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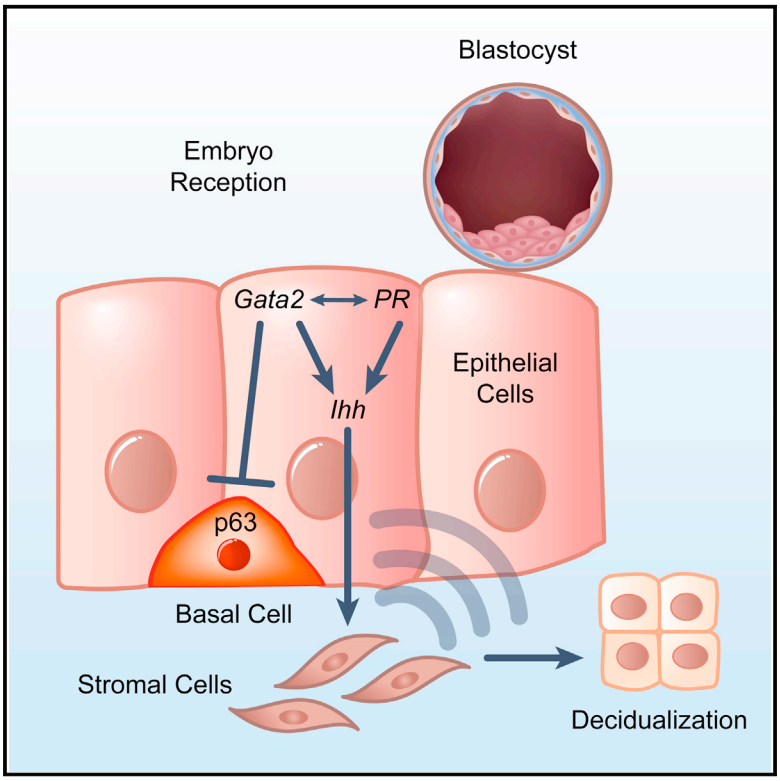
A *Gata2*-Dependent Transcription Network Regulates Uterine Progesterone Responsiveness and Endometrial Function

Graphical Abstract



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In Brief

Rubel et al. find that in the uterus, *Gata2* regulates the expression of progesterone receptor and its ability to modulate transcription of genes required for receptivity and support of embryo implantation. *Gata2* is critical for the uterus to maintain epithelial integrity and prevent stratification in response to an estrogen challenge.

Highlights

- *Gata2* regulates the ability of the uterus to support embryo implantation
- *Gata2* regulates uterine expression and action of the progesterone receptor
- *Gata2* regulates uterine epithelial differentiation
- A *Gata2* expression signature is present in the human endometrium

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A *Gata2*-Dependent Transcription Network Regulates Uterine Progesterone Responsiveness and Endometrial Function

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SUMMARY

Altered progesterone responsiveness leads to female infertility and cancer, but underlying mechanisms remain unclear. Mice with uterine-specific ablation of *GATA binding protein 2* (*Gata2*) are infertile, showing failures in embryo implantation, endometrial decidualization, and uninhibited estrogen signaling. *Gata2* deficiency results in reduced progesterone receptor (PGR) expression and attenuated progesterone signaling, as evidenced by genome-wide expression profiling and chromatin immunoprecipitation. GATA2 not only occupies at and promotes expression of the *Pgr* gene but also regulates downstream progesterone responsive genes in conjunction with the PGR. Additionally, *Gata2* knockout uteri exhibit abnormal luminal epithelia with ectopic TRP63 expressing squamous cells and a cancer-related molecular profile in a progesterone-independent manner. Lastly, we found a conserved *GATA2-PGR* regulatory network in both human and mice based on gene signature and path analyses using gene expression profiles of human endometrial tissues. In conclusion, uterine *Gata2* regulates a key regulatory network of gene expression for progesterone signaling at the early pregnancy stage.

INTRODUCTION

Central to women's health is the regulation of uterine function by the ovarian steroid hormones, estradiol (E2) and progesterone (P4), acting through their receptors, the estrogen receptor (ESR1), and progesterone receptor (PGR). PGR is critical for uterine function as shown in the uteri of *Pgr*-null (PRKO) female mice that are unable to support embryo implantation, undergo decidualization, or inhibit E2-induced endometrial epithelial proliferation with P4 (Lydon et al., 1995). Identifying modifier genes of PGR action and elucidating their interaction will facilitate a comprehensive understanding of mechanisms of P4 actions.

We have identified the transcription factor *GATA binding protein 2* (*Gata2*) as a potential modifier of uterine P4 signaling (Rubel et al., 2012a). Genetic ablation of *Gata2* regulates many biological processes, such as hematopoietic cell development (Tsai et al., 1994), vascular integrity (Johnson et al., 2012), adipocyte differentiation (Tong et al., 2000), and pituitary function (Charles et al., 2006). *Gata2* is expressed in the epithelium and stroma of the uterus and the epithelial expression coincides with that of *Pgr* (Rubel et al., 2012a). *Gata2* has been previously described as coordinating the transcriptional activity of other nuclear receptors including PGR (Böhm et al., 2009; Magklara and Smith, 2009; Nagayama et al., 2008). These findings suggest GATA2 maybe a modifier of P4 signaling in the uterus through interaction with PGR on the chromatin of P4 target genes.

We addressed the role of *Gata2* by using the Pgr^{Cre} mouse model to ablate *Gata2* in cells expressing *Pgr* in all compartments of the uterus (Soyal et al., 2005). Mice with *Gata2* ablation in the uterus were infertile, lacking both the ability to allow embryo implantation and subsequent uterine stromal decidualization. Importantly, the expression of P4 target genes and *Pgr* itself were deregulated. Combining cistromic analysis with transcriptomic analysis, we showed that most P4-regulated genes contained both PGR and GATA2 chromatin occupancy demonstrating a cooperative relationship between the two factors in controlling P4-mediated transcription in the mouse uterus. *Gata2*-ablated uteri showed alterations in epithelial morphology

resulting in a transition from a simple epithelium to a stratified squamous epithelium with increased TRP63 expression. Lastly, we present evidence that supports a conservation of the *GATA2-PGR* regulatory network in human uteri.

RESULTS

In Vivo Regulation of *PGR* Levels and Fertility by *GATA2*

We first examined the correlation between *GATA2* and *PGR* in the human endometrium. *GATA2* and *PGR* mRNA levels are positively correlated in human endometrial tissues from three independent cohorts, NCBI: GSE4888, GSE58144, and GSE51981 (Figures 1A and 1B), supporting a conservation of the expression between mouse and human. Given this inference and the role of GATA2 in transcription regulation, we hypothesized that *GATA2* may regulate P4 signaling through modulating *PGR* in uterus.

To test this hypothesis, we crossed mice with a floxed *Gata2* allele (*Gata2*$^{f/f}$) with the *Pgr*^{cre} mouse to ablate *Gata2* in PGR-positive cell lineages (*Pgr*^{cre}; *Gata2*$^{f/f}$, *Gata2*$^{d/d}$) (Charles et al., 2006; Soyal et al., 2005). *Gata2* deletion was confirmed by qRT-PCR (Figure 1C). Expression of other *Gata* genes did not show a compensatory increase in expression in *Gata2*$^{d/d}$ uteri (Figure 1C). In fact, *Gata2* is the most abundant *Gata* factor in uterus with its mRNA levels at least 200-fold more than the second-highest expressing *Gata* factor, *Gata6* (Figure 1C). The fertility of *Gata2*$^{d/d}$ mice was determined after a 6-month long breeding trial. Six *Gata2*$^{f/f}$ females delivered a total of 251 pups in 33 litters while all six *Gata2*$^{d/d}$ mice failed to generate any offspring, demonstrating that the *Gata2*$^{d/d}$ mice were infertile. To determine the cause of the infertility, *Gata2*$^{d/d}$ and *Gata2*$^{f/f}$ mice were analyzed on day 5.5 of pregnancy to assess if normal embryo implantation occurred. The *Gata2*$^{d/d}$ mice showed no embryo implantation sites compared to that of *Gata2*$^{f/f}$ that averaged seven to eight sites per mouse (Figures 1D and 1E; Table 1), demonstrating that loss of *Gata2* function leads to failure of embryo implantation.

The *Pgr*^{Cre} mouse also ablates genes in the granulosa cells of the preovulatory ovarian follicle and gonadotropes of the pituitary. To rule out an ovarian and/or pituitary cause of infertility, both *Gata2*$^{f/f}$ and *Gata2*$^{d/d}$ mice were subject to a superovulatory regimen of gonadotropins, and the results showed that *Gata2*$^{d/d}$ mice ovulated a similar numbers of eggs as control *Gata2*$^{f/f}$ mice (27 ± 3.0 versus 23.33 ± 4.37, respectively). Furthermore, serum P4 levels at Day 5.5 of pregnancy were similar in both the *Gata2*$^{d/d}$ mice and *Gata2*$^{f/f}$ mice (Table 1). Thus, the ability to ovulate and the normal serum P4 levels in combination with the fact that pituitary-specific *Gata2* knockout models are fertile (Charles et al., 2006) confirmed that the *Gata2*$^{d/d}$ infertility phenotype is not due to impairment of the hypothalamus-pituitary-ovarian axis but intrinsic to the uterus.

To demonstrate that the infertile phenotype of the *Gata2*$^{d/d}$ mice was of uterine origin, we assayed the ability of the uterus to undergo a decidual reaction in response to a regimen of E2 and P4. Although *Gata2*$^{f/f}$ mice showed typical increase in uterine weight, changes in uterine stromal cell shape and alkaline phosphatase staining in the stimulated horn compared to the unstimulated horn (Figures 1F and 1G), *Gata2*$^{d/d}$ mice failed to respond and showed no increase in uterine size, weight, stroma cell morphology, and alkaline phosphatase activities (Figures 1H, 1I, S1A, and S1B). In addition to these changes, the mRNA of genes known to be critical for decidualization, bone morphogenetic protein 2 (*Bmp2*), wingless-type MMTV integration site family, member 4 (*Wnt4*), follistatin (*Fst*), FK506 binding protein 4 (*Fkbp4*), FK506 binding protein 5 (*Fkbp5*), and prostaglandin-endoperoxide synthase 2 (*Ptgs2*) were significantly attenuated in the stimulated horn of *Gata2*$^{d/d}$ mice compared to the stimulated horn of *Gata2*$^{f/f}$ (Figure S1C). These findings collectively indicate an impaired decidualization phenotype because of *Gata2* deficiency.

Because the *Gata2*$^{d/d}$ phenocopied the *Pgr*-null mice (Lydon et al., 1995), we investigated if PGR expression was affected by *Gata2* deficiency. In order to control for variations of PGR levels caused by endogenous hormones, we performed PGR western blot analysis and immunostaining on uteri of ovariectomized (OVX) mice. *Gata2* deficiency led to a significant decreased expression of both PGR-A and PGR-B isoforms (Figure 1N) that could be seen in a reduction of PGR protein in the luminal and glandular epithelia at both baseline and in response to P4 stimulation (Figures 1J–1M). These results collectively indicate that *Gata2* is required for PGR expression.

Deregulated P4 Signaling in the Pre-implantation *Gata2*$^{d/d}$ Uterus

To determine whether the reduced PGR expression in *Gata2*$^{d/d}$ mice impaired P4 signaling, we assayed the expression of PGR target genes in OVX *Gata2*$^{d/d}$ and *Gata2*$^{f/f}$ uterus 6 hr following P4 (1 mg, subcutaneously [s.c.]) or vehicle (oil) treatment. *Gata2*$^{f/f}$ mice showed an increase in the expression of the PGR-regulated genes amphiregulin (*Areg*), cytochrome P450, family 26, subfamily A, polypeptide 1 (*Cyp26a1*), and Indian Hedgehog (*Ihh*) (Figures 2A–2C). In contrast, *Gata2*$^{d/d}$ mice showed a complete loss of P4-dependent induction of *Areg* and *Cyp26a1* (Figures 2A and 2B) as well as reduced *Ihh* expression at both baseline and in response to P4 (Figure 2C). These results indicate that the P4 signaling is attenuated on PGR-dependent marker genes in *Gata2*$^{d/d}$ mice.

The global impact of *Gata2* deficiency on P4 signaling was assayed by conducting microarray analysis on the uteri of OVX *Gata2*$^{f/f}$ and *Gata2*$^{d/d}$ mice with P4 treatment. Comparison between P4- versus vehicle-treated groups identified 1,861 P4 response genes (Figure 2D, green box; Table S1) in *Gata2*$^{f/f}$ mice, whereas only 139 genes responded to P4 stimulation in *Gata2*$^{d/d}$ mice (Figure 2D, red box; Table S2). Strikingly, P4 failed to regulate 1,814 (97%) of P4 response genes in *Gata2*$^{d/d}$ mice (Figure 2D, Venn diagram). Moreover, of the 47 P4 response genes that remained responsive to P4 stimulation in the *Gata2*$^{d/d}$ background, many genes exhibit diminished magnitude on change of gene expression in response to P4 (Figure 2E). We also identified 2,461 probes that represent the *Gata2*-dependent transcription profile in the uterus by comparing expression arrays of *Gata2*$^{f/f}$ and *Gata2*$^{d/d}$ mice under P4 treatment (Figure 2F; Table S3). Based on this profile, the Ingenuity Pathway Analysis (IPA) predicted inhibition of P4 signaling (activation *Z* score −4.05) and activation of P4 antagonist mifepristone (activation *Z* score 2.09) (Figure 2G). These

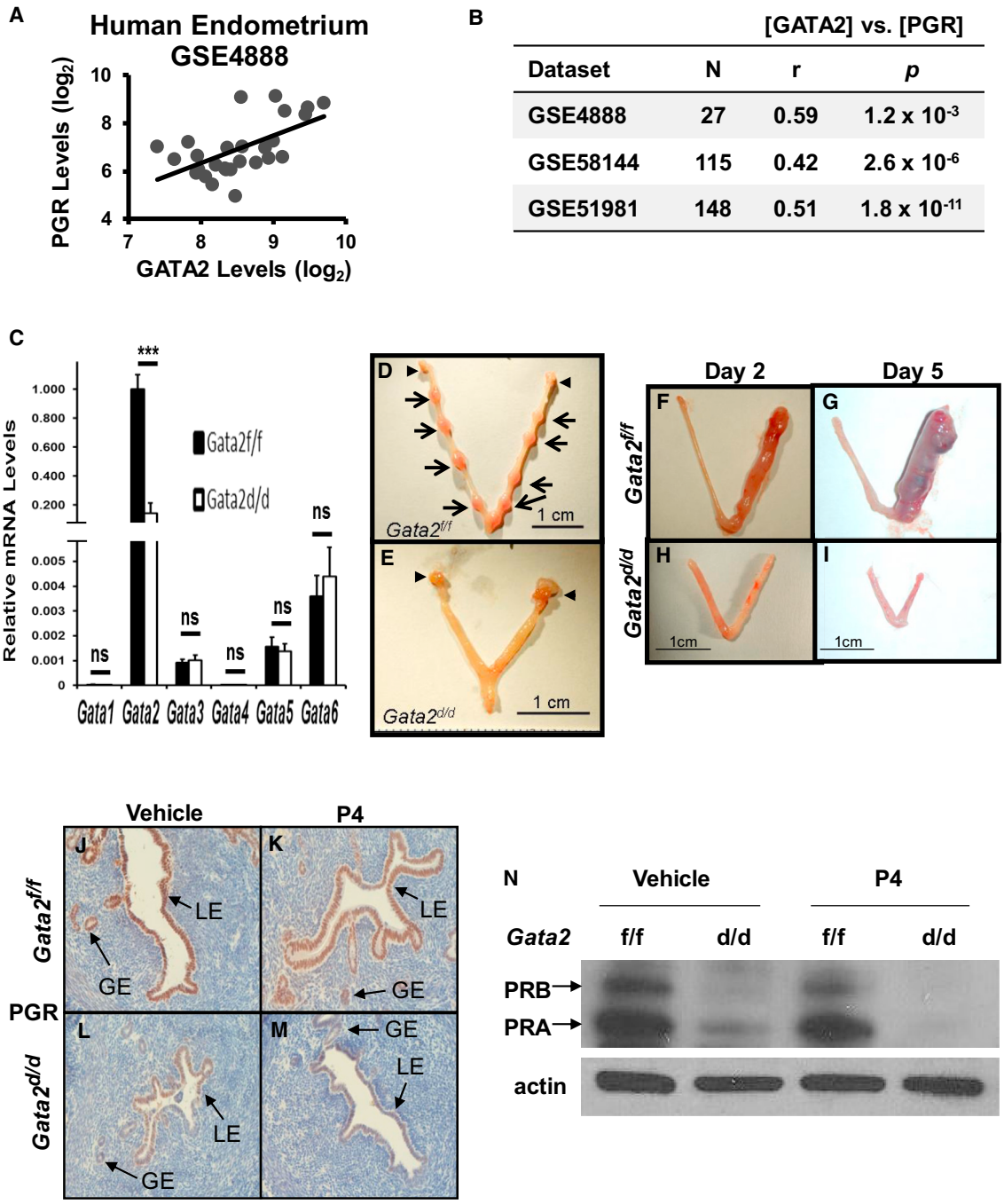


Figure 1. Regulation of PGR Expression by GATA2

(A and B) Correlation between *GATA2* and *PGR* mRNA levels in human tissues. N, number of samples; r, Pearson's correlation coefficient.

(C) mRNA levels of *Gata* factors in wild-type and *Gata2*^{dd} mouse uteri by qRT-PCR. N = 3 for each group.

(D and E) Uteri, pregnancy day 5.5 mice of denoted genotypes. Arrows mark the embryo implantation sites. Arrowheads denote ovaries.

(F–I) Morphology of the decidual response in day 2 and day 5 post decidual stimulation. Right horn was stimulated in all groups.

(J–N) PGR levels in uteri of OVX 6-week-old mice treated with 1 mg P4 or vehicle for 6 hr. (J–M) Immunohistochemical analysis of PGR protein. LE, luminal epithelium; GE, glandular epithelium. (N) Protein levels of both isoforms of the PGR by western blot. Error bars represent SEM. ***$p < 0.001$, ns, $p > 0.05$ by Student's t test.

See also Figure S1.

Table 1. Count of Embryo Implantation and Serum P4 Levels

Female Genotype	No. of Females with Implantation Sites	Average No. of Implantation Sites	Average Size of Decidual Balls (mm)	Serum Progesterone Levels (ng/mL)
Gata2$^{f/f}$ N = 10	6	7.67 ± 0.42 (6)	1.93 ± 0.06 (6)	32.94 ± 6.44
Gata2$^{d/d}$ N = 6	0	0	N/A	30.03 ± 6.29

Numbers in parenthesis denote the number of female mice used for analyses. N/A, not applicable.

results collectively indicate the requirement of *Gata2* to sustain PGR expression for P4 signaling.

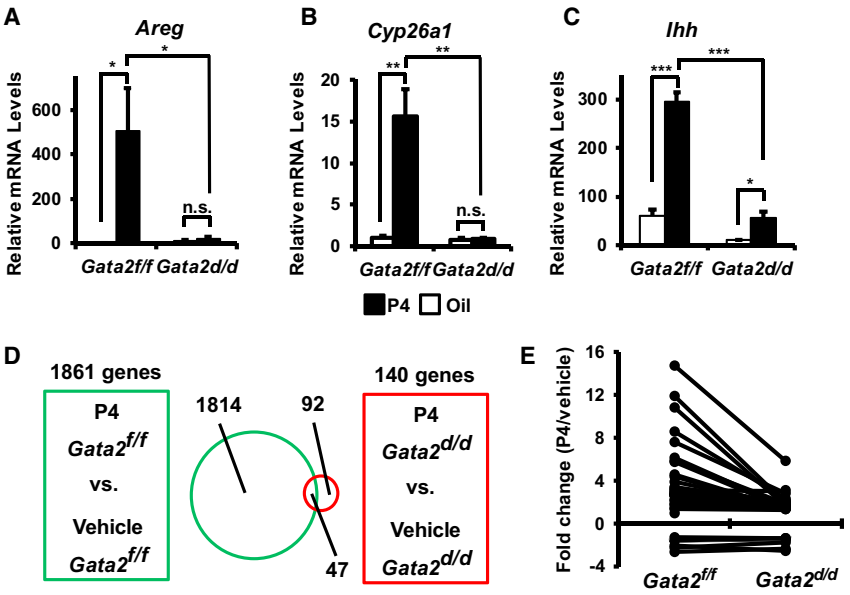
Suppression of uterine E2 signaling and E2-driven epithelial proliferation is a hallmark of P4-induced preparation for subsequent embryo implantation (Wetendorf and DeMayo, 2014). Compared with *Gata2*$^{f/f}$ uteri, *Gata2*$^{d/d}$ day 3.5 pseudopregnancy uteri showed an increased level of phosphorylation at serine 188 on ESR1 as well as increased expression of the target Mucin 1, transmembrane (MUC1) (Figure S2A) with no significant change in *Esr1* mRNA levels (Figure S2B). Thus, the *Gata2*$^{d/d}$ mice exhibited higher E2 signaling activities without elevated ESR1 levels. We also observed altered proliferation of the uterine epithelia in response to heightened E2 signaling after loss of *Gata2*. Normally, epithelial proliferation is high at day 2.5 and diminished at day 3.5 (Figures S2C and S2D). In contrast, uterine epithelial proliferation at day 3.5 remained as high as at day 2.5 in the *Gata2*$^{d/d}$ mice (Figures S2C and S2D). Taken together, these results demonstrate uninhibited E2 signaling and reduced P4 signaling as a result of *Gata2* deficiency.

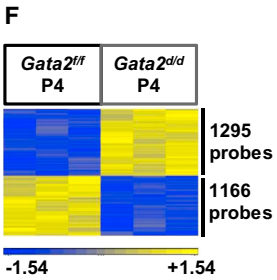
The mechanism by which *Gata2* regulates PGR expression was investigated by determining the ability of *Gata2* to regulate the expression of PGR at the level of *Pgr* gene transcription. *Pgr* mRNA levels was reduced in *Gata2*$^{d/d}$ uteri compared to controls (Figure 3A). Chromatin immunoprecipitation qPCR (ChIP-qPCR) analysis further shows enriched GATA2 occupancy at a region that consists of three predicted GATA binding motifs upstream and proximal to the *Pgr* promoter in uteri of OVX *Gata2*$^{f/f}$ mice 1 hr post P4 treatment (Figures 3B and 3C). This enrichment was absent in *Gata2*$^{d/d}$ mice and in the exon 4 negative control region devoid of GATA binding motifs (Figures 3B and 3C), supporting the finding of GATA2 occupancy at the *Pgr* locus. Results from luciferase reporter analysis in cultured epithelial cells showed that the *Pgr* promoter was stimulated with the full length *Gata2* construct and with the amino zinc finger deletion (ΔNT) construct but not with the carboxyl zinc finger deletion (ΔCT) construct (Figure 3D). This is consistent with a previous finding that the carboxyl zinc finger domain is involved in transcriptional regulation by GATA2 (Tong et al., 2000). The in vitro GATA2ΔCT mutant protein results support the in vivo functional loss of our *Gata2* mutant mice in which the carboxyl zinc finger domain is deleted upon cre-mediated excision. Interestingly, deleting the amino zinc finger domain of GATA2 did not affect transactivation activity by GATA2 in the *Pgr* promoter (Figure 3D), despite a previous report that both zinc fingers are important for suppressing the *PPARγ2* promoter (Tong et al., 2000). Our results demonstrate that GATA2 can enhance *Pgr* promoter activity with its carboxyl zinc-finger domain. Collectively, we find that *Pgr* is a direct downstream target of GATA2.

Significant Overlap between GATA2 and PGR Binding on Uterine P4 Response Genes

Due to the observed interdependence between PGR and GATA2 expression in uterine P4 signaling, we hypothesized that both transcription factors are required for the regulation of target gene expression. This hypothesis was tested by conducting chromatin immunoprecipitation sequencing (ChIP-seq) analysis for GATA2 on uterine chromatin isolated from OVX C57BL/6 mice treated with 1 mg P4 s.c. for 1 hr and immunoprecipitated for GATA2 (Rubel et al., 2012b). Whole uterine analysis resulted in over 22 × 10^6 tags mapping to unique locations in the mouse genome. A model-based analysis peak-finding algorithm (MACS) (Zhang et al., 2008) was utilized to normalize immunoprecipitated chromatin against uterine input generating a false discovery rate of essentially zero. This high-confidence cutoff produced 46,183 GATA2 binding intervals. Analysis for DNA motifs in the GATA2 cistrome revealed the expected GATA binding motifs as well as PGR binding motifs within the GATA2-occupying intervals (Figures 4A and 4B). To investigate whether this PGR binding motif enrichment in GATA2 occupying sites is uterus-specific, we compared GATA2 occupancy patterns and motif enrichment between two distinct tissue types, the uterus and HPC7 hematopoietic precursor cells (Wilson et al., 2010). Among 46,183 uterine and 9,243 HPC7 cell GATA2 occupying sites, GATA2 exhibited distinct cistrome profiles with 96.7% and 83.3% sites unique to uterus and HPC7 cells, respectively (Figure S3A). This finding is similar with a previous study in which GATA2 cistromes are significantly different between precursor cells and differentiated mast cells (Calero-Nieto et al., 2014). Motif analyses further revealed that the uterine GATA2 cistrome contained binding motifs enriched for nuclear receptors, such as PGR, glucocorticoid receptor (GR), and androgen receptor (AR) among others (Table S4), while such enrichment was not present in the HPC7 GATA2 cistrome (Table S4). Similarly, motifs of Sex Determining Region Y Box (SOX) factors are also enriched in the uterine, but not the HPC7 cell GATA2 cistrome (Table S4). It is worthy to note that SOX17 is also expressed in the uterine epithelial cells (Rubel et al., 2012b) where GATA2 is expressed and functions in the process of embryo implantation (Hirate et al., 2016). These findings revealed a uterine-specific GATA2 occupancy pattern that is associated with the presence of binding motifs of transcription factors, such as PGR and SOX17, that have been known to regulate uterine function.

Because PGR binding motifs are enriched in the GATA2 cistrome, we aligned the GATA2 location data with previous PGR ChIP-seq performed for mouse uterus (Rubel et al., 2012b). These PGR ChIP-seq data were also conducted on the uteri of OVX mice treated with 1 mg P4 for 1 hr. Through overlaying the GATA2- and PGR-occupying genes (defined by having at





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Predicted Regulator	Predicted Activation State	Activation Z-score	*p* value of overlap
Progesterone	Inhibited	-4.05	1.15 X 10^{-24}
Mifepristone	Activated	2.09	3.72 X 10^{-7}

Figure 2. Impaired P4 Signaling in *Gata2*-Deficient Uterus

OVX mice were treated with vehicle (oil) or 1 mg P4 for 6 hr.

(A–C) mRNA levels of PGR target genes assayed by qRT-PCR. n = 3 for each group.

(D and E) Gene expression profiling by microarray assays on denoted treatments and genotypes. (D) Venn diagram of numbers of P4-responsive genes in denoted genotypes. (E) Magnitude of P4-responsiveness on expression of the common P4-responsive genes between denoted genotypes in (D).

(F and G) Genome-wide expression profiles of *Gata2*-dependent genes in the presence of P4. (F) Heatmap of hierarchically clustered profiles. Low expression values are blue and high expression values are yellow. n = 3 for each group. (G) Predicted regulators of *Gata2*-dependent genes by the Ingenuity Pathway Analysis. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's t test.

See also Figure S2 and Tables S1, S2, S3, and S7.

least one ChIP interval within 25 kb of annotated gene boundaries) with P4-responsive genes derived from the microarray results, we found GATA2 occupancy in 1,478 (79%) and PGR occupancy in 1,013 (54%) P4 response genes (Figure 4C). Strikingly, GATA2 and PGR occupying sites are present together on 935 (50%) genes regulated by P4 (Figure 4C). We validated the GATA2 occupancy on 13 genes (15 locations total) that are previously identified to contain PGR binding sites (Rubel et al., 2012b). All validated sites were within known P4 responsive genes, and several of these GATA2 occupying locations directly overlapped with those of PGR occupying sites (Figure S3B). In summary, these findings suggest a major regulatory mechanism of P4 response genes jointly by GATA2 and PGR.

Joint Regulation of Transcription by GATA2 and PGR

The functionality of the overlap of PGR and GATA2 intervals in the regulation of gene expression was tested by conducting luciferase reporter analysis on putative enhancers containing overlapping PGR and GATA2 binding sites from chromatin locations in two P4 target genes, *Sox17* and *Ihh* (Figure 4D) (Lee et al., 2006; Rubel et al., 2012b). The GATA2-PGR intervals were subcloned into a luciferase vector containing a minimal promoter and then co-transfected with combinations of vectors expressing PGR-A, PGR-B, and GATA2 to evaluate the possible regulatory preferences of these factors on these putative enhancers of P4 target genes. The results showed that luciferase activities under the control of these putative enhancers increased in response to progestin (R5020) treatment in a *Pgr* isoform-specific manner (Figures 4E and 4F). No induction of these reporter genes was observed with cotransfection of the *Gata2* alone. However, when *Gata2* was cotransfected with the *Pgr* isoforms, these reporter plasmids showed a significant induction in luciferase activity in the presence of R5020 treatment (Figures 4E and 4F). These results are consistent with our hypothesis that PGR and GATA2 can coordinate the expression of these target genes and with the observation of GATA2 regulating PGR response genes in vivo (Figures 2C and S3C).

The ChIP-seq result also identified multiple GATA2 occupying sites in the *Pgr* locus (Figure S4A), including the proximal promoter region that contains predicted GATA binding motif and is corresponding to the *Pgr* promoter fragment in the luciferase construct (Figure S4B). Interestingly, the *Pgr* proximal promoter region exhibited a high GATA2 and low PGR occupying profile (Figure S4B). This occupancy pattern is consistent with the luciferase promoter assay result where GATA2 alone can enhance the *Pgr* promoter activity (Figure 3D), which is in contrast with the GATA2/PGR co-occupying enhancers where GATA2 co-activated transcription with PGR (Figures 4D–4F). Our results demonstrate that GATA2 employs two mechanisms to regulate P4 signaling through directly controlling *Pgr* gene transcription as well as co-regulating PGR downstream gene expression with PGR.

Progesterone-Independent Function of *Gata2* in Suppressing *p63*

The role of *Gata2* in hormone-independent gene regulation of uterine function was investigated by comparing the expression

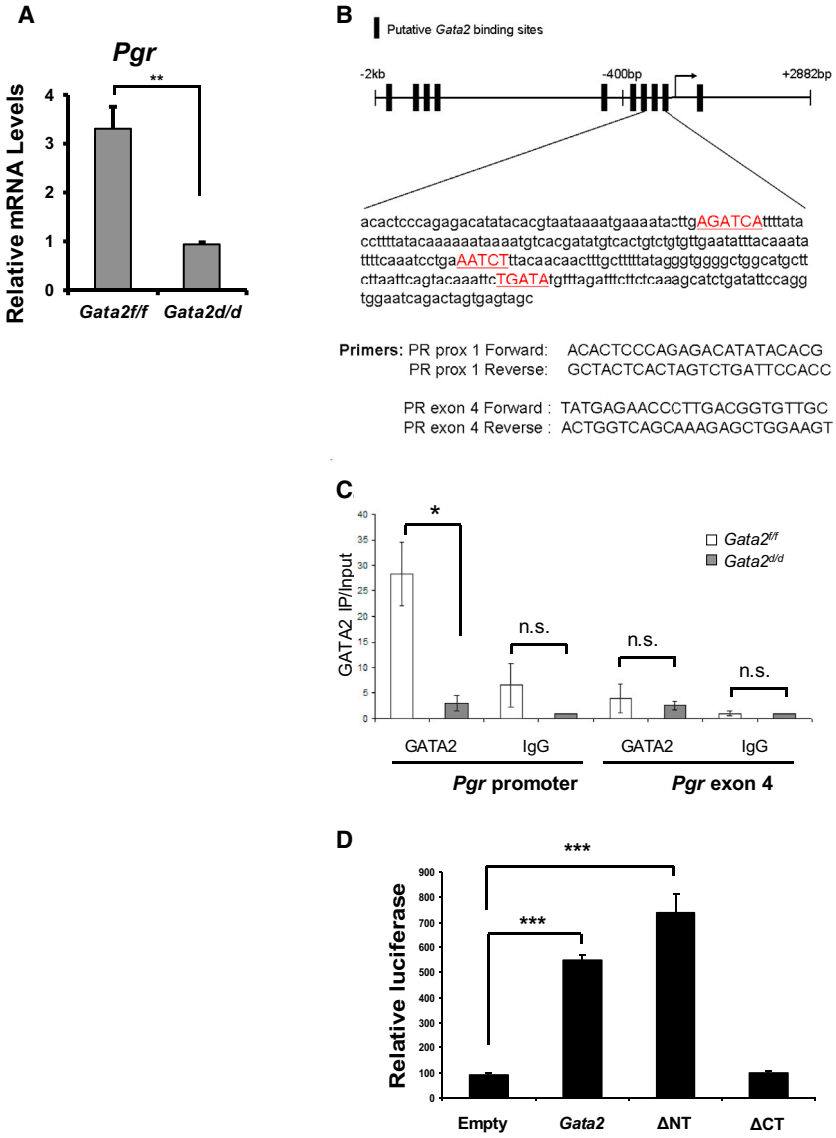

Figure 3. Binding and Regulation of *Pgr* by GATA2

(A) *Pgr* mRNA levels by qRT-PCR in uteri of OVX mice acutely treated with vehicle for 6 hr.

(B) Gata2 binding sites in the Pgr promoter. Red denotes GATA2 binding motifs. PCR primers used for ChIP-qPCR analysis are listed here.

(C) ChIP-qPCR analysis of GATA2 occupancy on *Pgr* proximal promoter in uteri 1 hr after P4 treatment in OVX mice.

(D) Luciferase reporter analysis of *Pgr* promoter activities in response to various GATA2 expression vectors. GATA2, full-length GATA2; ΔNT, GATA2 N-terminal zinc-finger deleted mutant; ΔCT, GATA2 C-terminal zinc-finger-deleted mutant. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test.

profiles of *Gata2*$^{d/d}$ and *Gata2*$^{f/f}$ treated only with vehicle. Microarray analysis revealed 426 genes upregulated and 535 downregulated (Table S5). IPA revealed enrichment in cancer and proliferation of keratinocytes/epidermal cells (Figure 5A), as evidenced by the *Gata2*$^{d/d}$ uteri exhibiting ectopic expression of TRP63 in a layer of cells, morphologically similar to basal cells, underneath the columnar luminal epithelia (Figure 5D). Because TRP63 is a molecular switch for squamous metaplasia (Koster et al., 2004) associated with in utero exposure to the synthetic E2 diethylstilbestrol (DES) (Franco et al., 2011; Goldberg and Falcone, 1999; Sassoon, 1999), we hypothesized that *Gata2* deficiency may increase susceptibility in developing squamous metaplasia in response to E2 signaling. Long-term treatment of OVX mice with E2 resulted in development of focal regions of TRP63-positive cells into squamous cell metaplasia as early as 2 weeks post treatment (Figure 5F), with *Trp63* expression over time (Figures 5G and S5). An increase in expression of keratin genes, including *Krt5* and *Krt15*, in *Gata2*$^{f/f}$ mice (Figures 5H and 5I) was also observed. These changes were not observed in the control mice (Figure 5E). In summary, our results revealed a *Gata2*-dependent and P4-independent mechanism in suppression of *Trp63* expression and a role in squamous metaplasia. This demonstrates that *Gata2* is critical for the uterine epithelium to maintain its differentiated state.

A Conserved *GATA2*-Dependent Regulatory Network in Human Endometrium

The clinical relevance of our findings in mouse models was evaluated by determining whether there is an association between the *GATA2* and P4 signaling in human uterine expression databases. We first identified human orthologs of *Gata2*-regulated mouse genes in the presence of P4 (Figure 2F). Based on these *GATA2* reporter genes originally derived from the mouse (Figure 6A, top panel), we then determined the human *GATA2* gene activities in individual endometrial tissue of 115 human samples (NCBI: GSE58144) (Koot et al., 2016). A positive correlation between *GATA2* levels and activities in human samples, just as in mouse, was observed (Figures 6A and S6A). Moreover, in the same cohort of human samples, we found *GATA2* activities are positively correlated with levels of *PGR* and of members of *PGR* signaling components *PTCH1*, *AREG*, *FKBP4*, *PTGS2*, and *SOX17* (Figures 6A and S6A). Taken together, these results suggest a conserved *GATA2* regulatory network in human and mouse endometrium.

Results from our studies and others revealed a regulatory network for female fertility (Figure 6B) that consists of GATA2, PGR, and SOX17 transcription factors (Guimarães-Young et al., 2016; Hirate et al., 2016; Rubel et al., 2012a, 2012b). To investigate this network in human endometrial samples, we

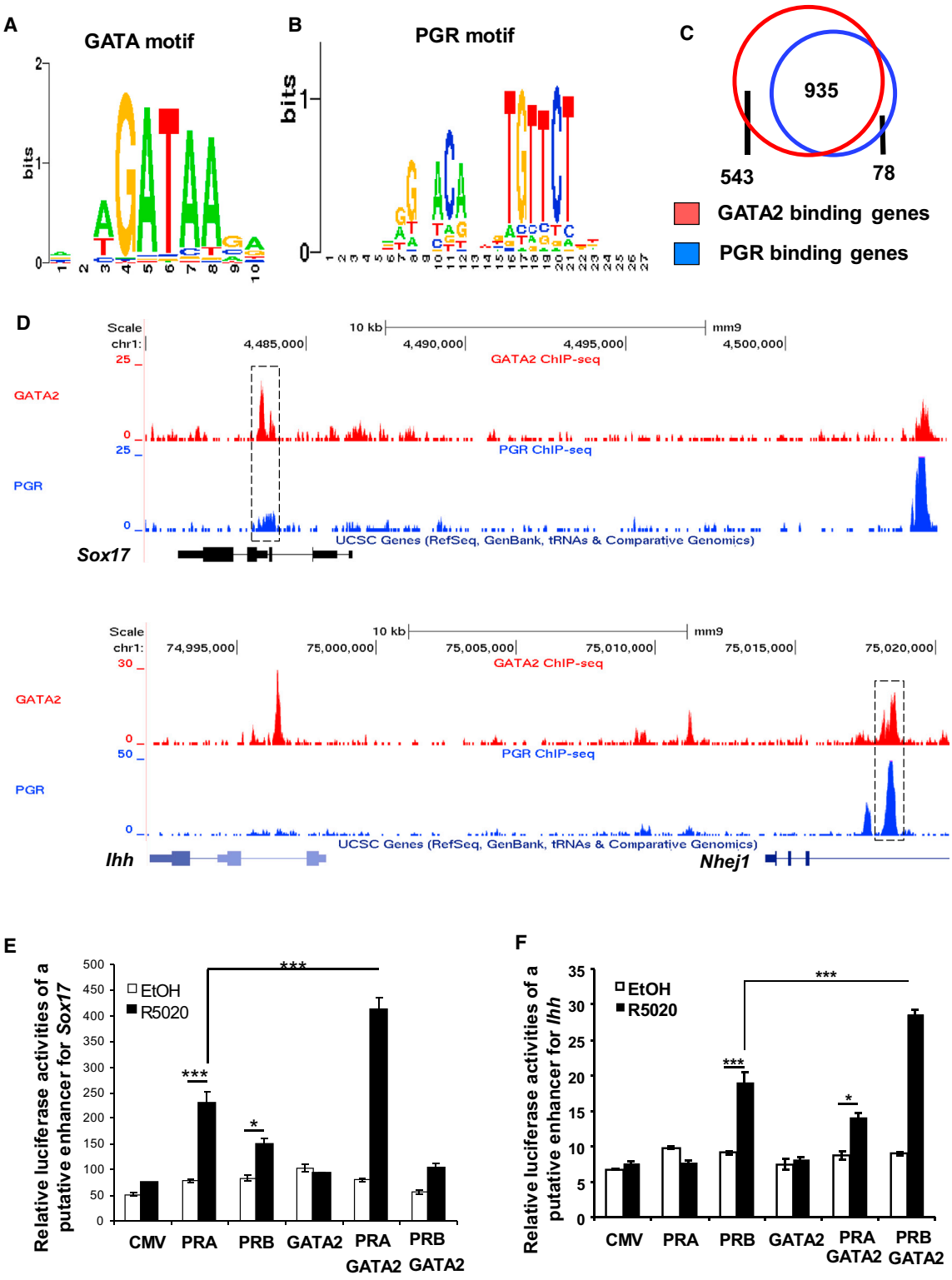

Figure 4. Binding of GATA2 and PGR at Loci of Uterine P4-Responsive Genes

(A and B) Enriched GATA2 and PGR binding motifs at GATA2 binding intervals that were identified by ChIP-seq in OVX mouse uteri 6 hr after P4 treatment.

(C) Venn diagrams of P4-regulated genes that contain binding sites for PGR or GATA2 within ±25 kb of gene boundaries.

(D) Occupancy of PGR and GATA2 in putative enhancers for *Sox17* and *Ihh* genes in P4-treated uteri. Blue indicates PGR while red indicates GATA2 occupancy. Brackets mark the regions used for luciferase reporter analysis.

(legend continued on next page)

performed path analyses on the NCBI: GSE58144 dataset using a structural equation modeling (Xiong et al., 2004). First, we examined a model in which GATA2 and PGR are mutually regulated and both transcription factors jointly modulate SOX17, based on mRNA levels of each gene (Figure 6C). This model significantly fits the data from NCBI: GSE58144 with all proposed paths (Figure 6C). The fit indices of the tested model all provided evidence of a close fit in which root-mean-square error of approximation (RMSEA) is <0.001, standardized root-mean-square residual (SRMR) is <0.001, comparative fit index (CFI) equals to 1.0, and Tucker Lewis fit index (TLI) equals to 1.0 (Figure S5B). Based on results of these indices, this model is considered not rejected by the human data. Additionally, both *GATA2* and *PGR* have significant direct effects on *SOX17* ($p < 0.05$). Overall, higher *GATA2* and *PGR* levels were associated with higher *SOX17* levels. Furthermore, using the *GATA2* signature (Figure 6A) and the *PGR* signature (derived from NCBI: GSE39920, Table S6) as molecular activities to replace *GATA2* and *PGR* levels in the model also yielded statistically significant results (Figures 6D and S6B). Collectively, these findings suggest the presence of a *GATA2-PGR-SOX17* regulatory network in the NCBI: GSE58144 dataset of human endometrial tissues.

Next, we tested the model of joint regulation of the P4 signaling by *GATA2* and *PGR*. Activities of human P4 signaling in NCBI: GSE58144 were generated based on a mouse P4 signature (Figure 2D). Model fitting of data from NCBI: GSE58144 via structural equation modeling shows statistical significance on all proposed paths ($p < 0.05$) (Figure 6E) while the model is considered not rejected by the human NCBI: GSE58144 data based on RMSEA, SRMR, CFI, and TLI indices (Figure S6B). In summary, these results suggest that, in humans, *GATA2* and *PGR* together regulate P4 signaling and form a network with *SOX17* to direct a transcription program for female fertility.

DISCUSSION

Our study demonstrated that GATA2 modulates PGR signaling via regulation of *Pgr* transcription and through GATA2-PGR interaction in transcriptional regulation of P4 response genes. These results are consistent with a previous study that shows that binding of GATA2 with PGR is essential for the expression of a known P4 target gene *Fkbp5* in mouse mammary cancer cells (Magklara and Smith, 2009). Thus, in conjunction with our previous findings on regulation of *Gata2* by PGR (Rubel et al., 2012a), we show the reciprocal regulation of *Gata2* and *Pgr* that allows both genes to be present to regulate the transcription of key genes involved in the preparation of the mouse uterus for embryo implantation. This finds further support from the model fitting analysis in human endometrial tissues, suggesting a conservation of the molecular mechanism across species. Several studies have provided evidences for the cooperative nature of GATA2 with the androgen receptor (AR) in androgen-induced gene response, while the expression of AR itself is also regulated by GATA2 (Perez-Stable et al., 2000; Wu et al., 2014). Given that androgen response elements (AREs) contain sequence homology with that of P4 response elements (PREs), our study highlights the amazing resemblance between GATA2-PGR and GATA2-AR in regulation of gene expression.

Recently, *GATA2* has been reported to be downregulated in endometriosis samples in humans in Dyson et al. (2014). They reported that *PGR* expression failed to respond to manipulation of *GATA2* levels, which is different from our observation from the in vivo mouse model. This discrepancy likely resulted from the cell type difference; we primarily observed the *Gata2-Pgr* interaction in epithelial cells while Dyson et al. (2014) conducted experiments in stromal cells. Owing to the fact that OVX mice are in the hormone-deprived state during which basal expression of PGR is restricted to the luminal epithelium and glands (Figures 1I and 1K), the epithelial derived P4 signaling is likely the primary source of acute P4 responses observed in our studies.

Interestingly, as a P4-independent phenotype, ablating *Gata2* in the uterus resulted in endometrial epithelial morphology alteration from simple columnar epithelium into stratified squamous epithelium, in association with increased TRP63 expression. TRP63, particularly the delta N isoform, has been suggested as a driver in the developmental switch from a simple epithelium to a squamous epidermal fate (Romano and Sinha, 2011). The stratified endometrial epithelial phenotype shown here is similar to exposure to DES in fetal or neonatal mice (Iguchi and Takasugi, 1987; Plapinger, 1981). A similar disruption of human uterine differentiation was observed when DES was mistakenly prescribed to pregnant women during 1947–1971 under the assumption of preserving the pregnancy. This exposure associated with increasing reproductive tract anomalies including vaginal adenosis and endometrial cancer in their daughters (Haney et al., 1979; Herbst et al., 1971). Due to the dependence of E2 during this process, we further demonstrated that loss of *Gata2* resulted in a squamous cell metaplasia phenotype in the glandular and luminal epithelium that was marked by the presence of TRP63 after chronic E2 treatment. Uterine disruption of a number of transcription factors, including Wnt signaling *Wnt7a*, *Wnt4*, *Wnt5a*, and *Ctnnb1* (Franco et al., 2011; Jeong et al., 2009; Mericskay et al., 2004; Miller and Sassoon, 1998), as well as Hox family members *Hoxa10*, *Hoxa11*, and *Hoxa13* (Benson et al., 1996; Zhao and Potter, 2001), also resulted in uterine epithelial morphological alteration. Future experiments will need to define the relationship of GATA2 with Wnt signaling in this process. Our results demonstrate that *Gata2* is critical for the maintenance of normal uterine epithelia differentiation.

The presence of P4- and GATA2-independent genes in the GATA2 and P4 targets, respectively, suggests involvement of additional mechanisms in controlling these two groups of genes. To explore potential signals that regulate expression of genes responded uniquely to P4 but not GATA2, we performed motif

(E and F) Luciferase reporter analyses of cotransfection analysis of denoted *cis*-acting elements in HEC-1A cells. Cotransfection with empty vector, PGR-A, PGR-B, or GATA2 expression vectors treated with vehicle or 10^{-8} M R5020 for 24 hr. Experiments were performed in triplicate. Error bars represent SEM. $^{*}p < 0.05$; $^{***}p < 0.001$ by Student's t test.
See also Figures S3 and S4; Table S4.

A

Diseases or Functions Annotation	*p*-Value	Activation z-score	# Molecules
abdominal cancer	7.32E-19	1.527	683
urogenital cancer	7.20E-11	1.932	354
proliferation of epithelial cells	1.05E-08	1.483	56
growth of tumor	7.48E-08	1.410	78
proliferation of keratinocytes	1.58E-05	2.380	20
proliferation of epidermal cells	2.27E-05	2.480	21

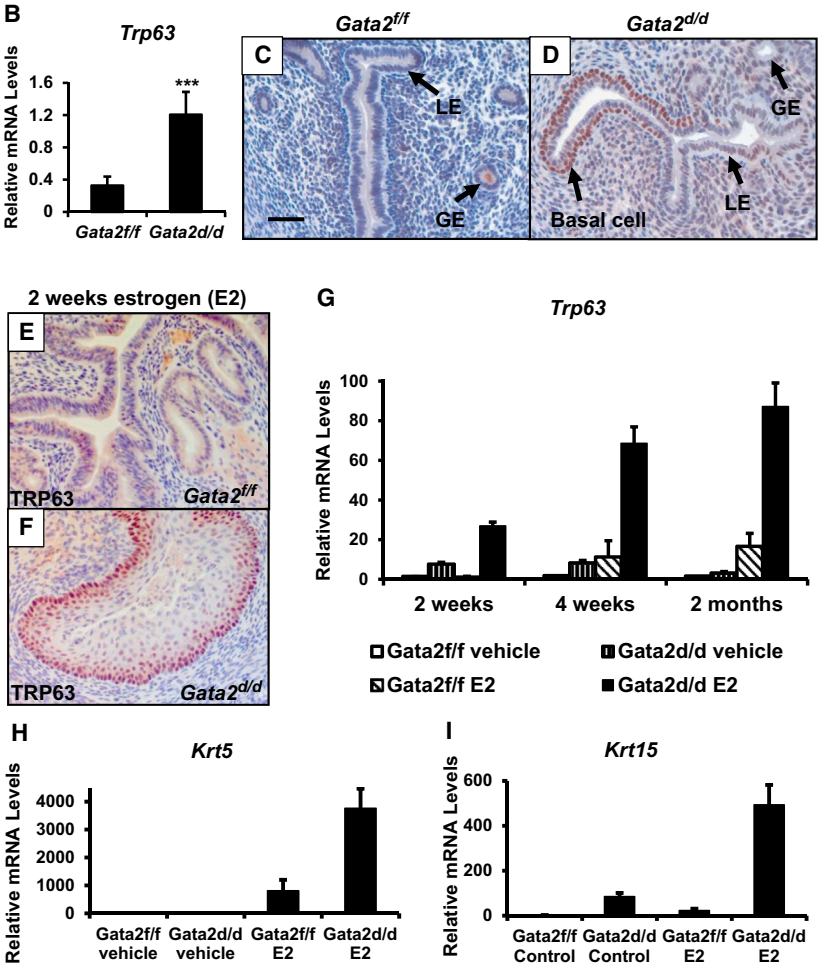

Figure 5. P4-Independent Functions of *Gata2*

(A) Enrichment of functional annotation in differentially expressed genes between *Gata2*$^{f/f}$ and *Gata2*$^{d/d}$ uteri of OVX, vehicle-treated mice by IPA analysis.

(B) mRNA levels of TRP63 in uterus of OVX mice.

(C and D) Immunostaining of TRP63 in uteri of OVX mice (control horn of decidualization treatment). Scale bar, 100 uM.

(E and F) Immunostaining of TRP63 in uteri of OVX mice treated with E2 for 2 weeks.

(G) qRT-PCR of uterine tissues of OVX mice treated with E2. n = 6 (*Gata2*$^{f/f}$, vehicle, 2 weeks), 6 (*Gata2*$^{d/d}$, vehicle, 2 weeks), 6 (*Gata2*$^{f/f}$, E2, 2 weeks), 6 (*Gata2*$^{d/d}$, E2, 2 weeks), 6 (*Gata2*$^{f/f}$, vehicle, 4 weeks), 6 (*Gata2*$^{d/d}$, vehicle, 4 weeks), 4 (*Gata2*$^{f/f}$, E2, 4 weeks), 6 (*Gata2*$^{d/d}$, E2, 4 weeks), 6 (*Gata2*$^{f/f}$, vehicle, 2 months), 4 (*Gata2*$^{d/d}$, vehicle, 2 months), 6 (*Gata2*$^{f/f}$, E2, 2 months), 5 (*Gata2*$^{d/d}$, E2, 2 months).

(H and I) qRT-PCR of uterine tissues of OVX mice treated with E2 for 2 months. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test.

See also Figure S5 and Table S5.

analysis on genes differentially expressed upon P4 stimulation in the *Gata2* knockout background (Figure 2D, red circle). Searching for known motifs in promoter regions of these genes revealed an enrichment of AP2 and RBPJ1 binding motifs (Table S7A). Given that the AP2 binding motif is also enriched in the PGR occupancy sites in T47D breast cancer cell lines (Yin et al., 2012), perhaps a conserved regulatory mechanism that consists of PGR and AP2 is present for a subset of PGR target genes. This result also suggests that PGR might work with multiple pioneer factors, such as GATA2 and AP2, to regulate various subsets of PGR downstream targets. On the other hand, we found a different profile of enriched motifs in promoter regions of genes downstream to GATA2 responsive genes without the need of P4 stimulation (Tables S5 and S7B). In this group, the binding motif for Forkhead box (FOX) proteins is enriched in the GATA2 occupying sites, which bears a resemblance of co-occupancy of GATA2 and FOXA1 in genomic loci of AR target genes in prostate cancer (Wu et al., 2014). Furthermore, the enrichment of SOX (Sex Determining Region Y Box) factor binding motifs also implicates a potential interaction between GATA2 and other uterine transcription regulators such as SOX17 (Garcia et al., 2007; Guimarães-Young et al., 2016; Hirate et al., 2016). These results indicate future investigations on potential mechanisms by which GATA2 and PGR work independently with other transcription regulators to direct gene expression in the uterus.

In summary, this study contributes to a more comprehensive understanding of P4 regulation through the identification of a role for the transcription factor GATA2 in uterine function. The morphological alteration of endometrial epithelium in response to *Gata2* deficiency suggests that *Gata2* may play a role in postnatal uterine development. Given that GATA2 activities can be modulated by small molecules (He et al., 2014), fully understanding the interactions of *Gata2* with the network of genes involved in the regulation of uterine function will have implications for the future treatment of female reproductive health and associated disorders.

EXPERIMENTAL PROCEDURES

Animals and Hormone Treatments

Mice were maintained in the designated animal care facility at Baylor College of Medicine (BCM) according to the Institutional Animal Care and Use Committee (IACUC) guidelines for the care and use of laboratory animals. All animal experiments were performed in accordance with an IACUC approved protocol. Detailed description of fertility assay, superovulation, embryo implantation

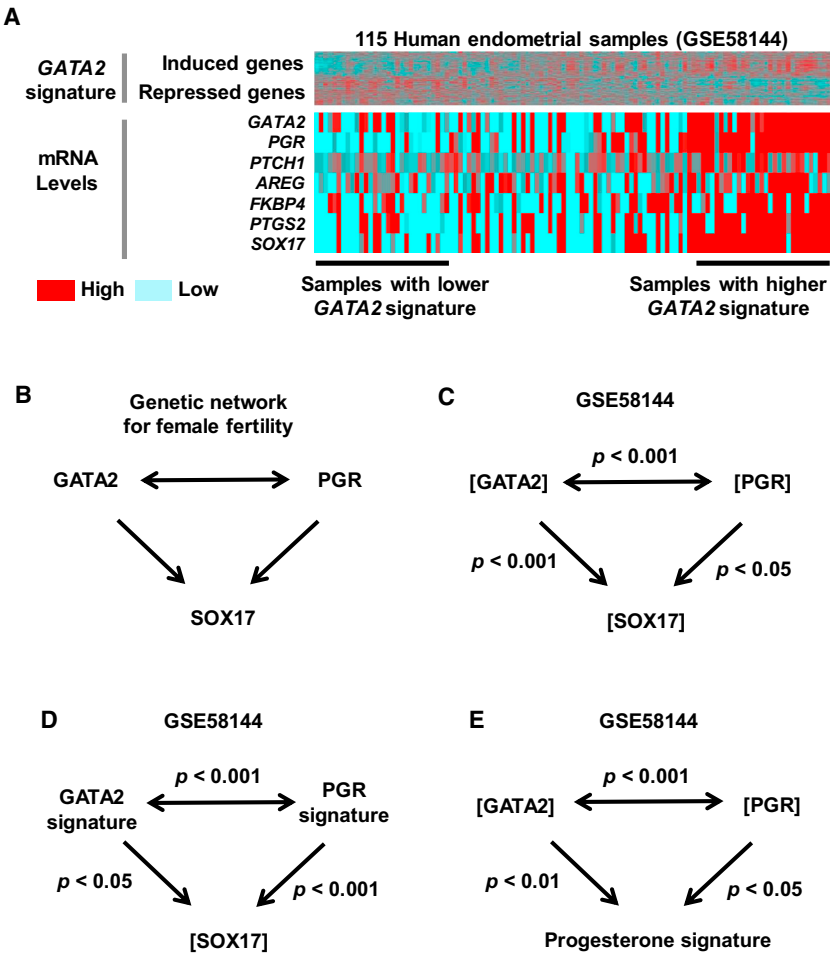

Figure 6. A GATA2-Dependent Regulatory Network in Human Endometrium

(A) Top: expression array data of 115 human endometrial tissue samples from NCBI: GSE58144, for genes in a transcriptional signature of GATA2, with samples ordered based on manifestation of the signature. Bottom: heatmap shows levels of genes of interest in individual samples corresponding to the order in the top panel.

(B) Network of transcription factors for regulation of female fertility.

(C and D) Path analysis of the genetic network by mRNA expression levels and gene signature in NCBI: GSE58144 through structural equation modeling. GATA2 and PGR signatures were derived from (A) and NCBI: GSE39920, respectively.

(E) Model fitting for regulation of P4 signaling by GATA2 and PGR. The P4 signature is based on a gene list described in the green box of Figure 2D. Brackets denote mRNA levels. p values are derived by path analysis based on linear statistic models.

See also Figure S6 and Table S6.

assessment, and artificial decidualization are listed in the Supplemental Information.

Microarray Analysis

Detailed sample preparation can be found in the Supplemental Information. All experiments were performed in triplicate with independent pools of RNA. The Partek Genomics Suite 6.6 software (Partek) was utilized to process raw data from CEL files. The Robust Multichip Analysis (RMA) algorithm with quantile for normalization and log2 transformation was applied to generate signal values of all samples. The one-way ANOVA model was used to compare expression profiles from different groups. Differentially expressed genes were defined using the filters of ANOVA unadjusted p value <0.01 and absolute fold change >1.3.

ChIP-Seq and Data Analysis

GATA2 and input ChIPs were performed by Active Motif on mouse uteri treated with P4 and with GATA2 antibody (sc-9008, Santa Cruz) as described in the Supplemental Information. The model-based analysis of ChIP-sequencing (MACS) peak finding algorithm was used to normalize ChIP against input control (Zhang et al., 2008). Specifically, a p value of 10^{-10} was used with this software to identify ChIP peaks in this work by comparing them to input. Genes associated with intervals were assessed using two increasingly less stringent requirements; if it was within 10 kb and 25 kb upstream or downstream of a gene, it was counted. Analysis of enriched motifs and CEAS were performed using the Cistrome Analysis Pipeline software (http://cistrome.org/ap/rootunder default settings (Liu et al., 2011) or using the HOMER (Heinz et al., 2010). For gene functional classifications, the web application tool Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) running default settings was used (Huang et al., 2009).

Signature Analysis

The publicly available human recurrent implantation failure (RIF) array dataset NCBI: GSE58144 was scored for manifestation of the mouse model-derived GATA2 signature, using published methods (Qin et al., 2013). In brief, a gene signature score (t score) was defined for each human sample as the two-sided t-statistic comparison of the high GATA2-signature with the low GATA2-signature genes' expression profile. Where multiple probes in NCBI: GSE58144 referred to the same gene, the probe with the highest variation was chosen to represent the gene. For each gene, the profiles were centered to the median across samples. Expression pattern was displayed as heatmap using Partek Genomics Suite 6.6 software.

Path Analysis on Human Data

To test the relationships among variables proposed in the research models, structural equation modeling was conducted to indicate the strength of influence among variables by getting an overall fit of model with the data. Goodness-of-fit tests were performed to determine whether the research models should be accepted or rejected. Models were analyzed using Mplus software version 7.11 (Muthén & Muthén). Based on the research models, the significance of direct and indirect relationships among variables was examined. All observed data were included when fitting the models. There was no missing data in the dataset.

The fit of the models was assessed using several methods including the root-mean-square error of approximation (RMSEA), along with a 90% confidence interval, the standard root-mean-square residual (SRMR), the Comparative Fit Index (CFI), and the Tucker-Lewis Fit Index (TLI) to determine the extent to which the relationships existing in the data are consistent with those proposed by the models (Hu and Bentler, 1998, 1999; MacCallum et al., 1996). To interpret the RMSEA, the general rule of thumb is that values <0.05 indicate close fit, values between 0.05 and 0.10 indicate marginal fit, and values >0.10 indicate poor fit (MacCallum et al., 1996). For both CFI and TLI, a value of 1 indicates perfect fit and the values >0.9 indicate adequate fit (Hu and Bentler, 1998, 1999). Values <0.08 for SRMR indicate a very good fit between the model and the data.

Histology

Alkaline phosphatase activity assay was performed on frozen sections. Immunohistochemistry analysis was carried out in paraformaldehyde-fixed, paraffin sections with PGR (A0098, Dako) and TP63 (sc-8431, Santa Cruz) antibodies. Detail information is in the Supplemental Information.

Biochemical, Molecular, and Cell Culture Assays

qRT-PCR was performed with TaqMan probes listed in Table S1. Statistical analyses were performed using t test or one-way ANOVA followed by Tukey's post hoc multiple range test with the Instat package from GraphPad (GraphPad Software). Western blot analysis was carried out with PGR antibody (sc-7208, Santa Cruz). Human endometrial epithelial cells were utilized for transient transfection assays. Detailed descriptions are listed in the Supplemental Information.

ACCESSION NUMBERS

The accession number for the microarray data and the ChIP-seq data reported in this paper is NCBI: GSE40661.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.093.

AUTHOR CONTRIBUTIONS

C.A.R., S.-P.W., and F.J.D. designed and executed experiments and wrote the paper. L.L. designed and performed path analyses on human data. T.W. and R.B.L. performed bioinformatic and signature analyses. All other authors carried out/supervised various aspects of experimental data collection.

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