Identification of cis-acting elements that regulate expression of the progesterone receptor gene PGR in the uterus.

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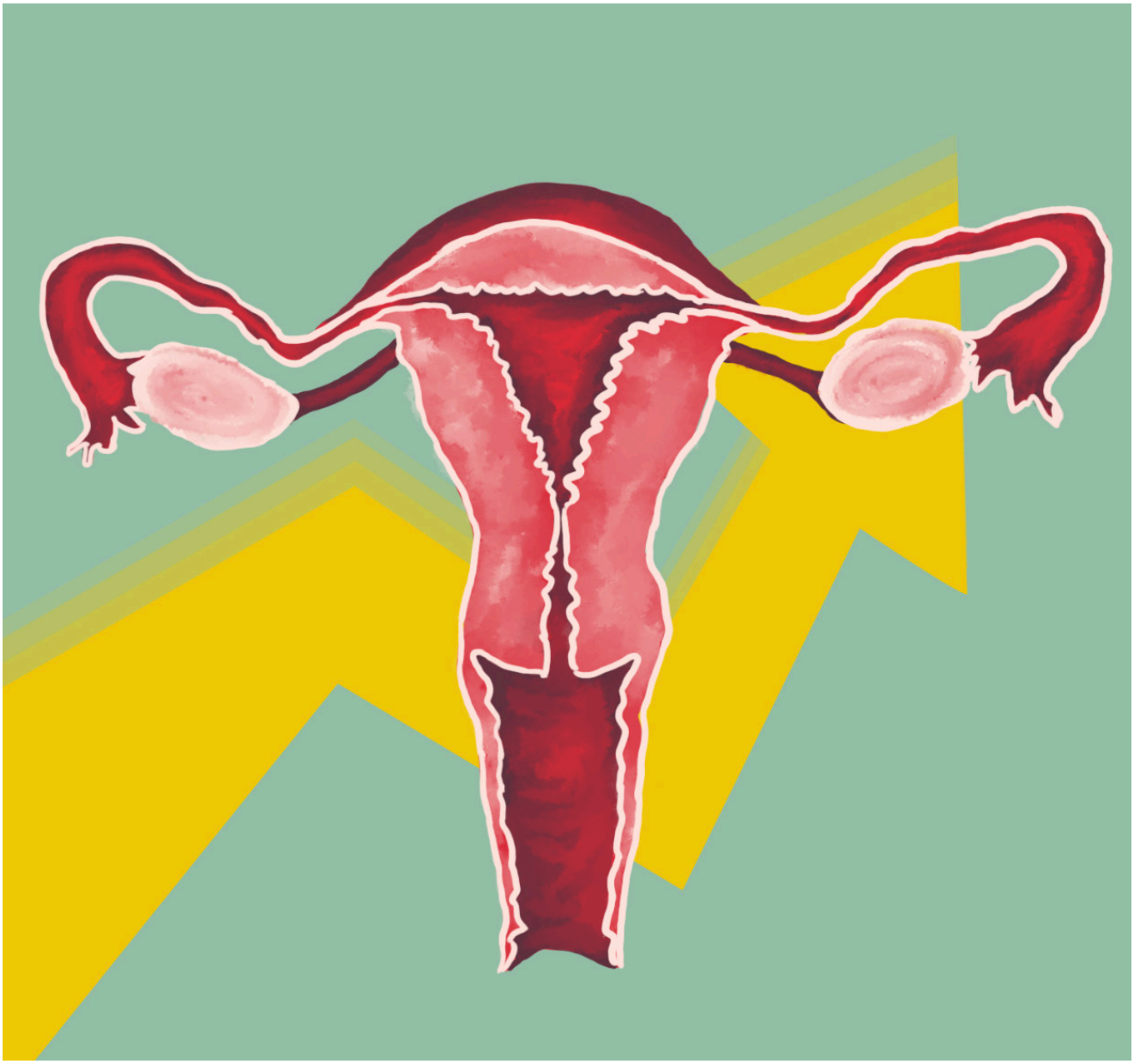
Article Synopsis

The hormone progesterone, alongside its receptor PGR, is a master regulator for female reproductive function, orchestrating processes like pregnancy establishment, maintenance, and labor. When PGR levels are misregulated, progesterone resistance can occur, which is an underlying factor in many reproductive pathologies including endometriosis and infertility. Despite the importance of PGR in the reproductive tract, very little is known about how it is regulated. In this study, elements of the genome that are critical for regulating PGR are identified to gain insight into how it becomes disrupted, and how we can restore its delicate balance.



Graphic by Gaby Dunn

Identification of cis-acting elements that regulate expression of the progesterone receptor gene PGR in the uterus.

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Abstract

The steroid hormone progesterone regulates a wide range of functions in the uterus, including menstruation, pregnancy establishment, pregnancy maintenance, and labor. The cellular effects of progesterone are mediated by the nuclear progesterone receptor (PGR). Progesterone resistance, a decreased cellular response to progesterone, is implicated in a wide range of pathologies including endometriosis, preterm birth, and endometrial cancer. Up to 10% of females within reproductive age are impacted by endometriosis, and no cure is available. This study hypothesizes that misregulation of PGR by upstream regulators is a potential cause of progesterone resistance. However, much remains unknown about the regulation of PGR expression, including the location of enhancers and the identity of transcriptional regulators that control PGR expression. In this study, candidate regulatory elements of PGR were identified by integrative analysis of genomic data, and their function was tested using the CRISPR activation system, with PGR mRNA levels as the readout. Using these methods, two PGR enhancers located at the PGR 3' untranslated region and 200 kbs upstream of the PGR transcription start site were identified. These enhancers permitted the identification of candidate upstream regulators of PGR expression in the uterus, including GATA2, ARID1A, KLF9, and ESR1. Activation of ESR1 increased PGR mRNA levels in the presence of estradiol in cultured uterine cells. Through the discovery of PGR regulators, we can improve our understanding of molecular mechanisms of progesterone resistance. Ultimately, these regulators could be evaluated in cases of progesterone resistance and targeted in the development of therapies.

INTRODUCTION

Progesterone is a steroid hormone synthesized by the ovaries, corpus luteum, and placenta (J. D. Graham & C. L. Clarke, 1997) and plays a central role in the regulation of biological processes throughout uterine tissues: the two major layers of the uterus, the endometrium and the myometrium. The myometrium is the smooth muscle component of the uterus that protects the developing fetus throughout gestation and generates contractile forces at the time of labor to deliver the fetus (Carsten, 1968). The endometrium forms the inner lining of the uterus, composed of the outer epithelium and inner stromal compartment. The epithelium thickens in anticipation of pregnancy and is the site of implantation if pregnancy is established. In the absence of pregnancy, menstruation occurs, resulting in the shedding of the endometrial lining (Critchley et al., 2020). If pregnancy is established, progesterone signaling triggers the differentiation of the stromal compartment into decidual cells (a process known as decidualization) that support embryo growth and maintain early pregnancy (Ng et al., 2020). Progesterone signaling is central to the two major layers of the uterus, the endometrium and the myometrium, regulating a host of biological processes including menstruation, ovulation, embryo implantation, uterine growth, and labor (J. D. Graham & C. L. Clarke, 1997).

Progesterone resistance, a decreased cellular response to progesterone throughout the uterus, is implicated in a wide range of pathologies including infertility, endometriosis, endometrial cancer, and adenomyosis (MacLean & Hayashi, 2022). Endometriosis occurs when endometrial tissue invades neighboring compartments, such as the fallopian tubes, ovaries, or vagina, causing chronic pelvic pain, infertility (Zondervan et al., 2018), nausea, fatigue, and bloating (WHO, 2023). The World Health Organization estimates that up to 10% of girls and women within reproductive age, or 190 million females worldwide, are impacted by endometriosis (WHO, 2023). To temporarily relieve pain, administration of hormone treatment to inhibit ovulation and menstruation, or surgical removal of lesions can be employed, but there is currently no cure (Vercellini et al., 2014). Furthermore, endometriosis can lead to subfertility or infertility, with the risk of infertility increasing two-fold in patients under the age of 35 with endometriosis compared to unaffected peers (Prescott et al., 2016). When endometrial tissues grow into the myometrial layer of the uterus, adenomyosis occurs which leads to heavy menstrual bleeding, increased risk of anemia, dyspareunia (pain during sex), decreased fertility, severe period cramps, and an enlarged uterus (Mildred R Chernofsky, 2016).

The cellular effects of progesterone are mediated by the nuclear progesterone receptor (PGR) which exists as two isoforms (PGRA and PGRB) encoded by the same gene via alternate promotors (Kastner et al., 1990; O'Malley & Schrader, 1972). Upon binding of progesterone to PGR, the dimerized receptor-ligand complex translocates to the nucleus and binds to the genome as a transcription factor (TF) to regulate a wide range of gene expressions (J. Graham & C. Clarke, 1997; J. D. Graham & C. L. Clarke, 1997; O'Malley & Schrader, 1972). The PGRA and B isoforms share the majority of their protein product structure. The conserved domains across the two include an N-terminal domain, a DNA binding domain, a ligand binding domain, and two transactivating domains (AF1 and AF2) that form the interface that interacts with coregulators (Mulac-Jericevic & Conneely, 2004). PGRB has an additional 164 amino acids at the N-terminal domain which codes for the transactivation domain, AF3, which is thought to explain the unique gene regulatory actions of each isoform throughout the uterus (Sartorius et al., 1994; Tora et al., 1988). In many cellular contexts, PGR isoforms coexist and interact via homo- or heterodimers, with the stoichiometric ratio of each isoform likely determining the genetic and molecular response to progesterone hormone signaling (DeMayo & Lydon, 2020).

PGR regulates a host of programs in the female reproductive tract required for embryo implantation, stromal decidualization, myometrial remodeling, and parturition. At ovulation, the newly formed corpus luteum secretes progesterone which antagonizes estrogen-induced proliferation of the endometrium (DeMayo & Lydon, 2020). Through the Pgr-Ihh-Nr2f2-Hand2 axis, the outer lining of the endometrial compartment, the epithelium, transitions to a receptive state, allowing for embryo implantation and subsequent invasion into the uterus (Oh et al., 2023). During this time, crosstalk between the stromal and epithelial compartments of the endometrium through the Pgr-Ihh-Nr2f2 axis regulates the differentiation of endometrial stromal cells into decidual cells, a process known as decidualization (Lee et al., 2010; Takamoto et al., 2002). Decidual cells create an immunotolerant environment and nourish the embryo until the placenta forms (Gellersen & Brosens, 2014). Throughout pregnancy, the myometrium undergoes drastic development, increasing in size up to 5-fold to accommodate the growing fetus (Carsten, 1968), while developing into a synchronous contractile unit capable of generating rhythmic and intense contractions (Csapo, 1981). Throughout gestation, high levels of progesterone ligand bind to PGRB, the dominant isoform at this time, to repress contractile genes and maintain a non-contractile myometrial state as the fetus develops (DeMayo & Lydon, 2020; Nadeem et al., 2016). At the time of parturition, PGRA levels surpass PGRB levels, and functional withdrawal of progesterone due to metabolism by 20αHSD enzyme sequesters PGRB in the cytosol, while unliganded PGRA activates pro-contractile genes to induce labor (Merlino et al., 2007; Nadeem et al., 2016).

PGR gene expression is regulated in a variety of ways including transcriptional regulation via cis- and

trans-acting factors, CpG methylation (Lapidus et al., 1996), histone modifications (Stratmann & Haendler, 2011), mRNA steady-state concentrations (Pei et al., 2018), PGR protein abundance (Lange et al., 2000), and post-transitional modifications (Abdel-Hafiz & Horwitz, 2014). Post-translational modifications play a role in regulating PGR stability and diversify its cellular activity through phosphorylation (Knotts et al., 2001; Lange et al., 2000), sumoylation (Chauchereau et al., 2003), acetylation (Daniel et al., 2010), and ubiquitination (Calvo & Beato, 2011). Trans-acting factors are proteins that bind to specific DNA sequences, known as cis-regulatory elements, to regulate gene expression. Cis-regulatory elements are non-coding DNA regions that regulate the expression of nearby genes; these include promotors that initiate transcription, enhancers that bind TFs to promote gene expression (Field & Adelman, 2020), silencers that bind TFs to decrease gene expression (Petrykowska et al., 2008), and insulators that isolate genomic regions into topologically associated domains (Burgess-Beusse et al., 2002; Yoon et al., 2007).

Progesterone unresponsiveness has been attributed to alterations in PGR isoform expression due to various origins including hypermethylation of PGR regulatory regions (Rocha-Junior et al., 2019; Wu et al., 2006), chronic uterine inflammation (Patel et al., 2017), PGR polymorphisms (Wieser et al., 2002), and changes in gene expression of PGR upstream regulators that have yet to be thoroughly studied (Kim et al., 2015; Lin et al., 2014).

Despite the significant role of progesterone signaling throughout the female reproductive system, much remains unknown about the upstream regulation of both PGR isoforms, including the location of cis-regulatory elements such as enhancers and the identity of trans-acting factors such as transcription factors. The status of histone acetylation marks helps to map the location of potential cis-regulatory elements such as enhancers. Enhancers can be active, marked by the epigenetic marker H3K27ac (Heintzman et al., 2009), or poised, marked by the epigenetic marker H3K27me3 (Rada-Iglesias et al., 2011). Poised enhancers do not regulate gene expression at all times, and instead are cell-type or developmental-stage specific (Rada-Iglesias et al., 2011).

The objective of this study was to identify cis-regulatory elements of PGR isoforms in the myometrium and use these elements to identify novel candidate PGR transcriptional regulators. In this study, candidate regulatory elements of PGR were identified by integrative analysis of H3K27ac marks, Hi-C data, and ATAC-seq data in human myometrial tissues and cells. H3K27ac marks map out the location of potential active enhancer marks, Hi-C data inform on the 3D folding structure of chromatin to delineate what regions of the DNA interact together, and ATAC-seq data identifies regions of the genome that are open and accessible for transcription. The function of candidate regulatory elements was tested using the CRISPR activation (CRISPRa) system. The activation system is comprised of a dead Cas9 enzyme that is mutated at the active site to inhibit its endonuclease activity (Chavez et al., 2015). The dead Cas9 enzyme is fused to a transcriptional activator which is directed by a guide RNA (gRNA) (Chavez et al., 2015). If the gRNA directs the complex to a regulatory element, RNA polymerase is recruited and drives gene expression.

gRNAs targeting around the PGR locus were designed and the single-cell RNA-seq with CRISPR perturbations (Perturb-seq) technology (Datlinger et al., 2017) and RT-qPCR were used to screen the gRNAs in human myometrial cells (hTERT-HM) and human endometrial stromal cells (THESC) to determine whether any gRNAs target regulatory elements of PGR. Results from Perturb-seq and RT-qPCR show that targeting two regions within the vicinity of the PGR locus, the PGR 3' untranslated region (UTR) and an enhancer located 200kbs from the PGR transcription start site (TSS), can successfully induce PGR expression in hTERT-HM cells and THESC. Candidate transcription factors that bind to these regulatory elements were identified using integration of Motif Enrichment and public ChIP-seq data. Motif Enrichment is a bioinformatical approach that identifies transcription factor binding sites that are enriched in a region of the genome and unlikely to be present due to chance, suggesting the potential binding of those transcription factors in that region. On the other hand, public ChIP-seq data is a publicly available database detailing the location of ChIP-determined TF binding sites across various experiments and tissues. Using

the integration of this data, candidate transcriptional regulators that bind to PGR cis-regulatory elements were identified. These include chromatin regulator ARID1A, endometrial epithelial and stromal cell regulators such as GATA2, NR2F2, and ESR1, and myometrial regulators including BRD4 and KLF9. Activation of ESR1 using CRISPRa in hTERT-HM cells results in a significant increase in PGR mRNA abundance.

By uncovering what cis-regulatory elements control the expression of PGR isoforms, we can identify non-coding mutations that lead to altered expression of *PGR*. Through the discovery of PGR regulators, we can improve our understanding of what genetic perturbations underly progesterone resistance. Ultimately, these cis-regulatory elements and transcriptional regulators could be targeted to screen for progesterone resistance or treat the underlying genetic abnormalities that lead to these disease phenotypes.

METHODS

Reagents and cell culture:

Telomerase-transformed human myometrial cells (hTERT-HM) (Condon et al., 2002) and telomerase-transformed human endometrial stromal cells (THESC) (Saleh et al., 2011) were maintained in DMEM/F-12 (Invitrogen, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco) and antibiotics (10 000 IU/mL penicillin, 10 000 IU/ mL streptomycin; Life Technologies, Grand Island, NY). Cell culture media was filtered using the 0.22um Rapid-Flow™ Sterile Disposable Filter Units (Nalgene). Cells were cultured and grown in a 5% CO_2 and 37°C incubator. Cells were plated at 20% confluency and the media was changed every three days. Cells were passaged before reaching 90% confluency.

gRNA design and plasmid construction:

To identify candidate proximal and distal regulatory elements of PGR, THESC or hTERT-HM cells were co-transduced with dCas9-VP64-p65-Rta (VPR) and guide RNAs (gRNA) (Chavez et al., 2015). Integration of active enhancer mark, H3K27ac, and HiC chromatin looping in human myometrial tissue and cells was used to identify candidate proximal and distal regulatory regions of PGR. 4-5 gRNAs per region were designed using the CHOPCHOP (Labun et al., 2019) and CRISPick tools (Doench et al., 2016). All gRNA expression vectors were synthesized by and acquired from VectorBuilder (VectorBuilder.com) and expressed neomycin resistance and GFP markers. IGI-P0492 pHR-dCas9-NLS-VPR-mCherry was a gift from Jacob Corn (Addgene plasmid # 102245; http://n2t.net/addgene:102245 ; RRID:Addgene_102245).

Lentivirus production and cell transduction:

To introduce plasmids encoding gRNAs and dCas9-VPR into the cells, viral transduction is performed which uses a lentivirus vector to integrate the plasmid sequences into the cell genome. To make lentivirus, plasmids were isolated from bacteria using Qiagen Plasmid Midi Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Plasmid quality was assessed by restriction digest and gel electrophoresis. Lentivirus was generated by transfection of the constructs and packaging vector into HEK293T at the NIEHS Viral Vector Core Facility. For viral transduction, cells were seeded at 20% confluency in 10cm plates, 24 hours before transduction. Cells were transduced with gRNA lentivirus at an MOI of 0.4, 2.0, or 4.0 and incubated for 24 hours before washing plates with PBS and replacing them with fresh media. MOI represents the ratio of the number of transducing lentiviral particles to the number of cells, with a higher MOI resulting in more integrations of the plasmid construct into the cells. Cells transduced with gRNA plasmid were selected for using 1mg/ml Geneticin (Gibco, Grand Island, NY) for 3 days (as determined by cell-specific kill curve). Cells were then transduced with dCas9-VPR at an MOI of 4.0, using the same method as above. After transduction with the second plasmid, cells positive for both plasmids were isolated using flow cytometry at the NIEHS Flow Cytometry Center.

Perturb-seq and Data Analysis:

gRNAs targeting candidate regulatory regions of PGR and two control gRNAs were pooled together to generate lentivirus carrying equimolar ratios of each gRNA. THESC and hTERT-HM cells were transduced with gRNA lentivirus at an MOI of 0.4. At an MOI of 0.4,

most cells receive just one gRNA. After transduction with dCas9-VPR at an MOI of 4.0, cells positive for both plasmids were isolated using flow cytometry. The cells received from flow cytometry were counted and examined for viability with trypan blue staining using a TC-20 cell counter (Bio-Rad). Approximately 16,500 live cells at 1×10^6 cells/ml concentration with 65% or above viability were loaded into the Single Cell Chip to generate single cell emulsion in Chromium Controller with Chromium Single Cell 3' Library & Gel Bead Kit v3.1 (10x Genomics, Cat. 1000268). Reverse transcription of mRNA and cDNA amplification were carried out according to the manufacture's instruction (10x Genomics, Cat. 1000268, Cat. 1000262 with 10x Genomics protocol CG000316). The amplified cDNA was separated into CRISPR sgRNA derived cDNA and transcriptome derived cDNA. The CRISPR sgRNA derived cDNA was used to make NGS sequencing libraries. The transcriptome derived cDNA was further fragmented to construct NGS libraries. Both libraries were then sequenced together with the molar ratio of 1 to 4 by the NIEHS Epigenomics and DNA Sequencing Core Laboratory according to the manufacture's instruction.

Raw data from the Perturb-seq assay was processed and analyzed at the NIEHS bioinformatics core. Briefly, the raw sequencing FASTQ files generated from both the transcriptome and CRISPR screening libraries were processed together by Cell Ranger software (version 4.0.0, 10× Genomics). The "cellranger count" pipeline used STAR for aligning the reads to the human reference, GRCh38 "refdata-gex-GRCh38-2020-A" (10X Genomics), and associated gene expression profile with gRNA identity by a unique barcode in each cell. Seurat software (version 3.6.3) was utilized to perform clustering analysis on the combined dataset (Satija 2015, PMID: 25867923). The SCTransform package was applied to normalize gene expression counts across cells (Hafemeister 2019, PMID: 31870423). The cells were clustered based on the number of unique gRNAs detected. Cell populations containing more than one unique gRNA were excluded from further analyses. The expression level of PGR in each cell containing the same gRNA was compared to cells containing the non-targeting control gRNA. A fold-change (FC) greater than 1.5 in PGR mRNA levels was defined as significant.

RT-qPCR assay:

In order to test for PGR expression in the transduced cells, RT-qPCR was employed. RNA was isolated from cells using the RNeasy mini RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 0.4 ug of RNA was reverse transcribed into complementary DNA (cDNA) using Moloney Murine Leukemia Virus reverse transcriptase (Thermo Fisher Scientific) with Random Hexamers (Invitrogen, Waltham, MA, USA) according to manufacturer protocol. For quantitative analysis of mRNA, SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) was used according to manufacturer instructions. Each reaction was performed in technical duplicates using the standard curve-based method (Larionov et al., 2005). Briefly, reaction samples were prepared to a total volume of 20ul with 5uM of each of the forward and reverse primers **(Table 1.1)**, 4ul of cDNA, and a final 1x concentration of the SYBR Green Supermix. Standards were made by pooling together 6ul of cDNA from each sample and diluting to 140ul by adding DNase-free water. Pooled cDNA was then diluted in DNase-free water to final relative concentrations of 100%, 25% and 10%. The reaction was heated to 95°C for 30 seconds, followed by 39 cycles of denaturation at 95°C for 15 seconds and annealing and elongation at 60°C for 30 seconds. Temperature cycles were performed on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Relative mRNA levels of PGR were compared to the negative control (non-targeting control) and positive control (PGR640A).

Table 1.1: Primers used for RT-qPCR

PGR	Forward	CTGGCATGGTCCTTGGAG
	Reverse	TCATTTGGAACGCCCACT
ESR1	Forward	CTGCAGGGAGAGGAGTTTGT
	Reverse	TCCAGAGACTTCAGGGTGCT
18S	Forward	GTAACCCGTTGAACCCCATT
	Reverse	CCATCCAATCGGTAGTAGCG

These primers were used in RT-qPCR to target the mRNA transcript of PGR, ESR1, and the housekeeping gene 18S to confirm upregulation of these genes by CRISPR activation.

Generating ESR1 expressing hTERT-HM cells:

To activate ESR1 expression, gRNAs were designed to target the promotor of the ESR1 gene using the same methods and tools as above. Plasmids were designed and lentivirus was produced as mentioned above. hTERT-HM cells were transduced with gRNA at an MOI of 4.0. Cells transduced with gRNA plasmid were selected for using 1mg/ml Geneticin (Gibco, Grand Island, NY) for 3 days. Cells were then transduced with dCas9-VPR at an MOI of 4.0, using the same method as above. After transduction with the second plasmid, cells positive for both plasmids were isolated using flow cytometry at the NIEHS Flow Cytometry Center. RT-qPCR and western blot were conducted to measure the levels of ESR1 mRNA and protein in cells.

Western Blot:

A western blot was employed to determine whether ESR1 was upregulated due to transduction. To isolate protein from cells, the RIPA Lysis and Extraction Buffer protocol was followed (Thermo Scientific) with the following specifications/modifications: approximately 300,000 cells were pelleted and lysed with 100ul of complete Pierce RIPA buffer. Protein was quantified using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) as instructed by the manufacturer. Western blot was conducted with 40ug of protein per sample. Protein lysates from MCF7 cells were used as positive controls, while protein lysates from HEK293T cells were used as negative controls. Western blots were run on Mini-PROTEAN TGX Pre-cast Protein gels (Bio-Rad, #4568094) with the Precision Plus Protein Dual Color Standards Ladder (Bio-Rad #1610374). Gels were transferred to nitrocellulose membrane using the Turbo-Blot transfer system (BioRad) according to the manufacturer's directions. The membrane was blocked with 5% milk (Santa Cruz Biotech, Santa Cruz, CA, USA) in TBST (20 mM Tris, pH 7.4 (Lonza, Morrisville, NC, USA), 140 mM NaCl (Lonza), 1% TWEEN-20 (Sigma) (Hewitt et al., 2022). ESR1 protein was detected by incubating with ESR1 specific antibodies **(Table 1.2)** diluted 1:1000 in 5% milk overnight at 4C. Bands were detected with secondary antibodies **(Table 1.2)** diluted 1:20,000 in 5% milk for 45 minutes and imaged using Odyssey Fc Imager (LI-COR Biosciences) using 800nm channel for 10 minutes, and 700nm channel for 30 seconds (to image ladder). The control protein (B-actin) was detected similarly as above with the antibodies and dilutions included in the table below.

Table 1.2: Antibodies used for Western Blot

Target Protein	Antibody	Dilution
ESR1	**Primary:** ERa (H-184) sc-7207, Lot#G2109 rabbit polyclonal IgG Santa Cruz	1:1000
	Secondary: Goat anti-rabbit 926-32211 Lot#DOO304-15.	1:20000
B-Actin (control)	**Primary:** Actin (I-19)-R sc-11616-R, Lot#DO406 Rabbit polyclonal IgG Santa Cruz.	1:50000
	Secondary: Goat anti-rabbit 926-32211 Lot#DOO304-15.	1:20000

These primary antibodies were used in western blot to detect ESR1 protein and the housekeeping control protein B-Actin to confirm upregulation of these genes by CRISPR activation. The secondary antibodies bind to the primary antibodies for visualization using fluorescence.

Identifying candidate PGR upstream regulators:

To identify candidate PGR upstream regulators, motif enrichment (JASAPR) and public ChIP-seq data (ReMap) were used to screen for potential transcription factors (TFs) that bind to the *PGR* enhancer. All TFs that either had enriched motifs in the enhancer (JASPAR) or were found to bind to the enhancer (ReMap) in at least one study were considered. The TF gene list was narrowed down by identifying genes with translated protein products present in the endometrium or myometrium of human biopsies using data from the Human Protein Atlas (Uhlen et al., 2015). If the protein product of the gene was not detected in myometrium or endometrium biopsies, it was eliminated. Next, candidate PGR regulators were further narrowed down by identifying TF genes with reproductive or embryonic lethal phenotypes in mouse knock-out models using data from MGI Jax. Only reproductive phenotypes in female mice were considered, which included anything related to decreased fertility or litter size, and abnormal uterus morphology. Lastly, a literature search was conducted to further narrow down the gene list; genes that have been discovered by previous studies to play important roles in the myometrium or endometrium or involved with endometriosis, decidualization, or parturition/labor were selected.

RESULTS

Identification of putative proximal and distal regulatory elements of PGR.

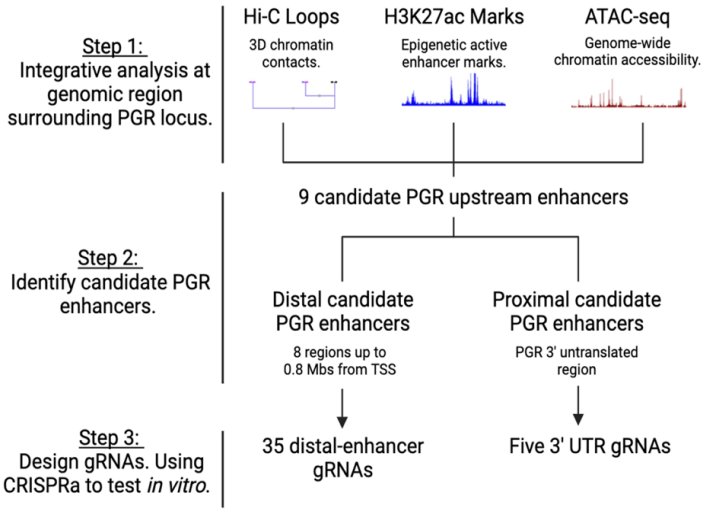

Figure 1: Workflow to identify and test candidate PGR enhancers.

To identify candidate cis-regulatory regions of *PGR*, integrative analysis of H3K27ac data, ATAC-seq data, and Hi-C looping data was used **(Figure 1)**. The following criteria were used to identify the candidate regions:

1. The presence of the active enhancer marks H3K27ac (Ji et al., 2015) in myometrial biopsy samples and the absence of H3K27ac

marks and ATAC-seq peaks in *in vitro* cultured myometrial cells. Upon the removal of myometrial cells from the body and culture *in vitro*, there is a rapid reduction in *PGR* expression levels. Epigenetic data indicates that there is a loss of H3K27ac marks in cultured myometrial cells at distinct regions around the PGR locus, in comparison to myometrial biopsies (unpublished observations). The loss of these marks at PGR enhancers promote chromatin condensing and gene silencing and may be responsible for this decrease in PGR expression.

2. The presence of chromatin interactions between the *PGR* promotor and distal regions of the genome, as identified by Hi-C data. Hi-C data identifies regions of chromatin that loop together, which allows regulatory elements residing within the loops to be brought closer together to associate with each other topologically for the regulation of gene expression (Palstra, 2009). It was arbitrarily defined that enhancer elements present in regions up to three connections away from the *PGR* transcription start site (TSS) will be considered for this study, covering a 1 Mb around the PGR TSS. Studies have shown that most enhancers lay within 1 Mb upstream or downstream of the gene TSS (Mora et al., 2016).
3. Significant ReMap peak. ReMap is a database of publicly available ChIP-seq data detailing the location of transcription factor (TF) binding sites across various experiments and tissues (Hammal et al., 2022). A significant ReMap peak indicates that a particular region of the genome is a TF hotspot, suggesting that it could be a regulatory domain.

Using these criteria, nine putative regulatory elements of PGR were defined, six regions upstream of the *PGR* TSS (denoted U1-6) and three regions downstream of the *PGR* TSS (denoted D1-3) **(Figure 2)**. The D1 region targets the 3' untranslated region (UTR) of *PGR*. 4-5 gRNAs were designed to target different locations within each region to ensure that each region has at least two working gRNAs, resulting in a total of 40 gRNAs. gRNAs were divided into two groups, a 3' UTR proximal gRNA pool with 5 gRNAs and a Distal-Enhancer gRNA pool with the remaining 35 gRNAs **(Figure 1)**.

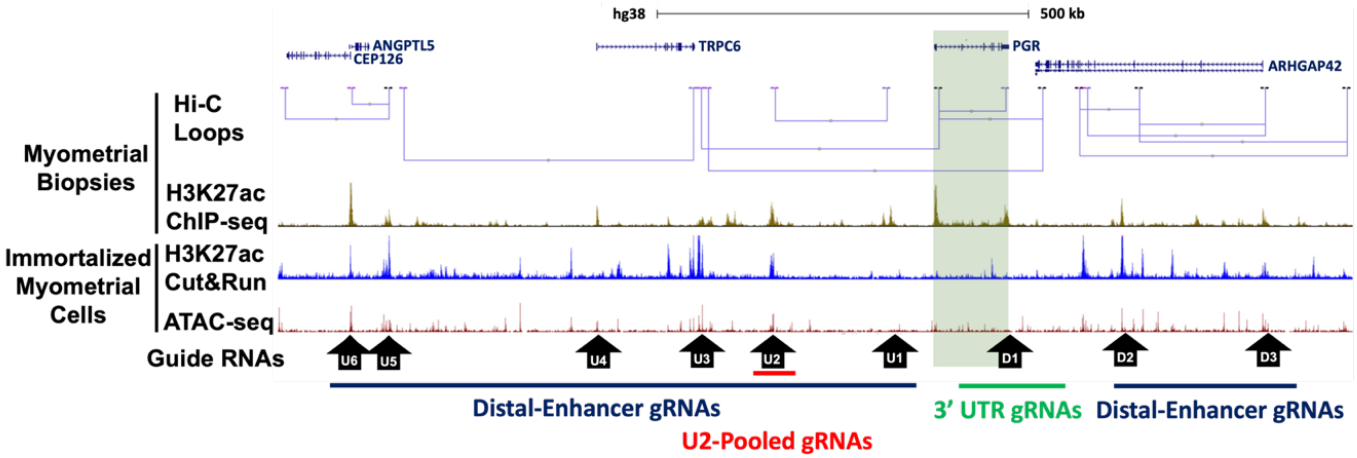

Figure 2: Integrative analysis for identification of putative proximal and distal regulatory elements of PGR. UCSC Genome Browser track view of the human PGR locus (highlighted in green) and surrounding regions marked with gRNA targeting locations (black arrows). Locations of gRNAs targeting distal-enhancer regions (blue) and 3' UTR (green) are highlighted.

Targeting distal PGR putative enhancers in uterine cells by RT-qPCR and Perturb-seq.

To test whether any gRNAs successfully target distal cis-regulatory elements of *PGR*, the Distal-Enhancer gRNA pool was introduced into uterine cells and *PGR* mRNA levels were measured with RT-qPCR. Transduction of hTERT-HM cells with dCas9-VPR and the Distal-Enhancer gRNA pool at a multiplicity of infection (MOI) of 4 resulted in a 1.89-fold-change (FC) of *PGR* mRNA levels (P-value < 0.05) **(Figure 3A)**. MOI refers to the ratio of viral transducing particles to cells. Transduction of THESC with dCas9-VPR and the Distal-Enhancer gRNA pool at an MOI of 4 resulted in a 1.88-FC of *PGR* mRNA levels (P-value < 0.05) **(Figure 3B)**. These results suggest that some of the gRNAs in the Distal-Enhancer gRNA pool can activate PGR expression, presumably by targeting cis-regulatory elements, and therefore, Perturb-seq was conducted to screen for functional cis-regulatory elements of *PGR* (Datlinger et al., 2017).

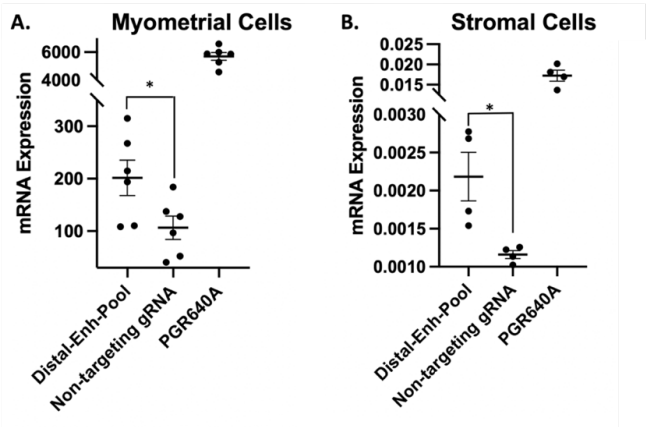

Figure 3: Targeting candidate PGR distal enhancers by CRISPR activation in mesenchymal lineage cells. Relative PGR mRNA levels measured by qRT-PCR in hTERT-HM cells **(A)** or T-HESC **(B)** that express denoted gRNAs with the CRIPSR activator, dCas9-VPR. N = 3 with technical duplicates **(A)**, N = 2 with technical duplicates **(B)**. Relative PGR mRNA levels were compared to PGR-promoter targeting (PGR640) or non-targeting gRNAs as controls. *P-value < 0.05 by unpaired t-test.

The perturb-seq assay combines CRISPR-Cas9 based gene perturbation and single-cell RNA sequencing to study the effects of specific genetic perturbations on gene expression in individual cells at a large scale (Datlinger et al., 2017). By identifying the gRNA introduced into individual cells and the level of *PGR* mRNA in those cells, Perturb-seq permits the identification of specific gRNAs that result in a significant upregulation of *PGR* mRNA compared to cells receiving the non-targeting control gRNA. For this study, significant upregulation was defined as an mRNA level FC greater than 1.5 which follows conventional practices. Both the Distal-Enhancer gRNA pool and 3' UTR gRNA pool were introduced into uterine cells hTERT-HM and THESC at a total MOI of 0.4, resulting in the majority of cells receiving a single gRNA. Using the transcriptome of each individual cell as a data point, the goal is to collect 7,000 data points per assay for an average of 100 data points for each individual gRNA to secure statistical power.

hTERT-HM cells transduced with both the Distal-Enhancer gRNA pool and 3' UTR gRNA pool and subjected to Perturb-seq did not yield any significant results, with no gRNAs resulting in a significant upregulation of *PGR* mRNA in comparison to the control. A total of 132 individual cells containing gRNA-21 were tested with an average *PGR* mRNA $\log_2$FC of 1.20 in comparison to non-targeting control (P-value = 0.03) **(Figure 4A)**. Perturb-seq in THESC identified significant upregulation of *PGR* mRNA in cells expressing gRNA-21 and gRNA-22 in comparison to the non-targeting control **(Figure 4B)**. A total of 102 individual cells containing gRNA-22 were tested with an average *PGR* mRNA $\log_2$FC of 2.00 in comparison to non-targeting control (P-value = 2.21×10^{-10}) **(Figure 4A)**. gRNA-21 and 22 correspond to gRNAs targeting the U2 putative enhancer region. To validate these results, RT-qPCR was conducted in hTERT-HM cells transduced with 5 pooled gRNAs targeting the U2 enhancer region (including gRNA-21 and 22). Transduction with these gRNAs resulted in a significant upregulation of *PGR* mRNA with a FC of 5.57 (P-value < 0.001) in comparison to non-targeting control **(Figure 4B)**.

A.

gRNA	Fold Change over Non-targeting Control (log2)	*P-value*	N
gRNA-21	1.20	0.03	132
gRNA-22	2.00	2.21×10^{-10}	102
Positive Control	3.17	2.70×10^{-35}	148

B.

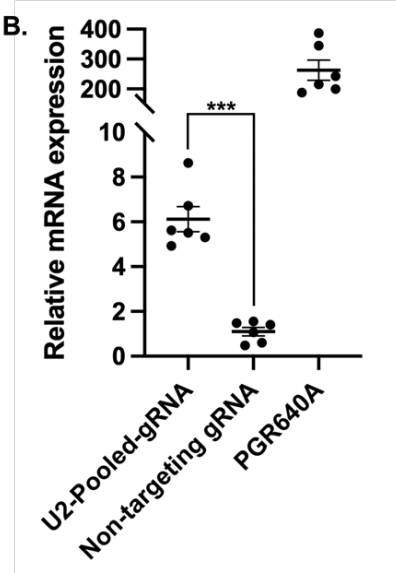

Figure 4: Using Perturb-seq to screen for candidate PGR distal enhancers.
(A) Relative PGR mRNA levels measured by RNA-seq in THESC cells that express denoted gRNAs with the CRIPSR activator, dCas9-VPR. Log2(FC) compares relative mRNA abundance in experimental samples over non-targeting control. N indicates the number of cells used for statistical analysis. **(B)** Relative PGR mRNA levels measured by qRT-PCR in hTERT-HM cells that express denoted gRNAs with the CRIPSR activator, dCas9-VPR (N = 3 with technical duplicates). *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001, ****P-value < 0.0001 by unpaired t-test.

Targeting the *PGR* 3' UTR in myometrial cells by RT-qPCR.

Studies have shown that strong H3K27ac marks are conserved at the *PGR* 3' UTR in both human and mouse uterine tissues, suggesting a functional role of this region (Li et al., 2021; Wu et al., 2022). Previous experiments in the lab have demonstrated concern over the lower sensitivity of Perturb-seq in comparison to manual RT-qPCR screening (unpublished observations). Additionally, there is Hi-C looping between the *PGR* promotor and 3' UTR, suggesting that these regions interact to regulate gene expression through activation or inhibition. There is also a significant reduction in H3K27ac marks and ATAC-seq signal at the *PGR* 3' UTR in myometrial cells in comparison to tissue, a possible reason for the downregulation of PGR in myometrial cells in comparison to tissue. The limited sensitivity of the Perturb-seq assay, alongside the FC cut-off, may have caused the regulatory activity of the *PGR* 3' UTR to be missed. Therefore, it was selected for manual screening.

In order to determine whether the *PGR* 3' UTR contains a *PGR* cis-regulatory sequence, hTERT-HM cells were transduced with dCas9-VPR and the 3' UTR gRNA pool, and *PGR* mRNA levels were determined by RT-qPCR. Transduction of hTERT-HM cells resulted in significant upregulation of *PGR* mRNA levels in a dose-dependent manner **(Figure 5A)**. At the lowest MOI of 0.4, *PGR* mRNA was upregulated 1.98-fold (P-value < 0.01), at an MOI of 2, PGR mRNA was upregulated 2.94-fold (P-value < 0.0001), and at an MOI of 4, *PGR* mRNA was upregulated 3.51-fold (P-value < 0.0001) **(Figure 5A)**. These results indicate that at least one of the gRNAs in the 3' UTR gRNA pool targets a cis-regulatory element of *PGR*. In order to determine which gRNAs in specific from the 3' UTR gRNA pool upregulate *PGR* mRNA levels, hTERT-HM cells were transduced with three individual gRNAs from the PGR 3' UTR gRNA pool. Transduction with each of these gRNAs resulted in significant upregulation of *PGR* mRNA, with gRNA D1D having the most significant upregulation with a FC of 2.88 (P-value < 0.0001) **(Figure 5B)**.

Identifying potential PGR upstream regulators that bind to PGR 3' UTR.

The identification of a *PGR* enhancer at the *PGR* 3' UTR opens the possibility of identifying upstream regulators of PGR that bind to this enhancer. Motif enrichment (JASAPR) and public ChIP-seq data (ReMap) were used to screen for potential TFs that bind to the *PGR* 3' UTR **(Figure 6)**. Candidate PGR regulators were first narrowed down by identifying genes that had lower levels of PGR expression in hTERT-HM cells in comparison to human myometrial biopsies using RNA-seq data from

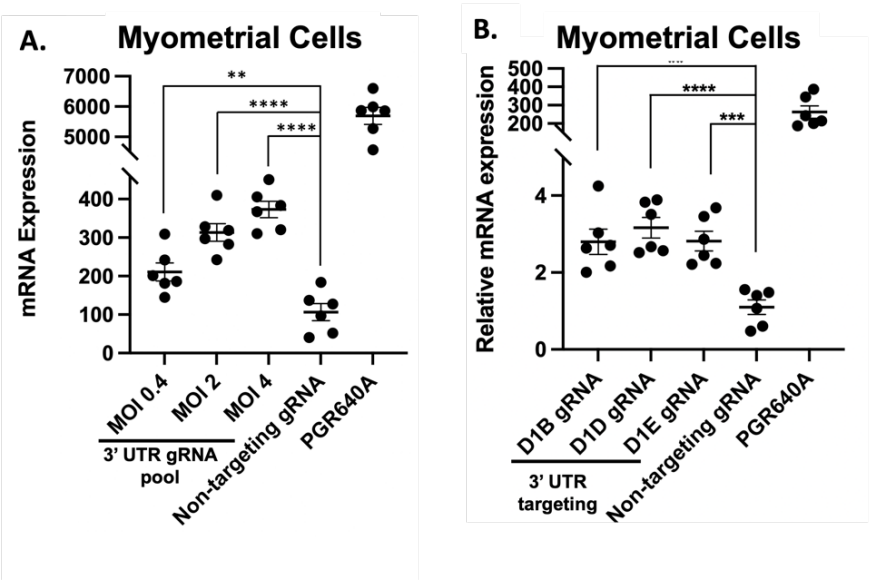

Figure 5: Targeting PGR 3' UTR by CRISPR activation in mesenchymal lineage cells. Relative PGR mRNA levels measured by qRT-PCR in hTERT-HM cells that express denoted gRNAs with the CRIPSR activator, dCas9-VPR (N = 3 with technical duplicates). Cells were transduced with gRNA at an MOI of 0.4, 2 and 4 **(A)** or MOI of 4 **(B)**. Relative PGR mRNA levels were compared to PGR-promoter targeting (PGR640) or non-targeting gRNAs as controls. **P-value < 0.01, ***P-value < 0.001, ****P-value < 0.0001 by unpaired t-test.

myometrial cells and tissue (PMID: 31908010 and unpublished observations). There were two reasons for this: (1) the hTERT-HM cells used in this study have lower levels of *PGR* mRNA in comparison to human myometrium biopsies, suggesting the possibility that upstream activators of PGR may be downregulated in hTERT-HM cells in comparison to tissue, and (2) the CRISPR activation assay has been thoroughly established in this cell line and will therefore be the main tool used to screen for candidate activators of PGR in subsequent studies. The TF gene list was further narrowed down by identifying genes with translated protein products present in the endometrium or myometrium of human biopsies using data from the Human Protein Atlas (Uhlen et al., 2015) **(Figure 6)**. If the protein product of the gene was not detected in the myometrium or endometrium biopsies, it was eliminated. Next, candidate PGR regulators were further narrowed down by identifying TF genes with reproductive or embryonic lethal phenotypes in mouse knock-out models using data from MGI Jax, implicating a major biological role for these genes in the uterus **(Figure 6)**. Lastly, a literature search was conducted to further narrow down the gene list; genes that have been discovered by previous studies to play important roles in the myometrium or endometrium or involved with endometriosis, decidualization, or parturition/labor were selected. The following genes were identified as potential activators of PGR in the endometrium: GATA2, FOXP1, ARID1A, NR2F2, ESR1, and MED1. The following genes were identified as potential activators of PGR in the myometrium: BRD4, MED12, NFATC, KLF9, and ESR1 **(Figure 6)**.

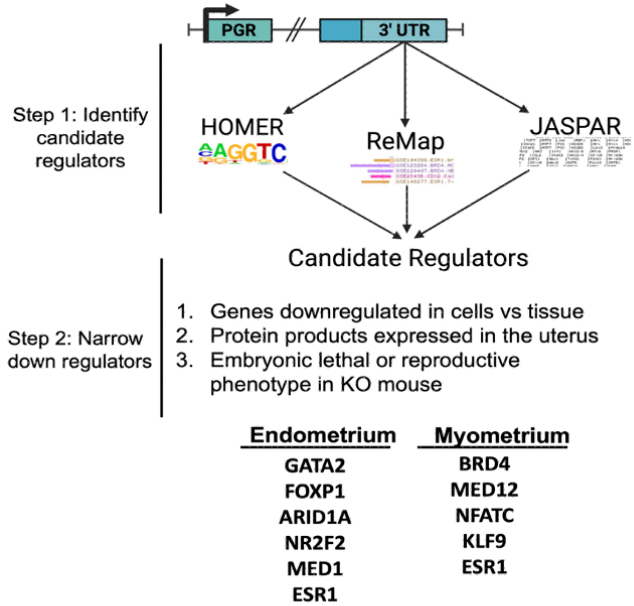

Figure 6: Strategy to identify PGR upstream regulators that act through the PGR 3'UTR in uterine mesenchymal cells.

Testing the effect of ESR1 expression on PGR mRNA levels in myometrial cells.

Because ESR1 is a known upstream regulator of *PGR* in the endometrium (Chantalat et al., 2020), the effects of its expression on *PGR* mRNA levels *in vitro* were tested. The use of ESR1 serves as a proof of principle on the methodology for future studies on the mechanism of action of ESR1 regulation of *PGR* transcription. Two gRNAs targeting the ESR1 promotor (ESR1-3 and ESR1-6) were introduced into hTERT-HM cells alongside dCas9-VPR. Western blot shows that hTERT-HM cells transduced with ESR1-3 gRNA express the ESR1 protein (66-kDa) in comparison to the unmanipulated positive control MCF7, a human breast cancer cell line (Comsa et al., 2015; Soule et al., 1973) **(Figure 7)**. A prominent band detected by the ESR1 antibody at roughly 46-kDa may be an ESR1 variant (Staub et al., 2005) **(Figure 7)**. Treatment of ESR1-expressing cells with the estrogen ligand Estradiol at a concentration of 10nM for 6 hours resulted in significant upregulation of *PGR* mRNA in hTERT-HM cells with a FC of 4.80 (P-value < 0.01) in comparison to non-targeting

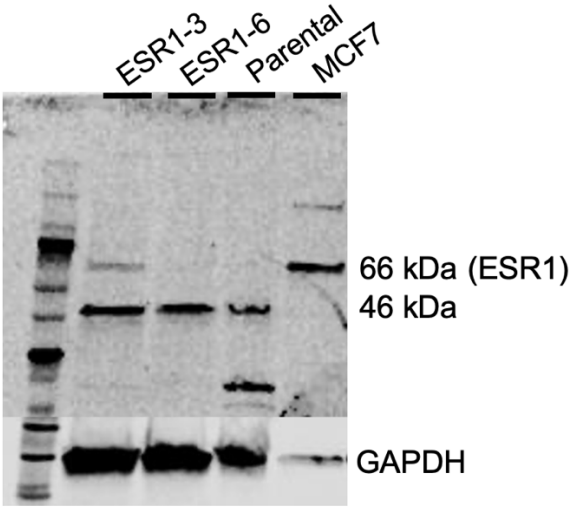

Figure 7: Myometrial cells genetically engineered to express ESR1.
Western Blot confirms the upregulation of ESR1 protein (66 kDa) in hTERT-HM cells expressing ESR1-3 gRNA alongside the CRISPR activator, dCas9-VPR. Protein levels from unmanipulated hTERT-HM cells and MCF7 cells serve as negative and positive controls for ESR1 presence. GAPDH is the loading control.

control **(Figure 8B, D)**. This finding supports ESR1 as an upstream regulator of *PGR*.

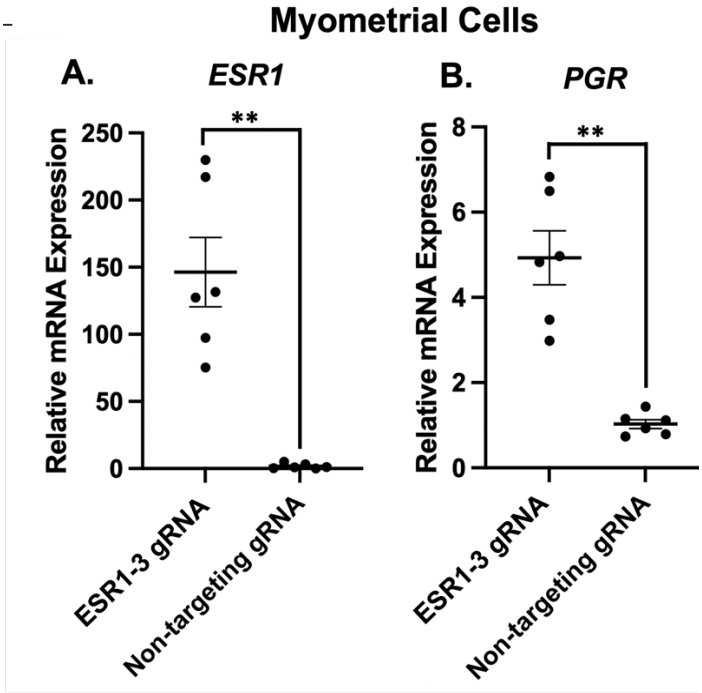

Figure 8: Testing the effect of PGR regulator, ESR1, in mesenchymal lineage cells.
Relative ESR1 **(A)** and PGR **(B)** mRNA levels measured by qRT-PCR in hTERT-HM cells that express denoted gRNAs with the CRIPSR activator, dCas9-VPR (N = 3 with technical duplicates), treated with 10 nM Estradiol for 6 hours. **(B)** Relative PGR mRNA levels were compared to PGR-promoter targeting (PGR640) or non-targeting gRNAs as controls. **P-value < 0.01, ***P-value < 0.001, ****P-value < 0.0001 by unpaired t-test.

DISCUSSION

PGR expression is regulated in a tissue and cell-specific manner via cis- and trans-acting factors, playing key roles in both the myometrium and endometrium for the regulation of embryo implantation, decidualization, parturition, and menstruation. Previous studies demonstrated that hypermethylation at regulatory regions around the PGR locus leads to altered PGR expression and progesterone resistance, resulting in impaired embryo implantation and

decidualization (Su et al., 2016). Through integrative analysis and CRISPR activation, this study identifies two new PGR enhancers located at the *PGR* 3' UTR (Region D1) and 200kbs upstream of the *PGR* TSS (Region U2). The identification of these enhancers revealed potential upstream regulators that control PGR isoform expression in the uterus, including GATA2, ARID1A, and KLF9. This study confirms that ESR1 gene expression is able to induce PGR expression in endometrial stromal cells, supporting previous studies. Moreover, this study shows that ESR1 expression is also able to induce PGR expression in myometrial cells, creating a new avenue to investigate the regulatory relationship between ESR1 and PGR in the myometrial compartment of the uterus.

Results from motif enrichment identified ESR1 as the most abundant TF binding motif in the *PGR* 3' UTR. ESR1 has been widely studied for its role in the endometrium, including signaling for embryo implantation and decidualization, as well as for its mis-regulation in patients with endometriosis (Chantalat 2020). For these reasons, the effect of liganded ESR1 on *PGR* mRNA levels was tested first as a proof of principle on the methodology. Furthermore, identifying ESR1 here supports the validity of the screening strategy in finding plausible targets. It has been shown that estrogen, the ESR1 ligand, acts as an upstream regulator of PGR in the uterus (Wendell 1971) by binding to estrogen response elements located in the regulatory regions of *PGR* (Savouret, 1991). After ovulation, estrogen acting through ESR1 in the endometrial stroma promotes the proliferation of endometrial epithelial cells and activates expression of PGR (Furuminato 2023). Subsequently, progesterone binding to PGR represses estrogen induced proliferation of epithelial cells, allowing for embryo implantation during the window of receptivity (Furuminato 2023). The effects of estrogen and ESR1 on *PGR* expression is dependent on co-regulators, such as SRC-1, allowing ESR1 to have both activation and inhibitory action on *PGR* expression (Oñate 1995). The results from this study support the fact that liganded ESR1 upregulates *PGR* mRNA expression in the stromal compartment of the uterus. The effects of estrogen and ESR1 on *PGR* expression in the myometrium have been much less studied. The results of this study show that in the myometrium, liganded ESR1 can also upregulate *PGR* mRNA levels.

Other candidate upstream PGR regulators identified through integrative analysis that bind to the *PGR* 3' UTR include key uterine regulators *GATA2*, *ARID1A*, and *KLF9*. Ablation of these genes in the mouse uterus results in infertility by interfering with key processes including embryo implantation, stromal decidualization (Rubel et al., 2016) (Kim et al., 2015), and parturition (Zeng et al., 2008). At the start of pregnancy, *GATA2* expression closely mirrors that of *PGR* expression (Rubel et al., 2012), and GATA2 ablation in the mouse uterus results in a significantly decreased expression of both PGRA and PGRB (Rubel et al., 2016), suggesting a potential activating role of GATA2 on *PGR* gene expression for pregnancy establishment. ARID1A encodes for a SWI/SNF subunit that plays a role in chromatin remodeling by breaking DNA-histone contacts (Mathur, 2018). ARID1A knock-out mice have a significant reduction in H3K27ac marks at the PGR locus, as well as a decrease in *PGR* expression levels (Asaka et al., 2023), suggesting epigenetic regulation of PGR by ARID1A through chromatin accessibility. *KLF9* expression has been reported in both the myometrium and endometrium compartments of the uterus (Pabona et al., 2015; Zeng et al., 2008). Significant reductions in both KLF9 and PGR levels in the endometrium have been recorded in patients with endometriosis (Pabona et al., 2015). These results alongside the results of this study strongly indicate a potential role of these genes in the regulation of *PGR* isoform expression in the uterus, and potentially in the rise of progesterone resistance phenotypes.

Future Studies:

The impact of these candidate regulators on *PGR* gene expression will be tested by activating their expression through CRISPR activation and looking at resulting changes in PGR mRNA levels through RT-qPCR and PGR isoform levels through western blot. The identified *PGR* regulators will be further examined for their genome occupancy in the *PGR* locus and the associated enhancers by the chromatin immunoprecipitation assay. Gain and loss of function assays of the identified *PGR* regulators will be carried

out in multiple lines of primary uterine cells to validate their functionality on *PGR* expression control.

Limitations:

With the use of the Perturb-seq assay, a PGR enhancer located 200kbs upstream of the *PGR* TSS was identified; however, the Perturb-seq assay was unable to detect the PGR cis-acting element at the 3' UTR which was identified through RT-qPCR. This data demonstrates a trade-off between efficiency and extensiveness of the Perturb-seq assay in comparison to manual RT-qPCR; with the sensitivity of sc-RNA-seq being insufficient as a comprehensive assay to survey all cis-acting elements in the vicinity of a gene. Furthermore, while the Perturb-seq assay was able to identify enhancers in stromal cells (THESC), no enhancers were identified in myometrial cells (hTERT-HM). This may be due to cell type specific changes in epigenomic signals at the *PGR* locus in myometrial cells in comparison to endometrial cells. The *PGR* promotor contains a CpG methylation site which has been shown to silence *PGR* expression due to hypermethylation, resulting in impaired embryo implantation and decidualization (Su et al., 2016). Furthermore, myometrial cells have a significant reduction in H3K27ac marks at the PGR promotor in comparison to myometrial tissue **(Figure 2)**, a likely factor contributing to the low gene expression of *PGR* in myometrial cells. It is possible that DNA methylation or histone deacetylation at the *PGR* promotor interfere with the induction of *PGR* expression by *PGR* enhancers, falling below the detection threshold for Perturb-seq. In order to boost up the signal-to-noise ratio, a stronger transcriptional activator than VPR may be used, such as SPH (Zhou et al., 2018) or CBP (Sajwan & Mannervik, 2019). Furthermore, two or more enhancers could be targeted simultaneously, or enhancers could be targeted in conjunction with the gRNA that targets the *PGR* promotor, allowing for the identification of more sensitive enhancers.

Though epigenome data from myometrial tissue was used to infer the location of putative upstream regulatory elements, this study exclusively relied on results from immortalized cell lines to test the activity of these regulatory elements. Due to the artificial conditions maintaining these cell lines, these cells may only offer a narrow window to capture *in vivo* biology. In the future, these studies will be replicated in multiple primary cell lines to validate the functionality of the enhancers on *PGR* expression control.

This study screened nine candidate enhancer regions located up to 600kbs away from the *PGR* TSS. The mechanism by which promotors communicate with distant enhancers, also known as "enhancer-promoter communication", has not yet been elucidated. One hypothesis is the looping model that postulates that enhancer-promotor communication occurs through chromatin loops that bring these elements within close proximity through the formation of topologically associated domains (Razin et al., 2023). Based on the looping model, this study arbitrarily identified putative enhancer regions up to three loop connections away from the *PGR* TSS. However, previous studies have shown that enhancers can regulate genes located up to 1Mb away, as seen for the SHH gene (Lettice et al., 2003), highlighting the limited scope of this experiment. Furthermore, due to the lower sensitivity of Hi-C looping data in tissue, key chromatin loops that inform on DNA interactions may have been missed.

Impact

Cis-regulatory elements and upstream transcription factor regulators play a pivotal role in the precise orchestration of gene expression and downstream gene networks. Cis-regulatory elements such as enhancers act as fine tuners for gene expression, allowing genes to respond to internal and external stimuli, such as the physical contact between the embryo and endometrium that triggers a cascade of gene expressions, allowing the uterus to support pregnancy. Understanding the regulatory elements of a gene is fundamental to deciphering the complex regulatory networks that sustain normal cellular function and development, as well as what goes wrong when disease phenotypes arise. Many disease phenotypes arise from mutations in the regulatory sequence of genes rather than the coding sequence, which can lead to alterations in the expression of a gene, rather than its translated protein product. For example, mis-regulation of PGR isoforms can alter the PGRB:PGRA ratio, a factor present in uterine pathologies including endometriosis, preterm birth,

and progesterone resistance (Mousazadeh et al., 2019; Nothnick, 2022; Peavey et al., 2021). By uncovering what cis-regulatory elements control the expression of PGR isoforms, we can identify non-coding mutations that lead to altered expression of *PGR*. Furthermore, through the discovery of upstream regulators of PGR, we can begin to decipher the underlying cause of altered *PGR* expression, and potentially, what gives rise to progesterone resistance. Ultimately, these regulators could be targeted to screen for progesterone resistance or treat the underlying genetic abnormalities that lead to these disease phenotypes.

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