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Estrogen mediated epithelial proliferation in the uterus is directed by stromal Fgf10 and Bmp8a

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Abstract

To define endometrial stromal-derived paracrine mediators that participate in estradiol-17β (E2)-induced epithelial proliferation, microarray analysis of gene expression was carried out in mouse uterine epithelial–stromal co-culture systems under the condition of E2 or vehicle (control). Our results demonstrated gene alteration by E2: in epithelial cells, we found up-regulation of 119 genes and down-regulation of 28 genes, while in stroma cells we found up-regulation of 144 genes and down-regulation of 184 genes. A functional enrichment analysis of the upregulated epithelial genes implicated them for proliferation, while upregulated stromal genes were associated with extracellular functions. Quantitative RT-PCR and *in situ* hybridization results confirmed differential gene expression in both cell cultures and ovariectomized uteri after the above treatments. Based on our identification of stromal secretory factors, we found evidence that suppression by siRNA specifically for *Bmp8a* and/or *Fgf10* in the stromal layer caused significant inhibition of proliferation by E2 in the co-culture system, suggesting Bmp8a and Fgf10 act as paracrine mediators during E2-dependent control of uterine proliferation. The localization of receptors and receptor activation signaling in epithelial cells in both the co-culture system and uteri was consistent with their involvement in ligand–receptor signaling. Interestingly, loss of *Bmp8a* or *Fgf10* also caused abrogation of E2-regulated epithelial receptor signaling in co-culture systems, suggesting that stroma-derived Fgf10 and Bmp8a are responsible for epithelial communication. Overall, stromal Fgf10 and Bmp8a serve as potential paracrine factors for E2-dependent regulation of epithelial proliferation in the uterus.

Keywords

Uterus; Estrogen; Epithelial; Stromal; Paracrine; Proliferation

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1. Introduction

Estrogen plays a primary role in various functions during the reproductive cycle and pregnancy. In mice, estrogen induces uterine epithelial cell proliferation and is essential for the maintenance of normal epithelial morphogenesis, cytodifferentiation, and secretory activity (Cunha and Lung, 1979). The general consensus is that estrogen exerts its effects by modifying gene expression through activation of its nuclear receptors in the uterus. Estrogen receptor 1 (alpha) (ESR1) is considered the primary receptor for estrogen (Couse and Korach, 1999), while estrogen receptor 2 (beta) (ESR2) has only limited functions (Chung and Das, 2011; Krege et al., 1998). Moreover, it has been widely recognized that estradiol-17β (E2) controls uterine epithelial proliferation via a cross-talk between the epithelial–stromal cell layers (Cooke et al., 1998), although the molecular mechanism by which stroma mediates this communication remains poorly understood. Based on previous tissue recombination studies, it has been demonstrated that the mitogenic response of uterine epithelial cells to E2 is mediated by stromal ESR1 (Cooke et al., 1997), suggesting the intimate involvement of stroma-derived paracrine mediators under the direction of E2/ESR1 signaling. Previously, many growth factors, including epidermal growth factor (Egf) (Nelson et al., 1991), insulin-like growth factor-1 (Igf-1) (Murphy and Ghahary, 1990), hepatocyte growth factor (Hgf) (Zarnegar and Michalopoulos, 1995), keratinocyte growth factor (Kgf) (Aaronson et al., 1990), transforming growth factor-α (Tgf-α) (Nelson et al., 1992), and fibroblast growth factors (Fgfs) (Li et al., 2011), have been proposed for stromal–epithelial communication in a variety of tissues. However, in the regulation of E2-induced uterine epithelial proliferation, direct evidence for the involvement of specific paracrine factor(s) has never been conclusively demonstrated. This is partly because analyzing interactions between different cell types at the molecular level requires a simple and defined condition where E2-directed control of proliferation remains intact.

Previously, we established a primary uterine epithelial–stromal co-culture system in mice as an alternative model to explore molecular aspects of uterine biology, especially ovarian hormonal regulation of cell-specific proliferation via cell–cell communication between the epithelial and stromal compartments (Chung and Das, 2011). More specifically, this co-culture system was derived from D4 of pseudopregnancy in mice. It was found to be fully supportive of paracrine interactions for cell-specific proliferation not only by E2, but also by E2 plus progesterone (P4), reflecting a cell specific proliferative response to ovarian hormone *in vivo*. Interestingly, this communication also supported cell-specific regulation of hormone receptors and hormone-dependent regulation of gene expression. Since we could molecularly suppress epithelial/stromal cell-specific factors in each compartment, we were able to establish our co-culture system as a great tool for exploring potential stromal paracrine factors that regulate E2-induced epithelial cell proliferation.

We used our co-culture system in the study described here to perform a comprehensive analysis on the differential expression profiles of genes involved in epithelial proliferation in order to define the potential regulators. Based on multiple approaches through validation of microarray results by both *in vitro* and *in vivo* studies, specific gene suppression, and analysis of receptor signaling activation, we are able to provide evidence to suggest that Fgf10 and Bmp8a may serve as potential stroma-derived paracrine factors that mediate

successful communication for the control of uterine epithelial proliferation under the direction of E2.

2. Materials and methods

2.1. Animals and uterine tissue collection

Adult CD1 (Charles Rivers Laboratory, Raleigh, NC) mice were housed in the animal care facility at Cincinnati Children's Hospital Medical Center according to the National Institutes of Health (NIH) and institutional guidelines for the use of laboratory animals. All protocols for the present study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) (approval number: 1D05043). In general, 8–10 week old adult mice were used to induce pseudopregnancy for cell culture studies or ovariectomized and rested for 10 days before they received injections of E2 or vehicle. Mice were given a single injection [(0.1 ml/mouse, subcutaneously (sc)] of E2 (100 ng/mouse) dissolved in sesame seed oil, and then uterine tissues were collected at 24 h. We also collected uterine tissues at 24 h from mice injected with oil as vehicle control (0.1 ml/mouse, sc) for comparison.

2.2. Primary co-culture of uterine epithelial and stromal cells

Uterine epithelial and stromal cells were isolated by enzymatic digestion of uterine tissues as described previously by us (Chung and Das, 2011). Epithelial sheets were grown on Matrigel coated cell culture inserts and the stromal cells were grown on cover slips in six-well plates. After initiation of the co-culture, the cells were depleted in phenol-red free DMEM/F12 media supplemented with charcoal-stripped 1% FBS (w/v) overnight prior to the initiation of treatment with E2 (10 nM) or vehicle (0.01% ethanol, v/v). Both cell layers were collected and analyzed separately after 24 or 48 h of treatment. Our previous study (Chung and Das, 2011) found that epithelial cells in the co-culture were significantly responsive to E2 for proliferation at a dose of 10 nM after 24 or 48 h, although maximum response was noted after 48 h of treatment. Thus, all studies described in the present report were carried out after 48 h of treatment.

2.3. Microarray and data analysis

Microarray hybridization and analysis were conducted according to the Affymetrix recommended protocols with help from the Microarray Core Facility at Cincinnati Children's Hospital Medical Center. In brief, RNAs were extracted from epithelial and stromal cell populations using Aurum total RNA Mini Kit according to the manufacturer's instructions (Bio-Rad, cat# 732-6820, Valencia, CA). Each treatment group for the epithelial or stromal cell populations was composed of three independent samples. The quality of total RNA was checked by the Agilent Bioanalyzer 2100 (Hewlett Packard) using the RNA 6000 Nano Assay. Biotin-labeled cRNAs were generated using the Affymetrix Whole Transcript Sense Target Labeling Assay (Affymetrix) and hybridized to GeneChip® mouse gene 1.0 ST array (Affymetrix Inc.). Affymetrix GeneChip Scanner 3000 7G was used to scan and quantitate the gene chips using default scan settings. The raw data have been deposited to the GEO database (accession number = GSE52399). Differentially expressed genes were selected based on the GeneSpring GX10 program, with a threshold of unpaired t-test (*P*-value ≤0.05), false discovery rate (FDR) ≤5%, and a fold change cut off of 2.0. Functional

enrichment analysis of the differentially expressed genes from microarray data analysis was performed using the ToppFun server (http://toppgene.cchmc.org) (Chen et al., 2009).

2.4. Quantitation of gene expression by real time-PCR

RNA (1 μg total RNA from each sample) was primed with random-hexamers in a volume of 20 μl and reverse transcribed into cDNA with MMLV Reverse Transcriptase (Promega, cat# M1701). Total RNA was extracted from three independent cell preparations for each group. The resulting cDNA was subjected to quantitative real time-PCR analysis. In brief, One-step RT-PCR was performed using Step One Plus real-time PCR system (Applied Biosystems, Grand Island, NY) and Fast SYBR Green Master Mix (Life Tech, Grand Island, NY, cat#4385610). Holding stage at 95 °C for 20 s, 40 PCR cycling stage consisting of denaturation at 95 °C for 3 s, annealing and extension at 60 °C for 30 s, and melting curve stage consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and final termination at 95 °C for 15 s. Melting curves for all products showed single peaks. The relative target gene expression was quantified by the ΔΔCt method (Livak and Schmittgen, 2001), using *ribosomal protein l7 (Rpl7*, housekeeping gene) for normalization.

2.5. Hybridization probes and *in situ* hybridization

Mouse-specific cDNA clones (in pGEM vector) were generated by RT-PCR. The authenticity for each of these clones was confirmed by nucleotide sequencing. For *in situ* hybridization, sense and antisense ^{35}S-labeled cRNA probes were generated. Frozen sections (10 μm) were hybridized with ^{35}S-labeled cRNA probes as described previously (Das et al., 1994). Sections hybridized with sense probes served as negative controls and showed no positive signals.

2.6. siRNA-driven perturbation of candidate genes

Stromal cells prior to the initiation of hormone treatment in the primary co-culture system were subjected to transfection with different siRNAs (at 100 nM) using the lipofectamine 2000 reagent (Invitrogen) for 6 h, according to the manufacturer's instructions. In brief, after incubation of control siRNA or gene specific siRNA with lipofectamine for 5 min at RT, mixture was added to stromal cells for transfection. After 48 h of culture in the presence of E2 (10 nM) or vehicle (0.01% ethanol, v/v), the experiment was terminated and analyzed for gene expression by RT-PCR or western blot studies. Mouse specific siRNAs for *bone morphogenetic protein 8a (Bmp8a)*: AACTATGAAGTTCACTGTAAA; *bone morphogenetic protein 8b (Bmp8b)*: CACCTTGACAACAGCCAGAAA; *fibroblast growth factor 10 (Fgf10)*: TTGTTTGATATTAATAGAGAA; *c-fos induced growth factor (Figf)*: AAGGGCAATGCTCATGAGTTA; or *glial cell line derived neurotrophic factor (Gdnf)*: TCGGTCGTTTGTGTTATACAA were purchased from Qiagen. In parallel studies, cells were also transfected with control siRNAs (AllStars Negative Control siRNA, Qiagen).

2.7. Immunofluorescence analysis

This protocol was followed as previously described (DeFalco et al., 2011). Briefly, cultured cells or frozen-tissue sections (12 μm) were directly fixed in 4% (w/v) paraformaldehyde overnight at 4 °C. After several washes in PBS + 0.1% Triton X-100 (PBTx), samples were

incubated in blocking solution (PBTx + 10% FBS + 10% BSA) for 1–2 hours at room temperature or overnight at 4 °C. Rabbit antibodies against p-FRS2 (1:200) (R & D system, cat# AF5126) and p-Smad1/5 (1:50) (Cell Signaling Technology, cat# 9516P) were diluted in blocking solution and applied to samples overnight at 4 °C. Fluorescent secondary antibodies were applied for 1 hour at room temperature. Cy3-conjugated secondary antibody (Jackson Immuno Research, 1:1000) was used for immunofluorescence. DAPI (Sigma-Aldrich) was used to stain nuclei. The samples were visualized by an inverted microscope (NIKON TE 2000U) with an optigrid structured light confocal system (Phylum) that has an all-motorized Z-focus device to capture high quality confocal images.

2.8. Analysis of cell proliferation

Cell proliferation was assessed by BrdU incorporation studies as previously described by us (Chung and Das, 2011). For studies with bromodeoxyuridine (BrdU) incorporation into DNA, mice were injected with BrdU (50 mg/kg, intraperitoneal) 2 h before they were sacrificed. Paraformaldehyde-fixed frozen tissue sections were stained with rat anti-BrdU (1:250) (catalog no. ab6326; Abcam, Cambridge, MA) for immunofluorescence analysis. BrdU positive counting of cells (%) was made based on ImageJ program [available at http://imagej.nih.gov/ij (NIH, USA)] for the number of BrdU positive cells per total number of cells, as described previously (Schmuck et al., 2014). Each experiment was performed in triplicate per treatment group.

2.9. Western blotting

This procedure was conducted as previously described (Chung and Das, 2011). Rabbit anti-Fgf10 (1:250) (GeneTex, cat# GTX10647) and Rabbit ant-Bmp8a (1:250) (GeneTex, cat# GTX102453) were used.

2.10. Statistical analyses

Data are presented here as mean ± SEM from at least three independent experiments that are based on independent cell isolations from different mice. Each experiment was performed in triplicate per treatment group. Statistical analysis in Microsoft Excel 2010 software was carried out by one-way ANOVA and a two-tailed Student's *t* test. $P \leq 0.05$ was considered to be significant.

3. Results

3.1. Microarray analysis identifies uterine epithelial and stromal genes differentially regulated by E2

Previously, we established a primary uterine epithelial–stromal co-culture system in mice as an alternative model to explore molecular aspects of uterine biology, especially hormonal regulation of cell-specific proliferation via cell–cell communication between the epithelial and stromal compartments (Chung and Das, 2011). We collected three independent pools of epithelial and stromal cell layers that were treated with either vehicle (control) or E2 for 48 h, in order to examine the effect of E2 on genes for epithelial proliferation in these cell populations. Total RNAs were isolated and subjected to gene expression profiling utilizing the GeneChip® mouse gene 1.0 ST array (Affymetrix Inc.), which offers whole genome

transcript coverage. Our analysis revealed that a total of 475 genes (P value ≤0.05) were differently expressed in the epithelial and stromal compartments by E2 as compared to vehicle (Tables S1 and S2, respectively). In the epithelial compartment, 119 or 28 genes exhibited up- or down-regulation, respectively, whereas in the stromal compartment, 144 or 184 genes were increased or decreased by E2, respectively, suggesting that an alteration of gene expression occurs during E2-dependent regulation of epithelial cell proliferation. Furthermore, cluster analysis of the microarray data for differential expression indicated that the genes were indeed segregated into two categories, as judged by distinct molecular signatures (Fig. S1). Overall, these results suggest that the gene expression profiles of E2-treated epithelial and stromal cells in the co-culture system are markedly different than oil-treated cell populations.

3.2. Multiple signaling networks in epithelial and stromal cell layers are differentially modified by E2

To better understand the functions of differentially expressed genes, a functional enrichment analysis was carried out separately for up- and down-regulated genes in the epithelial (Tables S3 and S4, respectively) and stromal (Tables S5 and S6, respectively) compartments using the ToppFun program (Chen et al., 2009). A summarized version of the predominantly altered epithelial and stromal signaling networks is represented in graphical form (Fig. 1). Our analysis primarily categorized the altered genes in terms of “Gene Ontology (GO)” (biological process, molecular function, and cellular component) in addition to functional categories including: “mouse phenotype,” “domain,” “pathway,” “interaction,” “transcription factor binding site,” “coexpression,” “microRNA,” “domain,” “drug,” “gene family,” and “disease” (Tables S3–S6). Under the GO: biological process category, the alteration of up-regulated genes in epithelial cells consisted of the following major subcategories: response to organic substances (21 genes), cell proliferation (20 genes), tissue development (18 genes), elevation of cytosolic calcium ion concentration (11 genes), and memory (5 genes) (Fig. 1 and Table S3). Conversely, down-regulated genes in epithelial cells largely revealed major sub-categories in locomotion (10 genes), regulation of multicellular organismal development (7 genes), and macrophage differentiation (3 genes) (Fig. 1 and Table S4). Under the GO: biological process category, the up-regulated genes in stromal cells were merely categorized into major subcategories that involved regulation of catalytic activity (24 genes), response to external stimulus (20 genes), blood circulation (11 genes), regulation of nucleotide biosynthesis and metabolic process (11 genes), and regulation of cell adhesion (9 genes) (Fig. 1 and Table S5). Major sub-categories for down-regulated genes in stromal cells were: regulation of multicellular organismal process (39 genes), cell proliferation (37 genes), response to wounding (35 genes), defense response (33 genes), response to external stimulus (30 genes), regulation of cell communication (29 genes), regulation of localization (29 genes), and circulatory system development (23 genes) (Fig. 1 and Table S6). Overall, it is interesting to note that cell proliferation genes are up-regulated in epithelial cells but down-regulated in stromal cells under the direction of E2 treatment, which is consistent with the fact that E2 promotes uterine cell proliferation (Dey et al., 2004; Huet-Hudson et al., 1989).

For the GO: molecular function category, down-regulated genes in epithelial cells primarily included receptor binding (7 genes), cytokine activity (4 genes), cytokine receptor binding (4 genes), and fibronectin binding (2 genes) (Fig. 1 and Table S3), while up-regulated genes represented only receptor binding (4 genes) (Fig. 1 and Table S4). Under the GO: molecular function category, up-regulated genes in stromal cells predominantly displayed receptor binding (8 genes), hormone binding (5 genes), peptide hormone binding (5 genes), and protein hormone binding activity (3 genes) (Fig. 1 and Table S5), while down-regulated genes mostly consisted of receptor binding (32 genes), cytokine activity (14 genes), endopeptide activity (13 genes), cytokine receptor binding (12 genes), metallopeptidase activity (10 genes), growth factor activity (9 genes), growth factor receptor binding (7 gene), chemokine activity (7 genes), and chemokine receptor binding (7 genes) (Fig. 1 and Table S6). Overall, those analyses suggest that multiple molecular signaling networks are affected in response to E2.

Under the GO: cellular component category, both up- and down-regulated genes predominantly included “extracellular region of proteins” as a major sub-category, with corresponding alteration of 16 up- or 13 down-regulated genes in epithelial cells and 25 up- or 48 down-regulated genes in stromal cells (Fig. 1 and Tables S3–S6). Overall, these results suggest that an increased number of extracellular gene products are largely targeted by both the epithelial and stromal cell compartments under the direction of E2, presumably to support an effective cell–cell paracrine communication during the progression of cell proliferation.

3.3. RT-PCR analysis validates microarray data

In order to validate the microarray results discussed earlier, we analyzed gene expression between three independent pools of RNAs for epithelial and stromal co-cultured samples after treatment of vehicle (control) or E2 for 48 h. Randomly selected genes from the up- or down-regulated gene lists for epithelial (Table S1) and stromal cells (Table S2) were analyzed by quantitative real time-PCR, as described in Section 2. The primers used for real time-PCR are indicated in Table S7. Consistent with our microarray data, the expression of *S100g* and *Greb1* was up-regulated, whereas that of *Gabrp*, *Mmp9*, *Cyp1b1*, and *Epha4* was down-regulated in epithelial cells by E2 as compared to control (Fig. 2A). Also, the stromal expression of *Hsd11b2*, *Spon2*, *Bmp8a*, and *Fgf10* was up-regulated, whereas that of *Mmp13* and *Hgf* was down-regulated by E2 as compared to control (Fig. 2B). These results suggest that our experimental strategy was able to selectively identify differentially expressed genes in the isolated uterine epithelial and stromal cells in the co-culture system during E2-induced epithelial cell proliferation.

3.4. Genes are appropriately up- or down-regulated by E2 in the ovariectomized uterus

To further evaluate whether E2-dependent genes are up- or down-regulated appropriately in the uterus, gene expression was analyzed by quantitative real time-PCR and *in situ* hybridization using ovariectomized mice following an injection of oil (vehicle/control) or E2 for 24 h. Consistent with our findings from microarray analysis in the co-culture system, our analysis revealed that E2 was able to up-regulate epithelial (*S100g* and *Greb1*) (Fig. 3A) and stromal (*Hsd11b2*, *Spon2*, *Bmp8a*, and *Fgf10*) (Fig. 4A) genes, as well as down-regulate

other epithelial (*Gabrp*, *Mmp9*, *Epha4*, and *Cyp1b1*) (Fig. 3A) and stromal genes (*Mmp13* and *Hgf*) (Fig. 4A) as compared to oil in the ovariectomized mouse uterus. Moreover, uterine cell-specific gene expression analyses also showed that epithelial up-regulated genes (*S100g* and *Greb1*) are primarily revealed with increased abundance of mRNAs in the epithelial cells by E2, as compared to oil in ovariectomized mice (Fig. 3B). In contrast, down-regulated genes (*Gabrp*, *Mmp9*, *Epha4*, and *Cyp1b1*) were predominantly exhibited in uterine epithelial cells by oil, although *Mmp9* and *Cyp1b1* also had expression in stroma and circular muscle cells (Fig. 3B). Interestingly, an injection of E2 caused downregulation of expression for these genes (Fig. 3B). We noted a differential upregulation by E2 in the uterus for cell-specific expression of stromal up-regulated genes (*Hsd11b2*, *Spon2*, *Bmp8a*, and *Fgf10*) (Fig. 4B). More specifically, the expression of *Hsd11b2*, *Spon2*, and *Fgf10* was strongly induced in endometrial stromal cells by E2, as compared to control. The expression of *Bmp8a* was weakly induced both in stromal and epithelial cells (Fig. 4B). Meanwhile, the expression of stromal downregulated genes (*Mmp13* and *Hgf*) was primarily high throughout the endometrial stroma, epithelial, and circular muscle cells by oil, but had strong repression by E2 in these cells (Fig. 4B). Overall, these data suggest that E2-dependent genes are appropriately controlled in the ovariectomized uteri.

3.5. Suppression of *Bmp8a* and/or *Fgf10* by siRNA leads to significant inhibition of E2-induced epithelial cell proliferation in the co-culture system

It is widely known that proliferation of uterine epithelial cells is regulated by estrogen through stromal cell mediated paracrine factors (Cooke et al., 1997). Based on our observations with the epithelial–stromal co-culture system, we selected five genes (*Bmp8a*, *Bmp8b*, *Figf*, *Gdnf*, and *Fgf10*) that exhibit stromal upregulation by E2 and possess growth regulatory potentials in different cell-types (Abud et al., 1996; Goldfarb, 1996; Guillou et al., 2011; Hogan, 1996; Orlandini et al., 1996; Reddi and Reddi, 2009; Shimasaki et al., 2004; Tee et al., 2010). To determine their roles in the co-culture system, we next wanted to examine whether gene-specific perturbation of these factors in the stromal layer affects E2-induced epithelial cell proliferation. Our analysis revealed that the suppression of *Bmp8a* or *Fgf10* mRNAs with siRNAs effectively caused a reduction of expression by 65% and 90%, respectively (Fig. 5A). This was also consistent with the analysis of protein levels for Bmp8a or Fgf10 (Fig, 5B). Interestingly, those suppression effects for *Bmp8a* or *Fgf10* either independently (Fig. 5C and D, respectively) or in combination (Fig. 5E) were detrimental to E2-induced epithelial cell proliferation, without affecting stromal cell status in the co-culture system, as determined by BrdU incorporation studies. However, the suppression for *Bmp8b*, *Gdnf*, or *Figf* did not affect the E2-induced epithelial cell proliferation (data not shown). Overall, these results suggest that both *Fgf10* and *Bmp8a* may potentially serve as paracrine mediators for the control of stroma-driven epithelial cell proliferation under the direction of E2.

3.6. Expressional analyses of Bmp8a and Fgf10 receptors reveal E2's regulatory role in uterine epithelial cells

In order to further define the paracrine interactions discussed earlier, we evaluated the expression of Bmp8a and Fgf10 receptors in the co-cultured cells (Fig. 6A, B), as well as in the ovariectomized uterus (Fig. 6C) after E2 or control. Bmp8a exerts its signal primarily via

type 1 and type 2 transmembrane serine/ threonine kinase receptors, such as Bmpr1a (Alk3), Bmpr1b (Alk6), and Bmpr2 (T-Alk) (Liu et al., 1995; Sieber et al., 2009; Suzuki et al., 1994), while Fgf10 mediates its cellular responses by binding to and activating high affinity receptors in a family of four receptor tyro-sine kinases (RTKs), as designated by Fgfr1-4 (Givol and Yayon, 1992; Jaye et al., 1992). Expression analysis of the receptors discussed earlier by quantitative real time-PCR revealed that the epithelial expression of *Bmpr1a*, *Bmpr1b*, and *Fgfr1* was up-regulated, while that of *Bmpr2* was downregulated by E2 over control (Fig. 6A). However, stromal expression of *Bmpr1a*, *Bmpr1b*, *Bmpr2*, *Fgfr3*, and *Fgfr4* was down-regulated by E2 over control (Fig. 6B). In contrast, *Fgfr2*, *Fgfr3*, and *Fgfr4* in epithelial cells, and *Fgfr1* and *Fgfr2* in stromal cells did not reveal any significant change (Fig. 6A, B). The cell-specific expression of those receptors was also examined by *in situ* hybridization studies in the uteri of ovariectomized mice after E2 or control. Our analysis revealed that *Bmpr1a* and *Bmpr1b* were primarily upregulated in epithelial cells by E2, as compared to oil, although *Bmpr1a* expression was also found in the circular muscle in both treatment groups (Fig. 6C). In contrast, *Bmpr2* was detected primarily in epithelial and stromal cells, and low levels were found in the circular muscle for both oil and E2 treatments (Fig. 6C). In case of *Fgfr2*, expression was predominant in the epithelial and circular muscle for both oil and E2 groups (Fig. 6C). In contrast, other genes (*Fgfr1*, *Fgfr3*, and *Fgfr4*) did not exhibit any signal in endometrial cells in either group (data not shown). Overall, these results suggest that appropriate regulation of receptors for uterine epithelial signaling mediated by stroma-derived Bmp8a and Fgf10 is feasible under the direction of E2.

3.7. Stromal-derived Fgf10 and Bmp8a essentially controls activation of receptor-mediated signaling events in epithelial cells under the direction of E2

Since stromal-derived Fgf10 and Bmp8a critically control E2-driven epithelial proliferation, and because their receptors exhibit in the epithelial compartment, we wanted to specifically analyze whether these stromal factors can elicit activation of receptor-mediated signaling in epithelial cells under the direction of E2. Previous studies showed that Fgf-mediated stimulation of Fgfrs prompts phosphorylation to specific tyrosine residues in a critical docking protein, Fgfr substrate2 (Frs2), which is necessary for the proper assembly of a distinct multi-protein complex leading to the activation of an intracellular signaling cascade for cell proliferation (Eswarakumar et al., 2005). Additionally, it is known that Bmp-mediated stimulation of type 2 receptors can induce phosphorylation of type 1 receptors, which in turn can phosphorylate Smads (Smad1/ 5/8) and induce heteromeric complex formation with Smad4 for translocation to the nucleus for gene transcription (Adelman et al., 2002; Attisano and Wrana, 2002; Massague et al., 2005; Sieber et al., 2009). Thus, in our initial analysis we monitored the phosphorylation status of Frs2 (p-Frs2) and Smad1/5 (p-Smad1/5) under the condition of E2-dependent proliferation. Indeed, our analysis showed upregulation of p-Frs2 or p-Smad1/5 primarily in co-culture system epithelial cells by E2 as compared to control (Fig. 7A, B, respectively). Strikingly, the suppression of stromal *Fgf10* or *Bmp8a* by siRNAs caused abrogation of both p-Frs2 and p-Smad1/5 signals in epithelial cells (Fig. 7A, B, respectively), suggesting that these signals may be linked to E2-induced epithelial cell proliferation in the uterus. Furthermore, it is interesting to note that these activated signals were also predominantly detected in uterine epithelial cells by E2 in the

ovariectomized mice (Fig. 7C), which is consistent with the analysis of protein levels for Fgf10 and Bmp8a by E2 in the uteri of ovariectomized mice (Fig. 7D), suggesting that such activation signaling events are indeed operational under the direction of E2 in both *in vitro* and *in vivo* conditions. Collectively, these results suggest that stromal-derived Fgf10 or Bmp8a predominantly directs stromal–epithelial communication for the control of epithelial cell proliferation via activation of receptor signaling events in the epithelial cells by E2.

4. Discussion

Our previously established primary uterine epithelial–stromal co-culture system (Chung and Das, 2011), in which epithelial cells display cell-specific proliferation under the direction of E2 (Dey et al., 2004; Huet-Hudson et al., 1989), led us to utilize microarray studies and bioinformatics analyses to further examine the effects of E2. We observed that genes involved in “cell proliferation” are specifically up-regulated in epithelial cells but down-regulated in stromal cells following E2 treatment. Furthermore, we found that up-regulated genes associated with alterations of “extracellular region of proteins” and “receptor binding” are specifically detected in both stromal and epithelial compartments, lending support to the paracrine/autocrine signaling mechanism upon binding to the appropriate receptors under E2. In this regard, it has been widely viewed that E2-dependent uterine epithelial cell proliferation is essentially controlled by stroma-mediated paracrine mediators (Chung and Das, 2011; Cooke et al., 1997; Cunha and Lung, 1979), although the specific potential factors in this regulation remain unknown. Our identification of several extracellular factors in the stromal compartment is likely to provide a major clue in elucidating the role of these unknown paracrine mediator(s). Among these factors, we further evaluated the roles of Bmp8a and Fgf10 for E2-dependent control of epithelial cell proliferation and the activation of epithelial receptor signaling mechanisms. Based on our parallel *in vitro* and *in vivo* studies for cell-specific expression of ligand–receptor signaling components and the analysis of E2-mediated effects in the co-culture system following molecular suppression of signaling mediators, we provided strong evidence to suggest that stromal-derived factors Bmp8a and/or Fgf10 possess growth regulatory potentials for the control of E2-dependent epithelial cell proliferation in the uterus; other genes may also be involved in an autocrine manner. Our gene array found the potent mitogens IGF1 and Fgf2 (Hawsawi et al., 2013; Zhang et al., 2012) to be up-regulated by E2 in epithelial cells (Table S1). Moreover, several microarray studies (Hewitt et al., 2003; Wall et al., 2013; Watanabe et al., 2003) have suggested that IGF1 is upregulated in the uterus under the direction of E2 in mice. In this regard, it has been shown that IGF1 is induced in uterine epithelial cells of the ovariectomized mouse by E2 (Kapur et al., 1992), and that IGF1 can serve as a potential regulator in controlling uterine epithelial cell proliferation (Murphy and Ghahary, 1990; Winuthayanon et al., 2010; Zhu and Pollard, 2007). On the other hand, it has also been suggested that IGF-1 may originate from the circulatory system (Sato et al., 2002). Although the role of IGF-1 and Fgf2 in E2-dependent epithelial proliferation was not evaluated in our study, it would be interesting to examine in the future.

After E2 treatment we observed that both *Bmp8a* and *Bmp8b* are expressed in stromal cells in the co-culture system (Fig. 2), and *Bmp8a* is further found in stromal cells of the adult ovariectomized uteri (Fig. 4B). In contrast, the expression of their receptors (*Bmpr1a*,

Bmpr1b, and *Bmpr2*) is primarily detected in epithelial cells both in the co-culture and adult ovariectomized uteri following Fig. 6A, C. Collectively, these results suggest that Bmp-specific ligand/ receptor signaling is operational in the control of stromal–epithelial communication during E2-regulated uterine biology. Both *Bmp8a* and *Bmp8b* are members of a large family of extracellular polypeptide signaling molecules related to transforming growth factor β (TGF-β) (Hogan, 1996), and play a variety of roles in cell proliferation, differentiation, survival, and fate determination (Dudley et al., 1995; Winnier et al., 1995; Zhang and Bradley, 1996; Zhao et al., 1996, 1998). *Bmp8A* and *Bmp8B* genes are organized closely on chromosome 4 and have high levels of sequence identity, especially in the pro-region. Although they share a similar expression pattern in different reproductive organs (Hogan, 1996), they also possess differential expression in the post-implantation uterus (Zhao and Hogan, 1996): *Bmp8a* is exclusively expressed in trophoblast cells during the progression of proliferation and differentiation in the placenta, while *Bmp8b* is robustly expressed during decidualization, a process that requires extensive stromal cell proliferation and differentiation (Zhao and Hogan, 1996).

Previous studies have shown that Fgf signaling ligand/receptor activation primarily controls epithelial cell proliferation, differentiation, and migration through regulation of epithelia/ mesenchymal interactions in many tissues, including the uterus, lung, mammary gland, prostate, and kidney (Abler et al., 2009; Celli et al., 1998; De Moerlooze et al., 2000; Jackson et al., 1997; Li et al., 2011; Steinberg et al., 2005). In this regard, it has been shown that *Fgfr2* is primarily expressed in epithelia and its signaling activation is predominantly controlled by Fgfs 1, 3, 7, and 10, which are mainly produced by the mesenchyme (Bellusci et al., 1997; Finch et al., 1995; Zhang et al., 2006). Recent studies by RT-PCR have shown that the expression of several *Fgf* ligands (*Fgf2, Fgf9*, and *Fgf18*) is increased in the estrogenized uteri without showing any cell-specific localization, while the localization of their receptors (*Fgfr1* and *Fgfr2*) is primarily observed in uterine epithelial cells, leading to the suggestion that they may participate in the control of epithelial/stromal interactions during the progression of epithelial cell proliferation (Li et al., 2011). However, in the present study, we observed that *Fgf10* is specifically induced in uterine stromal cells by E2 treatment (Figs 2B and 4B), while its receptor *Fgfr2* was primarily detected in epithelial cells, in both the co-culture system and the adult ovariectomized uteri (Fig. 6A and C). Overall, these results suggest that Fgf10/ Fgfr2 signaling may be specifically involved in the control of stromal–epithelial communication during E2-induced epithelial cell proliferation in the uterus.

Utilizing the direct molecular suppression approach with specific siRNAs for stromal *Bmp8a* and/or *Fgf10* expression within the co-culture system under E2 treatment (Fig. 5), our observations strongly suggest that both Bmp8a- and Fgf10-mediated signaling are essentially involved in either an independent or mutually interactive fashion for E2-regulated uterine proliferative response. In this vein, estrogen might stimulate multiple signaling components within the stroma to control E2-dependent regulation of uterine epithelial cell proliferation. It is worth mentioning that the targeted mutation of *Bmp8a* in mice does not show any defects in terms of female fertility (Zhao et al., 1998), while the loss of *Fgf10* in mice causes perinatal lethality, primarily due to the lack of lung development

(Min et al., 1998). While it remains unknown what effects the conditional deletion of *Fgf10* has on mouse uteri, uterine conditional deletion of *Fgfr2* by progesterone receptor-Cre has recently been reported in mice (Filant et al., 2014). Collectively, these results suggest that *Fgfr2* is critically important in postnatal uterine development specifically in terms of luminal epithelial cell stratification and in female fertility. On the same note, homozygous deletion of *Bmpr1a* (Mishina et al., 1995) or *Bmpr2* (Beppu et al., 2000) in mice is embryonically lethal, while conditional deletion of *Bmpr1a* by *Amhr2*-Cre (Edson et al., 2010) or homozygous inactivation of *Bmpr1b* (Yi et al., 2001) results in female sterility with an implication in ovarian follicular development, although the roles of these receptors in uterine estrogenic function remain unknown.

Earlier it had been shown that Fgf ligand/receptor signaling activation of phosphorylated Frs2 essentially controls cellular proliferation (Eswarakumar et al., 2005; Li et al., 2011). Additionally, phosphorylated Smad1/5 has been shown to control Bmp-mediated signaling and gene transcriptional regulation (Adelman et al., 2002; Attisano and Wrana, 2002; Massague et al., 2005; Sieber et al., 2009). Interestingly, we observed that both pFrs2 and pSmad1/5 are upregulated by E2 in uterine epithelial cells not only in the co-culture system but also in the ovariectomized mice (Fig. 7), suggesting that Bmp- and Fgf-mediated signaling plays a role in E2-dependent uterine epithelial cell function. Most importantly, the disappearance of the earlier phosphorylation signals following the suppression of stromal *Bmp8a/Fgf10* under E2 in the co-culture system (Fig. 7A, B) strongly supports the notion that epithelial accumulation of Bmp/Fgf-mediated signals may be necessary for E2-dependent regulation of uterine epithelial cell proliferation. It is indeed interesting to examine whether Fgf10 and/or Bmp8a proteins promote uterine epithelial cell proliferation. Based on our observations with the siRNA approach, it is possible that the addition of these proteins could enhance uterine epithelial cell proliferation both *in vivo* and *in vitro*. However, that is not within the scope of this paper and should be pursued in the future.

In conclusion, our study showed for the first time that Bmp8a and Fgf10 may be considered stroma-derived paracrine factors that are specifically involved in the regulation of E2-induced epithelial cell proliferation in the mouse uterus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2014.11.002.

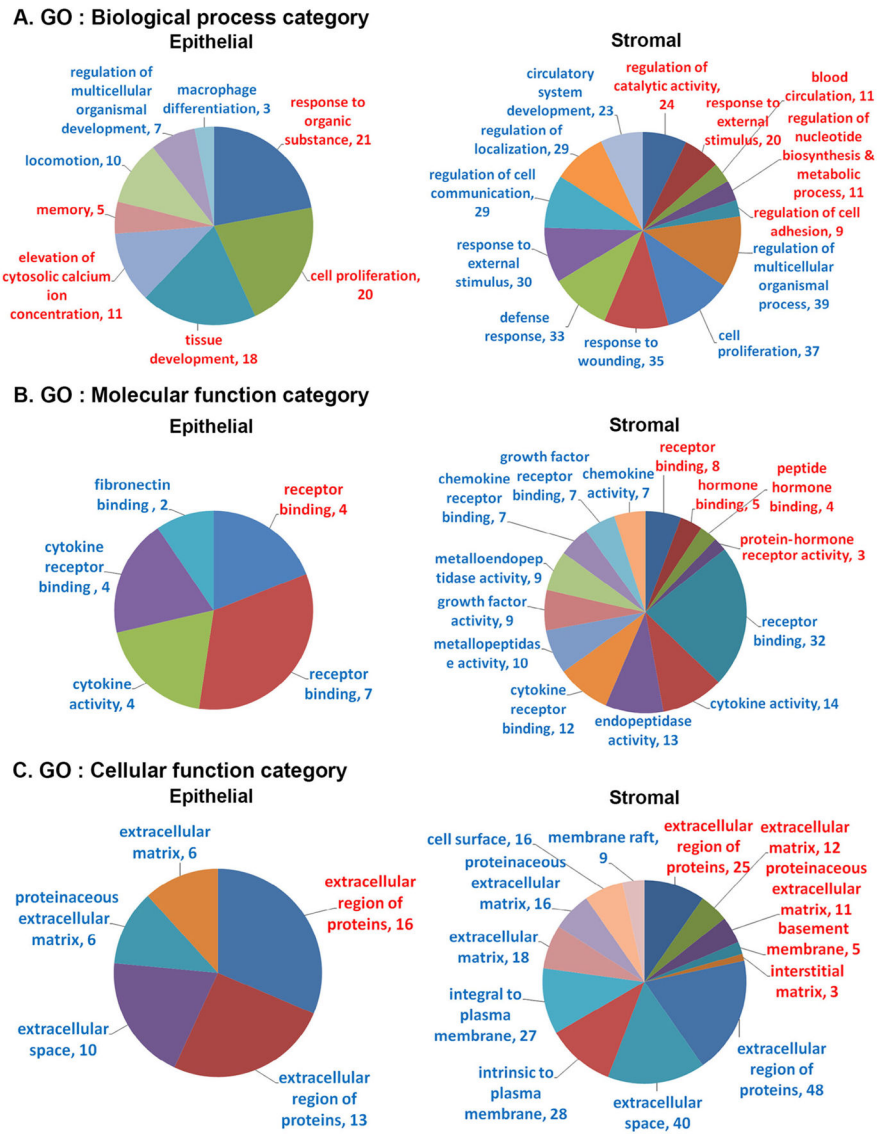

Fig. 1.

Functional categorization of differentially expressed genes in epithelial and stromal cell compartments. ToppFun analyses for the up (in red)- or down (in blue)-regulated genes in the epithelial and stromal cell compartments are shown on the basis of gene ontology (GO) categories: biological process (A), molecular function (B), and cellular function (C). Total number of genes modulated in each sub-category is indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

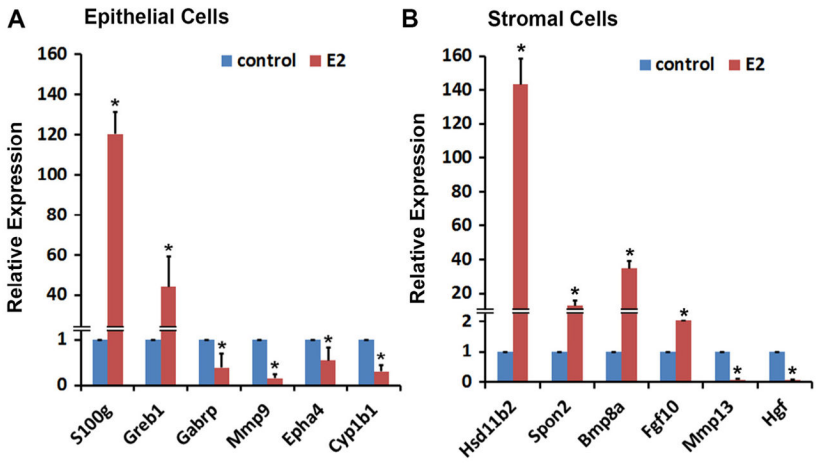

Fig. 2.

Analysis of expression for differentially modulated genes in epithelial (A) and stromal (B) compartments. Cells were analyzed in a co-culture system following treatment with either E2 or vehicle (control) for 48 h. Based on the microarray data, randomly selected up- or down-regulated genes were analyzed by quantitative real time-PCR as described in Section 2. Total RNA was extracted from three independent cell preparations for each group, and then subjected to real time-PCR. The relative target gene expression was quantified by the ΔΔCt method, using *Rpl7* for normalization. Standard errors are represented with bars. *, Significantly different ($p < 0.05$) against the control group. These experiments were repeated at least 2–3 times with similar results.

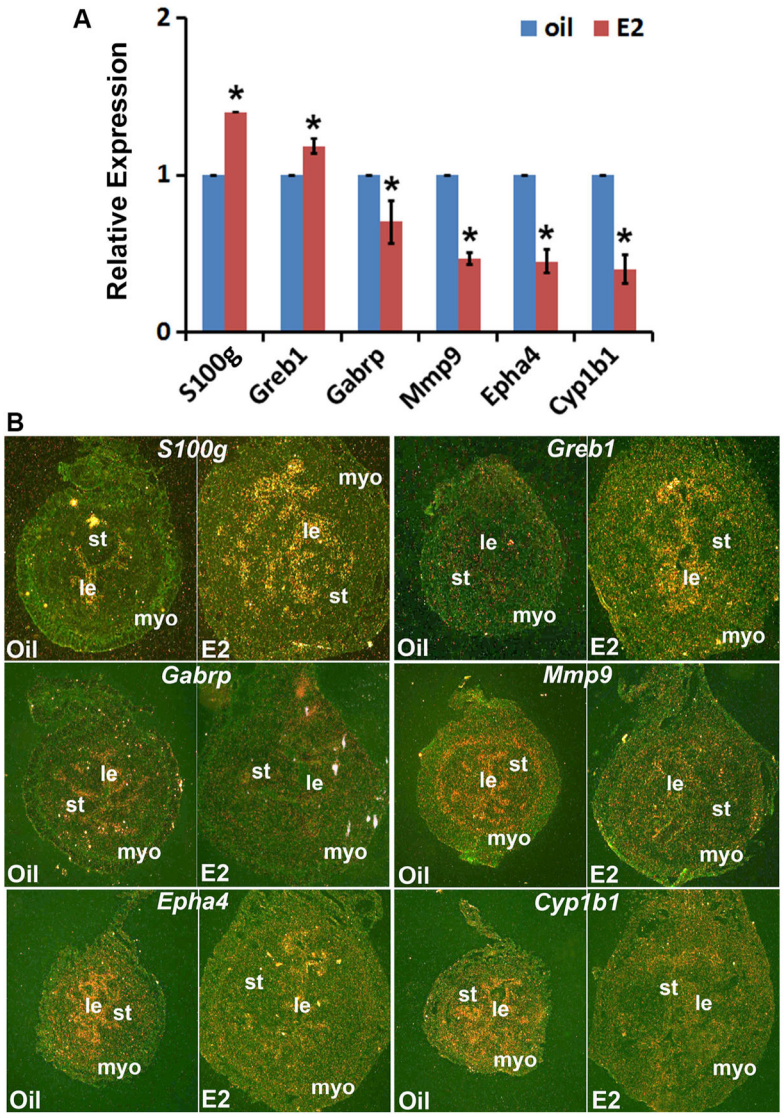

Fig. 3.

Analyses of uterine gene expression for epithelial-regulated genes in ovariectomized mice. Mice were injected with E2 or oil (as vehicle) for 24 h. Quantitative real time-PCR (A) and *in situ* hybridization (B) data are shown for select up- (*S100g* and *Greb1*) or down-regulated (*Gabrp*, *Mmp9*, *Epha4*, and *Cyp1b1*) genes. Relative target gene expression was quantified by the ΔΔCt method, using *Rpl7* for normalization. Standard errors are represented with bars. *, Significantly different ($p < 0.05$) between the groups. For *in situ* hybridization studies, frozen sections were hybridized with ^{35}S-labeled antisense or sense riboprobes, and RNase A resistant hybrids were detected by autoradiography. Sections were post-stained with hematoxylin and eosin. Dark-field photomicrographs of representative cross-sections hybridized with antisense probes are shown at 40×. Sections hybridized with corresponding sense probes did not show any positive signals (data not shown). le, luminal epithelium; s, stroma; myo, myometrium. These experiments were repeated at least 3 times with similar results.

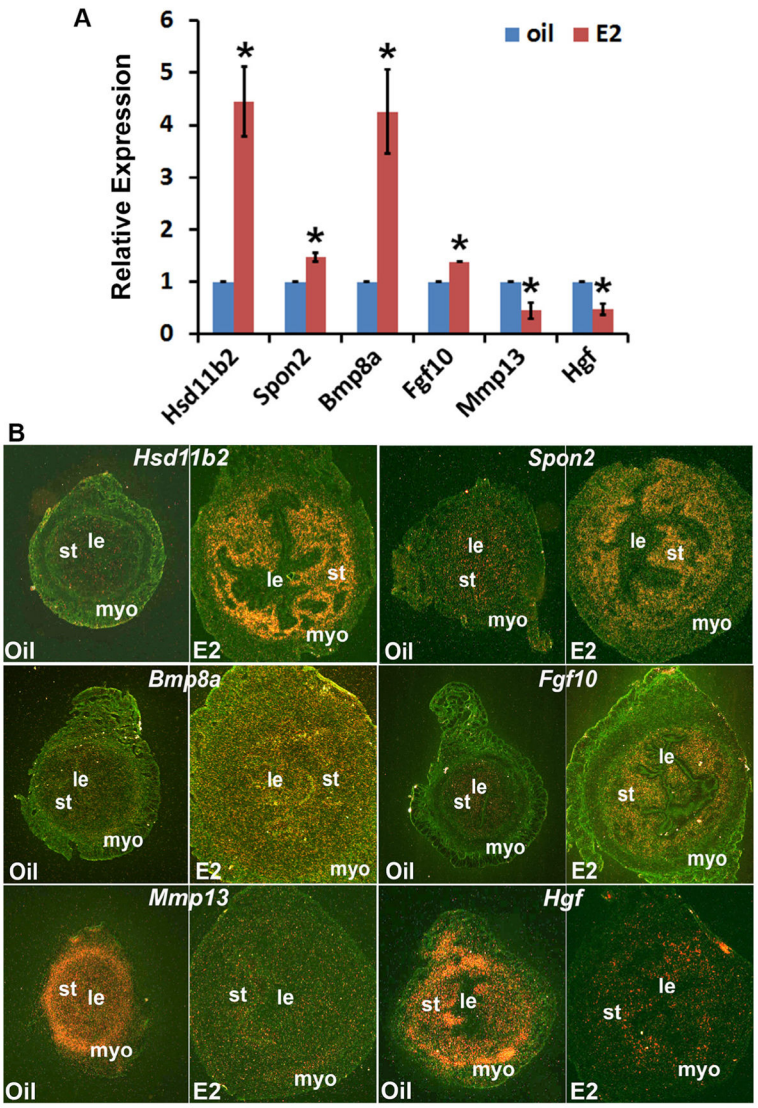

Fig. 4.

Analyses of uterine gene expression for stromal-regulated genes in the ovariectomized mice. Mice were injected with E2 or oil (as vehicle) for 24 h. Quantitative real time-PCR (A) and *in situ* hybridization (B) data are shown for select up- (*Hsd11b2*, *Spon2*, *Bmp8a*, and *Fgf10*) or down-regulated (*Mmp13* and *Hgf*) genes. Relative target gene expression was quantified by the ΔΔCt method, using *Rpl7* for normalization. Standard errors are represented with bars. *, Significantly different ($p < 0.05$) between the groups. For *in situ* hybridization studies, frozen sections were hybridized with ^{35}S-labeled antisense or sense riboprobes, and RNase A resistant hybrids were detected by autoradiography. Sections were post-stained with hematoxylin and eosin. Dark-field photomicrographs of representative cross-sections hybridized with antisense probes are shown at 40×. Sections hybridized with corresponding sense probes did not show any positive signals (data not shown). le, luminal epithelium; s, stroma; myo, myometrium. These experiments were repeated at least 3 times with similar results.

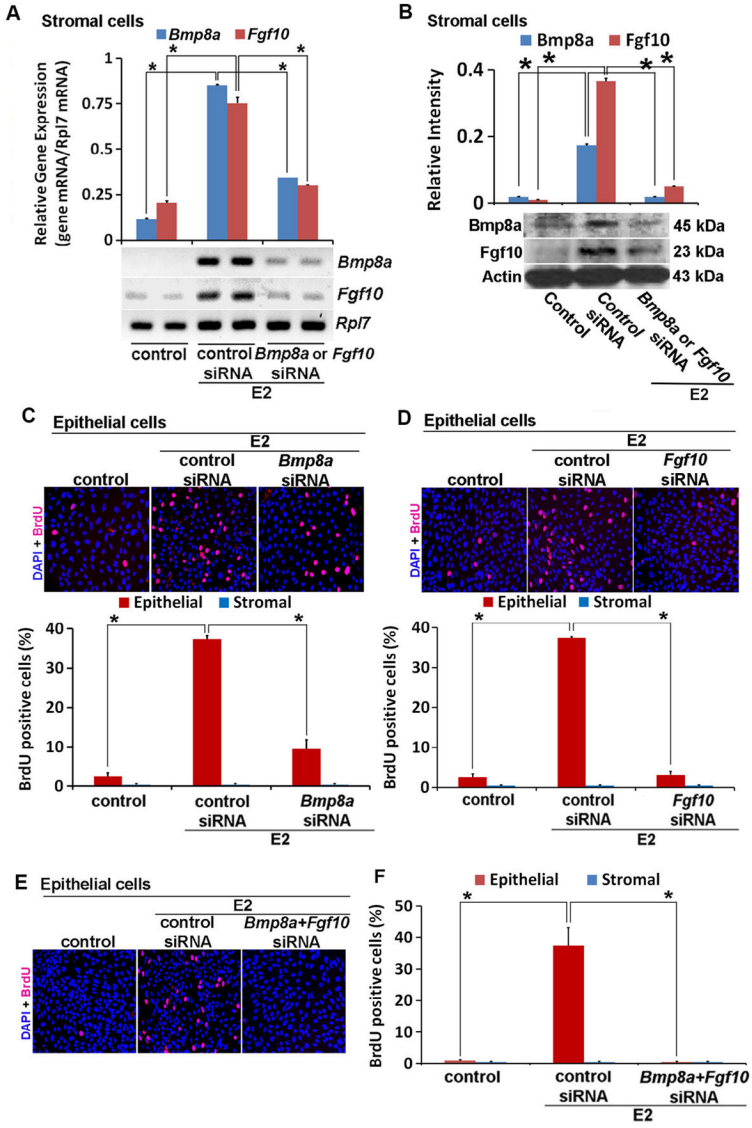

Fig. 5.

Effects of stromal-specific suppression of *Bmp8a* and/or *Fgf10* on epithelial proliferation in a co-culture system following treatment with either E2 or vehicle for 48 h. Stromal expression status for Bmp8a or Fgf10 at the mRNA (A) and protein (B) levels, following siRNA-mediated suppression, were confirmed by comparative RT-PCR and western blotting, respectively. For quantitative analyses, relative levels of gene expression were obtained after correction with *Rpl7* or actin. *, Significantly different ($p < 0.05$) between the groups. Confocal analysis of epithelial cell proliferation by incorporation of BrdU (red), following the stromal suppression of *Bmp8a* (C) or *Fgf10* (D) individually, or in combination (E) by siRNA-approach. DAPI (blue) was used to stain all nuclei. Quantitative analysis of cellular proliferation is presented based on counting of at least 1000 cells from each group. *, Significantly different ($p < 0.05$) between the groups. These experiments were repeated at least 3 times with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

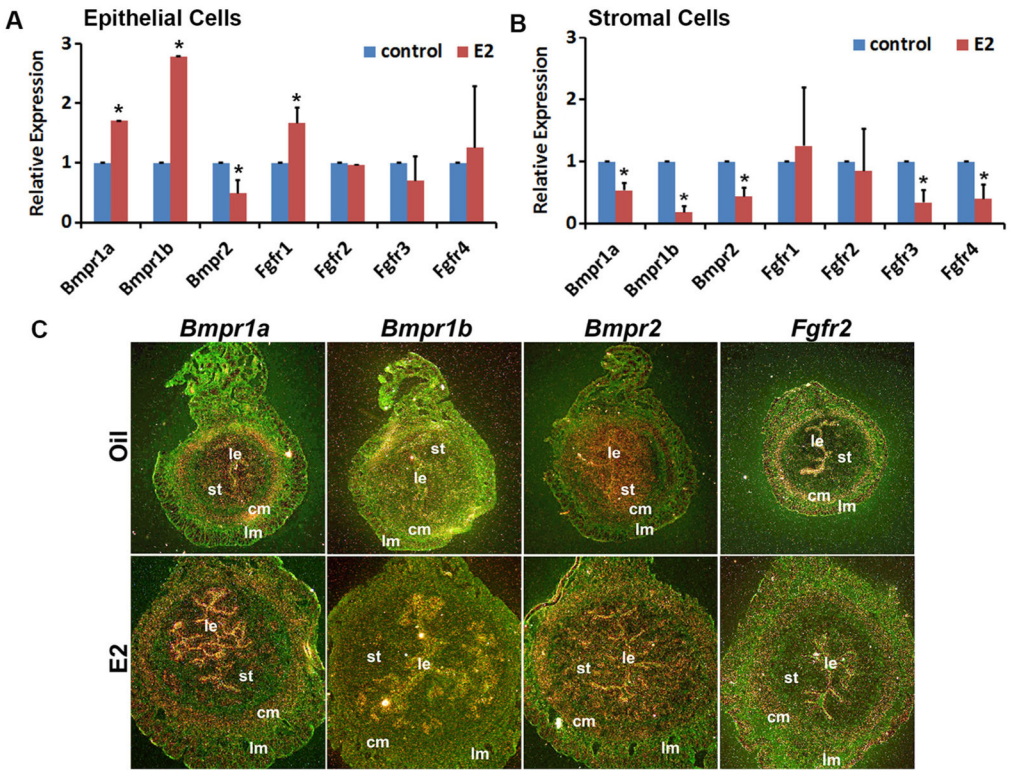

Fig. 6.

Analysis of receptor expression for Bmp and Fgf ligands. Expression in epithelial cells (A) or stromal cells (B) was analyzed by quantitative real time-PCR in a co-culture system following treatment with either E2 or vehicle (control) for 48 h. The relative target gene expression was quantified by the ΔΔCt method, using *Rpl7* for normalization. Standard errors are represented with bars. *, Significantly different ($p < 0.05$) between the groups. (C) *In situ* hybridization for gene expression on uterine sections following injections of E2 or oil (as vehicle) for 24 h in ovariectomized mice. le, luminal epithelium; s, stroma; cm, circular muscle; lm, longitudinal muscle. These experiments were repeated at least 3 times with similar results.

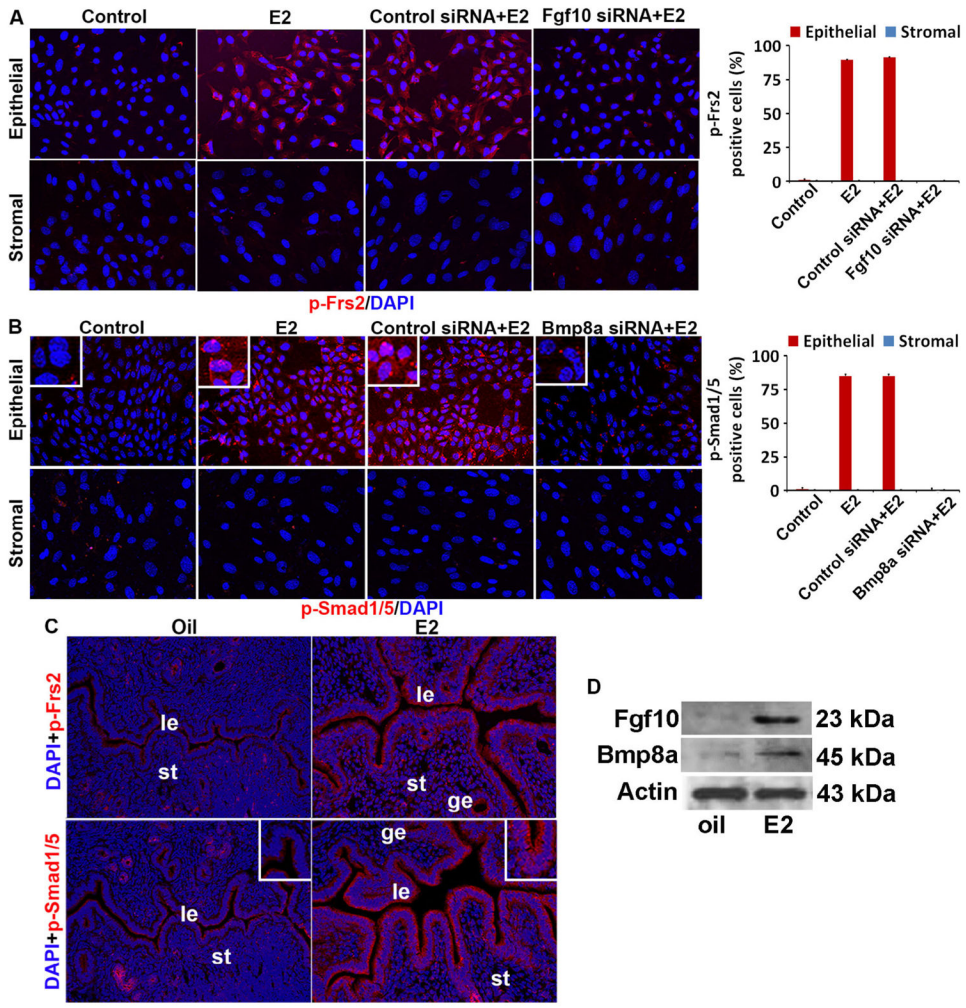

Fig. 7.

Functional analyses of the stroma-derived regulators Bmp8a or Fgf10 in control of uterine cell-specific receptor signaling by E2. Confocal analyses of expression for pFrs2 (A) or p-Smad1/5 (B) in epithelial and stromal cells in the co-culture system. Co-cultured cells were treated with vehicle (control) or E2 for 48 h. In addition, E2-treated cells were also subjected to siRNA-mediated transfection for control (A, B), *Fgf10* (A), or *Bmp8a* (B) 6 h prior to the initiation of hormone treatment. (C) Ovariectomized mice: Confocal analyses of expression for p-FRS2 or p-Smad1/5 on uterine sections of mice following the injections of E2 or oil (as vehicle) for 24 h. le, luminal epithelium; s, stroma; myo, myometrium. Note: Insets in (B) and (C) are shown at high magnification to indicate nuclear localization of p-Smad1/5 after E2 treatment. (D) Western blot analysis of protein expression for Bmp8a or Fgf10 in uterine extracts after oil (control) or E2 for 24 h. These experiments were repeated at least 3 times with similar results.