Stimulating the GPR30 Estrogen Receptor with a Novel Tamoxifen Analogue Activates SF-1 and Promotes Endometrial Cell Proliferation

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Introduction

Estrogens and selective estrogen receptor (ER) modulators such as tamoxifen are known to increase uterine cell proliferation. Mounting evidence suggests that estrogen signaling is mediated not only by ERα and ERβ nuclear receptors, but also by GPR30 (GPER, a seven transmembrane (7TM) receptor). Here, we report that primary human endometrial H-38 cells express high levels of GPR30 with no detectable ERα or ERβ. Using a novel tamoxifen analogue, STX, which activates GPR30 but not ERs, significant stimulation of the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways was observed in H-38 cells and in Ishikawa endometrial cancer cells expressing GPR30; a similar effect was observed in JEG3 choriocarcinoma cells. STX treatment also increased cellular pools of phosphatidylinositol (3,4,5) triphosphate, a proposed ligand for the nuclear hormone receptor SF-1 (NR5A1). Consistent with these findings, STX, tamoxifen, and the phytoestrogen genistein were able to increase SF-1 transcription, promote Ishikawa cell proliferation, and induce the SF-1 target gene aromatase in a GPR30-dependent manner. Our findings suggest a novel signaling paradigm that is initiated by estrogen activation of the 7TM receptor GPR30, with signal transduction cascades (PI3K and MAPK) converging on transcriptional output. We propose that this novel GPR30/SF-1 pathway increases local concentrations of estrogen, and together with classic ER signaling, mediate the proliferative effects of synthetic estrogens such as tamoxifen, in promoting endometriosis and endometrial cancers. [Cancer Res 2009;69(13):5415–23]

Abstract

Estrogens and selective estrogen receptor (ER) modulators such as tamoxifen are known to increase uterine cell proliferation. Mounting evidence suggests that estrogen signaling is mediated not only by ERα and ERβ nuclear receptors, but also by GPR30 (GPER, a seven transmembrane (7TM) receptor). Here, we report that primary human endometrial H-38 cells express high levels of GPR30 with no detectable ERα or ERβ. Using a novel tamoxifen analogue, STX, which activates GPR30 but not ERs, significant stimulation of the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways was observed in H-38 cells and in Ishikawa endometrial cancer cells expressing GPR30; a similar effect was observed in JEG3 choriocarcinoma cells. STX treatment also increased cellular pools of phosphatidylinositol (3,4,5) triphosphate, a proposed ligand for the nuclear hormone receptor SF-1 (NR5A1). Consistent with these findings, STX, tamoxifen, and the phytoestrogen genistein were able to increase SF-1 transcription, promote Ishikawa cell proliferation, and induce the SF-1 target gene aromatase in a GPR30-dependent manner. Our findings suggest a novel signaling paradigm that is initiated by estrogen activation of the 7TM receptor GPR30, with signal transduction cascades (PI3K and MAPK) converging on nuclear hormone receptors (SF-1/LRH-1) to modulate their transcriptional output. We propose that this novel GPR30/SF-1 pathway increases local concentrations of estrogen, and together with classic ER signaling, mediate the proliferative effects of synthetic estrogens such as tamoxifen, in promoting endometriosis and endometrial cancers. [Cancer Res 2009;69(13):5415–23]

Introduction

Biological effects of 17β-estradiol (17βE2) and other synthetic estrogens result from the activation and subsequent interaction of nuclear estrogen receptors (ER)α and ERβ with the genome. This classic or genomic mode of ER action is slow compared with rapid or nongenomic responses that can occur within minutes (1, 2). Several studies suggest that nongenomic responses to estrogen are mediated by two distinct mechanisms involving membrane ERs. The first invokes localization of nuclear ER at the plasma membrane (3–5), whereas the second incorporates the seven-transmembrane (7TM) receptor GPR30 (GPER; refs. 6, 7). Similar to ER, GPR30 is not only activated by a wide variety of estrogenic compounds including ER agonists, but also by partial antagonists such as tamoxifen (7–10). Although the potential contribution of GPR30 in breast cancer remains unclear (11), activation of GPR30 promotes proliferation of both uterine (12) and thyroid (9) cancer cells. Indeed, breast cancer cell lines including ER-positive (MCF-7) and ER-negative (SKBR3) lines strongly express GPR30 (7, 13). Moreover, a National Cancer Institute–sponsored survey of breast carcinomas reported that correlation between receptor expression and clinical outcomes are distinct for GPR30 and ER, with GPR30 expression strongly associated with HER2 expression and tumor progression (14).

Despite the lingering controversies surrounding the contribution of GPR30 to breast cancer, recent analyses of GPR30 knockout mouse firmly establish this 7TM receptor as an important player in mediating estrogen responses in vivo. Indeed, estrogen-induced thymic atrophy is severely compromised in GPR30 null mice (15). Other phenotypes, especially those involving reproduction, have yet to be reported in mice lacking GPR30. Regardless, the sum of these in vivo and in vitro data suggests strongly that physiologic responses initiated by natural and synthetic estrogens extend beyond the nucleus to include the 7TM receptor, GPR30.

Estrogenic ligand signaling through GPR30 is shown to have a number of rapid effects, including activation of the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways (7, 10, 16). Activation of these proliferative pathways by GPR30 is proposed to be propagated by trans-activation of the plasma membrane tyrosine kinase receptor epidermal growth factor (EGF) receptor (13). However, the fact that the GPR30-specific agonist G-1 (17) and EGF (18) stimulate proliferation in ER-negative SKBR3 breast cancer cells whereas E2 does not (18) suggests that GPR30 signaling may involve other mechanisms. Nonetheless, stimulation of MAPK and PI3K signaling pathways by GPR30 coincides with mechanisms proposed to activate NR5A nuclear receptors including steroidogenic factor 1 (SF-1) and liver receptor homologue 1 (LRH-1). Both of these transcription factors are important during development and in the adult due to their regulation of steroidogenesis and bile acid synthesis (19). Similar to other nuclear receptors, activation of the MAPK pathway and receptor phosphorylation by extracellular signal-regulated kinase (ERK) increases NR5A receptor activity (20). However, structural analyses of SF-1 and LRH-1 also revealed...
phosphatidylinositols including phosphatidylinositol-3,4,5-trisphosphate (PIP3) as a potentially unique class of ligands for this subfamily of nuclear receptors (21, 22).

Based on these collective findings, we hypothesized that GPR30 stimulation of PI3K- and MAPK pathways would increase the activity of SF-1/LRH-1. Unfortunately, distinguishing GPR30- from ER-specific effects is difficult in many cells. Therefore, synthetic compounds selective for GPR30, but not ERs, are useful tools for dissecting these signaling pathways. Two agonists proposed to activate membrane ERs include the GPR30-selective agonist G-1 (23), and the biphenylacrylamide compound STX that resembles 4-hydroxytamoxifen (OHT; ref. 24); no binding to nuclear ERs is exhibited by either compound. Although direct binding of STX to GPR30 has yet to be shown, primary hypothalamic neurons isolated from ERα and ERβ double knockout mice elicit a rapid and specific response to STX that mimics the response to a bovine serum albumin (BSA)-estradiol conjugate, suggesting that the biological response to STX is mediated through an estrogen membrane receptor such as GPR30 (25, 26).

To test the hypothesis that GPR30 mediates NR5A activation via estrogens, primary human endometriotic and immortalized uterine cancer cell lines were assayed with STX. Our results suggest a novel cellular signaling paradigm that is initiated by estrogen activation of the 7TM receptor GPR30, with signal transduction cascades (PI3K and MAPK) converging on nuclear hormone receptors (SF-1/LRH-1) to modulate their transcriptional output. Based on our findings, we speculate that such a pathway might contribute to the known proliferative effects of tamoxifen and other natural estrogens in the female reproductive tract.

**Materials and Methods**

**Plasmids and reagents.** Full-length human GPR30 (hGPR30) was isolated and expressed from a pIRESneo expression vector (Clontech Laboratories) harboring an NH2-terminal 3X-FLAG- or HA-epitope tag (FLAG-hGPR30/pIRESneo). Double-stranded RNAi oligonucleotides for GPR30, LRH-1, and GFP were cloned into the pSUPERneo vector (DNAengine). Full-length mouse SF-1 (mSF-1) was expressed from either HA or 3X FLAG tagged version in pCIneo or pcDNA3, respectively. 3X FLAG-mSF-1 S203A and A270W mutants were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Full-length human LRH-1 was obtained from human ductal carcinoma T47-D, and subcloned into a 3X-FLAG epitope-tagged pcDNA3 vector. ERs, and ERE TATA-Luc vectors, were generous gifts from S. Kato (University of Tokyo, Tokyo, Japan). Antibodies, chemicals, and oligonucleotides used are specified in Supplementary Tables S1 and S2.

**Cell culture and luciferase assays.** Ishikawa and JEG3 cells were maintained in DMEM/H-21, 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS), and 1X penicillin/streptomycin. Endometriotic stromal H-38 cells were maintained in DMEM high glucose 50%/F-12 50% mixed with 10% FBS and 1X penicillin/streptomycin. Ishikawa cells stably expressing HA-hGPR30 were grown in selective medium containing 300 μg/mL of geneticin (Invitrogen). Cells were seeded in phenol red–free medium with 5% charcoal-dextran–stripped (CDS) FBS and cotransfected in serum-free medium with indicated receptor or siRNA construct using FuGENE 6 (Roche). Drugs were added to cells 4 to 8 h before harvesting to achieve maximal stimulation. All luciferase assays were normalized to β-galactosidase activity and results expressed as relative luciferase units or fold activation with all data representing the mean ± SD from three independent experiments performed in triplicate.

**Proliferation studies.** For quantitative fluorescence-activated cell sorting (FACS) analysis, Ishikawa cells were cultured in phenol red–free

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**Figure 1.** Primary human ectopic H-38 endometrial cells express GPR30, SF-1, and CYP19A1, but no ERs. A, relative expression of GPR30 and nuclear ERs in primary H-38 endometrial cancer cells, and other cell lines including breast carcinoma cell lines: MCF-7, MDA-MB-231 (MDA), and T47-D; endometrial cancer cell line: Ishikawa; choriocarcinoma placental cell line: JEG3; primary endometrial cells: normal endometrium (Endo) and endometriotic cells (H-38). B, localization of endogenous GPR30 (calnexin, endoplasmic reticulum membrane marker) in H-38 cells, determined by immunocytochemistry and confocal microscopy. C, relative expression of SF-1 and CYP19A1 in T47-D, Ishikawa, normal endometrium, and H-38 cells.
medium containing 5% CDS FBS for 48 h. Cells were treated with drugs as indicated for 16 h, collected, permeabilized with 40% ethanol in PBS, and resuspended in staining solution (50 μM propidium iodide, 3.8 mM sodium citrate in PBS RNase solution). Cell-cycle data were collected with a Becton Dickinson FACScan Immunocytochemistry system and analyzed with ModFit LT (Verity Software House).

For cell proliferation assays, Ishikawa cells were seeded in six-well plates with phenol red–free medium containing 5% CDS FBS. Cells were washed and the medium changed to 1% CDS and drug, as indicated. Medium and drugs were renewed every 2 d. Cell number was counted in a hemocytometer.

**Immunocytochemistry and immunoblot analyses.** For phospho-Akt and phospho-ERK1/2 analyses, Ishikawa cells were transfected with hGPR30. For phospho-SF-1 analysis, JEG3 cells were transfected with 3X-FLAG-mSF-1. Following transfection, cells were serum starved in phenol red–free medium, except for endometriosis H-38 cells where 2% CDS FBS was added. Cells were treated with drugs as indicated, washed, and lysed in ice cold 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NaDeoxycholate, 1% Triton X-100, 0.1% SDS, Complete Protease Inhibitor Cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride, 1X Phosphatase Inhibitor Cocktail I, and II (Sigma-Aldrich). Equal amounts of total protein were probed with antibodies directed against phospho-Akt (Ser473; 1:1,000), Akt (1:1,000), phospho-ERK1/2 (1:1,000), ERK1/2 (1:1,000), pS6-1 (1:2,500), or M2-Flag (1:5,000). Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). For all Western blots, equivalent levels of protein were added to each lane, as determined by a Bradford protein assay.

H-38 cells were fixed in 4% paraformaldehyde before being permeabilized with PBS containing 0.1% Triton X-100. Cells were incubated with primary antibodies overnight at 4°C in PBS containing 5% BSA. Primary antibodies were as follows: anti-GPR30 (Lifespan Biosciences; 1:250) and anti-calnexin (Abcam; 1:250). Images were collected using a Zeiss Pascal confocal microscope.

**53P** orthophosphate in vivo labeling and high performance liquid chromatography analysis of PI3P. Ishikawa cells containing empty vector or stably overexpressing GPR30 were starved in serum- and phosphate-free medium for 2 h, were labeled with 500 μCi of [32P]-orthophosphate (Amersham Biosciences) for 2 h at 37°C in 5% CO2, and then treated with DMSO or drug for 15 min. Total lipids were extracted into chloroform, deacylated in 750 μL of methylamine/methanol/n-butanol/H2O (26:45:11:16) at 53°C for 50 min, and polar head groups extracted into 10 mM NH4H2PO4. Anion exchange high performance liquid chromatography was used to separate soluble glycerol-inositol head groups over a gradient of NH4H2PO4, and monitored using a Packard flow scintillation detector and compared with 32P-labeled standards (Amersham Biosciences, Sigma). All peaks were integrated using Pro-Star Varian Software, and PI(4)P levels or total radioactivity served as internal controls to quantify, both giving similar results.

**Results**

**Human ectopic endometrial cells express GPR30 and SF-1, but not ERs.** We initially examined GPR30 expression in primary human stromal endometriotic H-38 cells obtained from patients with endometriosis, because GPR30 is amplified in endometrial cells compared with normal endometrial cells due to elevation in transcripts encoding cytochrome p450 enzyme aromatase (Cyp19a1; ref. 29). Higher estrogen output by these cells is proposed to maintain endometrial cells in an ectopic location and contribute to endometrial cancer (30). Consistent with increased estrogen production in H-38 cells and the fact that Cyp19a1 is a known target of SF-1 (31), both Cyp19a1 and SF-1 transcripts are significantly elevated compared with primary endometrial cells (Fig. 1C).

To examine how H-38 endometriotic cells respond to estrogens in the absence of ERs, we used a variety of estrogens including STX and OHT. Although OHT is proposed to act as a partial estrogen agonist in the uterus (32, 33), STX is a novel diphenylacrylamide tamoxifen analogue (Fig. 2A) that has previously been linked to estrogen activation of a GPCR (25, 26). Indeed, gene profiling in Ishikawa cells suggests that STX activates a distinct subset of genes from the cluster of genes induced by both OHT and 17βE2 (Supplementary Fig. S1). Both OHT and STX induce rapid phosphorylation of Akt and ERK1/2 (Fig. 2B), showing that estrogen signaling is present in the apparent absence of any detectable ERα or ERβ (Fig. 1A). Interestingly, OHT and STX activated the MAPK and PI3K pathways significantly more than 17β estradiol (17βE2; Fig. 2B), possibly reflecting more robust activation of GPR30 in this cellular context, as noted by others (12, 17). Taken together, these data show that human endometriotic cells express high levels of GPR30 and activate proliferative signals in response to OHT and STX.

![Figure 2. Tamoxifen and the tamoxifen analogue STX activate PI3K and MAPK pathways in H-38 cells. A. chemical structures of STX and OHT. B. phospho-ERK1/2 (pERK1/2) and phospho-Akt (pAkt) were detected by immunoblotting using serum-depleted H-38 primary cells treated with negative control inhibitors (1 μM; 100 nM wortmannin (W) or 10 μM U0126 (U), 30 min pretreatment before adding STX), or with positive control drugs (EGF or insulin); total ERK1/2 and Akt show loading controls.](image-url)
STX fails to bind, antagonize, or activate ERα and ERβ. To determine the GPR30-dependent effects of STX, we used other relevant cell lines that could be more easily manipulated, and did not exhibit the low transfection efficiency and extremely high levels of SF-1 and Cyp19a as observed for H-38 cells. Given that the affinity of STX for ERα and ERβ is several logs lower (10^6) compared with 17βE2 (Fig. 3A; ref. 24), it is not surprising that STX was unable to activate or repress a classic estrogen response element luciferase reporter (ERE TATA-Luc). No activation was observed in human placental choriocarcinoma cells JEG3 cells transiently overexpressing ERα; these cells are ERα/β negative and GPR30 positive. A similar observation was observed in human endometrial cancer Ishikawa cells, which express endogenous ERβ (Fig. 3B). Consistent with data obtained in primary endometriotic H-38 cells, these results establish that STX signaling is independent of ERs and unlike OHT, STX neither activates nor antagonizes classic ERE-reporter constructs. Thus, although STX and OHT share similar structural features, STX fails to activate ERα/ERβ.

SF-1 transcriptional activation via estrogen stimulation of GPR30. We next assessed the potential link between GPR30 stimulation and activation of SF-1 or LRH-1. Ligand-stimulated GPR30 triggers activation of the MAPK pathway, which is then predicted to activate SF-1 by direct phosphorylation. GPR30 also stimulates the PI3K pathway, elevating cellular PIP3 levels (Fig. 2B; ref. 2), which is also predicted to activate NR5A receptors by direct binding of PIP3 to the ligand binding domain (21). Using JEG3 cells that express endogenous GPR30, but very low levels of SF-1 and no ER, we found that both 17βE2 and STX produced dose- and time-dependent activation of an aromatase promoter luciferase reporter (Aro-Luc), after addition of mSF-1 and human LRH-1 (Supplementary Figs. S2 and S3; Fig. 4A). STX failed to enhance the activity of estrogen-related receptor (ERRα; data not shown). Similar to other studies (9), we found that the estrogen antagonist, ICI182,780, failed to mimic STX (data not shown), despite the fact that ICI is reported to bind GPR30 directly (34). SF-1 mutants defective for phosphorylation (S203A) or with a disrupted ligand binding pocket (A270W) decreased or eliminated estrogen and STX stimulation of SF-1 transcriptional activity compared with wild-type SF-1, establishing the requirement for both Ser203 phosphorylation and a fully accessible ligand binding pocket for these estrogenic compounds to activate SF-1 (see Fig. 4B; ref. 21).

Inhibiting MAPK and PI3K signaling attenuates STX activation of SF-1 in JEG3 cells. Consistent with known effects of MAPK signaling on SF-1 and LRH-1, pharmacologic inhibitors of either MAPK/ERK kinase or PI3K (U0126 or wortmannin, respectively) effectively blocked 17βE2 and STX activation of SF-1 (Fig. 4C, left). Furthermore, STX treatment increased ERK activation (phospho-ERK1/2) and SF-1 phosphorylation to equivalent levels, indicating that MAPK and PI3K are critical signaling components for estrogenic compound activation of SF-1.
levels as observed with EGF (Fig. 4C, right). Importantly, siRNA directed against GPR30 abolished the ability of 17βE2 or STX to induce SF-1–dependent transcription (Fig. 4D, left), as well as the ability of STX to induce SF-1 phosphorylation (Fig. 4D, right). Interestingly, knocking down GPR30 consistently lowered basal levels of SF-1 phosphorylation, as shown in the DMSO control (−). Collectively, these data establish that SF-1 activation via estrogen and STX signaling is GPR30-dependent in JEG3 cells. Furthermore, receptor activation by STX and 17βE2 required phosphorylation of SF-1 and an accessible ligand-binding pocket.

STX activates SF-1 and increases PIP3 accumulation in Ishikawa cells. We next asked if STX stimulation of GPR30 is linked to NR5A receptor activation in Ishikawa endometrial cancer cells, where both GPR30 and nuclear ER signaling are suggested to promote proliferation (10, 12). In these cells, STX stimulation of SF-1 was amplified after increasing GPR30 expression transiently (Supplementary Fig. S3). Given this fact, we created a stable Ishikawa cell line expressing GPR30 comparable with levels observed in MCF-7 cells to fully characterize the pharmacologic effects of STX (Figs. 5A, right, and 1A). These cells exhibited SF-1–dependent activation of
the Aro-Luc reporter after 17βE2, OHT, and STX treatment, but not with the inactive 17αE2 (Fig. 5A, left). Consistent with effects in endometriotic H-38 cells, we observed robust activation of the MAPK and PI3K pathways after treatment with OHT and STX in Ishikawa cells expressing GPR30 (Supplementary Fig. S4; Fig. 5B). Ishikawa cells are ERβ positive and express lower levels of GPR30 and ERα than MCF-7 cells (refer back to Fig. 1A).

Previous descriptive studies using a fluorescent-tagged pleckstrin homology domain of Akt suggested that stimulation of GPR30 results in nuclear PIP3 accumulation (7, 23). To determine if STX similarly increased cellular pools of PIP3, we measured levels of this very scarce phospholipid after STX stimulation of Ishikawa cells stably expressing GPR30 (see Materials and Methods). A dose-dependent STX-induced increase in PIP3 accumulation was observed with a maximal response occurring at 1 μM drug (Fig. 5C and D); this same dose corresponded to the maximal stimulation of SF-1–dependent activation of the Aro-Luc reporter (refer to Figs. 4A and 5D). No accumulation of PIP3 was detected in cells treated with the inactive 17αE2 epimer, or with DMSO vehicle (data not shown). Taken together, these data show that estrogen signaling via GPR30 increases cellular levels of PIP3. Our ability to detect STX-induced increases in cellular PIP3, the least abundant cellular phosphatidylinositol, most likely reflects the fact that Ishikawa cells are negative for the lipid phosphatase PTEN, which removes the 3′ phosphate. Although others report that estrogen acting via ERα increases endogenous PIP3 in endothelial cells (35), our data using the selective compound STX suggest strongly that this effect on PI3K signaling might also be mediated by GPR30.

Effects of STX on cellular proliferation and aromatase (Cyp19a1) expression. Increased Ishikawa cell proliferation after 17βE2 or OHT treatment is attributed to the combined actions of both nuclear ERs and GPR30 (12). This prompted us to measure endogenous Cyp19a1 (aromatase) expression after STX treatment, given that both SF-1/LRH-1 are known to control expression of promoter II of Cyp19a1 (36). Indeed, we found that STX, OHT, and genistein, but not 17αE2, elevate endogenous Cyp19a1 transcripts in Ishikawa cells with induction greatly enhanced by GPR30 expression (Fig. 6A). Knockdown experiments using siRNA show that STX activation of Cyp19a1 depends on both GPR30 and LRH-1 (Supplementary Fig. S5; Fig. 6B). Finally, we observed a marked increase in Ishikawa cell proliferation as measured in two independent assays after treatment with STX, OHT, or the dietary...
estrogen genistein, but not with 17αE2 (Supplementary Fig. S6; Fig. 6). Overexpressing GPR30 amplified this response (Fig. 6C, right), although stimulation by 17βE2 was less robust than observed with STX. That STX elevates endogenous levels of the NR5A target aromatase and increases cell proliferation in a GPR30-dependent manner suggests that estrogen signaling via GPR30 might be an important pathway for increasing local levels of estrogen, and for promoting cellular proliferation in endometrial cells.

Discussion
Here, we provide data supporting a novel pathway linking estrogen stimulation of the membrane receptor GPR30 with activation of NR5A nuclear receptors and the target gene aromatase (Fig. 6D). Using human Ishikawa endometrial cancer cells or human primary endometriotic H-38 cells, we report that the GPR30-dependent tamoxifen-analogue STX rapidly activates both the PI3K and MAPK signaling pathways, and increases levels of cellular PIP3 in a dose-dependent manner. In Ishikawa cells, STX as well as OHT elevated endogenous levels of aromatase (Cyp19a1) and increased proliferation in a GPR30-dependent manner. These data suggest that STX activation of NR5A receptors depends on GPR30, but not nuclear ER signaling. Our study is now one of several suggesting that the physiologic effect of estrogen signaling in normal and disease states can be expanded beyond nuclear ER signaling to include a 7TM receptor. Using human primary H-38 cells, we provide further evidence that GPR30 alone is sufficient for estrogen signaling (Figs. 1A and 6D). Moreover, this novel
GPR30/SF-1 pathway shows how synthetic estrogen analogues such as OHT and STX increase local concentrations of estrogen, thereby amplifying estrogen signaling.

The link between estrogens and NR5A receptor activity is especially intriguing given that both SF-1 and LHR-1 are involved in steroid and estrogen production (37). When placed in the context of prior studies, our findings imply that GPR30 activation and up-regulation of SF-1 set up a feed-forward autocrine pathway for continuous synthesis of estradiol in tissues such as ectopic endometrium. Indeed, profiling studies comparing human ectopic and eutopic endometrium confirm that SF-1 and estradiol biosynthesis are amplified in ectopic disease tissue (38, 39). Mendelson and colleagues (40) found that immobilized human endometrial cells readily convert exogenously applied androstenedione to estradiol, with this androgen also promoting SF-1 recruitment to the CYP19A1 promoter. Regardless of whether androstenedione is supplied exogenously from the peritoneal cavity (41) or is synthesized de novo by ectopic endometrium, amplification of SF-1 ensures that local estrogen signaling increases dramatically in endometriosis.

Our model depicted in Fig. 6D also predicts that many dietary and environmental xenoestrogens could bind to GPR30, stimulate the PI3K/MAPK pathways, and ultimately activate SF-1. Indeed, in cellular assays, we find that the dietary estrogen genistein mimics STX, consistent with studies showing genistein to be the best competitive inhibitor of E2 binding to GPR30 (34). Given the crucial role of NR5A receptors in regulating early gonadal development, steroidogenesis, and peptide hormone signaling, genistein activation of GPR30 might be predicted to disturb genetic programs targeted by SF-1, and as a result alter reproductive and endocrine tissue development. Interestingly, although GPR30 is expressed in many endocrine tissues including the uterus, loss of GPR30 in mice does not impair reproduction, although GPR30 is expressed in many endocrine tissues including reproductive and endocrine tissue development. Interestingly, although GPR30 is expressed in many endocrine tissues including the uterus, loss of GPR30 in mice does not impair reproduction, suggesting that GPR30 is not an essential gene for urogenital development (15).

STX is similar to other synthetic and environmental estrogens that activate the PI3K and MAPK signaling pathways in a GPR30-dependent manner (42, 43). The activation of PI3K by STX is of particular interest given that phospholipids seem to be integrally associated with purified human SF-1 (44) and that phosphatidylinositols such as PIP3 are hypothesized to modulate SF-1 activity (19, 22). Indeed, we show that STX increased cellular PIP3 and that STX stimulation of Cyp19a depends on an intact ligand-binding pocket mutant. Taken together, it is tempting to speculate that STX activation of GPR30 increases PIP3 cellular pools to then activate SF-1 with additional receptor activation arising from MAPK signaling and increased SF-1 phosphorylation. That STX elevated cellular PIP3 pools and activated the PI3K pathway might also be relevant to the increased risk of endometrial ovarian cancer associated with endometriosis (45). Given that PTEN mutations are common in both uterine and ovarian cancers, as well as in endometriosis (45, 46), we speculate that activation of GPR30 in a PTEN-deficient genetic background would further amplify PIP3 signaling and promote tumor proliferation.

The role of GPR30 in endometrial cancer is now worth considering as cellular studies such as ours, and those surveying human cancer tissues are being reported. Initial studies linking GPR30 to endometrial proliferation showed that knockdown of GPR30 reduced estradiol and OHT-induced growth of Ishikawa and HEC1A endometrial cell lines, as well as induction of c-fos and ERK1/2 phosphorylation (10). Similarly, we find that both STX and genistein increase Ishikawa endometrial cell proliferation in a GPR30-dependent manner. More importantly, GPR30 is prominently expressed in primary human endometriotic H-38 cells. Thus, activation of GPR30 may underlie the higher risk of endometrium hyperplasia and/or cancer in postmenopausal women observed with chronic OHT treatment, but not with aromatase inhibitor therapy (47, 48). As such, development of drugs that specifically target this GPR30/SF-1 signaling axis might offer additional therapies in treating reproductive cancers in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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