF/FGFR signaling mechanism that controls activation of the PI3K/AKT1 pathway. Our findings suggest that caveolae functions as a focal center for organizing the proximal FGF2/FGFR1 signaling via FRS2α in placental endothelial cells because: 1) FRS2α has a caveolin binding motif (<sup>65</sup>YGYDSNLF<sup>72</sup>, aromatic amino acids underlined) [38] that is highly conserved among different species [40–42]; 2)

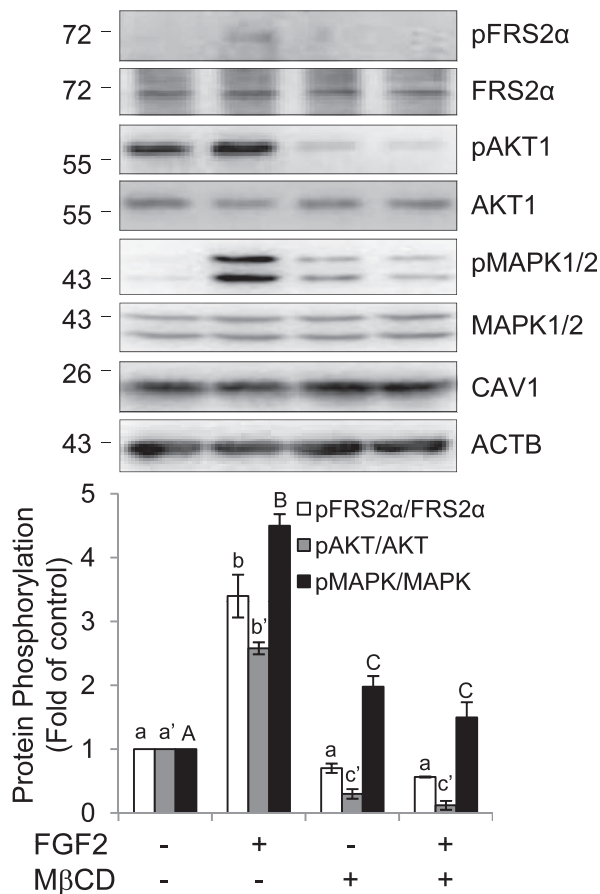


FIG. 6. Effects of caveolae disruption with M $\beta$ CD on FGF2-induced FRS2 $\alpha$  phosphorylation and activation of AKT1 and MAPK1/2 in oPAE cells. The cells were pretreated with or without 10 mM M $\beta$ CD for 1 h, followed by stimulation with 10 ng/ml FGF2 for 5 min. Total cellular proteins were prepared for analyzing phosphorylation of FRS2 $\alpha$ , AKT1, and MAPK1/2 by immunoblotting with specific antibodies. Total cav-1 and ACTB were measured by immunoblotting for a loading control. Experiments were repeated four times using cells from different pregnant ewes, and band intensities were quantified and expressed as mean  $\pm$  SD. Bars with different superscripts differ significantly ( $P < 0.05$ ).

FRS2 $\alpha$  cofractionates with cav-1 in the caveolae membranes; 3) FRS2 $\alpha$  colocalizes with cav-1 at the plasma membrane; and 4) FRS2 $\alpha$  coimmunoprecipitates with cav-1, which can be competed off with caveolin scaffolding domain peptide. Moreover, treatment with FGF2 rapidly reduces the levels of cav-1-bound FRS2 $\alpha$  and then returns to baseline in a time-dependent fashion, suggesting that FGF2 temporally regulates the dissociation of FRS2 $\alpha$  from cav-1. Consistently, we have shown more recently that FGF2 regulates a similar time-dependent change in FGFR1 association/dissociation with cav-1 in oPAE cells [33]. Thus, these findings suggest that binding to cav-1 renders FGFR1 and FRS2 $\alpha$  in an inactive state; upon FGF2 treatment, dissociation from cav-1 might be required for FGFR1 and FRS2 $\alpha$  activation. From this standpoint, cav-1 seems to act as an intrinsic switch to the spatiotemporal control of the proximal FGF/FGFR signaling via FRS2 $\alpha$  in the caveolae.

It is noteworthy that, contrasting with a previous report suggesting cofractionation of GRB2 and GAB1 with cav-1 in the caveolae membranes in other cell types [43], our current results do not support these two important proximal FGF/FGFR signaling proteins to be caveolar proteins. First, they are found to be mainly present in the noncaveolar compartments in resting oPAE cells. Second, the two proteins cannot be immunoprecipitated with the anti-cav-1 antibody. Third, they are constitutively associated with each other without binding to FRS2 $\alpha$  in resting cells. Fourth, they do not have the caveolin binding domain that provides a structural base for being a caveolar protein according to amino acid sequence analysis. However, our data suggest that GRB2 and GAB1 can be rapidly recruited to the caveolae, where they form a ternary complex with FRS2 $\alpha$  upon FGF2 stimulation. This notion is supported by the findings showing that disruption of integral caveolae with M $\beta$ CD reduces the FGF2-induced FRS2 $\alpha$  phosphorylation and blocks the FGF2-induced FRS2 $\alpha$ -GRB2-GAB1 ternary complex formation and AKT1 activation, without altering GRB2 association with GAB1 in oPAE cells (Fig. 5). In keeping with the fact that GAB1 is responsible for mediating PI3K/AKT1 activation via FGF/FGFR signaling [8, 12, 13], our findings allow us to conclude that caveolae functions as a platform for organizing the proximal FGF2/FGFR1 signaling via formation of the FRS2 $\alpha$ -GRB2-GAB1 ternary complex, thereby mediating the downstream activation

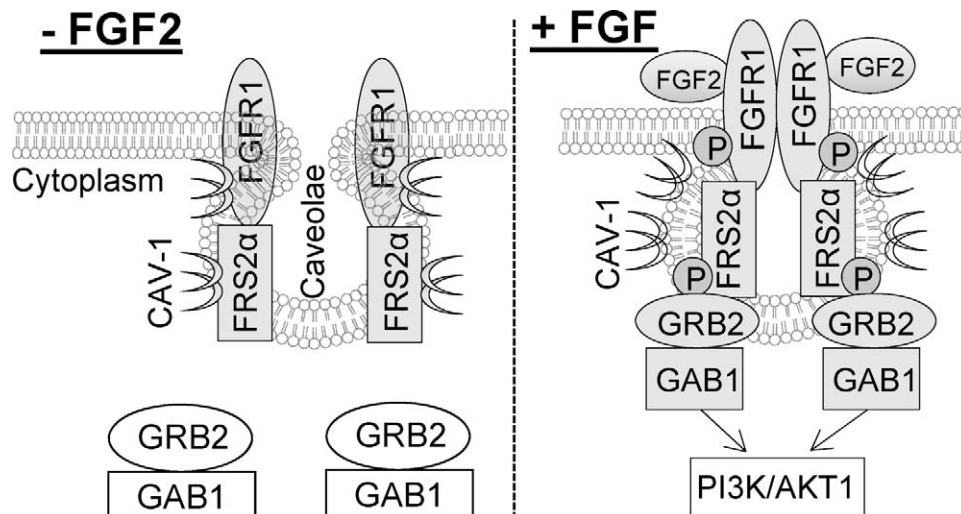


FIG. 7. Caveolae targeting of the proximal FGF2/FGFR1 signaling in oPAE cells via FRS2 $\alpha$ .



of the PI3K/AKT1 signaling pathway in placental endothelial cells. In parallel, caveolae disruption also inhibited MAPK1/2 activation (as shown above and in Feng et al. [33]), suggesting a critical role for caveolae in regulating the ERK2/1 pathway in placental endothelial cells. However, how the proximal FGF2/FGFR1 signaling to the MAPK1/2 pathway, likely via FRS2 $\alpha$ -GRB2-Sos [3, 4, 8], is organized in the caveolae is waiting for further investigation.

Together, we have demonstrated in the present study that the proximal FGF2/FGFR1 signaling via FRS2 $\alpha$  is compartmentalized in placental endothelial caveolae. In resting conditions, binding to cav-1 sequesters FGFR1 and FRS2 $\alpha$  in an inactive form. Upon FGF2 stimulation, FGFR1 and FRS2 $\alpha$  are temporally dissociated from cav-1, which facilitates their phosphorylation; the cytosolic GRB2-GAB1 complex is then recruited to the caveolae, where it forms a ternary complex with FRS2 $\alpha$  for mediating downstream signaling transduction cascades (Fig. 7).

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