Cell Biology
Genetics and Genomics

Assessment of the Histone Mark-based Epigenomic Landscape in Human Myometrium at Term Pregnancy

Reviewed Preprint
v2 • April 24, 2025
Revised by authors

Reviewed Preprint
v1 • June 19, 2024

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eLife Assessment

This **valuable** study employed a multi-omics approach to elucidate the regulatory mechanism underlying parturition and myometrial quiescence. The data presented to support the main conclusion are **solid**. This work will be of interest to both basic researchers who work on reproductive biology and clinicians who practice reproductive medicine.

https://doi.org/10.7554/eLife.95897.2.sa4

Abstract

The myometrium plays a critical role during pregnancy as it is responsible for both the structural integrity of the uterus and force generation at term. Emerging studies in mice indicate a dynamic change of the myometrial epigenome and transcriptome during pregnancy to ready the contractile machinery for parturition. However, the regulatory systems underlying myometrial gene expression patterns throughout gestation remain largely unknown. Here we investigated human term pregnant nonlabor myometrial biopsies for transcriptome, enhancer histone mark cistrome, and chromatin conformation pattern mapping. More than thirty-thousand putative enhancers with H3K27ac and H3K4me1 double positive marks were identified in the myometrium. Enriched transcription factor binding motifs include known myometrial regulators AP-1, STAT, NFkB, and PGR among others. Putative myometrial super enhancers are mostly colocalized with progesterone receptor occupying sites and preferentially associated with highly expressing genes, suggesting a conserved role of PGR in regulating the myometrial transcriptome between species. In

human myometrial specimens, inferred PGR activities are positively correlated with phospholipase C like 2 (*PLCL2)* mRNA levels, supporting that PGR may act through this genomic region to promote *PLCL2* expression. PGR overexpression facilitated *PLCL2* gene expression in myometrial cells. Using CRISPR activation, we assessed the functionality of a PGR putative enhancer 35-kilobases upstream of the contractile-restrictive gene *PLCL2*. In summary, results of this study serve as a resource to study gene regulatory mechanisms in the human myometrium at the term pregnancy stage for further advancing women's health research.

Introduction

The myometrium is the muscular component of the uterus. Throughout gestation, the myometrium maintains contractile quiescence until labor, where uterine contractility facilitates parturition. Pre-term birth, often characterized by aberrant myometrial activity before term, results in the termination of pregnancy prior to 37 weeks gestation. Complications associated with pre-term birth account for 35% of neonatal death and contribute to lifelong physical and socioeconomic multi-morbidities to surviving neonates and mothers (Chawanpaiboon, Vogel et al. 2019). It is therefore critical to understand the molecular mechanisms underlying the maintenance and shift of myometrial quiescence throughout gestation.

The myometrium undergoes extensive structural and functional remodeling in preparation for parturition through genomic regulatory mechanisms which influence gene expression throughout pregnancy. Major transcription factor families have been identified to contribute throughout the remodeling process. Studies on the role of steroid hormone receptors in myometrial remodeling suggest that the withdrawal of functional progesterone signaling at term results from a stoichiometric shift favoring the PGR-A isoform over PGR-B. This shift is associated with increased activation of estrogen receptor alpha (ESR1) expression at term (Mesiano, Chan et al. 2002) (Merlino, Welsh et al. 2007) (Nadeem, Shynlova et al. 2016).

Activator protein 1 (AP-1) complex subunits have been observed to act as PGR coregulators (Dai, Litman et al. 2003) and have dynamic expression patterns throughout gestation in both humans and rodent models. For example, FOS:JUN heterodimers are implicated to be critical for the initiation of labor through transcriptional regulation of gap junction proteins such as Gja1 (Gap junction alpha 1) (Mitchell and Lye 2001) (Mitchell and Lye 2005) (Balducci, Risek et al. 1993).

Contributing to the dynamic nature of the myometrial transcriptome during term is the epigenome and its reprogramming. Studies investigating epigenetic markers related to gene activation in the mouse myometrium have revealed that the promoters for contractility-driving genes, such as Gja1, are epigenetically activated well before the onset of labor (Shchuka, Abatti et al. 2020). Considering this, the myometrial epigenome's role in parturition disorders, such as preterm birth, has thus been under investigation. For example, it is known that altered DNA methylation is linked with preterm birth (Barcelona de Mendoza, Wright et al. 2017), including at the promoters of genes associated with myometrial contraction (Erickson, Myatt et al. 2023) and fetal membrane rupture (Wang, Ogawa et al. 2008). However, differential myometrial DNA methylation at CpG islands in the promoters of contractility-driving genes is not thought to be a major contributor to preterm birth (Mitsuya, Singh et al. 2014). Given that DNA methylation mediated gene regulation often occurs outside of CpG islands (Irizarry, Ladd-Acosta et al. 2009), there is still work to be done at this interface. Regardless, whether through epigenetic or transcriptomic gene regulatory programs, the molecular mechanisms underlying the regulation of genes critical for myometrial reprogramming and quiescent maintenance are still poorly understood.

In this study, we aim to improve our understanding of the cellular processes involved in shaping the myometrium's dynamic state of quiescence at pregnancy through the epigenetic and transcriptomic profiling of the human myometrium. Because of the relevance of genomic regulatory mechanisms in coordinating myometrial activity, we identified candidate cis-acting regulatory regions using chromatin immunoprecipitation sequencing (ChIP-Seq) assays of surrogate histone enhancer markers H3K27ac (Creyghton, Cheng et al. 2010) and H3K4me1 (Heintzman, Stuart et al. 2007) alongside chromatin conformation capture assays in term pregnant myometrial tissues. Using this data, we investigated cis-acting regulatory regions for the gene phospholipase C like 2, which encodes for the protein PLCL2 and has been implicated in the modulation of calcium signaling (Uji, Matsuda et al. 2002) and maintenance of myometrial quiescence (Peavey, Wu et al. 2021). Putative enhancers upstream of the PLCL2 transcriptional start site were subjected to functional assessment using CRISPR activation-based assays. Here, we identified a genomic region 35-kilobases upstream of the *PLCL2* transcriptional start site involved in *PLCL2* transcriptional regulation. Furthermore, we aimed to characterize this genomic region through the identification of candidate *PLCL2* transcriptional regulators co-localizing with this cis-acting element using integrative cistrome and transcriptome analysis and have demonstrated PGR to be a direct regulator for *PLCL2* expression. These findings build upon our understanding of myometrial remodeling throughout gestation and will be pertinent for the development of medical interventions aiming to address pre-term birth.

Results

Epigenomic Landscape in Term Pregnant Myometrial Specimens

To better understand the regulatory network shaping the myometrial transcriptome before labor, we analyzed transcriptome and putative enhancers in individual human myometrial specimens. Using RNA-seq, we identified actively expressed RNAs, while ChIP-seq for H3K27ac and H3K4me1 was used to map putative enhancers. Active genes were associated with nearby putative enhancers based on their genomic proximity. Additionally, chromatin looping patterns were mapped using Hi-C to further link active genes and putative enhancers within the same chromatin loops. The ChIP-seq assay identified an average of 44238 H3K27ac and 74325 H3K4me1 positive genomic regions in three term-pregnant, nonlabor myometrial biopsies (**Table 1**). An RNA-Seq assay revealed an average of 12157 active genes in these specimens that manifested expression levels of fragments per kilobase of transcript per million mapped reads (FPKM) greater or equal to 1 (**Table 1**). A High-throughput Chromosome Conformation Capture (Hi-C) assay further found a total of 27162 chromatin loops from two of the three myometrial specimens (**Table 1**, subject 1 and 3). We failed to identify chromatin loops in the second subject's biopsy due to limited sample availability. Together, these results delineate a map of H3K27ac and H3K4me1 positive signals in the human term pregnant myometrial tissue before the onset of labor, which we use as a resource to investigate the molecular mechanisms that prepare the myometrium for subsequent parturition.

When comparing the present study to previous findings (Dotts, Reiman et al. 2023), 8563 genomic regions carry common H3K27ac-positive histone marks. However, significant variations on the number and location of H3K27ac-positive intervals are present across the six samples among these two studies (Figure S1). Depending on the number of mapped intervals, the 8563 common regions constitute between 20.7% to 71.5% of total H3K27ac-positive intervals across the six myometrial specimens (Figure S1). Notably, the fraction of intervals commonly identified in the present study's three specimens ranges between 56.4% and 70.0% of each of their total H3K27ac-positive regions, while the Dotts dataset has a wider range between 28.0% and 74.0%. With a less stringent criterium, most the H3K27ac-positive intervals are found in at least two samples, ranging between 75.4% and 98.3% (Figure S1). These data together highlight the degree of variation on mapping the epigenome among specimens and datasets.

Subject	1	2	3
Number of H3K27ac intervals	47,223	39,465	46,026
Number of H3K4me1 intervals	72,091	74,511	76,374
Number of active genes	12,809	11,761	11,902
Number of chromatin loops	10,321	N.D.	16,841

Table 1.

Epigenome and transcriptome profile of the individual myometrium biopsy.

Active genes are defined as FPKM ≥ 1. N.D., not determined.

Putative Enhancers for Gene Regulation in the Human Myometrium

Since H3K27ac and H3K4me1 marked genome regions are associated with enhancers for gene regulation (Creyghton, Cheng et al. 2010) (Heintzman, Stuart et al. 2007), we define the regions that have overlapping H3K27ac and H3K4me1 marks as putative myometrial enhancers at the term pregnant nonlabor stage (Dataset S1). Among the three specimens, 13114 putative enhancers are commonly present (**Figure 1A**). A significant variance on the location of myometrial putative enhancers is also seen among biopsies as evident by the observation that these common putative enhancers make up less than half of the total putative enhancers in each individual specimen (**Figure 1A**). More than one-third of these 13114 common putative enhancers are located within a 100-kilobase vicinity of actively high-expressing genes (FPKM >= 15), while a much smaller fraction of them (13.3%) are associated with actively low-expressing genes (**Figure 1B**). Motif enrichment analysis on these common putative enhancers further reveals an overrepresentation of binding motifs for myometrial transcription factors AP-1, STAT5, and NFκB, steroid hormone receptors PGR and NR3C1, and smooth muscle transcription regulators SRF and ELK1 (**Figure 1C** and Dataset S2). These findings collectively suggest that the putative myometrial enhancers bring together smooth muscle and hormonal control programs for the regulation of myometrial gene expression at term pregnancy.

Super enhancers house hormone-dependent gene regulatory programs for female reproductive tract homeostasis (Shin, Willi et al. 2016) (Hewitt, Grimm et al. 2020). Across the three human subjects, 540 putative super enhancers are commonly identified in the term pregnant nonlabor myometrial specimens (**Figure 2A** and Dataset S3). More than 40% of the 540 putative super enhancers are located within a 100-kilobase distance to high-expressing genes (FPKM >= 15), while only 7.3% of putative myometrial super enhancers are found near low-expressing genes (5 > FPKM >=1) (**Figure 2B**). Compared with the regular putative enhancers, the putative myometrial super enhancers are found more frequently near active genes that are expressed at relatively higher levels (**Figure 1B** and **Figure 2B**). Notably, 76% of the putative super-enhancers co-localize with known PGR-occupied regions in human myometrial tissue, compared to 20% co-localization observed in regular enhancers (Figure S2). Further examining the myometrial active genes that are associated with putative super enhancers revealed an enrichment of gene functions in cytoskeleton organization, extracellular-receptor interaction, transcription regulation, mRNA stability, and estrogen signaling (**Figure 2C** and Dataset S4). Taken together, these results establish the association among the putative myometrial enhancers, the potential regulatory program within these super enhancers, and the cellular functions of the genes they may control.

Cis-acting elements for the control of the contractile gene *PLCL2*

We previously demonstrated the positive correlation of PLCL2 and PGR expression in a mouse model and PLCL2's function on negatively modulating oxytocin-induced myometrial cell contraction (Peavy et al., 2021). However, the mechanism that underlies PGR's regulation of PLCL2 remains unclear. Taking advantage of the mapped myometrial cis-acting elements, we aimed to identify the cis-acting elements that may contribute to the PLCL2 transcriptional regulation with a special interest on the PGR-related enhancers.

The Hi-C assay identified a chromatin loop with one end at the *PLCL2* transcription start site (TSS) and the other end at approximately 90-kilobases upstream of the TSS (**Figure 3A**). Within this chromatin loop, ChIP-Seq results revealed seven genomic regions that are marked with H3K27ac in all three myometrial specimens (**Figure 3A**). Six of the seven regions are also co-localized with previously published genome occupancy of transcription regulators curated by the ReMap Atlas (Hammal, de Langen et al. 2022) (**Figure 3A**). For the purpose of functional screening, we focus on H3K27ac signals instead of using H3K27ac/H3K4me1 double positive criterium to cast

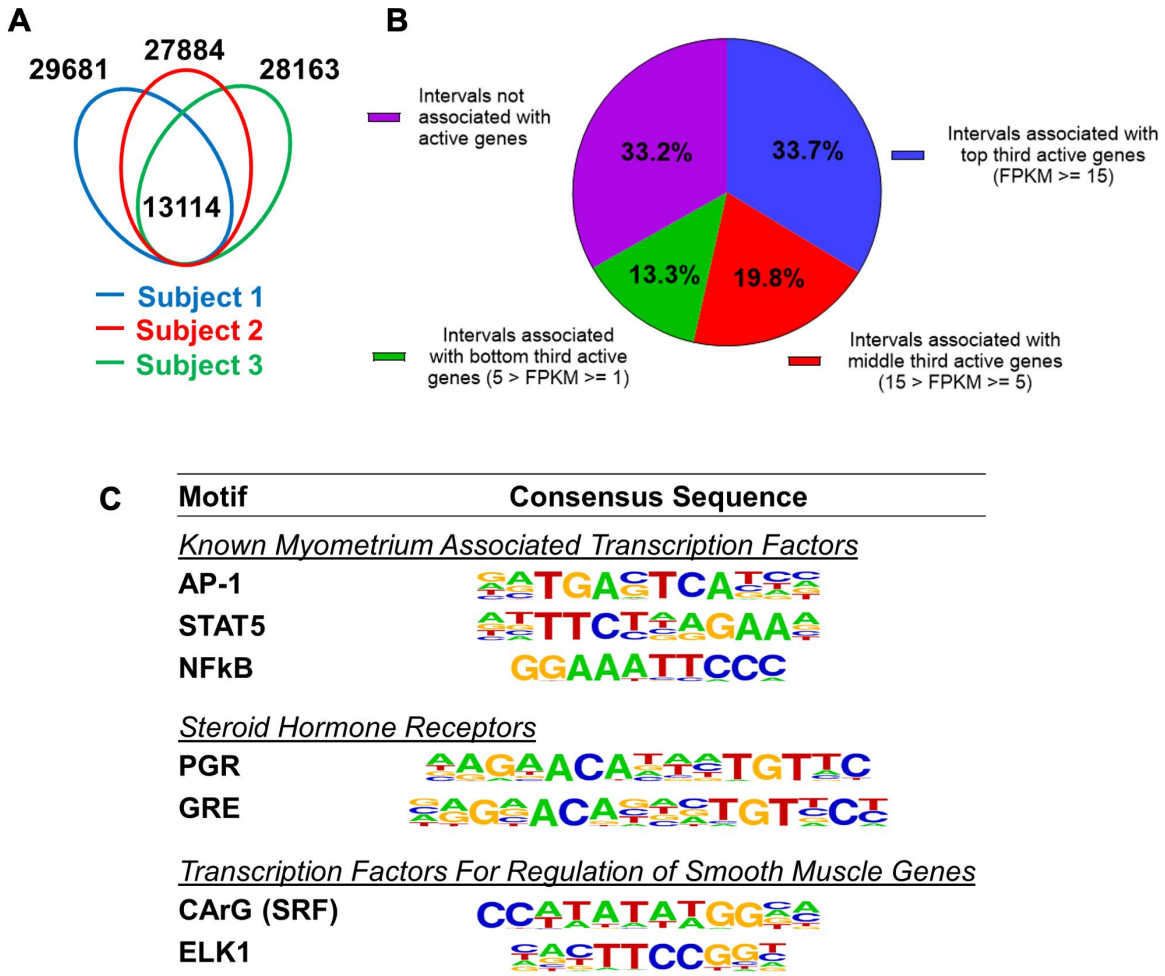
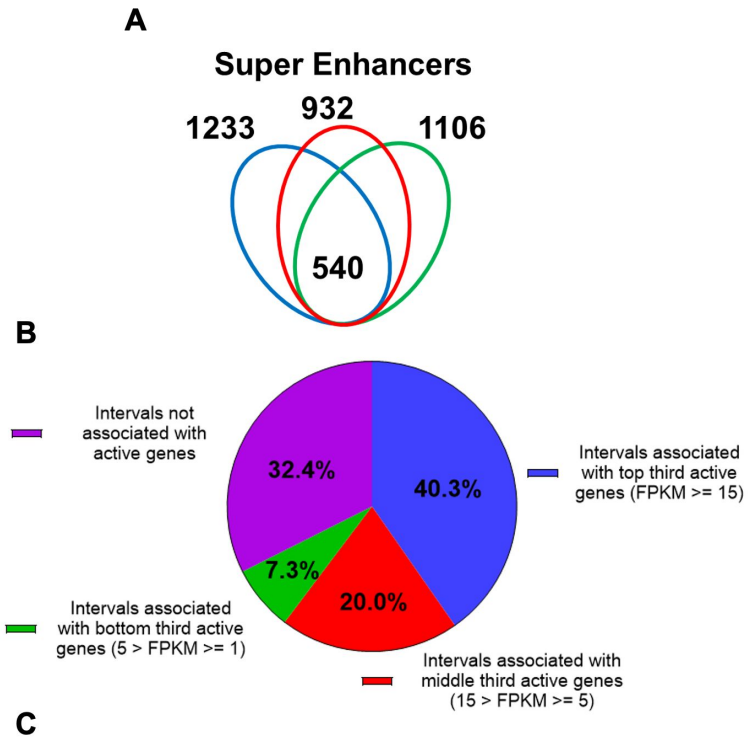


Figure 1.

Putative enhancers in term pregnant human myometrial tissues.

(A) Distinct and common putative enhancers in term pregnant myometrial biopsies from three subjects. Genomic regions with H3K27ac and H3K4me1 double positive histone marks are defined as putative active enhancers. (B) Association of commonly shared putative enhancers with active genes. The association between an interval and an active gene is defined by locating within 100 kb vicinity of each other. (C) Over-Represented transcription factor binding motifs in putative enhancers. A subset of enriched motifs that are relevant to myometrial homeostasis in the 13090 H3K4me1/H3K27ac-positive putative enhancer regions is displayed.



Gene Ontology Term	P-value	Genes
Actin cytoskeleton organization	9.22E-05	PTPN1, SHC1, ARHGEF17, ACTN1, FHL3, ACTN4, SSH1, PDLIM1, INF2, TYROBP, PDLIM2, CAPZB, MYH9, FLNB
ECM-receptor interaction	0.024366	COL1A1, COL4A1, COL6A2, THBS1, HSPG2, CD44
DNA-binding transcription factor activity	1.18E-03	ZFHX3, NFIX, SMAD3, ZNF581, EPAS1, RFX2, FOS, NR2F2, HIC1, FOXP1, ELK3, MNT, DBP, SP1, NFIC, KLF9, ATOH8, CREB3L2, RARA, ERF, HOXD9, MEF2D
3'-UTR-mediated mRNA destabilization	0.037196	ZFP36, ZFP36L2, ZFP36L1
Estrogen signaling pathway	0.004193	SHC1, SP1, GREB1, CREB3L2, RARA, FOS, ADCY7, HSPA1B, GNAI2

Figure 2.

Putative super enhancers in term pregnant human myometrial tissues.

(A) Number of super enhancers mapped in tissues of each individual human subject. (B) Association of commonly shared super enhancers with active genes in human myometrium. (C) Selected enriched gene ontology terms in the 346 active genes that are associated with super enhancers.

a wider net. Eight guide RNAs (gRNAs) were designed to target these seven candidate regions for CRISPR activation (CRISPRa) based screening of *PLCL2* gene regulation (**Figure 3A**). The two previously reported gRNAs (PLCL2-7 and PLCL2-8) were able to elevate the endogenous *PLCL2* mRNA levels significantly higher than the non-targeting gRNA in immortalized human myometrial cells (hTERT-HM) (**Figure 3B**) (Peavey, Wu et al. 2021). gRNAs PLCLC2-1, PLCL2-3, PLCL2-5, and PLCL2-6 also significantly increased *PLCL2* expression above the levels of the non-targeting group (**Figure 3B**). Moreover, results from the Perturb-Seq assay found that the PLCL2 gene ranks high in the differentially expressed gene list of the PLCL2-5 gRNA expressing cells compared with the non-targeting gRNA expressing cells (Dataset S4), in line with the qRT-PCR assay finding in which the gRNA PLCL2-5 induced *PLCL2* expression to the greatest extent among the upstream non-coding genome targeting gRNA (**Figure 3B**). Moreover, gRNA PLCL2-5 was capable of mediating the increase of the *PLCL2* transcript abundance in another uterine mesenchymal cell type T-HESC (**Figure 3C**). Collectively, these findings support the PLCL2-5 targeted genomic region as a cis-acting element for regulation of the *PLCL2* gene.

PGR as an upstream regulator for the *PLCL2* gene in the human myometrium

After determining its cis-acting element role, the PLCL2-5 targeted genomic region was further examined to identify upstream regulators that control myometrial *PLCL2* expression. This was achieved by first identifying transcription factor occupancy in this genome region in any tissues/cells that are documented in public databases (ReMAP Atlas) (Hammal, de Langen et al. 2022), followed by filtering for factors that have known mRNA and/or protein expression in hTERT-HM cells and tissues using publicly available transcriptomic and histological data including the Human Protein Atlas (Human Protein Atlas *proteinatlas.org*) (Uhlen, Fagerberg et al. 2015) and published RNA-Seq datasets (Stanfield, Amini et al. 2019) (Wu, Anderson et al. 2020). The resulting candidate upstream regulators for the myometrial *PLCL2* gene are documented in **Table 2**. Since the *PLCL2* mRNA abundance is much lower in the hTERT-HM cells than in the myometrial tissues, these candidate upstream regulators were further grouped into candidate activators and repressors based on an assumption that a candidate activator would be expressed higher in human myometrial tissue, and a candidate repressor being expressed higher instead in the hTERT-HM cells, holding the *PLCL2* mRNA levels down. Candidate activators for the *PLCL2* gene include known myometrial regulators such as PGR, estrogen receptor alpha (ESR1), and AP-1 (**Table 2**). Interestingly, the mediator complex protein subunit MED1, cohesion complex members RAD21 and SMC3, and many epigenomic modifiers are among the list of the candidate repressors (**Table 2**). These findings not only provide candidates for subsequent functional assessment, but also highlight potential pathways for future investigations on the regulation of the contractile gene *PLCL2* expression in the myometrium.

We previously demonstrated the regulation of mouse *Plcl2* gene by the myometrial PGR (Wu, Wang et al. 2022). To further test whether such a regulatory mechanism is also present in the human, the CRISPRa technology was employed to increase the expression of PGR mRNA and proteins in hTERT-HM cells (**Figure 4A** and **Figure 4B**). Given that the PGR640 gRNA led to higher PGR protein production (**Figure 4B**), this gRNA was utilized to stimulate myometrial *PGR* expression for subsequent experiments. Indeed, RT-qPCR results showed that PGR overexpression increased *PLCL2* mRNA abundance in hTERT-HM cells (**Figure 4C**), demonstrating a consistent finding with the mouse model.

To further explore this regulatory relationship in the human myometrial tissue, we examined the degree of correlation between the inferred PGR activity and the *PLCL2* transcript abundance in myometrial biopsies. Gene expression profiles of myometrial specimens from 13 proliferative-phase, 6 secretory-phase, 14 post-menopausal, and 10 Provera-treated human subjects were determined by the RNA-Seq assay (Dataset S6) to take advantage of the wide spectrum of

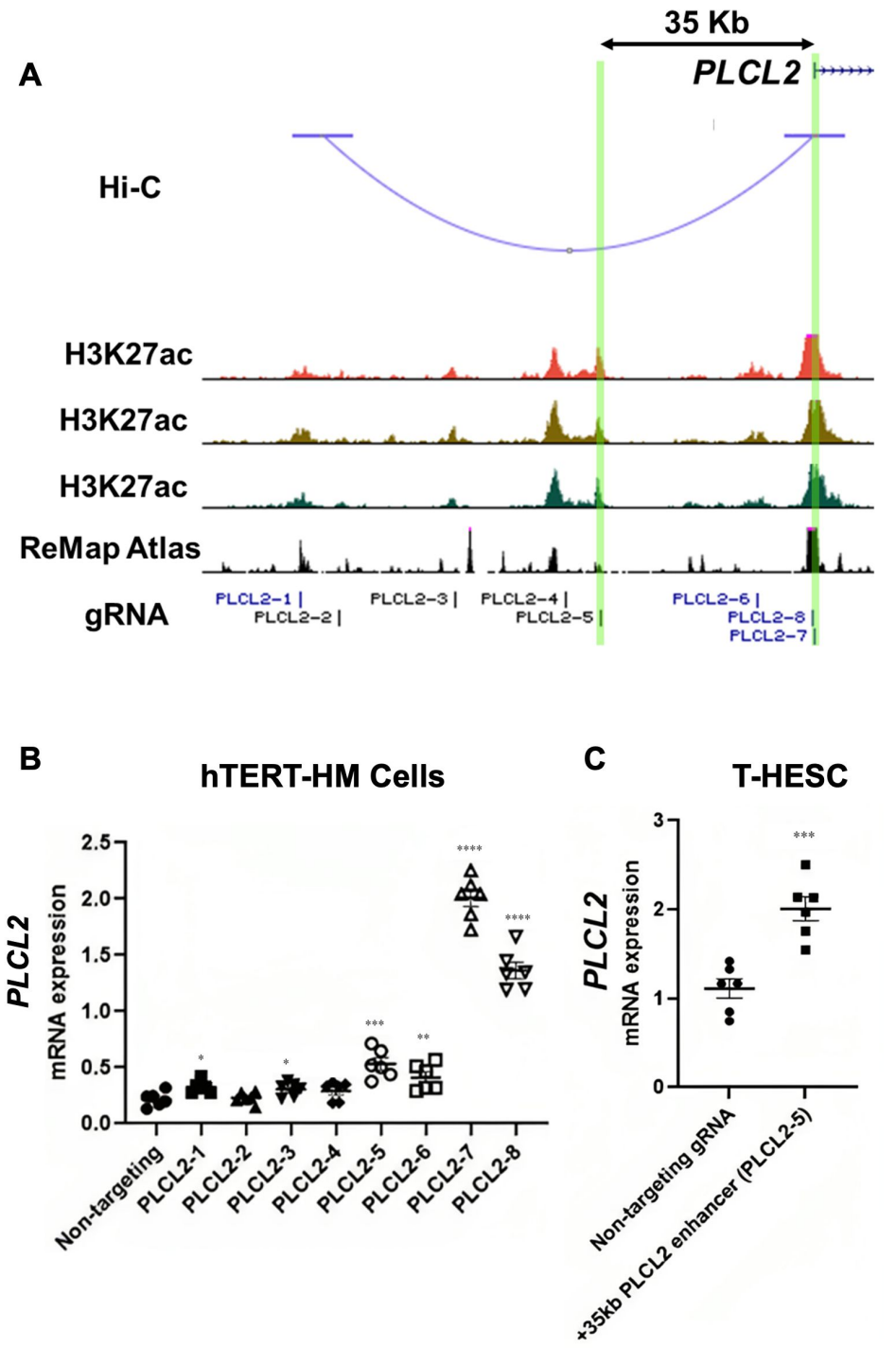


Figure 3.

Identification of enhancers for the *PLCL2* gene.

(A) UCSC Genome Browser track view of the human PLCL2 locus marked with gRNA targeting locations. (B-C) Relative PLCL2 mRNA levels measured by qRT-PCR in hTERT-HM cells (B) or T-HESC cells (C) that express denoted gRNAs with the CRISPR activator (N=3 with technical duplicates). ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ by unpaired t-test.

Candidate Activators	Description
PGR	Steroid Hormone Receptor
ESR1	
FOS, JUN	AP-1 Transcription Factor Subunit, bZIP Transcription Factor
JUN	
JUNB	
JUND	
MAF	bZIP Transcription Factor
BHLHE40	bHLH Transcription Factor, Involved in CLOCK gene regulation
TCF21	bHLH Transcription Factor
MAX	bHLHZ Transcription Factor
FOXO1	Forkhead Box Transcription Factor
MRTFB	Myocardin Family, SRF Trancriptional Co-Activator
STAT3	Transcriptional Regulator
ERG	ETS Transcriptional Regulator

Candidate Repressors	Description
ARID1A	SWI/SNF Family of Epigenetic Modifiers
BRD2	Epigenetic Modifier, Chromatin Reader
BRD4	
KMT2A	Component of MLL Epigenetic Modification complex
SMARCB1	Component of BAF Epigenetic Modification Complex
ZMYM3	Component of Epigenetic Modification Complexes
CREB1	bZIP Transcription Factor, Interacts with Epigenetic Modifiers
CREBBP	CREB Binding Protein, Epigenetic Modifier
HDAC2	Histone Deacetylase, Epigenetic Modifier
HDAC3	
CLOCK	bHLH Transcription Factor Family, Rhythmic Epigenetic Modifier
MED1	Mediator Complex Subunit
RELB	NF-KB Transcription Factor Subunit
CHD4	Component of NuRD Epigenetic Modification Complex
RAD21	Cohesin Complex Member
SMC3	
REST	KLF Sliencing Transcription Factor
TCF4	bHLH Transcription Factor
ZNF687	Zinc Finger Protein, Transcriptional Regulator

Table 2.

Candidate upstream regulators that mediate PLCL2-5 enhancer's regulatory effect on PLCL2 expression.

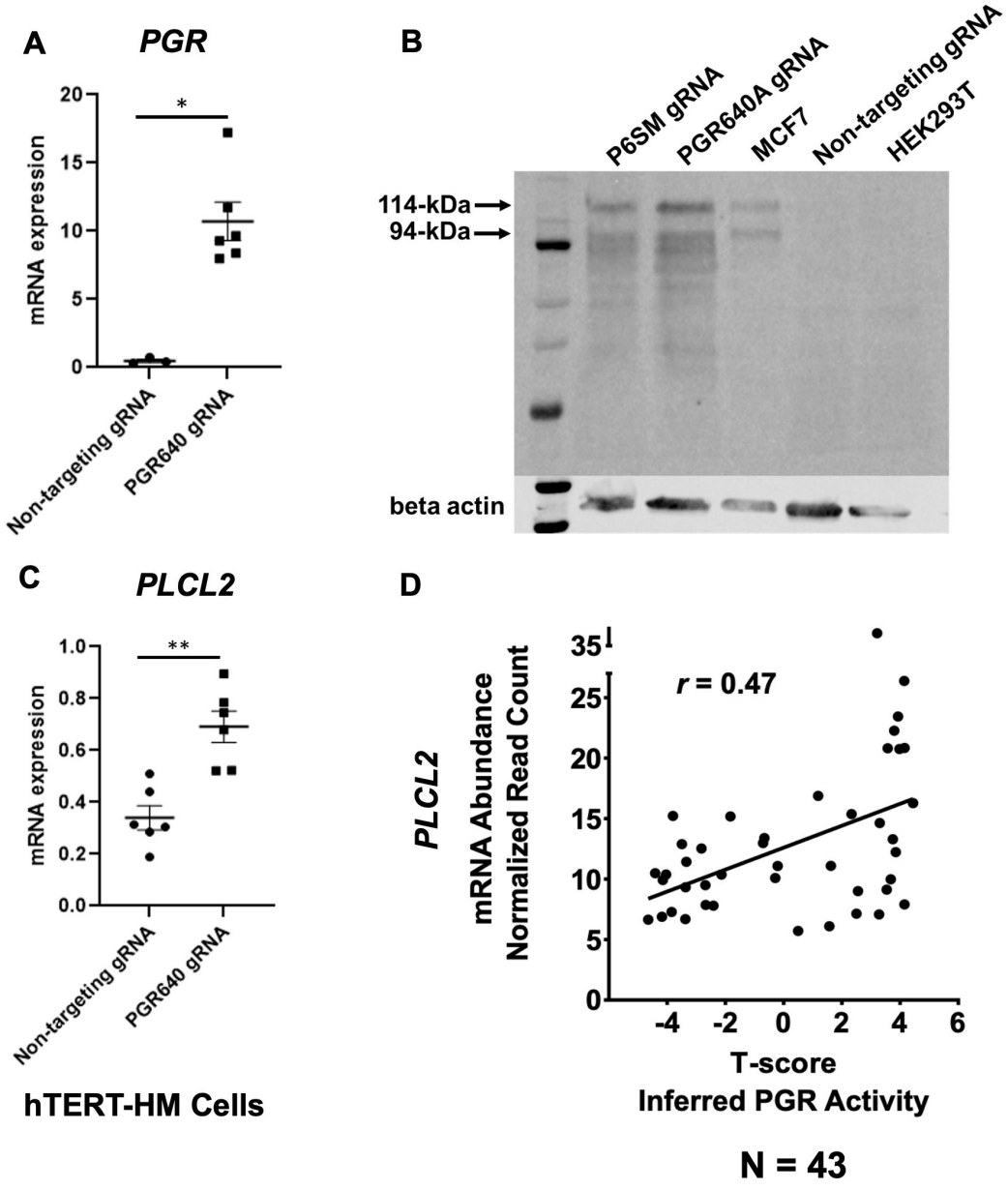


Figure 4.

PGR regulation of *PLCL2* expression.

(A) *PGR* mRNA abundance in hTERT-HM cells with the PGR-promoter targeting (PGR640) or non-targeting gRNAs under the CRISPR activation system. (N=3 with technical qPCR duplicates). (B) Protein abundance of in hTERT-HM cells with the PGR-promoter targeting (PGR640 and P6SM) or non-targeting gRNAs under the CRISPR activation system. Protein extracts from unmanipulated MCF7 and HEK293T cells serve as positive and negative control for the PGR presence. (C) *PLCL2* mRNA abundance in in hTERT-HM cells with the PGR-promoter targeting (PGR640) or non-targeting gRNAs under the CRISPR activation system. (N=3 with technical qPCR duplicates). (D) Pearson correlation between *PLCL2* mRNA levels and inferred PGR activities (T-Scores) in 43 human myometrial specimens. **, $p < 0.01$; *, $p < 0.05$ by by Mann-Whitney test (A) and unpaired t-test (C).

progesterone signaling activities in these samples. Inferred myometrial PGR activities, represented as T-scores (Dataset S6), were derived from this normalized gene expression matrix of 43 human myometrial specimens with the previously published mouse myometrial *Pgr* gene signature (Wu, Wang et al. 2022) (Li, Bushel et al. 2021). A Pearson correlation analysis found a positive correlation (r = 0.47) between the T-scores and normalized *PLCL2* mRNA levels in these 43 specimens (**Figure 4D**). Together, these in vitro and in vivo findings collectively support that PGR is a *PLCL2* activator, likely acting through the 35-kb upstream cis-acting element.

Discussion

Temporally and spatially regulated gene expression is the foundation of tissue phenotype manifestation. Shchuka and colleagues utilize the mouse model to show that the myometrial epigenome is readily programed for parturition-associated gene induction days ahead of the term pregnancy (Shchuka, Abatti et al. 2020), laying the ground for subsequent laboring event. We chose to study the human myometrium at the term pregnant nonlabor stage in order to examine the association between the epigenome landscape and the gene expression pattern not only for preparing the myometrium for parturition, but also for maintaining myometrial quiescence. We also performed a mullti-omic study on a myometrial specimen from each individual human subject to better align all the datasets together, reflecting specimen and subject individuality. While the variation among specimens is substantial, we were able to identify myometrial cis-acting elements commonly shared among three individuals. As a proof of principle, we subsequently used this information to functionally screen for genomic regions that may mediate the regulation of the contractile-suppressing gene *PLCL2*. The H3K27ac marked 35-kilobase upstream region as a result of the screening then serves as a bridge to identify PGR as one of the upstream regulators for modulating *PLCL2* gene expression in human myometrium. These findings showcase the value of these datasets as a resource for future investigations on the mechanisms of myometrial gene regulation.

Variations across cistromic datasets for the human myometrium are significant (**Figure 1** and Figure S1) (Dotts, Reiman et al. 2023). Contributing factors likely include but are not limited to subject-to-subject differences and batch effects from sample preparations. Taking the H3K27ac marked genomic regions as an example, regions commonly identified in the specimens of this study's three subjects account for 55.9%, 67.7%, and 57.9% of total H3K27ac-positive intervals in each individual sample, despite our best attempt to reduce the batch effect. Heterogeneity on cell type compositions, likely due to the sampling location, could be a major contributing factor to the variation. It is also speculated that the genomic regions with subject-to-subject variances could be transiently present and thus the signal could not be captured across samples. In addition, the underlying health conditions, medications, and environmental challenges among others may also affect the epigenomic profile. Variations could also arise from differences in the detection threshold. We identified an average of 44238 H3K27ac marked regions per specimen, while Dotts and colleagues found nearly 19000 per sample (Dotts, Reiman et al. 2023). Reagent and sample handling could confound with biological variances of specimens leading to this difference. Future investigations using standardized sample preparation protocols at the single cell resolution may help to reduce this variation.

Results from the CRISPRa-based Perturb-Seq assay demonstrate the capability of this technology to screen for cis-acting elements that are in topological vicinity of the gene of interest. This assay is particularly useful on genes that are expressed at low levels in the screening platform, i.e., the cells. The dCas9VPR activator can generate satisfactory signal-to-noise ratios to be detected by single cell RNAseq. The advantage of assaying with this system is that each individual cell serves as a container to generate data points instead of relying on multiple wells on cell culture plates. Multiplexing with multiple gRNAs can be achieved by using the gRNA sequence as a unique barcode of the cell, enabling the simultaneous collection of numerous data points for each

individual gRNA. In the present study, 56.7% of tested cells carried one species of detectable gRNAs, permitting the study of the resulting effect under a single gRNA. Moreover, another 27.0% of assayed cells have more than one species of detectable gRNAs, which open the possibility to study interactions between different cis-acting elements. However, we failed to use this approach for the purpose of identifying target genes of a putative enhancer of interest (Dataset S4). Challenges such as difficulties on defining the criteria of the associated genes and unsatisfactory signal-to-noise ratios on genes already at high baseline levels should be considered in future experimental designs. Taken together, results of the present study support the use of the CRISPRa-based Pertub-Seq assay under a defined scope.

PLCL2 and its paralog PLCL1 are catalytically inactive members of the phospholipase C family functioning as negative regulators of calcium signaling (Kanematsu, Takeuchi et al. 2005) (Takenaka, Fukami et al. 2003) (Uji, Matsuda et al. 2002). Both of them are expressed in human myometrial tissues and are able to attenuate oxytocin-induced muscle cell contraction (Peavey, Wu et al. 2021). *PLCL1* mRNA abundance is higher in the myometrium at term pregnancy than the non-gravid stage, while *PLCL2* transcript levels remain comparable between the two stages (Wu, Anderson et al. 2020). Data from cultured human endometrial stromal cells and the genetically engineered mouse model reveal progesterone signaling as the upstream regulator of *PLCL1* and *PLCL2*, respectively (Muter, Brighton et al. 2016) (Peavey, Wu et al. 2021). The present study further demonstrates the PGR-dependent regulation of the *PLCL2* gene in the human myometrial cells, suggesting that this pathway is conserved between human and mouse. Importantly, the identification of the cis-acting element 35-kilobase upstream of the *PLCL2* gene opens an avenue to investigate the impact of interactions between PGR and other myometrial transcription regulators on the mechanism of action that controls the myometrial contractile machinery. The future findings in this space could provide insight on progesterone signaling modification and identify progesterone signaling modifiers for the development of novel therapy that targets parturition disorders.

Materials and Methods

Collection of myometrial specimens

Permission to collect human tissue specimens was prospectively obtained from individuals undergoing hysterectomy or cesarean section for benign clinical indications (H-33461). Gravid myometrial tissue was obtained from the margin of the hysterotomy in women undergoing term cesarean sections (>38 weeks estimated gestational age) without evidence of labor. Non-gravid myometrial tissue was collected from pre-menopausal women undergoing hysterectomy for benign conditions. Specimens from gravid women receiving treatment for pre-eclampsia, eclampsia, pregnancy-related hypertension, or pre-term labor were excluded.

Human Cell Lines

Human immortalized myometrial cells (hTERT-HM) (Condon, Yin et al. 2002) cells and human immortalized endometrial cells (T-hESC) (ATCC, CRL-4003) were cultured 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 incubated at 5% CO_2 and 37°C.

RNA Isolation and RT-qPCR

RNA was isolated from cells using TRIzol Reagent (Invitrogen) and the RNeasy mini RNA isolation kit (Qiagen, Hilden, Germany). cDNA was prepared 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, Krause and Miller 2005). Relative expression of genes of interest were normalized to the 18S rRNA. Briefly, reaction samples were prepared to a volume of 20ul with 5uM of both forward and reverse primers, cDNA, and a final 1x concentration of the SYBR Green Supermix. 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^{TM} Real-Time PCR Detection System (Bio-Rad).

Western Blot Assay

Protein was isolated from cells using the RIPA Lysis and Extraction Buffer (Thermo Scientific) with the following specifications: 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, and 40ug of protein were loaded per lane on a Mini-PROTEAN TGX Pre-cast Protein gel (Bio-Rad, #4568094) with the Precision Plus Protein Dual Color Standards Ladder (Bio-Rad #1610374). Protein lysates from MCF7 cells were used as positive controls, while protein lysates from HEK293T cells were used as negative controls. Gels were transferred to nitrocellulose membrane using the Turbo-Blot transfer system (BioRad) according to the manufacturer's instructions. 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). PGR protein was detected using Monoclonal Mouse Anti-Human Progesterone receptor clone PgR 1294 diluted 1:1000 in milk overnight at 4C. Bands were detected using Donkey Anti-Mouse 926-32212 (Lot # C60524-15) diluted 1:20,000 in milk for 45 minutes.

Blots were 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 using the following antibodies: Actin (I-19)-R sc-11616-R (Lot#DO406) Rabbit polyclonal IgG Santa Cruz (1:1000 dilution) and Goat anti-rabbit 926-32211 (Lot#DOO304-15) (1:20,000 dilution).

RNA-Seq Library Preparation

RNA-Seq libraries were prepared according to the Illumina TruSeq Stranded mRNA protocol Document # 1000000040498 v00. Libraries were sequenced using the Illumina NextSeq 500 and NovaSeq 6000 systems. The summary table is available in Dataset S7.

ChIP-Seq Library Preparation

The ChIP-Seq assay was performed by the Active Motif service laboratory using snap-freeze human myometrial specimens. The H3K27Ac ChIP reactions were conducted with 10 µg of tissue chromatin and 4 µg of H3K27Ac antibody (Active Motif, cat # 39133). The H3K4me1 ChIP reactions were carried out by using 10 ug of tissue chromatin and 4 ul of H3K4me1 antibody (Active Motif, cat # 39297). Libraries were prepared by a custom Illumina library with the standard Illumina PE adaptors (Bentley, Balasubramanian et al. 2008). Libraries were sequenced using the Illumina NextSeq 500 and NovaSeq 6000 systems.

Hi-C Library Preparation

Snap-freeze human myometrial specimens were shipped to the Arima Genomics and the Active Motif service laboratories for preparation of the Hi-C library using the Arima-HiC Kit (Arima Genomics A510008), protocol version A160132 v00. Libraries were sequenced in the NIEHS on the Illumina NovaSeq 6000 platform.

CRISPR Activation (CRISPRa) assay

Acquisition of guide RNA expression vectors for CRISPRa assay

All gRNA expression vectors were synthesized by and acquired from VectorBuilder. gRNA expressing vectors for PGR targeting (PGR640A and P6SM), non-targeting control, and the empty backbone were purchased from VectorBuilder under the catalog numbers VB191117-1498rhp (PGR640), VB210824-1268wmh (P6SM), VB191117-1500trj, and VB190918-1522gwq, respectively.

Acquisition of dCas9-VPR-mCherry for CRISPRa assay

IGI-P0492 pHR-dCas9-NLS-VPR-mCherry was a gift from Jacob Corn (Addgene plasmid # 102245; *http://n2t.net/addgene:102245*; RRID:Addgene_102245).

Production of Lentiviruses

All lentiviruses were packaged in HEK293T/17 cells (ATCC # CRL-11268) according to published method in Current Protocols in Neuroscience (Chen, Haam et al. 2019). Briefly, 293T cells were transiently transfected with pMD2G, psPAX2 and a transfer vector containing the desired gene using Lipofectamine 2000. Supernatant was collected 48 hours post transfection and concentrated by centrifugation at 50,000 g for 2 hours. Pellets were resuspended in PBS and used for infection. To determine titer, HEK293T/17 cells were infected with lentiviral samples. Five days post infection, Qiagen DNeasy Blood & Tissue Kits was used to isolate chromosomal DNA from infected cells. All titers were determined by performing droplet digital PCR (ddPCR) to measure the number of lentiviral particles that integrated into the host genome.

CRISPRa viral transduction

hTERT-HM cells were infected with lentiviral gRNA expression vectors with a multiplicity of infection of 4 (MOI = 4) and with dCas9-VPR-mCherry at an MOI = 4. gRNA expression vector and dCas9-VPR-mCherry expression vectors, following lentiviral transduction, confer GFP and mCherry fluorescent markers, respectively.

Fluorescence Activated Cell Sorting for CRISPRa assay

Cells were examined using a BD FACSAriaII cell sorter (Becton Dickinson Biosciences, San Jose, CA) equipped with FACSDiVa software. Initially, a “scatter” gate was set on a forward scatter (FSC-A) versus side scatter (SSC-A) dot plot to isolate the principal population of cells free of debris. Subsequently, cells were consecutively gated on a side scatter height (SSC-H) versus width (SSC-W), then a forward scatter height (FSC-H) versus width (FSC-W) dot plot to isolate single cells. GFP expressing cells were excited off a 488 nm laser and detected using a 525/50 nm filter. mCherry expressing cells were excited off a 561 nm laser and detected using a 610/20 nm filter. Double positive cells were collected based off non-transfected controls and used for further cultural/biochemical analysis.

Perturb-Seq library preparation

The cells in suspension 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 following 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 with the parameters recommended in the manufacture's instruction.

Human enhancer perturb-seq data analysis

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, 10X 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 guide RNA (gRNA) identity by unique barcode in each cell. Seurat software (version 3.6.3) was utilized to perform clustering analysis on the combined dataset (Satija, Farrell et al. 2015). We applied the SCTransform package to normalize gene expression counts across cells (Hafemeister and Satija 2019). The cells were clustered based on number of unique gRNA type detected. The cell populations containing more than one type of gRNA were excluded from further analyses. We applied two approaches to determine the association between gRNAs and target genes:

1. For each cell cluster having gRNA with known target genes, such as PLCL1 and PLCL2, we compared the expression of target genes in these clusters to the cluster with scramble control gRNA. We defined individual gRNA activation as a fold change greater than 1.5.
2. For cell clusters having gRNAs with unknown target genes, we identified differentially expressed genes by performing pair-wise comparisons between each cluster and the cluster with scramble control gRNA. This analysis was conducted using the "FindAllMarkers" function in the Seurat package. Target genes were defined as those with a fold change greater than 1.5 and an adjusted p-value less than 0.05.

Additional perturb-seq data analysis is provided in Dataset S9, with a summary table available in Dataset S7

ChIP-Seq

The raw ChIP-seq reads (51-bp, single-end) were filtered with average quality scores greater than 20. Adaptor sequences were trimmed from reads using cutadapt (v1.12). Then the reads were aligned to the human reference genome (hg38) using Bowtie (v1.1.2) (Langmead, Trapnell et al. 2009), requiring unique mapping and allowing up to 2 mismatches per read (-m 1 -v 2). Duplicated reads with identical sequences were removed using Picard tools. To visualize the read coverage, BigWig files were generated from the bedgraph files of each sample using bedGraphToBigWig. These bigWig files were displayed as custom tracks on the UCSC genome browser.

The normalization of sequencing depth across all ChIP-seq datasets were achieved by down sampling to 20 million uniquely mapped reads per sample. The initial peaks of each sample were identified using MACS2 with a cutoff of adjusted p-value < 0.0001 (Zhang, Liu et al. 2008). The gene associated with each peak was predicted by searching the transcription start site (TSS) of nearby genes within a 100Kb range using HOMER (Heinz, Benner et al. 2010). The summary table is available in Dataset 7.

Identification of union peaks between H3K27ac and H3K4me1 peaks

The initial union peaks between H3K27ac and H3K4me1 in each sample were identified using the "merge" function of BedTools. The common union peaks from multiple samples were used for downstream analysis.

Identification of union peaks for term pregnant myometrial specimens

Additional H3K27ac ChIP-seq and related input data were obtained from GEO (GSE202027; patient 1, 4, and 7). The same ChIP-seq analysis pipeline described above was applied to those samples. To obtain a comprehensive set of peaks across all H3K27ac ChIP-seq datasets, the peak intervals from each dataset were merged using BedTools.

Identification of super enhancers

H3K27ac-positive enhancers were defined as regions of H3K27ac ChIP-seq peaks in each sample. The enhancers within 12.5Kb were merged by using bedtools merge function with parameter "-d 12500". The combined enhancer regions were called super enhancers if they were larger than 15Kb. The common super enhancers from multiple samples were used for downstream analysis (Whyte, Orlando et al. 2013).

Colocalization of super enhancers and PGR genome occupancy was compared by calling peaks from previously published PGR ChIP-seq data (GSM4081683 and GSM4081684). The percentages of enhancers and super enhancers that manifest PGR occupancy were calculated by overlapping the genomic regions in each category with PGR occupancy regions.

RNA-Seq analysis of Term Myometrial Specimens

The raw paired-end reads (51-bp, paired-end) were initially processed by applying a filter with an average quality score of 20. Adaptor sequences were subsequently removed from reads using cutadapt version 1.12. The processed reads were then aligned to the human reference genome (hg38) using the STAR aligner version 2.5.2b. The gene expression levels in each sample were determined by counting the total number of paired-end reads mapped to each gene using DESeq2 R package version 1.12.4 (Love, Huber and Anders 2014). The gene expression count matrix was normalized based on ratio of total mapped read pairs in each sample to 56.5 million. Only the genes with average normalized count across all samples larger than one were used for further analysis. The Bioconductor package edgeR was applied to the gene expression count matrix to detect differentially expressed genes between groups of interest (Robinson, McCarthy and Smyth 2010). The false discovery rate (FDR) of differentially expressed genes was estimated using the Benjamini and Hochberg method.

RNA-Seq analysis of Provera Treated Human Myometrial Specimens

The raw paired-end reads (76-bp, paired-end) were processed by applying a filter with an average quality score of 20. Adaptor sequences were removed from reads using cutadapt (v1.12). The processed reads were then aligned to the human reference genome (hg38) using the STAR aligner (v2.5.2b). Fragments Per Kilobase Million (FPKM) was calculated for each gene in each sample by using Cufflinks version 2.0.2.

Hi-C

The raw reads (51-bp, paired-end) generated from HiC library were mapped to human reference genome (hg38; Genome Reference Consortium Human GRCh38 from December 2013) using HiCUP version 0.7.1 (Wingett, Ewels et al. 2015). The uniquely mapped di-tag passing quality filtering with distance larger than 10Kb were used for downstream analysis. Chromatin loops were identified using "hiccups" function of Juicer version 1.8.9 with default parameters (Durand,

Shamim et al. 2016). The A/B compartments were predicted at 100Kb resolution using "eigenvector" function of Juicer. The Hi-C quality control data are provided in Dataset S8, and the summary table is available in Dataset 7.

DNA-binding factor motif enrichment analysis

Enriched motifs were identified by HOMER (Hypergeometric Optimization of Motif EnRichment) v4.11 with default background sequences matching the input sequences (Heinz, Benner et al. 2010).

Inferred myometrial PGR activities and the correlation analysis

The inferred PGR activities were represented by the T-score, which was derived by inputting the mouse myometrial *Pgr* gene signature, based on the differentially expressed genes between control and myometrial *Pgr* knockout groups at mid-pregnancy (Wu, Wang et al., 2022), into the SEMIPs application (Li, Bushel et al., 2021). The T-scores were computed using this signature alongside the normalized gene expression counts (FPKM) from 43 human myometrial biopsy specimens.

Acknowledgements

We thank the following National Institute of Environmental Health Sciences (NIEHS) Core facilities for exceptional technical supports: Epigenomic and DNA Sequencing Core, Viral Vector Core, Flowcytometry Core, and Integrative Bioinformatics Supportive Group. We thank Ms. Olivia Emery for her technical assistance. This study is partly supported by the Intramural Research Program of the National Institute of Environmental Health Sciences Z1AES103311 (FJD) and by the Gaine Research Foundation GRF-2018-01 (LL). EQ is the awardee of the NIH Intramural Training Program Fellowship. SMR is the recipient of the NIEHS Scholar Connect Program Fellowship.

Additional information

Author Contribution

SPW, LL, and FJD conceived the study. MLA provided crucial reagent. SPW, EQ, SMR, and XX performed experiments. SPW, TW, EQ, and SMR conducted data analyses. SPW, TW, EQ, XX, MLA, and SMR wrote the initial draft.

Data Availability

All the Nest Generation Sequencing Data were deposited to the National Center for Biotechnology Information Gene Expression Omnibus under the accession number GSE244735 (*https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE244735*).

Additional files

Dataset_S1. *H3K27ac and H3k4me1 double positive enhancers in term pregnant not in labor human myometrial specimens.*

Dataset_S2. *Enrichment of known transcription factor binding motifs in putative myometrial enhancers.*

Dataset_S3. *Super enhancers in term pregnant not in labor human myometrial specimens.*

Dataset_S4. *Active genes associated with super enhancers in the term nonlabor myometrium.*

Dataset_S5. *CRISPRa dependent gene expression patterns in hTERT-HM cells and gRNA information. DEG, differentially expressed genes between denoted gRNAs.*

Dataset_S6. *Normalized gene expression counts across the 43 human myometrial specimens and the PGR T-Scores of individual specimens.*

Dataset_S7. *Summary table of sequencing datasets.*

Dataset_S8. *Summary table of Hi-C quality control metrics.*

Dataset_S9. *Cell counts per gRNA and protospacer call frequencies per cell for Perturb-seq analysis.*

Supplementary Figure 1. *Subject variations on H3K27ac-positive histone marks.*

Supplementary Figure 2. *PGR occupancy in myometrial enhancers. (A) Percentages of enhancers and super enhancers that manifest PGR occupancy. PGR genome occupancy data was previously published in NCBI GEO accession numbers GSM4081683 and GSM4081684.*

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Reviewer #1 (Public review):

Summary:

The use of a multi-omics approach to elucidate the regulatory mechanism underlying parturition and myometrial quiescence adds novelty to the study. The identification of myometrial cis-acting elements and their association with gene expression, particularly the regulation of the PLCL2 gene by PGR opens the door to further investigate the impact of PGR and other regulators.

Strengths:

(1) Multi-Omic Approach: The paper employs a comprehensive multi-omic approach, combining ChIP-Seq, RNA-Seq, and CRISPRa-based Perturb-Seq assays, which allow for a thorough investigation of the regulatory mechanisms underlying myometrial gene expression.
(2) Clinical Relevance: Investigating human myometrial specimens provides direct clinical relevance, as understanding the molecular mechanisms governing parturition and myometrial quiescence can have significant implications for the management of pregnancy-related disorders.
(3) Functional work: For functional screening, They have used CRISPRa-based screening of PLCL2 gene regulation using immortalized human cell-line hTERT-HM and T-hESC to add more dimension to the work which strengthens their finding of PGR-dependent regulation of the PLCL2 gene in the human myometrial cells.

Weaknesses:

(1) Variability in epigenomic mapping: The significant variations in the number and location of H3K27ac-positive intervals across different samples and studies suggest potential challenges in accurately mapping the myometrial epigenome. This variability may introduce uncertainty and complicate the interpretation of results.
(2) Sample specificity: The study focuses on term pregnant nonlabor myometrial specimens, limiting the generalizability of the findings to other stages of pregnancy or labor.
(3) Limited Understanding of Regulatory Mechanisms: While the study identifies potential regulatory programs within super-enhancers, the exact mechanisms by which these enhancers regulate gene expression and cellular functions in the myometrium remain unclear. Further mechanistic studies are needed to elucidate these processes.
(4) Discordant analysis: Why regular enhancers are being understood in terms of motif enrichment of transcription factors and super-enhancers in terms of pathways enriched for active genes? This needs a clear reason.

https://doi.org/10.7554/eLife.95897.2.sa3

Reviewer #2 (Public review):

Summary:

In "Assessment of the Epigenomic Landscape in Human Myometrium at Term Pregnancy" the authors generate a number of genome-wide data sets to investigate epigenomic and transcriptomic regulation of the myometrium at term pregnancy. These data provide a useful resource for further evaluation of gene regulatory mechanisms in the myometrium and include the first Hi-C data published for this tissue. There is a comparison to previously published histone modification data and integration with RNA-seq to highlight potential enhancer-gene regulatory relationships. The authors further investigate putative enhancers

upstream of the PLCL2 gene and identify a candidate region that may be regulated by the PGR (progesterone receptor) signaling.

Strengths:

The strengths of this study are in the multi-omics nature of the design as several genome-wide data sets are generated from the same patient samples. Extending this type of approach in the future to a larger number of samples will allow for additional investigation into gene regulation as correlation between epigenomic features and gene expression across a larger number of samples can reveal regulatory relationships.

Weaknesses:

One of the most interesting aspects of this study is the generation of the first Hi-C data for the human pregnant myometrium, however, there is minimal description in the results section of the Hi-C data analysis and the only data shown are the number of loops identified and one such loop that includes the PLCL2 promoter shown in figure 3A. The manuscript would benefit from a more extensive analysis of the Hi-C data, for example, the analysis of TADs (topological associating domains) would be interesting to add and could be used to evaluate to what extent H3K27ac domains and putative regulated genes fall within the same TAD.

The authors present some convincing evidence on the transcriptional regulation of the PLCL2 gene using Perturb-Seq to identify putative upstream enhancer regions and PGR over-expression showing PGR can act as an activator. These two experiments on their own are interesting, however, they are not as mechanistically integrated as they could be to clarify the molecular mechanisms. Deletion of the putative enhancer upstream of PLCL2 followed by over-expression of PGR would clarify the mechanistic relationship between the proposed enhancer, PGR and PLCL2 expression. Does PGR act through the proposed enhancer? In addition, reporter assays using this proposed enhancer region with and without increased expression of PGR and mutation of any PRE sequences would also provide mechanistic insight. Although CRISPRa and Perturb-Seq can be used to identify potential regulatory regions, the best approach to verify the requirement for a particular enhancer in regulating a specific gene is a deletion approach.

Comments on revisions:

The authors have addressed my comments that were directly sent to them, however, my comments in the public review, specifically the superficial nature of the Hi-C analysis were not addressed.

In addition, many of the comments to reviewer 3 were unaddressed and declared out of the scope of this study, as these were points of accuracy in the data analysis they are very much in scope.

I hope the authors reconsider presenting a more thorough analysis.

https://doi.org/10.7554/eLife.95897.2.sa2

Reviewer #3 (Public review):

In this manuscript, Wu et al. investigate active H3K27ac and H3K4me1 marks in term pregnant nonlabor myometrial biopsies, linking putative enhancers and super enhancers to gene expression levels. Through their findings, they reveal the PGR-dependent regulation of the PLCL2 gene in human myometrial cells via a cis-acting element located 35-kilobases upstream of the PLCL2 gene. By targeting this region using a CRISPR activation system, they

were able to increase the elevate the endogenous PLCL2 mRNA levels in immortalized human myometrial cells.

This research offers novel insights into the molecular mechanisms governing gene expression in myometrial tissues, advancing our understanding of pregnancy-related processes.

Major comments:

(1) A more comprehensive analysis of the epigenetic and transcriptomic data would have strengthened the paper, moving beyond basic association studies. Currently, it is challenging to assess the quality and significance of the data as much of the information is lacking.

Strengths:

- The combination of ChIP-Seq, RNA-Seq, and CRISPRa Perturb-Seq approaches to investigate gene regulation and expression in myometrial cells.
- The use of CRISPR activation system to specifically target cis-acting elements.

Weaknesses:

- The manuscript would strongly benefit from a deeper analysis of the Omic datasets. Furthermore, expanding figures/graphs to effectively contextualize these datasets would be greatly beneficial and would add more value to this research.
- Limited sample size, coupled with variability in results and overall lack of details, compromises the robustness of result interpretation.
- Additional efforts are needed to dissect the proposed regulatory mechanisms.
- While the discussion provided helpful context for understanding some of the experiments performed, it lacked interpretation of the results in relation to the existing literature.

Comments on revisions:

The authors have improved the manuscript by enhancing its readability and organization. Tables were added to present key information more clearly, and figures were refined for better visualization. Additionally, more details were included, particularly in the methods and bioinformatics analyses sections, ensuring a more comprehensive and precise presentation of the data.

However, in many cases, reviewers' questions and concerns were addressed in the response to reviewers rather than incorporated into the manuscript, or it was noted that these points would be explored in future studies.

https://doi.org/10.7554/eLife.95897.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) Sample size: If the sample size of the study is increased, more confidence and new insights can be inferred about myometrial enhancer-mediated gene regulation in term pregnancy. Such a small sample size (N = 3) limits the statistical power of the study. As mentioned in the manuscript they failed to identify chromatin loops in the second subject's biopsy is observed due to a limited sample.*

We agree with the reviewer's comment about the sample size. We sincerely hope the result of this study would increase the interest of stakeholders to fund future projects in a larger scale.

> *(2) Figure quality: There is a lack of good representations of the results (e.g., screenshots of tables as figure panels!) as well as missing interpretations that might add value to the manuscript.*

Figure 1B and 2B have been converted to the pie chart format.

> *(3) Definition of super-enhancer: The definition of super-enhancer is not clear. Also, the computational merging of enhancers to define super-enhancers should be described better.*

Added more details about tool and parameter setting in the Method section of "Identification of super enhancers":

"Identification of super enhancers

H3K27ac-positive enhancers were defined as regions of H3K27ac ChIP-seq peaks in each sample. The enhancers within 12.5Kb were merged by using bedtools merge function with parameter "-d 12500". The combined enhancer regions were called super enhancers if they were larger than 15Kb. The common super enhancers from multiple samples were used for downstream analysis."

Reference:

Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, Rahl PB, Lee TI, Young RA. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell. 2013 Apr 11;153(2):307-19. doi: 10.1016/j.cell.2013.03.035. PMID: 23582322; PMCID: PMC3653129.

> *(4) Assay-Specific Limitations: Each assay employed in the study, such as ChIP-Seq and CRISPRa-based Perturb-Seq, has its limitations, including potential biases, sensitivity issues, and technical challenges, which could impact the accuracy and reliability of the results. These limitations should be addressed properly to avoid false-positive results and improve the interpretability of the results.*

The major limitations of the CRISPRa-based Perturb-Seq protocol in this study are the use of the hTERT-HM cells and the two-vector system for transduction. While hTERT-HM cells are a much easier platform in terms of technical operation, primary human myometrial cells are generally considered retaining a molecular context that is closer to the in vivo tissues. Due to the limitation on the efficiency of having two vectors simultaneously present in the same cell, hTERT-HM cells are much more affordable and operationally feasible to conduct the experiment. Future advancements on the increase of viral vector payload capacity may overcome this challenge and open the venue to perform the assay on primary human myometrial cells.

> *(5) Sample collection and comparison: There is mention of matched gravid term and non-gravid samples whereas no description or use of control samples was found in the results. Also, the comparison of non-labor samples with labor samples would provide a better understanding of epigenomic and transcriptomic events of myometrium leading to laboring events.*

The description has been updated:

“Collection of myometrial specimens

Permission to collect human tissue specimens was prospectively obtained from individuals undergoing hysterectomy or cesarean section for benign clinical indications (H-33461). Gravid myometrial tissue was obtained from the margin of the hysterotomy in women undergoing term cesarean sections (>38 weeks estimated gestational age) without evidence of labor. Non-gravid myometrial tissue was collected from pre-menopausal women undergoing hysterectomy for benign conditions. Specimens from gravid women receiving treatment for pre-eclampsia, eclampsia, pregnancy-related hypertension, or pre-term labor were excluded.”

> *(6) Lack of clarity:*
>
> *(6a) It is written as 'Chromatin Conformation Capture (Hi-C)'. I think Hi-C is Histone Capture and 3C is Chromosome Conformation Capture! This needs clear writing.*

As the reviewer suggested, to make it clear, we have changed the text “A high throughput chromatin conformation capture (Hi-C) assay” to “A High-throughput Chromosome Conformation Capture (Hi-C) assay”.

> *(6b) In multiple places, 'PLCL2' gene is written as 'PCLC2'.*

Corrected as suggested.

> *(6c) What is the biological relevance of considering 'active' genes with FPKM {greater than or equal to} 1? This needs clarification.*

In RNA-seq analysis, the gene expression levels are often quantified using FPKM (Fragments Per Kilobase of transcript per Million mapped reads). Setting a threshold of FPKM for defining "active" genes in RNA-seq analysis is biologically relevant, because it helps to distinguish between genuinely expressed genes and background noise. It helps researchers focus on genes, which are more likely to have a significant biological impact. A common threshold for defining "active" genes is FPKM ≥ 1. Genes with FPKM values below this threshold may be transcribed at very low levels or could be background noise.

> *(6d) The understanding of differentially methylated genes at promoters is underrated as per the authors. But, why leaving DNA methylation apart, they selected histone modification as the basis of epigenetic reprogramming in terms of myometrium is unclear.*

DNA methylation indeed plays a crucial role in evaluating the impact of cis-acting elements on gene regulation. Large-scale studies, such as the comprehensive analysis of the myometrial methylome landscape in human biopsies (Paul et al., JCI Insight, 2022, PMID: 36066972), have provided valuable insights. When integrated with histone modification and chromatin looping data, contributed by our group and collaborators, future secondary analyses leveraging machine learning are poised to further elucidate the mechanisms underlying myometrial transcriptional regulation.

> *(6e) How does the identification of PGR as an upstream regulator of PLCL2 gene expression in human myometrial cells contribute to our understanding of progesterone signaling in myometrial function?*

In a previous study, we demonstrated a positive correlation between *PLCL2* and *PGR* expression in a mouse model and identified PLCL2's role in negatively modulating oxytocin-induced myometrial cell contraction (Peavy et al., PNAS, 2021, PMID: 33707208). The present

study builds on this by providing evidence for a direct regulatory mechanism in which PGR influences *PLCL2* transcription, likely through a cis-acting element located 35 kb upstream. These findings suggest that PLCL2 acts as a mediator of PGR-dependent myometrial quiescence prior to labor, rather than merely participating in a parallel pathway. Further in vivo studies are necessary to delineate the extent to which PLCL2 mediates PGR activity, particularly the contraction-dampening function of the PGR-B isoform.

> *(7) Grammatical error: The manuscript has numerous grammatical errors. Please correct them.*

Corrections have been made as suggested.

> *(8) Use of single-cell data: Though from the Methods section, it can be understood that single-cell RNA-seq was done to identify CRISPRa gRNA expressing cells to characterize the effect of gene activation, some results from single-cell data e.g., cell clustering, cell types, gRNA expression across clusters could be added for better elucidation.*

As reviewer suggested, we have prepared a file “PerturbSeq_summary.xlsx” (Dataset S9) to provide additional results of perturb-seq data analysis. It includes 2 spreadsheets, “Cell_per_gRNA” for clustering and “Protospacer_calls_per_cell” for gRNA expression across clusters.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) The following are a number of grammatical issues in the abstract. I suggest having a careful read of the entire manuscript to identify additional grammatical issues as I may not be able to highlight all of these issues.*
>
> *(1a) "The myometrium plays a critical component during pregnancy." change component to role.*
>
> *(1b) "It is responsible for the uterus' structural integrity and force generation at term," à replace "," with "."*
>
> *(1c) Also, I suggest rephrasing the first 2 sentences to: The myometrium plays a critical role during pregnancy as it is responsible for both the structural integrity of the uterus and force generation at term.*
>
> *(1d) "Here we investigated the human term pregnant nonlabor myometrial biopsies for transcriptome, enhancer histone mark cistrome, and chromatin conformation pattern mapping." Remove "the", and modify to "Here we investigated human term pregnant".*
>
> *(1e) Missing period and sentence fragment, "PGR overexpression facilitated PLCL2 gene expression in myometrial cells Using CRISPR activation the functionality of a PGR putative enhancer 35-kilobases upstream of the contractile-restrictive gene PLCL2.*

Corrections have been made as suggested.

> *(2) Sentence fragment: Studies on the role of steroid hormone receptors in myometrial remodeling have provided evidence that the withdrawal of functional progesterone signaling at term is due to a stoichiometric increase of progesterone receptor (PGR) A to B isoform-related estrogen receptor (ESR) alpha expression activation at term. (Mesiano, Chan et al. 2002) (Merlino, Welsh et al. 2007) (Nadeem, Shynlova et al. 2016).*

The statement has been updated:

"Studies on the role of steroid hormone receptors in myometrial remodeling suggest that the withdrawal of functional progesterone signaling at term results from a stoichiometric shift favoring the PGR-A isoform over PGR-B. This shift is associated with increased activation of estrogen receptor alpha (ESR1) expression at term (Mesiano, Chan et al. 2002) (Merlino, Welsh et al. 2007) (Nadeem, Shynlova et al. 2016)."

> *(3) FOS:JUN heterodimers are implicated to be critical for the initiation of labor through transcriptional regulation of gap junction proteins such as Cx43 (Nadeem, Farine et al. 2018) (Balducci, Risek et al. 1993).*
>
> *Use Gja1 (Gap junction alpha 1) as the current correct gene, not Cx43.*
>
> *Also, several references predate Nadeem, Farine et al. 2018 and are more appropriate to use as references for the role of Ap-1 proteins in regulating Gja1; PMID: 15618352 and PMID: 12064606 were the first to show this relationship in myometrial cells.*

The statement has been updated as suggested:

"FOS:JUN heterodimers are implicated to be critical for the initiation of labor through transcriptional regulation of gap junction proteins such as *GJA1* (Nadeem, Farine et al. 2018) (Balducci, Risek et al. 1993)"

> *(4) Define PLCL2 on first use.*

Updated as suggested.

> *(5) There are a number of issues with this section, "Matched sSpecimens of gravid myometrium were collected at the margin of hysterotomy from women undergoing clinically indicated cesarean section at term (>38 weeks estimated gestation age) without evidence of labor. Specimens of healthy, non-gravid myometrium were also pecimens were collected from uteri removed from pre-menopausal women undergoing hysterectomy for benign clinical indications."*

The description has been updated:

"Collection of myometrial specimens

Permission to collect human tissue specimens was prospectively obtained from individuals undergoing hysterectomy or cesarean section for benign clinical indications (H-33461). Gravid myometrial tissue was obtained from the margin of the hysterotomy in women undergoing term cesarean sections (>38 weeks estimated gestational age) without evidence of labor. Non-gravid myometrial tissue was collected from pre-menopausal women undergoing hysterectomy for benign conditions. Specimens from gravid women receiving treatment for pre-eclampsia, eclampsia, pregnancy-related hypertension, or pre-term labor were excluded."

> *(6) Enriched motifs were identified by HOMER (Hypergeometric Optimization of Motif EnRichment) v4.11 (Heinz, Benner et al. 2010).*

Please clarify what background is used for motif enrichment.

We used the default background sequences generated by HOMER from a set of random genomic sequences matching the input sequences in terms of basic properties, such as GC content and length. We have added more details in the Method section:

"DNA-binding factor motif enrichment analysis

Enriched motifs were identified by HOMER (Hypergeometric Optimization of Motif EnRichment) v4.11 with default background sequences matching the input sequences (Heinz, Benner et al. 2010)."

> *(7) "Six of the seven regions are also co-localized with previously published genome occupancy of transcription regulators curated by the ReMap Atlas"*

Please clarify if this Atlas includes myometrial tissues or not and clarify the cell types included in the atlas.

According to the UCSC Genome Browser and the reference by Hammal et al. (2022), the current ReMap database includes PGR ChIP-seq data from human myometrial biopsies, available under NCBI GEO accession number GSE137550, alongside data from various other cell and tissue types. ReMap provides valuable insights into potential functional cis-acting elements in the genome from a systems biology perspective. However, tissue specificity requires independent validation.

> *(8) "Notably, 76% of the putative super-enhancers are co-localized with known PGR-occupied regions in the human myometrial tissue (Figure S2). This is significantly higher than the 20% co-localization in the regular enhancer group (Figure S2)."*
>
> *Because there is a huge difference in the size of the putative super enhancer regions and the isolated enhancers this comparison is not appropriate as conducted. The comparison needs to account for the difference in size of the regions. Please provide P values for significance statements.*

We acknowledge the reviewer's concern that our initial statement was overstated and potentially misleading, given the substantial difference in size between putative super-enhancer regions and regular enhancers. Rather than emphasizing the enrichment, it would be more accurate to simply describe our observation that super-enhancers encompass more PGR-occupied regions.

Here is the updated version:

"Notably, 76% of the putative super-enhancers co-localize with known PGR-occupied regions in human myometrial tissue, compared to 20% co-localization observed in regular enhancers (Figure S2)."

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) Title is extremely misleading, as here we do not get a view of the epigenomic landscape, but rather sparce data related to H3K27ac and H3K4me (focusing on enhancers) and chromatin conformation associated with the PLCL2 transcription start site (TSS).*

As suggested, the title is modified to "Assessment of the Histone Mark-based Epigenomic Landscape in Human Myometrium at Term Pregnancy".

> *(2) Improve the first result paragraph by providing a clear rationale for the experiments and their objectives, as well as introducing the samples used. Rather than simply listing approaches and end results in Table 1, offer concise explanations for the experiments alongside the supporting data presented in detailed figures. Using appropriate figures/graphs to effectively contextualize these datasets would be greatly appreciated by readers and would add more value to this research. Currently, it is difficult for us to assess and appreciate the quality of the data.*

The following statement is included in the beginning of the Result section:

"To better understand the regulatory network shaping the myometrial transcriptome before labor, we analyzed transcriptome and putative enhancers in individual human myometrial specimens. Using RNA-seq, we identified actively expressed RNAs, while ChIP-seq for H3K27ac and H3K4me1 was used to map putative enhancers. Active genes were associated with nearby putative enhancers based on their genomic proximity. Additionally, chromatin looping patterns were mapped using Hi-C to further link active genes and putative enhancers within the same chromatin loops."

> *(3) The statistics for every sequencing approach need to be provided for each sample (e.g., RNA-seq: number of total reads, number of mapped reads, % of mapped reads; ChIP-Seq: number of mapped reads, % of mapped reads, % of duplicates).*

We have generated the summary table of each dataset included in this study (Dataset S7) [NGS-summary.xls].

> *(4) Figure S1: The rationale behind comparing the Dotts study and yours regarding H3K27ac-positive regions needs to be better defined. Why is this performed if the data will not be used afterwards? What are the conserved regions associated with vs the ones that are variable? Is this biologically relevant? Why not use only the regions conserved between the 6 samples, to have more robust conclusions?*

The purpose of comparing our data with the Dotts dataset is to highlight the degree of variation across studies. In this study, we focused on addressing specific biological questions using our own dataset rather than developing methodologies for meta-analysis. Future advancements in meta-analysis techniques could leverage the combined power of multiple datasets to provide deeper insights.

> *(5) Perhaps due to a lack of details, I am unable to ascertain how the putative myometrial enhancers were defined. In Dataset S1, it is stated, "we define the regions that have overlapping H3K27ac and H3K4me1 marks as putative myometrial enhancers at the term pregnant nonlabor stage (Dataset S1)". Within Dataset S1, for subjects 1, 2, and 3, H3K27ac and H3K4me1 double-positive enhancers are shown in term pregnant, non-labor human myometrial specimens, with approximately 100 regions corresponding to 131 (sample 1), 127 (sample 2), and 140 (sample 3) common peaks. However, in Figure 1a, reference is made to the 13114 putative enhancers commonly present across the three specimens. Is Dataset S1 intended to represent only a small fraction of the 13114 putative enhancers? Detailed analyses need to be conducted and better showcased.*

Dataset S1 has been updated to list all 13,114 putative enhancers.

> *(6) For the gene expression analyses of RNA-seq data, FPKM values were utilized. However, it is unclear why the gene expression count matrix was normalized based on the ratio of total mapped read pairs in each sample to 56.5 million for the term myometrial specimens. I would recommend exercising caution regarding the use of FPKM expression units, as samples are normalized only within themselves, lacking cross-sample normalization. Consequently, due to external factors unaccounted for by this normalization method, a value of 10 in one sample may not equate to 10 in another.*

We value the reviewer's input. This question will be addressed in future secondary data analyses with suitable methodologies, as it is beyond the scope of this study.

> *(7) In Figure 1b, the authors have categorized their 12157 active genes into 3 bins based on FPKM values: >5 FPKM >1, >15 FPKM >5, and >15 FPKM. However, in the text, they describe these as 'actively high-expressing genes (FPKM >= 15)'. I would advise caution regarding the interpretation of these values, as an FPKM of 15 is not typically associated with highly expressed genes. According to literature and resources such as the Expression Atlas, an FPKM of 15 is generally considered to represent a low to medium expression level.*

We appreciate the reviewer's feedback. This question will be revisited during secondary data analyses using appropriate methodologies, as it falls outside the scope of the present study.

To increase readability and clarity, we modified the sentence as following: More than 40% of the 540 putative super enhancers are located within a 100-kilobase distance to high-expressing genes (FPKM >= 15), while only 7.3% of putative myometrial super enhancers are found near low-expressing genes (5 > FPKM >=1) (Figure 2B).

> *(8) Out of the 12157 active genes, approximately two-thirds have an FPKM >15. Was this expected? How does this correspond to what is observed in the literature, particularly in other similar studies (https://pubmed.ncbi.nlm.nih.gov/30988671/ ; https://pubmed.ncbi.nlm.nih.gov/35260533/) .*

This is indeed an intriguing question that merits further exploration in future secondary analyses.

> *(9) It is also surprising to see that for the motif enrichment analysis (Fig. 1C), the P-values are small. This is probably because the percentage of target sequences with the motif is very similar to the percentage of background sequences with the motif. For instance, for selected genes in Figure 1C: AP-1 (50.68% vs. 46.50%), STAT5 (28.08% vs. 25.04%), PGR (17.90% vs. 16.12%), etc. Can one really say that you have a biologically relevant enrichment for values that are so close between target sequences and background sequences?*

Reviewer's comment is noted. Biological relevance shall be experimentally examined though wet-lab assays in future studies.

> *(10) For Figure 2, again not convinced that FPKM >= 15 can be used to say: Compared with the regular putative enhancers, the putative myometrial super-enhancers are found more frequently near active genes that are expressed at relatively higher levels (Figure 1B and Figure 2B). A higher threshold should be used if they want to say this.*

To compare the association of putative enhancers with active genes expressed at different levels, we categorized the active genes into three groups based on their FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values. These groups are defined as follows: the top third active genes (FPKM ≥ 15), the middle third active genes (5 ≤ FPKM < 15), and the bottom third active genes (1 ≤ FPKM < 5). By "active genes expressed at relatively higher levels," we refer specifically to the top third active genes with FPKM values of 15 or higher, indicating their relatively higher expression levels compared to the other groups of active genes.

> *(11) More detailed explanations and methods are needed regarding how the data for Figure S2 was obtained.*

The following details were added to the methods section:

"Colocalization of super enhancers and PGR genome occupancy was compared by calling peaks from previously published PGR ChIP-seq data (GSM4081683 and GSM4081684). The percentages of enhancers and super enhancers that manifest PGR occupancy were calculated by overlapping the genomic regions in each category with PGR occupancy regions."

> *(12) In Figure 2C, there is no information provided on the genes used to obtain the results. It would be helpful to include examples of these genes, along with their expression values, for instance.*

The expression levels of the 346 active genes that are associated with myometrial super enhancers are included in Dataset S4, along with results of the updated gene ontology enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) of Knowledgebase v2024q4. Selected pathways of interest are listed in updated Figure 2C.

> *(13) The linking of PLCL2-related data to the first part of the story is lacking, and the rationale behind it is missing. This entire section should be more detailed, and the data should be expanded to better reflect the context.*

As suggested, we included the following statement at the beginning of the section "Cis-acting elements for the control of the contractile gene PLCL2":

"We previously demonstrated the positive correlation of PLCL2 and PGR expression in a mouse model and PLCL2's function on negatively modulating oxytocin-induced myometrial cell contraction (Peavy et al., 2021). However, the mechanism underlies the PGR regulation of PLCL2 remains unclear. Taking advantage of the mapped myometrial cis-acting elements, we aimed to identify the cis-acting elements that may contribute to the PLCL2 transcriptional regulation with a special interest on the PGR-related enhancers."

The context is that our results provide additional evidence to support a direct regulation mechanism of PGR on the *PLCL2* transcription, likely though the 35-kb upstream cis-acting element. This finding suggests that PLCL2 likely plays a mediator's role of PGR dependent myometrial quiescence before laboring rather than a mere passenger on a parallel pathway. Further studies using in vivo models are needed to determine the extent of PLCL2 in mediating PGR, especially PGR-B isoform's contraction-dampening function.

> *(14) The entire Hi-C data should be presented to allow for the assessment of its quality and further value.*

The revised manuscript has included the Hi-C quality control summary in Dataset S8 [HiC-QC-Summary.xlsx].

> *(15) The authors state: "For the purpose of functional screening, we focus on H3K27ac signals instead of using H3K27ac/H3K4me1 double positive criterium to cast a wider net." However, it is unclear how many of the targeted regions contained H3K27ac/H3K4me1 peaks. Were enhancers or super-enhancers targeted, and if so, how did they compare to H3K27ac sites?*

The numbers of H3K27ac/H3K4me1 double positive peaks are recorded in Figure 1A. Compared to the numbers of H3K27ac intervals (Table 1), the H3K27ac/H3K4me1 double positive peaks are 62.9%, 70.7%, and 61.2% of corresponding H3K27ac intervals in each individual specimen.

> *(16) For the first set of data (Table 1), the authors state, "Together, these results reveal an epigenomic landscape in the human term pregnant myometrial tissue before the onset of labor, which we use as a resource to investigate the molecular mechanisms that prepare the myometrium for subsequent parturition." While it is acknowledged that an epigenetic landscape exists in all tissues, there is a lack of clarity regarding this landscape in the current manuscript, as we are only presented with a table containing numbers.*

This sentence has been revised to: “Together, these results delineate a map of H3K27ac and H3K4me1 positive signals in the human term pregnant myometrial tissue before the onset of labor, which we use as a resource to investigate the molecular mechanisms that prepare the myometrium for subsequent parturition.”

> *(17) For S1, the authors conclude: These data together highlight the degree of variation in mapping the epigenome among specimens and datasets. This conclusion seems somewhat perplexing, and I find myself in partial disagreement. Firstly, providing a clear rationale for this section would strengthen the conclusions. It's important to consider what factors may contribute to this variability. It could simply be attributed to differences in experimental settings, such as variations in samples, protocols used, antibodies, sequencing departments, or overall data quality. Deeper analyses of the data could have provided more information.*

We agree with the reviewer that deeper analyses are needed in order to extract more information among studies. However, appropriate methods for meta-analyses should be carefully evaluated and employed for this purpose. We humbly believe that such a task should belong to future studies that may combine available datasets for secondary analyses, leveraging the collective contribution of the reproductive biology community.

> *(18) In the methods section, please include an explanation of how enhancers and super-enhancers were defined or add appropriate citations for reference.*

Added more details about tool and parameter setting in the Method section of “Identification of super enhancers”.

“Identification of super enhancers

H3K27ac-positive enhancers were defined as regions of H3K27ac ChIP-seq peaks in each sample. The enhancers within 12.5Kb were merged by using bedtools merge function with parameter “-d 12500”. The combined enhancer regions were called super enhancers if they were larger than 15Kb. The common super enhancers from multiple samples were used for downstream analysis.”

Reference:

Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, Rahl PB, Lee TI, Young RA. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell. 2013 Apr 11;153(2):307-19. doi: 10.1016/j.cell.2013.03.035. PMID: 23582322; PMCID: PMC3653129.

> *(19) Additional description on the "Inferred myometrial PGR activities and the correlation analysis "method section should be included to enhance clarity and understanding.*

The description has been updated:

"The inferred PGR activities were represented by the T-score, which was derived by inputting the mouse myometrial *Pgr* gene signature, based on the differentially expressed genes between control and myometrial *Pgr* knockout groups at mid-pregnancy (Wu, Wang et al., 2022), into the SEMIPs application (Li, Bushel et al., 2021). The T-scores were computed using this signature alongside the normalized gene expression counts (FPKM) from 43 human myometrial biopsy specimens."

> *(20) How was the qPCR analysis performed? Was the ddCT method utilized, and was a reference gene used for control? Additional information would be beneficial.*

Quantifying relative mRNA levels was performed via the standard curve method.

The following details were added: "Relative levels of genes of interest were normalized to the 18S rRNA."

> *(21) Regarding the RNA-Seq analysis of Provera-treated human Myometrial Specimens, the continued use of FPKM is not ideal due to potential differences in RNA composition between libraries. Additionally, clarification is needed on why Cufflinks 2.0.2 was used, considering it is no longer supported.*

FPKM (Fragments Per Kilobase of transcript per Million mapped reads) is used in RNA-Seq analysis, because it allows for the normalization of gene expression data, accounting for differences in gene length and sequencing depth, and facilitates comparability across different genes and libraries. This makes it one of the essential tools for accurately measuring and comparing gene expression levels in various biological and clinical research contexts.

CuffLinks was once a popular tool for analyzing RNA-seq data, transcriptome assembly, and DEG identification. Its usage has declined in recent years due to the emergence of newer and more advanced tools. The main reason is that it was used for RNA-seq analysis at early stage of this study a few years ago. For the purpose of comparison and consistency, we continued using this tool for later RNA-seq analysis. If we start a new project now, we will choose newer tools, such as HISAT2, Salmon, and DEseq2.

> *(22) Overall, sentence structure and typos need to be corrected across the text. Here are some examples:*
>
> *Line 17: at term, emerging studies.*
>
> *Line 20-22: Here we investigated the human term pregnant nonlabor myometrial biopsies for transcriptome, enhancer histone mark cistrome, and chromatin conformation pattern mapping.*
>
> *Line 30-32: PGR overexpression facilitated PLCL2 gene expression in myometrial cells Using CRISPR activation the functionality of a PGR putative enhancer 35-kilobases upstream of the contractile-restrictive gene PLCL2.*
>
> *Line 66-70: However, the role of differential myometrial DNA methylation at contractility-driving gene promoter CpG islands in preterm birth is not thought to be major (Mitsuya, Singh et al. 2014), but given that DNA methylation-mediated gene regulation often occurs outside of CpG islands (Irizarry, Ladd-Acosta et al. 2009), there is still work to be done at this interface.*
>
> *Line 80-83: Putative enhancers upstream of the PLCL2, a gene encoding for the protein PLCL2 which has been implicated in the modulation of calcium signaling (Uji, Matsuda et al. 2002) and maintenance of myometrial quiescence (Peavey, Wu et al. 2021),*

> *transcriptional start site were subject to functional assessment using CRISPR activation based assays.*
>
> *Line 290 : sSpecimens*

We appreciate the reviewer's kind efforts and have made changes accordingly.

https://doi.org/10.7554/eLife.95897.2.sa0