

The Transcriptional Repressor GATAD2B Mediates Progesterone Receptor Suppression of Myometrial Contractile Gene Expression

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Running Title: GATAD2B mediates anti-inflammatory action of PR in myometrium

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ABSTRACT

The mechanisms whereby progesterone (P₄) acting via progesterone receptor (PR) inhibits *proinflammatory/contractile* gene expression during pregnancy are incompletely defined. Using immortalized human myometrial (hTERT-HM) cells stably expressing wild-type PR-A or PR-B (PR_{WT}), we found that P₄ significantly inhibited IL-1 β induction of the NF- κ B target genes, *COX-2* and *IL-8*. P₄/PR_{WT} transrepression occurred at the level of transcription initiation and was mediated by decreased recruitment of NF- κ B p65 and RNA Pol II to *COX-2* and *IL-8* promoters. However, in cells stably expressing a PR-A or PR-B DNA-binding domain mutant (PR_{mDBD}), P₄-mediated transrepression was significantly reduced, suggesting a critical role of the PR-DBD. ChIP analysis of hTERT-HM cells stably expressing PR_{WT} or PR_{mDBD} revealed that P₄ treatment caused equivalent recruitment of PR_{WT} and PR_{mDBD} to *COX-2* and *IL-8* promoters, suggesting that PR inhibitory effects were not mediated by its direct DNA binding. Using immunoprecipitation, followed by MS, we identified a transcriptional repressor, GATA Zinc Finger Domain Containing 2B (GATAD2B), that inter-

acted strongly with PR_{WT}, but poorly with PR_{mDBD}. P₄ treatment of PR_{WT} hTERT-HM cells caused enhanced recruitment of endogenous GATAD2B to *COX-2* and *IL-8* promoters. Further, siRNA knockdown of endogenous GATAD2B significantly reduced P₄/PR_{WT} transrepression of *COX-2* and *IL-8*. Notably, GATAD2B expression was significantly decreased in pregnant mouse and human myometrium during labor. Our findings suggest that GATAD2B serves as an important mediator of P₄/PR suppression of *proinflammatory* and *contractile* genes during pregnancy. Decreased GATAD2B expression near term may contribute to the decline in PR function leading to labor.

INTRODUCTION

The actions of progesterone (P₄) are mediated by progesterone receptors (PR), PR-A and PR-B, which are products of a single gene and ligand-activated members of the nuclear receptor (NR) family. Classically, binding of P₄ to PR induces conformational changes in PR, followed by its dimerization and translocation from the cytoplasm into the nucleus where it either directly binds to progesterone response elements (PRE) in PR-

target gene promoters or tethers to other transcription factors bound to their respective response elements in the promoter regions of target genes in order to modulate their transcriptional activity. Transcription factors with which PR interacts include specificity protein 1 (Sp1) (1), nuclear factor κ B (NF- κ B) (2), and activator protein 1 (AP1) (3). After binding to PREs, or tethering to other DNA-bound transcription factors, PRs recruit coactivator/corepressor complexes, which possess activities for histone modification and chromatin remodeling leading to the induction or repression of target gene expression (4). In the pregnant uterus, P₄ plays a critical role through its actions to maintain myometrial quiescence throughout most of gestation.

Both term and preterm labor are associated with an increased inflammatory response. This is characterized by elevated concentrations of proinflammatory cytokines (*e.g.*, IL-1 β , IL-6) in amniotic fluid (5) and infiltration of the myometrium, cervix, and fetal membranes by neutrophils and macrophages (6-8). The invading immune cells secrete proinflammatory cytokines and chemokines (9), resulting in activation of NF- κ B and other proinflammatory transcription factors in the myometrium (7, 10). Activated NF- κ B, in turn, increases contraction associated protein (*CAP*) gene expression (*e.g.* connexin 43 (*CX43/GJA1*) (7, 11), oxytocin receptor (*OXTR*) (12)), as well as the expression of other inflammation-associated genes, such as prostaglandin F₂ α receptor (13), and cyclooxygenase-2 (*COX-2/PTGS2*) (14, 15).

The mechanisms whereby P₄/PR maintains uterine quiescence during pregnancy are diverse and likely include the direct antagonistic interaction of PR with proinflammatory transcription factors such as NF- κ B or AP-1 (15, 16), and other genomic actions, including increased expression of inhibitor of κ B α (I κ B α) and MAPK phosphatase 1 (MKP-1/DUSP1), which act to block activation of proinflammatory transcription factors (15, 17). We also observed that P₄/PR blocks myometrial contractility by increasing expression of the gene encoding the transcriptional repressor, ZEB1, which binds to and suppresses expression of *CX43* and *OXTR* (18).

The finding in rodents that circulating levels of maternal P₄ decline precipitously near term (19) led to the concept that term labor is associated

with P₄ withdrawal. However, in humans and guinea pigs, circulating P₄ levels remain elevated throughout pregnancy and into labor, as do myometrial levels of PR (20). Notably, even in mice, maternal P₄ levels at term remain well above the K_d for binding to PR (21). These findings have led to the concept that parturition in all species is initiated by a concerted series of biochemical events that act to impair PR function and antagonize its ability to maintain myometrial quiescence. Some of the mechanisms postulated to contribute to the functional withdrawal of progesterone/PR prior to labor at term include a decrease in PR coregulators (22-25), increased expression of the inhibitory PR isoform, PR-C, an increase in the ratio of PR-A to PR-B (10, 26-28), and enhanced local metabolism of P₄ to inactive products (29). However, the details of how these mechanisms are integrated to orchestrate the functional withdrawal of P₄/PR during late gestation remain unknown.

To better understand the mechanism(s) responsible for the decline of PR function prior to labor at term, in the present study, we observed that the DNA-binding motif of PR plays an important role in P₄-mediated inhibition of endogenous proinflammatory genes. We further observed that transrepressive activity of P₄/PR occurred at the level of transcription initiation and was mediated by decreased recruitment of NF- κ B p65 and RNA Pol II to the *COX-2* and *IL-8* promoter regions. Thus, we postulated that nuclear proteins interacting with the PR DNA-binding motif may play an important role in P₄/PR mediated transrepression. Using mass spectrometry to identify proteins that differentially interacted with PR_{WT} vs. the PR_{DBD} mutants, we identified a transcriptional repressor, GATAD2B, which interacted with the PR DNA binding motif and served an important role in P₄/PR suppression of *proinflammatory* and *CAP* gene expression during pregnancy. We propose that during late gestation, a decrease in GATAD2B expression contributes to the decline in PR function, and thereby contributes to the initiation of labor at term.

RESULTS

Inhibitory Effects of P₄ on NF- κ B-mediated Reporter Activity in HEK-293 Cells is Lost by Mutagenesis of the PR DBD. To further define mechanisms underlying P₄/PR mediated anti-inflammatory responses, we first identified the

functional domain(s) of PR important for these effects using transiently transfected HEK-293 cells. HEK-293 cells were used because they are easily transfectable and lack endogenous PR, but contain cofactors required for transcriptional activity of transfected steroid receptors. Because sumoylation of nuclear receptors has been shown to play an important role in anti-inflammatory activity (30, 31), we used point mutagenesis to generate a PR-B K388R mutant in which the PR sumoylation site was disrupted (31-33). Previously, it was reported that the PR DBD contributed to P_4 /PR transrepressive activity on NF- κ B p65-mediated transactivation in transfected cells; when the entire DBD was deleted, the P_4 /PR mediated repressive activity was lost (2). To avoid causing major changes in PR structure, in the present study, we generated point mutations in two functional motifs within the DBD of PR. These included PR-B A604T, a point mutation in the D-box of the DBD, important for receptor dimerization and PR-B_{mDBD}, a triple mutation of the P-box, required for direct DNA-binding (34). To test PR transrepression activity, an NF- κ B mediated reporter assay was used.

As shown in Figure 1A, when HEK-293 cells were transiently transfected with a luciferase reporter plasmid containing five NF- κ B consensus binding sites, treatment with IL-1 β significantly induced luciferase activity compared to vehicle. Treatment with IL-1 β + P_4 did not affect luciferase activity since PR is not expressed in the HEK-293 cells. When cells were cotransfected with PR-B_{WT} expression vector and reporter plasmids, treatment with P_4 + IL-1 β caused a significant decrease in luciferase activity compared to IL-1 β alone. By contrast, in cells cotransfected with PR-B_{mDBD}, P_4 -mediated repressive activity was completely lost. While in cells cotransfected with expression vectors for PR-B mutants K388R or PR-B A604T, P_4 repression of IL-1 β -induced luciferase activity was still evident, the fold-repression was reduced significantly compared to effects of PR-B_{WT} (Fig. 1B). Taken together, these findings suggest that the sumoylation site, dimerization site and DNA binding motif of PR may play a role in P_4 -repression of NF- κ B mediated transcription. However, PR transrepression activity was most severely disrupted by mutation of the PR DNA-binding motif. To further study transrepressive efficacy of the PR-A isoform and effects of DBD mutations, HEK-293 cells were cotransfected with

expression vectors encoding either PR-A_{WT}, PR-B_{WT}, or the corresponding DBD mutants. As can be seen in Figure 1C and D, when cells expressed PR-A_{WT} or PR-B_{WT}, co-treatment with P_4 significantly inhibited IL-1 β -induced luciferase activity to a similar extent. As observed for PR-B_{mDBD}, mutation of the PR-A DBD (PR-A_{mDBD}) completely blocked transrepression activity. To confirm that P_4 /PR-mediated repression of NF- κ B activity was direct, rather than through an indirect mechanism, such as induction of I κ B α (15,43), a mammalian one-hybrid reporter assay was used. Using this assay, HEK-293 cells were cotransfected with Gal4-p65 CTD expression vector (35), encoding a chimeric protein comprised of the C-terminal transactivation domain (CTD, amino acids 286–551) of NF- κ B p65 fused to the DBD of Gal4, a GAL4 luciferase reporter and PR_{WT} or PR_{mDBD} expression vectors. The Gal4-DBD, containing a nuclear localization sequence, binds to Gal4 binding sites; luciferase activity is transactivated via the p65-transactivation domain. As shown in Figure 1E, treatment with P_4 significantly repressed luciferase activity compared to vehicle when cells were cotransfected either with PR-B_{WT} or PR-A_{WT} expression vectors. By contrast, cotransfection of PR-B_{mDBD} or PR-A_{mDBD} expression vectors failed to exert transrepressive activity. These data suggest that P_4 /PR directly repressed p65 transactivation and that the PR DNA binding motif plays an important role in transrepression activity.

PR DNA-Binding Motif Mediates P_4 Repression of NF- κ B-regulated Gene Expression in Stably Transfected Human Myometrial Cells – To evaluate the roles of PR-A and PR-B and their DNA-binding motifs in P_4 -mediated repression of endogenous *proinflammatory* and *CAP* gene expression, we utilized immortalized human myometrial cells (hTERT-HM) that lack endogenous PR to develop cell lines stably expressing either GFP alone or GFP conjugated PR-B_{WT} or PR-A_{WT} or their corresponding DNA-binding domain mutants (PR_{mDBD}) (Fig. 2A). These cells maintain characteristics of myometrial smooth muscle cells (36) and contract in response to a number of contractile stimuli (18, 37). As shown in the representative immunoblot (Fig. 2B), PR expression levels in the four genetically modified stable hTERT-HM cell lines were comparable.

mRNA levels of *proinflammatory* and *CAP* genes in the cell lines cultured in the absence or presence of IL-1 β \pm P₄ for 2 h were analyzed by RT-qPCR. As shown in Figure 3, in cells stably expressing either PR-B_{WT} or PR-A_{WT}, co-treatment with P₄ significantly reduced IL-1 β -induced COX-2 and IL-8 expression (Fig. 3A, E), compared to IL-1 β alone. Although both PR-B_{WT} and PR-A_{WT} had the capacity to repress COX-2 and IL-8 expression, the repressive activity mediated by PR-B_{WT} was significantly greater than that of PR-A_{WT} (Fig. 3B, F). In cells stably expressing the PR-B_{mDBD} mutant, P₄-mediated repression activity was significantly reduced compared to cells expressing PR-B_{WT}, while in cells expressing the PR-A_{mDBD} mutant, P₄ no longer could repress IL-1 β induced COX-2 and IL-8 mRNA expression (Fig. 3B, F). Similar findings were obtained for endogenous mRNA expression of the proinflammatory genes, CCL-2 and IL-6 (data not shown).

To determine whether the P₄/PR-mediated transrepression activity occurred at the level of transcription initiation, the uridine analogue, 5-ethynyluridine (EU), was used to label and isolate newly synthesized nascent RNA; levels of heterogeneous nuclear RNA (hnRNA) were analyzed by RT-qPCR. The hTERT-HM cells stably transfected with GFP or GFP conjugates of PR-A_{WT}, PR-B_{WT}, or the corresponding DBD mutants were cultured for only 40 min \pm IL-1 β \pm P₄ to ensure that the level of hnRNA for a given gene was representative of transcriptional initiation. When cells were treated with IL-1 β alone, COX-2 (Fig. 3C) and IL-8 (Fig. 3G) hnRNA levels were significantly increased compared to vehicle treated cells. In both PR-B_{WT} and PR-A_{WT} expressing cells, co-treatment with P₄ significantly reduced COX-2 and IL-8 hnRNA levels (Fig. 3C, G) suggesting that P₄/PR inhibition of IL-1 β -induced expression is at the level of transcription initiation. In cells expressing PR-B_{mDBD}, P₄ retained its ability to repress transcription initiation, but transrepression activity was significantly reduced compared to cells expressing PR_{WT} (Fig. 3D, H). However, in PR-A_{mDBD} expressing cells, P₄ completely lost its ability to repress transcription (Fig. 3D, H). Taken together, P₄/PR mediated transrepression activity occurs at the level of transcription initiation and the DNA binding motif of PR appears to play a critical role. Moreover, PR-B

appears to have more profound transrepressive activity than PR-A on IL-1 β -mediated activation of proinflammatory gene expression. Since levels of PR expression in the four genetically modified hTERT-HM cell lines were comparable (Fig. 2B), the decreased repressive activities of PR-B_{mDBD}, PR-A_{WT} and PR-A_{mDBD} relative to PR-B_{WT}, cannot be attributed to differences in PR expression.

The PR-DBD Inhibits IL-1 β -induced Recruitment of NF- κ B p65 and RNA Pol II to Promoter Regions of Proinflammatory Genes – To further define the mechanisms whereby P₄/PR represses transcription of proinflammatory genes in human myometrial cells, chromatin immunoprecipitation (ChIP) was used to analyze effects of IL-1 β \pm P₄ on recruitment of RNA polymerase II (RNA pol II), NF- κ B p65 and PR to the COX-2 and IL-8 promoters. hTERT-HM cells stably expressing GFP or GFP conjugates of PR-A_{WT} and PR-B_{WT} and corresponding DBD mutants were cultured \pm IL-1 β \pm P₄ for 40 min. In all cell lines, IL-1 β significantly stimulated recruitment of RNA pol II to a genomic region surrounding the COX-2 (Fig. 4A) and IL-8 (Fig. 4B) transcription start sites (TSS); co-treatment with P₄ significantly decreased recruitment of RNA pol II in cells stably expressing PR-B_{WT}, PR-A_{WT} and PR-B_{mDBD}. However, in cells expressing PR-A_{mDBD}, P₄ failed to inhibit recruitment of RNA pol II (Fig. 4A, B). Similar effects were observed for recruitment of NF- κ B subunit p65 to NF- κ B response elements of the COX-2 and IL-8 promoters (Fig. 4C, D). Thus, while P₄ significantly inhibited IL-1 β -induced recruitment of p65 to the NF- κ B response elements of the COX-2 and IL-8 promoters in PR-A_{WT}, PR-B_{WT} and PR-B_{mDBD} expressing cells, the inhibitory effects of P₄ were consistently not observed in hTERT-HM cells stably expressing PR-A_{mDBD} (Fig. 4C, D). Taken together, these findings suggest that P₄/PR transrepression activity is mediated, in part, by inhibition of recruitment of endogenous NF- κ B p65 and RNA Pol II to promoter regions of *proinflammatory* genes. Interestingly, when we used ChIP to analyze recruitment of PR-A_{WT}, PR-B_{WT}, PR-A_{mDBD} and PR-B_{mDBD} to the genomic regions containing NF- κ B response elements in the COX-2 (Fig. 4E) and IL-8 (Fig. 4F) promoters in the stably transfected hTERT-HM cells, PR recruitment was observed in cells treated with IL-1 β +P₄ (Fig. 4E, F). Moreover,

PR recruitment was unaffected by mutagenesis of the PR-A or PR-B DBD, compared to the corresponding PR_{WT} (Fig. 4E, F). By contrast, P₄-mediated recruitment of PR-A and PR-B to the promoter of *FKBP5*, a direct PR target gene, was completely abrogated by mutation of their respective DBDs (Fig. 4G). This indicates that inhibitory effects of PR on *proinflammatory* and *CAP* gene expression are not mediated by direct DNA binding. Our findings, therefore, suggest that nuclear proteins that interact with the PR-DBD may play an important role in P₄/PR trans-repression activity.

GATAD2B (GATA Zinc Finger Domain Containing 2B)/P66β Selectively Interacts with the PR-DNA-Binding Domain – One of the mechanisms proposed to cause a decline PR function during late gestation in myometrium is a decrease in PR coregulators (22). As mentioned, previous findings suggested that the DNA-binding motif of PR may serve an important role in P₄/PR trans-repression activity by interacting with nuclear corepressors (2). Based on the present findings, we hypothesized that whereas PR-B_{WT} had the capacity to interact with such corepressors, the PR-A DBD mutant did not. Thus, to identify novel PR coregulators that mediate P₄/PR anti-inflammatory activity, we sought to identify nuclear proteins that interacted with PR-B_{WT} but not with PR-A_{mDBD}.

To obtain relatively large amounts of cell protein, we isolated nuclear extracts from HEK-293 cells stably expressing GFP alone, PR-B_{WT}-GFP and PR-A_{mDBD}-GFP treated with IL-1β + P₄ for 30 min. Antibodies to GFP were used to co-immunoprecipitate GFP-interacting proteins, followed by mass spectrometry analysis to identify proteins that had higher binding activity for PR-B_{WT} vs. PR-A_{mDBD} (Table S1). Mass spectrometry values of PR-B_{WT} and PR-A_{mDBD}-interacting proteins were normalized to those interacting with GFP alone. Proteins were selected with spectral counts >3 and a normalized ratio of PR-B_{WT} vs. PR-A_{mDBD} of >2.5. Using these criteria, 146 proteins were identified that interacted more strongly with PR-B_{WT} than PR-A_{mDBD}. Interacting proteins were further selected because of their known roles in transcriptional regulation. One of the proteins of potential interest that preferentially interacted with PR-B_{WT} was GATAD2B, a component of the nucleosome remodeling and deacetylase (NuRD) complex (38, 39). Four

unique peptides corresponding to GATAD2B were identified (Fig. 5A). The MS/MS spectrum of the most abundant GATAD2B peptide (468-478) is shown (Fig. 5B).

Using co-immunoprecipitation assays in HEK-293 (Fig. 6A, left panel) and hTERT-HM cells (Fig. 6A, right panel) stably transfected with WT and DBD mutant forms of PR-A and PR-B, we confirmed findings from mass spectrometry analysis indicating that PR-B_{WT} specifically interacted with GATAD2B, while the association between PR-B_{mDBD} and GATAD2B was weaker (Fig. 6A left and right panels). Notably, PR-A_{WT} also interacted more strongly with GATAD2B than did PR-A_{mDBD} (Fig. 6A left and right panels). To further validate the direct interaction between PR and GATAD2B in the hTERT-HM cells, a Duolink proximity ligation assay was used. As can be seen in Figure 6B, when hTERT-HM cells were treated with IL-1β + P₄ for 30 min, there was abundant red fluorescent signal in the nuclei of cells expressing PR-B_{WT} or PR-A_{WT}. This suggests a direct in vivo interaction of PR_{WT} and GATAD2B. By contrast, the intensity of red fluorescent signal in cells expressing PR-B_{mDBD} or PR-A_{mDBD} was significantly lower compared to PR_{WT}-expressing cells, suggesting that mutations in the DNA-binding motif of PR disrupted the interaction between PR and GATAD2B (Fig. 6B).

Using ChIP, we observed that P₄ treatment of hTERT-HM cells expressing PR-B_{WT} or PR-A_{WT} markedly enhanced recruitment of endogenous GATAD2B to the NF-κB response element (Fig. 6C, E) and transcription start site (TSS) (Fig. 6D, F) regions of the *COX-2* and *IL-8* promoters. However, GATAD2B recruitment in response to P₄ was abolished in hTERT-HM cells expressing the corresponding PR-A/-B_{mDBD} (Fig. 6C-F). Taken together, we have identified a nuclear protein, GATAD2B, which directly interacts with PR through the PR DNA-binding motif. Notably, PR also may exert its inhibitory effects by recruiting GATAD2B to transcription factors bound to other *cis*-acting elements (e.g. AP-1) in the *proinflammatory* gene promoters, not amplified by the primers used in this study. Previously, PR was reported to promote recruitment of corepressor p54nrb to an AP-1 response element in the *Cx43/Gjal* promoter (23).

GATAD2B Plays an Important Role in Mediating P₄/PR Suppression of Proinflammatory and

CAP Genes in the Pregnant Myometrium – To explore the role of GATAD2B in P_4 /PR mediated transrepression activity, PR-B_{WT} and PR-A_{WT} expressing hTERT-HM cells were transfected with siRNA for GATAD2B or with a non-targeting siRNA, as control. The day after transfection, the cells were serum-starved by culturing them in DMEM-F12 medium containing 1% charcoal-stripped serum for another 48 h. The cells were then treated with IL-1 β + P_4 for 2 h and harvested for mRNA expression analysis. As can be seen in Figure 7A-F, in cells expressing PR-B_{WT} or PR-A_{WT}, siRNA-mediated knockdown of GATAD2B, significantly reduced P_4 /PR mediated repression of IL-1 β induced COX-2 (Fig. 7A-C) and IL-8 (Fig. 7D-F) mRNA expression compared to cells transfected with non-targeting siRNA. Knockdown of GATAD2B also blocked P_4 /PR mediated suppression of endogenous COX-2 protein, which was clearly evident in cells transfected with nontargeting siRNA (Fig. 7G). Interesting, we also observed that knockdown of endogenous GATAD2B resulted in up-regulation of the basal levels of IL-8 and COX-2 mRNA expression (Fig. 7A-F), as well as levels of COX-2 protein expression (Fig. 7G). Notably, levels of NF- κ B p65 phosphorylated at Ser536, an NF- κ B activation mark, were unaffected by GATAD2B knockdown (Fig. 7H), suggesting that the increased levels of COX-2 and IL-8 mRNA were not due to activation of NF- κ B signaling. Together, these findings suggest that GATAD2B, not only plays a role as corepressor of P_4 /PR mediated anti-inflammatory activity, but also suppresses NF- κ B target gene expression in P_4 /PR independent manner. The finding that knockdown of endogenous GATAD2B markedly reduced, but did not abolish P_4 /PR repression of IL-1 β and COX-2 expression, suggests the possible role of other corepressors. A number of corepressors that interacted far more strongly with PR-B_{WT} than with PR-A_{mDBD}, identified by MS, including C-terminal-binding proteins 1 and 2, and CNOT1 (Table S1), are the subject of ongoing investigations.

To determine the expression pattern of GATAD2B in human myometrium during pregnancy and labor, we used RT-qPCR to analyze mRNA levels in biopsies of term pregnant myometrium from women who were either in spontaneous labor (IL) or not in labor (NIL) at term. As

can be seen in Figure 8A, GATAD2B mRNA levels were significantly decreased in the IL samples compared to NIL samples. This was inversely correlated with IL-8 (Fig. 8C) mRNA levels, which were significantly increased in the IL myometrial tissues compared to NIL tissues. To determine whether this pattern of GATAD2B expression is conserved from human to mouse, we analyzed GATAD2B protein levels in myometrial tissues from timed pregnant mice at 15.5 days post-coitum (dpc), 17.5 dpc, 18.5 dpc, and during active labor (19 dpc), signaled by the birth of the first pup. Shown are bar graphs representing combined scanned values from immunoblots of tissue lysates from three, or more, independent myometrial samples per gestational timepoint. GATAD2B protein levels were significantly reduced at 17.5 dpc and 18.5 dpc, and continued to decline during labor, compared to 15.5 dpc (Fig. 8D). In the same series of tissues, COX-2 (Fig. 8E) and IL-6 (Fig. 8F) protein levels remained relatively unchanged between 15.5 and 18.5 dpc and were upregulated significantly during active labor. IL-8 levels in mouse myometrium are not shown because they were below detectable levels in mouse myometrium. Taken together, our findings suggest that GATAD2B may play a key role in mediating P_4 /PR suppression of *proinflammatory* and *CAP* gene expression in the pregnant myometrium. A decline in myometrial GATAD2B expression during late gestation may contribute to the reduction in P_4 /PR-mediated anti-inflammatory activity and the increase in *proinflammatory* and *CAP* gene expression leading to parturition.

DISCUSSION

The objective of this study was to further define the mechanisms that mediate the inhibitory actions of P_4 /PR on expression of *proinflammatory* and *CAP* genes in the pregnant myometrium. We (15, 17, 40) and others (28, 41-43) previously obtained compelling evidence that P_4 /PR maintains uterine quiescence through its anti-inflammatory actions. We also demonstrated that P_4 /PR represses *CAP* gene expression via upregulation of the inhibitory transcription factor, ZEB1 (18, 44). Recently, it was observed that P_4 acting through PR-B recruits Jun/Jun homodimers and the corepressor complex, P54^{mtb}/Sin3A/HDAC, to the promoter of the *CAP* gene *CX43* (45) to inhibit its expression. Near term, it is suggested that the

increased metabolism of P_4 by 20 α -hydroxysteroid dehydrogenase (29) and an inflammation-induced increase in PR-A/PR-B ratio (26, 46, 47) causes PR-A to become unliganded and to recruit Fra2/JunD heterodimers (45). This switch may allow PR-A to serve as an activator of *CX43* expression. However, the detailed molecular mechanism(s) by which PR exerts all of its repressive actions on proinflammatory and *CAP* genes remains incompletely defined.

It was first of interest to identify the domain(s) of PR required for its anti-inflammatory actions. Using an NF- κ B mediated reporter assay in HEK-293 cells transiently transfected with PR_{WT} and PR-A and PR-B mutants, we observed that PR sumoylation, dimerization and DNA binding motifs all play an important role in P_4 -mediated repression of NF- κ B transactivation. However, mutations in the PR DBD caused the greatest decline in PR anti-inflammatory activity. To further study the role of the DNA binding motif in P_4 /PR mediated transrepression activity, we created hTERT-HM immortalized myometrial cells stably expressing wild-type and DNA binding domain mutant forms of PR-A and PR-B. Interestingly, we found that P_4 /PR repressed *proinflammatory* and *CAP* gene expression at the level of transcription initiation; this involved a block in IL-1 β -induced recruitment of NF- κ B p65 and RNA Pol II to the *IL-8* and *COX-2* promoters. Whereas, both PR-B_{WT} and PR-A_{WT} had the ability to repress IL-1 β induced initiation of transcription, PR-B_{WT} had significantly higher repressive activity compared to PR-A_{WT}. Furthermore, while P_4 -mediated repression of IL-1 β -induced mRNA and hnRNA expression was moderately decreased in hTERT-HM cells expressing PR-B_{mDBD}, in cells expressing PR-A_{mDBD}, P_4 completely lost its transrepression activity. Similarly, ChIP analysis revealed that in hTERT-HM cells expressing PR-A_{mDBD}, the effect of P_4 to inhibit p65 and Pol II recruitment to the *COX-2* and *IL-8* promoters observed in the other PR-expressing cell lines was prevented. Taken together, these results suggest that the unique 164 amino acids at the N-terminus of PR-B (BUS region), which is lacking in PR-A plays an important role in P_4 -mediated transrepression function. The BUS region may act cooperatively with the DBD (common to PR-A and PR-B) to promote PR-B-mediated suppression of proinflammatory genes. ChIP revealed that both WT

and DBD mutant forms of PR-A and PR-B were equivalently recruited to promoters of *COX-2* and *IL-8* genes in cells treated with IL-1 β + P_4 . By contrast, P_4 -mediated recruitment of PR-A and PR-B to the promoter of *FKBP5*, a direct target of PR, was abrogated by mutation of their respective DBDs (Fig. 4G). These compelling findings suggest that the inhibitory effects of P_4 /PR on inflammatory gene expression are not mediated by direct DNA binding, but rather by tethering to other transcription factors. We hypothesize that the loss of transrepression activity of the PR-A DBD mutant resulted from decreased interaction of PR with proteins that contain a corepressor function.

To identify novel nuclear proteins that interact with the PR DNA binding motif and serve as a corepressor function for P_4 /PR-mediated anti-inflammatory actions, mass spectrometry (complex mixture ID) analysis was utilized. To select relevant proteins that interact with the PR DNA binding motif and serve as PR corepressors in myometrial tissues, we used three criteria to narrow down candidates: 1) For PR DNA binding motif-dependent interactions, we selected proteins that manifested ≥ 3 -fold greater binding to PR-B_{WT} compared to PR-A_{mDBD}; 2) To identify PR interacting proteins with known corepressor function, DAVID bioinformatics resource (48, 49) was used; 3) To identify proteins known to be expressed in human myometrium, RNA expression levels of proteins of interest were analyzed using published datasets (50). GATAD2B was one of the nuclear proteins identified using all three criteria.

Human GATAD2B, also known as p66 β , is a member of the transcription repressor Mi-2/nucleosome remodeling and deacetylase (NuRD) complex (51). The NuRD complex contains six major members including, CHD3/4 ATPase, HDAC (histone deacetylases) 1/2, MBD (methyl-CpG-binding domain) 2/3, RBBP (retinoblastoma-binding proteins) 7/4, MTA (metastasis-associated) 1/2/3 and p66 α/β . The NuRD complex is responsible for silencing of methylated genes by nucleosome remodeling and histone deacetylation. The N-terminal conserved CR1 region of GATAD2B is required for interaction with MBD2/3, MTA2, HDAC1/2, and RBBP7/4, and also plays an important role in GATAD2B-mediated repression activity (38, 39, 52). Using the Duolink proximity ligation assay, we observed a direct protein-protein interaction between PR and GATAD2B in

human myometrial cells; mutations in the PR DNA binding motif not only disrupted the PR-GATAD2B physical interaction but also abrogated P₄-induced recruitment of GATAD2B to the COX-2 and IL-8 promoters. Thus, these findings suggest that the PR DNA binding motif plays an important role in PR-GATAD2B interaction. In hTERT-HM cells, knockdown of GATAD2B expression by RNA interference resulted in a significant loss of P₄/PR-mediated repression activity, supporting the functional role of GATAD2B in P₄/PR mediated transrepression. Interestingly, knockdown of GATAD2B also caused an increase in endogenous levels of COX-2 and IL-8 mRNA expression in untreated hTERT-HM cells. However, levels of NF- κ B p65 phosphorylated at Ser536, an NF- κ B activation mark, were unaffected by GATAD2B knockdown (Fig. 7H), suggesting that the increased levels of COX-2 and IL-8 mRNA were not caused by activation of NF- κ B signaling. Collectively, these data suggest that GATAD2B also may play a role in repression of NF- κ B mediated gene expression independent of P₄ and PR.

As summarized in Figure 8G, we have identified a novel PR interacting factor, GATAD2B, which binds to the DNA binding motif of PR tethered to inflammatory transcription factors (*e.g.* NF- κ B) bound to the promoter regions of proinflammatory genes (*e.g.* COX-2, IL-8). We propose that GATAD2B serves a role in P₄-mediated transrepression of these proinflammatory genes by recruiting transcriptional repressors (*e.g.* the NURD complex). Importantly, GATAD2B expression levels were found to be reduced in myometrial biopsies of women in labor, compared to not in labor, and in pregnant mouse myometrium at during late gestation and labor. Thus, we suggest that decreased expression of GATAD2B causes a derepression of *proinflammatory* and *CAP* genes and contributes to the functional P₄/PR withdrawal leading to parturition (Fig. 8G).

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture – Human immortalized myometrial (hTERT-HM) cells were maintained in DMEM-F12 (Life Technologies, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (FBS). HEK-293 cells were maintained in DMEM (Life Technologies) containing 7% (vol/vol) FBS. Cells were cultured and grown in a 95% air: 5% CO₂ atmosphere at

37°C. For RNA and protein expression experiments, cells were seeded in maintenance medium; the next day, cells were incubated with phenol red-free medium supplemented with 1% charcoal-stripped serum (Life Technologies) for another 24 h prior to treatment. For treatment with various reagents, cells were incubated in phenol red-free medium with 1% charcoal stripped FBS for times indicated. Progesterone (Sigma, St. Louis, MO), IL-1 β (Cell Signaling, Beverly, MA), and all other reagents used were of the highest quality available from commercial sources. Antibodies used for western blotting include: progesterone receptor (R&D Systems, Minneapolis, MN), GATAD2B (Bethyl, Montgomery, TX), GAPDH and COX-2 (Abcam, Cambridge, England).

Cloning and Generation of Stably Transfected PR Expressing Cells – Wild-type PR-B (PR-B_{WT}) expression vector was a gift from Dr. Donald McDonnell at Duke University. hPR-A_{WT} was generated by PCR using hPR-B as a template with primers to amplify the region from the first ATG of PR-A to the stop codon. PR-B- and PR-A-DBD mutants (PR_{mDBD}) carrying a triple mutation (G584E / S585G / V589A) in the P-box were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).

To generate recombinant lentiviruses, PR-B_{WT}, PR-B_{mDBD}, PR-A_{WT} and PR-A_{mDBD} were subcloned into pLVX-GFP IRES Puro vector (Clontech, Palo Alto, CA) to create N-terminal green fluorescent protein (GFP)-conjugated PR expression constructs. High titer lentiviruses were generated according to Lenti-X system specifications (Clontech). pLVX-GFP IRES Puro plasmid was used to create a ‘GFP only’ recombinant virus and served as a control.

To generate cells stably expressing PR, human immortalized myometrial cells (hTERT-HM cells) (36) or HEK-293 cells were infected with the recombinant lentiviruses. Viral suspensions were titrated by infecting a fixed number of cells with various volumes of the viral suspension. The proportion of infected cells was determined using fluorescence microscopy for the detection of GFP expression. Cells with a low proportion of infection (10–30%) were selected for further study, because they contained approximately one integrant per host genome, thereby minimizing overexpression. GFP-positive cells were isolated by flow cytometry and used to establish the cell lines

used thereafter. PR protein expression levels in the stable cell lines were routinely verified by immunoblotting (18) using PR-A/B Rabbit mAb (Cell Signaling, A/B (D8Q2J) XP® #8757).

Reporter Assay and RNA Interference – For luciferase reporter assays, HEK-293 cells were seeded in 24-well plates and transfected using FuGENE HD transfection reagent (Roche, Indianapolis, IN) with luciferase reporter constructs pGL4.32[luc2P/NF- κ B-RE/Hygro] (Promega, Madison, WI), and Renilla luciferase plasmid to normalize for transfection efficiency. One day post transfection, cells were treated with DMSO, IL-1 β (10 ng/mL) or IL-1 β plus P₄ (100 nM) for 6 h. Cells from each experiment were harvested in Passive Lysis Buffer (Promega). Firefly luciferase and Renilla luciferase activities were assayed using a Dual-Luciferase assay system (Promega). Relative luciferase activities were calculated by normalizing Firefly luciferase activity to Renilla luciferase activity within the same samples to correct for transfection efficiencies.

For RNA interference (RNAi) experiments, small inhibitory RNA (siRNA) oligonucleotides against human GATAD2B and silencer-negative control oligonucleotides purchased from Life Technologies were transfected using the Neon® Transfection System (Life technologies).

RT-qPCR Assay – Total RNA was extracted using an RNeasy Mini kit (Qiagen, Foster City, CA). Approximately one μ g of RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). For quantitative analysis of mRNA, the CFX384TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used along with iTaq SYBR Green Supermix with ROX (Bio-Rad) or Taqman PCR master mix (Applied Biosystems) for detection of PCR products. Relative arbitrary units were determined by comparative cycle times (Ct) of each transcript to Ct of acidic ribosomal phosphoprotein P0 (36B4) (endogenous control) and calculated using ($2^{-\Delta\Delta Ct}$) method. Taqman primers were obtained from Applied Biosystems. Primer sets are listed in Table 1.

Chromatin Immunoprecipitation (ChIP) – ChIP assays were performed using the Chromatin Immunoprecipitation (ChIP) Assay kit (Millipore, Billerica, MA), according to the manufacturer's recommendations and as described previously (15). Antibodies used for the ChIP assay included

human PR-A/-B, p65 NF- κ B subunit, non-immune IgG (Cell Signaling), RNA pol II (Millipore) and GATAD2B (Bethyl). Purified DNA was analyzed by qPCR. Primer sets are listed in Table 1.

Duolink Proximity Ligation Assay – hTERT-HM cells were cultured on collagen coated coverslips and treated with IL-1 β (10 ng/mL) + P₄ (100 nM) for 30 min. The cells were then incubated with blocking solution (provided with Duolink In Situ PLA probe, Sigma) for 30 min at 37°C, followed by a 2 h incubation at 37°C with primary mouse antibody against human PR (R&D Systems) and rabbit antibody against human GATAD2B (Bethyl). Cells were washed twice using Wash Buffer A (Duolink In Situ Reagents, Sigma) and incubated with the appropriate anti-species 2° antibodies to which oligonucleotides had been conjugated (anti-rabbit PLA probe PLUS and anti-mouse PLA probe MINUS, Sigma) for 1 h at 37°C. The cells were then treated with Duolink ligation-ligase solution for 30 min at 37°C. Finally, cells were incubated with the Duolink amplification-polymerase solution for 60 min at 37°C, followed by washing and mounting on slides with Duolink Mounting Medium with DAPI. Images were captured using a Nikon TE2000-U Fluorescence Microscope. Six images were acquired for each sample and quantified using BlobFinder software (53). Briefly, images were converted to 8 bit for segmentation for each channel. Intensity per cell was calculated by expressing normalized red fluorescent PLA signals with cell number which was determined by DAPI signals in that image.

Human Subjects and Tissue Acquisition – Lower uterine segment myometrial tissues were biopsied from pregnant women undergoing repeat cesarean section. Informed consent was obtained in writing from each woman before surgery using protocols approved by the Institutional Review Board of the University of Texas Southwestern Medical Center in accordance with the Donors Anatomical Gift Act of the State of Texas. Myometrial biopsies were collected from two groups of patients: (i) pregnant women who underwent cesarean section prior to the onset of labor at term with no evidence of infection, and; (ii) pregnant women in active labor at term undergoing cesarean section. Myometrial smooth muscle was dissected from each biopsy, flash-

frozen in liquid nitrogen, and stored at -80°C for subsequent protein and mRNA analysis.

Timed-Pregnant Mice and Myometrial Tissue Collection – All animal studies were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. Outbred virgin CD1/ICR mice were acquired from Harlan Laboratories (Harlan USA, Houston, TX). Mice were time mated as previously described (54). Briefly, males and females were housed together between 1800 and 0600 h. Pregnancy was determined by the presence of a vaginal plug that morning and designated as 0.5 days post-coitum (dpc). Mice were maintained on a diurnal 12-h light/12-h dark cycle with access to standard chow and water ad libitum. Timed-pregnant mice were euthanized at approximately 1000 h on 15.5, 16.5, 17.5, and 18.5 dpc and during labor (in labor; IL) following the delivery of the first pup using isoflurane anesthetic inhalation (Baxter Healthcare Corp., Puerto Rico) and cervical dislocation. Maternal myometrial tissues were isolated as described (54). All tissues were flash frozen in liquid N_2 , and stored at -80°C until analyzed.

Immunoblot Analysis – Whole cell protein extracts were isolated from flash-frozen myometrial tissues homogenized in 1X RIPA buffer containing proteinase and protease inhibitors (Sigma). Homogenates were centrifuged at 5000 xg for 10 min at 4°C and supernatants were stored at -80°C . Protein concentrations were quantified using the Bradford assay (BCA Protein Assay Kit, Pierce). Equivalent amounts of myometrial protein were added to 4X NuPage LDS Sample Buffer (Invitrogen). Proteins were resolved by SDS-PAGE under reducing conditions (Bio-Rad) and transferred onto a polyvinylidene difluoride (PVDF) membrane using the XCell SureLock Electrophoresis System (Bio-Rad). Membranes were blocked for 1 h at room temperature in 5% (wt/vol) nonfat dry milk in 1X PBS-T (Phosphate-buffered saline containing 0.1% Tween 20), washed with 1X PBS-T and incubated at 4°C overnight with primary antibody against GATAD2B/p66 β (A301-281A, Bethyl Laboratories; 1:750), IL-6 (sc-57315, Santa Cruz Biotechnology, 1:1200), COX-2 (ab15191, Abcam, 1:1000), and β -actin (ab8227, Abcam, 1:5000), to correct for loading and transfer. Membranes were washed and incubated for 1 h at room temperature

in blocking buffer containing horseradish peroxidase-conjugated goat anti-rabbit-IgG (ab')₂ fragment (GE NA9340V, GE Healthcare), for GATAD2B, COX-2, and β -actin detection, or with m-IgG κ BP-HRP (sc-516102, Santa Cruz Biotechnology) for IL-6 detection. Enhanced Chemiluminescent Substrate (Thermo Scientific) was used according to the manufacturer's protocol. Because GATAD2B (66 kDa) and COX-2 (69 kDa) proteins are of similar molecular weight, membranes were stripped following GATAD2B immunoblotting and re-probed using antibody against COX-2. Densitometric analysis of immunoreactive bands were quantified using ImageJ software (NIH). Corrected values from scanned immunoblots of myometrial GATAD2B, COX-2 and IL-6 protein levels during late gestation and labor were combined and analyzed.

Mass Spectrometry – To identify novel nuclear proteins that interact with the PR DNA binding motif and serve a corepressor function for P_4 /PR mediated anti-inflammatory actions, we utilized mass spectrometry analysis of HEK-293 cells stably expressing GFP-conjugated PR-B_{WT} or PR-A_{mDBD} treated with IL-1 β (10 ng/mL) plus P_4 (100 nM) for 30 min. The stable HEK-293 cell lines were utilized for these studies because hTERT-HM cells grow slowly in culture and would likely yield insufficient amounts of nuclear protein. Moreover, in cotransfection experiments, the HEK-293 cells respond to P_4 and IL-1 β in a manner similar to the stably transfected hTERT-HM cells – *i.e.* PR-B_{WT} markedly represses NF- κ B transcriptional activity, whereas, PR-A_{mDBD} lacks this repressive effect (Fig. 1). Nuclear protein fractions were isolated from these cell lines, as described (55), and immunoprecipitated with anti-GFP magnetic beads (MBL International, Nagoya, Japan) at 4°C for 24 h. After washing the beads, supernatants were collected and the immunoprecipitated nuclear proteins were loaded and run 5-10 mm into the resolving portion of an SDS-PAGE gel. The gel was stained with SimplyBlue SafeStain (Invitrogen). The stained area for each sample was then excised and sent to the UT Southwestern Proteomics Core for complex mixture ID analysis. Gel bands were digested overnight with trypsin (Promega) following reduction and alkylation with DTT and iodoacetamide (Sigma-Aldrich). Digested samples were extracted from

the gel and cleaned by solid-phase extraction with Oasis HLB plates (Waters). The samples were then reconstituted and analyzed by LC/MS/MS, using a Q Exactive mass spectrometer (Thermo) coupled to an Ultimate 3000 RSLC-Nano liquid chromatography system (Dionex). Proteins interacting with PR-B_{WT} or PR-A_{mDBD} were identified and quantified as described below.

To analyze Mass Spec data, raw MS data files were converted to a peak list format and analyzed using the central proteomics facilities pipeline (CPFP), version 2.0.3 (56, 57). Peptide identification was performed using the X!Tandem (58) and open MS search algorithm (OMSSA) (59) search engines against the human database from Uniprot. Fragment and precursor tolerances of 20 ppm and 0.1 Da were specified; three missed cleavages were allowed. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was set as a variable modification. Label-free quantitation of proteins interacting with PR-B_{WT} or PR-A_{mDBD} across samples was performed using SING normalized spectral index software (60).

Co-Immunoprecipitation (Co-IP) Assays – Co-IP assays were performed to validate interactions *in vivo* between PR and GATAD2B. Total cell proteins were isolated in cell lysis buffer (Cell Signaling Technology, Cat # 9803S) from hTERT-HM or HEK-293 cells stably expressing GFP-conjugated PR-B_{WT}, PR-A_{WT}, PR-B_{mDBD} or PR-A_{mDBD} that were previously treated with IL-1 β (10 ng/mL) + P₄ (1 nM) for 12 h. Cell extracts (1500 μ g) were then precleared with 80 μ l Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Cat # sc-2003) for 30 min and immunoprecipitated with 100 μ l anti-GFP magnetic beads for 24 h (MBL International Corporation, Cat # D153-11). The beads were washed with cell lysis buffer 3 \times and 3 \times with lysis buffer containing 2 mM DTT and 500 mM NaCl. The eluted samples were then subjected to immunoblotting analysis of PR and

GATAD2B using antibodies for PR-A/B (Cell Signaling #8757 and GATAD2B (Bethyl Laboratories, #A301-281A) with input as control.

Data Analysis – Differences between groups were analyzed using either the student's t test or by one-way ANOVA, where appropriate, using GraphPad Prism, version 7.0 (GraphPad software Inc., San Diego, CA, USA.). Statistical significance was set as *P* value of < 0.05.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest concerning the contents of this article.

AUTHOR CONTRIBUTIONS

CCC contributed to the conceptual design, conducted the experiments, interpreted the data and wrote the paper. APM analyzed expression of GATAD2B, COX-2 and IL-6 proteins in pregnant mouse myometrium (Figure 8D - F), reviewed Mass Spectrometry data with Proteomics Core, edited manuscript, extended statistical analyses, revised figures. IH analyzed expression of PR (Fig. 2B) and performed co-immunoprecipitation assays in stably transfected HEK-293 and hTERT-HM cell lines (Fig. 6A). RWL conducted the Duolink proximity ligation assays in hTERT-HM cells (Fig. 6B). CRM contributed to the conceptual design of the studies, reviewed and interpreted the data and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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²**The abbreviations used are:** (GATAD2B) GATA Zinc Finger Domain Containing 2B; (P₄) progesterone; (PR) progesterone receptor; (PRE) progesterone response element; (hTERT-HM cells) human telomerase reverse transcriptase immortalized human myometrial cell line; (TSS) transcription start site; (qPCR) quantitative PCR; (h36B4) human ribosomal phosphoprotein P0; (EU) 5-Ethynyl Uridine; (DAPI) 4',6-diamidino-2-phenylindole; (_mDBD) DNA binding-defective mutant; (RNA Pol II) RNA polymerase II; (SINQ) spectral index quantitation software; (IL-1 β) human interleukin 1 β ; (DMSO) dimethyl sulfoxide.

FIGURE LEGENDS

Figure 1. Progesterone/PR-mediated repression of NF- κ B reporter activity in HEK-293 cells is lost by mutagenesis of the PR DBD. A, HEK-293 cells were cotransfected with NF- κ B-luciferase reporter constructs, Renilla luciferase plasmid and expression vectors of PR-B_{WT} and PR-B mutants, including PR-B-K388R (sumoylation mutant), PR-B-A604T (dimerization mutant) and PR-B_{mDBD} P-box mutant. One day after transfection, cells were treated with DMSO (V), IL-1 β (10 ng/mL) or IL-1 β plus P₄ (100 nM) for 6 h. B, Repression activity/fold-repression of data shown in Panel A was calculated by dividing the ‘fold induction’ for PR-B_{WT} and each PR-B mutant in response to IL-1 β by the fold-induction in response to IL-1 β + P₄. C, To further study the effects of DBD mutations on transrepression activity of PR-B and PR-A, HEK-293 cells were cotransfected with PR-B_{WT}, PR-A_{WT} or the corresponding DBD mutants and with NF- κ B-luciferase reporter constructs and Renilla luciferase plasmid. One day after transfection, cells were treated with DMSO (V), IL-1 β (10 ng/mL) or IL-1 β plus P₄ (100 nM) for 6 h. D, Repression activity of PR-B_{WT} and PR-A_{WT} and corresponding DBD mutants shown in Panel C was calculated for cells transfected with each PR isoform and mutant by dividing the ‘fold induction’ in response to IL-1 β by the fold-induction in response to IL-1 β + P₄. E, HEK-293 cells were cotransfected with Gal4-p65 CTD expression vector, GAL4 luciferase reporter and PR_{WT} or PR_{mDBD} expression vector, as indicated. One day after transfection, cells were treated with DMSO (V) or P₄ (100 nM) for 24 h. Cells from each experiment were then harvested and firefly luciferase and Renilla luciferase activities were assayed. Relative luciferase activities were calculated by normalizing Firefly luciferase activity to Renilla luciferase activity in the same samples to correct for transfection efficiencies. Data for experiments shown in Panels A-E are the mean \pm SEM of three replicate determinations for each treatment group. *, significant ($p < 0.05$) difference between samples.

Figure 2. WT and mutant PR-A and PR-B isoforms are expressed at equivalent levels in stably transfected hTERT-HM cells. A, hTERT immortalized human myometrial cells were stably transformed using recombinant lentiviruses expressing PR-B_{WT}, PR-B_{mDBD}, PR-A_{WT} and PR-A_{mDBD} conjugated to GFP at their N-terminal ends. PR-B- and PR-A-DBD mutants (PR_{mDBD}) contained a triple mutation (G584E / S585G / V589A) within the P-box region of the DBD. B, Immunoblotting of total cell lysates revealed that levels of PR expression in the four stably transfected cell lines were equivalent.

Figure 3. The PR DNA-binding motif plays an important role in P₄-mediated transrepression of COX-2 and IL-8 promoter activity in human myometrial cells. hTERT-HM myometrial cells lines stably expressing either PR-B_{WT}, PR-B_{mDBD}, PR-A_{WT} or PR-A_{mDBD} and cultured in the absence or presence of IL-1 β \pm P₄ were utilized. RT-qPCR was used to analyze expression of endogenous COX-2 (A-D), and IL-8 (E-H). A, B, E, F, Cells were treated with DMSO vehicle (V), IL-1 β (10 ng/mL) or IL-1 β + P₄ (100 nM) for 2 h. Total RNA was extracted and reverse transcribed and the relative abundance of COX-2 (A) and IL-8 (E) mRNA transcripts was determined by qPCR. C, D, G, H, To examine the effects of P₄ on IL-1 β induction of nascent COX-2 and IL-8 hnRNA expression, the uridine analog 5-ethynyluridine (EU) (200 μ M) was added to the medium at the time that cells were placed in DMSO, IL-1 β or IL-1 β + P₄. After 40 min of treatment, total RNA was extracted and the EU-labelled nascent RNA was isolated and reverse transcribed. The relative abundance of nascent COX-2 (C) and IL-8 (G) mRNA transcripts was determined by qPCR. B, D, F, H, Fold-repression activity was calculated by comparing the levels of RNA expression in cells treated with IL-1 β alone to the RNA levels after treatment with IL-1 β plus P₄. Data shown in Panels A-H are the mean \pm SEM., significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) difference between samples.

Figure 4. Recruitment of endogenous RNA Pol II and NF- κ B p65 to the COX-2 and IL-8 promoters was reduced by mutagenesis of the PR DBD in P₄-treated hTERT-HM cells, whereas, recruitment of PR was unaffected. ChIP analysis was used to study effects of P₄ on recruitment of RNA polymerase II (A,B), p65 (C,D) and PR (E,F,G) to promoters of COX-2 (A,C,E), IL-8 (B,D,F) and FKBP5 (G) genes. The myometrial cells were treated with DMSO (V), IL-1 β (10 ng/mL) or IL-1 β plus P₄ (100 nM) for 40 min prior to harvest and formalin treatment. qPCR was used to quantify the recruitment of RNA Pol II to

the TSS, and p65 and PR to the NF- κ B-RE using specific primers (Table 1). Fold recruitment is depicted as percent input relative to the corresponding vehicle-treated sample. Data are the mean \pm SEM of three replicate determinations for each treatment group. *, Significant ($p < 0.05$) difference between samples.

Figure 5. Mass spectrometry identification of GATAD2B/p66 β as a PR-B_{WT} interacting protein. A, Sequence of GATAD2B; four tryptic peptides identified by mass spectrometry analysis are highlighted. B, MS/MS spectrum of the most abundant tryptic peptide of GATAD2B, ALQEQEIEQR, with fragment ion assignments labeled.

Figure 6. PR interacts with GATAD2B and recruits GATAD2B to the COX-2 and IL-8 promoters in response to P₄ treatment. A, Co-immunoprecipitation assays (Co-IP) were performed in stably transfected HEK-293 (left panel) and in hTERT-HM cells (right panel) cells to validate interaction between PR and GATAD2B. Total cell proteins were isolated from cells stably expressing GFP-conjugated PR-B_{WT}, PR-A_{WT}, PR-B_{mDBD} or PR-A_{mDBD} that had been treated with IL-1 β (10 ng/mL) + P₄ (1 nM) for 12 h and immunoprecipitated with anti-GFP magnetic beads. The eluted samples were collected for immunoblotting analysis of PR and GATAD2B with input as control. B, Duolink proximity ligation assays were used to detect the direct in vivo interaction between PR and GATAD2B. hTERT-HM myometrial cells stably expressing PR-A_{WT} or PR-B_{WT} or the corresponding DBD mutants were treated with IL-1 β (10 ng/mL) + P₄ (100 nM) for 40 min. Intensity of fluorescent signal per cell was calculated by normalizing red fluorescent PLA signals to cell number, which was quantified by DAPI signals (blue) in that image. Data are expressed as mean \pm SEM for each treatment group (N = 6 fields per sample). C-F, ChIP assays were used to analyze the recruitment of GATAD2B to an NF- κ B response element or TSS in the COX-2 (C, D) and IL-8 gene (E, F) promoters. The myometrial cells were treated with DMSO (V), or P₄ (100 nM) for 40 min. qPCR was used to quantify the recruitment of each factor to the promoter using specific primers (Table 1). Fold recruitment is depicted as percent input relative to the corresponding vehicle treated sample. Data are the mean \pm SEM of three replicate determinations for each treatment group. *, Significant ($p < 0.05$) difference between samples.

Figure 7. GATAD2B plays an important role in P₄-mediated transrepression activity. A-F, Effects of siRNA-mediated knockdown of GATAD2B on P₄ transrepression activity. hTERT-HM cells stably expressing PR-B_{WT} or PR-A_{WT} were transfected with 20 nM siRNAs targeting GATAD2B or non-targeting siRNA (siNT) control. Data are the mean \pm SEM for each treatment group. *, Significant ($p < 0.05$) difference between samples. GATAD2B siRNA transfection markedly reduced endogenous GATAD2B protein expression (G). After transfection, the cells were synchronized in phenol red-free medium supplemented with 1% charcoal stripped FBS for another 48 h. The cells were then treated with DMSO vehicle (V), IL-1 β (10 ng/mL) or IL-1 β + P₄ (100 nM). Effects of GATAD2B knockdown on COX-2 (A-C) and IL-8 (D-F) mRNA were analyzed after hormonal treatment for 2 h. C, F, Repression activity was calculated by comparing the levels of mRNA expression in cells treated with IL-1 β alone to mRNA expression after treatment with IL-1 β + P₄. Effects of siRNA-mediated knockdown of GATAD2B on COX-2 (G) and phospho-p65 at Ser536 (H) proteins were assayed by immunoblotting after 4 h or 2 h of hormonal treatment, respectively. Data are the mean \pm SEM of three replicate determinations for each treatment group. *, Significant ($p < 0.05$) difference between samples.

Figure 8. GATAD2B is significantly decreased in human and mouse myometrium during labor. RT-qPCR was used to analyze GATAD2B (A), COX-2 (B) and IL-8 (C) mRNA expression in lower uterine segment myometrium of women in spontaneous labor (IL, n=12) or not in labor (NIL, n=20) at term. The Mann-Whitney U test was used to evaluate differences in GATAD2B, IL-8 and COX-2 expression between the NIL vs. IL groups. P values are shown for each dataset. Immunoblotting was used to analyze GATAD2B (D), COX-2 (E) and IL-6 (F) protein expression in three or more matched series of timed pregnant mouse myometrium between 15.5 dpc and labor (n \geq 3 mice at each gestational timepoint). Each immunoblot was analyzed for β -actin as a control for loading and transfer. Scanned immunoblots were quantitated by densitometric analysis followed by correction for loading and transfer variances using ImageJ software. Combined data from scanned immunoblots are shown as bar graphs (arbitrary units,

AU). Data are the mean \pm SEM for each group. For one-way ANOVA, GATAD2B: $F(3,12) = 10.57$, $P = 0.0011$; COX-2: $F(3,12) = 9.372$, $P = 0.0018$; IL-6: $F(3,8) = 8.219$, $P = 0.0079$. Bonferroni multiple comparison test was carried out post-hoc using 15.5 dpc levels for comparison: $**P < 0.01$, $***P < 0.001$; $n = 4$ mice at 15.5 dpc, 3 mice at 17.5 and 18.5 dpc, and 6 mice in labor. G, Schematic model of the putative role of GATAD2B in suppression of myometrial contractile gene expression during pregnancy and labor.

Table 1

Gene	Primers (5' to 3')
For mRNA assay	
Human 36B4	F-tgcatcagtacccattctatca; R-aaggtgtaatccgtctccacaga
Human CCL-2	F-cagccagatgcaatcaatgcc; R-tggaatcctgaaccacttct
Human COX-2	F-gcccagcacttcacgcatcag; R-agaccaggcaccagaccaaagacc
Human IL-6	F-agtgaggaacaagccagagc; R-catttggtggtgggtcagg
Human IL-8	F-cggaaggaaccatctcactg; R-agcactccttggaactg
Human GATAD2B	F-ctgtacgcaccacaacacc; R-cgctgacatggaacagaaga
Human PgR	F-tcagtgggcagatgctgtattt; R-gccacatggtaaggcataatga
Mouse 36B4	F-cactggtctaggacccgagaag; R-ccacggagacctctaaaagc
Mouse COX-2	F-cagccaggcagcaaatcc; R-tgtaaggggtgcaaaactg
Taqman primers	
Mouse 36B4	Mm0197446190_gh
Mouse IL-6	Mm00446190_m1
Mouse GATAD2B	Mm00522590_m1
For hnRNA assay	
Human hnCOX-2	F-agtttccgctctgacctct; R-tgatcagtgttgggaaa
Human hnIL-8	F-tgaggtcaagggtaggaga; R-cagctctgccagctacttcc
For ChIP assay	
Human COX-2-NF- κ B	F-ccgccagatgtcttttcttc; R-aaccttactcgccccagtct
Human COX-2-TSS	F-ctgggtttccgattttctca; R-gacgctcactgcaagtcgta
Human IL-8-NF- κ B	F-aagaaaactttcgtcatactccg; R-tggcttttatatcatcacctac
Human IL-8-TSS	F-tcgtggaatttcctctgaca; R-gccagcttgaagtcattgtt
Human FKBP5-PRE	F-ctcttccaaatctcactgaaatg; R-ataacaaccctgatgattgctg

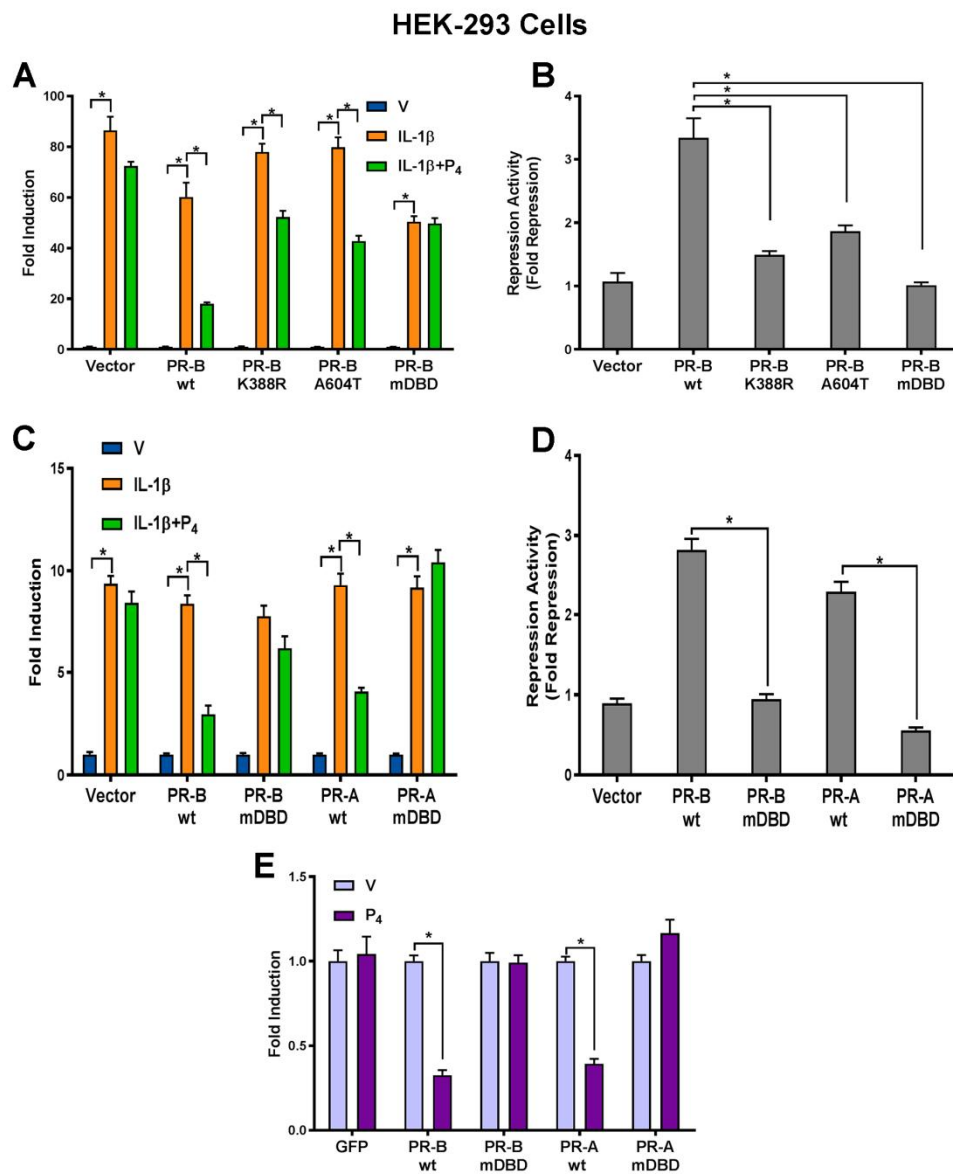


Figure 1

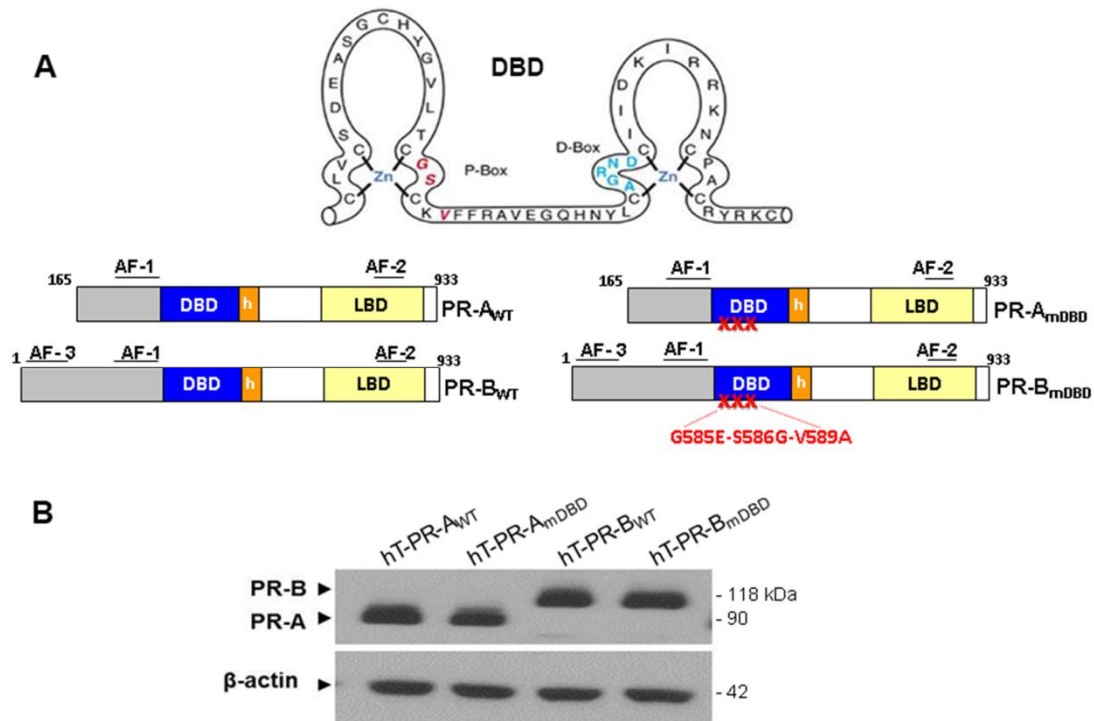


Figure 2

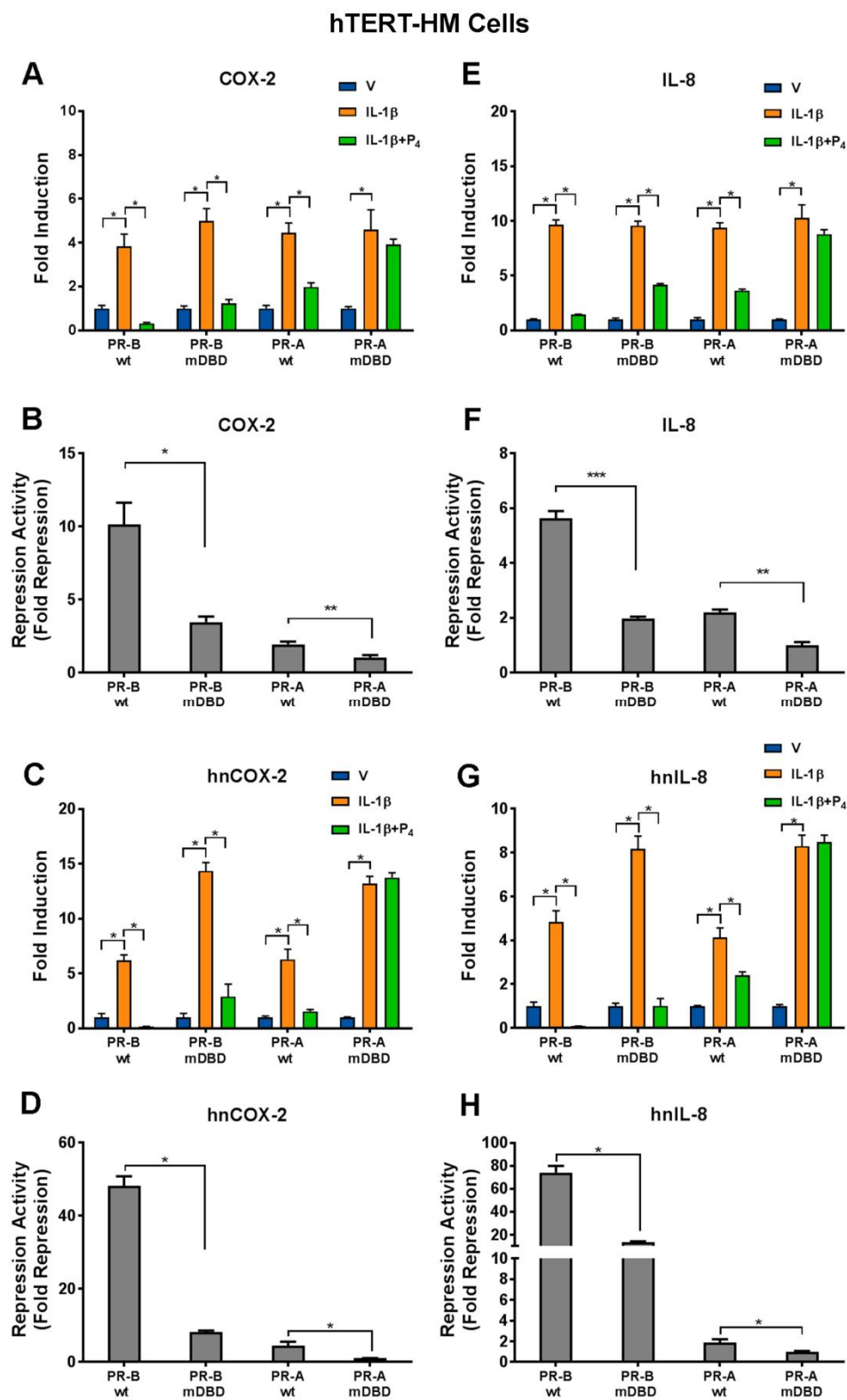


Figure 3

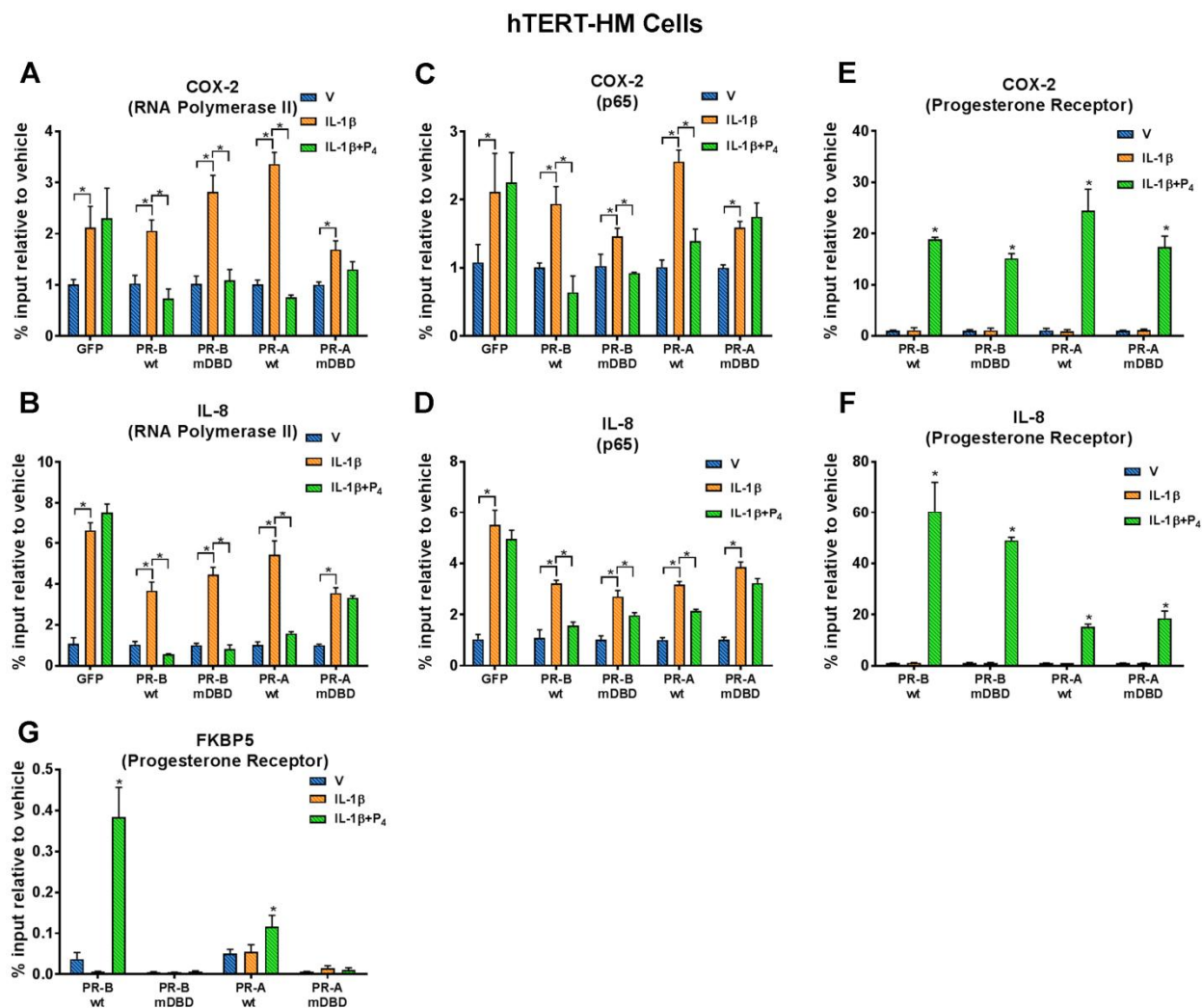


Figure 4

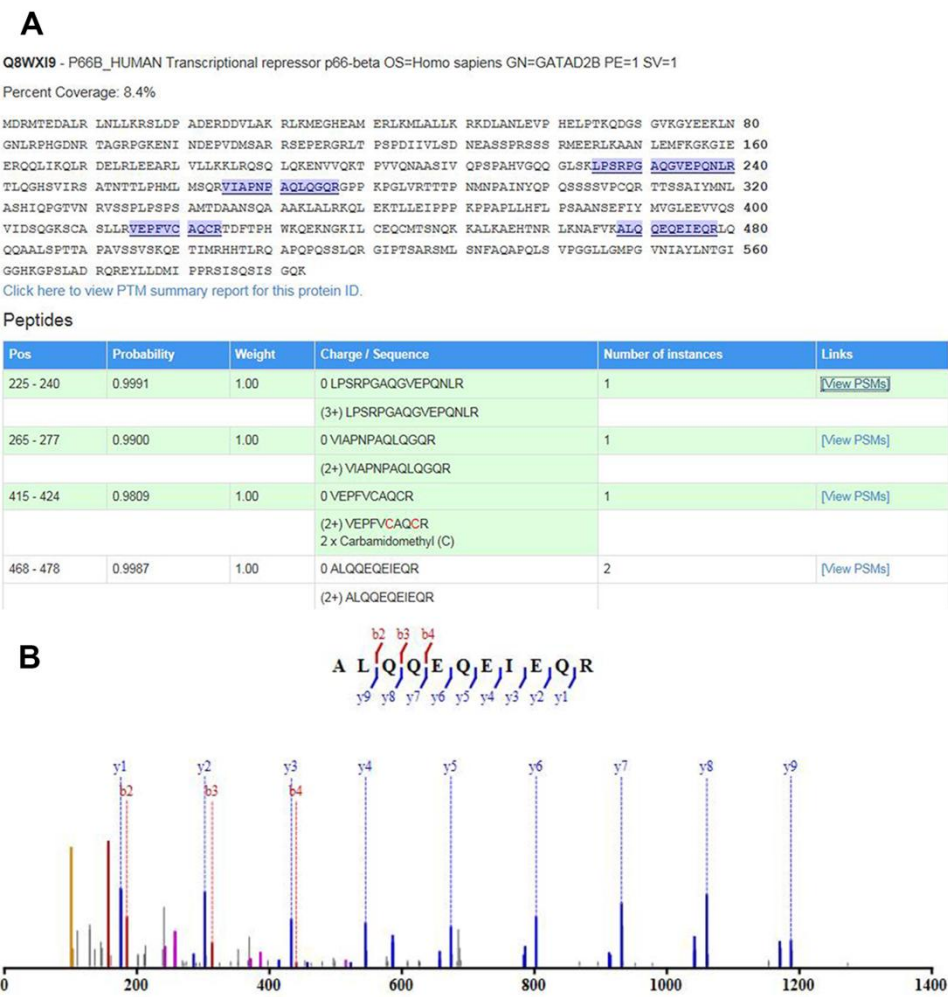


Figure 5

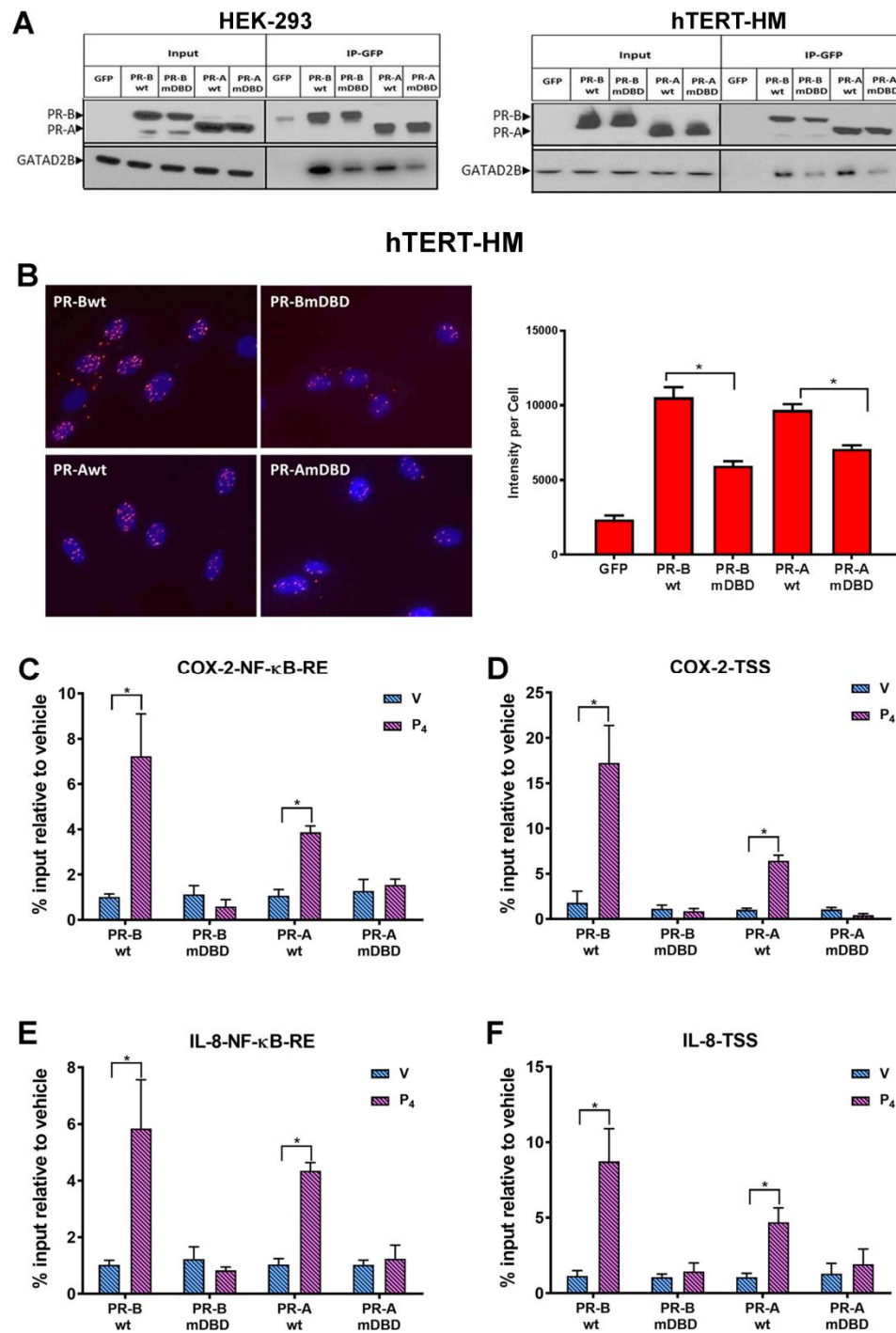


Figure 6

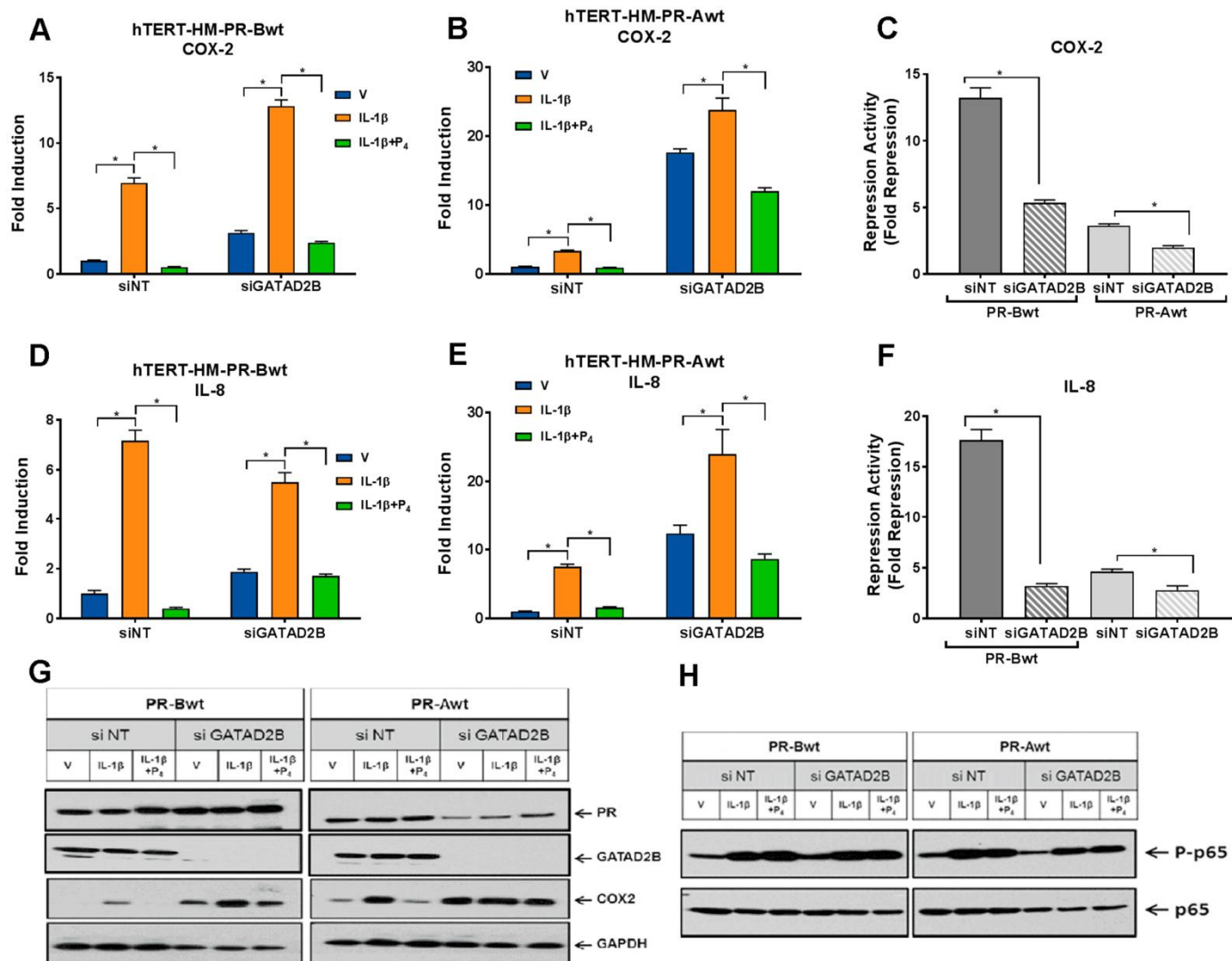


Figure 7

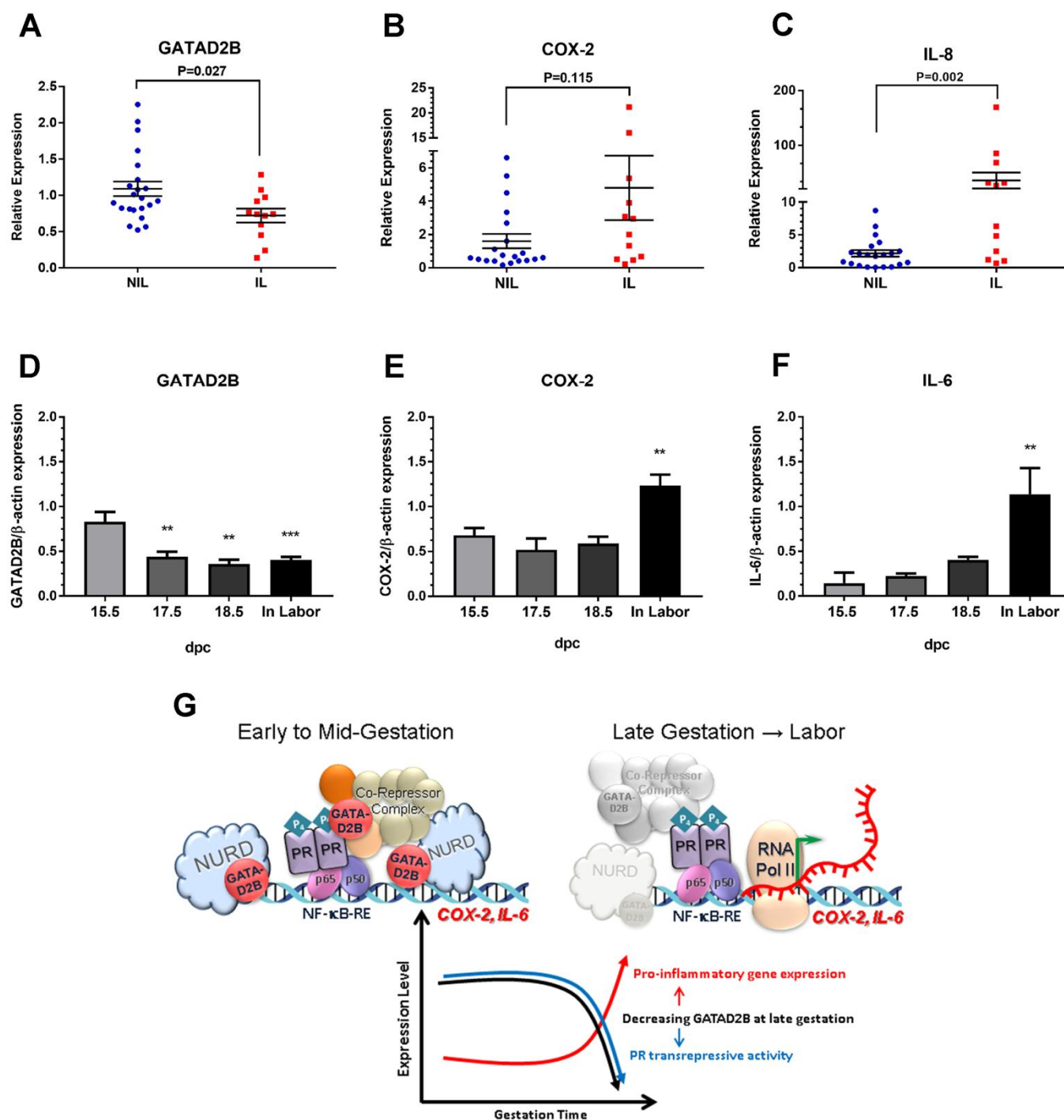


Figure 8

The transcriptional repressor GATAD2B mediates progesterone receptor suppression of myometrial contractile gene expression

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