Interplay between polypyrimidine tract binding protein-associated splicing factor and human myometrial progesterone receptors

A J Tyson-Capper, E A Shiells and S C Robson

The Medical School, Institute of Cellular Medicine, Newcastle University, 3rd Floor, William Leech Building, Newcastle upon Tyne NE2 4HH, UK

(Correspondence should be addressed to A J Tyson-Capper; Email: a.j.tyson-capper@ncl.ac.uk)

Abstract

The precise molecular mechanisms controlling progesterone receptor (PR)-mediated gene regulation within the human myometrium in pregnancy and in labour remain poorly defined. PR recruit different nuclear co-activators/co-repressors to mediate receptor-specific transcription regulation and expression of PR, and these co-factors may alter within the myometrium during pregnancy and labour. The aims of this study were to test the hypotheses that i) the human splicing and transcription factor, polypyrimidine tract binding protein-associated splicing factor (PSF), is spatially and temporally regulated in the myometrium during pregnancy and labour; ii) PSF influences the expression of myometrial PR and iii) the action of PR in regulating specific hormone response target genes in the human myometrium may involve PSF. Immunoblotting indicated that PSF expression is significantly up-regulated within the human myometrium as pregnancy progresses, in particular within the upper uterine region, and levels remain elevated in labour. Co-immunoprecipitations and DNA-binding assays show that PSF directly interacts with nuclear PR and glucocorticoid receptor (GR) and specific co-regulatory proteins, all of which have defined roles as co-activators or co-repressors in gene regulation. Over-expression and inhibition of PSF by transient transfection and RNAi respectively alters expression of myometrial PR and GR and may influence expression of two PR/GR-target genes, cyclooxygenase-2 and histone deacetylase-2. These findings are suggestive of a role for myometrial PSF as a nuclear co-regulator in the regulation of specific hormone receptor genes and their target hormone response genes.

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Introduction

The steroid hormone progesterone is pivotal in the establishment and maintenance of pregnancy by promoting myometrial quiescence and may have protective effects against endometrial cancer (Dai *et al.* 2002, Druckmann & Druckmann 2005). In addition, progesterone has multiple biological effects outside of the reproductive system, having smooth muscle relaxant, anti-inflammatory and immunoregulatory properties (Szekeres-Bartho *et al.* 2001). The diverse biological effects of progesterone are mediated primarily by the progesterone receptors (PR) and also nuclear protein co-factors that interact with the PR (Kastner *et al.* 1990, McKenna *et al.* 1999, Leonhardt *et al.* 2003).

The two major nuclear PR subtypes, PR-A (98 kDa) and PR-B (114 kDa), are independently regulated from defined promoter regions within a single gene (Kastner *et al.* 1990). PR-A lacks the first 164 amino acid sequence of PR-B. In addition, there may be spliced variants of PR present in myometrium, and other tissues, and membrane-bound receptors (mPR- α and mPR- β) have been reported (Karteris *et al.* 2006). Nuclear PR are ligand-activated transcription factors; on hormone

binding, they undergo phosphorylation, dimerise and in many cases bind to progesterone-responsive elements (PRE) present within the regulatory promoter regions of target genes. When bound to DNA, PR may interact with the transcription machinery directly or indirectly via nuclear co-activators or co-repressors to activate or repress target genes. However, the presence of a PRE does not necessarily predict progestin responsiveness. PR can also mediate its effect independently of PREs, through the protein–protein interactions of PR with other sequence-specific transcription factors. Although PR-A and PR-B have similar ligand and DNA-binding affinities, they can have different transcriptional activities (Yudt *et al.* 2006). PR-B is often described as the transcriptional activator of progesterone-responsive genes whereas PR-A primarily serves as a transcriptional repressor. In the presence of specific agonists and antagonists, PR-A can also act as a trans-dominant repressor of both PR-B and oestrogen receptors (ER; Vegeto *et al.* 1993).

Myometrium is a unique developmentally regulated smooth muscle organ in that the lower segment contracts during pregnancy and the upper expands and relaxes, whereas in labour the upper region

contracts and the lower relaxes. These physiological changes are due in part to the differential expression of specific subsets of genes within the functionally distinct regions. Although circulating progesterone levels do not fall prior to or during labour in the human, progesterone withdrawal is considered to activate the uterine smooth muscle to initiate labour (Astle *et al.* 2003, Mesiano & Welsh 2007). Changes in the expression and signalling events of myometrial PR are likely to contribute to functional progesterone withdrawal. There is some evidence to suggest that expression ratios of myometrial PR-A and PR-B (Pieber *et al.* 2001, Merlino *et al.* 2007), or PR-B and PR-C (Condon *et al.* 2006), may alter in labour and we have recently reported a decrease in PR-B levels at term and in labour (Samalecos & Gellersen 2008, Tyson-Capper & Robson 2008). It is also conceivable that expression of different nuclear co-activators/co-repressors changes within the myometrium during pregnancy and at the onset of labour (Condon *et al.* 2003, O'Malley 2008).

The nuclear protein PSF was originally identified as an essential factor in mammalian pre-mRNA splicing mediating its role in RNA processing via two RNA recognition motifs (Patton *et al.* 1993). PSF has since been shown to also possess a proline/glutamine-rich domain typical of the DNA-binding domains associated with transcriptional regulation (Urban *et al.* 2002). PSF contributes to the repression of oncogenic genes and is recruited to activated promoters of specific genes, such as the P450scc gene (Urban & Bodenbun 2002, Rosonina *et al.* 2005). In 293T cells, PSF functions as a transcriptional co-repressor via interactions with the thyroid hormone receptors (TR) and the retinoic acid receptors (RXR; Mathur *et al.* 2001). Dong *et al.* (2005, 2007) subsequently proposed that PSF mRNA expression increases in the rat myometrium during pregnancy and that PSF binds to PR and the androgen receptor (AR) in 293T cells and may interfere with PR/AR binding to DNA response elements. PSF may also play a role in regulating polyadenylation and associates directly with RNA polyadenylation elements within the PR-regulated gene, COX-2, suggesting additional roles in post-transcriptional gene regulation (Hall-Pogar *et al.* 2007).

Therefore, in this study, we investigated whether PSF may play a role in PR gene regulation within the human myometrium in pregnancy and labour.

Materials and methods

Tissues and cell culture

Human primary myometrial smooth muscle tissue and cultured cells were used in this study. Myometrial biopsies were obtained from non-pregnant (NP; $n=14$), preterm (28–34 weeks, $n=6$), term pregnant, non-labouring (39–40 weeks, $n=14$) and labouring

women ($n=14$) undergoing hysterectomies for benign conditions or caesarean sections. NP samples were taken from the middle of the uterine corpus. Samples from the upper segment (corpus) of the myometrium were taken from within the uterine cavity using laparoscopic biopsy forceps (Richard Wolf Endoscopes, Wimbledon, UK) introduced through the lower segment incision, avoiding the site of the placental bed; lower segment samples were taken close to the cervix. Written consent was obtained from all women, and ethical approval granted by the Newcastle and North Tyneside Health Authority Ethics Committee. Primary myocyte isolation and culture was undertaken as previously described and cultured with complete α -valine medium to restrict the growth of contaminating fibroblast cells (Phaneuf *et al.* 1997, Pollard *et al.* 2002).

Cell culture experiments

Transfection experiments on primary myometrial cells were undertaken on sub-culture passages 1–2 to ensure that endogenous PR expression was not lost. Myometrial cell monolayers were transfected, at 60–70% confluency (in the absence of antibiotics) as previously described (Pollard *et al.* 2002, Tyson-Capper *et al.* 2005) using Mirus LT-1 (Cambridge Bioscience, Cambridge, UK) cationic-lipid transfection reagent with OptiMEM medium (Invitrogen) and a GTP-tagged-PSF plasmid (kindly provided by Dr Yaron Shav-Tal, NY, USA) or empty plasmid as a control (data not shown). Transfection efficiencies were in the range of 40–45% for all experiments, as determined by light microscopy after transfection with a β -galactosidase encoding plasmid, pcDNA3.1 LacZ (Invitrogen; data not shown). In specific experiments, as indicated, cultured cells were treated with lipopolysaccharide (LPS) 1 μ g/ml or trichostatin A (TSA) 330 nM or progesterone 1 μ M (Sigma-Aldrich) for 24 h. RNA interference (siRNA) was undertaken to suppress PSF using a pool of three target-specific PSF-siRNA, control siRNA and cationic-lipid transfection reagents (Insight Biotechnology Ltd, Middlesex, UK; Applied Biotechnologies/Ambion, Warrington, UK) according to the manufacturer's recommended protocol; cells were harvested 4 days post-transfection. Note, all transfection experiments with and without additional treatments were performed in duplicate and repeated at least three times.

Western immunoblotting and RT-PCR

Myometrial tissue and cell lysates were prepared as previously described (Pollard *et al.* 2000, Tyson-Capper *et al.* 2005). Protein concentration of tissue/cell lysates, nuclear and cytoplasmic fractions was assayed using the DC protein assay kit (Bio-Rad). SDS-PAGE was

performed using 10–12% polyacrylamide gels and proteins transferred to nitrocellulose membranes as described (Pollard *et al.* 2000). Membranes were stained/de-stained in Ponceau-S and re-probed for GAPDH to ensure equal protein loading. All antibodies used in this study are detailed in Table 1. Immunoreactive bands were detected by enhanced chemiluminescence, ECL (Amersham, GE Healthcare, Buckinghamshire, UK) and data obtained where a linear relationship existed between the amount of protein loaded and the intensity of the ECL signal from the immunoblots. ECL signals were quantified by scanning densitometry and data subsequently analysed using a one-way ANOVA with Bonferroni's multiple comparison or *t*-test. RT-PCR was undertaken using 1 µg total RNA isolated from individual transfection experiments; PCR amplification was performed under standard conditions with an initial hot start at 94 °C followed by 20–26 cycles at 94 °C (1 min), 50 °C (30 s), and 72 °C (1 min; details of the primers are shown in Table 2).

Immunofluorescent staining

Immunofluorescent staining for endogenous PSF was undertaken on unfixed cultured primary myometrial cells prepared from term pregnant myometrium. Slides were treated at room temperature with PBS containing 10% normal goat serum and then incubated with a monoclonal anti-PSF primary antibody overnight at 4 °C (Table 1). Cells were washed three times in PBS and then incubated with a FITC-conjugated goat-anti-mouse secondary antibody (Jackson ImmunoResearch, Peterborough, UK) for

30 min. Following further washes, a DAPI Vectashield mountant (Vector Laboratories, Ltd, Peterborough, UK) was applied.

Co-immunoprecipitations

Nuclear and cytoplasmic extracts prepared from cultured myometrial cells (200 µg) were first pre-cleared with protein A/G agarose beads (Santa Cruz, Insight Biotechnologies) and then incubated with 1–3 µg capture antibody (Table 1), as indicated, overnight at 4 °C. Reactions containing a rabbit IgG as the capture antibody were included as controls. Each reaction was next incubated with 40 µl protein A/G agarose beads for 2 h and unbound proteins removed by six washes in 1× high stringency binding buffer (the first three rinses were supplemented with 10% BSA) and centrifugation at 25 *g*. Proteins were dissociated from the A/G agarose beads using 2× SDS-loading buffer and incubation at 95 °C. Immunoprecipitated proteins and nuclear extracts (ne) were separated by SDS-PAGE and immunoblotting as described above. The antibodies used to detect PSF-binding proteins have been previously tested for their specificity by other researchers and ourselves (Zhang & Dufau 2002, Phillips *et al.* 2005).

DNA affinity precipitation assay

Double-stranded biotin-labelled oligonucleotides containing the individual monocyte elongation factor (MEF2), Sp1 (PR-B promoter), Sp1/ERE (PR-A promoter) sequence motifs were designed for use in

Table 1 Antibodies used in this study

	Product code	Use and concentration	Source
Protein target			
PSF	P-2860	WB, IF (1:1500); IP, ChIP (2–3 µg)	Sigma–Aldrich
PR	sc-539	WB (1:250)	Insight Biotechnology, Middlesex, UK
PR-B	NCL-PGR-B	IP (2–3 µg), WB (1:1000)	Novacastra, Leica, UK
PR-A/PR-B	NCL-PGR-AB	IP (2–3 µg) WB (1:1000)	Novacastra
MEF2	sc-10794	WB (1:300)	Insight Biotechnology
GR	611226	IP (2–3 µg), WB (1: 2500)	BD Biosciences, Oxford, UK
Sp1	ab13370	WB (1:300)	Abcam, Cambridge, UK
Sp-3	sc-644	WB (1:300)	Insight Biotechnology
Sin3a	sc-5299	WB (1:300)	Insight Biotechnology
HDAC1	ab19845	WB (1:1000)	Abcam
HDAC2	ab7029	WB (1:1000)	Abcam
Ac-K	ab3879	IP (2–3 µg), WB (1:1000)	Chemicon Europe Ltd, Hampshire, UK
COX-2	sc-7951	WB (1: 300)	Insight Biotechnology
RbAb48	sc-12434	WB (1: 500)	Abcam
GFP	g1544	WB: (1:4000)	Sigma–Aldrich
GAPDH	sc-25778	WB: (1:3000)	Insight Biotechnology

Details of the antibodies used for western immunoblotting, co-immunoprecipitations, dual-labelled immunofluorescent staining and chromatin immunoprecipitations experiments. WB, western immunoblotting; IP, immunoprecipitations; IF, immunofluorescence; ChIP, chromatin immunoprecipitations. The amount of antibody used in specific experiments is shown in brackets.

Table 2 Sequences of primers and oligonucleotides used in this study

	DNA sequence (5' to 3')	Position	Use	Accession number
Primer or oligonucleotide				
PR-B sense	CCTGAAGTTTCGGCCATACC	+ 135 to + 331	RT-PCR	NM_000926
PR-B antisense	AGCAGTCCGCTGTCTTTTC		RT-PCR	
GAPDH sense	CTGCCGTCTAGAAAACC	+ 840 to + 1060	RT-PCR	NM_002046
GAPDH antisense	GCCAAATTCGTTGTCATACC		RT-PCR	
Bio-MEF2 wt	TATTTACTAAAAATTGGAACACATTCC	− 1310 to 1285	DAPA	NM_000926
Bio-MEF2-mu	TATTTAGT ACGT ATTGGAACACATTCC		DAPA	
Bio-Sp1 wt (PR-B)	GGGCTTTGGGGGGGGCCTCCCTAGAG	− 799 to − 773	DAPA	NM_000926
Bio-Sp1-mu (PR-B)	GGGCTTTT ACA ACGGCCTCCCTAGAG		DAPA	
Bio-Sp1 wt (PR-A)	GGTCGGGGGGGGGAGGGCGGCGC-TGG TCAGCTCCT	− 179 to − 142	DAPA	NM_000926
Bio-Sp1-mu (PR-A)	GGTCGGGG TACAT GGG ACTTCTAC CGCTG-CACAGCTCCT		DAPA	
PR promoter 1 sense	ACTGAGGATGTTATGATGTAG	− 1375 to − 1244	ChIP-PCR	NM_000926
PR promoter 1 antisense	AAGTTTGGATGTTGTGTGCC		ChIP-PCR	
PR promoter 2 sense	GCCAACTCCAGAGTTTCAG	− 884 to − 673	ChIP-PCR	NM_000926
PR promoter 2 antisense	TCTCATTGAGAATGCCACC		ChIP-PCR	
PR promoter 3 sense	CGAGTTAGGAGACGAGATC	− 296 to − 106	ChIP-PCR	NM_000926
PR promoter 3 antisense	TGAATGTGGCTGGACCGG		ChIP-PCR	
COX-2 promoter 1 sense	CGACCCTCTAAAGACGTACA	− 495 to − 292	ChIP-PCR	U04636
COX-2 promoter 1 antisense	TGTCTTTTCTTCTTCGAGTG		ChIP-PCR	
COX-2 promoter 2 sense	GAAAGCAACTTAGCTACAAAG	− 702 to − 474	ChIP-PCR	U04636
COX-2 promoter 2 antisense	TCGACCCTCTAAAGACGTACAG		ChIP-PCR	
Bio-Consensus PRE wt	GATCCTGTACAGGATGTTCTAGCTACA		DAPA	
Bio-Consensus PRE-mu	GATCCT CA ACAGGAT CA TCTAGCTACA		DAPA	

DNA sequences for the primers used in RT-PCR and ChIP-PCR experiments and oligonucleotides used for DNA affinity precipitation assays (DAPA). Positions are derived from + 1 bp from (ATG) transcription start sites. Only one of each pair of complementary oligonucleotides is shown. Bio, biotinylated oligonucleotides; wt, wild-type oligonucleotides; mu, mutant oligonucleotides with nucleotide substitutions in binding site motifs, shown underlined in bold.

DNA affinity precipitation assay (DAPA; Table 2). Oligonucleotides for a consensus PRE were also included in the study. Oligonucleotides with the individual motifs mutated were included as controls. Oligonucleotides (0.25 µg/reaction) were incubated with 50 µg myometrial nuclear extract in 400 µl binding buffer (12 mM HEPES (pH 7.9), 4 mM Tris-HCl, 60 mM KCl, 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol) for 1 h on ice with gentle agitation. Forty microlitres streptavidin magnetic beads (Active Motif, Rixensart, Belgium) equilibrated in binding buffer were added to each reaction and samples incubated for a further 2 h at 4 °C to form DNA/protein/streptavidin magnetic bead complexes. Unbound proteins were removed by washing magnetic beads, six times in 1 ml 1× binding buffer. Magnetic beads were next re-suspended in 30 µl SDS-PAGE-loading buffer to dissociate the DNA/protein complexes. Proteins were analysed by SDS-PAGE and immunoblotting as described above.

Chromatin immunoprecipitations

Myometrial cells (3×10⁷) were cultured in OptiMEM medium (Invitrogen Limited) and chromatin immunoprecipitation (ChIP) reactions were performed using the ChIP-express enzymatic kit (Active Motif). Briefly,

cells (1–3×10⁷) were rinsed in PBS and then 1% formaldehyde for 12 min to cross-link proteins to the DNA. The anti-PSF monoclonal antibody was incubated with chromatin overnight at 4 °C and then cross-linking reversed and proteins removed by proteinase K. DNA was purified by phenol–chloroform extraction and precipitated in 100% ethanol. Ten microlitre aliquots (and input DNA diluted 1:10) were used for PCR. PCR was performed using primers that amplify specific regulatory regions within the promoters of PR and the PR/GR-target gene COX-2 (Table 2). PCR conditions were 5 min (95 °C), followed by 36 cycles of 4 min (95 °C), 20 s (59 °C), 45 s (72 °C) and then 5 min at 72 °C. ChIP analysis with an RNA polymerase II antibody and irrelevant antibody, together with positive and negative primers for PCR, were included. ChIP experiments were performed in duplicate and repeated twice.

Results

Expression of PSF in human myometrium during pregnancy and labour

We investigated the expression of human myometrial PSF during pregnancy and in labour by immunoblotting analyses. Levels of PSF protein were

determined in NP, third trimester (28–34 weeks, TT), term (P) and labouring (L) tissue biopsies. Paired tissue biopsies from upper (US) and lower (LS) uterine segments were included to define whether there are spatial variations in the expression of PSF within the different regions of the uterus. Immunoblotting with the monoclonal anti-PSF antibody indicated that PSF expression is increased at term but there is no further increase during labour (Fig. 1A). Equal loading of protein was confirmed by Ponceau-S staining (data not shown) and re-probing membranes for GAPDH, as shown (lower panel). Quantification by densitometric analysis, as shown in Fig. 1B, indicated that PSF expression increased significantly in the upper uterine region at term (P-US, $P<0.001$) and in labour (L-US,

$P<0.001$) when compared with NP tissues. The data also suggest that PSF levels were significantly higher in the upper uterine segment at term and in labour than those found in the lower uterine segment (P-US versus P-LS, $P<0.05$; L-US versus L-LS $P<0.01$). Data also indicated that levels of myometrial PSF increase in preterm tissue samples (from 28 to 34 weeks; Fig. 1C), in particular within the upper uterine region (NP versus PT-US, $P<0.001$, Fig. 1D).

The effect of PSF on nuclear receptors in human myometrial cells

To examine whether PSF inhibits expression of PR, we over-expressed GFP-tagged PSF in primary cultures of human myometrial cells by transient transfection; a two- to threefold increase in total PSF expression was calculated (Fig. 2A). Confocal microscopy of live unfixed cells after transfection indicated that exogenous GFP-tagged PSF localises to nuclear speckles in a similar manner to endogenous PSF (Fig. 2B), as previously reported (Dye & Patton 2001, Shav-Tal *et al.* 2001).

Results presented in Fig. 2C indicate that exogenous PSF has a significant inhibitory effect ($P<0.001$) on the expression of myometrial PR; lysates from cells over-expressing PSF (+PSF) are shown in lanes 1 and 3 and lysates from non-transfected cells (–PSF) are shown in lanes 2 and 4. The inhibitory effect of exogenous PSF on PR-B protein expression was also verified using a PR-B-specific PR antibody (Fig. 2D) and at the mRNA level by RT-PCR using primers that only amplify the unique mRNA sequence of PR-B (Fig. 2E). Over-expression of PSF had no effect on the oestrogen receptors, ER α and ER β (Fig. 2F, upper panels, lanes 1 and 3) but did appear to some extent increase

Figure 1 Expression of human myometrial PSF at term and in labour. (A) Detection of PSF protein expression by western immunoblotting using (50 µg) samples prepared from non-pregnant myometrium and paired samples from the upper (US) and lower (LS) segments of the uterus from term pregnant (not in labour) and labouring myometrium. Equal loading of protein was confirmed by re-probing membranes for GAPDH. (B) Densitometric analysis of PSF expression was undertaken to quantify levels and spatial changes in myometrial PSF expression. Data are shown for non-pregnant (NP); term upper (P-US) and lower (P-LS) and labouring: upper (L-US) and lower (L-LS) samples. Analysis was undertaken using a one-way ANOVA and a Bonferroni's multiple comparison test ($n=14$ for each set of samples): NP versus P-US, $P<0.001$; NP versus P-LS, $P>0.05$; NP versus L-US, $P<0.001$; NP versus L-LS, $P>0.05$; P-US versus P-LS, $P<0.05$; L-US versus L-LS, $P<0.01$. Data, presented as arbitrary units, are the mean $\pm$ s.e.m. ($n=14$ for all tissues). (C) Expression of PSF in non-pregnant myometrium and paired samples from the US and LS of the uterus from 28, 29, 32, 33 and 34 weeks gestation: (D) Densitometric analysis: NP versus PT-US, $P<0.001$, NP versus PT-LS, $P>0.05$.

expression of GR within myometrial cells, although this was not statistically significant ($P > 0.05$; Fig. 2F, lanes 1 and 3). We next investigated whether inhibition of endogenous PSF by siRNA (Fig. 3A, upper panel) had any effect on myometrial PR-B and GR expressions. As shown, the levels of PR increased and GR decreased respectively as a direct consequence of reducing

endogenous PSF within myometrial cells by ~60% (Fig. 3A); interestingly, a similar effect was observed when we treated cultured myometrial cells with the HDAC inhibitor, TSA (Fig. 3B). Equal loading of protein was confirmed by Ponceau-S staining (data not shown) and re-probing membranes for GAPDH, as shown.

Nuclear co-regulatory proteins that interact with PSF

Co-immunoprecipitation experiments (Co-IP) were employed to identify potential co-regulatory proteins that may interact with PSF within the nuclei of cultured myometrial cells. The results presented in Fig. 4A are from Co-IP experiments with anti-PSF as the capture antibody and immunoblotting analyses using a range of specific antibodies to different nuclear transcription factors and co-regulators, as indicated, to detect proteins that potentially interact with PSF. PSF appeared to bind Sp proteins, Sp1 and Sp3, class I histone deacetylases, HDAC1 and HDAC2, MEF2, Sin3A, PR and GR; PSF did not directly bind to nuclear RbAp48 or polymerase II subunits (data not shown). Interestingly, acetylated proteins in the 55–65 kDa range, as detected by immunoblotting using an acetylated lysine antibody, bind with PSF in myometrial cell extracts as do both PR and GR (Fig. 4A). Evidence to show that PSF interacts with both nuclear hormone receptors was also obtained by repeating the Co-IP experiments with PR-B-specific or GR-specific antibodies as the capture antibodies and using anti-PSF as the detection antibody (Fig. 4B). Co-IPs using rabbit IgG as the capture antibody were included as controls.

PSF interacts with two DNA-binding motifs within the PR promoter

Previous studies have reported that the promoter regions for PR-B and PR-A contain functional consensus Sp1 sites (PR-B has one Sp1 site and PR-A has two Sp1 sites and a half ERE site; *Petz et al.* 2004). We have also identified a novel potential MEF2-binding site (CTA(A/T₆)) within the PR promoter sequence located at –1340 nucleotides upstream from the first transcription start site (GeneBank accession number NM_000926). To determine whether this MEF2 site may be functional, double-stranded biotin-labelled oligonucleotides were designed containing the MEF2 DNA sequence and DAPA performed. Data indicate that MEF2, HDAC1, HDAC2, mSin3A, RbAp48 and PSF may form part of multi-protein complexes bound to a MEF2

consensus sequence (Fig. 5A) but not the mutated MEF2 sequence. Results from DAPA using the oligonucleotides containing DNA sequences for the different Sp1-binding sites indicate that PSF, Sp1, Sp3, HDAC1, HDAC2 and RbAp48 may form part of a multi-protein complex bound to the Sp1-binding site within the PR-B promoter (Fig. 5B) and PSF, Sp1, Sp3, HDAC1, HDAC2 and ER β form part of the multi-protein complex bound to the Sp1/half ERE-binding site within the PR-A promoter (Fig. 5C).

To further investigate the potential interactions of PSF with the PR promoter, we employed ChIP with the anti-PSF monoclonal antibody and chromatin prepared from cultured human pregnant myometrial cells. Three regions of the PR promoter were analysed, the –1375 to –1244 DNA sequence (which contains the MEF2-binding site); the –884 to –673 sequence (which contains the Sp1-binding site within the PR-B promoter) and the –296 to –106 sequence (which contains the two Sp1/half ERE sites within the PR-A promoter). Primers flanking these regulatory regions (Table 2) were used to amplify their target sequences by PCR. Data as shown in Fig. 5E provides further evidence to suggest that PSF may bind to two regulatory regions, containing Sp1-binding sites, within the PR promoter.

PSF interacts with consensus PRE

The observation that PSF interacts with specific isoforms of PR in the nuclei of myometrial cells raises the possibility that PSF has the potential to affect PR binding to its hormone response element (HRE). To address this, DAPA experiments, as described above, were undertaken using biotin-labelled oligonucleotides for a consensus PRE and a mutated PRE. Results demonstrated that PSF and specific isoforms of PR (114 kDa, 98 kDa and a small 30–40 kDa PR subtype) bind/complex to the consensus PRE oligonucleotide; these findings were verified using several different PR-specific antibodies (Fig. 6A). Both HDAC1 and HDAC2 were found to be present within the PRE-bound multi-protein complex (Fig. 6A). In addition,

Figure 2 Effect of PSF on nuclear hormone receptor expression in myometrial cells. (A) Immunoblotting using anti-PSF and anti-GFP antibodies shows over-expression of PSF after transient transfection in primary myometrial cultures with a GFP-tagged PSF expression plasmid. GFP-tagged PSF was visualised by immunofluorescence and confocal microscopy using live unfixed cells post transfection; immunostaining of non-transfected fixed myometrial cells using a specific monoclonal anti-PSF antibody and DAPI. (C) Immunoblotting showing the effect of exogenous PSF on the expression of PR-B, and PR-A. Data are shown in duplicate using cell lysates from two separate transient transfection experiments; lysates prepared from cells over-expressing PSF (+PSF) are shown in lanes 1 and 3 and lysates from non-transfected cells (–PSF) are shown in lanes 2 and 4. Equal loading is shown by re-probing membranes with anti-GAPDH. (D) Effect of exogenous PSF on PR-B expression using a PR-B-specific monoclonal antibody (NCL-PGR-B, Table 1). GAPDH controls for each sample are shown in lower panel, lanes 1–4. (E) RT-PCR using primers that amplify the unique mRNA sequence of PR-B was undertaken to evaluate the effect of exogenous PSF on PR-B mRNA expression; RT-PCR using primers for GAPDH were included as controls. (F) The effect of exogenous PSF on ER α , ER β and GR expression; lysates prepared from cells over-expressing PSF (+PSF) are shown in lanes 1 and 3 and lysates from non-transfected cells (–PSF) are shown in lanes 2 and 4. The bars show the mean $\pm$ S.E.M. of values from four independent experiments.

Figure 3 Inhibition of PSF increases expression of myometrial PR-B and decreases expression of GR. (A) Primary myometrial cells were transfected with a pool of three target-specific siRNA (40–80 pmols) targeted against PSF, irrelevant control siRNA (con) or vehicle (lipid) only for 4 days. PSF proteins levels were assessed by immunoblotting (top panel) and expression of PR-B, GR and GAPDH mRNA, analysed by RT-PCR (lower panels). (B) Effect of HDACi (TSA) on PR-B, GR and GAPDH; myometrial cells were treated with TSA (330 nM) for 24 h and levels of protein assessed by immunoblotting.

data from DAPA experiments indicate that GR present in myometrial nuclear extracts binds directly to the consensus PRE but not the mutated PRE (Fig. 6A).

The effect of PSF on the PR/GR-target genes COX-2 and HDAC2

We next went on to look into whether PSF regulated specific PR- and GR-target genes *in vivo*. The two candidate PR/GR-target genes chosen for this preliminary investigation were COX-2 and HDAC2 (Davies *et al.* 2006). Transient transfections to over-express PSF in cultures of pregnant myometrial cells were undertaken, as described above. The data shown in Fig. 6B (compare lanes 1 and 3 to lanes 2 and 4) indicate that over-expression of PSF (resulting in a two- to threefold increase in the total levels of PSF) inhibits expression of COX-2 protein ($P < 0.001$; in the absence of LPS), but appears to increase the protein levels of HDAC2 in cultured myometrial cells ($P < 0.05$). Equal loading of protein was confirmed by re-probing membranes for GAPDH, as shown. The inhibitory effect of exogenous PSF on COX-2 expression was also observed at the mRNA level (Fig. 6C, compare lanes 1 and 3 to lanes 2 and 4). Over-expression of PSF had no inhibitory effect on COX-2 protein levels, in the presence of LPS (Fig. 6D). Since PSF has previously been shown to directly bind to activated promoters (Rosonina *et al.* 2005), we then went on to analyse whether PSF interacts with any regulatory regions within the promoter of the COX-2 gene. Two regions of the COX-2 promoter were analysed by ChIP PCR analysis: the DNA sequence from –495 to –292 (which contains a proximal NFκB-binding site (Appleby *et al.* 1994) and an upstream –702 to –474 sequence (which contains a CRE-binding site). Data suggest that, in

the presence of progesterone, PSF may bind to a specific NFκB-binding site within the COX-2 promoter (Fig. 6E) but not a consensus CRE-binding site.

Discussion

The precise molecular mechanisms controlling PR-mediated gene regulation within the human myometrium in pregnancy and in labour are poorly defined but are likely to be important as functional withdrawal of progesterone is thought to be facilitated by changes in the expression and signalling events of myometrial PR. It is known that PR recruit different nuclear co-regulators to mediate receptor-specific transcription regulation, and expression of these co-factors may change within the myometrium prior to the onset of labour (Condon *et al.* 2003, Vienonen *et al.* 2004). We show for the first time that nuclear human myometrial PSF levels increase significantly with advancing pregnancy and remain elevated in labour and that over-expression of myometrial PSF inhibits expression of PR-B *in vitro*. This observation is in keeping with previous evidence that report PSF mRNA increases in rat myometrium at term and PSF inhibits PR function (Dong *et al.* 2005). Protein:DNA-binding assays undertaken in this study demonstrate that endogenous PSF interacts with at least two regulatory regions within the promoter of the human PR gene; these findings were also validated by ChIP analyses. The observation that PSF directly binds to regulatory regions within the PR promoter is intriguing and further studies will evaluate whether these protein:DNA interactions are functionally relevant.

It is widely accepted that transcription and pre-mRNA processing are tightly coupled interdependent events that include recruitment of RNA polymerase II to activated promoters together with a complex interplay of DNA- and RNA-binding co-regulatory proteins (Auboeuf *et al.* 2002, 2004). A direct association between the strength of promoter-bound activators and the efficiency of pre-mRNA splicing and 3'-end cleavage has been recognised. With relevance to the present study, PSF has been shown to specifically facilitate the ability of a transcriptional activator (VP16) to promote efficient co-transcriptional splicing and 3'-end cleavage (Rosonina *et al.* 2005). Moreover, a recent study by Guillouf *et al.* (2006) has reported that another transcription factor, Sp1-1/PU.1, mediates alternative pre-mRNA splicing events of a gene (E1A), whose transcription it regulates, in a promoter-binding dependent manner. This latter study clearly demonstrated that Sp1-1/PU.1 modulates splicing as a consequence of its ability to bind DNA. It is known that naturally occurring alternative promoters can influence splicing events and, although purely

Figure 4 Co-regulatory proteins that interact with PSF within myometrial nuclei. Co-immunoprecipitations (Co-IP) were undertaken to identify potential PSF-binding proteins by using anti-PSF as a capture antibody and immunodetection analyses using specific antibodies to different nuclear transcription factors and co-regulators. IP, capture antibody; WB, immunodetection antibody; ne, nuclear extract. (A) In myometrial nuclei, PSF directly binds to Sp1, Sp3, HDAC1, HDAC2, MEF2, Sin3A, PR, GR and acetylated proteins in the 55–65 kDa range, as detected using an acetylated lysine antibody. (B) Co-IP with PR-B-specific or GR-specific antibodies as the capture antibodies and immunoblotting using the anti-PSF antibody were employed to further validate whether PSF directly binds to PR and GR; the presence of PR-B homodimers within myometrial nuclei was also observed. Co-IP using rabbit IgG as the capture antibody included as controls are shown. Experiments were performed a minimum of three times, with similar results.

speculative, it is conceivable that PSF may influence co-transcriptional and splicing decisions of PR in a promoter-binding-dependent manner.

The transcriptional co-repressor activity of PSF with type II hormone receptors, such as TR and RXR in 293T cells, occurs via its interactions with Sin3a and through the recruitment of class I histone deacetylases (Mathur *et al.* 2001). In the present study, we also provide evidence to suggest that PSF binds to Sin3a, HDAC1, HDAC2, Sp1 and Sp3 in human myometrial nuclei and complexes of these regulatory proteins interact with Sp1-binding sites within the PR promoter. Multi-protein complexes similar to these have been documented. In one such study investigating the silencing of the PR-target gene, LH receptor (LHR) in JAR cells, a HDAC–mSin3A–RbAp48–protein complex interacts directly with the Sp1/Sp3 sites present within the LHR promoter (Zhang & Dufau 2002). Interestingly, we found that PSF may interact with the transcription factor MEF2 in uterine smooth muscle cells. MEF2 functions either as a transcriptional activator or

repressor to regulate various muscle-specific genes (McKinsey *et al.* 2002). MEF2 represses the expression of specific target genes in a calcium-dependent manner, an activity that is usually associated with its interactions with class II histone deacetylases (Han *et al.* 2005). MEF2 also interacts with, and is acetylated by, p300 (Ma *et al.* 2005). In this study, we have identified a novel MEF2 DNA-binding motif within the promoter of the PR gene and observe that MEF-2, PSF and class I histone deacetylases, HDAC1 and HDAC2, all complex with the MEF2 motif. Whether PSF or MEF2 play a role in tethering co-regulatory proteins to the PR promoter and/or influences expression of myometrial PR *in vivo* remains to be elucidated.

Over-expression of PSF in human myometrial cell cultures also resulted in a modest increase in the expression of GR. This latter observation conflicts with previous studies that describe PSF as a co-repressor of hormone receptor gene expression (Mathur *et al.* 2001, Dong *et al.* 2005). However, over-expression of nuclear proteins by transfection can disturb the overall nuclear

Figure 5 DNA binding of PSF and other nuclear proteins to specific sequences within the human PR promoter. (A) DAPA using 5' biotin-labelled double-stranded oligonucleotide, containing the DNA sequence for a potential MEF2-binding site within the PR promoter motif (CTA(A/T)₆) was employed and shows that PSF, MEF2, RbAp48, HDAC1, HDAC2 form multi-protein complexes at the MEF2-binding site. (B and C) Precipitated protein complexes identified using DAPA with a 5' biotin-labelled double-stranded oligonucleotides for the (B) Sp1-binding site within the PR-B promoter and (C) Sp1/half ERE-binding site within the PR-A promoter include PSF, Sp1, HDAC1, HDAC2, RbAp48 and PSF, HDAC1, HDAC2, ERβ, respectively. (D) ChIP analysis was undertaken using myometrial cells (3×10^7) cultured with progesterone (10 μ M) and treated with 1% formaldehyde to cross-link proteins to the DNA. Three regions of the PR promoter were then analysed by PCR to investigate PSF-DNA binding; the -1375 to -1244 DNA sequence (MEF2-binding site); the -884 to -673 sequence (Sp1-binding site within the PR-B promoter) and the -296 to -106 sequence (Sp1/half ERE site within the PR-A promoter). Experiments were performed a minimum of three times, with similar results.

environment within cultured cells and lead to anomalies when interpreting data. Consequently, reducing PSF expression by RNA interference in non-transfected cells was also undertaken to validate

whether PSF genuinely decreases and increases myometrial expression of PR and GR respectively. More importantly, in this study we show that endogenous PSF directly binds to both PR and GR within myometrial nuclei and may form part of a multi-protein complex bound to a consensus PRE. Our results cannot, therefore, exclude the possibility that PSF has the potential to compete or affect binding of nuclear co-regulatory proteins involved in hormone response signalling.

The observation that PR and GR both interact with the same consensus PRE, while novel in human myometrial cells, is not surprising. These findings highlight the complexity and promiscuity between PR- and GR-mediated gene regulation as both hormone receptors are capable of binding the same HRE (Horie-Inoue *et al.* 2006). Our results also reinforce the hypothesis that differential recruitment of specific co-activators/co-repressors is likely to be a key step in remodelling of chromatin and transcription regulation at specific promoters of myometrial hormone response genes (Li *et al.* 2003). Other factors such as changes in the expression, acetylation and phosphorylation of specific nuclear co-activators/co-repressors, including PSF, may also influence PR and GR binding to their respective HRE. Published data, combined with our data, support this possibility in that levels of specific PR co-activators decrease within the myometrium at term (Condon *et al.* 2003) and potential PR co-repressors such as PSF increase (Dong *et al.* 2005, Tyson-Capper & Robson 2007). In addition, class 1 histone deacetylases (Harper *et al.* 2007) and MEF2 are both spatially and temporally regulated within the myometrium during pregnancy and labour (unpublished data).

Our data from binding studies suggest that HDAC1 and HDAC2 interact with PSF in myometrial nuclei and complex to a consensus PRE. This observation is consistent with previous studies reporting that HDAC1 and HDAC2 are commonly found together within the same multi-protein complexes and contribute to the remodelling of chromatin at specific promoters. Interestingly, treatment of cultured myometrial cells with the HDAC inhibitor, TSA, reversed the inhibitory and stimulatory effects of PSF on PR and GR expressions respectively. Since changes in acetylation status is a contributing factor to HDAC binding, the acetylation status of the PSF-bound HDACs should also be assessed in the future. A direct link between PR- and GR-mediated transcription and changes in histone acetylation/deacetylation has been described (Qiu *et al.* 2006, Aoyagi & Archer 2007); this is particularly relevant for this study as evidence now indicates that HDAC1 can act as a co-activator for GR and hormone stimulation of GR can lead to progressive acetylation of HDAC1 *in vivo* (Qiu *et al.* 2006).

Figure 6 A role for PSF in PR-mediated gene regulation. (A) DAPA using 5' biotin-labelled double-stranded oligonucleotides for a consensus PRE and mutated PRE (details are shown in Table 2) with 50 µg myometrial nuclear extract. The precipitated protein complexes bound to the PRE were identified by immunoblotting using antibodies against PSF, PR (C20, top panel; NCL-PGR-AB, second panel), HDAC1, HDAC2 and GR. No protein binding was observed using the mutated PRE. (B) The effect of over-expressing PSF on candidate PR/GR-target genes, COX-2 and HDAC2. Results are shown in duplicate using samples from two separate transient transfections; samples over-expressing PSF (+PSF) are shown in lanes 1 and 3 and samples from non-transfected cells (-PSF) are shown in lanes 2 and 4. Equal protein loading was confirmed by re-probing membranes for GAPDH. Bars show the mean $\pm$ S.E.M. of values from four independent experiments. (C) RT-PCR indicates that exogenous PSF also inhibits COX-2 mRNA (upper panel); RT-PCR using primers for GAPDH were included as controls. (D) Myometrial cells over-expressing PSF (and non-transfected cells) were treated with LPS (1 µg/µl) or vehicle (lipid) for 24 h and levels of COX-2 expression analysed by immunoblotting. (E) ChIP analysis was undertaken using pregnant myometrial cells (3×10^7) cultured with progesterone (10 µM) and treated with 1% formaldehyde to cross-link proteins to the DNA. Two regions of the PR promoter were analysed by PCR; DNA sequences from -495 to -292 (which contains a proximal NFκB-binding site) and an upstream -702 to -474 sequence. Experiments were performed a minimum of three times, with similar results.

To determine whether PSF binding to PR and GR may have functional implications, we carried out a preliminary investigation to see whether exogenous PSF has the potential to influence expression of two PR/GR-target genes, HDAC2, and an inducible gene, COX-2. Expression of COX-2, and prostaglandin production, increases in myometrium and foetal membranes prior to labour and promotes uterine activity (Slater *et al.* 1999,

Challis *et al.* 2000). Our data suggest that over-expression of PSF decreases COX-2 expression in myometrial cell cultures, in the absence of LPS but is insufficient to interfere with the activation of COX-2 expression by LPS. However, since PSF levels also increase with advancing pregnancy, it is unlikely that PSF influences myometrial COX-2 expression *in vivo*. This paradox may be explained, at least in part, by the observation that

exogenous PSF appears to increase the expression of myometrial GR in cultured cells, which, unlike PR, express high levels of GR upon culturing (unpublished data). In keeping with this, previous studies have shown that COX-2 is a GR- and dexamethasone-repressed gene in a range of different cell types (Ristimäki *et al.* 1996, Nishimori *et al.* 2004, Wang *et al.* 2004) and HDAC2 a dexamethasone/progesterone-activated gene (Davies *et al.* 2006). Moreover, recent evidence has suggested that at term, in amnion cells (Mitchell *et al.* 2007), GR binds directly to the same proximal region of the COX-2 promoter as we have demonstrated for PSF in this study. It is also plausible that exogenous PSF may influence expression and/or activity of other negative-regulatory proteins that control transcriptional or post-transcriptional regulation of COX-2.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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