The Essential Role of GATA6 in the Activation of Estrogen Synthesis in Endometriosis

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Abstract

Endometriotic stromal cells synthesize estradiol via the steroidogenic pathway. Nuclear receptor subfamily 5, group A, member 1 (NR5A1) is critical, but alone not sufficient, in activating this cascade that involves at least 5 genes. To evaluate whether another transcription factor is required for the activation of this pathway, we examined whether GATA Binding Protein 6 (GATA6) can transform a normal endometrial stromal cell (NoEM) into an endometriotic-like cell by conferring an estrogen-producing phenotype. We ectopically expressed GATA6 alone or with NR5A1 in NoEM or silenced these transcription factors in endometriotic stromal cells (OSIS) and assessed the messenger RNAs or proteins encoded by the genes in the steroidogenic cascade. Functionally, we assessed the effects of GATA6 expression or silencing on estradiol formation. In OSIS, GATA6 was necessary for catalyzing the conversion of progesterone to androstenedione (CYP17A1; $P < .05$). In NoEM, ectopic expression of GATA6 was essential for converting pregnenolone to estrogen (HSD3B2, CYP17A1, and CYP19A1; $P < .05$). However, simultaneous ectopic expression of both GATA6 and NR5A1 was required and sufficient to confer induction of all 5 genes and their encoded proteins that convert cholesterol to estrogen. Functionally, only simultaneous knockdown of GATA6 and NR5A1 blocked estradiol formation in OSIS ($P < .05$). The presence of both transcription factors was required and sufficient to transform endometrial stromal cells into endometriotic-like cells that produced estradiol in large quantities ($P < .05$). In summary, GATA6 alone is essential but not sufficient for estrogen formation in endometriosis. However, simultaneous addition of GATA6 and NR5A1 to an endometrial stromal cell is sufficient to transform it into an endometriotic-like cell, manifested by the activation of the estradiol biosynthetic cascade.

Keywords

endometriosis, GATA6, NR5A1, steroidogenesis, estrogen

Introduction

Endometriosis is a benign gynecologic condition associated with infertility and pelvic pain that affects up to 10% of women of reproductive age. The disorder is defined by growth of endometrial tissue outside the uterus, which leads to inflammation.¹ The most well-accepted hypothesis regarding etiology is that endometrial tissue shed by retrograde menstruation migrates, adheres, and grows on extrauterine peritoneal surfaces.^{1,2} A key pathologic feature of pelvic endometriosis is the survival of tissue that is endometrial in origin on lower abdominal tissues followed by an intense inflammatory process and tissue remodeling.³ The stromal cell component of endometriosis comprises the bulk of the pathologic tissue and is thought to play a central role.³ Ectopically located endometriotic stromal cells look histologically similar to (eutopic) endometrial cells but strikingly differ from them with respect to production of estrogen and certain cytokines.³ Thus far, the critical genes that confer this steroidogenic and inflammatory transformation have not been defined.

Estrogen plays a central role in the development and persistence of endometriosis by promoting the growth of ectopic tissue.³ Steroidogenic genes that drive the biosynthesis of estradiol are abnormally active in endometriotic stromal cells, leading to local estradiol production that is a hallmark feature of the disease.⁴ In differentiated steroidogenic tissues such as the

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adrenal, gonads, and placenta, the metabolic pathways governing steroidogenesis are tightly controlled by an array of tissue-specific transcription factors.⁵ However, the molecular mechanisms underlying the aberrant steroidogenic phenotype in endometrial stromal cells are not fully understood. It has previously been demonstrated that DNA methylation is responsible for altered expression of nuclear receptor subfamily 5, group A, member 1 (NR5A1) in endometriotic cells.⁶ Recent work suggests that epigenetic regulation by another transcription factor, GATA Binding Protein 6 (GATA6), may be also be involved.⁷ The functional role of GATA6 for the transformation of an endometrial stromal cell to an endometriotic phenotype, however, has not been demonstrated to date.

An intriguing feature of endometriosis is that endometriotic stromal cells have the ability to synthesize estradiol from cholesterol via the activation of the steroidogenic cascade. The NR5A1 is a nuclear receptor important for sexual development and differentiation of steroidogenic tissues and has been established as a key factor regulating steroidogenesis.^{8,9} The NR5A1 is expressed in steroidogenic tissues, specifically the adrenal cortex and gonads,⁸ and coordinates the expression of multiple steroidogenic pathway genes, including the steroidogenic acute regulatory protein (StAR),¹⁰ the 3- β -hydroxysteroid dehydrogenases (HSD3B1 and HSD3B2), and the cytochrome P450 steroid hydroxylases (family 11, subfamily A, polypeptide 1 [CYP11A1]; family 17, subfamily A, polypeptide 1 [CYP17A1]; and family 19, subfamily A, polypeptide 1 [CYP19A1]).¹¹ Consistent with its action in regulating steroidogenesis, NR5A1 is expressed in endometriotic stromal cells but is absent in healthy eutopic endometrial stromal cells and has been strongly implicated in the pathogenesis of endometriosis.^{6,8} Although NR5A1 plays a role in the steroidogenic cascade, it is not sufficient to activate the entire pathway.^{12,13}

The GATA family of transcription factors is a highly conserved group of 6 zinc finger proteins that control stem cell regulation and tissue development.¹³⁻¹⁶ GATA 1, 2, and 3 regulate cell lineage fate during hematopoiesis,¹⁷ while GATA 4, 5, and 6 govern cell fate and stemness in tissues originating from the endoderm and mesoderm, including the gonads.¹⁸ GATA4 and GATA6 are commonly expressed in steroidogenic tissues and are essential for regulating steroidogenic genes.^{13,19} Similar to NR5A1, GATA6 expression is significantly higher in endometriotic tissue than in normal endometrial stromal cells (NoEMs).⁷ Previous studies have demonstrated that GATA6 plays a role in regulating the expression of StAR,²⁰ CYP11A1,²⁰ HSD3B2,²¹ and CYP17A1.²² Because the GATA factors are expressed in steroidogenic tissue along with NR5A1, it has been proposed that these factors work synergistically to regulate gene expression in certain tissues.¹³ In the adrenal, NR5A1 and GATA6 concomitantly increase transcription of steroidogenic enzymes, including StAR, CYP11A1, HSD3B2, and CYP17A1.^{14,20-22} Moreover, NR5A1 and GATA6 physically interact to accomplish these effects.²³

To date, the minimum set of essential transcription factors that are sufficient to transform an endometrial cell into an endometriotic stromal cell has not been defined. In this study,

we sought to demonstrate the endometriotic phenotype both via messenger RNA (mRNA) and protein expression of the steroidogenic genes in the estrogen synthetic cascade. Functionally, we determined the roles of these transcription factors in the production of estradiol. Given the potential roles of GATA6 and NR5A1 in steroidogenesis, we sought to determine whether ectopic expression of GATA6, with or without NR5A1, is sufficient to transform an endometrial stromal cell into an endometriotic-like cell, manifested by the activation of the estradiol biosynthetic cascade.

Materials and Methods

Ethical Approval

The Northwestern University Institutional Review Board for Human Research (1375-005) approved this study. Prior to undergoing surgery, each participant provided written informed consent.

Tissue Collection

In participants who underwent ovarian surgery and were found to have endometriomas, primary endometriotic stromal cells (OSIS) were isolated from endometriotic tissue taken from cyst walls postoperatively ($n = 5$; mean age: 42 years). Histological examination confirmed endometriosis was present in each sample. In participants who underwent hysterectomy for benign indications and did not have evidence of endometriosis, NoEMs were isolated from eutopic endometrial tissue obtained following surgery ($n = 5$; mean age: 41 years). No participants had received hormonal therapy preoperatively. Human foreskin fibroblast cells (BJ cells), a nonimmortalized stromal cell line with a normal diploid karyotype, were obtained from ATCC and used as controls (cat. CRL-2522; Manassas, Virginia; $n = 7$).

Stromal Cell Isolation and Culture

Enzymes for tissue processing were obtained from Sigma (St Louis, Missouri). Cell culture media, trypsin, and supplements were from Gibco, unless otherwise stated (Thermo Fisher Scientific, Waltham, Massachusetts). Cell plastics were from TPP (Trasadingen, Switzerland). Both OSIS and NoEM cells were isolated from endometriotic and eutopic endometrial tissue as previously described.²⁴⁻²⁶ Glandular and stromal elements were rinsed with Hank buffered saline solution (HBSS), dissected away, minced, and digested in 10 mL of HBSS supplemented with 2 mM CaCl_2 , 5 mg/mL collagenase (cat C0130), 0.2 mg/mL DNaseI (cat D5025), and 2 mg/mL hyaluronidase (cat H3506) at 37°C for 30 minutes. Cells were pelleted, resuspended in HBSS, and then passed through sterile 70- and 20- μm sieves to progressively filter out epithelial cells. The OSIS cells or NoEM cells were again pelleted and transferred to complete medium (Dulbecco's Modified Eagle's Medium [DMEM]/F12 with 10% fetal bovine serum [FBS], 100 IU/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 2.5 $\mu\text{g/mL}$ amphotericin B) and propagated on 150-mm dishes. The cells were maintained in

complete medium, the media was changed every 48 hours, and the cells were kept in a humidified atmosphere at 37°C with 5% CO₂ at 37°C. Once the cells grew to confluence, the cells were either trypsinized and split into 60-mm dishes with 400 000 cells per dish or were trypsinized and resuspended in DMEM/F12 with 10% FBS and 10% dimethyl sulfoxide (DMSO), and cryopreserved at -80°C. When it was necessary for frozen cells to be used for experiments, the cells were thawed and placed onto a 150-mm dish and maintained in medium as described above.

Skin fibroblasts were grown and maintained as recommended in Minimum Essential Medium (MEM) (ATCC) supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. The cells were propagated in 150-mm dishes. Once confluent, the cells were trypsinized and split into 60-mm dishes with 400 000 cells per dish.

Prostaglandin E₂ Treatment

OSIS, NoEM, and BJ cells were treated with 100 nM prostaglandin E₂ (PGE₂), either 48 hours (2 days) posttransfection or 120 hours (5 days) posttransduction, for 16 hours. For treated OSIS and NoEM cells, complete media was replaced with serum-free, phenol red-free DMEM/F12 with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B, 16 hours before cells were isolated. For treated BJ cells, complete media was replaced with serum-free, phenol red-free MEM with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B, 16 hours before cells were isolated.

siRNA Transfection

For depletion experiments, OSIS cells were transiently transfected with Silencer Select small interfering RNAs (siRNAs) (Supplemental Table 1) from Ambion (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) as previously described.⁷ Briefly, pairs of siRNAs targeting *NR5A1* and *GATA6* expression were tested for specificity and optimized for capacity to deplete expression of their respective targets in comparison with scrambled siRNA. On the day of transfection, OSIS cells were trypsinized and resuspended at 400 000 cells/60-mm dish in 4 mL of antibiotic-free DMEM/F12 with 10% FBS. The siRNAs were resuspended in RNase-free water and then complexed with Lipofectamine (0.11 nmol of total siRNA in complex with 15 µL of reagent) in a total volume of Opti-MEM reduced serum media (Thermo Fisher Scientific) for 15 minutes before being added to the individual plates of OSIS cells. Cells were replenished with complete medium 12 hours after transfection. Treatment with vehicle or PGE₂ was initiated 48 hours posttransfection and continued for 16 hours.

Adenoviral Transduction

For adenoviral transduction, NoEM and BJ cells were plated at 400 000 cells/60 mm dish in complete medium at a

multiplicity of infection (MOI) of 10 with adenoviral particles (Vector Biolabs, Malvern, Pennsylvania) carrying an empty vector (AdControl), human *NR5A1* (AdNR5A1), human *GATA6* (AdGATA6), or a combination of *NR5A1* and *GATA6* (combination group), each under the direction of the cytomegalovirus (CMV) promoter. The virus was commercially prepared and initially titrated against HEK293 cells. Our stock was monitored over time by PCR and immunofluorescence following transduction. For our cell types, we subsequently transduced different MOIs of the adenovirus to optimize expression and evaluate cytotoxicity and cell viability. The optimal cell viability was at an MOI of 10. Treatment with vehicle or PGE₂ was initiated 16 hours before isolation of total genomic DNA, RNA, and protein.

Nucleic Acid Isolation

Total genomic DNA and total RNA from each plate of cells were isolated with AllPrep DNA/RNA columns (Qiagen, Valencia, California). Total DNA and RNA quantity was analyzed using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA and RNA were stored at -20°C.

Reverse Transcription and qPCR

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted to analyze gene expression following siRNA transfection or adenoviral transduction. Complementary DNA (cDNA) was prepared from 1 µg total RNA using Q-script cDNA SuperMix (Quanta Biosciences, Gaithersburg, Maryland). Quantitative PCR was performed on the cDNA generated from 25 ng equivalents of total RNA using final primer and probe concentrations of 500 nM and 250 nM, respectively. Amplification was carried out using Taqman Universal Master Mix (Thermo Fisher Scientific) on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific).^{26,27} TATA-binding protein (*TBP*) was used as a reference gene to normalize gene expression. Relative fold change in the mRNA level was calculated by calibrating the normalized ΔC_T for each gene following treatment to the average normalized value observed in NoEM cells with vehicle treatment and is expressed as fold change relative to vehicle-treated NoEM. Additional comparisons were made in the fold changes between experimental groups; these comparisons are expressed as percentage change. Error bars are included in the graphs that demonstrate the findings from these experiments. These error bars represent standard error of the mean for each experimental group. Independent experiments that studied cells from different participants were used to compute standard error of the mean. These findings therefore represent biologic variance. Primers used to assess expression of *NR5A1*, *GATA6*, and steroidogenic genes (*STAR*, *CYP11A1*, *HSD3B2*, *CYP17A1*, and *CYP19A1*) are listed in Supplemental Table 2.

Protein Preparation and Immunoblotting

Cells were washed with PBS prior to preparing whole cell lysates. Cells were then lifted and homogenized in 100 μ L RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 1% NP-40) supplemented with protease inhibitor cocktail (Sigma). Lysates were then sonicated and bicinchoninic acid assay (BCA) assays were performed to quantify protein concentration. Equal amounts of protein were resolved on NuPAGE Novex 4% to 12% Bis-Tris Plus Gels (Thermo Fisher Scientific). Transfer and membrane blocking were performed as previously described.²⁸ Membranes were incubated with primary antibodies against NR5A1, GATA6, and the steroidogenic proteins (StAR, HSD3B2, CYP17A1, CYP19A1) in 2.5% nonfat milk overnight at 4°C (Supplemental Table 3). Three commercially available antibodies were unable to reliably detect CYP11A1 in 2 control tissues (human ovary and placenta). As such, we were unable to assess CYP11A1 protein levels in any of the experimental groups. For all other proteins, the membranes were then washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. Detection was performed using Luminata Crescendo HRP substrate (Millipore, Billerica, Massachusetts), SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), or HyGLO Quick Spray (Denville Scientific Inc, Holliston, Massachusetts). For each individual experiment with OSIS, NoEM, or BJ cell lysates, the 8 treatment groups were run together on 10-well gels. Proteins were quantified using Image Studio Software (version 5.2.5; LI-COR, Inc, Lincoln, Nebraska). All values for protein quantification were normalized against GAPDH.

Detection of Estradiol Production

Sixteen hours after treatment with PGE₂, 1 mL of media was aspirated from each OSIS, NoEM, and BJ plate and stored at -20°C. Organic phase extraction and enzyme immunoassays (EIAs) were performed according to the manufacturer's instructions to assess estradiol production (Cayman Chemical, Ann Arbor, Michigan). To eliminate matrix effects due to the media, 0.5 mL of each collected media sample was extracted 3 times with 2 mL of methylene chloride. The combined organic phases from each extraction were dried under nitrogen at room temperature. Dried samples were resuspended in 250 μ L of the provided EIA sample buffer, also used for standards. Recovery was calculated at >95%. Estradiol standards, which spanned from 6.6 to 4000 pg/mL, were all detectable above blank matrix. All samples fell within the quantitative range of the standards. All data were collected using a SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, California). Error bars are included in the graphs that demonstrate the findings from these experiments. These error bars represent standard error of the mean for each experimental group.

Figure 1. Fold change difference in messenger RNA (mRNA) expression compared to untreated controls between normal endometrial stromal cells (NoEMs) and endometriotic stromal cells (OSIS) treated with vehicle (control) or prostaglandin E₂ (PGE₂). Bars indicate a statistically significant difference in expression between NoEM and OSIS. Asterisks (*) indicate a statistically significant difference between NoEM and OSIS control groups as well as a statistically significant interaction between vehicle control and PGE₂-treated groups.

Statistical Analyses

For qPCR data between NoEM and OSIS treated with or without PGE₂, differences were assessed by 2-way analysis of variance (ANOVA). When no significant interaction was present, a main effect was considered significant for a *P* value of <.05. When an interaction was detected, multiple comparisons were made using Tukey test and were considered significant for adjusted *P* values <.05. Given the strong and consistent interaction between PGE₂ treatment and disease status (Figure 1) in the depletion and transduction studies using OSIS, NoEM, and

BJ cells, only differences across the PGE₂-treated groups were assessed by 2-way ANOVA. Tukey test was then used to make multiple comparisons. Data were analyzed using GraphPad Prism (version 6.01; GraphPad Software, Inc, La Jolla, California).

For EIA data, normalized data were fit to a 4-parameter logistic fit plot using SoftMax Pro 7 (Molecular Devices). Differences across the PGE₂-treated groups were assessed by 2-way ANOVA, and multiple comparisons followed by Tukey test.

Results

As shown previously in our laboratory and confirmed by experiments demonstrated in Figure 1, both *NR5A1* and *GATA6* expressions were significantly higher in OSIS compared to NoEM ($P < .05$).^{7,29} The steroid-producing capacity of endometriotic stromal cells is comparable to ovarian granulosa cells. As shown in Figure 1, the steroidogenic genes were largely dependent on PGE₂ within endometriotic cells; in contrast, expression levels of *NR5A1* and *GATA6* varied by disease status but not by PGE₂ treatment. Figure 1 demonstrates the baseline molecular signature of the primary cell culture model for the mechanistic experiments in this study. Figures 2 through 4 demonstrate the results of these experiments that were conducted to define the relative roles of the steroidogenic transcription factors *NR5A1* and *GATA6* in endometriotic estradiol production.

Depletion of *NR5A1* and *GATA6* Regulates Steroidogenic Gene Expression in OSIS

To examine the roles of *NR5A1* and *GATA6* on steroidogenesis in the context of disease, we utilized siRNAs to selectively deplete each transcription factor individually, as well as together, in OSIS. Figure 2 shows the decrease in endogenous mRNA and protein levels, for *NR5A1* and *GATA6*, following depletion with specific siRNAs. For each gene, mRNA levels were reduced by more than 5-fold, and protein expression was effectively abolished, by gene-specific siRNAs compared to scrambled controls.

Figure 2A demonstrates how the gene expression of *StAR*, *CYP11A1*, *HSD3B2*, *CYP17A1*, and *CYP19A1* changed at the mRNA level when *NR5A1* and *GATA6* were depleted alone or in combination. In the presence of PGE₂, *StAR* expression decreased more than 70% compared to scrambled control upon *NR5A1* depletion ($P < .05$), but *GATA6* depletion did not significantly alter *StAR* expression. *CYP11A1* was similarly decreased in response to silencing *NR5A1* (57%, $P < .05$), while loss of *GATA6* had little effect. The expressions of *HSD3B2*, *CYP17A1*, and *CYP19A1* were all markedly decreased when either *NR5A1* or *GATA6* was silenced alone or in combination. *HSD3B2* expression decreased 89% with loss of *NR5A1* ($P < .05$), 79% with loss of *GATA6* ($P < .005$), and 95% when the combination of the two were depleted ($P < .001$). While *CYP17A1* decreased when *NR5A1* (80%)

Figure 2. Change in steroidogenic gene expression in primary endometriotic stromal cells (OSIS) experimental groups following depletion of nuclear receptor subfamily 5, group A, member 1 (*NR5A1*) and *GATA6*. A, Change in messenger RNA (mRNA) expression in OSIS experimental groups (normalized to untreated normal endometrial stromal cells, NoEMs): xControl (scrambled small interfering RNAs [siRNA] control), xNR5A1 (*NR5A1* silenced), xGATA6 (*GATA6* silenced), xNR5A1, and *GATA6* (*NR5A1* and *GATA6* silenced). N = 5. Bars indicate a statistically significant difference in expression between prostaglandin E₂ (PGE₂)-treated groups. B, Immunoblots for OSIS treatment groups. Image is a representative blot from 4 experimental replicates.

and *GATA6* (96%) were silenced individually, the expression of *CYP17A1* only reached significance when *NR5A1* and *GATA6* were depleted together, resulting in a 99% reduction ($P < .05$). Finally, *CYP19A1* decreased significantly when

NR5A1 ($P < .001$), GATA6 ($P < .05$), or the combination of the two were depleted ($P < .0005$), leading to 84%, 52%, and 88% reductions in expression, respectively.

Figure 2B demonstrates similar changes in the protein expression of steroidogenic genes in response to NR5A1 and GATA6 depletion by siRNA. Quantification of the differences in protein levels in PGE₂-treated OSIS cells transfected with scrambled control or siRNA directed to NR5A1 and GATA6 alone and in combination is shown in Supplemental Figure 1. The StAR, HSD3B2, CYP17A1, and CYP19A1 were all readily detectable in OSIS. Baseline expression of StAR was increased by PGE₂. The StAR was only modestly affected by loss of GATA6 but decreased significantly when NR5A1 ($P < .005$) was depleted. The loss of both NR5A1 and GATA6 effectively abolished StAR expression ($P < .05$). Expression of HSD3B2 was less affected by PGE₂. Both scrambled control and NR5A1 depleted cells had similar expression of HSD3B2, which was lost when GATA6 was depleted. There was a non-significant decrease in CYP17A1 expression when NR5A1 was silenced; however, expression significantly decreased when GATA6 ($P < .05$) or the combination of NR5A1 and GATA6 was silenced ($P < .05$). CYP19A1 showed consistent upregulation by PGE₂. There was a nonsignificant decrease in CYP19A1 expression compared to scrambled control when NR5A1 or GATA6 was silenced; however, CYP19A1 expression decreased significantly when the combination of NR5A1 and GATA6 was depleted ($P < .05$). Three commercially available antibodies were unable to specifically detect CYP11A1 in control tissues (placenta and ovary); as such, we were unable to determine the effect of NR5A1 or GATA6 silencing on expression of CYP11A1 protein in OSIS.

Overexpression of NR5A1 and GATA6 Regulates Steroidogenic Genes in NoEM

We next tested whether introducing NR5A1 and GATA6 into healthy NoEM could coordinate the induction of steroidogenic genes. Using adenoviral transduction to express NR5A1 and GATA6 either individually or together, Figure 3A shows the increase in NR5A1 and GATA6 mRNA expression following adenoviral transduction in NoEM. Figure 3B confirms a concomitant increase in protein expression of NR5A1 and GATA6.

Figure 3A demonstrates the effect of NR5A1 and GATA6 overexpression on the mRNA levels of *StAR*, *CYP11A1*, *HSD3B2*, *CYP17A1*, and *CYP19A1*. Overexpression of GATA6 alone did not affect *StAR* expression. However, *StAR* expression increased significantly from the control group when NR5A1 was transduced ($P < .05$) and when NR5A1 and GATA6 were transduced together. This was further increased by PGE₂ treatment in both groups, which showed increases in expression of more than 21 000% compared to the control group ($P < .05$). *CYP11A1* expression was similarly affected by NR5A1 overexpression, increasing nearly 450% in the presence of PGE₂ ($P < .005$). The combination of NR5A1 and GATA6 overexpression led to a 665% increase in *CYP11A1*

Figure 3. Change in steroidogenic gene expression in normal endometrial stromal cells (NoEMs) experimental groups following overexpression of nuclear receptor subfamily 5, group A, member 1 (NR5A1) and GATA6. A, Change in messenger RNA (mRNA) expression in NoEM experimental groups: AdControl (control), AdNR5A1 (NR5A1 transduced), AdGATA6 (GATA6 transduced), AdNR5A1, and GATA6 (NR5A1 and GATA6 transduced). N = 5. Bars indicate a statistically significant difference in expression between prostaglandin E₂ (PGE₂)-treated groups. B, Immunoblots for NoEM treatment groups. Image is a representative blot from 4 experimental replicates.

expression in PGE₂-treated NoEM, which was significantly relative to the PGE₂-treated control group ($P < .0001$), but not the PGE₂-treated NR5A1 overexpressing group. Neither NR5A1 nor GATA6 overexpression alone induced the expression of *HSD3B2*; however, the combination of NR5A1 and GATA6 led to a striking increase in expression of 572 000% ($P < .05$). Expression of *CYP17A1* increased slightly in response to NR5A1 overexpression, but the change in

CYP17A1 expression was not statistically different from controls for this group or with GATA6 overexpression. By contrast, the combination of NR5A1 and GATA6 overexpression significantly increased *CYP17A1* expression to over 281 000% relative to PGE₂-treated controls ($P < .001$) and by 368% relative to PGE₂-treated cells transduced with NR5A1 alone ($P < .005$). *CYP19A1* expression changes in response to NR5A1 and GATA6 overexpression were similar to those of *CYP17A1*. Individually, NR5A1, but not GATA6, overexpression modestly increased *CYP19A1* expression, but these changes were not significantly different from the PGE₂-treated controls. The combination of NR5A1 and GATA6 overexpression increased *CYP19A1* expression by 21 000% relative to PGE₂-treated controls ($P < 0.05$) and by 422% relative to PGE₂-treated cells transduced with NR5A1 alone ($P < .05$).

Figure 3B illustrates similar changes in steroidogenic gene expression at the protein level with overexpression of NR5A1 and GATA6 alone or in combination. Quantification of protein levels in PGE₂-treated NoEM cells transduced with a null control or adenovirus expressing NR5A1 or GATA6 alone or in combination is shown in Supplemental Figure 2. The PGE₂ treatment did not induce detectable levels of StAR or HSD3B2 in NoEM transduced with the control adenovirus. CYP17A1 and CYP19A1 proteins were faintly detectable but were not significantly affected by PGE₂. Compared to PGE₂-treated controls, StAR expression increased significantly when transduced with either NR5A1 ($P < .005$) or the combination of NR5A1 and GATA6 ($P < .05$), but was undetectable in cells transduced with GATA6. HSD3B2 was undetectable at the protein level in cells transduced with NR5A1 or GATA6. However, expression of HSD3B2 increased significantly when NR5A1 and GATA6 were transduced together ($P < .01$). Similarly, no significant difference in CYP17A1 expression was observed in cells transduced with control, NR5A1, or GATA6; however, there was a significant increase in expression when NR5A1 and GATA6 were transduced together ($P < .0001$). CYP19A1 was readily detected in PGE₂-treated cells transduced with NR5A1, but not in cells transduced with GATA6. Again, when NR5A1 and GATA6 were transduced together, the expression of CYP19A1 increased significantly compared to PGE₂-treated controls ($P < .0005$) and was significantly higher than in cells transduced with NR5A1 alone ($P < .05$). CYP11A1 protein levels could not be examined.

NR5A1 and GATA6 Support Estradiol Production in Endometrial Cells

Based on the observation that changes in NR5A1 and GATA6 levels elicited striking changes in the expression of steroidogenic genes coordinating estradiol synthesis, we next determined the functional consequences of manipulating these transcription factors on estradiol synthesis by OSIS and NoEM (Figure 4). Baseline estradiol concentrations in the OSIS cells transfected with scrambled siRNA (control) averaged 108 ± 24.9 pg/mL (Figure 4A). The PGE₂ treatment had little effect

Figure 4. A, Differences in estradiol (E₂) production in OSIS experimental groups. N = 4. Asterisk (*) indicates significantly lower concentration compared to other groups tested ($P < .05$). B, Differences in estradiol (E₂) production in normal endometrial stromal cells (NoEMs) experimental groups. N = 4. Asterisk (*) indicates a significantly higher concentration compared to other groups tested ($P < .05$). C, Differences in estradiol (E₂) production in skin fibroblast (BJ) experimental groups. N = 4.

on estradiol synthesis in OSIS. Estradiol concentrations fell to 90.4 ± 22.6 pg/mL with NR5A1 silencing, but did not achieve statistical significance. Silencing GATA6 alone also had little effect on estradiol. However, estradiol levels dropped to 54.6 ± 15.6 pg/mL when both NR5A1 and GATA6 were silenced ($P < .01$).

In NoEM cells transduced with null control adenovirus, baseline estradiol concentrations averaged 19.1 ± 4.3 pg/mL and were not affected by PGE₂ treatment (Figure 4B). The overexpression of NR5A1 in NoEM generated a

Figure 5. Roles of GATA6 and nuclear receptor subfamily 5, group A, member 1 (NR5A1) in the steroidogenic cascade.

nonsignificant increase in estradiol production (55 ± 11.7 pg/mL), which was increased to 64.4 ± 16.7 pg/mL by PGE₂ treatment. GATA6 transduction combined with PGE₂ treatment yielded a slight increase in estradiol (48.2 ± 32.4 pg/mL) over control cells. The NoEM transduced with both NR5A1 and GATA6 showed a significant increase in estradiol production: 255 ± 57.3 pg/mL in vehicle-treated cells and 520 ± 122 pg/mL in PGE₂-treated cells.

Expression of Steroidogenic Genes and Estradiol Production in Skin Fibroblasts

The changes in steroidogenic potential seen in NoEM transduced with NR5A1 and GATA6 prompted us to question whether these genes could induce the steroidogenic pathway in other cell types. Therefore, we utilized skin fibroblasts (BJ cells), a nontransformed, chromosomally normal, human skin stromal cell obtained from male foreskin. Changes in steroidogenic gene expression were examined following adenoviral transduction of NR5A1 and GATA6 alone or in combination, identical to the study performed in NoEM cells. As demonstrated in Supplemental Figure 3, mRNA and protein levels of *NR5A1* and *GATA6* in BJ cells increased following adenoviral transduction into BJ cells. Changes in steroidogenic gene expression in BJ cells were similar to our findings in NoEM. Expression of *Star* and *CYP11A1* was not significantly higher when NR5A1 and GATA6 were transduced together compared with transduction of NR5A1 alone. However, *HSD3B2*, *CYP17A1*, and *CYP19A1* expression increased significantly ($141\,000\%$ $P < .01$, $101\,000\%$ $P < .005$, and 1475% $P < .005$, respectively) when NR5A1 and GATA6 were transduced together. Supplemental Figure 3B shows similar changes in protein expression of these genes. Although steroidogenic gene expression was induced in BJ cells in response to NR5A1 and GATA6 overexpression, there was no detectable difference in estradiol production, with or without PGE₂ treatment (Figure 4C). The average across all BJ samples was 26.9 ± 1.85 pg/mL.

Discussion

These findings suggest that GATA6 alone is essential but not sufficient for transforming an endometrial stromal cell into a cell that functions similarly to an endometriotic stromal cell in regard to de novo estrogen formation. The simultaneous addition of GATA6 and NR5A1 to an endometrial stromal cell supports the activation of the entire estradiol biosynthetic

cascade (Figure 5). In endometriotic cells, silencing experiments demonstrated that GATA6 was necessary to confer PGE₂ induction of the enzymatic protein and its mRNA levels (CYP17A1) that catalyzes the conversion of progesterone to androstenedione. However, ectopic expression of GATA6 in endometrial stromal cells was essential for PGE₂ induction of the 3 mRNAs (*HSD3B2*, *CYP17A1*, and *CYP19A1*) that encode 3 proteins with 4 enzymatic activities for the conversion of pregnenolone to estrogen. GATA6 alone was not sufficient for the activation of all 5 genes (*STAR*, *CYP11A1*, *HSD3B2*, *CYP17A1*, and *CYP19A1*) for the conversion of cholesterol to estrogen. Simultaneous ectopic expression of both GATA6 and NR5A1 was required and sufficient to confer PGE₂ induction of these 5 genes and their encoded proteins.

The collective presence of both GATA6 and NR5A1 was required and sufficient to aid endometrial cells in producing estradiol in large quantities, which is critical to the development and persistence of endometriosis. Endometrial stromal cells appear to be unique in their capacity for endometriotic-like transformation via the addition of these 2 transcription factors, as the addition of the same factors to skin fibroblasts, which served as controls, induced steroidogenic gene expression but did not give rise to increased basal or PGE₂-stimulated estradiol formation. Because basal levels of estradiol were readily detectable in skin fibroblasts transfected with an empty vector, we interpreted these findings as the lack of a functional capacity for transformation to an endometriotic cell phenotype.

We acknowledge that there are some limitations in this study. The in vitro approach is a potential limitation. The in vivo demonstration of the transformation of a primary human endometrial cell to a functional endometriotic phenotype, however, is technically quite challenging. Additionally, steroidogenesis or estrogen biosynthesis from cholesterol is a remarkable aspect of endometriotic cells, but does not define endometriosis. Endometriotic cells also display other pathologic properties such as resistance to apoptosis and cytokine production, which were not assessed in this study. These additional features of an endometriotic cell remained outside of the scope of this study and will be investigated in the future. We anticipate that the stable transfection of a primary endometrial stromal cell with GATA6 and NR5A1 may produce a valuable model system to study many aspects of this enigmatic disease.

In conclusion, GATA6 is essential in conferring an endometriotic phenotype in an endometrial stromal cell. GATA6 alone, however, is not sufficient and needs to cooperate with NR5A1 to activate the entire estradiol biosynthetic cascade, which is one of the indispensable features of endometriotic cells. In this

study, we demonstrated the activation of mRNA and protein expression of a series of genes in estradiol synthesis, as well as the actual production of estradiol in an endometrial cell transformed to a cell with an endometriotic phenotype. Both GATA6 and NR5A1 were required and sufficient for the production of estradiol, and this is critical to the development and persistence of endometriosis. Given that ectopic expression of these transcription factors in an endometrial stromal cell can permit it to behave like a steroid-producing endometriotic cell, this strategy can be used to generate high-fidelity model systems of endometriosis. As an understanding of the impact of GATA6 improves, insight into new diagnostic and treatment strategies may also be gained.

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Supplemental Material

Supplementary material is available for this article online.

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