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Authors

Erikson, David W
Chen, Joseph C
Piltonen, Terhi T
et al.

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ORIGINAL ARTICLE

Inhibition of epidermal growth factor receptor restores decidualization markers in stromal fibroblasts from women with endometriosis

David W. Erikson[1], Joseph C. Chen[1], Terhi T. Piltonen[1,2], Marco Conti[1], Juan C. Irwin[1], Linda C. Giudice[1]

[1] Center for Reproductive Sciences and Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California – San Francisco, San Francisco, California - USA

[2] Department of Obstetrics and Gynecology and Medical Research Center, University of Oulu and Oulu University Hospital, Oulu - Finland

Purpose: *Decidualization comprises specific biochemical and morphological changes in uterine endometrium essential for establishment of pregnancy. This process is abnormal in women with endometriosis, a disorder in which endometrial-like tissue is present outside the uterus. The aim of this study was to restore cAMP-induced decidualization marker expression in endometrial stromal fibroblasts from women with endometriosis by using chemical inhibitors to PI3K/AKT/mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK) and epidermal growth factor receptor (EGFR) signaling pathways in vitro.*

Methods: *Endometrial stromal fibroblasts (eSF) from women with (eSF$_{endo}$) and without (eSF$_{non-endo}$) endometriosis were treated with inhibitors to EGFR tyrosine kinase (gefitinib), mTOR (rapamycin) and MAPK kinase 1/2 (MEK1/2) (UO126) during 8-bromoadenosine 3',5'-cyclic monophosphate (8-br-cAMP)–stimulated decidualization. Decidualization was assessed by evaluating expression of insulin growth factor binding protein 1 (IGFBP1), prolactin (PRL) and forkhead box protein O1A (FOXO1A) by quantitative real-time PCR.*

Results: *Gefitinib restored expression of decidualization markers in eSF$_{endo}$ to levels consistent with those in eSF$_{non-endo}$. Elevated levels of phosphorylated mTOR in eSF$_{endo}$ were reduced to levels found in eSF$_{non-endo}$, by gefitinib during treatment with 8-br-cAMP. Additional gene expression analyses suggested dysregulation of EGFR negative feedback regulators in eSF$_{endo}$.*

Conclusions: *Results implicate EGFR signaling as an underlying cause for aberrant cAMP-induced decidualization in women with endometriosis, and provide a potential target for management of infertility associated with the disease. The reduction of p-mTOR levels in eSF$_{endo}$ during 8-br-cAMP treatment suggests cooperation between EGR and protein kinase A signaling in the regulation of mTOR in eSF.*

Keywords: *Decidualization, Endometriosis, Epidermal growth factor receptor, Fibroblast, Mammalian target of rapamycin, Mitogen-activated protein kinase*

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INTRODUCTION

Endometriosis is a chronic estrogen-dependent disorder affecting 6%-10% of reproductive aged women (1, 2). It is characterized by extrauterine endometrial tissue that elicits an inflammatory response mainly in the pelvis (3), contributing to chronic pelvic pain and associated infertility due to ovulatory dysfunction, poor egg quality, abnormal

uterine endometrium and/or compromised embryo implantation (1, 4). Ectopic endometrial lesions (outside the uterus) and eutopic endometrium (within the uterus) of women with this disease are stimulated by estradiol, but are resistant to the action of progestins and progesterone (P_4) (5, 6). The latter regulates nociceptive pain thresholds in innervated endometriosis lesions (7) and, within the uterus, is essential for endometrial epithelial secretory transformation and stromal fibroblast decidualization, critical for the establishment and maintenance of pregnancy. Thus, resistance to P_4 and progestins can have a major impact on pain relief, disease control and fertility success of affected women.

Several signaling pathways have been implicated in these aspects of the pathophysiology of progesterone resistance and compromised stromal fibroblast decidualization, including protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and phosphatidylinositide-3 kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) (8). Understanding involvement and cross-talk among these pathways, and whether pathway-specific inhibitors can restore endometrial cell function or biomarkers, is of great interest as they hold promise to mitigate endometrial cellular dysfunction in the setting of disease.

Constitutive activation of PI3K/AKT and MAPK pathways has been reported in both eutopic and ectopic endometrial tissues in endometriosis (9). We previously found higher levels of phosphorylated (p)-ERK1/2 in stromal fibroblasts from women with severe endometriosis versus women without disease *in vivo* (2). Higher levels of phosphorylated (p)-mTOR have been found in ovarian endometrioma tissues (10), and active AKT1 is elevated in eutopic endometrium of women with versus without endometriosis (11). In addition, mRNA expression of epidermal growth factor receptor (EGFR), which is upstream of both PI3K/AKT and MAPK pathways, is increased in eutopic endometrium in the early secretory phase in severe versus mild disease (12). These data suggest a role for EGFR in increased activation of these pathways within the stromal compartment of the uterus in women with endometriosis. Consistent with this notion is the finding that members of the EGFR family *HER1*, *HER2* and *HER3* exhibit greater mRNA expression in whole eutopic endometrium from women with versus without disease (13).

When treated with decidualizing stimuli (P_4, progestin and/or 8-bromoadenosine 3',5'-cyclic monophosphate [8-br-cAMP]), eutopic endometrial stromal fibroblasts (eSF) from women with endometriosis (eSF$_{endo}$) do not express appreciable amounts of the decidual marker insulin growth factor binding protein 1 (IGFBP1) due to inherent insensitivity to PKA pathway stimulation (14, 15). Treatment of ectopic eSF from ovarian endometriomas with small molecule inhibitors of AKT or PI3K during *in vitro* decidualization increased expression of IGFBP1 and its upstream transcriptional regulator forkhead box 1 (FOXO1) (16). We previously demonstrated that treatment of stromal fibroblasts from eutopic endometrium of women without endometriosis (eSF$_{non-endo}$) with 8-br-cAMP resulted in up-regulation of *IGFBP1*; whereas eSF$_{endo}$ were refractory to 8-br-cAMP (14). In addition, the inhibition of MAPK kinase 1/2 (MEK1/2) with UO126 did not restore 8-br-cAMP up-regulation of *IGFBP1* in these cells from women with disease (2). Thus, the limited use of small molecule inhibitors, reported in a few studies, has met with fair success in restoring decidualization marker expression in eSF$_{endo}$.

Herein, we hypothesized that a combination of overactive PI3K/AKT/mTOR, MAPK and EGFR signaling pathways is causative of aberrant 8-br-cAMP-induced decidualization marker expression in eSF from women with endometriosis. We treated eSF with the selective chemical inhibitors gefitinib (EGFR tyrosine kinase inhibitor), rapamycin (mTOR inhibitor) and UO126 concomitantly with 8-br-cAMP, and performed multiplex and quantitative real-time polymerase chain reaction (qPCR) analyses to investigate their effects on decidualization marker expression. Our results indicate that inhibition of EGFR tyrosine kinase activity restored expression of specific decidual markers in eSF$_{endo}$ to levels in eSF$_{non-endo}$.

MATERIALS AND METHODS

Human endometrial samples

Human eutopic endometrial tissue samples were obtained in accordance with the guidelines of the Declaration of Helsinki, from 24- to 42-year-old women undergoing endometrial biopsy or hysterectomy for diagnosis or treatment of pelvic pain, fibroids, prolapse and/or endometriosis (Tab. I). No ectopic lesions or tissues were collected from patients or used in the present study. Presence or absence of endometriosis was confirmed by laparoscopic visualization and histological analysis of peritoneal lesions. Staging of endometriosis

TABLE I - HUMAN PARTICIPANT CHARACTERISTICS

Subject ID	Phase	Age	Procedure	Ethnicity	Diagnosis	Analysis
S01	PE	28	Laparoscopic supracervical hysterectomy	White	Dysmenorrhea, menometrorrhagia, ovarian cyst	qPCR
S02	PE	42	Supracervical hysterectomy	White	Symptomatic fibroids, adenomyosis	qPCR, multiplex
S03	SE	24	EBx	Asian	Natural cycle donor	qPCR, multiplex
S04	PE	37	Tubal ligation, EBx	Asian	Undesired fertility	qPCR
S05	PE	39	Tubal ligation, EBx	Asian	Undesired fertility	qPCR, multiplex
S06	SE	37	Operative laparoscopy, EBx	White	Endometriosis, stage IV, pelvic adhesion, adenomyosis	qPCR, multiplex
S07	PE	30	Operative laparoscopy, EBx	Unknown	Endometriosis, stage IV, pelvic pain, uterine bleeding	qPCR
S08	PE	32	Operative laparoscopy, EBx	White	Endometriosis, stage IV, pelvic pain, uterine bleeding, dysmenorrhea	qPCR, multiplex
S09	PE	42	Operative laparoscopy, EBx	White	Endometriosis, stage IV, pelvic pain, pelvic adhesions	qPCR
S10	SE	40	Operative laparoscopy, EBx	Asian	Endometriosis, stage IV, pelvic pain, uterine bleeding	qPCR, multiplex

EBx = endometrial biopsy; PE = proliferative endometrium; qPCR = quantitative real-time reverse transcription polymerase chain reaction; SE = secretory endometrium.

was defined according to the Revised American Society for Reproductive Medicine classification system (17). No patients used hormone treatments within 3 months of surgery. Samples were obtained through the UCSF NIH Human Endometrial Tissue and DNA Bank with appropriate institutional review, approvals and written informed consent from all participating subjects, as approved by the University of California San Francisco Committee on Human Research. Endometrial samples were processed for cell culture experiments as described below.

eSF isolation and culture

eSF from non-endometriosis (eSF$_{non-endo}$) (n = 5) and endometriosis (eSF$_{endo}$) (n = 5) subjects were isolated by digesting endometrial tissue samples with collagenase followed by filtration as previously described (18, 19). Isolated eSF were cultured in growth medium (phenol red-free medium of 3:1 high-glucose phenol red-free DMEM/MCDB-105, 0.676 mM sodium pyruvate, 10% charcoal-stripped fetal bovine serum [FBS], 1% penicillin-streptomycin mix, 50 µg/mL gentamycin, 5 µg/mL insulin), with medium replaced every 2-3 days for up to 6 passages. All cells used in the experiments described herein were from the fourth passage. For qPCR and multiplex experiments, confluent cells were incubated for 24 hours in low-serum medium (3:1 high-glucose phenol red-free DMEM/MCDB-105, 0.75 mM sodium pyruvate, 50 µg/mL gentamycin, 2% FBS) before treatment. For multiplex experiments, cells were incubated in either EGF (20 ng/mL) for 30 minutes or 8-br-cAMP (0.5 mM) for 60 minutes with inhibitors to mTOR (rapamycin; 10 nM), MEK1/2 (UO126; 10 µM) or EGFR Tyr1173 Tyr992 (gefitinib; 10 µM) alone or in combination. These time points were determined by treatment of eSF with either EGF or 8-br-cAMP and then observing the time point at which optimal p-ERK1/2 activation

(EGF) or inhibition (8-br-cAMP) occurred (2, 20). All inhibitors were delivered in dimethyl sulfoxide (DMSO), and all treatments were adjusted to contain 0.1% DMSO. Cells were then harvested for protein analysis as described below. For decidualization, cells were treated with 0.5 mM 8-br-cAMP for 96 hours in low-serum medium, conditions previously determined optimal for decidualization (2, 14), with or without the inhibitors listed above. Media were refreshed after 48 hours. Optimal inhibitor concentrations were determined using previously published data on effective doses (2, 21, 22). Samples without treatment were collected at baseline (t = 0) to serve as controls. After 96 hours of treatment, cells were harvested for RNA or protein isolation as described below. All sample treatments were performed in duplicate in independent experiments to ensure reproducibility of results.

Quantitative RT-PCR

Total RNA was isolated from cultured, treated eSF using the NucleoSpin RNA kit (Macherey-Nagel, Bethlehem, PA, USA) following the manufacturer's instructions. Total RNA was quantified by UV spectrophotometry (Nanodrop, Wilmington, DE, USA), and reverse transcription was performed using random primers and the iScript RT reagents following the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). mRNA levels were determined by qPCR using the Mx 3005 Pro (Stratagene, La Jolla, CA, USA). Primer sets used are listed in Supplemental Table I. cDNA (10 ng) was amplified by SYBR Supermix (Thermo Fisher Scientific, Waltham, MA, USA) using the following protocol: (i) DNA polymerase activation at 95°C for 15 minutes; (ii) 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 60 seconds. The absence of primer dimers was determined through dissociation curve analysis by performing a cycle of denaturation at 95°C for 1 minute followed by 35 cycles of increasing temperature for 33 seconds from 65°C to 97°C at the end of each analysis. Relative expression was calculated using the relative standard curve method (23), where a standard curve is created for each primer set using pooled cDNAs from the entire experimental sample set, and efficiency of the cDNA standards is between 90% and 110%. Samples were assayed in duplicate, and relative gene expression was normalized with beta actin (ACTNB) and t = 0 controls within each group to control for time in culture.

Multiplex assays

Following treatment, cells were washed in phosphate-buffered saline (PBS) and lysed with RIPA buffer containing a protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 μM Bestatin, E-64 14 μM, 1 μM Leupeptin, 0.3 μM Aprotinin) (Millipore, Bedford, MA, USA). After centrifugation to clear cellular debris (10,000g, 10 minutes, 4°C), protein quantity was evaluated by Bradford assay (Bio-Rad) using the manufacturer's instructions. Milliplex MAP multiplex protein assays were performed according to the manufacturer's protocol (Millipore). Briefly, cell lysates (3 μg protein/well) were diluted 1:1 with Milliplex MAP Assay Buffer 2. The filter plate was pre-wetted with Assay Buffer 2, and the buffer removed by vacuum filtration. Milliplex MAPmates were combined and 25 μL was added per well (Suppl Tab. II). After adding 25 μL of diluted cell lysate to the appropriate wells, the plate was incubated overnight at 4°C with shaking. The lysate was removed by vacuum filtration, washed 3 times with Assay Buffer 2, and 25 μL of detection antibody was added for 1 hour at room temperature. Diluted streptavidin-phycoerythrin (SAPE) was then added after filtration, the plate was incubated for 15 minutes at room temperature, and amplification buffer was added before a final incubation of 15 minutes. The SAPE/amplification buffer mix was removed by filtration, 150 μL of Assay Buffer 2 was added, and the plate was analyzed on a BioPlex 200 (Bio-Rad). The ratio of phospho protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was calculated for each target.

p-EGFR multiplex assays

Analysis of p-EGFR (Tyr1173) was performed using a multiplex approach similar to that for other phospho protein targets described above. After treatment and total protein extraction from cells, lysates were diluted in cell lysis buffer to a concentration of 10 μg/50 μL and incubated with BioPlex Pro magnetic cell signaling beads (Bio-Rad) for p-EGFR (Tyr1173) and GAPDH overnight at 4°C with shaking. Beads were washed and incubated for 30 minutes with antibodies for p-EGFR and GAPDH at room temperature, washed and further incubated with SAPE for 10 minutes at room temperature. Beads were then washed, resuspended in Bead Resuspension Buffer and analyzed on a BioPlex 200 (Bio-Rad). The ratio of phospho protein to GAPDH was calculated for p-EGFR.

Data analysis

All quantitative data were subjected to analyses of variance (ANOVA) using general linear model procedures available with SAS (SAS 2012-2013; SAS Institute, Cary, NC, USA). For transcript comparisons, data were generated from fold change of each transcript to ACTNB transcripts using individually determined standard curves. For protein comparisons fold change to GAPDH protein was performed. Statistical models considered variation due to the main effect of inhibitor treatment, including no treatment vehicle control (DMSO). Treatment effects were identified by performing a set of preplanned contrasts to include comparisons of specific groups for each target transcript or protein (24). In all cases, significance was set at a p value of <0.05.

RESULTS

To prove/disprove our central hypothesis that overactive EGFR signaling in eSF$_{endo}$ is causative of the compromised decidualization response observed in these cells, we pursued the following: (i) analyzed EGFR pathway activation, including downstream ERK1/2 and mTOR, in eSF to determine activation status of these pathways in disease; (ii) demonstrated compromised eSF decidualization response on a background of increased EGFR signaling (12, 13), and recreated this effect in eSF$_{non-endo}$ by activating EGFR; and (iii) used small molecule inhibitors to EGFR, mTOR and MEK1/2 in an attempt to restore the decidualization response in eSF$_{endo}$.

eSF from women with endometriosis do not decidualize in response to cAMP

Previous data have demonstrated that eSF$_{endo}$ exhibit an altered response to PKA stimulation (14), which manifests in reduced decidualization capacity (2, 15). Our objective in this experiment was to demonstrate compromised decidualization response by eSF on a presumed background of increased EGFR signaling (12, 13). After treatment with cAMP for 96 hours, we measured expression of 3 key decidualization genes by PCR, *IGFBP1*, *PRL* and *FOXO1A*, and calculated fold changes against expression levels at t = 0. *IGFBP1* expression increased over 100,000-fold in eSF$_{non-endo}$, while there was a minimal increase in eSF$_{endo}$

(Fig. 1A). *PRL* expression increased over 35,000-fold in eSF$_{non-endo}$, with a small increase in eSF$_{endo}$ (Fig. 1B). We also observed a significant increase in *FOXO1A* expression in eSF$_{non-endo}$, but little increase in eSF$_{endo}$ (Fig. 1C). These data demonstrate a limited capacity of eSF$_{endo}$ to respond to cAMP, and suggest that overactive EGFR found in these subjects could be responsible for aberrant decidualization in eSF$_{endo}$.

Components of the EGFR signaling pathway are activated in endometriosis

To better understand the role of signaling in aberrant decidualization, we analyzed mTOR, EGFR and ERK phosphorylation by 8-br-cAMP at 60 minutes, a time point found to decrease ERK1/2 phosphorylation in eSF$_{non-endo}$ (2). Contrary to our hypothesis, there were no significant differences in p-EGFR at baseline (t = 0), and p-EGFR increased in eSF$_{non-endo}$ when treated with 8-br-cAMP for 60 minutes, with no concomitant increase in eSF$_{endo}$ (Fig. 2A). p-mTOR was elevated in eSF$_{endo}$ at t = 0 and increased in both eSF$_{non-endo}$ and eSF$_{endo}$ when treated with cAMP, with levels in diseased cells remaining significantly higher than in nondiseased cells after 60 minutes of treatment (Fig. 2B). Finally, p-ERK1/2 was also elevated in eSF$_{endo}$ at t = 0, and decreased in eSF$_{non-endo}$ while remaining elevated in eSF$_{endo}$ with 60 minutes of cAMP treatment (Fig. 2C). These data indicate differences in response of EGFR and mTOR to cAMP and subsequent phosphorylation states that may affect the onset of decidualization in eSF.

EGF inhibition of decidualization in eSF from women without endometriosis is restored by gefitinib

Previous experiments have described the inhibition of cAMP-induced decidualization by EGF in eSF (25). The objective of this experiment was to inhibit decidualization in eSF$_{non-endo}$ by stimulating EGFR with EGF. Figure 3 shows *IGFBP1* mRNA expression after 96-hour treatment with EGF + 8-br-cAMP and compared with 8-br-cAMP alone. Results showed that expression of *IGFBP1* in eSF$_{non-endo}$, when treated with EGF + 8-br-cAMP was equal to that in eSF$_{endo}$ and similar to levels found in eSF$_{endo}$ treated with 8-br-cAMP alone. When the EGFR tyrosine kinase inhibitor gefitinib was added to EGF + 8-br-cAMP, *IGFBP1* expression in eSF$_{non-endo}$ was fully restored and allowed par-
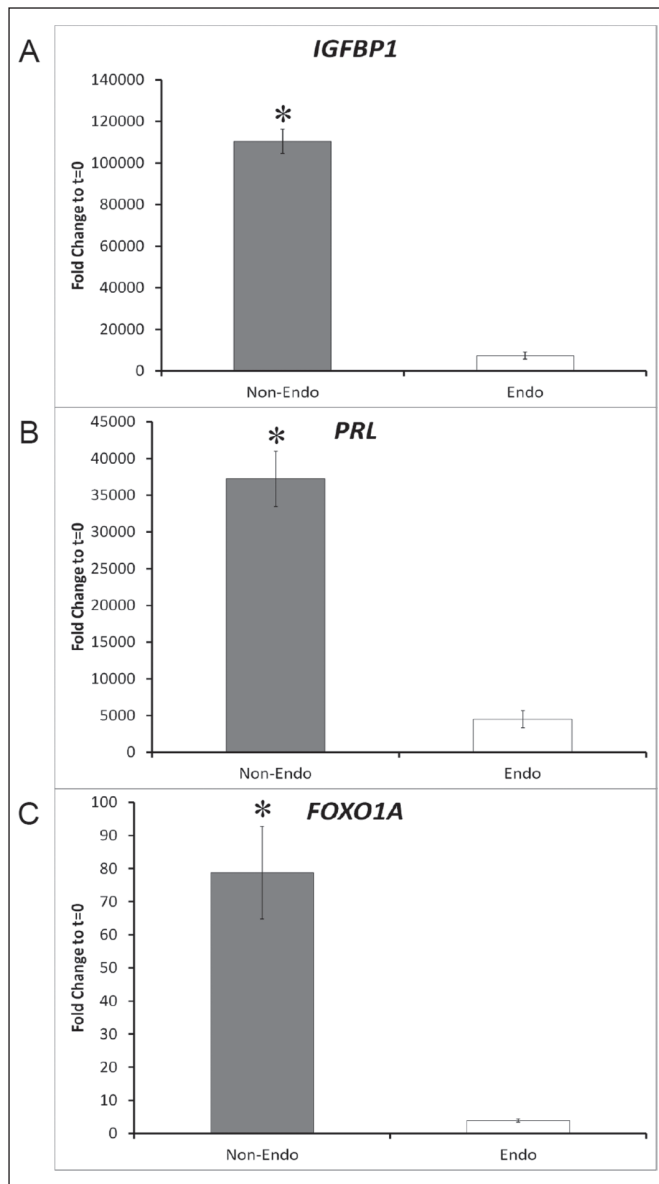
Fig. 1 - *Endometrial stromal fibroblasts from women with endometriosis do not decidualize in response to 8-br-cAMP. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) or with endometriosis (Endo) were cultured for 96 hours in low-serum medium (DMEM-MCDB105 + 2% FBS) with 0.5 mM 8-br-cAMP to induce decidualization. Separate cells were collected prior to 8-br-cAMP treatment and served as baseline (t = 0 + control). After 96 hours, cells were harvested for total RNA extraction and subjected to quantitative real-time PCR analysis for **A)** insulin-like growth factor binding protein 1 (IGFBP1); **B)** prolactin (PRL); and **C)** forkhead box protein 01A (FOXO1A). Gene expression data were calculated using the standard curve method and normalized to ACTNB levels in each sample. All data were then normalized to t = 0 to control for time in culture. Data are represented as average fold change to t = 0 ± s.e.m. Asterisks indicate significant differences by ANOVA (p<0.05).*

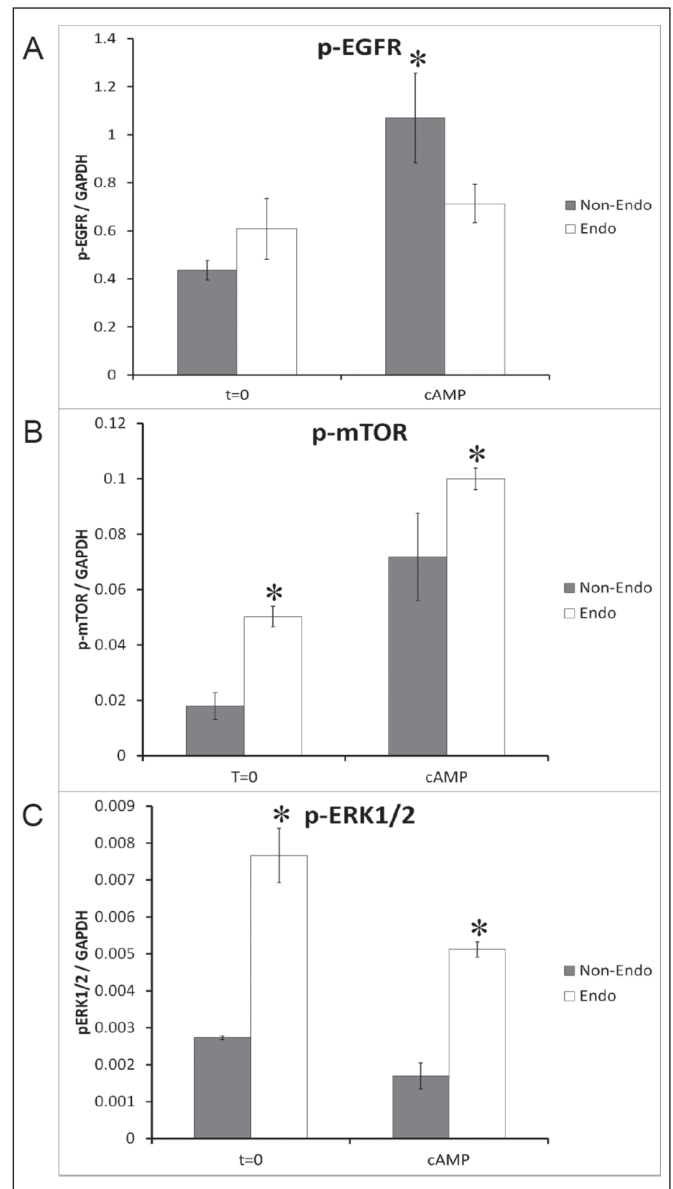**Fig. 2 -** *8-br-cAMP regulation of cell signaling activation in endometrial stromal fibroblasts. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) and with endometriosis (Endo) were cultured for 60 minutes in low-serum medium (DMEM-MCDB105 + 2% FBS) containing 0.5 mM 8-br-cAMP. Cells (n = 3 each Non-Endo and Endo) were harvested for total protein extraction and subjected to multiplex protein analysis for **A)** phosphorylated-epidermal growth factor receptor (p-EGFR); **B)** phosphorylated-mammalian target of rapamycin (p-mTOR); and **C)** phosphorylated-extracellular-signal-regulated kinase 1/2 (p-ERK1/2). Phosphorylated-protein values were normalized to GAPDH levels for each sample. Data are represented as average ratios ± s.e.m. Asterisks indicate significant differences by ANOVA (p<0.05) where Non-Endo and Endo values were compared within treatment (t = 0 or cAMP).*
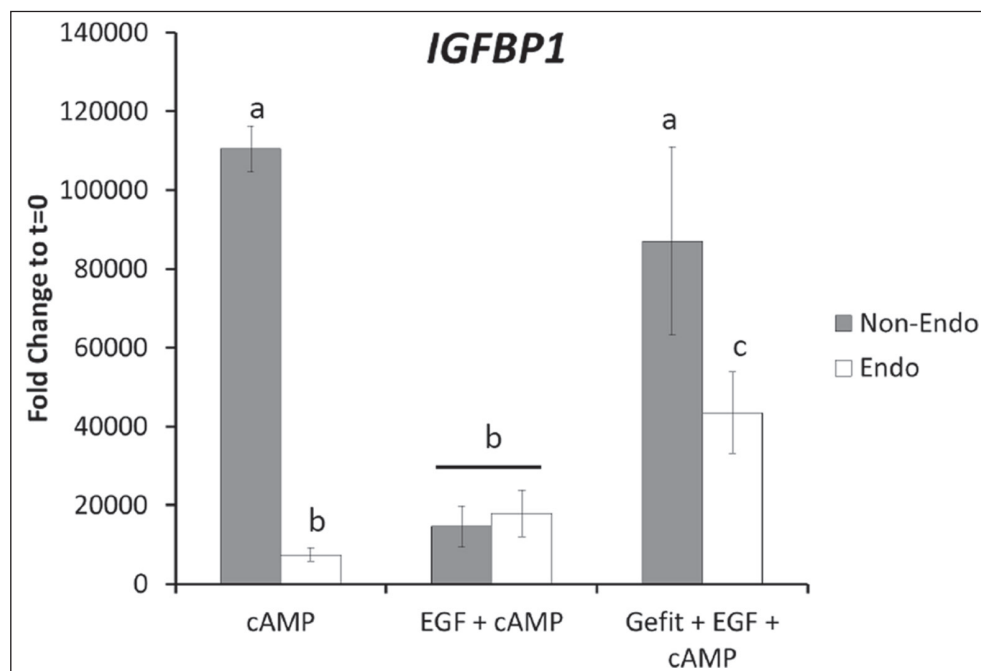
Fig. 3 - *EGF inhibition of decidualization in endometrial stromal fibroblasts is restored by gefitinib. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) or with endometriosis (Endo) were cultured for 96 hours in low-serum medium (DMEM-MCDB105 + 2% FBS) with 0.5 mM 8-br-cAMP, 0.5 mM 8-br-cAMP + 20 ng/mL EGF or 8-br-cAMP + EGF + 10 µM gefitinib. Inhibitors were delivered in DMSO, and all treatments including cAMP were adjusted to contain 0.1% DMSO. Cells were harvested for total RNA extraction and subjected to quantitative real-time PCR for insulin-like growth factor binding protein 1 (IGFBP1). Relative gene expression was calculated using the standard curve method and normalized to ACTNB levels in each sample. Data are represented as average fold change to t = 0 ± s.e.m. Different letters indicate significant differences by 2-way ANOVA ($p<0.05$).*

tial restoration of *IGFBP1* expression in eSF$_{endo}$. These results indicate that overactivation of EGFR in disease could be a cause for decreased decidualization in response to 8-br-cAMP, and that blockade of EGFR tyrosine kinase with gefitinib restores decidualization marker expression in eSF$_{endo}$.

EGF increases activation of mTOR in endometriosis

We treated eSF with EGF for 30 minutes alone or with inhibitors to specific signaling pathways, to evaluate EGFR, ERK and mTOR activation (in a pilot study, 30 minutes was found to be the optimal time for EGF activation of ERK1/2 in eSF – data not shown). p-EGFR increased similarly in eSF$_{non-endo}$ and eSF$_{endo}$ with all treatments, except for gefitinib + EGF which decreased p-EGFR in eSF independent of disease status (Fig. 4A). p-ERK1/2 was also detected in similar levels in eSF$_{non-endo}$ and eSF$_{endo}$, except for t = 0 where ERK1/2 activation was increased in eSF$_{endo}$ versus eSF$_{non-endo}$. Treatment with both gefitinib and UO126 inhibited ERK activation by EGF (Fig. 4B). mTOR phosphorylation was increased in eSF$_{endo}$ versus eSF$_{non-endo}$ by EGF in all treatments, including treatment with EGF in combination with gefitinib (Fig. 4C). These data demonstrate that EGF

is able to activate EGFR, ERK and mTOR pathways in eSF, independent of disease state. They also demonstrate that while gefitinib is able to decrease activation of both EGFR and ERK by EGF, the same was not evident with p-mTOR, suggesting an alternate route of activation of mTOR by EGF that is EGFR-independent.

Gefitinib restores decidualization marker expression in endometriosis

We treated eSF with inhibitors to EGFR, mTOR and MEK1/2 during cAMP treatment to determine their effect on restoring decidualization marker expression, and investigated the roles of these signaling pathways on the decidualization process in eSF. *IGFBP1* expression remained decreased in eSF$_{endo}$ versus eSF$_{non-endo}$ with 8-br-cAMP and rapamycin + 8-br-cAMP, and UO126 + 8-br-cAMP. In contrast, when treated with gefitinib, levels of *IGFBP1* increased in eSF$_{endo}$ to levels equal to that found in eSF$_{non-endo}$ (Fig. 5A). Similar results were also found with 2 other decidualization markers, *PRL* (Fig. 5B) and *FOXO1A* (Fig. 5C). These results indicate that inhibition of EGFR, but not downstream mTOR or ERK pathways, is able to restore cAMP-induced decidualization marker expression in eSF$_{endo}$.
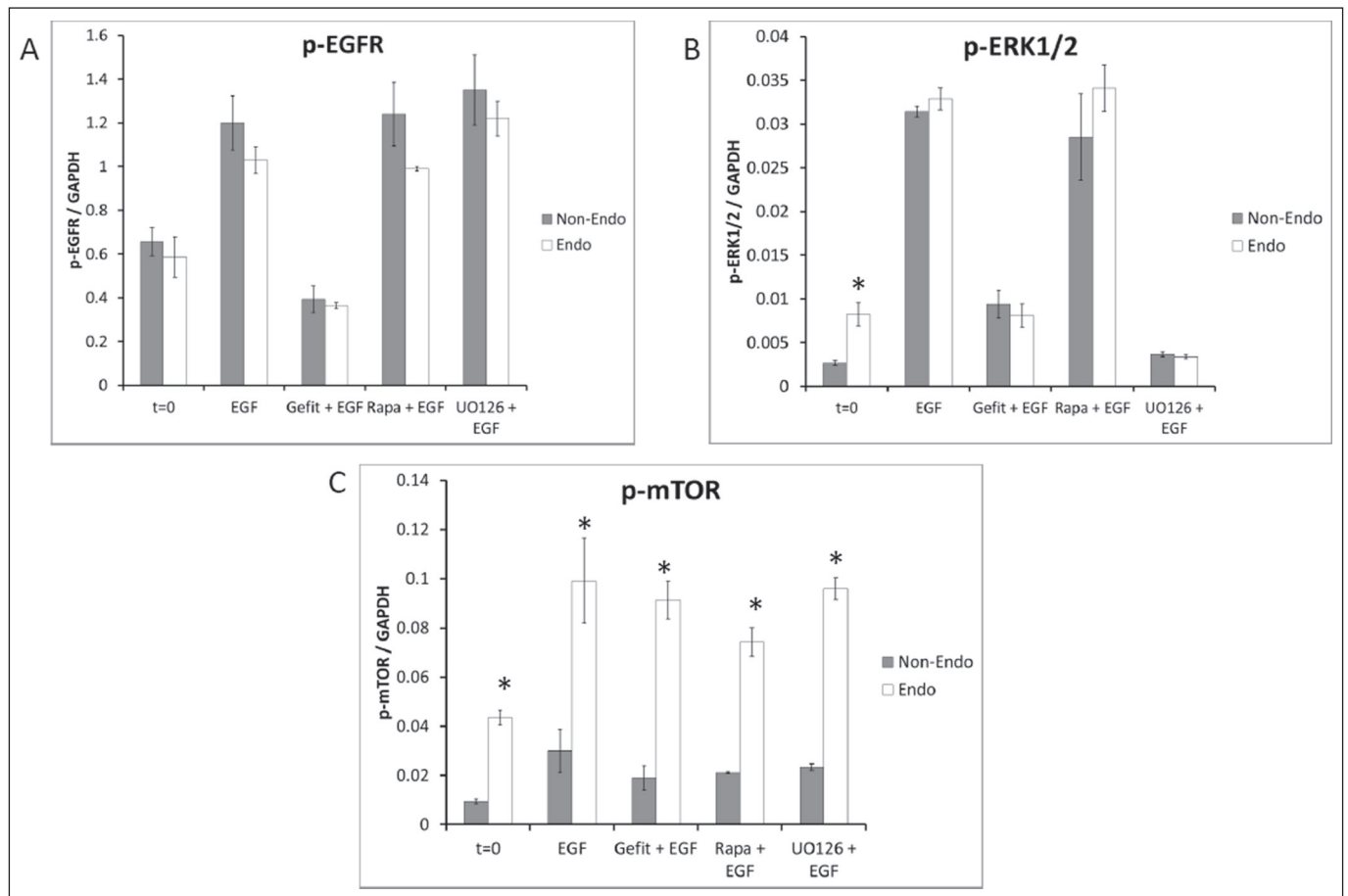
Fig. 4 - *EGF regulation of cell signaling activation in endometrial stromal fibroblasts. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) and with endometriosis (Endo) were cultured for 30 minutes in low-serum medium (DMEM-MCDB105 + 2% FBS) containing 20 ng/mL EGF, EGF + 10 μM gefitinib, EGF + 10 nM rapamycin, or EGF + 10 μM UO126. Inhibitors were delivered in DMSO, and all treatments including cAMP were adjusted to contain 0.1% DMSO. Cells were harvested for total protein extraction and subjected to multiplex protein analysis including A) phosphorylated-epidermal growth factor receptor (p-EGFR); B) phosphorylated-extracellular-regulated-kinase 1/2 (p-ERK1/2); and C) phosphorylated mammalian target of rapamycin (p-mTOR). Phosphorylated-protein values were normalized to GAPDH levels for each sample. Data are represented as average ratios ± s.e.m. Asterisks indicate significant differences between Non-Endo and Endo within treatment by ANOVA (p<0.05).*

Gefitinib affects mTOR activation in endometriosis

Because gefitinib restored decidualization marker expression in eSF$_{endo}$, we investigated signaling pathway activation when EGFR tyrosine kinase was inhibited with gefitinib. p-EGFR was increased in eSF$_{non-endo}$ versus eSF$_{endo}$ with 8-br-cAMP treatment, but increased in eSF$_{endo}$ versus eSF$_{non-endo}$ with rapamycin + 8-br-cAMP (Fig. 6A). There were no differences in p-ERK1/2 between eSF$_{non-endo}$ and eSF$_{endo}$ with any treatment, aside from the t = 0 time point and 8-br-cAMP treatment, as observed earlier (Fig. 6B). UO126 did not decrease p-ERK1/2 levels in the presence of 8-br-cAMP, as it did when cells were treated with EGF,

suggesting the possibility of direct activation of ERK1/2 by PKA stimulation in eSF (Fig. 6B). Activation of mTOR was higher at t = 0 in eSF$_{endo}$ as described earlier, and remained elevated in eSF$_{endo}$ versus eSF$_{non-endo}$ with 8-br-cAMP, rapamycin + 8-br-cAMP, and UO126 + 8-br-cAMP treatment. However, when cells were treated with gefitinib + 8-br-cAMP, the treatment that restored decidualization marker levels in eSF$_{endo}$, p-mTOR levels were equal in eSF$_{non-endo}$ and eSF$_{endo}$, although levels in eSF$_{endo}$ with this treatment were lower than those found in eSF$_{endo}$ treated with 8-br-cAMP alone (Fig. 6C). Thus, these data demonstrate that inhibition of the tyrosine kinase domain of EGFR during cAMP treatment results in decreased levels of p-mTOR in
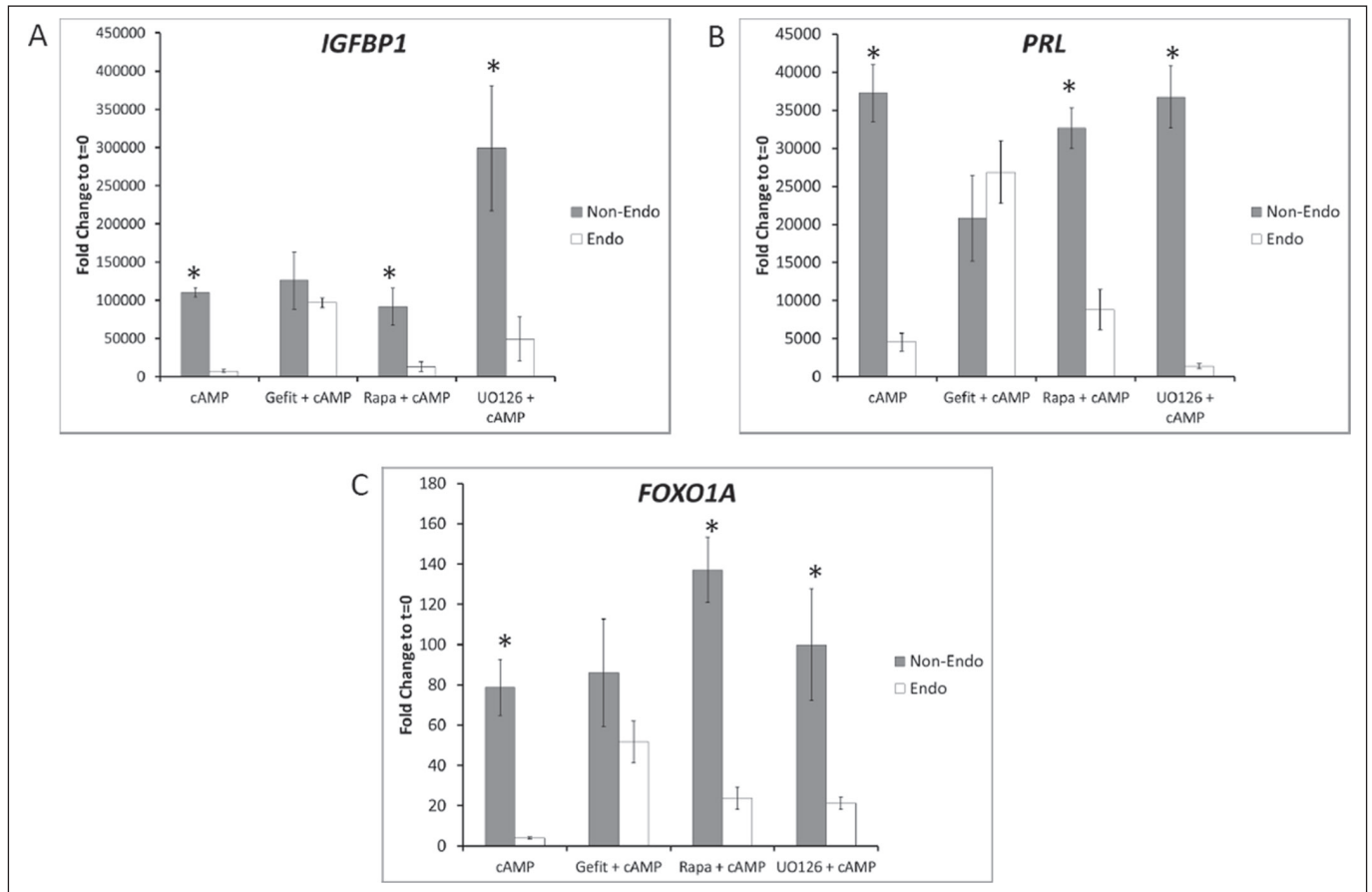
Fig. 5 - *Gefitinib restores decidualization marker expression in endometrial stromal fibroblasts from women with endometriosis. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) and with endometriosis (Endo) were cultured for 96 hours in low-serum medium (DMEM-MCDB105 + 2% FBS) containing 0.5 mM 8-br-cAMP, cAMP + 10 µM gefitinib, cAMP + 10 nM rapamycin, or cAMP + 10 µM UO126. Inhibitors were delivered in DMSO and all treatments including cAMP were adjusted to contain 0.1% DMSO. Cells were harvested for total RNA extraction and subjected to quantitative real-time PCR analysis for **A)** insulin-like growth factor binding protein 1 (IGFBP1); **B)** prolactin (PRL); and **C)** forkhead box protein 01A (FOXO1A). Gene expression data were calculated using the standard curve method and normalized to ACTNB levels in each sample, and normalized to t = 0 to control for time in culture. Data are represented as average fold change to t = 0 ± s.e.m. Asterisks indicate significant differences by ANOVA (p<0.05).*

eSF_{endo} to levels equal to $eSF_{non-endo}$, suggesting that EGFR-mTOR signaling is critical during cAMP-induced decidualization in eSF. When combined with the data above showing EGF stimulating mTOR through EGFR-independent means, the data suggest interplay of an EGFR tyrosine kinase and PKA signaling in the regulation of mTOR activation in eSF.

EGF receptor and negative feedback regulator expression is dysregulated in eSF_{endo}

Because differences in EGFR activation between $eSF_{non-endo}$ and eSF_{endo} were minimal, we evaluated expression of several EGF-ligand binding receptors and negative feedback

regulators to determine possible alternate regulation of EGFR signaling. Figure 7A shows expression of receptors *EGFR* and *ERBB2*, and negative regulators of EGFR, *ERRFI1* and *PTPRK* at t = 0. Levels of *EGFR*, *ERBB2* and *ERRFI1* mRNA were all higher in eSF_{endo} versus $eSF_{non-endo}$; levels of *PTPRK* did not differ between $eSF_{non-endo}$ and eSF_{endo}. However, after 96 hours of 8-br-cAMP treatment, levels of *EGFR*, *ERBB2*, *ERRFI1* and *PTPRK* mRNA all significantly increased in $eSF_{non-endo}$ versus eSF_{endo} (Fig. 7B). These data suggest some level of involvement of EGFR in decidualization in $eSF_{non-endo}$; however, when stimulated with cAMP, eSF_{endo} do not properly regulate activation of EGFR, as suggested by the lower levels of *ERRFI1* and *PTPRK* expression. Thus, increased
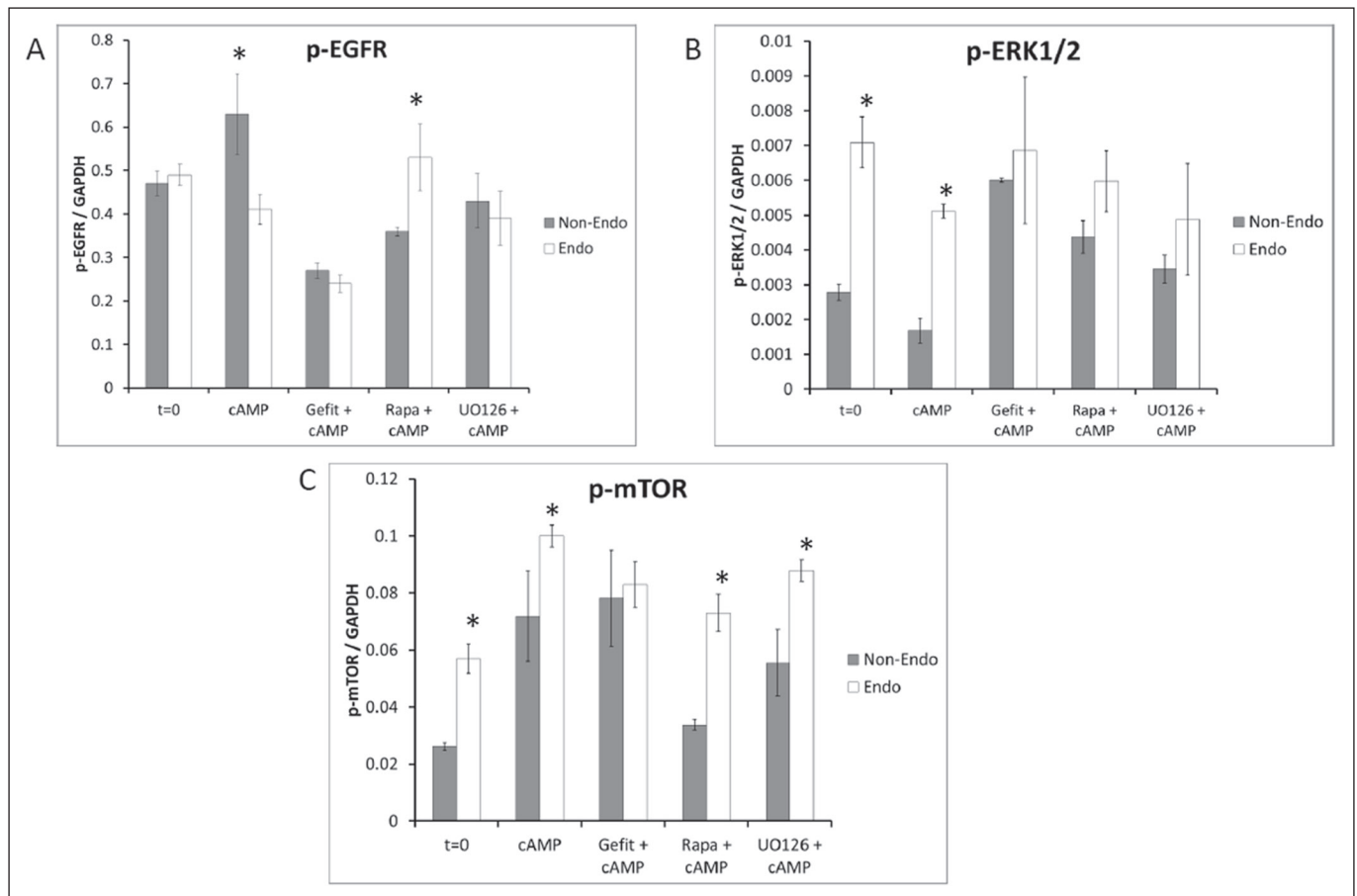
Fig. 6 - *8-br-cAMP regulation of cell signaling pathways in the presence of chemical inhibitors. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) and with endometriosis (Endo) were cultured for 60 minutes in low-serum medium (DMEM-MCDB105 + 2% FBS) containing 0.5 mM 8-br-cAMP, cAMP + 10 µM gefitinib, cAMP + 10 nM rapamycin, or cAMP + 10 µM UO126. Inhibitors were delivered in DMSO, and all treatments including cAMP were adjusted to contain 0.1% DMSO. Cells were harvested for total protein extraction and subjected to multiplex protein analysis for **A)** phosphorylated-epidermal growth factor receptor (p-EGFR); **B)** phosphorylated-extracellular-regulated-kinase 1/2 (p-ERK1/2); and **C)** phosphorylated-mammalian target of rapamycin (p-mTOR). Phospho-protein values were normalized to GAPDH levels for each sample. Data are represented as average ratios ± s.e.m. Asterisks indicate significant differences between Non-Endo and Endo within treatment by ANOVA ($p < 0.05$).*

activation of EGFR may not be causative of aberrant decidualization in eSF_{endo}, as levels of p-EGFR are actually higher in $eSF_{non-endo}$ following 8-br-cAMP treatment. Rather, decreased decidualization in response to cAMP may be due to sustained unopposed activation of EGFR in eSF_{endo}.

Expression of select EGFR ligands are increased in endometriosis

In addition to evaluating EGF family receptors and negative regulators, we evaluated mRNA expression of several EGFR ligands. Expression of *AREG* and *HBEGF* was higher in $eSF_{non-endo}$ than eSF_{endo} at t = 0, while *TGFA* expression oc-

curred at similar levels in $eSF_{non-endo}$ and eSF_{endo} at t = 0 (Fig. 8A). However, with 8-br-cAMP treatment, *AREG* increased in eSF_{endo} versus $eSF_{non-endo}$ and expression was inhibited in eSF independent of disease with UO126 + 8-br-cAMP (Fig. 8B). *TGFA* increased dramatically in eSF_{endo} versus $eSF_{non-endo}$ with 8-br-cAMP, gefitinib + 8-br-cAMP, and UO126 + 8-br-cAMP; rapamycin + 8-br-cAMP decreased expression of *TGFA* in eSF_{endo} (Fig. 8C). Finally, *HBEGF* expression was not different between $eSF_{non-endo}$ and eSF_{endo} except with the combination of rapamycin + 8-br-cAMP, where expression was elevated in eSF_{endo} versus $eSF_{non-endo}$ (Fig. 8D). These data demonstrate the possibility that autocrine/paracrine activation of EGFR by TGFA or AREG with cAMP treat-
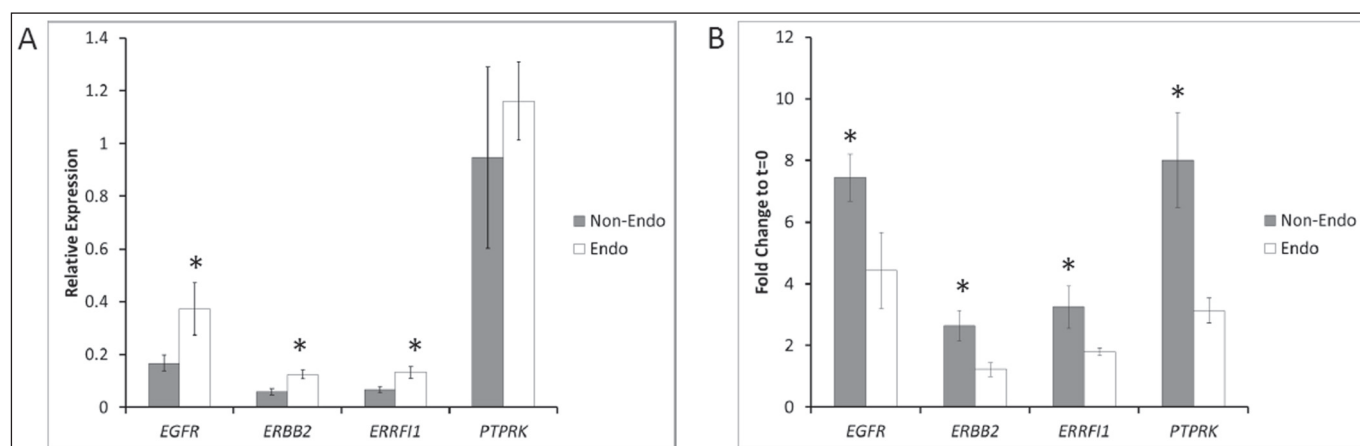
Fig. 7 - *EGF receptor and negative regulator expression in endometrial stromal fibroblasts. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) or with endometriosis (Endo) were cultured for 96 hours in low-serum medium (DMEM-MCDB105 + 2% FBS) with 0.5 mM 8-br-cAMP. Cells were harvested for total RNA extraction and subjected to quantitative real-time PCR analysis for epidermal growth factor receptor (EGFR), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2), ERBB receptor feedback inhibitor 1 (ERRFI1), and receptor-type tyrosine-protein phosphatase kappa (PTPRK) at t = 0 **(A)** and after 96-hour 8-br-cAMP treatment **(B)**. Gene expression data were calculated using the standard curve method and normalized to ACTNB levels in each sample, and normalized to t = 0 to control for time in culture. Data are represented as average fold change to t = 0 ± s.e.m. Asterisks indicate significant differences by ANOVA (p<0.05).*

ment could result in EGFR activation in eSF$_{endo}$. The different expression patterns of these ligands depending on pathway inhibition also provide insight into the complexity of their regulation in eSF. The similar levels of *AREG* in eSF$_{non-endo}$ and eSF$_{endo}$ with gefitinib, as well as the higher levels of *TGFA* in eSF$_{endo}$ versus eSF$_{non-endo}$ with gefitinib suggest that dysregulation of EGFR signaling occurs downstream of ligand receptor binding.

No significant differences in p-AKT, p-PTEN, p-P70S6K, cleaved PARP or active caspase 3 between eSF$_{non-endo}$ and eSF$_{endo}$ were observed regardless of treatment time or inhibitors present (Suppl Figs. 1 and 2). Simultaneous treatment with any 2-inhibitor combination did not have an additive effect on decidual marker gene expression (Suppl Fig. 3).

DISCUSSION

Herein, we used selective chemical inhibitors to block the EGFR, mTOR and MAPK signaling pathways in an effort to induce decidualization marker expression in eSF from women with endometriosis. Using gefitinib, an inhibitor of the EGFR tyrosine kinase domain (Tyr1173 and Tyr992), we were able to restore gene expression of 3 key markers of decidualization: *IGFBP1*, *PRL* and *FOXO1A*. To our knowledge, this is also the first study providing evidence of the dysregulation of protein tyrosine phosphatase recep-

tor type kappa (PTPRK), a negative feedback regulator of EGFR, during 8-br-cAMP-induced decidualization in eSF. These observations are crucial to understanding the decidualization process in eSF and its aberrant regulation in endometriosis.

Decidualization

Decidualization is a complex process that involves a number of autocrine/paracrine loops involving cytokines and growth factors and other secreted molecules including interleukin-11 (IL-11), PRL, IGFBP1 and HBEGF (26). Previously, studies have demonstrated a blunted expression of specific decidual markers in response to activation of the cAMP/PKA pathway in eSF from women with endometriosis versus those without disease (2, 14, 15). We also previously determined that there is no defect in cAMP hydrolysis in eSF$_{endo}$, indicating that the diminished response of these cells to cAMP/PKA stimulation may be due to insensitivity of certain genes to PKA stimulation or a difference in the regulation of cell signaling pathways in eSF$_{endo}$ (14). In the present study, *IGFBP1*, *PRL* and *FOXO1A* expression increased in eSF$_{endo}$ to levels consistent with those found in eSF$_{non-endo}$, by treatment with the EGFR tyrosine kinase domain inhibitor gefitinib (Fig. 5), suggesting dysregulation of EGFR signaling in eSF$_{endo}$ during cAMP-induced decidualization.
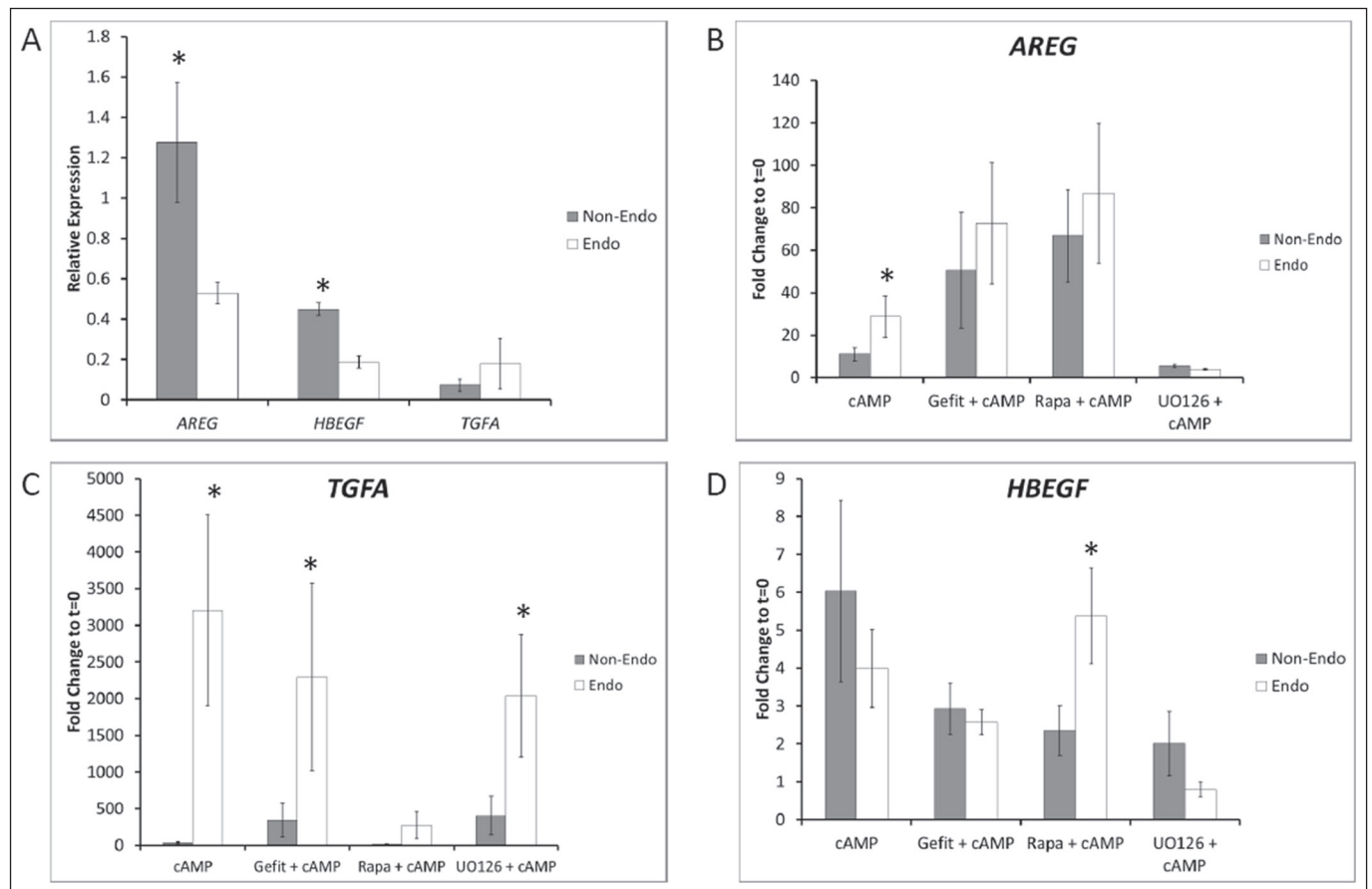
Fig. 8 - *Expression of select EGFR ligands in endometrial stromal fibroblasts. Endometrial stromal fibroblasts from women without endometriosis (Non-Endo) and with endometriosis (Endo) were cultured for 96 hours in low-serum medium (DMEM-MCDB105 + 2% FBS) containing 0.5 mM 8-br-cAMP, cAMP + 10 µM gefitinib, cAMP + 10 nM rapamycin, or cAMP + 10 µM UO126. Inhibitors were delivered in DMSO, and all treatments including cAMP were adjusted to contain 0.1% DMSO. Cells were harvested for total RNA extraction and subjected to quantitative real-time PCR analysis for amphiregulin (AREG), heparin-binding epidermal growth factor-like growth factor (HBEGF), and transforming growth factor alpha (TGFA) at t = 0 **(A)** or after 96-hour treatment with 8-br-cAMP **(B-D)**. Gene expression data were calculated using the standard curve method and normalized to ACTNB levels in each sample, and normalized to t = 0 to control for time in culture. Summary of 8-br-cAMP-induced pathways in decidualization of human endometrial stromal fibroblasts in women without endometriosis (Normal) and with disease (Endometriosis). **A)** Relative expression data calculated at t = 0. **B-D)** Average fold change to t = 0 ± s.e.m. Asterisks indicate significant differences by ANOVA (p<0.05).*

We detected elevated levels of p-ERK1/2 and p-mTOR in eSF$_{endo}$ when compared with normal counterparts prior to treatment with 8-br-cAMP, both of which are downstream of EGFR in their respective pathways. We previously found an increase in *EGFR* gene expression and gene expression of selected EGFR ligands in eSF from women with severe endometriosis versus women with mild disease (12), while another study showed decreased expression of EGFR in eSF from women with disease (27). A decreased response to EGF by eSF$_{endo}$ (28) and an inhibitory effect of EGF on 8-br-cAMP-induced decidualization (25) have also been reported. These data collectively suggest it is possible that

several mechanisms are operating with regard to EGFR and other ERBB receptors in eSF$_{endo}$, which may be affected by increased copy number or higher levels of tyrosine kinase activity. It is also possible that negative regulators of EGFR, including ERRFI1 and PTPRK, do not respond to stimuli in eSF$_{endo}$ as they do in eSF$_{non-endo}$, thus affecting down-regulation of EGFR tyrosine kinase. Evidence for dysregulation at different levels within the EGFR system is apparent in the current study from the impaired regulation of *EGFR*, *ERBB2*, *ERRFI1* and *PTPRK* expression by 8-br-cAMP in eSF$_{endo}$, which remained essentially nonresponsive to 8-br-cAMP (Fig. 7). Collectively, these data coupled

with restoration of decidual marker expression in eSF$_{endo}$ following treatment with gefitinib, demonstrate that the EGFR signaling system plays a key role in the aberrant expression of decidualization markers seen in endometriosis.

EGFR inhibitors

Our study showed lower levels of *ERRFI1* expression in 8-br-cAMP-treated eSF$_{endo}$ compared with their normal counterparts, consistent with its known altered expression in endometrium from women with endometriosis (26, 29-31). ERRFI1 has also previously been shown to be involved in regulation of uterine homeostasis in response to E2 in mice (31), and knockdown of ERRFI1 in human bronchiolar epithelial leads to an increase in both p-EGFR and p-AKT (32), resulting in increased cell proliferation. The current study also revealed the dysregulation of another negative receptor of EGF signaling in eSF$_{endo}$, receptor-type protein tyrosine phosphatase kappa (PTPRK), to our knowledge not previously reported. PTPRK acts as a regulator of EGFR activity by dephosphorylating the receptor at tyrosines 1068 and 1173 (33), and its silencing increases basal and EGF-stimulated EGFR tyrosine kinase phosphorylation and downstream ERK activation. In the current study, 8-br-cAMP-stimulated levels of both *ERRFI1* and *PTPRK* were markedly increased by gefitinib, and showed less prominent changes with the other inhibitors, but invariably with a persistent blunted response in eSF$_{endo}$ compared with controls. Alternatively, it is possible that the effect of gefitinib on dephosphorylation of p-EGFR Tyr1173 resulted in decreased expression of *PTPRK* in eSF$_{endo}$. The same effect was not seen in eSF$_{non-endo}$, however, indicating differences in how eSF respond to these stimuli in the context of disease. Combined, these results indicate that, regardless of EGFR expression or activation, eSF$_{endo}$ may demonstrate a compromised capacity to regulate EGFR activity during 8-br-cAMP decidualization.

EGFR ligands

The expression of several EGFR ligands was evaluated to investigate the potential for autocrine or paracrine activation of EGFR signaling in eSF$_{endo}$. In the present study, 8-br-cAMP increased *TGFA* and *AREG* expression selectively in eSF$_{endo}$. While similar observations in endometriosis have not been reported, the interaction of TGFA with EGFR in an autocrine manner has been implicated in the progression of a number of cancers, presumably by stimulating tumor cell proliferation (34). This raises the possibility that endogenous TGFA acts as an autocrine/paracrine ligand stimulating EGFR signaling that inhibits decidualization. However, changes in *TGFA* or *AREG* expression did not correlate with decidualization marker changes consistently across treatments. Addition of rapamycin in combination with 8-br-cAMP reduced *TGFA* expression, but this was not correlated with increased expression of decidualization markers in eSF$_{endo}$. Conversely, 8-br-cAMP in combination with gefitinib had no effect on *TGFA* expression, but increased expression of decidualization markers in eSF$_{endo}$. Therefore, increased availability of EGFR ligand alone does not seem to account for the inhibition of decidualization marker expression in eSF$_{endo}$, suggesting a potential dysregulation of the EGFR signaling pathway residing downstream from ligand-receptor binding.

mTOR and decidualization markers

Another observation made in this study is the relationship between mTOR activation and expression of decidualization marker genes. Levels of p-mTOR were constitutively elevated in eSF$_{endo}$ versus eSF$_{non-endo}$ and in all cases when treated with 8-br-cAMP (Fig. 6). The one exception occurred when eSF were treated with gefitinib in addition to 8-br-cAMP, which decreased p-mTOR in eSF$_{endo}$ to levels equal to those in eSF$_{non-endo}$. Addition of gefitinib to 8-br-cAMP treatment was also when we observed equivalent levels of expression of *IGFBP1*, *PRL* and *FOXO1A* in eSF$_{endo}$ and eSF$_{non-endo}$ (Fig. 5). This would suggest that inhibition of EGF signaling in eSF$_{endo}$ causes a sufficient decrease in the elevated levels of p-mTOR for decidualization to occur. cAMP or its analogues are not known to directly activate the mTOR signaling pathway. A study previously performed in thyroid cells demonstrated that cAMP, in combination with TSH, could activate mTORC1, which then led to downstream activation (35). This activation of mTOR by the cAMP/TSH combination occurred independent of AKT phosphorylation, suggesting direct activation of mTOR by cAMP/TSH. This observation, coupled with our own data, suggests that cAMP may stimulate mTOR signaling through nonclassic yet still unclear mechanisms. Thus, it is possible that the dysregulation of EGFR signaling we propose is occurring downstream of ligand-receptor binding results in hyperactivation of mTOR in eSF$_{endo}$ that is normalized to that of eSF$_{non-endo}$ with inhibition of the EGFR tyrosine kinase domain.

Summary of observations

EGF is known to signal via multiple pathways including ERK1/2 and mTOR via AKT. It is evident that in eSF$_{non-endo}$ 8-br-cAMP activates mTOR, but not ERK1/2 in our model. In eSF$_{endo}$ 8-br-cAMP has no effect on constitutively high levels of p-ERK1/2, but activates mTOR such that it remains higher than in eSF$_{non-endo}$. This differential activation of mTOR correlates with the differential expression of decidualization markers in 8-br-cAMP-treated eSF$_{non-endo}$/eSF$_{endo}$, and further, parallels changes in eSF$_{non-endo}$ in response to inhibitor treatments. The involvement of EGFR is inferred from the effects of gefitinib, which normalizes decidualization marker expression in eSF$_{endo}$ and at the same time reduces mTOR activation to normal eSF$_{non-endo}$ levels, while nonselectively activating p-ERK1/2 in both eSF$_{non-endo}$ and eSF$_{endo}$. This is consistent with the known involvement of mTOR signaling in protein synthesis versus MAPK pathway in proliferation. Despite these parallel observations, there are inconsistencies in the model. Activation of mTOR by 8-br-cAMP is associated with decidualization marker expression in eSF$_{non-endo}$, but rapamycin has no effect on decidualization marker expression. In addition, overactive mTOR correlates with no expression of decidualization markers. This suggests that cAMP signaling in eSF$_{non-endo}$ does not depend on mTOR to induce decidualization marker expression. While it is not entirely clear from these data how constitutively active EGFR may prevent decidualization marker expression in eSF$_{endo}$, inhibition of EGFR tyrosine kinase with gefitinib restores expression of 3 classic decidualization markers to similar levels to those found in eSF$_{non-endo}$. This posits overactive EGFR as key in the aberrant decidualization response of eSF$_{endo}$ to 8-br-cAMP through dysregulation of downstream pathways.

Clinical translation

Endometriosis-related infertility is presumably due, in part, to endometrium that is nonreceptive to embryo implantation, as well as poor egg quality and reduced ovarian reserve due to intrapelvic inflammation. Current therapies have focused on a variety of fertility medications, surgery or assisted reproductive technologies to achieve pregnancy, which are costly and can result in treatment-related side effects (1). Treatment of endometriosis-related pain has largely relied on the use of drugs such as combined contraceptive steroids, progestogens, GnRH agonists and aromatase P450 inhibitors to block ovarian estradiol synthesis and/or action on endometrial tissue, prevent retrograde menstruation and provide a less optimal environment for lesion survival and growth (36). Most of these current medical therapies affect ovulation and endometrial cyclicity, thus having no positive effect of fertility in women with disease, and in fact delay fertility potential. Furthermore, no drug that has been used to date has proven to be effective at restoring infertility in subfertile endometriosis patients versus placebo (36). The data presented herein demonstrating that the use of gefitinib restores 8-br-cAMP-induced decidualization marker expression in women with endometriosis indicate that chemical inhibitors of EGFR tyrosine kinase activation warrant further exploration as potential treatments for endometriosis-related infertility. The use of an EGFR inhibitor could be one alternative, as it may not have negative effects on reproductive functions, although this remains undetermined.

Summary and conclusions

Herein, we have demonstrated the restoration of the decidualization markers IGFBP1, PRL and FOXO1A in eSF from women with endometriosis by inhibiting EGFR tyrosine kinase activity with gefitinib. These experiments have also provided insight into the roles of EGFR, mTOR and MAPK signaling in 8-br-cAMP-induced decidualization. Future studies are warranted to investigate the involvement of these pathways in the complex process of decidualization, and evaluate the potential for, and safety of, EGFR inhibitors in the clinical management of endometriosis-related infertility.

Address for correspondence:
Linda C. Giudice, MD, PhD, MSc
Distinguished Professor and Chair
Department of Obstetrics, Gynecology
and Reproductive Sciences
University of California – San Francisco
505 Parnassus Avenue M1496
San Francisco, CA 94143-0132, USA
Linda.Giudice@ucsf.edu

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