Increased progesterone receptor A expression in labouring human myometrium is associated with decreased promoter occupancy by the histone demethylase JARID1A


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Abstract: Progesterone regulates female reproductive function predominantly through two nuclear progesterone receptors (PRs), PR-A and PR-B. During human parturition myometrial PR expression is altered to favour PR-A, which activates pro-labour genes. We have previously identified histone H3 lysine 4 trimethylation (H3K4me3) as an activator of myometrial PR-A expression at labour. To further elucidate the mechanisms regulating PR isoform expression in the human uterus at labour, we have (i) determined the methylation profile of the cytosine-guanine dinucleotides (CpG) island in the promoter region of the PR gene and (ii) identified the histone-modifying enzymes that target the H3K4me3 mark at the PR promoters in term and preterm human myometrial tissues obtained before and after labour onset. Bisulphite sequencing showed that despite overall low levels of PR CpG island methylation, there was a significant decrease in methylated CpGs with labour in both preterm (P<0.05) and term (P<0.01) groups downstream of the PR-B transcription start site. This methylation change was not associated with altered PR-B expression, but may contribute to the increase in PR-A expression with labour. Chromatin immunoprecipitation revealed that the histone methyltransferase, SET and MYND domain-containing protein 3 (SMYD3), bound to the PR gene at significantly higher levels at the PR-A promoter compared with the PR-B promoter (P<0.010), with no labour-associated changes observed. The H3K4 demethylase, Jumonji AT-rich interactive domain 1A (JARID1A), also bound to the PR-A, but not to the PR-B promoter prior to term labour, and decreased significantly at the onset of labour (P=0.014), providing a mechanism for the previously reported increase in H3K4me3 level and PR-A expression with labour. Our studies suggest that epigenetic changes mediated by JARID1A, SMYD3 and DNA methylation may be responsible, at least in part, for the functional progesterone withdrawal that precipitates human labour.

Key words: parturition / myometrium / progesterone receptors / epigenetics

Introduction

Progesterone maintains pregnancy by blocking myometrial contraction, and its blocking effect is withdrawn at term (Csapo, 1956). In most mammalian species, progesterone withdrawal is preceded by a precipitous drop in circulating maternal progesterone levels prior to the onset of labour. Humans and higher primates, however, progress to labour without a decrease of progesterone concentration in the maternal plasma. Nonetheless, the administration of progesterone receptor (PR) antagonist such as RU486 increases myometrial contractility and induces labour (Avrech et al., 1991; Chwalisz et al., 1995; Neilson, 2000). Therefore, it has been proposed that progesterone withdrawal in humans at labour is ‘functional’ such that circulating progesterone is unable to exert its activity to maintain myometrial quiescence. One proposed mechanism for this functional withdrawal is through changes in the levels of nuclear PR isoforms, which lead to a decrease in uterine responsiveness to the relaxatory effects of progesterone (Mesiano, 2004).

There are two major forms of the nuclear PR in humans, PR-A and PR-B, which are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors. Upon progesterone binding, the receptors undergo conformational changes and interact with progesterone-responsive genes. PR-A is a truncated version of PR-B, lacking the first 164 N-terminal amino acids. Both isoforms are products of messenger RNAs (mRNAs) transcribed from a single
gene that is controlled by two promoters (Kastner et al., 1990; Gian-grande and McDonnell, 1999). PR-A and PR-B are activated upon pro-gesterone binding, and regulate the transcription of different gene cohorts. Studies in progesterone target cells have demonstrated that PR-B is the principal activator of progesterone-responsive genes, whereas PR-A represses the transcriptional activity of PR-B (Tung et al., 1993; Vegeto et al., 1993). Thus, the capacity for progesterone to affect gene expression depends not only on its circulating levels, but also on the relative level of PR-A to PR-B in target cells. During human pregnancy myometrial PR-A concentrations are relatively low compared with PR-B, and the PR-B dominant state favours relax-ation and cervical closure by suppressing contraction-associated pro-teins (CAPs) and pro-inflammatory genes while stimulating anti-inflammatory gene expression (Tan et al., 2012). At term parturition, myometrial PR-A expression is increased relative to PR-B and the increased PR-A:PR-B ratio promotes labour by inhibiting the anti-inflammatory actions of PR-B, while stimulating the expression of pro-inflammatory and CAP genes (Vegeto et al., 1993; Richer et al., 2002; Merlino et al., 2007; Tan et al., 2012). We have previously shown that prostaglandin PGF2α selectively increased PR-A, but not PR-B expression in a pregnant human myometrial cell line (PHM1-31), leading us to propose that increased PGF2α production by the gestational tissues induces functional progesterone withdrawal by increasing myometrial PR-A expression (Madsen et al., 2004). This model would explain how administration of PGF2α induces the full parturition cascade. However, the mechanism(s) by which the differential expression of the PR isoforms are regulated by prostaglandins or other factors influencing myometrial contractility is/are yet to be elucidated.

Studies with progesterone-responsive cancer cells have suggested that the chromatin region comprising the PR gene is subject to modifications that influence the activity of the PR-A and PR-B promoters. These modifications may include cytosine methylation of DNA at cyto-sine-guanine dinucleotides (CpG) and post-translational histone mod-ifications. The PR gene has a CpG island (>50% GC content) in its 5′ regulatory regions and there is evidence that CpG methylation at these locations is associated with isoform-specific expression in cancer cells (Kastner et al., 1990; Lapidus et al., 1996; Ferguson et al., 1998; Sasaki et al., 2001; Liu et al., 2003; Xiong et al., 2005; Wu et al., 2006; Gaudet et al., 2009). To date, no studies have determined the methylation status of these CpG islands in pregnant human myometrium, or assessed the possibility that the differential expression of myometrial PR isoforms prior to the onset of labour may be due to isoform-specific gene methylation at the promoter regions. We have previously reported that trimethylation of histone H3 lysine 4 (H3K4me3) is increased at both PR promoters during active labour, and this increase is significantly higher at the PR-A promoter compared with PR-B (Chai et al., 2012). H3K4 trimethylation marks the transcription start sites (TSS) of actively transcribed genes, and is associated with RNA polymerase II (Pol-II) binding (Bernstein et al., 2002; Schneider et al., 2004; Schubeler et al., 2004; Kim et al., 2005; Miao and Natarajan, 2005; Eisenberg and Shilatifard, 2010). In addition, we detected high levels of histone acetylation, another modification associated with transcrip-tion, at the PR gene in term myometrial samples with significantly higher enrichment at the PR-A than at the PR-B promoter (Chai et al., 2012). These data indicate that both PR promoters are in a transcriptionally active chromatin state in term myometrium with the PR-A promoter primed by higher H3K4me3 and histone acetylation levels for further activation at labour.

In breast cancer cells PR gene transcription is controlled by estrogen via the nuclear receptor ESRI, a process that involves several histone-modifying enzymes as co-factors (Hervouet et al., 2013). These include the SET and MYND domain-containing protein 3 (SMYD3), which acts as a co-activator of ER-mediated transcription methylating H3K4 at the estrogen responsive element (ERE) in the promoter regions of target genes. Down-regulation of SMYD3 expression resulted in the reduction of H3K4 methylation and the repression of ER target genes demonstrating the functional role of SMYD3 in ER-dependent gene transcription (Hamamoto et al., 2006; Kim et al., 2009). Further-more, H3K4 methylation can be reduced by the Jumonji AT-rich inter-active domain 1A (JARID1A) histone demethylase (HDM), which removes the methyl groups from methylated H3K4 (Klose et al., 2007; Zhou et al., 2010), and has been associated with transcriptional repres-sion (Pasini et al., 2008). Stratmann and Haendler recently demonstrated that JARID1A specifically removes H3K4 trimethylation and is involved in the regulation of PR gene expression in MCF-7 cells (Stratmann and Haendler, 2011). Sequence analysis identified several SMYD3 motifs (5′-CCCTCC-3′ motif) and JARID1A (5′-CCGCC-3′ motif) binding sites located close to the TSS of the PR-A promoter (Kim et al., 2009; Stratmann and Haendler, 2011), illustrated in Fig. 1. This suggests that SMYD3 and JARID1A may interact at the PR-A regulatory region to determine the level of H3K4 methylation and PR-A promoter activity in the human myometrium.

The aim of the present study was to further elucidate the epigenetic mechanisms that contribute to the control of PR isoform expression in the pregnant human myometrium. We examined the methylation of CpG dinucleotides in the 5′ regulatory regions of the PR gene in preterm and term human myometrium using bisulphite sequencing. We also measured the expression and localization of SMYD3 and JARID1A in pregnant myometrium, and determined the binding of these histone modifiers to the PR gene to determine whether the labour-associated up-regulation of H3K4me3 at the PR-A promoter is the result of the differential localization of the two enzymes to the PR-A and PR-B regulatory regions of the gene.

Materials and Methods

SYBR Green PCR Master Mix, Taqman Universal PCR Master Mix and 20× human 18s ribosomal RNA (rRNA) probe/primer mix were from AppliedBio-systems (Carlsbad, USA). Ampicillin, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), GenElute Plasmid Miniprep Kit, Iura broth (LB) medium, salmon sperm DNA and Superscript III First-Strand Synthesis System were purchased from Sigma (St Louis, USA). Complete Mini Protease Inhibitor Cocktail tablets were from Roche Applied Science (Indianapolis, USA). EcoRI I, Isosylpropyl β-D-thiogalactopyranoside (IPPG), LB agar, S.O.C. medium and PCR primers were from Invitrogen (Carlsbad, USA). EpiTect Bisulphite Kit, QIAamp DNA Mini Kit, RNaseasy Mini kit and TopTaq Master Mix were obtained from Qiagen (Hilden, Germany). pcGEM-T Easy Vector System II and Wizard SV Gel and PCR Clean-Up System were from Promega (Madison, USA). The TURBO DNA-free kit was purchased from Ambion (Austin, USA). Magna ChiP protein A magnetic beads were from Millipore (Kilsyth, Australia). Anti-SMYD3 (ab16027) was from Abcam (Cambridge, USA). Anti-JARID1A for ChiP (A300-897A) was from Bethyl Laboratories (Montgomery, USA), while anti-JARID1A used in immunohisto-chemistry (NB110-40499) was from Novus Biologicals (Littleton, USA).
Tissue specimens

All specimens were obtained after informed patient consent and the study was approved by the Human Research Ethics Committees of the Hunter New England Health Services and the University of Newcastle. Myometrial tissue was collected from singleton preterm (32–36 gestation weeks) or term (38–40 gestation weeks) Caesarean deliveries in the absence of labour (NIL) or during active labour (L). Tissues from the upper margin of the lower uterine segment were collected on ice, snap frozen in liquid nitrogen and stored at $-80\, ^\circ\, C$. The indications for Caesarean deliveries in the absence of labour were previous Caesarean section, placenta praevia, fetal distress and breech presentation. Preterm Caesarean deliveries have been performed for indications that did not affect myometrial contractility, such as placenta praevia, pre-eclampsia and fetal distress. No signs of labour onset, such as, uterine contractions or cervical changes were evident at the time of surgery. Labouring myometrial tissues were from women undergoing emergency Caesarean delivery for breech presentation, placenta praevia, fetal distress and delayed or failure to progress. No significant differences were detected in the maternal age, gravidity or parity of the patient population used in this study. Women who had an induced labour or who had a history of infection, clinical or histological signs of intrauterine inflammation were excluded from the study.

Total RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted from 30 mg of tissue using the RNeasy Mini kit according to the manufacturer’s protocol. Following extraction, 0.5–1 $\mu g$ of RNA was treated with 1 $\mu l$ DNase I to remove contaminating genomic DNA using the TURBO DNA-free kit. RNA concentration was determined by absorbance at 260 nm, and 0.5–1 $\mu g$ of total RNA was reverse transcribed to cDNA using the Superscript III First-Strand Synthesis System and random hexamer primers. Target cDNAs were amplified in triplicate using an ABI 7500 Sequence Detector. Final volume of each PCR reaction was prepared to 20 $\mu l$ with 10 $\mu l$ of $2\times$ SYBR Green PCR Master Mix for PR total, PR-B, SMYD3 and JARID1A primers, or with 10 $\mu l$ of TaqMan Universal PCR Master Mix for the 18s rRNA (reference gene) primer/probe mix. The PCR primer sequences used in the assays are shown in Table I.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5′–3′)</th>
<th>Location</th>
<th>GeneBank #</th>
</tr>
</thead>
</table>
| PR total | F: GTGGGAGCTGTAAGGTCTTAA  
R: AACGATGCAGTCATTTCTTCA | 1927/2008 | NM000926 |
| PR-B | F: TCGGACACCTTGCTGAAGT  
R: CAGGGCCGAGGGAAGTAG | 867/934 |  |
| SMYD3 | F: GGCAACCTGCAGCTACATCAAG  
R: CTCATAATATCAAAAAAGCGTCTCAGA | 1254/1327 | NM022743.2 |
| JARID1A | F: GTGGGCAATGGGAAACAAA  
R: CCGTTGTCTCATTTCATGTAA | 1112/1209 | NM001042603 |

Figure 1  Schematic illustration of the promoter regions of the PR gene. Top panel: ‘TSS’ indicates transcriptional start site; grey arrow represents the half-ERE site; open arrows represent the SMYD3-binding motifs (5′-CCCTCC-3′); black arrows represent the JARID1A-binding motifs (5′-CCGCC-3′). Second panel: location of the CpG island in the PR gene promoter region. Third panel: locations of PCR amplicons of bisulphite-treated DNA. Fourth panel: locations of PCR amplicons of DNA purified from chromatin immunoprecipitation.

Cellular localization of SMYD3 and JARID1A

Myometrial biopsies collected from term Caesarean deliveries were dissected from connective tissue, fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Paraffin sections (5 $\mu m$) were incubated for 30 min at 60°C, deparaffinized in xylene and rehydrated in graded ethanols. The slides were transferred through tris-buffered saline-Tween (TBS-T), pH 7.5 (20 mM Tris, 150 mM sodium chloride, 0.1% Tween 20) washes and separated into two groups depending on the protein to be detected: (i) SMYD3 or (ii) JARID1A. The slides were heated in an RHS-1 microwave vacuum histoprocessor (Milestone) for 10 min at 110°C in (i) sodium citrate buffer, pH 6.0 (10 mM sodium citric acid, 0.05% Tween 20) for SMYD3 antibody (Abcam) and (ii) Tris–EDTA buffer, pH 9.0 (10 mM Tris, 1 mM EDTA, 0.05% Tween 20) for JARID1A antibody (Novus Biologicals). The slides were then cooled down to room temperature, washed with TBS-T and blocked with 0.1% bovine serum albumin (BSA) for 1 h at room
temperature, washed again with TBS-T and then incubated with the appropriate primary antibody (each at 1:300 diluted in 0.1% BSA) overnight. Rabbit IgG was included in place of primary antibody as a negative control for each condition. Following overnight incubation, the slides were washed and incubated with secondary antibody (Alexa Fluor 488 anti-rabbit antibody from Invitrogen) for 90 min at room temperature (1:2500 diluted in 0.1% BSA), then washed with TBS-T and counterstained with ProLong Gold anti-fade reagent with DAPI (Invitrogen) and mounted with coverslips. Fluorescent images were captured using a Nikon C2 confocal microscope.

**Genomic DNA extraction, bisulphite treatment, end-point PCR, sub-cloning and sequencing**

Genomic DNA was extracted from <25 mg of tissue (preterm NIL, n = 3; preterm L, n = 3; term NIL, n = 4; term L, n = 4) using the QiAmp DNA Mini kit following the manufacturer’s protocol. DNA of 100–500 ng was subjected to bisulphite conversion using the EpiTect Bisulfite kit according to the manufacturer’s instructions. Bisulphite-modified PR promoter regions were amplified in PCR reactions using the primer sequences shown in Table II, illustrated in Fig. 1. The primers for the bisulphite-converted PR-A promoter region (MePRA) were designed using the Sequenom Epidesigner software (http://www.epidesigner.com/index.html), while the primers used for amplifying the bisulphite-modified region downstream of the PR-B promoter (MePRB) have been previously described (Wu et al., 2006). End-point PCR of bisulphite-treated DNA was performed in a 50 µl reaction containing 2.5 µl of 2× TopTaq Master Mix, 200 or 400 nM primer, template and water. A non-converted DNA template was used as a negative control in the PCR reactions. PCR products were visualized in a 1.5% agarose gel run in TBE buffer and bands of the expected size were purified using the Wizard SV Gel and PCR Clean-Up System. Purified PCR products were cloned into pGEM-T easy vector and transformed into competent Escherichia coli JM109 cells. Transformated products were plated on LB agar plates containing 100 µg/ml of ampicillin, 80 µg/ml of X-Gal and 0.5 mM of IPTG. Ten individual colonies from each transformed product were selected using the blue-white screening technique, and cultured overnight in LB medium containing 100 µg/ml of ampicillin. Plasmids were purified from cultured colonies using the GenElute Plasmid Miniprep Kit as per the manufacturer’s instructions, and inserts were verified by EcoR I digestion. Plasmids with the correct insert size were sequenced in both directions by the Australian Genome Research Facility and sequences were analysed using the BiQ Analyzer software.

**ChIP and real-time PCR**

ChIP was performed as previously described (Chai et al., 2012). Briefly, 500 µl of sonicated chromatin was incubated overnight at 4°C with antibodies against rabbit IgG (as control immunoprecipitation), SMYD3 or JARID1A. Immune complexes were precipitated at 4°C for 1.5 h with 10% Magna beads and 2 µg of salmon sperm DNA. The beads were washed sequentially, immune complexes were eluted and cross links reversed. The recovered DNA was purified using the Wizard SV Gel and PCR Clean-Up System, and input DNA quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Purified DNA samples were diluted (1:5000 for input and 1:5 for IP samples) and 5 µl of each was used in real-time PCR. Each PCR reaction was prepared to a total volume of 20 µl, containing 2× SYBR Green PCR Master Mix, primers, template and water. Primers used were as described previously (Chai et al., 2012), and their locations illustrated in Fig. 1. Real-time PCR was performed using an ABI 7500 Sequence Detector.

**Data analysis**

All mRNA abundance data were expressed relative to the 18s rRNA reference gene, and relative mRNA abundance was calculated using the formula $2^{-\Delta C_t}$, where Ct is the mean threshold cycles of PCR done in triplicate and delta Ct ($\Delta C_t$) is the Ct difference between the reference and target mRNAs (Schmittgen and Livak, 2008). The relative abundance of PR-A mRNA was estimated by subtracting the relative abundance of PR-B mRNA from that of PR total. Methylation levels from bisulphite sequencing data were calculated by aligning sequencing reads to the positions in the PR gene using the BiQ Analyzer software, and the percentage of Cs and Ts determined. Relative enrichment of a sequence site by ChIP was calculated using the equation $2^\Delta C_t$ (CtIP – Ctinput) and expressed as a percentage of input normalized to a downstream control site.

**Statistical analysis**

STATA 11.0 software (Statacorp) was used for all statistical analyses in this study. Differences between the CpG methylation of PR-A and PR-B in each clinical group were assessed using the Student’s t-test. Differences of relative mRNA abundance between clinical groups were determined after log-transformation using ANOVA (one-way). 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 associations between ChIP-enriched DNA sequences and labour status. mRNA expression data were presented in box plots. Bisulphite sequencing and ChIP data were expressed as mean ± SD. Differences were considered statistically significant if $P < 0.05$.

**Results**

**mRNA expression of PR isoforms in pregnant human myometrium**

We determined the expression of PR-A and PR-B in myometrial tissues (preterm NIL, n = 3; preterm L, n = 3; term NIL, n = 5; term L, n = 6) by measuring their relative mRNA levels, using 18s rRNA as a reference gene. Consistent with previous findings (Chai et al., 2012), we found lower expression of PR-B mRNAs in the preterm than in the term groups ($P < 0.002$). PR-B mRNA abundance showed no labour-associated changes with preterm or term labour (Fig. 2A). PR-A mRNA abundance was similar in the preterm and term NIL groups (Fig. 2B):

**Table II Bisulphite primer sequences.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5′–3′)</th>
<th>Location</th>
<th>GeneBank #</th>
</tr>
</thead>
<tbody>
<tr>
<td>MePRA</td>
<td>F: GAGAGTTAAGGTTAGGAGTTGATTA&lt;br&gt;R: CTACCTCCAAACCCCTTTATAACCT</td>
<td>552/1039</td>
<td>NM000926</td>
</tr>
<tr>
<td>MePRB</td>
<td>F: AGTATGAGGTTAGTGAAGATT&lt;br&gt;R: TCACAACTCCAAACCTTTATAACCT</td>
<td>170/390</td>
<td></td>
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however, it was significantly higher in the term L group \((P<0.005)\) compared with the term NIL group, indicating that the PR-A:PR-B expression ratio increased with term labour in agreement with previous findings \((\text{Merlino et al., 2007})\).

**SMYD3 and JARID1A mRNA expression and localization in pregnant human myometrium**

To date, there have been no reports on the expression of the histone methyltransferase SMYD3 or the HDM JARID1A in human myometrium. Therefore, we measured SMYD3 and JARID1A mRNA levels in myometrial samples by qRT–PCR, and detected both mRNAs in all gestational groups. Both histone-modifying enzymes were expressed at lower levels in the preterm than in the term samples \((\text{SMYD3, } P<0.000; \text{ JARID1A, } P<0.000)\), with no labour-associated changes observed \((\text{Fig. 2C and D})\). In addition, fluorescence immunohistochemical analyses showed strong SMYD3 staining mainly in the nuclei of labouring myometrium tissue \((\text{Fig. 3C})\), whereas JARID1A localization was predominantly peri-nuclear in the same tissue \((\text{Fig. 3G})\).

**Methylation of the PR CpG island in pregnant human myometrium**

To assess whether the changes in myometrial PR mRNA expression during pregnancy and labour involve changes in PR gene methylation, we determined the methylation status of the CpG island within the PR gene by bisulphite sequencing. The location of the CpG island and the two regions analysed for methylation are indicated in Fig. 1. The region downstream of the PR-B TSS has been studied in cancer cells and its methylation was found to repress PR-B expression \((\text{Sasaki et al., 2002; Wu et al., 2006; Mc Cormack et al., 2008})\). We designed primers to analyse an additional region containing 30 CpG sites surrounding the PR-A TSS. There were no significant differences in per cent methylation between the two regions in any of the clinical groups \((\text{Fig. 4})\); therefore pooled methylation values from both regions were used as an overall measure of the PR CpG island methylation level. Overall, the PR CpG island was hypomethylated \((<10\% \text{ CpG methylation})\) in all clinical groups \((\text{Fig. 5 and Table III})\), and no change in the methylation level of the CpG island was detected in the region surrounding the PR-A TSS with either gestational age or labour. However, despite the low levels of methylation, we found a significant decrease in the number of methylated CpGs in the region downstream of the PR-B TSS with both preterm \((P<0.05)\) and term labour \((P<0.01, \text{Fig. 4})\).

**SMYD3 occupancy at the PR promoters in term human myometrium**

SMYD3 is a methyltransferase capable of establishing the gene-activating H3K4me3 mark at TSS as part of the RNA Pol-II complex \((\text{Hamamoto et al., 2004})\). Both the PR-A and the PR-B promoters are marked with
H3K4me3 in the human myometrium, and the modification level increases at the PR-A promoter with labour, when the expression of this PR isoform is enhanced (Chai et al., 2012). SMYD3 binds to the DNA sequence motif 5′-CCCTCC-3′ (Kim et al., 2009), and we have identified six CCCTCC motifs within 400 bp upstream and downstream of the PR-A TSS (Fig. 1). ChIP analyses demonstrated significant binding of SMYD3 in the proximity of the PR-A TSS (P < 0.001, Fig. 6) relative to an upstream control site. Lower, but significant SMYD3 binding was also detected close to the PR-B TSS, where no recognition DNA sequence motifs were found (P < 0.01, Fig. 6). There were no labour-associated differences in SMYD3 occupancy in the PR gene regions analysed.

JARID1A occupancy at the PR promoters in term human myometrium

JARID1A is a demethylase that removes the H3K4me3 mark and renders its target genes transcriptionally inactive. Stratmann and Haendler have demonstrated that JARID1A binds at the PR-A promoter, decreases H3K4 trimethylation and reduces PR expression in the MCF-7 breast cancer cell line, thus effectively regulating PR expression (Stratmann and Haendler, 2011). In line with these observations, the PR-A promoter contains a half-ERE site and three copies of the JARID1A recognition sequence 5′-CCGCC-3′ (Fig. 1), which may localize the enzyme to the proximity of H3K4me3 at the PR-A promoter region. We have previously reported that the H3K4me3 level and PR-A expression changed with labour status (Chai et al., 2012), and, as JARID1A mRNA was highly expressed in term myometrium (Fig. 2D), we determined the level of JARID1A binding at the PR promoters in term labouring (L) and non-labouring (NIL) myometrium. ChIP analyses showed that, regardless of labour status, JARID1A occupancy was absent from the PR-B promoter, as the relative enrichment measured at sites B1, B2 and B3 were not significantly different from that of the 5′ UTR control site (Fig. 7). Conversely, in the absence of labour, JARID1A binding at the PR-A promoter was significantly higher at amplicon sites A1 (P = 0.000) and A2 (P = 0.036), compared with the 5′ UTR control site (Fig. 7), whereas after labour onset, binding declined to background levels (P = 0.014). Thus, term labour was associated with a decrease in H3K4 demethylase occupancy at the PR-A promoter consistent with the increased H3K4 trimethylation and transcriptional activity previously observed (Chai et al., 2012).

Discussion

Progesterone is essential for the maintenance of pregnancy, and the withdrawal of this hormone from the maternal circulation leads to the onset of labour (Csapo, 1956; Mesiano et al., 2002). In women, however, circulating levels of progesterone do not fall before term or preterm labour (Smith, 2007), which has led to the proposal that the removal of progesterone in pregnant women is functional and occurs as a result of the decrease of the progesterone responsiveness of the myometrium. Several mechanisms for a functional progesterone withdrawal have been proposed (Allport et al., 2001; Condon et al., 2003; Renthal et al., 2010; Williams et al., 2012), and strong evidence suggests that one of them involves the elevated expression of the nuclear PR isoform PR-A relative to PR-B in the pregnant human myometrium (Mesiano et al., 2002). It has been postulated that an increase in the PR-A:PR-B ratio results in the
Methylation status of individual CpGs at the 5′ regulatory regions of the PR gene in preterm and term human myometrium, collected in the absence of labour (NIL) or during active labour (L). Preterm NIL, n = 3; preterm L, n = 3; term NIL, n = 4; term L, n = 4. PCR-amplified products from bisulphite-treated myometrial DNA were subcloned into pGEM-T plasmid and 8 to 10 clones from each tissue sample were sequenced. Location of CpG sites is indicated in the top panel. Open circles represent unmethylated CpG and black circles methylated CpG.

Figure 4 Methylation status of individual CpGs at the 5′ regulatory regions of the PR gene in preterm and term human myometrium, collected in the absence of labour (NIL) or during active labour (L). Preterm NIL, n = 3; preterm L, n = 3; term NIL, n = 4; term L, n = 4. PCR-amplified products from bisulphite-treated myometrial DNA were subcloned into pGEM-T plasmid and 8 to 10 clones from each tissue sample were sequenced. Location of CpG sites is indicated in the top panel. Open circles represent unmethylated CpG and black circles methylated CpG.
Epigenetic regulation of PR isoform expression

withdrawal of progesterone action by antagonizing the effects of PR-B (Pieber et al., 2001; Mesiano et al., 2002), the isoform responsible for maintaining pregnancy by inhibiting expression of contraction-associated proteins (Challis et al., 2000) and inflammatory mediator production in the myometrium (Romero et al., 2007). In addition, liganded PR-A and PR-B up-regulate pro-inflammatory and anti-inflammatory genes, respectively, in myometrial cells (Tan et al., 2012). The present study corroborates previous findings (Mesiano et al., 2002) demonstrating an increase in the levels of PR-A in term labouring myometrium (Fig. 2), leading to an increased PR-A:PR-B expression ratio (Bisits et al., 2006). However, Ferguson et al. have demonstrated that removal of DNA methylation by S’aza-2’deoxycytidine (a DNA methyltransferase inhibitor) was not alone sufficient to restore PR gene expression in MDA-MB-231 cells, but that the histone acetyltransferase SRC-1A, responsible for catalyzing histone acetylation, was additionally required for reactivation of the PR gene (Ferguson et al., 1998). We have previously reported that labouring tissue is associated with increased histone acetylation, with a greater increase at the PR-A promoter (Chai et al., 2012), therefore it is possible that the increased histone acetylation interacts with the decrease in DNA methylation to lead to the increased PR expression at the time of labour, with a greater increase in PR-A.

We reported in a previous study (Chai et al., 2012) that a labour-associated increase of H3K4 trimethylation occurred at the PR-A promoter in term myometrium, which correlated with an increase in PR-A mRNA levels. H3K4 trimethylation is considered a gene-activating histone modification (Berger, 2007), which is found almost exclusively at gene promoters, providing docking sites for transcription complexes that catalyse histone acetylation, which in turn leads to increased chromatin accessibility for other transcriptional activators (Eissenberg and Shilatifard, 2010). In line with this concept, we also found higher levels of histone acetylation at the PR-A promoter in myometrium at term (Chai et al., 2012). Thus H3K4 trimethylation appears to be a key step up-regulating PR-A promoter activity at labour, which has been addressed in further experiments.

A dynamic balance of histone methyltransferases and demethylases determines the pattern of histone methylation at gene promoters (Yokoyama et al., 2011; Liu et al., 2012). The lysine-specific H3K4 methyltransferase SYMD3 and demethylase JARID1A are known co-regulators of ESR1 target genes, and there is evidence of their involvement in PR gene transcription (Kim et al., 2009; Stratmann and Haendler, 2011). Sequence analyses identified potential binding motifs for both SYMD3 and JARID1A in the PR-A promoter (Fig. 1), including a half-ERE site, which is a common binding site for both histone-modifying
enzymes. As estrogens are thought to play a role in the initiation of labour (Smith, 2007) and PR is an estrogen target gene, histone-modifying enzymes may provide a possible mechanistic link by which estrogen dominance occurs in conjunction with functional progesterone withdrawal in the labouring myometrium (Mesiano et al., 2002). In agreement with this, we have detected both SYMD3 and JARID1A expression in the myometrium, which significantly increased at term (Fig. 2).

We next examined the association of the two histone-modifying enzymes with the PR gene promoters. ChIP analyses detected significant binding of SMYD3 near the TSS of both PR-B (site B3) and PR-A (sites A1, A2 and A3) (Fig. 6). We found significantly higher enrichment of SMYD3 at the PR-A promoter, compared with the PR-B promoter, but no labour-associated differences. Strong binding of SMYD3 is in line with the hypermethylation of H3K4 at the PR-A promoter reported previously (Chai et al., 2012), but the lack of a labour-associated increase suggested that increased promoter occupancy by this enzyme was not responsible for the enhanced H3K4 trimethylation at term labour.

We performed fluorescence immunohistochemistry to localize SMYD3 and JARID1A in the pregnant human myometrium. The subcellular distribution of SMYD3 has been reported to be both cytoplasmic and nuclear in cancer cells (Hamamoto et al., 2006; Yamamoto et al., 2011; Liu et al., 2013), being mainly cytoplasmic when cells are arrested at G0/G1, but accumulating in the nucleus in the S phase and G2/M (Hamamoto et al., 2006). We found that in term labouring myometrial

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**Figure 6** Enrichment of histone methyltransferase SMYD3 at the PR gene in term human myometrial samples collected in the absence of labour (White bars n = 4) or after labour onset (black bars n = 4). Data are presented as the mean ± SD. Significance levels are denoted by the letters on top of the bars. Bars marked with different letters are significantly different, while bars with at least one identical letter show no significant difference.

**Figure 7** Enrichment of HDM JARID1A at the PR gene in term human myometrial samples collected in the absence of labour (White bars n = 3) or after labour onset (Black bars n = 3). Data are presented as the mean ± SD. Significance levels are denoted by the letters on top of the bars, and letters shared in common indicate no significant difference, while bars marked with different letters are significantly different.
tissue, SMYD3 was present mainly in the nuclei (Fig. 3) consistent with the ChIP results showing SMYD3 binding to the PR promoters at term (Chai et al., 2012).

The H3K4me3 demethylase JARID1A preferentially targets genes that possess the CCGCCC-binding motif (Patsialou et al., 2005; Tu et al., 2008) and the half-ERE site to which Pol-II also binds (Stratmann and Haendler, 2011). This is consistent with our results showing significant JARID1A binding at sites A1 and A2 of the PR-A promoter region, which contains two copies of this sequence (Fig. 1). Importantly, the occupancy of this region by JARID1A was markedly reduced during active labour (Fig. 7), suggesting that H3K4 demethylase activity is diminished at the PR-A promoter in the myometrium at labour.

Early cellular localization studies by Kim et al. (1994) reported that JARID1A was expressed exclusively as a nuclear protein in tumour cell lines. In contrast, another member of the Jumonji C domain-containing HDM, JMJD1A, has been shown to be localized in both the cytoplasm and the nucleus in a large proportion of normal tissues and clinical tumour samples, and depending on the circumstances, localization shifted to the nucleus or to the cytoplasm (Yang et al., 2009). Our results show that in term myometrium, JARID1A was predominantly peri-nuclear (Fig. 3), which is in line with the observation that JARID1A binding to the PR-A promoter decreased significantly with the onset of labour (Fig. 7).

Our findings suggest that the histone methyltransferase SMYD3 may be responsible for the establishment of the H3K4me3 mark at the PR promoters in term myometrium. The presence of the demethylase JARID1A at the PR-A promoter indicates that the H3K4me3 mark is dynamic in this region. The loss of the demethylase, but not the methyltransferase, at the PR-A promoter in labour, would result in unopposed histone methylation by SMYD3 and elevated H3K4 trimethylation, histone acetylation and an increase in PR-A expression. It is not clear what controls JARID1A targeting to the PR-A promoter, since the expression of the enzyme does not change with labour, but this may well be estrogen driven.

We observed lower expression of PR-B in the preterm than in the term non-labouring myometrium samples (Fig. 2A), equal amounts of PR-A mRNA in the preterm and term groups (Fig. 2B) and found no difference in PR isoform mRNA expression associated with preterm labour (Fig. 2A and 2B). This is in variance with the PR protein data reported by Merlino et al. (2007), who found an increase of PR-A protein abundance at term compared with preterm in the absence of labour and no change of PR-B protein between the preterm and term non-labouring samples. Our preterm samples, however, were collected at a later gestation (32–36 weeks) than those analysed by Merlino et al. (24–33 weeks), and further PR isoform mRNA and protein analyses of carefully timed preterm samples may resolve this disparity in data.

In summary, we have uncovered an epigenetic mechanism that may be responsible for the elevated PR-A:PR-B expression ratio in term myometrium during functional progesterone withdrawal. The key component of this process is the loss of the H3K4me3-selective demethylase JARID1A from the PR-A promoter. It is reasonable to surmise that this leads to increased H3K4 trimethylation and H3 acetylation at the PR-A promoter region enhancing its transcriptional activity and eventually increasing the PR-A:PR-B ratio. Blocking SMYD3 activity and/or JARID1A dissociation from the PR-A promoter has the potential of altering myometrial PR isoform expression to maintain myometrial quiescence by enhancing progesterone responsiveness.

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Authors’ roles

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

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Conflict of interest

None declared.

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