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ORIGINAL RESEARCH

The study of progesterone action in human myometrial explants

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STUDY HYPOTHESIS: Myometrial explants represent a superior model compared with cell culture models for the study of human myometrial progesterone (P4) signalling in parturition.

STUDY FINDING: Gene expression analysis showed myometrial explants closely resemble the *in vivo* condition and the anti-inflammatory action of P4 is not lost with labour onset.

WHAT IS KNOWN ALREADY: Circulating P4 levels decline before the onset of parturition in most animals, but not in humans. This has led to the suggestion that there is a functional withdrawal of P4 action at the myometrial level prior to labour onset. However, to date, no evidence of a loss of P4 function has been provided, with studies hampered by a lack of a physiologically relevant model.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Myometrial biopsies obtained at Caesarean section were dissected into explants after a portion was immediately snap frozen ($t = 0$). Microarray analysis was used to compare gene expression of $t = 0$ with paired (i) explants, (ii) passage 4 myometrial cell cultures or (iii) the hTERT myometrial cell line. Western blotting and chemokine/cytokine assays were used to study P4 signalling in myometrial explants.

MAIN RESULTS AND THE ROLE OF CHANCE: Gene expression comparison of $t = 0$ to the three models demonstrated that explants more closely resemble the *in vivo* status. At the protein level, explants maintain both P4 receptor (PR) and glucocorticoid receptor (GR) levels versus $t = 0$ whereas cells only maintain GR levels. Additionally, treatment with 1 μM P4 led to a reduction in interleukin-1 (IL-1) β-driven cyclooxygenase-2 in explants but not in cells. P4 signalling in explants was PR-mediated and associated with a repression of p65 and c-Jun phosphorylation. Furthermore, the anti-inflammatory action of P4 was maintained after labour onset.

LIMITATIONS/REASONS FOR CAUTION: There is evidence of basal inflammation in the myometrial explant model.

WIDER IMPLICATIONS OF THE FINDINGS: Myometrial explants constitute a novel model to study P4 signalling in the myometrium and can be used to further elucidate the mechanisms of P4 action in human labour.

LARGE SCALE DATA: Data deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gvmpggkurbgxfqf&acc=GSE77830.

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Key words: progesterone withdrawal / nuclear receptors / myometrial explants / smooth muscle / parturition / myometrial cells / hTERT

Introduction

The seminal papers of Csapo *et al.* and Frydman *et al.* demonstrated that progesterone (P4) is essential for human pregnancy, the former showing that P4 is responsible for the maintenance of early pregnancy and that its removal results in miscarriage (Csapo *et al.*, 1973) and the latter, that blocking P4 can result in the onset of labour (Frydman *et al.*, 1991). However, while in most animals labour follows a precipitous fall in peripheral P4 levels, no such fall occurs in humans and non-human primates. This has led to the concept of a myometrial functional P4 withdrawal and several theories have been proposed to explain it. The most widely accepted is that there is a change in the balance of expression of the

P4 receptor (PR), which is comprised of two main isoforms: PR-B, which mediates the effects of P4, and PR-A, which antagonises PR-B-mediated P4 signalling, but is also transcriptionally active in its own right. An increase in the PR-A:PR-B ratio at the time of labour onset has been demonstrated in myometrial samples obtained at the time of labour at the mRNA and protein level (Mesiano et al., 2002; Merlino et al., 2007). Furthermore, in PR-A-dominant myometrial cells, P4 enhances pro-inflammatory gene expression (Tan et al., 2012). Another theory suggests that uterine quiescence is maintained throughout pregnancy by a PR-mediated inhibition of the actions of the pro-inflammatory transcription factor NF-κB (Kalkhoven et al., 1996), possibly via the NF-κB inhibitor IκBα (Hardy et al., 2006), but that with the onset of labour, inflammation-induced NF-κB represses PR action bringing about a uterine switch to a contractile phenotype (Allport et al., 2011). A third theory suggests that changes to PR co-regulator expression may cause labour onset (Condon et al., 2003). All three mechanisms have the common end result of a loss of myometrial sensitivity to P4 action and the onset of labour. Efforts to use a mouse model to study this question have been limited, as PR-A knock-out mice are infertile, while no apparent change in ovarian and uterine function was observed in PR-B knock-out mice (Mulac-Jericevic et al., 2000, 2003).

Although increasingly controversial (O'Brien et al., 2007; Norman et al., 2016), supplementation with P4 or 17 alpha-hydroxyprogesterone caproate has been shown, at least in some studies, to reduce the risk of preterm labour in high-risk singleton pregnancies (da Fonseca et al., 2003; Meis et al., 2003). The mechanism involved is uncertain, but since labour is widely accepted to be an inflammatory event (Bollapragada et al., 2009), it is assumed that P4 acts to maintain pregnancy by repressing inflammation. On that basis, the ability of P4 to repress inflammation-induced cyclooxygenase-2 (COX-2) expression has been widely used as a model of P4 action. COX-2 expression is driven by the inflammatory transcription factors NF-κB and activator protein-1 (AP-1) (Soloff et al., 2004; Khanjani et al., 2011, 2012; Lim and Lappas, 2014). Several studies have investigated whether P4 inhibits NF-κB and AP-1 activation to repress COX-2 expression, but these have typically been performed in primary cell cultures or cell lines and have involved the over-expression of PR, NF-κB and/or AP-1 (Bamberger et al., 1996; Kalkhoven et al., 1996; Hardy et al., 2006). Primary cultures of uterine smooth muscle cells have been shown to maintain structural and functional characteristics (Lee et al., 2012; Mosher et al., 2013), but are possibly not an optimal model for the study of P4 action as high doses of P4 (10 μM) are required to bring about a reduction in IL-1β-driven COX-2 (Lei et al., 2012). Previous work by our group using this model has demonstrated that P4 signals via the glucocorticoid receptor (GR) to reduce COX-2 (Lei et al., 2012) via MAPK phosphatase-1 (MKP-1) (Lei et al., 2015a); however, this may be because PR levels are lower in primary cells compared with tissue.

To overcome the limitations of myometrial cell culture, we have developed an explant-based model for the *ex vivo* study of myometrial function. We compared gene expression in tissue snap frozen at the time of Caesarean section ($t = 0$) to myometrial explants, passage 4 myometrial cells and the hTERT myometrial cell line. We subsequently compared PR protein levels in $t = 0$, myometrial explants and passage 4 myometrial cells before using the explant model to study P4 action in myometrial samples before and after the onset of labour to test the hypothesis that a functional withdrawal of P4 action occurs with the onset of labour.

Materials and Methods

Ethical approval

The Brompton and Harefield Research Ethics Committee approved this project.

Myometrial biopsies

Myometrial biopsies were obtained from women at term (≥37 weeks) following informed consent at the time of planned or emergency Caesarean section. Women with multiple pregnancy, gestational diabetes mellitus, pre-eclampsia and obstetric cholestasis were excluded. In addition, labouring women were recruited to the study if labouring spontaneously and requiring an emergency Caesarean section due to fetal distress or a breech presentation. Cervical dilatation was used to categorise labour into early (≤3 cm) or established (>3 cm). Biopsies were collected into sterile universal bottles containing phosphate-buffered saline (PBS) and were processed immediately. Samples used were as follows: term no-labour (TNL): $n = 35$; term early labour (TEaL): $n = 8$ and term established labour (TEsL): $n = 8$.

Explant culture

Biopsies were dissected into 3 × 3 × 3 mm^3 pieces (explants) with any obvious vasculature excised. They were placed in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich Ltd, Dorset, UK) supplemented with penicillin–streptomycin (Sigma-Aldrich Ltd) or immediately snap-frozen in liquid nitrogen ($t = 0$). Depending on the experimental protocol, explants were either untreated or immediately treated for 6 h with vehicle control (ethanol ± DMSO), progesterone (100, 500 nM, 1, 5 and 10 μM; Sigma-Aldrich Ltd), mifepristone (RU486, 1 μM; Sigma-Aldrich Ltd) or onapristone (ZK299, 1 μM; Arno Therapeutics, Flemington, NJ, USA). They were subsequently treated for a further 24 h with interleukin-1 (IL-1)β (1, 10, 20, 50 and 100 ng/ml; Sigma-Aldrich Ltd), at which point all tissues were snap frozen in liquid nitrogen and stored at −80°C. The media in which explants were cultured were also stored at −80°C.

Cell culture

Biopsies were digested in a mixture of collagenases as previously described (Sooranna et al., 2004) and passaged by trypsinization in 0.25% (w/v) trypsin containing 0.02% (w/v) EDTA (Sigma-Aldrich Ltd) when confluent. Once confluent at passage 4, cells were incubated overnight in 1% (v/v) charcoal and dextran-stripped fetal calf serum (1% DCC-FCS) supplemented with penicillin–streptomycin. They were then cultured and treated using the same protocol as explant cultures and were placed at −80°C once the experiment was completed. Supernatants were stored at −80°C.

The myometrial hTERT cell line was cultured in the same conditions as myometrial cells with overnight incubation in 1% DCC-FCS once cells reached confluence. Once thawed, cells were not passaged beyond passage 5.

RNA extraction

Total RNA was extracted using a Trizol® Plus RNA Purification kit (Thermo Fisher Scientific, Ambion, Abgene Ltd, West Sussex, UK) with on-column DNase treatment prior to elution, all as per the manufacturer's protocol. Bead homogenization in Precellys® tubes (Stretton Scientific Ltd, Derbyshire, UK) was used for tissue lysis with two 20 s cycles at 5000 rpm; cells were lysed directly with Trizol® added to the culture plate. The concentration and purity of RNA was determined by spectrophotometry and integrity

was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA).

Microarray analysis

Whole-genome transcriptome analysis was conducted by hybridizing six biological samples of total RNA per condition to Affymetrix Human Gene 2.1 ST Arrays Strips (Affymetrix, Santa Clara, CA, USA). A minimum RIN score of 8 was used as cut-off for inclusion in the microarray analysis. All steps were conducted at the Nottingham Arabidopsis Stock Centre. Gene expression data were analysed using Partek Genomics Suite 6.6 software (Partek Incorporated, St Louis, MO, USA). The raw CEL files were normalized using the RMA background correction with quantile normalization, log base 2 transformation and mean probe-set summarization with adjustment for GC content. Differentially expressed genes (DEG) were identified by a two-way ANOVA, and *P*-values were adjusted using the FDR (false-discovery rate) method to correct for multiple comparisons. DEG were considered significant if *P*-value was ≤ 0.05 at a fold change (FC) of >2 with FDR <0.05.

Quantitative RT–PCR

Following quantification, 1 μg RNA was reverse transcribed with oligo dT random primers using MuLV reverse transcriptase (Life Technologies Ltd, Paisley, UK). Primer sets were designed and obtained from Invitrogen (Table I). Quantitative PCR was performed using SYBR Green (Roche Diagnostics Ltd, West Sussex, UK) using the previously described cycling protocol (Lei *et al.*, 2015a) and amplicon yield was monitored during cycling in a Rotor-Gene Sequence Detector (Qiagen Ltd, West Sussex, UK). The abundance of mRNA for sequences of interest was expressed relative to the constitutively expressed GAPDH and 18S rRNA. Here we present the data normalized to GAPDH.

Protein extraction and western blotting

Protein was extracted from explants using bead homogenization in pre-cooled Precellys® tubes (Stretton Scientific Ltd) containing lysis buffer (New England Biolabs, Hertfordshire, UK) supplemented with protease (Roche Diagnostics Ltd) and phosphatase inhibitors (Thermo Fisher Scientific, Abgene Ltd, Epsom, UK). Tissues were immediately homogenized by mechanical disruption using two 20 s cycles at 5000 rpm. Protein from cells was extracted via direct lysis using the same lysis buffer mixture as for explants. The supernatant was separated from tissue debris by centrifugation at 15 890*g* for 10 min at 4°C. Protein concentrations were determined by DC protein assay (Bio-Rad laboratories Ltd, Hertfordshire, UK) and bovine serum albumin (Sigma-Aldrich Ltd) was used for reference standards.

Samples in NuPAGE® LDS Sample Buffer (Life Technologies Ltd) were denatured at 75°C for 10 min and 20 μg of total protein for each sample was electrophoresed through a 4–20% polyacrylamide gel (Bio-Rad laboratories Ltd). Transfer was carried out onto a polyvinylidene fluoride membrane (Bio-Rad laboratories Ltd) using the Trans-Blot® Turbo Transfer system (Bio-Rad laboratories Ltd), followed by blocking in 5% non-fat dried milk powder (AppliChem GmbH, Germany) dissolved in 0.1% Tween–Tris buffered saline (TBS–T) for 1 h at room temperature. The membrane was incubated overnight at 4°C with primary antibody followed by incubation for 2 h at room temperature with secondary antibody (Table II). Clarity Western ECL substrate (Bio-Rad laboratories Ltd) was used for detection. Protein band size was determined using Precision Plus Protein Standards ladder (Bio-Rad laboratories Ltd). All protein abundance data were expressed relative to the amount of constitutively expressed GAPDH after 1 h incubation at room temperature (Table II).

Chemokine/cytokine assays

Human Bio-Plex© Pro™ chemokine/cytokine assays (Bio-Rad laboratories Ltd) were used to measure the concentrations of IL-1α, IL-1β, IL-6, IL-8, C–C motif chemokine 2 (CCL2), CCL5, CCL11, CCL20, C–X–C motif chemokine 1 (CXCL1), CXCL2, intercellular adhesion molecule 1 (ICAM) and leukaemia inhibitory factor (LIF) in explant culture media. These were performed according to the manufacturer's instructions and read using a Bio-Plex© 200 reader and Bio-Plex Manager© v6.1 software (Bio-Rad laboratories Ltd). Data were normalized to tissue weights.

Table I Primer sets used for quantitative RT–PCR.

Name	Forward (F) and Reverse (R) primer sequence (5′-3′)	Genbank/ EMBL Accession no.	Product size (bp)
GAPDH	F: tgatgacatcaagaaggtggtgaag R: tccttggaggccatgtaggccat	BC014085	240
18S rRNA	F: aaacggctaccacatccaag R: cctccaatggatcctcgtta	X03205.1	155
PTGS2	F: tgtgcaacacttgagtggct R: actttctgtactgcgggtgg	AY151286	297
IL8	F: gccttcctgattttgcagc R: cgcagtgtggtccactctca	NM_000584	150
OXTR	F: agaagcactcgcgcctctt R: aggtgatgtcccacagcaact	NM_000916	102
PGR	F: agcccacaatacagcttcgag R: tttcgacctccaaggaccat	NM_000926	253
IL1B	F: gctgaggaagatgctggttc R: tccatatcctgtccctggag	NM_000576	240
NR3C1	F: cttccagaaccatggtagcc R: tacgaaactccacccaaagg	NM_002425	166
TPM1	F: ccacgctctcaacgatatga R: cagtgtgtgcctggctctaa	NM_000366	215
TNFAIP3	F: aagggtgtctgagcaggaga R: tgcttggtaggagaggagga	NM_001270507	163
IL6	F: agtgaggaacaagccagagc R: gaggtgcccatgctacattt	NM_000600	246
ACTA2	F: ttcaatgtcccagccatgta R: gaaggaatagccacgctcag	NM_001141945	222
MYLK	F: ggccacgatgaaacagattt R: ggcatgattgatgcacattt	NM_005965	172
PTGER3	F: cgccatgtcttcatcacatc R: atgtgatcctggcagaaagg	NM_000957	199
GJA1	F: aattcagacaaggcccacag R: catggcttgattccctgact	NM_000165	214
ESR1	F: tccaactgcatttcctttcc R: ttggaacatggcagcattta	NM_000125	201
IL1A	F: aatgacgccctcaatcaaag R: tgggtatctcaggcatctcc	NM_000575.4	226

Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Normality was determined via a Kolmogorov–Smirnov test for up to six replicates or a Shapiro Wilks test for more than six replicates. Normally distributed data were subsequently analysed using a paired *t* test for the comparison of two groups or an ANOVA followed by Bonferroni's multiple comparison test post hoc testing for three groups or more. Data that were not normally distributed were analysed using a Wilcoxon matched pairs test or a Kruskal–Wallis followed by Dunn's

Table II Primary and secondary antibody table.

Peptide/protein target	Name of antibody	Manufacturer, Catalog Number	Species raised in; monoclonal or polyclonal	Dilution used
COX-2	COX-2 (C20)	Santa Cruz Biotechnology sc-1745	Goat polyclonal	1:2000
PR	Progesterone Receptor Clone 16	Leica Biosystems, NCL-L-PGR-312	Mouse monoclonal	1:500
GR	GR (E-20)	Santa Cruz Biotechnology, sc-1003	Rabbit polyclonal	1:1000
Phosphorylated (p)-p65 (Ser536)	Phospho-NF-κB p65	Cell Signaling, 3031	Rabbit polyclonal	1:1000
p-cJun (Ser63)	p-c-Jun (KM-1)	Santa Cruz Biotechnology, sc-822	Mouse monoclonal	1:5000
p-c-Fos (Ser32)	Phospho-c-Fos (D82C12)	Cell Signaling, 5348	Rabbit monoclonal	1:1000
p-ERK1/2 (Thr202/Thr204)	Phospho-p44/p42 MAPK (Erk1/2)	Cell Signaling, 9101	Rabbit polyclonal	1:5000
p-p38 (Thr180/Tyr182)	Phospho-p38 MAP Kinase	Cell Signaling, 9211	Rabbit polyclonal	1:1000
p-JNK (Thr183/Tyr185)	Phospho-SAPK/JNK	Cell Signaling, 9251	Rabbit polyclonal	1:1000
p65	NFκB p65 (F-6)	Santa Cruz Biotechnology, sc-8008	Mouse monoclonal	1:1000
c-Jun	c-Jun (60A8)	Cell Signaling, 9165	Rabbit monoclonal	1:1000
c-Fos	c-Fos	Cell Signaling, 4384	Rabbit, polyclonal	1:1000
ERK1/2	p44/42 MAPK (Erk1/2)	Cell Signaling, 9102	Rabbit, polyclonal	1:1000
p38	p38 MAPK	Cell Signaling, 9212	Rabbit, polyclonal	1:1000
JNK	SAPK/JNK	Cell Signaling, 9252	Rabbit, polyclonal	1:1000
IκBα	IκBα (C-21)	Santa Cruz Biotechnology, sc-371	Rabbit polyclonal	1:500
MKP-1	MKP-1 (C-19)	Santa Cruz Biotechnology, sc-370	Rabbit polyclonal	1:500
GAPDH	Anti-glyceraldehyde-3-phosphate dehydrogenase	Millipore, MAB374	Mouse monoclonal	1:20 000
Rabbit IgG	Anti-rabbit IgG, HRP-linked antibody	Cell Signaling, 7074	Goat polyclonal	1:2000
Mouse IgG	Anti-mouse IgG, HRP-linked antibody	Cell Signaling, 7076	Horse polyclonal	1:2000

multiple comparisons post hoc testing for three groups or more. $P < 0.05$ was considered statistically significant.

Results

The myometrial explant gene expression profile closely resembles the *in vivo* status

Biopsies obtained from non-labouring women at elective Caesarean section at term were divided into three: (i) dissected and immediately snap frozen ($t = 0$), (ii) dissected for myometrial explants and (iii) processed for cell culture. Explants, myometrial cells at passage 4 (the typical passage our group uses for experiments) and the hTERT cell line were cultured for a period of 30 h without treatment. Gene expression analysis via microarray demonstrated that explants most closely resemble $t = 0$ (Fig. 1A). Upon direct comparison between explants and $t = 0$, 1444 genes varied significantly whereas the corresponding number for passage 4 myometrial cells was 3840 and for hTERT 4603 (Fig. 1B). A total of 555 genes varied commonly upon comparing all 3 groups to $t = 0$ with gene ontology analysis demonstrating higher enrichment scores for functions including 'immune response', 'inflammatory response' and 'leukocyte migration' (Supplementary Table SI). Furthermore, explants shared 119 genes exclusively with passage 4 myometrial cells and 137 genes with hTERT; the equivalent figure for the passage 4 myometrial cells and hTERT overlap was 2111 (Fig. 1B). Of the 633 genes uniquely up-regulated in the explant group, the most common gene ontology groups pertained to glucose metabolism including 'glycolysis', 'gluconeogenesis' and 'glucose metabolic process' (Supplementary Table SII). Overall, the degree of variability on comparing $t = 0$ to each of the three groups was least for explants (Fig. 1C) followed by passage 4 myometrial cells (Fig. 1D) and hTERT (Fig. 1E).

A second set of biopsies and hTERT cultures were used to validate the microarray results via quantitative RT–PCR. Microarray trends were preserved for a panel of genes of interest including those associated with reproductive function (*PTGS2*, *OXTR*, *PGR*, *GJA1*) and smooth muscle phenotype (*ACTA2*, *MYLK*) (Table III). Overall, 15 genes of interest were chosen for validation of microarray results with 3 comparisons performed per gene (explants versus $t = 0$, passage 4 myometrial cells versus $t = 0$, hTERT versus $t = 0$); microarray and RT–PCR data followed the same trend in 39 of 45 cases (86.7%).

Nuclear receptor levels

Subsequent experiments focused on comparing explants and passage 4 myometrial cells to $t = 0$, as the same biopsy could be utilized in matched experiments and these two groups most closely resembled $t = 0$ based on the microarray results. A separate set of biopsies obtained from non-labouring women were divided into (i) $t = 0$, (ii) myometrial explants and (iii) myometrial cells, and utilized to assess key nuclear receptor levels on the protein level. Although the level of both PR isoforms tended to decline, there was no significant difference between $t = 0$ and explants. However, the levels of PR were observed to be significantly lower in myometrial cells at passage 4 than in either $t = 0$ tissue or explants (Fig. 2A and B). The PR-A:PR-B ratio was

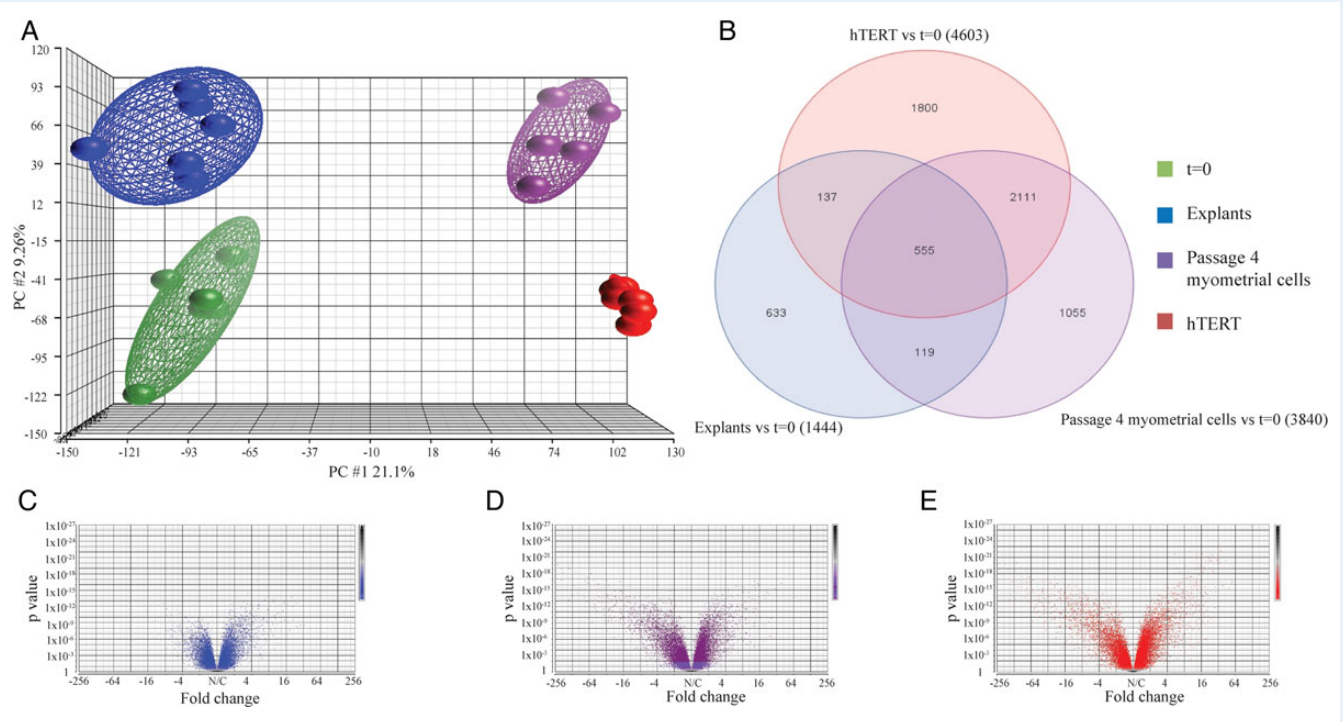

Figure 1 The myometrial explant gene expression profile most closely resembles the physiological status. Myometrial tissue obtained from term non-labouring women was divided into three portions: (i) snap frozen at the time Caesarean section ($t = 0$), (ii) finely dissected into 3 × 3 × 3 mm^3 explants and (iii) digested with a collagenase mixture to isolate cells for myometrial cell culture. Explants were cultured in Dulbecco's Modified Eagle Medium without treatment for 30 h. After overnight incubation with 1% (v/v) charcoal and dextran-stripped fetal calf serum, the media of myometrial cells at passage 4 and hTERT cells was refreshed and cells incubated for a further 30 h without treatment. RNA was extracted as described in Methods. Whole-genome transcriptome analysis was conducted by hybridization to Affymetrix Human Gene 2.1 ST array strips and analysed using Partek Genomics Suite 6.6 software. DEG was identified by two-way ANOVA, and *P*-values were adjusted using the FDR (false-discovery rate) method to correct for multiple comparisons. DEG were considered significant if *P*-value was $P \leq 0.05$ at a fold change of >2 with FDR <0.5, $n = 6$. (**A**) principal component analysis (PCA) plot, (**B**) Venn diagram of genes varying significantly versus $t = 0$ that are common and unique to each model, (**C**) Volcano plot of myometrial explants, (**D**) Volcano plot of passage 4 myometrial cells (**E**) Volcano plot of hTERT.

similar in explants and cells although this was significantly raised compared with $t = 0$ ($P = 0.0327$ and $P = 0.0067$, respectively) (Fig. 2C). No statistically significant difference was observed in GR levels between $t = 0$, explants and cells (Fig. 2A and D).

Progesterone-repression of IL-1β-induced COX-2

Previous group data demonstrated that 10 μM was the minimum P4 dose causing a significant reduction in IL-1β-driven COX-2 in passage 4 myometrial cells. Based on dose–response experiments, the myometrial explant IL-1β EC50 was defined as 10 ng/ml and the P4 IC50 P4 was 1 μM (Supplementary Fig. S1).

In order to directly compare P4 sensitivity, explants and cells originating from the same biopsy were treated with 10 ng/ml IL-1β ± 1 or 10 μM P4. Compared $t = 0$, basal COX-2 levels were significantly raised in explants ($P = 0.0313$) and not in cells. Although not significant, the addition of IL-1β showed a trend towards a greater increase in COX-2 levels in myometrial cells than in explants ($P = 0.07$; Fig. 3). The IL-1β-induced increase in COX-2 protein levels was statistically significantly reduced by pre-incubation with 1 μM P4 in myometrial explants and 10 μM P4 in cell cultures (Fig. 3).

P4 acts via PR in myometrial explants and represses IL-1β-induced activation of p65 and AP-1

The mixed PR/GR antagonist RU486 reversed the repressive effect of 1 μM P4 on IL-1β-induced COX-2 expression in myometrial explants (Fig. 4). The more PR-selective inhibitor ZK299 at the PR-specific dose of 1 μM (Kohmura *et al.*, 2000), also reversed the P4 effect (Fig. 4).

IL-1β increased the phosphorylation of ERK ($P = 0.0156$) and p38 ($P = 0.0313$) as well as the transcription factor targets c-Jun ($P = 0.0078$) and p65 ($P = 0.0078$; Fig.5) with no alteration to levels of total protein. Although a trend was observed, IL-1β did not significantly increase the phosphorylation of JNK or c-Fos (Fig. 5). Pre-incubation with 1 μM P4 reduced the IL-1β induced increase in p65 and c-Jun phosphorylation in association with a reduction in total protein levels. Interestingly, there was no reduction in MAPK phosphorylation, nor any change in MKP-1 or IκBα levels following P4 treatment alone or in combination with IL-1β (Fig. 5). P4 treatment alone did not drive the MAPKs, c-Jun or p65 phosphorylation, but did lead to a decrease in total p65 levels and an increase in c-Fos phosphorylation (Fig. 5).

Table III Quantitative RT–PCR validation of selected genes.

Gene name	Model	Quantitative RT–PCR fold change	Quantitative RT–PCR direction of change	Microarray fold change	Microarray direction of change
PTGS2	Explants	7.02 ± 3.34	↑	24.55 ± 0.45	↑
	Myometrial cells	2.43 ± 0.65	↑	2.21 ± 0.41	↑
	hTERT	1.73 ± 0.29	↑	2.47 ± 0.02	↑
IL8	Explants	200.53 ± 43.89	↑	28.53 ± 0.45	↑
	Myometrial cells	−4.51 ± 0.07	↓	−1.22 ± 0.14	↓
	hTERT	14.26 ± 0.05	↑	9.92 ± 0.46	↑
OXTR	Explants	−11.93 ± 0.05	↓	−1.23 ± 0.21	↓
	Myometrial cells	−34.74 ± 0.01	↓	−7.74 ± 0.42	↓
	hTERT	−9.09 ± 0.02	↓	−3.31 ± 0.20	↓
PGR	Explants	−2.89 ± 0.04	↓	−1.76 ± 0.08	↓
	Myometrial cells	−4.43 ± 0.04	↓	−3.35 ± 0.45	↓
	hTERT	−18.47 ± 0.00	↓	−8.21 ± 0.08	↓
IL1B	Explants	2.53 ± 0.09	↑	2.64 ± 0.40	↑
	Myometrial cells	−35.06 ± 0.01	↓	−1.31 ± 0.11	↓
	hTERT	18.90 ± 0.80	↑	18.44 ± 0.36	↑
NR3C1	Explants	−2.19 ± 0.04	↓	1.39 ± 0.03	↑
	Myometrial cells	−1.64 ± 0.08	↓	1.28 ± 0.14	↑
	hTERT	−1.23 ± 0.04	↓	1.81 ± 0.04	↑
TPM1	Explants	−1.37 ± 0.10	↓	−4.40 ± 0.05	↓
	Myometrial cells	−3.05 ± 0.04	↓	−2.77 ± 0.16	↓
	hTERT	1.33 ± 0.06	↑	−1.48 ± 0.08	↓
TNFAIP3	Explants	4.09 ± 1.30	↑	6.44 ± 0.19	↑
	Myometrial cells	−13.44 ± 0.01	↓	−5.92 ± 0.12	↓
	hTERT	−2.99 ± 0.04	↓	−1.08 ± 0.12	↓
IL6	Explants	20.12 ± 4.27	↑	15.21 ± 0.21	↑
	Myometrial cells	1.66 ± 0.46	↑	2.03 ± 0.31	↑
	hTERT	8.32 ± 1.59	↑	9.47 ± 0.11	↑
ACTA2	Explants	−10.48 ± 0.03	↓	−1.79 ± 0.14	↓
	Myometrial cells	−15.08 ± 0.02	↓	−1.49 ± 0.24	↓
	hTERT	−44.47 ± 0.00	↓	−2.83 ± 0.07	↓
MYLK	Explants	−8.98 ± 0.02	↓	−1.40 ± 0.03	↓
	Myometrial cells	−13.14 ± 0.01	↓	−1.14 ± 0.19	↓
	hTERT	−124.56 ± 0.00	↓	−3.58 ± 0.06	↓
PTGER3	Explants	−3.59 ± 0.09	↓	−1.10 ± 0.08	↓
	Myometrial cells	−10.37 ± 0.03	↓	−5.57 ± 0.21	↓
	hTERT	−653.11 ± 0.00	↓	−24.47 ± 0.04	↓
GJA1	Explants	1.06 ± 0.18	↑	1.58 ± 0.10	↑
	Myometrial cells	2.10 ± 0.19	↑	2.56 ± 0.11	↑
	hTERT	1.49 ± 0.11	↑	1.39 ± 0.03	↑
ESR1	Explants	−1.58 ± 0.10	↓	1.30 ± 0.17	↑
	Myometrial cells	1.41 ± 0.23	↑	−1.01 ± 0.13	↓
	hTERT	−3.56 ± 0.02	↓	−1.32 ± 0.08	↓
IL1A	Explants	3.36 ± 0.83	↑	2.71 ± 0.35	↑
	Myometrial cells	−1.55 ± 0.04	↓	−1.26 ± 0.10	↓
	hTERT	18.24 ± 3.60	↑	17.09 ± 0.19	↑

Myometrial tissue obtained from non-labouring women at term was snap frozen at the time Caesarean section ($t = 0$), finely dissected into 3 × 3 × 3 mm^3 explants or digested with a collagenase mixture to isolate the myometrial cells. Explants were left in culture without treatment for 30 h. After overnight incubation with 1% charcoal and dextran-stripped fetal calf serum, the media of myometrial cells at passage 4 and hTERT myometrial cells was refreshed and cells were left in culture for 30 h without treatment. RNA was extracted, cDNA was synthesized and quantitative RT–PCR was performed as described in Methods. Data presented as mean fold change ± SEM; $n = 6$.

Lack of a functional withdrawal of P4 action in myometrial explants

To determine whether there was any evidence of a functional P4 withdrawal with the onset of labour, we obtained myometrium from women before the onset of labour, in early labour (≤3 cm), during which the cervix effaces, begins to dilate and contractions become regular and strong, and in established labour (>3 cm), during which the cervix dilates more rapidly and contractions are regular and strong. The increase in COX-2 levels induced by IL-1β was similar in all 3 groups (Supplementary Fig. S2). Pretreatment with 1 μM P4, reduced

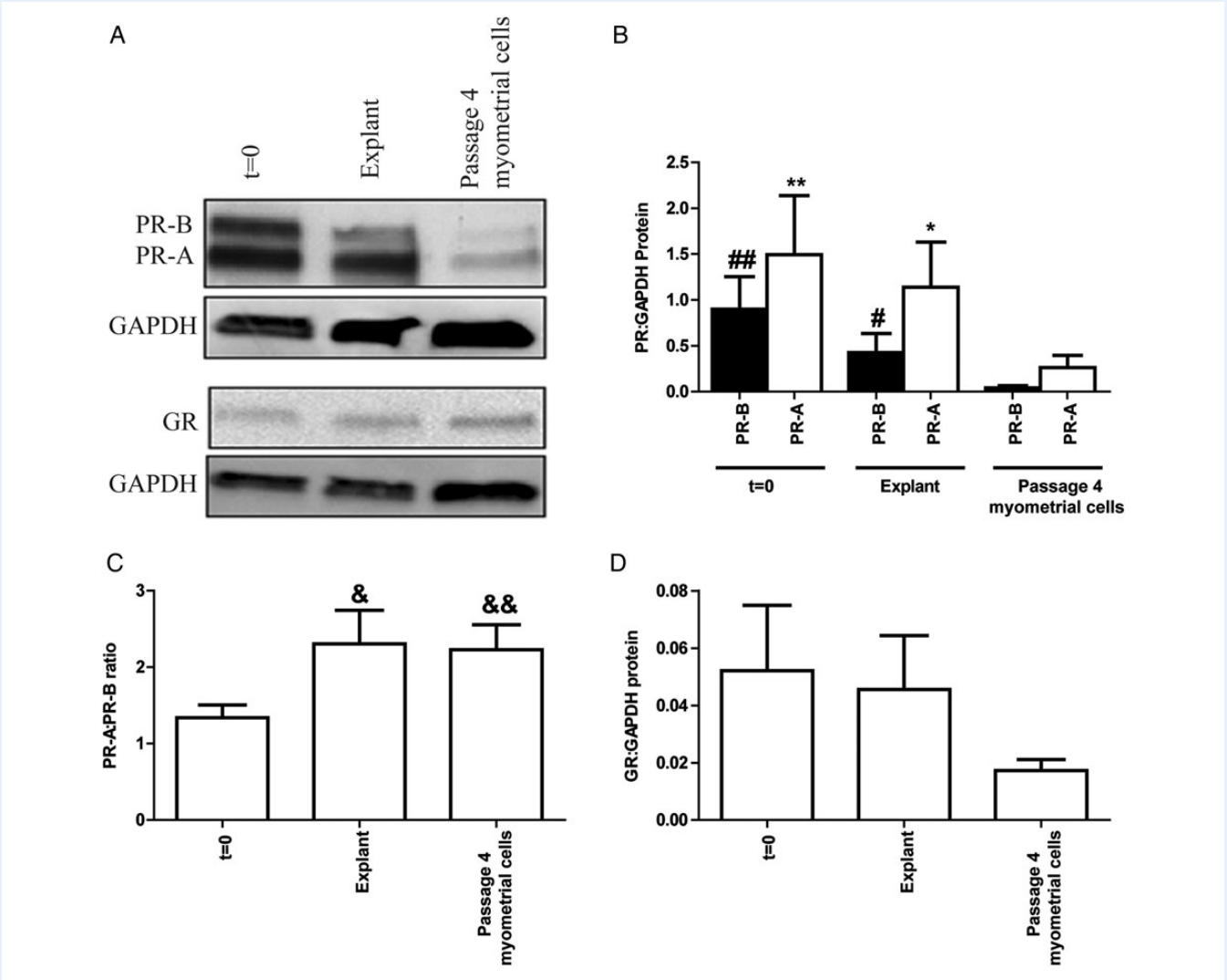

Figure 2 Myometrial explants maintain nuclear receptor levels in culture. Myometrial tissue obtained from term non-labouring women was divided into three portions: (i) snap frozen ($t = 0$), (ii) finely dissected into $3 \times 3 \times 3$ mm^3 explants and (iii) digested with a collagenase mixture to isolate the cells for myometrial cell culture. Explants were treated immediately with ethanol vehicle for 30 h. After overnight incubation with 1% (v/v) charcoal and dextran-stripped fetal calf serum, myometrial cells at passage 4 were treated with ethanol vehicle for 30 h. Protein was extracted and quantified. Western blotting for the progesterone receptor (PR) isoforms (PR-A and PR-B) and glucocorticoid receptor (GR) was performed as described in Methods. (**A**) Representative western blots of PR and GR levels, (**B**) densitometric analysis of PR levels, (**C**) analysis of PR-A:PR-B ratio, (**D**) densitometric analysis of GR levels. The data are expressed as mean + SEM. Normality was tested using a Shapiro–Wilks test followed by the Wilcoxon-signed rank testing. $^{\#}P < 0.05$ cells versus explants for PR-B; $^{\#\#}P < 0.01$ cells versus $t = 0$ for PR-B; $^{*}P < 0.05$ cells versus explants for PR-A; $^{**}P < 0.01$ cells versus $t = 0$ for PR-A; $^{\&}P < 0.05$ $t = 0$ versus explants; $^{\&\&}P < 0.01$ $t = 0$ versus cells, $n = 8–9$.

the expression of IL-1β-driven COX-2 in explants from patients in both early and established labour (Fig. 6). Treatment with P4 alone was not associated with a significant change in COX-2 levels compared with control (Fig. 6).

The role of P4 was further studied by quantification of a panel of pro-inflammatory cytokines in the tissue culture media of explants obtained from non-labouring women as well as women in early and established labour. In the first instance, using non-labouring samples, we sought to identify which cytokines were driven by IL-1β in our model and, if so, whether P4 was able to significantly reduce these levels. We identified a list of chemokines/cytokines fulfilling these two criteria comprised of CXCL2, IL-6 and IL-8 (Fig. 7A–C, Supplementary Fig. S3). CCL11 is not shown as it was not within the detection range of the assay. In addition, even though LIF was originally included in the list above, it was not detectable in the labouring culture media.

Next we determined whether the effect of IL-1β was altered by labour status by calculating the delta change (Δ) between control and IL-1β treated samples. As with COX-2, we found that the effect of IL-1β was similar in all 3 groups for CXCL2, IL-6 and IL-8 (Fig. 7D–F). We then assessed whether P4 treatment alone altered the release of pro-inflammatory cytokines into the medium in either of the labouring groups and found that P4 had no effect on cytokine levels (Fig. 7A–C).

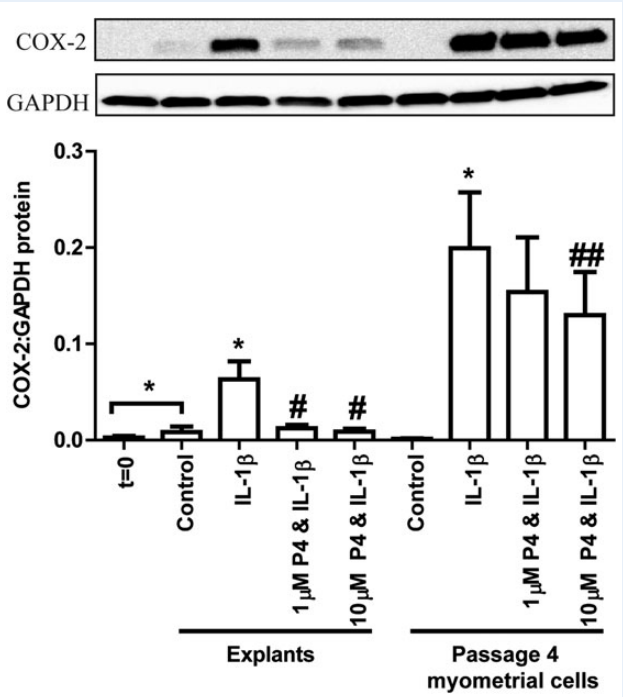

Figure 3 Explants respond to 1 μM progesterone (P4) in paired biopsies whereas myometrial cells do not. Myometrial tissue obtained from term non-labouring women was divided into three portions: (i) snap frozen ($t = 0$), (ii) finely dissected into $3 \times 3 \times 3$ mm^3 explants and (iii) digested with a collagenase mixture to isolate cells for myometrial cell culture. Explants were immediately pre-treated for 6 h with ethanol vehicle, 1 or 10 μM P4 followed by a 24 h treatment with IL-1β (10 ng/ml). After overnight incubation with 1% (v/v) charcoal and dextran-stripped fetal calf serum, myometrial cells at passage 4 were pre-treated for 6 h with ethanol vehicle, 1 or 10 μM P4 followed by a 24 h treatment with IL-1β (10 ng/ml). Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2) was performed as described in Methods. A representative western blot is shown at the top of the figure with densitometric analysis below. The data are expressed as mean + SEM. Normality was tested using a Kolmogorov–Smirnov test followed by comparison of control versus IL-1β by the Wilcoxon-signed rank testing or paired *t* testing depending on the data distribution; $^{*}P < 0.05$ versus control in that group. The IL-1β, 1 μM P4 & IL-1β and 10 μM P4 & IL-1β conditions in each group were compared by ANOVA followed by Bonferroni's Multiple Comparison Test; $^{\#}P < 0.05$ versus IL-1β in that group; $^{\#\#}P < 0.01$ versus IL-1β in that group, $n = 4–8$.

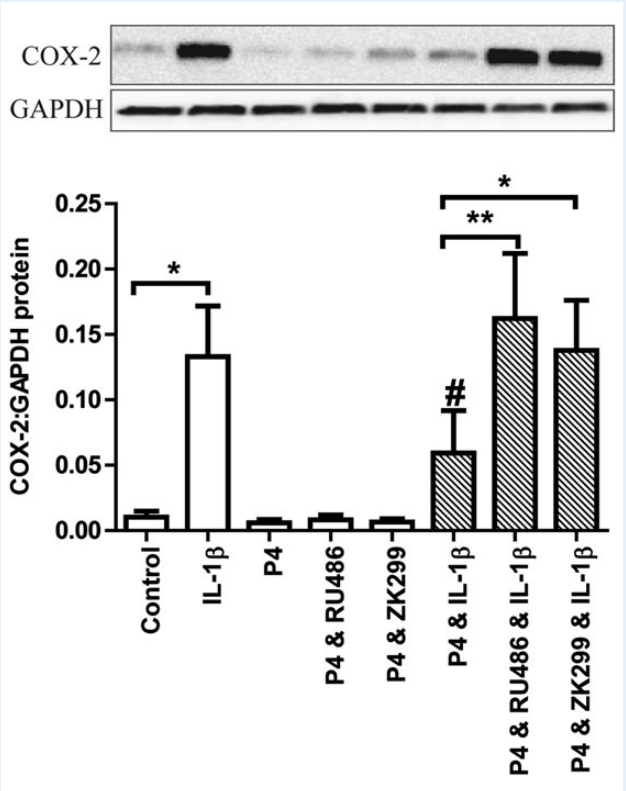

Figure 4 Mifepristone (RU486) and onapristone (ZK299) reverse the progesterone (P4)-mediated reduction in IL-1β-driven cyclooxygenase-2 (COX-2) in myometrial explants. Myometrial tissue obtained from term non-labouring women was finely dissected into $3 \times 3 \times 3$ mm^3 explants. These were immediately pre-treated for 6 h with ethanol and DMSO vehicle, 1 μM P4 ± 1 μM RU486 or 1 μM ZK299 followed by a 24 h treatment with IL-1β (10 ng/ml). Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2) was performed as described in Methods. A representative western blot is shown at the top of the figure with densitometric analysis below. The data are expressed as mean + SEM. Normality was tested using a Kolmogorov–Smirnov test. Paired *t* tests were used to compare control versus IL-1β and IL-1β versus P4 & IL-1β ($^{\#}P < 0.05$). The shaded groups were compared with a Friedman test followed by Dunn's Multiple Comparison Test; $^{*}P < 0.05$; $^{**}P < 0.01$, $n = 6–7$.

Finally, we confirmed that there was no difference in the ability of P4 to repress the IL-1β-induced increase in CXCL2, IL-6 and IL-8 levels in the labouring samples (Fig. 7G–I).

Discussion

In this study, we sought to establish a model that reflects the *in vivo* situation more accurately than the current *in vitro* cell models and to use this model to study P4 signalling in the myometrium.

As expected, we demonstrated that none of the *in vitro* models overlapped with the *in vivo* ($t = 0$) condition in the principal component analysis (PCA) plot; however, the explant gene expression profile was most closely associated with it. The separation between $t = 0$ and both myometrial cells and the hTERT cell line was much more marked. This was supported by the distinct distribution of top genes shown in the volcano plots of the three models compared with $t = 0$. Importantly, this pattern was preserved on examining genes relevant to reproductive function and parturition; for example the explant levels of PR (*PGR*), oxytocin receptor (*OXTR*) and connexin-43 (*GJA1*) did not vary significantly compared with $t = 0$. In contrast, PR RNA levels were significantly lower in both passage 4 myometrial cells and hTERT. Indeed, the same pattern was observed for PR on the protein level with no significant change in explants versus $t = 0$, but a significant reduction in myometrial cells. In addition, *OXTR* and *GJA1* RNA levels were significantly lower and higher, respectively, in myometrial cells. Although COX-2 (*PTGS2*)

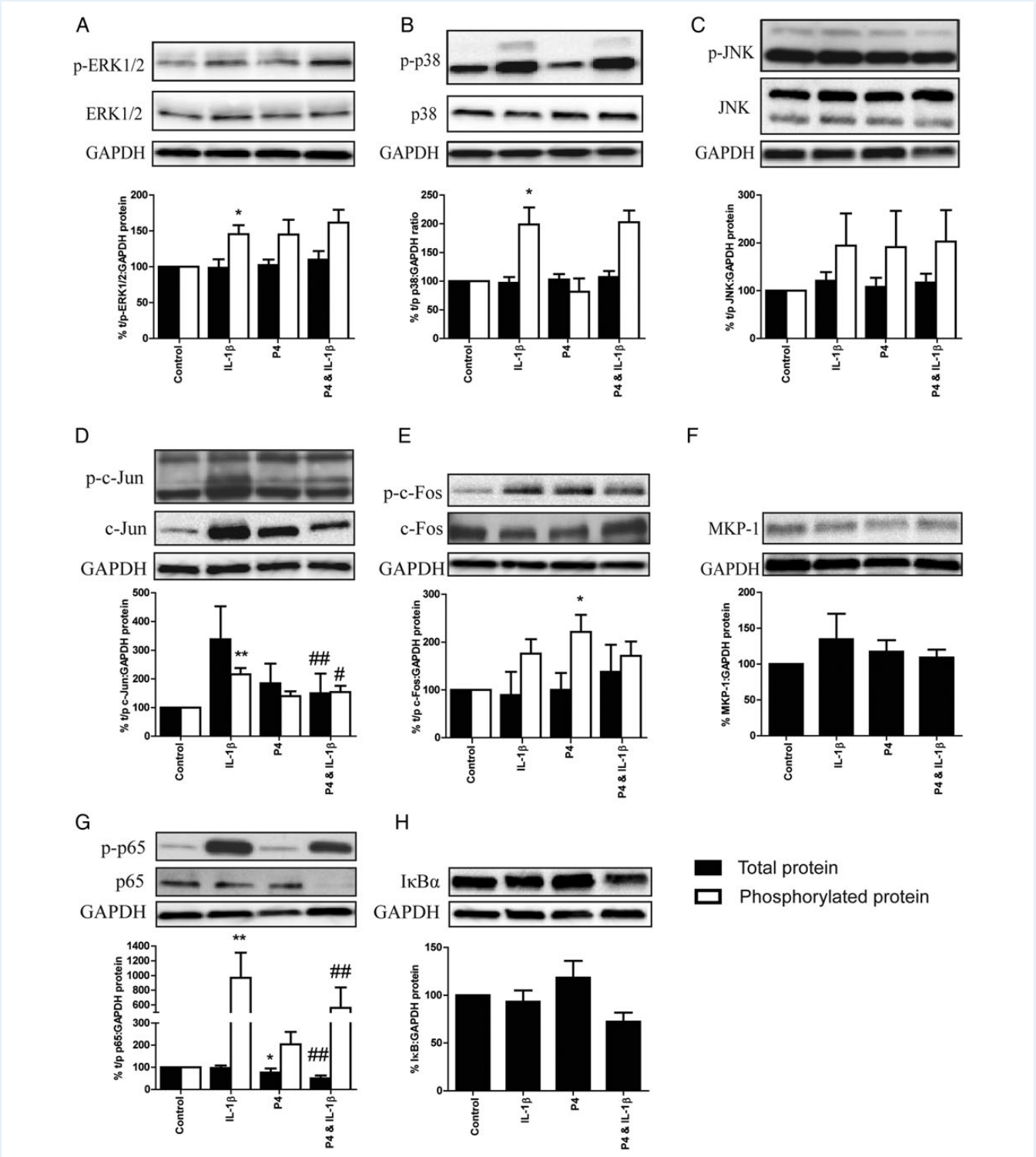

Figure 5 Progesterone (P4) reduces the phosphorylation of pro-inflammatory transcription factors in myometrial explants. Myometrial tissue obtained from term non-labouring women was finely dissected into 3 × 3 × 3 mm^3 explants. These were immediately pre-treated for 6 h with ethanol or 1 μM P4 followed by a 30 min treatment with IL-1β (10 ng/ml). Protein was extracted and quantified, and western blotting for total protein ± phosphorylated protein (where applicable) for (**A**) ERK1/2, (**B**) p38, (**C**) JNK, (**D**) c-Jun, (**E**) c-Fos, (**F**) MKP-1, (**G**) p65 and (**H**) IκBα was performed as described in Methods. Representative western blots are shown above each densitometric analysis plot. The data are normalized to control and expressed as mean + SEM. Normality was tested using a Kolmogorov–Smirnov for six replicates or a Shapiro–Wilk test for more than six replicates. Wilcoxon-signed rank testing was used to compare between pairs; $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus IL-1β; $^{\#\#}P < 0.01$ versus IL-1β. $n = 6–9$. Black bars: total (t) protein; clear bars: phosphorylated (p) protein.

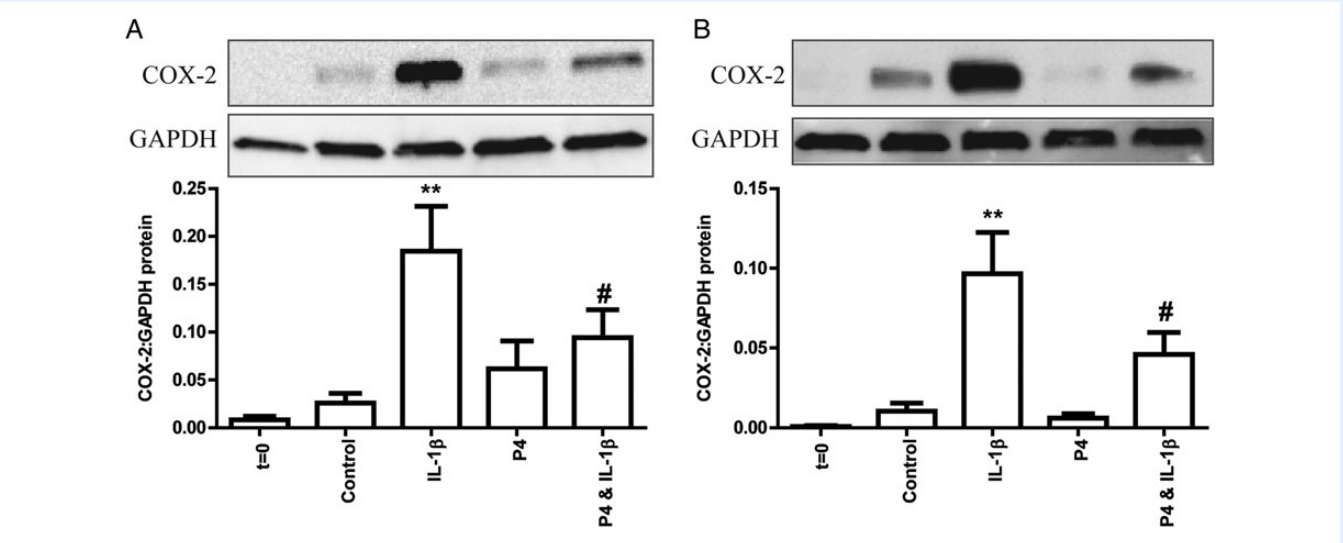

Figure 6 Progesterone (P4) maintains its anti-inflammatory role throughout labour. Myometrial tissue obtained from women in term (**A**) early labour and (**B**) established labour was finely dissected into 3 × 3 × 3 mm^3 explants after a portion was immediately snap frozen ($t = 0$). Explants were immediately pre-treated for 6 h with ethanol vehicle or 1 μM P4 followed by a 24 h treatment with IL-1β (10 ng/ml). Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2) was performed as described in Methods. Representative western blots are shown above each densitometric analysis plot. The data are expressed as mean + SEM. Normality was tested using a Shapiro–Wilk test. A Wilcoxon signed rank test was used for non-normally distributed data and a paired *t* test for normally distributed data. $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$ versus IL-1β. $n = 8$.

levels were significantly elevated in explants as well as hTERT, upon comparing this up-regulation in relation to COX-2 levels after IL-1β treatment, the effect was negligible with a signal-to-noise ratio of 17.7 (FDA, 2003). Indeed, it was noted that genes associated with inflammation were elevated in all three models as evidenced by gene ontology analysis (Supplementary Table SI). We also determined that the expression pattern of smooth muscle markers such as alpha smooth muscle actin (*ACTA2*) and myosin light chain kinase (*MYLK*) remained unaltered in explants whereas both were significantly reduced in hTERT.

We noted with interest that the uniquely up-regulated genes in the explant group versus $t = 0$ comprised glucose metabolism pathways. We hypothesized that this was the result of the explants being cultured in DMEM without any additional nutrient supplementation and hence utilizing alternative biochemical pathways to produce glucose. Although high lactate levels have been associated with dystocia (Quenby *et al.*, 2004) and hence it is plausible that glucose metabolism may play a role in the success of the parturition, further study is required. In an attempt to mimic the physiological conditions as closely as possible, we did supplement culture media with P4, estradiol and/or the cAMP agonist forskolin, but found that this had no effect on restoring PR levels even closer to $t = 0$ and hence these conditions were not incorporated into the final experimental model used (data not shown).

To our knowledge, this is the first microarray study directly comparing gene expression in pregnant myometrium at $t = 0$ with that of myometrial explants, passage 4 myometrial cells and hTERT. One study comparing non-pregnant myometrium at $t = 0$ to matched primary cell culture reported changes in expression of more than 1000 genes (Zaitseva *et al.*, 2006) supporting our finding of marked changes in gene expression in primary cell culture systems. Some gene expression studies focused on gene expression in myometrial explants (Cordeaux *et al.*, 2010) or cultured myometrial cells (Lei *et al.*, 2015b; Chandran *et al.*, 2016) in the presence or the absence of progestins and hence cannot be compared with our study. Other authors (Rehman *et al.*, 2003; Bollapragada *et al.*, 2009; Mittal *et al.*, 2010; Chan *et al.*, 2014) have sought to compare the gene expression profile of myometrium from non-labouring and labouring patients.

One possible limitation of this gene expression analysis was that it was conducted via microarray and not RNA-Seq, the latter being widely accepted as a superior platform with better concordance with quantitative RT–PCR (Wang *et al.*, 2014). However, microarray and quantitative RT–PCR share high correlation and for the purpose of this study, microarray was deemed appropriate. One notable case of poor concordance between microarray and quantitative RT–PCR data was GR (*NR3C1*), where the microarray indicated up-regulation in all three models whereas quantitative RT–PCR showed down-regulation. We sought to clarify our findings by undertaking protein level analysis and confirmed that GR levels do not vary significantly in explants or passage 4 myometrial cells compared with $t = 0$.

On the protein level, we demonstrated that the treatment of explants with 1 μM P4 significantly reduces inflammation, whereas in passage 4 myometrial cells it does not. Intriguingly, this occurs despite the fact that the PR-A:PR-B ratio increases in explant culture versus $t = 0$. Interestingly, the response to IL-1β is greater in cultured myometrial cells and this could reflect the architectural difference of the explants and cells. For consistency, we opted to treat both myometrial explants and cells with the same dose of IL-1β; however, it could be argued that this stimulus is supraphysiological and that corresponding *in vivo* events are much subtler. Nevertheless, no similar evidence of increased sensitivity to P4

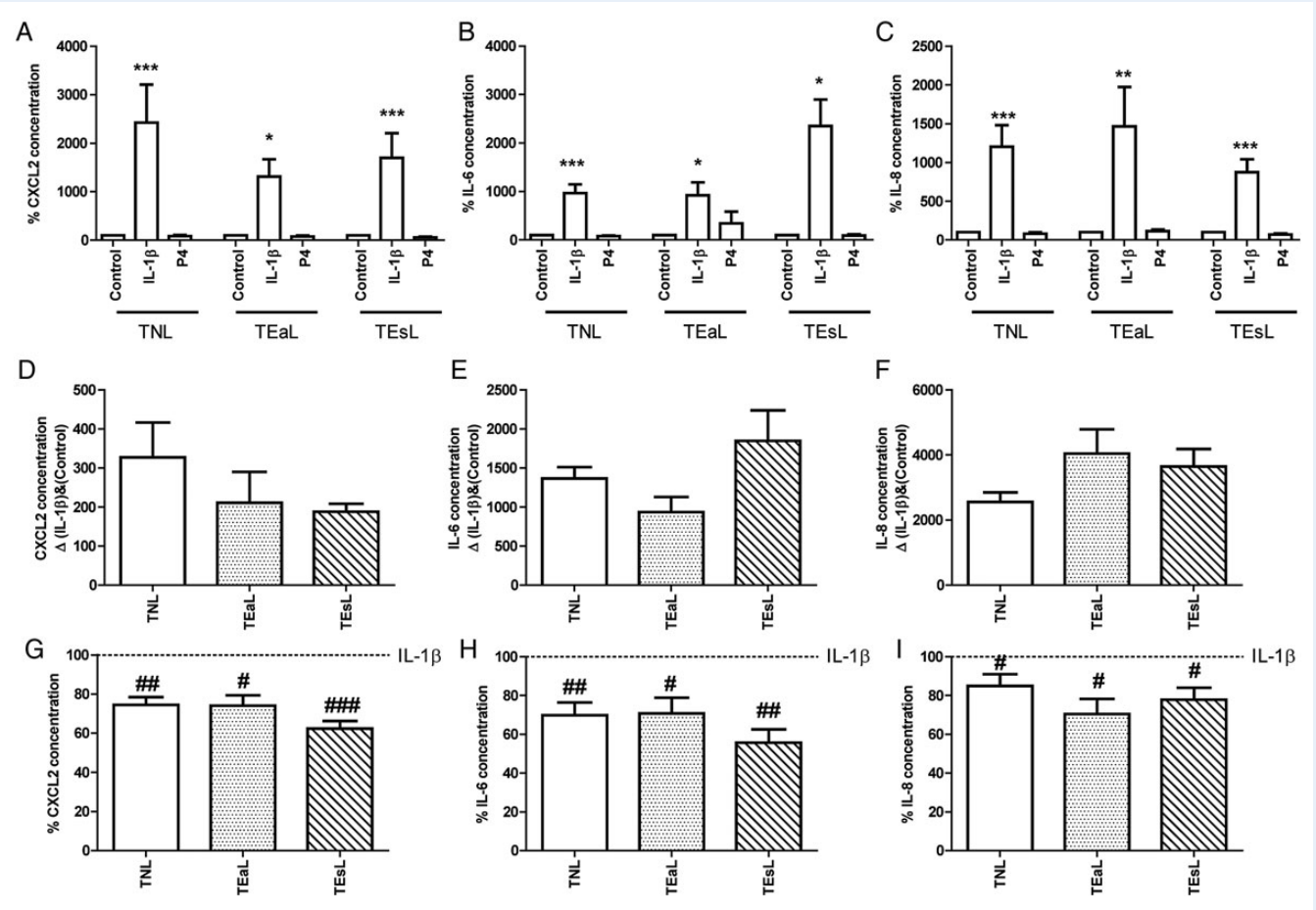

Figure 7 Progesterone does not become pro-inflammatory with labour onset and it reduces the expression of proinflammatory cytokines/chemokines irrespective of labour status. The media from explant cultures was used to run human Bio-Plex© Pro™ chemokine/cytokine assays for (**A**, **D**, **G**) C–X–C motif chemokine 2 (CXCL2), (**B**, **E**, **H**) IL-6 and (**C**, **F**, **I**) IL-8 as per the manufacturer's protocol. Data were normalized to tissue weight. The data in (A–C) are standardized to control and expressed as mean + SEM. The data in (D–F) are expressed as mean + SEM of the delta (Δ) between IL-1β and control groups. The data in (E–I) are standardized to IL-1β and expressed as mean + SEM. Normality was tested using a Kolmogorov–Smirnov for six replicates or a Shapiro–Wilk test for more than six replicates. For groups of three, a Kruskal–Wallis test was performed followed by Dunn's Multiple Comparison Test for non-normally distributed data and an ANOVA followed by Bonferroni's Multiple Comparison Test was performed for normally distributed data. Wilcoxon-matched pair testing was used for non-normally distributed paired data and paired *t* tests for normally distributed data. For comparisons to vehicle control within matched labour status group, statistical significance is indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. For comparisons to IL-1β within matched labour status group, statistical significance is indicated as follows: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, $n = 6–16$. Labour status groups are labelled as TNL (term no labour), TEaL (term early labour) and TEsL (term established labour).

treatment was observed in cells, suggesting that explants do not just differ to myometrial cells in their cellular organization, but also in their overall sensitivity to different treatments.

Even though other studies have shown that passaging of primary cells does not affect the myometrial phenotype (Mosher *et al.*, 2013), they have crucially not included tissue at $t = 0$. Our data suggest that there are marked functional differences between explants and passage 4 myometrial cell cultures. Explants contain a heterogeneous collection of cell types, including myometrial smooth muscle, vascular smooth muscle, endothelial cells, stromal fibroblasts and blood cells which may all contribute to the phenotype which, upon comparison to cell culture systems which only contain one cell type, most closely resembles $t = 0$. Indeed, by virtue of its similarity to the *in vivo* status, we believe that the study of myometrial function on the explant level, with its variety of cell types, is superior to cell culture systems. Nevertheless, we have observed by immunohistochemistry for alpha smooth muscle actin that smooth muscle cells predominate (data not shown) and additionally that explants contract spontaneously and after treatment with oxytocin (data not shown).

We have shown that both RU486 and ZK299 are capable of reversing the effect of P4. Although neither drug is a pure PR antagonist (Miner *et al.*, 2003), 1 μM ZK299 has previously been shown to reverse the effect of P4 but not that of dexamethasone (Kohmura *et al.*, 2000), suggesting that P4, in contrast to our observations from myometrial cell cultures (Lei *et al.*, 2012), may signal via PR in explants. The reason for this may lie in the difference in nuclear receptor levels in the two models, with the explant model more closely resembling the *in vivo* state. Trial of other 'specific' PR and GR antagonists

demonstrated that they have non-specific actions in myometrial explants (data not shown).

Consistent with previous findings (Lei *et al.*, 2012, 2015a; Hardy *et al.*, 2006), we show that P4 is able to repress the IL-1β-induced activation of two major pro-inflammatory transcription factors: NF-κB and AP-1. Both of these transcription factors have previously been shown to be required for IL-1β-mediated up-regulation of COX-2 in gestational tissues (Allport *et al.*, 2000). In support of our previous findings (Lei *et al.*, 2015a) and in contrast to other reports in the literature (Hardy *et al.*, 2006), the mechanism of P4 action does not appear to be via an increase in IκBα. Further, the reduction in c-Jun phosphorylation does not seem to be mediated via an increase in MKP-1 (Lei *et al.*, 2015a). Rather, it appears that total p65 and c-Jun levels are reduced by P4 treatment. These data suggest that the *in vivo* mechanism of P4 action may differ markedly compared with the *in vitro* models.

Our data indicate that with the onset of labour, P4 does not become pro-inflammatory as suggested by other groups (Allport *et al.*, 2000; Tan *et al.*, 2012), nor does it lose its anti-inflammatory action in myometrial explants. Furthermore, the explant sensitivity to IL-1β does not alter with labour status, nor does the ability of P4 to down-regulate the IL-1β response suggesting that there is no functional withdrawal of P4 action, at least in terms of the ability of P4 to repress inflammation. It should however be noted that these represent *ex vivo* observations and may not be reflective of *in vivo* events. Nevertheless, our data suggest that should a functional P4 withdrawal occur *in vivo*, then it is unlikely to involve COX-2 and at least the inflammatory chemokine/cytokines examined in the present study. It does remain possible that there is a withdrawal of other P4-mediated functions that lead to the onset of labour.

In conclusion, this study has established the validity of using an explant model to study myometrial P4 signalling. We provide evidence that P4 acts via PR to reduce IL-1β-induced COX-2 synthesis in association with a reduction in NF-κB and AP-1 activation. Furthermore, we show that P4 is able to repress the expression IL-1β-induced genes even after the onset of labour, suggesting that, at least in this regard, there is no functional withdrawal of P4 action.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

E.X.G. designed the study, recruited patients, performed the experiments and wrote the manuscript. K.L., P.F.L., S.R.S and M.R.J contributed to the design of the study and data interpretation. A.Y. contributed to the design of the study. B.R.H. assisted with chemokine/cytokine assay data acquisition. M.C. and S.T.M. carried out microarray data acquisition and analysis. All authors assisted with drafting of the article and approved the final version to be published.

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Conflict of interest

None declared.

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