The Mammalian Target of Rapamycin-p70 Ribosomal S6 Kinase but Not Phosphatidylinositol 3-Kinase-Akt Signaling Is Responsible for Fibroblast Growth Factor-9-induced Cell Proliferation*

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Fibroblast growth factor-9 (FGF9) is a potent mitogen that stimulates normal and cancer cell proliferation through the signaling mechanism is not fully understood. In this study, we aimed to unravel the signaling cascades mediate FGF9 actions in human uterine endometrial stromal cell. Our results demonstrate that the mitogenic effect of FGF9 is transduced via two parallel but additive signaling pathways involving mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase. Activation of mTOR by FGF9 induces p70 ribosomal S6 kinase (S6K1) phosphorylation, cyclin expression, and cell proliferation, which are independent of phosphatidylinositol 3-kinase and Akt. Coimmunoprecipitation analysis demonstrates that mTOR physically associates with S6K1 upon FGF9 treatment, whereas ablation of mTOR activity using RNA interference or pharmacological inhibitor blocks S6K1 phosphorylation and cell proliferation induced by FGF9. Further study demonstrates that activation of mTOR is regulated by a phospholipase Cγ-controlled calcium signaling pathway. These studies provide evidence to demonstrate, for the first time, that a novel signaling cascad involving phospholipase Cγ, calcium, mTOR, and S6K1 is activated by FGF9 in a receptor-specific manner.

Fibroblast growth factor-9 (FGF9), 1 a potent mitogen and survival factor for numerous cell types (1–6), belongs to the heparin-binding polypeptide family that consists of at least 23 structurally related proteins (7, 8). Expression of FGF9 plays critical roles in sex determination, bone formation, neuron development, lens fiber differentiation, and gap junction formation (4, 9–12). Null-allele mice lacking FGF9 exhibit severe lung hypoplasia and die shortly after birth (4). On the other hand, aberrant expression of FGF9 is associated with the development of several human diseases including prostate cancer, brain tumor, and endometriosis (3, 6, 13). Despite its well-studied biological functions, the signaling mechanism of FGF9 remains to be identified.

The signaling of FGF is mediated via complex interactions between specific members of the FGF family and one or more FGF receptor isoforms. Receptors for FGF (FGFR1 to -4) are tyrosine kinase receptors consisting of two intracellular tyrosine kinase domains, a single transmembrane domain, and an extracellular portion that contains three immunoglobulin-like (Ig) domains. The third Ig domain (Ig III), which exerts the highest impact on FGF receptor binding specificity and tissue-specific expression patterns, is the region in which alternative splicing occurs. Three different splice variants (designated as IIIa, IIIb, and IIIc) have been identified for FGFR1 and FGFR2, whereas only the IIIb and IIIc variants have been detected for FGFR3 (14–17). As of yet, no splice variant for FGFR4 has been identified. The splicing variant “IIIa” of FGF is a secreted protein, whereas IIIb and IIIc are both membrane-bound receptors containing mutually exclusive Ig III domains. It is generally believed that the IIIb isoform of FGFRs is expressed in epithelial lineages, whereas the IIIc variant is restricted to mesenchymal origin (18–20).

Activation of FGFR triggers several intracellular signaling cascades. These include phosphorylation of Src and phospholipase Cγ (PLCγ), leading finally to activation of PKC as well as activation of Crk and Shc (21). The adaptor protein, FRS2, serves as an alternative link of FGFR to the activation of PKC and, in addition, activates the Ras signaling cascade. FRS2 contains multiple tyrosine phosphorylation sites in the C-terminal tail that serve as binding sites for the Grb2 and for the Src homology 2 domain-containing protein tyrosine phosphatase, Shp2 (22–24). Furthermore, phosphatidylinositol 3-kinase (PI3K)/Akt/p70 ribosomal S6 kinase (S6K1) cascade also play important roles in mediating FGF signaling. Activation of PI3K by FGF-FGFRs has been demonstrated in studies using biochemical, pharmacological, and genetic approaches (25–28). Results from these studies suggested that PI3K can be activated directly via association with FRS or indirectly via an Erk-dependent pathway (27, 29).

Although the signaling pathways of FGFR have been investigated extensively, most studies failed to distinguish individual signaling being activated by specific isoforms of FGFR or particular members of the FGF family. For example, most studies on FGF receptor-mediated signal transduction have
been carried out using FGFR1 as the prototypical FGFR, which
is bound by many members of FGF family. Others using an
overexpression system to characterize the signaling pathways
of a given splice variant have had trouble in interpreting the
significance of their data, since it is not obtained under phys-
iological conditions (30, 31). We previously reported that endo-
metrial stroma expresses predominantly the FGFR2IIIc splice
variant by quantitative reverse transcription-PCR analysis and
immunohistostaining and that FGFR9 potently stimulates
endometrial stromal cell proliferation (5). This unique feature
of the endometrial stromal cell makes it the ideal model for
investigation of the signaling pathway of FGFR9 devoid of
necessary interference from other FGFRs or forced overexpres-
sion of FGFR in a cell that normally does not express such
receptor. Results from this study should provide novel infor-
mation in dissecting the complex FGF-FGFR signaling network
and bring new insight in understanding physiological and
pathological functions of FGFR9.

**EXPERIMENTAL PROCEDURES**

**Materials—**Primary antibodies (anti-Akt, anti-phospho-Akt (Ser473),
anti-phospho-Akt (Thr308), anti-Erk1/2, anti-phospho-Erk1/2 (Thr202/204),
anti-S6K1, anti-phospho-S6K1 (Thr421/Ser422), anti-mTOR, anti-phospho-mTOR (Ser2448),
anti-phospho-mTOR (Ser2441), anti-TSC2, anti-phospho-TSC2 (Thr1465),
anti-PI3K and anti-PI3Kγ were from Cell Signaling Technologies (Beverly,
MA); anti-β-actin was from Oncogene Research Products (Cambridge,
MA); anti-proliferating cell nuclear antigen was from Zymed Laborato-
ries (South San Francisco, CA); and anti-cyclin A, anti-cyclin D1, anti-
PI3K 110α, anti-total PKC, and anti-PDK-1 were from Santa Cruz
Biotechnology, Inc. (Santa Cruz, CA). The small interference RNA
(siRNA) of mTOR, SignalSilence™ mTOR siRNA, was purchased from
Cell Signaling. Cell culture materials (phenol red-free Dulbecco’s mod-
eified Eagle’s medium/F-12, fetal bovine serum, and antibiotics) were
purchased from Hyclone.

**Tissue Collection and Stromal Cell Purification—**Eutopic endome-
trial tissues from patients of reproductive age undergoing hysterectomy
for leiomyoma or ovarian pathology (n = 25) were collected as previ-
ously described (5). Human ethics approval was obtained from the
Clinical Research Ethics Committee at The National Cheng Kung
University Medical Center, and informed consents were obtained from the
patients. Tissues were immersed in Hank’s solution supplemented with
HEPES and antibiotics and transported to the laboratory for further
processing. The biopsies were minced and subjected for isolation
of stromal cells as previously described (32, 33). Purity of the cell
was determined by immunostaining with vimentin (stromal cell-specific)
and cytokeratin (epithelial cell-specific) specific antibodies as previ-
ously described (32). The stromal cell population was free of epithelial
cell contamination.

**Cell Cultures—**Stromal cells were cultured in culture medium con-
sisting of Dulbecco’s modified Eagle’s medium/F-12, 10% FCS, penicillin
(100 μg/ml), streptomycin (100 units/ml), and fungizone (50 μg/ml) in a
humidified atmosphere with 5% CO₂ at 37 °C. The medium was
changed every other day. When the cells reached confluence, they were
subcultured in phenol red-free Dulbecco’s modified Eagle’s medium/ F-
12 medium supplemented with 10% FCS, penicillin (100 μg/ml),
streptomycin (100 units/ml), and fungizone (50 μg/ml) until 70% con-
fluence was reached. After serum starvation for 36 h, the cells were
treated with vehicle, FGFR9 (50 ng/ml) in the presence or absence of
different inhibitors under serum-free, phenol red-free medium condi-
tions. Stromal cells were serum-starved for 36 h

**PI3K Kinase Activity Assay—**Serum-starved stromal cells were stim-
ulated with 50 ng/ml FGF-9 for 5 min at 37 °C. The samples were
lysed for 20 min on ice in 200 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM
NaCl, 0.1% (w/v) SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 5
mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leu-
peptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM Na₂VO₃, 2.5 mM
sodium pyrophosphate). Immunoprecipitation was performed using anti-
phospho-Akt, anti-phospho-TSC2, or an irrelevant mouse IgG at 4 °C over-
night. After washing 3 times with cold PBS-Tween, the immune com-
plexes were recovered by protein-A Sepharose and resuspended in
20 μl of IEF buffer (40 mM Tris-HCl, pH 6.7). The samples were
subjected to SDS-PAGE separation. Proteins were transferred onto
an Immobilon-P membrane and subjected to Western blot using
primary antibodies directed against phospho-Akt (Ser473), total Akt,
phospho-Akt (Thr308), Erk1/2, or phospho-Erk1/2 (Thr202/Tyr204), and
anti-GAPDH or -tubulin. The membranes were then incubated with
appropriate horseradish peroxidase-conjugated secondary antibodies
and visualized using ECL (Amersham Biosciences). The gels were
digitized and analyzed using ImageJ software.

**PI3K Inhibitors—**Five randomized primary human endometrial stromal
biopsies were minced and subjected for isolation of stromal cells as
previously described (32, 33). Purity of the cell was determined by
immunohistostaining and that FGF9 potently stimulates
endometrial stroma expresses predominantly the FGFR2IIIc splice
variant by quantitative reverse transcription-PCR analysis and
immunohistostaining and that FGFR9 potently stimulates
endometrial stromal cell proliferation (5). This unique feature
of the endometrial stromal cell makes it the ideal model for
investigation of the signaling pathway of FGFR9 devoid of
necessary interference from other FGFRs or forced overexpres-
sion of FGFR in a cell that normally does not express such
receptor. Results from this study should provide novel infor-
mation in dissecting the complex FGF-FGFR signaling network
and bring new insight in understanding physiological and
pathological functions of FGFR9.
autofluorescence determined by exposing the tissue to 5 mM manganese to quench cytosol Fura-2 fluorescence. Intracellular free calcium was estimated after subtracting background and autofluorescence according to the established formula.

**Statistical Analysis**—The data were expressed as means ± S.E. Data were analyzed using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA). Duncan’s procedure was used to test the difference between individual treatment groups, whereas Dunnet’s test was applied to compare treatment groups versus control once significance was found by F test. p < 0.05 was considered statistically significant.

**RESULTS**

**FGF9-induced Stromal Cell Proliferation Is Partially Mediated by the Ras-Erk Signaling Pathway**—To investigate the mitogenic effect of FGF9, endometrial stromal cells were cultured in serum-free, phenol red-free Dulbecco’s modified Eagle’s medium/F-12 for 36 h and treated with FGF9 to induce cell proliferation. The addition of FGF9 (50 ng/ml) induced a greater than 10-fold increase in [3H]thymidine incorporation over basal level (Fig. 1A). Concordantly, expression of cell cycle regulators such as cyclin D1 (marker of G1 to S phase) and cyclin A (marker of S to G2 phase) was time-dependently up-regulated by treatment with FGF9. Elevation of cyclin D1 induced by FGF9 was first evident after treatment with FGF9 for 12 h and sustained for at least 48 h (Fig. 1B). Up-regulation of cyclin A was induced between 12 and 24 h after FGF9 treatment and remained elevated until 48 h (Fig. 1B). Together, this demonstrates that FGF9 can promote cell cycle progression, and the process is unremitting.

To investigate the signaling pathway that governs FGF9-induced stromal cell proliferation, cells were pretreated with selective inhibitors for Ras and MEK. Cell proliferation induced by FGF9 was inhibited by pretreatment with MEK inhibitor, PD98059, or Ras inhibitor, FPT, in a dose-dependent manner (Fig. 2, A and B). Interestingly, disruption of Ras-Erk signaling pathway using FPT or PD98059 cannot completely inhibit FGF9-induced stromal cell proliferation without causing cell death (Fig. 2, A and B, and data not shown).

To verify that incomplete inhibition of FGF9-induced stromal cell proliferation by Ras and MEK inhibitors was not due to incomplete blockage of Ras-MEK signaling, phosphorylation and nuclear translocation of MEK substrate, Erk1/2, was investigated. The addition of FGF9 to cultured stromal cells induced rapid and transient phosphorylation of Erk1/2 and nuclear translocation (Fig. 2D). In contrast, p38 mitogen-activated protein kinase and c-Jun N-terminal kinase were not phosphorylated by treatment with FGF9 (data not shown). Pretreatment with Ras inhibitor, FPT (10 μM), or MEK inhibitor, PD98059 (10 μM), inhibited Erk1/2 phosphorylation induced by FGF9 (Fig. 2E). Interestingly, expression of cyclin D1 was only partially inhibited by pretreatment with FPT and PD98059 (Fig. 2F). Thus, it is likely that the Ras-Erk cascade is only responsible for a portion of FGF9 actions, and one or more pathway(s) may exist to transduce FGF9 signaling.

**FGF9 Action Is Independent of PI3K and Akt Activation**—It is generally believed that PI3K-S6K1 is an important downstream effector system of peptide growth factors. To test whether PI3K-S6K1 signaling is involved in FGF9-induced cell proliferation, we first used PI3K inhibitor, LY294002, to block FGF9-induced stromal cell proliferation. Treatment with LY294002 dose-dependently inhibited FGF9-induced stromal cell proliferation (Fig. 3A). We next examined whether the downstream effector, S6K1, was activated by FGF9. Treatment of endometrial stromal cell with FGF9 induced phosphorylation of S6K1 at Thr389 and Thr421/Ser424 within 5 min and peaked at 15 min (Fig. 3B). The phosphorylation of S6K1 disappeared after 30 min of treatment (Fig. 3B). Phosphorylation of S6K1 was accompanied with nuclear translocation (Fig. 3B), although the significance remains unknown. Pretreatment with LY294002 (10 μM) abrogated FGF9-induced S6K1 phosphorylation and nuclear translocation induced by FGF9 (Fig. 3C).

Phosphorylation of Akt is usually accompanied by the activation of PI3K. Therefore, we tested whether Akt was phosphorylated by FGF9 treatment and served as an upstream effector for S6K1. To our surprise, Akt was not phosphorylated after the addition of FGF9 even after 60 min (Fig. 4A and data not shown). To confirm whether the human endometrial stromal cell was able to utilize Akt as a signaling molecule, EGF (10 ng/ml), IGF-1 (10 ng/ml), insulin (10 μg/ml), or FCS (10%) was added to the cell. A side-by-side experiment showed that EGF, IGF-1, insulin, and FCS all induced Akt phosphorylation on Ser473 and Thr308 by 5 min, whereas FGF9 was not able to exert such an effect (Fig. 4B).

The phosphorylation status of Akt might not entirely correlate with its enzyme activity; therefore, we directly evaluated the ability of Akt to phosphorylate its downstream substrates, forkhead transcription factor (FOXO) and tuberin (TSC2). Direct phosphorylation of FOXO by Akt resulted in cytoplasmic retention and inactivation. We therefore examined the subcellular localization of FOXO3a as an indicator of Akt activation. Treatment with serum (10% FCS) or IGF-1 (10 ng/ml) caused FOXO3a phosphorylation and cytosolic retention (Fig. 4C). In contrast, FOXO3a was accumulated in the nucleus under serum-free conditions or treated with 50 ng/ml FGF9 (Fig. 4C). In a parallel experiment, phosphorylation of TSC2 by Akt was evaluated. It has been shown that phosphorylation of TSC2 relieves inhibition of mTOR, which is a direct indicator for Akt-mediated mTOR activation (35). As shown in Fig. 4D, treatment with IGF-1 (10 ng/ml) markedly induced TSC2 phosphorylation, which can be blocked by LY294002 and wortmannin. In contrast, FGF9 failed to induce TSC2 phosphorylation (Fig. 4D). Taken together, these results provided further evidence to support that Akt was not activated by FGF9 in human endometrial stromal cells.

It is obvious that FGF9 does not activate the PI3K-dependent Akt, but whether PI3K is activated remains unanswered despite the effectiveness of LY294002 in blocking S6K1 phos-
phorylation and DNA synthesis. We therefore decided to determine whether PI3K was phosphorylated by FGF9 treatment. The PI3K protein was immunoprecipitated by anti-phosphotyrosine antibody and then blotted with anti-p85 subunit of PI3K. The result demonstrated that IGF-1 induced robust PI3K phosphorylation, whereas FGF9 failed to induce any PI3K phosphorylation (Fig. 4E). Again, both EGF and serum could induce PI3K phosphorylation, but the extent was much less than that of IGF-1 (Fig. 4E). Finally, we directly measured the kinase activity of PI3K. In concordance with other data, treatment with FGF9 did not induce any measurable kinase activity of PI3K (Fig. 4F). In contrast, PI3K kinase activity was significantly elevated by IGF-1 treatment and by the addition of 10% FCS to a lesser extent (Fig. 4F). Taken together, these results provided direct evidence to support that PI3K is not involved in FGF9-induced stromal cell proliferation.

**FIG. 2.** FGF9-induced cell proliferation is partially inhibited by the Ras-Erk signaling pathway. A and B, serum-starved stromal cells were preincubated for 30 min with different doses of PD98059 or FTP and subsequently treated with FGF9 for 22 h. At the final 4 h, [3H]thymidine was added to culture medium. Cells were then harvested and subjected to a [3H]thymidine incorporation assay. Data are means ± S.E. from five independent experiments using different batches of cells performed in triplicate. Different letters denote significant difference between means of each group (p < 0.05). C, stromal cells were serum-starved and were subsequently incubated with FGF9 for the indicated times. Equal amounts of proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against phospho-ERK1/2 and subsequently reprobed with anti-Erk1/2 antibody. Four independent experiments were repeated using different batches of cells, and the results were identical. D, a representative Western blot shows phosphorylation and nuclear translocation of Erk1/2 after FGF9 treatment. Cells were serum-starved and then treated with FGF9 for 15 min. Cytosolic and nuclear fractions were collected and subjected to Western blot analysis as described. Four independent experiments were repeated using different batches of cells, and the results were similar. E, a representative Western blot shows that phosphorylation of Erk-induced by FGF9 was blocked by pretreatment with PD98059 (10 μM) and FTP (10 μM). Four independent experiments were repeated using different batches of cells, and the results were identical. F, a representative Western blot shows that induction of cyclin D1, cyclin A, and cyclin B1 by FGF9 was inhibited by pretreatment with PD98059 (10 μM) and FTP (10 μM). Four independent experiments were repeated using different batches of cells, and the results were identical.

**FIG. 3.** S6K1 is activated by treatment with FGF9 in human endometrial stromal cell. A, stromal cells were treated as described in the legend of Fig. 1 except that different doses of LY294002 were administered. Data are means ± S.E. from five independent experiments using different batches of cells performed in triplicate. Different letters denote significant difference between means of each group (p < 0.05). B, serum-starved stromal cells were treated with FGF9 for 5, 15, or 30 min, and phosphorylation and nuclear translocation of S6K1 was determined by blotting with specific antibody. Equal amounts of cytoplasmic or nuclear proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form (Thr421 and Ser424) of the S6K1 (p(T/S)). Membrane was stripped and reprobed with anti-phospho-S6K1 (Thr^{421}), anti-S6K1, and anti-β-actin (for cytoplasmic fraction) anti-proliferating cell nuclear antigen (for nuclear fraction) antibodies, respectively. C, serum-starved stromal cells were preincubated for 30 min with LY294002 (10 μM) and subsequently treated with FGF9 for 15 min. Equal amounts of cytoplasmic or nuclear proteins were analyzed as described above.
**Phosphorylation of S6K1 Induced by FGF9 Is Mediated by mTOR**—To address what is the upstream effector leading to S6K1 phosphorylation, we immunoprecipitated stromal cell lysates with anti-S6K1 antibody and blotted with antibodies against several potential candidates. The result demonstrated that mTOR directly associated with S6K1 in cells treated with FGF9 (Fig. 5A). The physical association may be with phosphorylated form S6K1, since the level of total S6K1 was not different between FGF9-treated and control cells (Fig. 5A). Western blot analysis demonstrated that mTOR was phosphorylated on Ser2481 and Ser2448, which are critical sites for mTOR kinase activity, within 5 min after FGF9 treatment and remained phosphorylated even up to 60 min (Fig. 5B). To confirm that activation of mTOR resulted in S6K1 phosphorylation, stromal cells were pretreated with rapamycin, a selective inhibitor for mTOR at 30 min before FGF9 treatment. Pretreatment with rapamycin (10 nM) specifically inhibited S6K1 phosphorylation (Fig. 5C), providing evidence to support that mTOR is an upstream effector of S6K1.

An alternative approach using RNA interference technique was conducted to evaluate effect of mTOR on S6K1 phosphorylation. Transfection with mTOR-specific siRNA knocked down about 80% of mTOR, whereas transfection with nonrelated siRNA did not affect expression of mTOR (Fig. 5D). The expression of eIF4B was not affected by either mTOR siRNA or control siRNA (Fig. 5D). Although mTOR siRNA did not completely ablate mTOR expression, it was sufficient to blunt FGF9-induced mTOR phosphorylation (Fig. 5D). Concordantly, mTOR siRNA blocked FGF9-induced S6K1 phosphorylation without altering the expression level of S6K1 (Fig. 5F). Taken together, these results provided evidence showing that mTOR is an upstream effector of S6K1 in FGF9-treated stromal cells.

Finally, we aimed to determine whether blockade of mTOR activity is sufficient to inhibit FGF9-induced cell proliferation. Treatment with rapamycin dose-dependently (from 0.1 nM to 1 μM) inhibited stromal cell proliferation (Fig. 5F). Nevertheless, rapamycin still cannot completely inhibit FGF9-induced stromal cell proliferation without causing cell death (Fig. 5F).

**Activation of mTOR by FGF9 Is Mediated through PLCγ**—So far, we have demonstrated that mTOR is activated by FGF9 treatment and is able to induce phosphorylation of S6K1, but the upstream effector of mTOR remains uncharacterized. Activation of PLCγ is another signaling pathway that mediates FGF actions. To determine whether PLCγ is involved in mTOR activation, we first examined whether PLCγ could be phosphorylated by treatment with FGF9. Fig. 6A shows that phosphorylation of PLCγ can be detected by 2.5 min after treatment with FGF9. FGF9-induced PLCγ phosphorylation was still evident at 5 min after FGF9 treatment but disappeared after 15 min. The role of PLCγ on FGF9 action was further supported by evidence that pretreatment with a selective PLCγ inhibitor, U73122 (10 μM), effectively inhibited FGF9-induced mTOR phosphorylation and cell proliferation (Fig. 6, B and C).

**Activation of PLCγ results in conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-di phosphate (PIP2) to phosphatidylinositol-4-phosphate (PIP2).** PLCγ activation results in the recruitment of PI3K to the plasma membrane, leading to the activation of mTOR. This activation is mediated through the serine/threonine kinase AKT, which phosphorylates the proline-rich motif of mTOR at Ser2448 and Ser2481. These phosphorylation sites are critical for the activation of mTOR. Activation of mTOR results in the phosphorylation of S6K1, which is a downstream effector of mTOR.

**Fig. 4. Activation of S6K1 by FGF9 is independent of PI3K and Akt.** A, stromal cells were serum-starved and incubated with FGF9 for the indicated times. Equal amounts of proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Akt. The blot was subsequently stripped and reprobed with an antibody against total Akt and anti-β-actin antibodies, respectively. A positive control using papillary thyroid carcinoma (PTC) shows phosphorylation of Akt at both Ser and Thr residues. Four independent experiments were repeated using different batches of cells, and the results were identical. B, serum-starved stromal cells were treated with FGF9 (50 ng/ml), EGF (10 ng/ml), IGF-1 (10 ng/ml), insulin (10 μg/ml), or 10% FCS for 5 min, and phosphorylation of Akt was determined by blotting with specific antibodies as described above. Four independent experiments were repeated using different batches of cells, and the results were identical. C, serum-starved stromal cells were treated with control medium (Con), FGF9 (50 ng/ml), IGF-1 (10 ng/ml), or 10% FCS (serum) for 1 h. Cells were then fixed, stained with anti-FOXO3a antibody, and visualized under an epifluorescent microscope. The experiment was repeated three times using different batches of cells with similar results.

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Inositol bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, which causes PKC activation and calcium influx, respectively. To determine whether PKC is involved in FGF9-induced mTOR phosphorylation, we first examined translocation of PKC to the plasma membrane, a sign of PKC activation. FGF9 failed to induce PKC translocation from cytosol to plasma membrane, whereas others were undetected under our conditions. By including inhibitors that selectively block PLC, we demonstrated that pretreatment with W7 (calmodulin inhibitor, 10 μM), KN93 (CaMK inhibitor, 10 μM), or EDTA (calcium chelator, 10 mM) attenuated FGF9-induced phosphorylation of mTOR on Ser2481 (p < 0.05, Fig. 7D) but not on Ser2448 (p > 0.05, data not shown). Phosphorylation of S6K1 after FGF9 treatment was also reduced by pretreatment with inhibitors that selectively block PLCγ, calmodulin, or CaMK, or by depletion of calcium (Fig. 7D). Together, these evidences demonstrate that PLCγ-mediated calcium signaling may play a role in FGF9-induced mTOR and subsequently S6K1 activation.

It has been reported that LY294002 can block growth factor-induced calcium influx (36–38). We thus decided to determine whether the effect of LY294002 on inhibiting FGF9-induced cell proliferation is mediated via blocking calcium signaling. Stromal cells were pretreated with LY294002 or wortmannin for 30 min and then subjected to calcium measurement. Pretreatment with LY294002 dose-dependently inhibited FGF9-induced calcium influx, whereas pretreatment with wortmannin had no such effect (Fig. 7E). As a positive control, IGF-1 also induced significant increase in intracellular free calcium concentrations. Again, LY294002 effectively blocked IGF-1-in}-
duced calcium influx whereas wortmannin was not effective (Fig. 7F). The effective dose of LY294002 (0.1 μM) was much lower than that on inhibiting PI3K activity (10 μM), suggesting that its effect on blocking calcium influx was not mediated via suppression of PI3K activity.

**FGF9-induced Ras-Erk and mTOR-S6K1 Pathways Are Parallel to Each Other**—We have thus far identified that both Ras-Erk and PLCγ-calcium-mTOR-S6K1 were activated by FGF9. It has been reported that PI3K-S6K1 and Erk pathways can cross-talk to each other. Since mTOR is a PI3K-like kinase, we sought to investigate whether the mTOR and Ras-Erk can mutually affect each other. Fig. 8A shows that inhibition of Ras-Erk signaling pathway by treatment with FPT (Figs. 4F and 5F) or rapamycin (Fig. 8B) or ablation of mTOR by RNAi (Fig. 8C) failed to prevent Erk1/2 phosphorylation, whereas PD98059 alone only exerted partial inhibitory effect on FGF9-induced cell proliferation (Fig. 8E). Combined treatment with U73122 and PD98059 or rapamycin and PD98059 completely abrogated FGF9-induced basal level of S6K1 phosphorylation (Fig. 8D). Taken together, these data demonstrated that Ras-Erk and mTOR-S6K1 signaling pathways independently and additively control FGF9 action in human endometrial stromal cells.

**DISCUSSION**

Fibroblast growth factor-9 plays critical roles in many physiological and pathological processes including sex determination, embryonic development, neuron degeneration, cancer formation, and endometriosis. The mitogenic effect of FGF9 is mediated via binding to its high affinity receptors, although the cellular and molecular mechanisms remain to be determined. This study shows that FGF9 stimulates cell proliferation via two parallel but additive pathways, the Ras-Erk and mTOR-S6K1 cascades in human uterine endometrial stromal cells (Fig. 9). We demonstrate that FGF9-induced cell proliferation...
is independent of the well established PI3K-Akt signaling pathway utilized by most peptide growth factors such as IGF-1, EGF, and other FGFs (25–28). Instead, our data provide evidence to show that activation of S6K1 is directly mediated by mTOR. Importantly, we further demonstrate that PLCγ and calcium-dependent kinase are involved in FGF9-induced mTOR phosphorylation. To our knowledge, this is the first report to characterize the signal pathways responsible for FGF9 function under a physiological condition and to demonstrate a novel signaling cascade involving PLCγ, calcium, mTOR, and S6K1 in a ligand receptor-specific manner.

It has been established that the Ras-mitogen-activated protein kinase pathway plays an important role in signaling via FGF receptors (23). The adapter protein Grb2 links FGF receptors with the Ras signaling pathway by binding to the guanine nucleotide-releasing factor Sos through its Src homology 3 domains and to tyrosine-phosphorylated receptors or docking molecules via its Src homology 2 domain (39, 40). Our current results agree with this notion by showing that Ras-ERK cascade is activated by treatment with FGF9. Nevertheless, we found that activation of Ras-ERK signaling only accounts for partial FGF9-induced stromal cell proliferation.

Activation of PI3K-Akt-S6K1 signaling cascade by growth factor not only mediates cell survival but also controls DNA synthesis (41). Surprisingly, we found no sign of PI3K activation and Akt phosphorylation upon FGF9 treatment in human endometrial stromal cells indicating that S6K1 was phosphorylated at Thr389, Thr421, and Ser424. The inability of FGF9 to utilize the PI3K-Akt pathway is not due to a lack of either signal molecule in the endometrial stromal cell but rather to specific ligand-receptor interaction. Four lines of evidence show that the PI3K-Akt signaling cascade is intact and functional in endometrial stromal cells. First, treatment of endometrial stromal cells with IGF-1 or serum significantly increases PI3K phosphorylation and kinase activity. Second, serum or other peptide growth factors such as EGF, IGF-1, and insulin effec-
tively cause Akt phosphorylation. Third, phosphorylation of TSC2, a hallmark of Akt activity, is markedly elevated after IGF-1 treatment. Fourth, cytosolic retention of FOXO3a caused by Akt-dependent phosphorylation (42) is evident after IGF-1 or serum treatment, suggesting that the enzymatic activity of Akt is intact in the endometrial stromal cells. Most notably, the cytosolic retention of FOXO3a after IGF-1 treatment was conducted in primary cultured cells without forced overexpression of Akt. These results clearly show that FGF9, via binding to its high affinity receptor (likely to be FGFR2IIIC), stimulates endometrial stromal cell proliferation independently of the PI3K-Akt signaling cascade.

At first glance, the findings that FGF9-induced S6K1 phosphorylation and DNA synthesis can be inhibited by LY294002, a putatively selective PI3K inhibitor, are contradictory to the conclusion that PI3K is not activated by FGF9 in endometrial stromal cell. This disparity leads us to ponder that the target of LY294002 may not be PI3K per se. Our hypothesis is supported...
by reports that LY294002 and wortmannin can inhibit activities of PI3K and mTOR (43, 44) and that LY294002 but not wortmannin can suppress receptor tyrosine kinase-induced calcium influx (37, 38). Our current results demonstrate that LY294002 is a much more effective inhibitor at blocking calcium influx than at inhibiting PI3K activity, whereas wortmannin is only effective in suppressing PI3K activity. These results provide another possibility that the effect of LY294002 on FGF9-induced cell proliferation may be mediated via inhibiting the calcium signaling pathway. Nevertheless, the molecular target of LY294002 remains undetermined and merits further investigation.

mTOR (also known as FRAP, RAFT, and RAPT) is a member of phosphatidylinositols kinase-related kinase family that plays a central role in controlling cellular growth and DNA synthesis (45, 46). Recent studies have demonstrated that mTOR directly phosphorylates S6K1 in vitro (47–49) and in vivo (47) and that rapamycin, a selective mTOR inhibitor, inhibits growth factor-induced S6K1 phosphorylation (50). To test whether mTOR is the direct activator of S6K1 in FGF9-treated stromal cells, we used anti-S6K1 antibody to bring down the immunocomplex and blotted with anti-mTOR antibody. Our results show that mTOR physically associates with S6K1 upon FGF9 treatment. Furthermore, ablation of mTOR by siRNA completely blocks FGF9-induced S6K1 phosphorylation. Results from the current study and previous reports (47–49) provide clear evidence to show that mTOR is the direct upstream activator of S6K1 in response to FGF9 signaling. Concordantly, rapamycin inhibited FGF9-induced cyclin A, D1, and B1 expression and [3H]thymidine incorporation similar to, if not more potent than, that inhibited by LY294002. All of this evidence implicates mTOR as the alternative signaling molecule that mediates FGF9-induced S6K1 phosphorylation and thus DNA replication.

The upstream effectors that activate mTOR have been extensively investigated in the past couple years (50). It has been shown that the availability of nutrients (amino acid levels) and phosphatidic acid can regulate mTOR function (51–53). In addition, Akt can activate mTOR either by direct phosphorylation or via inactivation of its repressor, TSC2 (35, 54–58). However, none of the above mentioned molecules seems to be a likely candidate to activate mTOR in FGF9-treated endometrial stromal cells. The activation of PLCγ by FGF9 provides a new direction to test whether its downstream effectors, PKC and calcium, can activate mTOR. mTOR undergoes multiple phosphorylations. The phosphorylation of Ser2448 and Ser2481 under various stimuli is used to reflect the activity of mTOR (59–62). Our results show that inhibitor for PLCγ attenuates FGF9-induced mTOR phosphorylation on Ser2481 and its ability to induce S6K1 phosphorylation. Further study suggests that calcium but not the PKC pathway is involved in phosphorylation of Ser2481, which is concordant with a recent report demonstrating that activation of α1-adrenergic receptor by phenylephrine induces mTOR phosphorylation on Ser2481 in calcium-dependent and PKC-independent (59). Another mTOR residue, Ser2448, was also phosphorylated after FGF9 treatment but the phosphorylation appears to be independent of calcium signaling. The underlying mechanism is not clear but it has been reported that amino acid induces mTOR phosphorylation is not mediated by Akt, Erk, or calcium signaling pathways in primary culture rat adipocytes (60). Furthermore, though Ser2448 is a consensus phosphorylation site for Akt, recent studies reveal that Ser2448 can be phosphorylated even when PI3K/Akt is not activated and that phosphorylation of this site might not be sufficient for mTOR kinase activity (61, 62). Taken together, these lines of evidence implicate that phosphorylation of mTOR induced by FGF9 might also be mediated by other signaling molecules. Further investigation is warranted to identify the involvement of other possible signaling molecules in FGF9 signaling and unravel the mechanism responsible for mTOR phosphorylation.

It has been reported that Erk is one of the downstream effectors of PI3K/Akt pathway and that Erk may regulate the activation of S6K1 (63, 64). However, our data demonstrated that these two signaling pathways are parallel but additive in mediating FGF9-induced stromal cell proliferation by four lines of evidence. First, blockage of either pathway only results in partial inhibition of FGF9-induced [3H]thymidine incorporation, whereas simultaneous disruption of both pathways completely inhibits FGF9 action. Second, phosphorylation of Erk is not affected by treatment with LY294002, U73122, rapamycin, W7, or KN93. Third, ablation of mTOR by siRNA does not influence Erk phosphorylation. Fourth, the addition of Ras or MEK inhibitors fail to inhibit S6K1 phosphorylation.

The complexity of FGF-FGFR signaling has hampered researchers in dissecting functions of an individual member of FGF family. For example, FGF2 can bind to FGFR1, FGFR2IIc, FGFR3IIc, and FGFR4 with high affinity and FGFR1 can sever as common receptor for FGF1, -2, -4, -5, and -6 and more (65). In light of an elegant study by Ornitz et al. (65), showing distinct binding affinity and/or specificity of FGFs to FGFRs, it is reasonable that FGF9 may utilize a distinct signaling pathway, since it does not bind FGFR1. The human uterine endometrial stromal cell is an excellent system to characterize the signaling pathway of FGF, because the predominantly receptor isoform in this cell type is FGFR2IIc (5). Therefore, it provides a clean system to study the signaling pathway activated by the ligand, such as FGF9 and an exclusive isotype of the receptor devoid of interference by other variants. Although it may be argued that the signaling pathways from different FGFRs are similar, giving a high degree of homology at the amino acid level, some caveats still exist due to the biochemical nature of the ligand-receptor interaction. Thus, different members of FGFR family may utilize distinct signal pathways to exert their specific actions. For example, a recent report by Portnoy et al. shows that FGF7 stimulates alveolar cell proliferation through Erk and PI3K/Akt pathways by binding to FGFR2IIb (66). In the current study, our results show that FGF9 follows the PLCγ-calcium-mTOR-S6K1 cascade, clearly demonstrating that this is different from the conventional PI3K-Akt signaling pathway. In sum, the results presented here provide compelling evidence to demonstrate specific ligand-mediated FGFR signaling, which is important for understanding signaling pathways by different FGFRs and the consequence of their physiological and pathological functions.

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The Mammalian Target of Rapamycin-p70 Ribosomal S6 Kinase but Not Phosphatidylinositol 3-Kinase-Akt Signaling Is Responsible for Fibroblast Growth Factor-9-induced Cell Proliferation

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