Mol. Hum. Reprod. Advance Access published February 27, 2012

Term myometrium is characterised by increased activating epigenetic modifications at the progesterone receptor-A promoter

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Abstract

Term human myometrial expression of PR-A is increased relative to PR-B, and as PR-A is a repressor of progesterone action mediated through PR-B, this increase may mediate the withdrawal of progesterone action and precipitate labour onset. PR-A and PR-B expression is regulated by two separate promoters of the PR gene. We hypothesised that epigenetic histone modifications at the two promoters contribute to the labour-associated regulation of PR-A and PR-B expression in term myometrium. PR total, PR-B and PR-A mRNA levels were determined using qRT-PCR, and ChIP was used to determine the level of activating and repressive histone modifications at the PR-A and PR-B promoters in human myometrial samples not in labour (n=4) and in labour (n=4). Chromatin extracts were immunoprecipitated with antibodies against activating (aH3, aH4 and H3K4me3), and repressive (H3K9me3, H3K27me3 and H3R2me2a) histone modifications. PR-A mRNA levels increased during labour while PR-B mRNA levels remained constant resulting in an increase of PR-A/PR-B mRNA ratio, as expected. Regardless of labour status, significantly higher levels of the activating histone modifications were found at the PR-A promoter compared to the PR-B promoter ($P<0.001$). H3K4me3 increased significantly at both promoters with labour onset ($P=0.001$). Low levels of the repressive histone modifications were also present at both promoters, with no labour-associated changes observed. Our data indicate that the PR-A promoter is epigenetically marked for activation in term myometrium more extensively than the PR-B promoter, and that labour is associated with an increase in H3K4me3 activating modification, consistent with the previously described increase in PR protein at this time.

Keywords: Progesterone receptors / myometrium / labour / histone modifications / epigenetic

Introduction

The blocking effect of progesterone on myometrial contractility maintains a state of quiescence during human pregnancy and diminishes at term (Csapo 1956). In most mammalian species, progesterone withdrawal is achieved by a dramatic drop in maternal progesterone levels prior to the onset of labour. In humans and higher primates, however, the circulating levels of progesterone remain high at birth, yet the administration of progesterone receptor (PR) antagonists increases myometrial contractility and induces labour (Avrech et al. 1991; Chwalisz et al. 1995; Frydman et al. 1988; Neilson 2000). Therefore, in these species, progesterone withdrawal before birth is thought to be 'functional' and caused by a decrease of target tissue responsiveness to progesterone.

Progesterone exerts its genomic actions through its nuclear receptors (nPRs), which are members of a superfamily of ligand-activated transcription factors. In humans, the nPRs exist as two major isoforms (PR-A and PR-B), both comprising domains involved in DNA binding, hormone binding and transactivation (Leonhardt and Edwards 2002). The PR-A isoform is a truncated version of the full-length PR-B, lacking in the first 164 N-terminal amino acids that are essential for one of the transactivation sites. Both isoforms are translated from distinct messenger RNAs (mRNAs) transcribed from a single gene controlled by two separate promoters (Giangrande and McDonnell 1999; Kastner et al. 1990). PR-A and PR-B are functionally distinct receptors, since the suite of genes they regulate show little overlap (Richer et al. 2002). In addition, PR-A has been shown to repress PR-B-mediated transcription on promoters where PR-A was inactive (Vegeto et al. 1993). Both PR isoforms are expressed in the pregnant human myometrium and during pregnancy the PR-A:PR-B expression ratio favours myometrial quiescence and cervical closure due to PR-B dominance (Merlino et al. 2007). Furthermore, myometrial PR-A expression is increased relative to PR-B at term labour, and it has been postulated that

increased PR-A mediates the withdrawal of progesterone action by antagonising the effects of PR-B. This change in PR isoform ratio results in a decrease of myometrial progesterone responsiveness triggering labour and delivery (Merlino et al. 2007; Mesiano et al. 2002; Mesiano et al. 2011; Pieber et al. 2001). These data, therefore, highlight the importance of understanding the regulation of PR isoform expression. We have previously demonstrated that PR isoform expression can be differentially regulated in the immortalised pregnant human myometrial cell line PHM1-31, where $PGF_{2\alpha}$ (a labour-stimulating prostaglandin) selectively increased PR-A, but not PR-B mRNA levels (Madsen et al. 2004). However, the exact mechanism by which this is achieved is still unknown. In the present study we have explored the possibility that regulation occurs through a change in epigenetic status in term human myometrial tissue.

Transcriptional activation of genes depends on (i) the establishment of an open chromatin structure that is permissive of gene expression, and (ii) the subsequent recruitment of transcription factors (TFs) and co-regulatory proteins that stimulate gene activity (Freedman 1992; Kishimoto et al. 2006). Cytosine methylation of DNA at CpG dinucleotides and post-translational modifications of histones (including acetylation and methylation) are part of an epigenetic regulatory system that controls the establishment of permissive or repressive chromatin structure. There is increasing evidence, stemming mainly from studies in breast and endometrial cancers, that the expression of PRs can be regulated by certain epigenetic events within the PR gene (Ferguson et al. 1998; Gaudet et al. 2009; Lapidus et al. 1996; Liu et al. 2003; Mc Cormack et al. 2008; Sasaki et al. 2001; Sasaki et al. 2002; Sasaki et al. 2003; Wu et al. 2006; Xiong et al. 2005; Xu et al. 2004). Xiong et al (Xiong et al. 2005) reported that hypermethylation of the PR-B promoter silenced its gene expression in adenocarcinoma cells, which could be restored through DNA demethylation in conjunction with histone acetylation. An earlier study by Condon et

al (Condon et al. 2003) found that the myometrial expression of several histone acetyltransferases (HATs), which catalyse histone acetylation, decreased with labour in pregnant women. In the present investigation we hypothesised that epigenetic histone modifications are involved in the regulation of PR isoform expression in the pregnant human myometrium. The aims were (1) to determine the presence of activating and repressive histone modifications at the PR-A and PR-B promoters in pregnant myometrial tissue and (2) to determine if there are any labour-associated changes in the levels of these histone modifications. The activating modifications investigated were histones H3 and H4 acetylation (aH3 and aH4 respectively), and histone H3 lysine 4 trimethylation (H3K4me3), while the repressive modifications included histone H3 lysine 9 trimethylation (H3K9me3), histone H3 lysine 27 trimethylation (H3K27me3), and asymmetrical histone H3 arginine 2 dimethylation (H3R2me3a) (An 2007; Binda et al. 2010; Kirmizis et al. 2007; Kouzarides 2007).

Materials and Methods

TRIzol, Superscript III First-Strand Synthesis System, and PCR primers were purchased from Invitrogen (Carlsbad, USA) and DNase I kit was obtained from Ambion (Austin, USA). 2X SYBR Green PCR Master Mix, Taqman Universal PCR Master Mix and 20X human 18s ribosomal RNA (rRNA) probe/primer were from Applied Biosystems (Carlsbad, USA). Complete Mini Protease Inhibitor Cocktail tablets were supplied by Roche Applied Science (Indianapolis, USA). All antibodies (rabbit IgG: AP101; aH3: 06-599; aH4: 06-598; H3K4me3: 07-473; H3K9me3: 07-442; H3K27me3: 07-449; H3R2me2a: 07-585) used in the chromatin immunoprecipitation (ChIP) assays and Magna protein A-agarose beads were from Millipore (Kilsyth, Australia), salmon sperm DNA was

purchased from Sigma (St Louis, USA), while the Wizard SV Gel and PCR purification kits were obtained from Promega (Madison, USA).

Tissue Specimens

All specimens were obtained with informed patient consent and the study approved by the Human Research Ethics Committees of the John Hunter Hospital and the University of Newcastle. Tissue biopsies of human myometrium were collected from singleton term caesarean deliveries in the absence of labour (NIL, n=4) and during active labour (IL, n=4). Tissue from the upper margin of the lower uterine segment was collected on ice, snap-frozen in liquid nitrogen, and stored at -80°C until required. Non-labouring tissues were collected from elective Caesarean deliveries, the indications for which included previous Caesarean section and breech presentation. No signs of labour onset, such as, uterine contractions or cervical changes were evident at the time of surgery. Labouring tissues were from women undergoing emergency Caesarean delivery for breech presentation or fetal distress. There were no significant differences in the gestational age at delivery, maternal age, gravidity or parity of the patient population used in these studies and all newborn infants were normal. Women who had an induced labour or who had a history of infection or histological chorioamnionitis were excluded from the study. For each myometrial sample collected, 0.1 gram of tissue was used for total RNA extraction and 1 gram for ChIP analyses.

Total RNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR

Total RNA was extracted using *TRIzol* reagent according to the manufacturer's protocol. RNA samples (NIL, n=4; IL, n=3) were treated with DNase, and 1μg of total RNA was reversed transcribed to cDNA using the SuperScript III First-Strand Synthesis System. Target cDNA samples were amplified in triplicate using SYBR Green PCR Master Mix in

an ABI 7500 Sequence Detector. Total reaction volume was prepared to 20µl with 10µl of SYBR Green PCR master mix (2x) for the PR primers, or with 10µl of TaqMan Universal PCR master mix for the 18s rRNA (reference gene) primer. The PCR primers used were as shown in Table I.

Tissue Extraction for ChIP

One gram of tissue (in ~0.5cm^3 pieces) was thawed and fixed in phosphate-buffered saline (PBS) containing 1% formaldehyde for 15 minutes on a rotator at room temperature. Tissues were washed in cold PBS containing 125mM glycine and homogenized in 20ml of swelling buffer (125mM glycine, 2mM EDTA, 0.5mM EGTA, 10mM Tris pH8.0, 10mM DTT, 0.1mM PMSF, 1x protease inhibitor cocktail) using a Polytron homogenizer with six 30s bursts and 30s cooling periods. 0.5% (v/v) Triton X-100 and 0.1mM PMSF were added, and samples were further homogenized with a Potter type glass-teflon homogenizer. The homogenate was pressed through a stainless steel mesh (380µM pore size) and centrifuged at 2500g for 15 minutes at 4°C. The pellet was suspended in 2ml ice-cold Buffer 1 (0.25% Triton X-100, 10mM EDTA, 0.5mM EGTA, 10mM HEPES pH6.5, 10mM DTT, 0.1mM PMSF, 1x protease inhibitor cocktail), stood on ice for 10 minutes, centrifuged at 12000g for 1 minute at 4°C, and supernatant discarded. The pellet was resuspended in 1ml of ice-cold Buffer 2 (200mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM HEPES pH6.5, 10mM DTT, 0.1mM PMSF, 1x protease inhibitor cocktail), stood on ice for 10 minutes, centrifuged at 12000g for 1 minute at 4°C, the supernatant discarded and the pellet processed by ChIP.

Chromatin Immunoprecipitation

Each pellet was resuspended in 400µl of cold lysis buffer (1% SDS w/v, 10mM EDTA, 50mM Tris pH8.0, 0.1mM Na_3VO_4, 1x protease inhibitor cocktail) and sonicated (10

cycles of 10s sonication with 50s cooling periods in a salt/ice slurry) using a MISONIX XL-2000 (Qsonica) at power setting 15. The sonicate was centrifuged at 12000g for 10 minutes at 4°C, a 50µl aliquot was kept as a non-immunoprecipitated (NIP) input control, and 100µl aliquots (IP) were diluted with 900µl Buffer 4 (1.12% Triton X-100, 0.11% Na deoxycholate, 1mM EDTA, 0.56mM EGTA, 170mM NaCl, 10mM Tris pH8.0, 1mM PMSF). Each aliquot (except NIP) was pre-cleared with 4µg of salmon sperm DNA and 40µl 10% Magna beads at 4°C for 2 hours on a rotator. IP samples were centrifuged at 12000g for 1 minute at 4°C, supernatants were pooled and 500µl aliquots were incubated with antibodies (anti-rabbit IgG, anti-aH3, anti-aH4, anti-H3K9me3 at 3µg, and anti-H3K4me3 and anti-H3K27me3 at 1µg) at 4°C overnight. Immune complexes were captured by incubating with 2µg of salmon sperm DNA and 20µl 10% Magna beads for 1.5 hours at 4°C. The beads were washed sequentially with 1ml of TSE I (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris pH8.0), 1ml of TSE II (0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl, 20mM Tris pH8.0) and 1ml of Buffer 3 (0.25M LiCl, 1% NP-40 v/v, 1% Na deoxycholate, 1mM EDTA, 10mM Tris pH8.0), with 10 minutes incubations on ice. After each wash, samples were centrifuged at 12000g for 1 minute and supernatant discarded. The beads were washed a further three times with 1ml of cold TE buffer (10mM Tris pH8.0, 1mM EDTA). The immune complexes were eluted from the beads by three washes of 1 x 150µl and 2 x 100µl elution buffer (1% SDS, 0.1M $NaHCO_3$) for 10 minutes at 65°C each. Cross-links were reversed by incubating pooled supernatants at 65°C overnight, including the NIP input control. The recovered DNA was purified using the Wizard SV Gel and PCR purification kit, and the input DNA was quantitated using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA).

Quantitative Real-time PCR (qPCR)

Input DNA (NIP) samples were diluted 1:5000 and all IP DNA samples were diluted 1:5 and 5µl of each was used in real-time PCR reactions. The PCR reaction was performed in a total volume of 20µl, containing 10µl of 2x SYBR green PCR master mix, primers (Table II), template and water. Primers specific to different regions on promoters of PR-A and PR-B (Figure 1) were designed using Primer Express software from Applied Biosystems and primer specificity was checked using BLAST. Two control primer pairs were also included; an upstream 5' untranslated region (5'UTR) and a downstream region in the PR gene that were not part of the promoter sequences. Optimal primer concentrations were determined in preliminary experiments and subsequently used between 400 to 600nM. The amplification efficiency of each primer pair was determined using the LinRegPCR 11.0 software (HFRC) (Ramakers et al. 2003). All primers used were deemed efficient with values ranging from 1.92 to 2.05, indicating that each primer pair amplifies with comparable efficiency close to the theoretical maximum (2.0) as expected after optimisation. Dissociation curve analyses were also performed to ensure that a single amplicon at the expected melting temperature (Tm) was generated. Amplifications were performed using an ABI PRISM 7500 Sequence Detector. No template controls were included to detect primer-dimer formation, and dissociation curve analyses were performed to monitor the homogeneity of amplicons. A calibrator template (sonicated NIP DNA from human myometrial cells) was included to monitor the amplification efficiencies of each primer pair, and also to correct for plate-to-plate variation.

Data Analysis

All mRNA abundance data were expressed relative to the 18S rRNA reference gene. The relative mRNA abundance was calculated using the formula $2^{-\Delta Ct}$, where Ct is the mean threshold cycles of PCR done in triplicate and delta Ct (ΔCt) is the Ct difference between

the reference and target mRNAs (Schmittgen and Livak 2008). The relative abundance of PR-A mRNA was calculated by subtracting the relative abundance of PR-B mRNA from that of PR total.

In each ChIP assay, control immunoprecipitation using rabbit IgG was included to measure non-specific background. Relative abundance of an immunoprecipitated factor (ΔCt) at a specific sequence site was calculated using the equation, $2^{\Lambda}(Ct^{(IP)} - Ct^{(input)})$. The relative enrichment of an immunoprecipitated factor was expressed as a percentage of input normalised to the downstream control site. The relative abundance of the upstream and downstream control sites served as a measure of non-specific DNA background.

Statistical Analysis

STATA 11.0 software (Statacorp) was used for all statistical analyses. Differences of the relative mRNA abundance of PR-B and PR-A between NIL (n=4) and IL (n=3) groups were assessed using the Wilcoxon rank sum test. The mRNA data are expressed as mean ± SD (Figure 2). Two separate mixed-effects linear regression models were used to assess square root- or log-transformed data from the ChIP experiments. A random intercept term was included in each regression model to adjust for repeated measurements (at each primer site for each patient), in order to assess the association of an immunoprecipitated factor with primer sites and labour status. The first model used the relative enrichment of a factor at the 5'UTR upstream control site as the reference group, and the second model used the PR-B primer site as the reference group. ChIP data were presented in box-plots (Figures 3 and 4), with PR-B and PR-A representing pooled data of the three sites on each promoter. Breakdown occupancy of each histone modification at individual sites was also presented in Figures 3 and 4. For both the mRNA and ChIP analyses, differences were considered statistically significant if $P<0.05$.

Results

mRNA expression of myometrial PRs

In order to determine the expression of PRs in the tissue samples collected for ChIP analysis, the relative mRNA abundance of PR total, PR-B, and PR-A were measured. While the mRNA levels of PR-B remained constant with labour (Figure 2a), PR-A mRNA levels increased significantly during labour ($P<0.05$). Consequently, the PR-A:PR-B mRNA expression ratio was significantly higher in labour (Figure 2b). These results are consistent with previous findings (Merlino et al. 2007; Mesiano et al. 2002) and indicate that the myometrial tissues collected for histone modification analyses were a representative set of samples exhibiting the change of PR-A:PR-B expression ratio that occurs at term labour.

Histone H3 and H4 acetylation

For both aH3 and aH4, very low levels of these acetylated forms were detected at the 5'UTR region of the PR gene. Relative enrichment of aH3 was significantly higher at both the PR-B ($P<0.001$) and PR-A ($P<0.001$) promoters compared to the 5'UTR control region (Figures 3a and 3d). A separate regression model showed that the aH3 enrichment level was significantly higher at the PR-A promoter, when compared to the PR-B promoter ($P<0.001$). A similar enrichment pattern was observed for H4 acetylation (Figures 3b and 3e), with significantly higher levels of aH4 at the PR-A promoter ($P<0.001$). No significant labour-associated changes were detected in the relative enrichment levels of aH3 ($P=0.182$) and aH4 ($P=0.360$).

Histone-H3K4 trimethylation

Trimethylation of histone H3K4 is a well-characterized activating histone modification involved in the recruitment of coactivator proteins to marked gene promoters (Kouzarides 2007; Sims et al. 2007; Vermeulen et al. 2007). Therefore, we measured the levels of the H3K4me3 modification at the PR promoters. Low levels of H3K4me3 were detected at the 5'UTR control region (Figures 3c and 3f). H3K4me3 enrichment was significantly higher at both PR-B ($P<0.001$) and PR-A ($P<0.001$) promoters when compared to the 5'UTR control site. Similar to acetylated H3 and H4, significantly higher levels of H3K4me3 were also detected at the PR-A promoter ($P<0.001$), compared to the PR-B promoter. Moreover, the relative levels of H3K4me3 at both promoters were significantly increased in association with labour ($P=0.001$).

Histone-H3K9 trimethylation

Trimethylated histone H3K9 is an epigenetic modification commonly associated with inactive gene promoters and DNA methylation. Overall, low levels of H3K9me3 were found at all sites (Figures 4a and 4c). However, significantly higher levels of H3K9me3 were detected at the PR promoters ($P<0.001$) when compared to the 5'UTR control region, indicating that this repressive modification was also present at the myometrial PR gene. The H3K9me3 modification was significantly more abundant at the PR-A promoter ($P<0.001$) compared to the PR-B promoter. No labour-associated changes were detected ($P=0.821$).

Histone-H3K27 trimethylation

Trimethylation of H3K27 is a well-characterised repressive histone modification that often accompanies the activating H3K4me3 at dually marked ("bivalent") promoters (Barski et al. 2007). For this reason, enrichment levels of H3K27me3 were measured. Very low

levels of H3K27me3 were detected at all sites (Figures 4b and 4e). However, small but statistically significant higher levels of this modification were measured at the PR promoters (*P<0.001*) compared to the 5'UTR control region. There were no labour-associated changes (*P=0.542*).

Histone-H3R2 asymmetric dimethylation

Given the reported interaction of H3R2 asymmetric dimethylation with H3K4 trimethylation (Guccione et al. 2007; Kirmizis et al. 2007), whereby this arginine modification contributes to transcriptional repression by inhibiting H3K4me3, we measured this specific repressive mark at the PR promoters and its changes with labour. Compared to the 5'UTR control region, H3R2me2a levels were significantly higher at both PR-B (*P<0.005*) and PR-A (*P<0.001*) promoters (Figures 4c and 4f), with no labour-associated changes detected (*P=0.960*).

Discussion

Progesterone plays a dominant role throughout pregnancy by maintaining uterine quiescence during gestation. Withdrawal of progesterone results in the transition of the quiescent myometrium into a more contractile state, which is a key process of parturition. In humans, progesterone withdrawal is functional and has been shown to be associated with a decrease in myometrial progesterone responsiveness (Zakar and Hertelendy 2007). Many different studies have been performed to elucidate the mechanisms of progesterone withdrawal in humans at term, and suggestions including changes in transcription factor co-activators (Condon et al. 2003), nuclear factor kappa B (NFkB) (Allport et al. 2001) and metabolism of progesterone by target tissues (Sheehan et al. 2005), have been proposed. Progesterone responsiveness also depends on the level and the ratio of the PR isoforms since the two isoforms target distinct sets of genes and high levels of PR-A act as a

dominant inhibitor of PR-B transcriptional activity (Chalbos and Galtier 1994; Giangrande and McDonnell 1999; McDonnell et al. 1994; Scarpin et al. 2009; Tung et al. 1993; Wen et al. 1994). Previous studies have shown that the PR-A:PR-B mRNA and protein ratios increase in labouring human myometrium and these increases are associated with increased expression of contraction-associated proteins (CAPs) (Mesiano et al. 2002). A labour-associated increase in the PR-A mRNA levels (Figure 2a) and PR-A:PR-B ratio (Figure 2b) was also apparent in the sample set collected for the present study. However, in contrast to a previous report (Mesiano et al. 2002), we did not detect a significant change in PR-B mRNA levels between non-labouring and labouring myometrium (Figure 2a), possibly because our newly designed primer pair has better defined specificity for PR-B mRNA. Our mRNA data is also consistent with protein expression studies, which reported that PR-A protein expression was increased in labouring myometrium while PR-B protein expression remained constant (Merlino et al. 2007). Thus the selective increase in PR-A expression (both mRNA and protein) indicates the crucial involvement of this isoform in the orchestration of human labour.

The focus of the present study was to assess the involvement of epigenetic histone modifications in the regulation of the PR-A and PR-B promoters in term human myometrium. Based on the observation that epigenetic events can play a role in PR gene regulation by altering expression levels in cancer-derived cells (Sasaki et al. 2001; Sasaki et al. 2002; Sasaki et al. 2003; Xiong et al. 2005), we hypothesised that the changing expression of PR isoforms in human myometrium at labour may be underpinned by epigenetic histone modifications at their promoters. We therefore investigated the involvement of histone modifications in the differential regulation of the PR-A and PR-B promoters in term human myometrial samples. Two major histone modifications of interest

are the acetylation and methylation of histones, specifically on histone H3 where most modifications are observed.

The ChIP technique was optimised for use with fresh or snap-frozen myometrial samples, thereby allowing the detection of histone-DNA interactions *in vivo*. We assessed the acetylation status of histones H3 and H4 at the PR promoters in term myometrial tissues obtained in the absence and presence of labour. Significant levels of these gene activating modifications were found at both promoters (Figure 3a and 3b), with higher levels at the PR-A promoter than at the PR-B promoter. However, histone acetylation levels remained similar on both promoters prior to and after labour onset indicating that this epigenetic modification may not be directly responsible for the increased expression of PR-A at labour. While histone acetylation is involved in direct chromatin reorganisation (An 2007), H3K4me3 serves as an activating modification that can be recognised by specific proteins resulting in the recruitment of downstream coregulators (Sims et al. 2007). High levels of H3K4me3 were detected at both the PR-A and PR-B promoters (Figure 3c), with significantly higher levels measured at the PR-A promoter compared to the PR-B. We observed elevated trimethylation of H3K4 at both promoters with labour, suggesting a possible role for H3K4me3 in the mechanism responsible for the labour-associated up-regulation of PR-A. It is unclear why this activating modification did not promote increased PR-B gene activity with labour, but studies by Pavri et al (Pavri et al. 2006) established that H3K4me3 itself has no direct effect on transcription, and Sims et al (Sims et al. 2007) demonstrated that H3K4me3 is recognised by the human CHD1 protein and facilitates pre-mRNA maturation. Any requirement for promoter-specific factors for labour-associated H3K4me3 recognition in the myometrium remains to be established.

We also determined the levels of repressive histone modifications (H3K9me3, H3K27me3 and H3R2me2a) at the myometrial PR promoters (Figure 4). Modest, but significant levels of these repressive histone modifications were detected with no difference between the promoters and regardless of labour status. Further work will be needed to determine whether the presence of activating and repressive histone H3 methylations in the same chromatin samples indicate that the PR promoters are bivalently marked in the myometrium or that the activating and repressive modifications separate populations of alleles. Nevertheless, the absence of significant changes in repressive modifications with labour and between promoters suggests that the corresponding differences in expression levels are not driven by altered levels of these repressive modifications.

This is the first report so far of histone modifications at the PR gene in human myometrial tissue. Our results suggest that both PR-A and PR-B promoters are in a permissive chromatin state, primed by activating histone modifications that may underpin the observed isoform-specific differences and labour-associated changes in term human myometrium *in vivo*. Identification of the specific histone modifying enzymes involved in the epigenetic regulation of PR-A and PR-B could provide new insights into the mechanisms of functional withdrawal of progesterone at term, potentially contributing towards more efficient designs of progestin-based interventions to prevent preterm birth.

Acknowledgements

We would like to thank the patients and staff of the John Hunter hospital, and in particular, midwives, Ms Anne Wright and Ms Sally Graham for their work in subject recruitment, and Ms Julia Smith and Dr Patrick McElduff for support in statistical analyses.

Funding

This study was supported by the National Health and Medical Research Council of Australia (Project Grant #631026), the John Hunter Hospital Charitable Trust and the University of Newcastle, Australia.

Authors' roles

S.Y.C. in execution of experiments, acquisition, analysis and interpretation of data, as well as manuscript drafting. R.S. involved in supervision of project and article revision. T.Z. assisted in experimental design, statistical analyses and article revision. C.M. assisted in initial experimental design. G.M. is responsible for conception and design of study, overall supervision of project and article revision. All authors participated in the final approval of version to be published.

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Figure Legends

Figure 1: Schematic illustration of the target sites of primers designed specifically to the PR-A and PR-B promoters (3 primer pairs on each promoter), including an upstream 5'UTR and a downstream control. For primer sequences, refer Table II.

Figure 2: Expression of progesterone receptor isoforms (PR-B, PR-A) in term human myometrium. (a) Relative mRNA abundance of PR-B and PR-A in myometrial samples collected prior to labour onset (NIL, n=4) or after labour onset (IL, n=3), expressed relative to the 18s rRNA reference gene. (b) PR-A:PR-B expression ratio before and after labour onset. Data are presented as the mean ± SD. The asterisk (*) denotes a significant difference ($P<0.05$).

Figure 3: Enrichment levels of activating histone modifications at the progesterone receptor (PR) gene in term human myometrial samples collected prior to labour (NIL, n=4), and after labour onset (IL, n=4). Activating histone modifications (a) acetyl-histone H3, (b) acetyl-histone H4, and (c) trimethyl-histone H3K4, at the 5'UTR upstream control site, as well as the PR-B and PR-B promoters. Relative enrichment at individual primer sites shown in (d) acetyl-histone H3, (e) acetyl-histone H4 and (f) trimethyl-histone H3K4. As assessed by mixed-effects linear regression models, all activating histone modifications were significantly higher on the PR-A promoter compared to the PR-B promoter ($P<0.001$). Trimethyl-H3K4 increased significantly with labour at both promoters ($P=0.001$). No labour-associated changes were detected for aH3 and aH4.

Figure 4: Enrichment levels of repressive histone modifications at the progesterone receptor (PR) gene in term human myometrial samples collected prior to labour (NIL, n=4), and after labour onset (IL, n=4). Repressive histone modifications (a) trimethyl-H3K9, (b) trimethyl-H3K27, and (c) asymmetric-dimethyl-H3R2, at the 5'UTR upstream control site, as well as the PR-B and PR-B promoters. Relative enrichment at individual primer sites shown in (d) trimethyl-H3K9. (e) trimethyl-H3K27 and (asymmetric-dimethyl-H3R2. As assessed by mixed-effects linear regression models, low levels of repressive histone modifications were enriched at all sites, with no labour-associated changes detected.

Table I: cDNA primer sequences and locations for PR total and PR-B.

Primer	Primer Sequence (5' – 3')	Location	Gene Bank #
PR total	(F) GTGGGAGCTGTAAGGTCTTCTTTAA	1927/2008	NM000926
	(R) AACGATGCAGTCATTTCTTCCA		
PR-B	(F) TCGGACACCTTGCCTGAAGT	867/934	
	(R) CAGGGCCGAGGGAAGAGTAG		

Table II: ChIP primer sequences and locations.

Primer	Primer Sequence (5' – 3')	Location	Gene Bank #
5'UTR control	(F) ACCAACCAAAATCAAGCCAAA	-2352/-2203	AY525610.1
	(R) CAAACCCAAGACATCAAATGACA		
Downstream control	(F) CATCGTTGATAAAATCCGCAGAA	2570/2637	AY525610.1
	(R) TGCCAGCCTGACAGCACTT		
B1	(F) GATAATGTAGCCAAATGTCTTCCTCTG	-314/-148	NM000926
	(R) GGATGGAACCTCATAAGCATATTACG		
B2	(F) ACGGGTGGAAATGCCAACT	-112/-52	NM000926
	(R) GCCCCTCCCTACCCCAAT		
B3	(F) GAGGAGGAGGCGTTGTTAGAAA	-32/34	NM000926
	(R) CAAGGCTTACCCCGATTAGTGA		
A1	(F) CTGACGACAGGATGGAGGC	539/653	NM000926
	(R) GAGGAGAAAGTGGGTGTTGAATGT		
A2	(F) CGGTCCAGCCACATTCAAC	620/666	NM000926
	(R) ATAGGGGCAGAGGGAGGAGA		
A3	(F) TCGGACACCTTGCCTGAAGT	867/934	NM000926
	(R) CAGGGCCGAGGGAAGAGTAG		

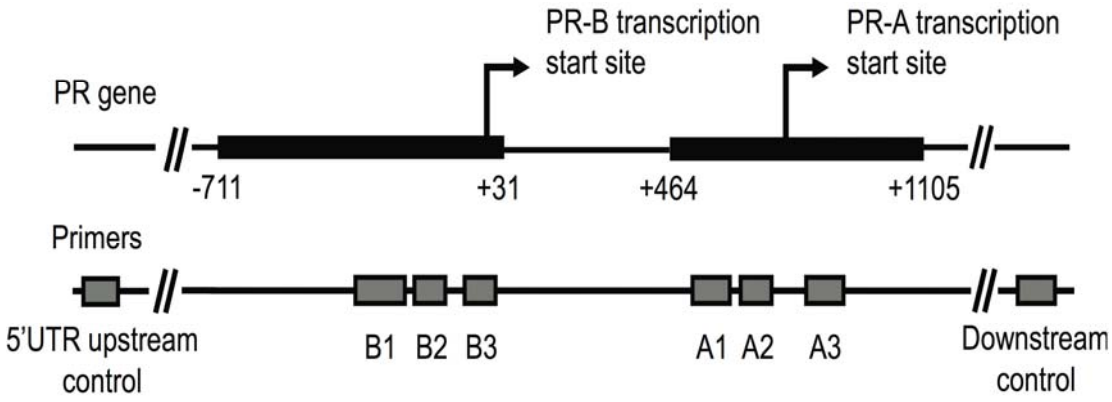

Fig. 1

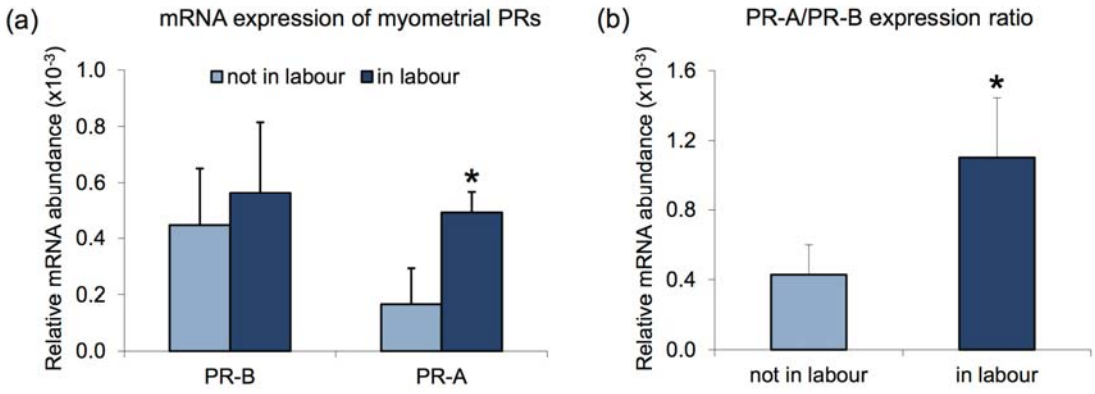

Fig. 2

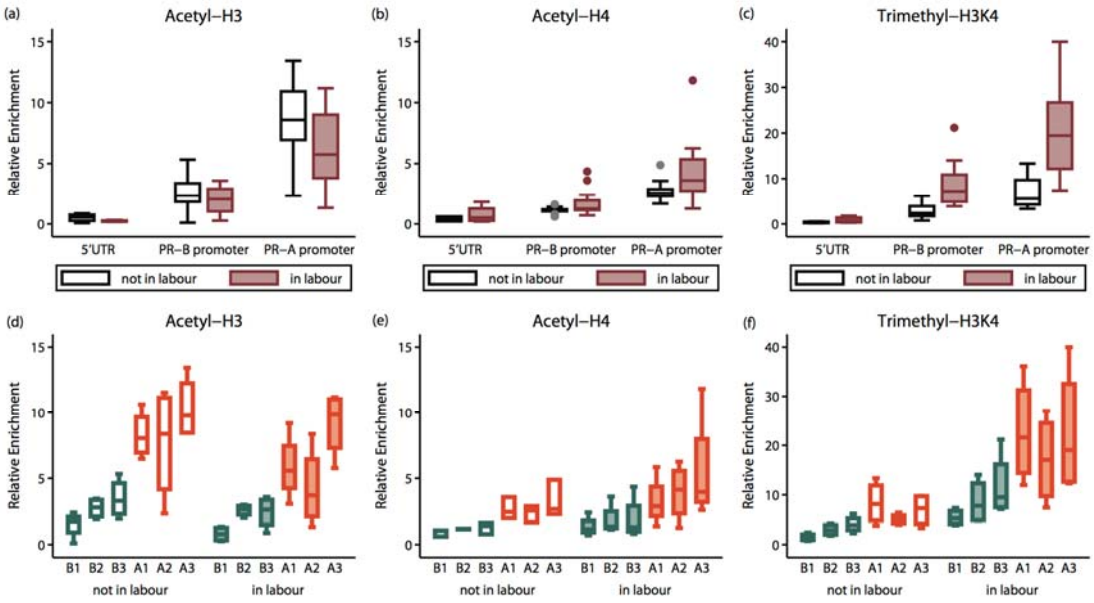

Fig. 3

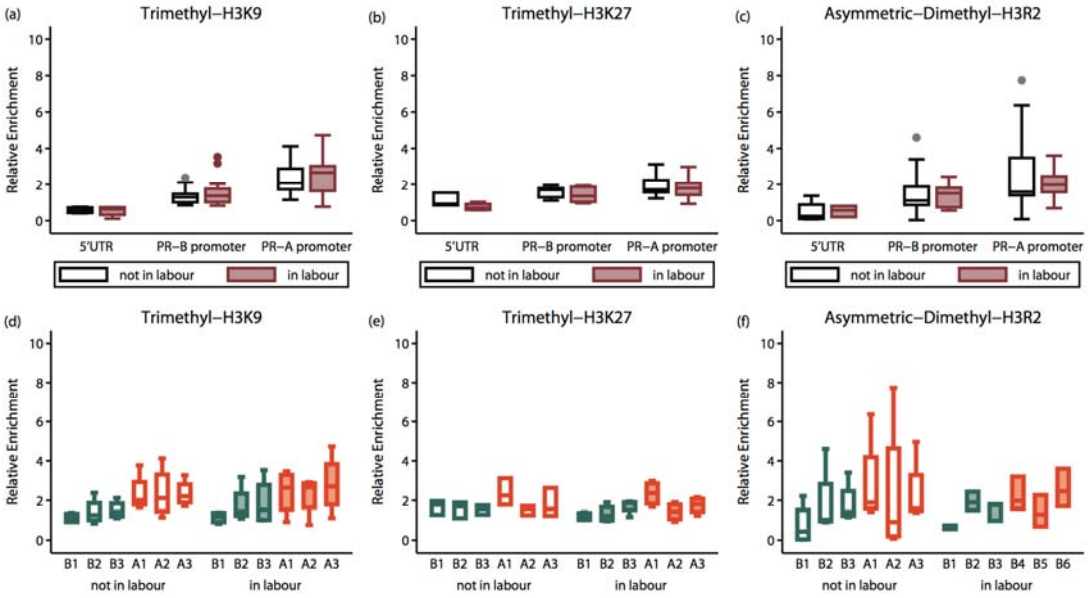

Fig. 4