

Regulated expression of putative membrane progesterin receptor homologues in human endometrium and gestational tissues

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Abstract

Rapid non-genomic actions of progesterone are implicated in many aspects of female reproduction. Recently, three human homologues of the fish membrane progesterin receptor (mPR) have been identified. We combined bioinformatic analysis with expression profiling to define further the role of these mPRs in human reproductive tissues. Sequence analysis confirmed that the mPRs belong to a larger, highly conserved family of proteins, termed 'progesterin and adiponectin receptors' (PAQRs). A comparison of the expression of mPR transcripts with that of two related PAQR family members, PAQRIII and PAQRIX, in cycling endometrium and pregnancy tissues revealed markedly divergent expression levels and profiles. For instance, endometrial expression of *mPRα* and *γ* and *PAQRIX* was cycle-dependent whereas the onset of

parturition was associated with a marked reduction in myometrial *mPRα* and *β* transcripts. Interestingly, *mPRα* and *PAQRIX* were most highly expressed in the placenta, and the tissue expression levels of both genes correlated inversely with that of the nuclear PR. Phylogenetic analysis demonstrated that *PAQRIX* belongs to the mPR subgroup of proteins. We also validated a polyclonal antibody raised against the carboxy-terminus of human *mPRα*. Immunohistochemical analysis demonstrated more intense immunoreactivity in placental syncytiotrophoblasts than in endometrial glands or stroma. The data suggest important functional roles for *mPRα*, and possibly *PAQRIX*, in specific reproductive tissues, particularly those that express low levels of nuclear PR.

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Introduction

Progesterone is required for all aspects of female reproductive function, including sexual behaviour, gonadotrophin secretion, ovulation, blastocyst implantation and maintenance of pregnancy (Mulac-Jericevic *et al.* 2000, Conneely *et al.* 2002, Mulac-Jericevic & Conneely 2004). The human uterus is a major target of progesterone action. During the menstrual cycle, the postovulatory rise in circulating progesterone induces coordinated expression of gene sets in the endometrium that initially define a limited period of uterine receptivity (implantation window) and subsequently control differentiation of the stromal compartment (decidualization) (Brosens *et al.* 1999, Gellersen & Brosens 2003). This decidual process is critical for trophoblast invasion and the formation of a functional

placenta. In pregnancy, progesterone ensures a relative state of myometrial quiescence, and the onset of parturition is thought to involve inhibition of progesterone signalling, resulting in 'functional progesterone withdrawal' (Astle *et al.* 2003, Condon *et al.* 2003, Mesiano 2004).

Progesterone regulates the expression of specific gene networks in the female reproductive tract, and other target tissues, predominantly through activation of the nuclear progesterone receptors (PR-A and PR-B), members of the steroid/thyroid hormone receptor superfamily of transcription factors (Aranda & Pascual 2001, Brosens *et al.* 2004). However, not all effects of progesterone are genotrophic, and, like other steroid hormones, progesterone has rapid effects on cell-signalling pathways independently of transcription (Bramley *et al.* 2002, Losel & Wehling 2003, Thomas *et al.* 2004). These non-genomic steroid effects

are thought to be initiated at the cell surface by unique membrane receptors, although there is compelling evidence that some of the rapid, post-transcriptional progesterone actions involve activation of 'classical' steroid receptors (Boonyaratankornkit *et al.* 2001). Recently, Zhu and co-workers identified and characterized three novel, putative membrane progesterin receptors (mPR α , β and γ ; also termed PAQRVII, PAQRVIII and PAQRV respectively) in a variety of vertebrate species, including man (Zhu *et al.* 2003a,b, Thomas *et al.* 2004). Interestingly, these conserved mPRs are structurally unrelated to nuclear PR but resemble seven-transmembrane domain G protein-coupled receptors (GPCRs). The three membrane progesterin receptors (mPRs), encoded by different genes, belong to a large and nearly ubiquitous family of proteins found in both prokaryotes and eukaryotes (Lyons *et al.* 2004). This family has been named the PAQR (progesterin and AdipoQ-receptor) family of proteins because several of its members are steroid or adiponectin (AdipoQ) receptors (Lyons *et al.* 2004).

Initial studies indicated that the expression of mPRs is highly tissue-specific, with mPR α being the predominant isoform in reproductive tissues, mPR β in neural tissues, and mPR γ in the gastrointestinal tract (Zhu *et al.* 2003a). The signalling pathways modulated by the human mPRs are yet to be defined. However, Zhu and co-workers demonstrated that progesterone transiently activated the MAP kinase signalling cascade and lowered intracellular cAMP levels in human MDA-MB-231 breast cancer cells stably transfected with the sea-trout homologue of the human mPR α (Zhu *et al.* 2003b). Furthermore, the effect of progesterone on cAMP levels was reversed by preincubation with pertussis toxin, suggesting that the sea-trout mPR activates an inhibitory G protein. Interestingly, intracellular cAMP levels are a major determinant of progesterone responses in both endometrium and myometrium (Pohnke *et al.* 1999, Christian *et al.* 2002a,b, Phillips *et al.* 2002, Gellersen & Brosens 2003, MacDougall *et al.* 2003), suggesting that non-genomic and genomic progesterone effects in the female reproductive tract are intimately intertwined and determined by the relative tissue expression of the various mPRs and nuclear PRs.

To provide insights into the potential roles of mPRs in the female reproductive tract, we have profiled the expression of mPR α , β and γ in the endometrium throughout the menstrual cycle and in placenta, fetal membranes, and myometrium before and after the onset of labour. In addition, we have cloned and characterized two related PAQR family members, termed PAQRIII and PAQRIX, and determined their expression in progesterone target tissues. Comparative analysis of the relative expression of mPRs, PAQRIII, and PAQRIX transcripts demonstrated that mPR α is the most abundantly expressed, regulated PAQR family member in all tissues examined, except for non-pregnant endometrium, which relatively expresses more mPR β mRNA.

Materials and Methods

Characterization of the mPR homologues PAQRIX and PAQRIII

The Genbank human genome assembly (Build 28) was downloaded from UCSC (<http://genome.ucsc.edu/>) and used to generate a set of six frame translations and GENSCAN predictions (Burge & Karlin 1997). PSI-BLAST profiles were derived from a multiple alignment of known mPRs (Altschul *et al.* 1997). The profile was used to query the six frame translations and human gene predictions. Associated genomic regions were then extracted with 5 kb of sequence either side of the original match and analysed by GENEWISE (Birney *et al.* 2004), seeded with the closest related mPR sequence as the query protein. GENEWISE predictions were further hand-crafted with EST data and comparative genomic data from mouse in a modified Genotator-based genomic workbench (Harris 1997). Multiple sequence alignments were generated with ClustalW and visualized with JalView or GeneDoc (www.psc.edu/biomed/genedoc) (Chenna *et al.* 2003, Clamp *et al.* 2004). Transmembrane regions were predicted with a combination of TMHMM (Krogh *et al.* 2001), TMPRED (www.ch.embnet.org/Software/TMPRED_form.html) and MEMSAT transmembrane prediction programs (McGuffin *et al.* 2000).

Tissue specimens

Endometrial biopsies were obtained from 45 normally cycling women, aged 24–40 years, awaiting *in vitro* fertilization (IVF) treatment for either tubal or male-factor infertility. All women had regular menstrual cycles and were not on hormonal treatment at the time of biopsy. Samples taken within 10 days of the start of the last menstrual period were classed as 'early to midproliferative', those taken between days 11 and 14 as 'late proliferative', those taken between days 15 and 23 as 'early to midsecretory', and samples obtained on day 24 or later as 'late secretory' endometrium. Myometrial biopsies were obtained from 36 pregnant women undergoing Caesarean section for fetal distress, breech presentation, previous section, placental praevia, maternal request or failure to progress. Biopsies were collected from women from the upper margin of the lower uterine segment before or after the onset of labour. Labour was defined as regular contractions (<3 min apart) plus membrane rupture and cervical dilation (>2 cm) with no augmentation (oxytocin or prostaglandin administration). In addition, we sampled the placenta, chorion and amnion from 18 pregnancies. Tissues were immersed in RNAlater (Ambion) and stored at -80°C until use. Informed, written consent was obtained from all patients, and the study received approval from the local research ethical committee of each participating centre.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from tissues by using TRIzol (Invitrogen) according to the manufacturer's instructions. Equal amounts of total RNA (1 µg) were treated with DNase (Invitrogen), and cDNA was prepared by using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. The resulting first-strand cDNA was used as a template in the real-time quantitative PCR.

Cloning of mPR α , β and γ , PAQRIII and PAQRX

The human (h)mPR α cDNA (coordinates -97/+1079 relative to the translational start codon) was amplified by RT-PCR on RNA from the human breast cancer cell line T47D with the following primers: 5'-GCTGAAGTTT GCCTGACACCATC-3' (hmPRalpha-5) and 5'-CCC AACTATACCTCCCTCCCTACC-3' (hmPRalpha-3). Human mPR γ cDNA (-79/+1061 relative to the start ATG) was amplified by RT-PCR on RNA from T47D cells, using primers 5'-AGGCCATGTTAGAGCTTT GAGTGA-3' (hmPRgamma-5) and 5'-CCGGCTTAT GGTGGTTAGGATG-3' (hmPRgamma-3). PCR products were cloned into pCR-BluntII-TOPO (Invitrogen) to yield pCR-Blunt/hmPR α and pCR-Blunt/hmPR γ respectively. Mammalian hmPR expression vectors with C-terminal V5-tag, pcDNA3.1/V5/hmPR α and pcDNA3.1/V5/hmPR γ were generated as follows. The hmPR α insert (-97/+1037) was amplified from template pCR-Blunt/hmPR α , using an SP6 primer (sense) and an antisense primer 5'-TTTTCTCGAGTTGGTCTTCTG ATCAAGTTTG-3' (hmPRalpha-3X), which mutates the stop codon and adds a 3' XhoI site (underlined). Similarly, the hmPR γ insert (-79/+989) was amplified from template pCR-Blunt/hmPR γ , using an SP6 primer (sense) and an antisense primer 5'-TGGTCTCGAGGT TTCTTTTTTATGTAATTCTGG-3' (hmPRgamma-3X) to mutate the stop codon and add a 3' XhoI site. Amplicons were digested with BamHI and XhoI and cloned into the respective sites of pcDNA3.1/V5-His (Invitrogen). An expression vector for human mPR β with C-terminal V5-tag, pcDNA3.1/V5/hmPR β , was generated by PCR on an EST clone in BluescriptR (GenBank Accession no. BC030664; MRC-GeneService), using a T7 primer (sense) and an antisense primer 5'-GGTTCTCGAGGAATCTTCTTGGTCAGTCTG-3' (hmPRbeta-3X), which mutates the stop codon and adds a 3' XhoI site. The PCR product, comprising positions -149/+1062 (relative to the start codon) of the hmPR β cDNA, was cut with EcoRI and XhoI and inserted into the respective sites of pcDNA3.1/V5-His. Eukaryotic expression vectors for hmPR α with N-terminal HA-tag were constructed as follows. From the vector pDISPLAY (Invitrogen), the transmembrane domain was deleted by digestion with BsmI/NotI and blunt-end religation

(pDISPLAY-TMD). The hmPR α cDNA was amplified with *Pfu* polymerase (Promega) on template pCR-Blunt/hmPR α , using a 5'-primer which mutates Met-1 and Met-3 and adds a 5' ApaI site (underlined) (5'-AAA AGGGCCCTGGCCAGAACTC-3'; hmPRalpha-Apa), and 3'-primer hmPRalpha-3 (see above). After digestion with ApaI, the fragment was inserted into the ApaI and SmaI sites of pDISPLAY-TMD. The resultant construct, pSP-HA/hmPR α , encompasses bases +10/+1079 relative to the start codon of hmPR α cDNA, preceded by the Igk signal peptide and a HA-tag. The latter two epitopes were removed by excision with EcoRV/ApaI and replaced by a double-stranded oligonucleotide which encodes a HA-tag with an added start ATG and carries a 5' EcoRV and a 3' ApaI overhang. The double-stranded fragment resulted from annealing of the following oligonucleotides: Eco-HA-Apa-s: 5'-ATC ACCATGTACCCATACGATGTTCCAGATTACGC TGGGGCC-3', Eco-HA-Apa-as: 5'-CCAGCGTAAT CTGGAACATCGTATGGGTACATGGTGAT-3' (HA-tag underlined). This construct, pHA/hmPR α , encodes the hmPR α cDNA from the fourth codon, tagged with an N-terminal HA epitope. PAQRX was cloned by PCR into pCR4-TOPO TA cloning vector (Invitrogen) using genomic cDNA as template and the following primers: 5'-TGGGCACAAAGGCTCCGA-3' (hmPAQRX-5) and 5'-CAAGGCGGAGGCTCACTT-3' (hmPAQRX-3). PAQRIII was cloned by PCR using testis cDNA as template with the following primers, 5'-ATGCAT CAGAAGCTGCTGAAG-3' (hmPAQRIII-5) and 5'-TCACAAATGTGAAACATAGTC-3' (hmPAQRIII-3), and subcloned into the pGEMTEasy vector (Promega). All plasmids were checked by sequence analysis.

Real-time quantitative PCR (RTQ-PCR)

Table 1 shows the specific primer sets for mPR α , β and γ ; nuclear PR; PAQRIII; PAQRX; PRL; and L19 transcripts, designed with Primer Express 1.5a (Applied Biosystems, Brackley, UK). The primers for the nuclear PR were designed to detect both PR-A and PR-B transcripts. RTQ-PCR was performed for 40 cycles (95 °C for 15 s, 60 °C for 1 min), after initial 10-min incubation at 95 °C, using an ABI Prism 7700 sequence detection system (Applied Biosystems). For quantification of mPR α , β and γ ; nuclear PR; PRL; and L19, RTQ-PCR was performed with SYBR Green (Applied Biosystems). Each reaction contained 12.5 µl SYBR Green and 1 µl cDNA in a final volume of 25 µl. For quantification of PAQRIII and PAQRX, RTQ-PCR was performed with a double-labelled fluorogenic probe that hybridizes to the target sequence between the two primer recognition sites (Eurogentec, Seraing, Belgium). The probe sequences for PAQRIII and PAQRX were as follows: 5'-AGT CCC AGA GCG GTA CTT TCC AGG ACA-3' and 5'-CAT TAT CGG CCA CAG CCA

Table 1 Sequence of primers used for real-time quantitative PCR and respective fragment size

	Forward (5'–3')	Reverse (5'–3')	Size of amplicon (bp)
Transcript			
mPR α	ACC ATC AAC CAG GCC CTA GTC	TGG GCA GGG AGC TAA GAT CA	68
mPR β	CAC AAC GAG GTG GTC AAC OT	CGG CAA AGG CCC AGA AT	76
mPR γ	GGT GCC GTC AAC CTC TTC A	CATACACGTTCCCGGATG	65
PAQR IX	CCA GCC GGG TCT TTT CG	TGA GGA AGG TGA AGA TGT GGA A	64
PAQR III	TCT TGC TTT CCT ATT CTA CAT TTC CA	TGG CTT GAT CCG AGG TAG TTT AG	67
Nuclear PR	TTAATACAATTCCTTTGGAAGGGC	CAAGCTACATTAGAGAGCTCATCAAGG	84
L19	GCG GAA GGG TAC AGC AAA T	GCA GCC GGC GCA AA	77
Prolactin	AAG CTG TAG AGA TTG AGG AGC AAA C	TCA GGA TGA ACC TGG CTG ACT A	76

CCA GCT–3' respectively. Each reaction contained 1 μ l cDNA in a final volume of 25 μ l with primer and 100 nM probe. L19, a non-regulated ribosomal housekeeping gene, served as an internal control and was used to normalize for differences in input RNA. Relative expression was determined by the standard curve method. All measurements were performed in triplicate. Product purity was confirmed by agarose gel analysis. To compare the relative expression of mPR α , β and γ ; PAQR III; and PAQR IX, standard curves were generated, using comparable amounts of the following serially diluted plasmids: pcDNA3.1/V5/hmPR α , pcDNA3.1/V5/hmPR β , pcDNA3.1/V5/hmPR γ , pCR4-TOPO TA/hmPAQR IX, and pGEMTEasy/hmPAQR III. These standard curves were subsequently used to calculate the relative abundance of each transcript in pooled tissue samples.

Confocal microscopy and immunohistochemistry

An affinity-purified anti-peptide antibody was raised against the predicted last 19 amino acids (LTAFLLSQLVQRKLDQKTK) of the carboxy-terminus of human mPR α (Cambridge Research Biochemical, Cambridge, UK) and used for detection of overexpressed wild-type or tagged mPR α in COS-1 cells and endogenous mPR α in human placental tissues. COS-1 cells cultured on chamber slides were fixed in acetone/methanol (1:1 v/v). Primary antibodies and dilutions were as follows: rabbit anti-mPR α , 1:100; mouse anti-V5 (Invitrogen), 1:500; and mouse anti-HA (Covance, New Orleans, LO, USA), 1:200. Secondary antibodies used were FITC-conjugated anti-rabbit antiserum (1:20) (Dako, Glostrup, Denmark) and Alexa fluor 594 goat anti-mouse IgG (1:200) (Molecular Probes, Invitrogen). Images were acquired with a Zeiss Meta 512 confocal microscope. Paraffin-wax-embedded, formalin-fixed placental specimens were examined for *in vivo* mPR α immunoreactivity. All specimens were obtained from uncomplicated term deliveries. In addition, we stained proliferative and secretory endometrial biopsies from normal fertile women. Sections of 5 μ m, placed on 1% w/v polylysine slides, were deparaf-

finized, dehydrated, and exposed to 0.3% v/v H₂O₂ for 30 min. Immunostaining was carried out with anti-human mPR α antibody diluted 1:20, biotinylated anti-rabbit IgG diluted 1:100 and peroxidase-labelled streptavidin (ABC Kit, Vector Laboratories, Peterborough, UK). In control slides, the primary antibody was either peptide preabsorbed or omitted.

Statistical analysis

Data were analysed with the Statistical Package for Social Sciences, Version 12.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed by one-way ANOVA, followed, when appropriate, by Tukey's HSD post-hoc test when variance was assumed to be equal, or the Games–Howell test when variance was assumed to be unequal. Kolmogorov–Smirnov and Levene tests were used to check for normality and homoscedascity respectively. In some cases, logarithmic transformations were applied to obtain normality and homoscedascity. Variables that were not normally distributed were compared by the Kruskal–Wallis test followed, when appropriate, by the Mann–Whitney test with the Bonferroni correction for paired comparisons. Results are expressed as mean \pm standard error of the mean (S.E.M.). A *P* value of <0.05 was considered statistically significant.

Results

Identification and cloning of the mPR homologue INSP122 (PAQR IX) and INTP047 (PAQR III)

The putative mPRs, α , β and γ , form part of the much larger PAQR family of proteins which is conserved from yeast through to man (Lyons *et al.* 2004). We carried out an exhaustive search of unannotated human genomic sequences, using a PSI-BLAST profile derived from the known mPRs to scan sets of human gene predictions. Two human genomic regions were identified which did not coincide with known genes and represented potentially two novel members of the mPR family. The first

region mapped to human chromosome 3q23. A single coding exon gene of 1134 bp, encoding a protein of 377 amino acids (aa), was identified, which we originally named *INSP122*. The human *INSP122* prediction was supported by equivalent predictions in the mouse and rat genomes, the human prediction sharing 93% sequence identity over the full length of the prediction with the rodent sequences. The second region mapped to human chromosome 4q21. A human gene prediction of 311 aa sharing 32% sequence identity to the human CGI-45 amino-acid sequence was identified and originally named *INTP047*. A mouse orthologue encoded by BC024094 was used to refine the prediction. *INTP047* was also supported by human high-through-put cDNA sequences AK055774, BI461746 and 77 human ESTs. The prediction contains six exons, the first exon overlaps with a CpG island, suggesting transcription starts at this exon (data not shown). Subsequently, *INSP122* and *INTP047* appeared in the public databases as *PAQR IX* (GenBank Accession no. AY424287) and *PAQR III* (GenBank Accession no. AY424281) respectively, and to avoid unnecessary confusion, we have adopted the GenBank nomenclature for these gene products. As shown in Fig. 1, multiple amino-acid sequence alignments confirmed that *PAQR III* and *PAQR IX*, as well as the mPRs, belong to the PAQR family of proteins. To determine whether specific PAQR family members are selectively expressed in the human female reproductive tract, we compared the expression profiles of *PAQR III* and *PAQR IX* to those of mPR α , β and γ .

Expression of mPR homologues in cycling endometrium

Endometrial samples, obtained from women awaiting IVF treatment for male-factor or tubal infertility, were used to examine the expression of mPR α , β and γ ; *PAQR III*; and *IX* during the various stages of the menstrual cycle. Expression of PRL mRNA was used to ensure that the samples had been allocated to the appropriate phase of the cycle. RTQ-PCR demonstrated low expression of PRL mRNA in proliferative phase biopsies but markedly increased expression of this differentiation marker in samples attributed to the secretory phase of the cycle (data not shown). The various PAQR genes were expressed at mRNA level in human endometrium, but only the expression of mPR α and γ , and *PAQR IX* transcripts varied significantly according to the phase of the cycle. As shown in Fig. 2, the postovulatory rise in progesterone coincided with a significant induction of mPR α mRNA and reduced expression of mPR γ and *PAQR IX* transcripts. The downregulation of mPR γ and *PAQR IX* mRNA expression was gradual, only the levels in late-secretory phase biopsies differing significantly from those obtained in the proliferative phase of the cycle ($P < 0.05$ and $P < 0.01$ respectively).

Expression of mPR homologues in gestational myometrium, fetal membranes and placenta

There is a general consensus that progesterone is critical for myometrial quiescence throughout pregnancy. We hypothesized that changes in myometrial mPR expression may play an integral role in the shift from a quiescent to a contractile state (Karalis *et al.* 1996, Astle *et al.* 2003, Condon *et al.* 2003, Mesiano 2004). To provide evidence for this hypothesis, we examined the expression of the five PAQR family members in myometrial biopsies obtained at the time of Caesarean section. Biopsy samples were grouped depending upon whether they were obtained (1) before or after 37 weeks' gestation ('preterm' and 'term' respectively) and (2) before or after the onset of regular uterine contractions ('non-labour' and 'labour' respectively). As shown in Fig. 3, the onset of parturition at term coincided with an approximately 50% drop in mPR α and β mRNA expression. Interestingly, mPR α , but not β mRNA, levels also declined upon preterm labour, although the differences were not statistically significant ($P > 0.05$). In contrast, the onset of labour, term or preterm, was not associated with statistically significant changes in mPR γ , *PAQR III* or *PAQR IX*. Furthermore, the onset of labour at term did not significantly alter the expression of any of the PAQR genes in placenta, chorion or amnion with the exception of mPR α mRNA, which was found to be more highly expressed in amnion before the onset of parturition ($P < 0.05$; data not shown).

Comparative analysis of the relative expression of nuclear PR and mPR homologues

To determine the likely target tissues for non-genomic progesterone action, we first compared the relative expression of the mPRs, *PAQR III* and *PAQR IX* transcripts in biopsy samples pooled according to the tissue of origin. In addition, we compared the pattern of tissue expression of mPRs with that of the nuclear PR. The primers for the nuclear PR were designed to recognize both PR-A and PR-B transcripts. As shown in Fig. 4, the relative expression of mPR α and *PAQR IX* transcripts differed dramatically among the tissues examined. For instance, the abundances of mPR α and *PAQR IX* mRNAs were approximately 1560-fold and 779-fold higher in placental samples than endometrium ($P < 0.001$). In fact, the expression of both genes was significantly higher in the placenta than fetal membranes and pregnant myometrium. Interestingly, a reverse pattern of expression was observed for the nuclear PR mRNA, which was found to be 14-fold more highly expressed in the myometrium than the placenta ($P < 0.05$). While there were significant tissue-dependent variations in the expression levels of mPR β and γ and *PAQR III*, the differences were much less pronounced than those observed for mPR α and *PAQR IX*. Notably, with the exception of mPR γ mRNA, the



Figure 1 Multiple alignment of the transmembrane spanning regions of PAQRX, PAQRIII, mPRs (α , β and γ) and the adiponectin receptors AdipoR1 and AdipoR2. Predicted transmembrane regions are underlined. Conserved residues are in grey or in black if 100% identical within the alignment.

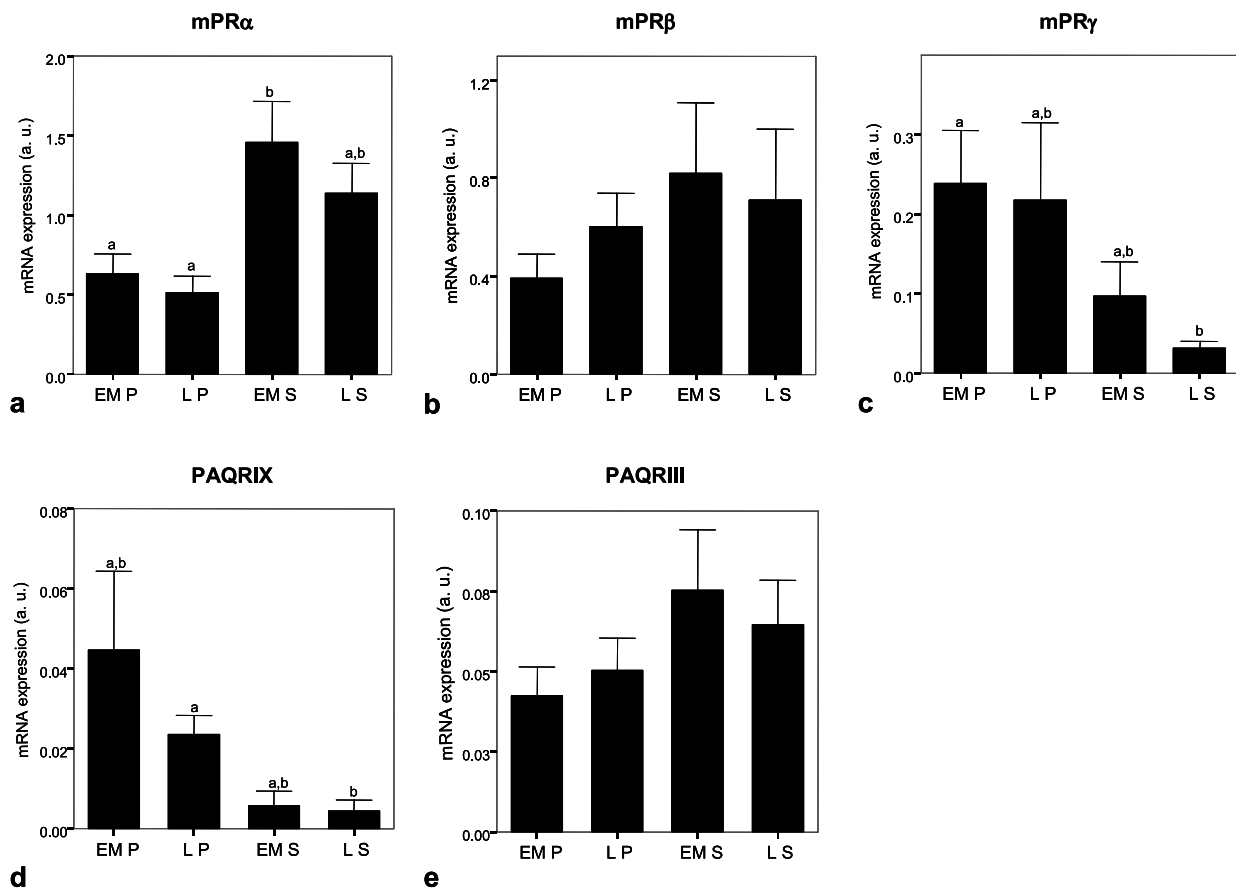


Figure 2 Cycle-dependent expression of mPR homologues in human endometrium. The endometrial expression of mRNA encoding mPR α (a), β (b) and γ (c); PAQRX (d); or PAQRIII (e) was examined in tissue samples obtained from different phases of the cycle: early-midproliferative (EMP; $n=15$), late proliferative (LP; $n=10$), early-midsecretory (EMS; $n=10$) and late secretory (LS; $n=10$). The mRNA levels are expressed in arbitrary units (a.u.), and the data represent the means \pm S.E.M. Different letters above the error bars indicate that those groups are significantly different from each other at $P<0.05$.

expression of PAQR transcripts was lowest in cycling endometrium.

Next, we aimed to determine the relative abundance of the mPR transcripts within each target tissue. To achieve this, we first amplified the coding region of each of the five PAQR family members by RT-PCR and cloned the resultant cDNAs in an expression or cloning vector. Standard curves for each of the PAQR cDNAs were generated by RTQ-PCR amplification of known amounts of plasmid and used to calculate the abundance of each transcript within pooled tissue samples. Table 2 shows the abundance of mPR β and γ , PAQRIII and PAQRX transcripts relative to mPR α mRNA expression in the various tissues examined. The results confirmed that mPR α is the most abundant transcript in fetally derived tissues. Conversely, mPR β mRNA was approximately three times more abundant in the endometrium, although the relative expression levels of both mPR α and β were very low when compared with other tissues. PAQRIII

was the predominant, albeit non-regulated, transcript in myometrium and endometrium.

Immunohistochemical analysis

We sought to determine the cellular expression pattern of mPR α in human reproductive tissues. For this purpose, we raised an anti-peptide antibody against the last 19 amino acids of the predicted carboxy-terminus of human mPR α . This antibody was validated in COS-1 cells transiently transfected with an empty control vector (pcDNA3.1) or an expression vector encoding either for the wild-type human mPR α (pcDNA3.1/hmPR α), a carboxy-terminally V5-tagged mPR α (pcDNA3.1/V5/hmPR α), or an amino-terminally HA-tagged mPR α (pcDNA3.1/HA/hmPR α). As shown in Fig. 5, the carboxy-terminal antibody was specifically capable of recognizing expressed wild-type and amino-terminally HA-tagged mPR α proteins in COS-1 cells. No immuno-

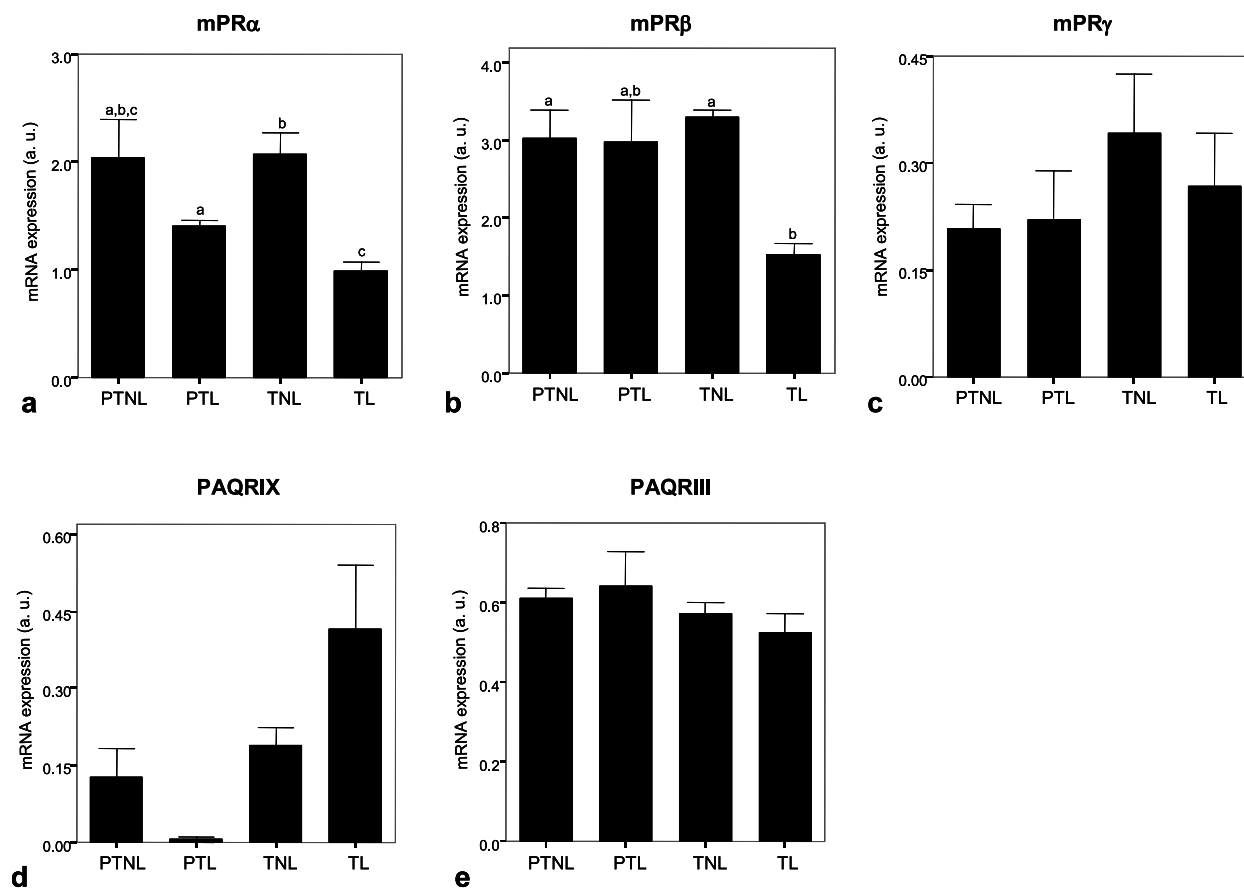


Figure 3 Expression of mPR homologues in human myometrium during pregnancy before and after the onset of labour. The expression of mRNA encoding mPR α (a), β (b) and γ (c); PAQRX (d); or PAQRIII (e) was examined in preterm non-labour (PTNL; $n=9$), preterm labour (PTL; $n=4$), term non-labour (TNL; $n=13$) or term labour (TL; $n=10$) lower uterine segment, myometrial samples. The mRNA levels are expressed in arbitrary units (a.u.), and the data represent the means \pm S.E.M. Different letters above the error bars indicate that those groups are significantly different from each other at $P<0.05$.

reactivity was observed with this antibody in cells transfected with pcDNA3.1/V5/hmPR α , suggesting that the carboxy-terminal V5-tag obscures the anti-mPR α antibody epitope, although the transfected protein was detectable with a V5 antibody. Furthermore, no mPR α immunoreactivity was detected in COS-1 cells transfected with pcDNA3.1/V5/hmPR β or pcDNA3.1/V5/hmPR γ (data not shown). Notably, overexpressed human mPR α did not localize to the plasma membrane in COS-1 cells but rather to an intracellular tubuloreticular network, which often included the nuclear envelope.

Having validated the specificity of our anti-human mPR α antibody, we examined the expression of endogenous mPR α in term placental tissues. As shown in Fig. 6, strong mPR α immunoreactivity was restricted to the syncytiotrophoblast at the periphery of term chorionic villi. In contrast, there was little or no mPR α staining in the stroma of the villi (Fig. 6a). Despite much lower mRNA levels, human endometrium was also immunopositive for

mPR α . Staining was weak in proliferative samples and largely confined to the epithelial cell compartment (Fig. 6d). In the secretory phase, staining became more pronounced in the glands, and immunoreactivity was now detectable in a subpopulation of the stromal cells (Fig. 6e).

Overexpressed carboxy-terminally V5-tagged mPR α and amino-terminally HA-tagged mPR α were readily identified on Western blot analysis using anti-V5 and anti-HA antibodies respectively. However, no specific bands were detectable with the anti-peptide human mPR α antibody, indicating that this antibody recognizes mPR α only in its native state (data not shown).

Discussion

Accumulating evidence suggests that rapid non-genomic progesterone actions are important for several key reproductive events, including female reproductive behaviour,

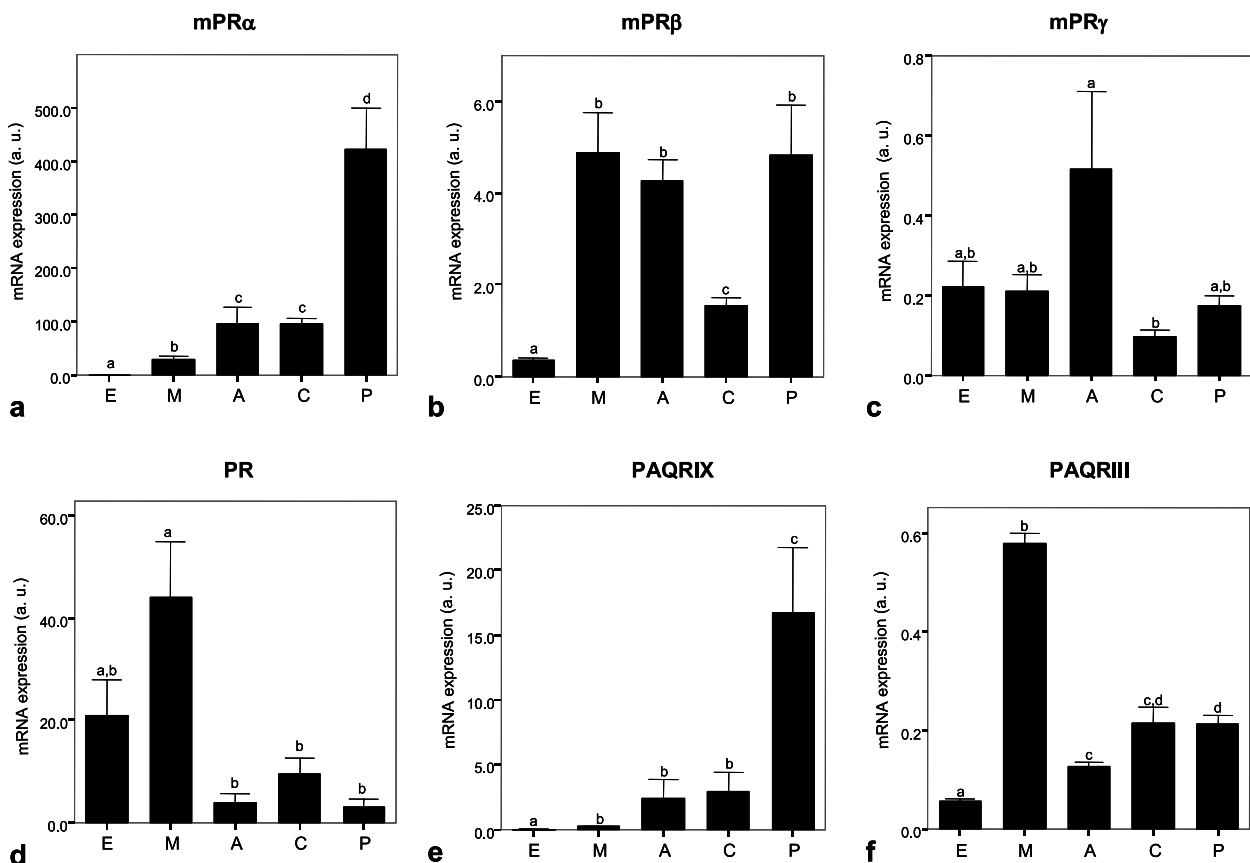


Figure 4 Tissue-specific expression of mPR homologues in the female reproductive tract. Comparative analysis of the relative expression of mRNA encoding mPR α (a), β (b) and γ (c); nuclear PR (d); PAQRX (e); and PAQRIII (f) in human endometrium (E), myometrium (M), amnion (A), chorion (C) and placenta (P). Tissue samples were pooled for analysis. The mRNA levels are expressed in arbitrary units (a.u.), and the data represent the means \pm S.E.M. Different letters above the error bars indicate that those groups are significantly different from each other at $P < 0.05$.

initiation of the acrosomal reaction of sperm during conception, and T-cell immunosuppression during pregnancy (Osman *et al.* 1989, Ehring *et al.* 1998, Krebs *et al.* 2000, Losel & Wehling 2003). However, it is not known whether non-genomic signalling contributes to or modulates physiological progesterone responses that are dependent upon activation of the nuclear PR, such as ovulation,

endometrial decidualization, or myometrial quiescence during pregnancy. Furthermore, the mechanisms involved in rapid progesterone signalling are poorly understood. Several studies have shown the existence of a variety of distinct but poorly characterized, putative, membrane-associated, progesterone-binding proteins in different tissues and species, although other lines of evidence suggest

Table 2 The relative abundance of mPR transcripts in progesterone target tissues. The abundance of mPR β , mPR γ , PAQRX and PAQRIII transcripts relative to mPR α mRNA levels was calculated as described in the Materials and Methods section in pooled tissue samples

	mPR β /mPR α	mPR γ /mPR α	PAQRX/mPR α	PAQRIII/mPR α
Tissue				
Endometrium	3.02	0.93	0.07	29.40
Myometrium	0.44	<0.01	<0.01	3.69
Amnion	0.15	<0.01	0.11	0.30
Chorion	0.05	<0.01	0.06	0.44
Placenta	0.04	<0.01	0.16	0.15

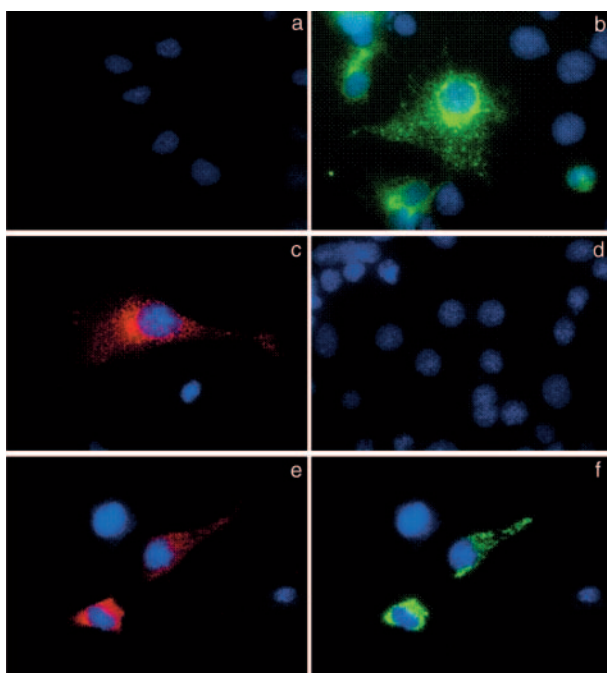


Figure 5 Validation of the anti-peptide human mPR α antibody. Confocal images of COS-1 cells transiently transfected with the empty control vector pcDNA3.1 (a) or an expression vector encoding for wild-type mPR α (b), a carboxy-terminally V5-tagged mPR α (c and d), and an amino-terminally HA-tagged mPR α (e and f). Transfected cells were stained with anti-peptide human mPR α antibody (a, b, d and f), anti-V5 antibody (c), or anti-HA antibody (e). Original magnification $\times 200$.

that some of the non-genomic progesterone responses are either mediated by 'classical' GPCRs or involve cytoplasmic activation of the nuclear PR (Gerdes *et al.* 1998, Boonyaratankornkit *et al.* 2001, Bramley *et al.* 2002, Losel & Wehling 2003, Losel *et al.* 2004, Peluso 2004, Thomas *et al.* 2004). Therefore, the characterization of a bona fide mPR in the spotted sea trout and the subsequent cloning of three human homologues (mPR α , β and γ) represented an important discovery that suggests new opportunities to unravel the interplay between genomic and non-genomic progesterone signalling (Hammes 2003, Zhu *et al.* 2003a,b). It also makes it possible to determine whether impaired membrane progesterone signalling can be implicated in reproductive failure or pregnancy disorders. In addition, the fact that multiple homologues to the fish mPRs were identified in other vertebrates suggested that additional family members could exist.

Phylogenetic and structural analyses have shown that the mPRs belong to a larger family of proteins, termed PAQRs, which include 11 mammalian members (PAQR1–XI), haemolysin III from *Bacillus cereus* (Baida & Kuzmin 1996), and YOL002c and related yeast genes from *Saccharomyces cerevisiae* (Karpichev *et al.* 2002). Family members can also be identified in *Arabidopsis*, *Drosophila*, *Caenorhabditis elegans*, *Xenopus*, zebra fish, fugu and chicken genomes. The predominant feature of the family is a core region containing seven transmembrane domains and four highly conserved sequence motifs that have recently been postulated to be involved in metal binding (Lyons *et al.* 2004). The mechanisms by which the PAQR

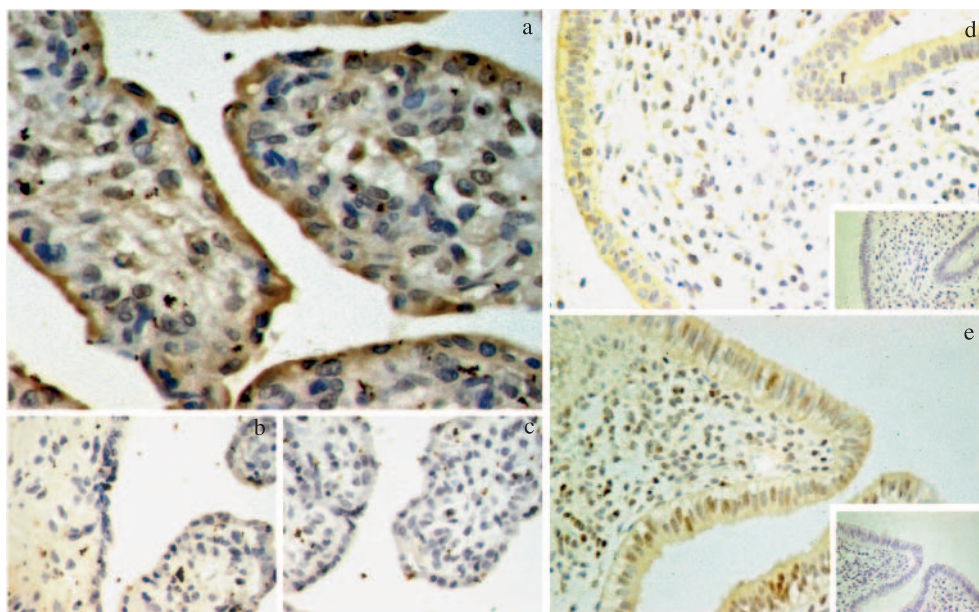


Figure 6 Localization of mPR α in placental and endometrial tissue. Tissue sections were stained with anti-human mPR α antibody. mPR α immunoreactivity localizes to syncytiotrophoblast in term placental villi (a). In control sections, this primary antibody was either peptide preabsorbed (b) or omitted (c). Under the same experimental conditions, mPR α immunoreactivity was also observed in proliferative (d) and secretory (e) endometrium. The inserts show control sections without primary antibody. Original magnification $\times 100$.

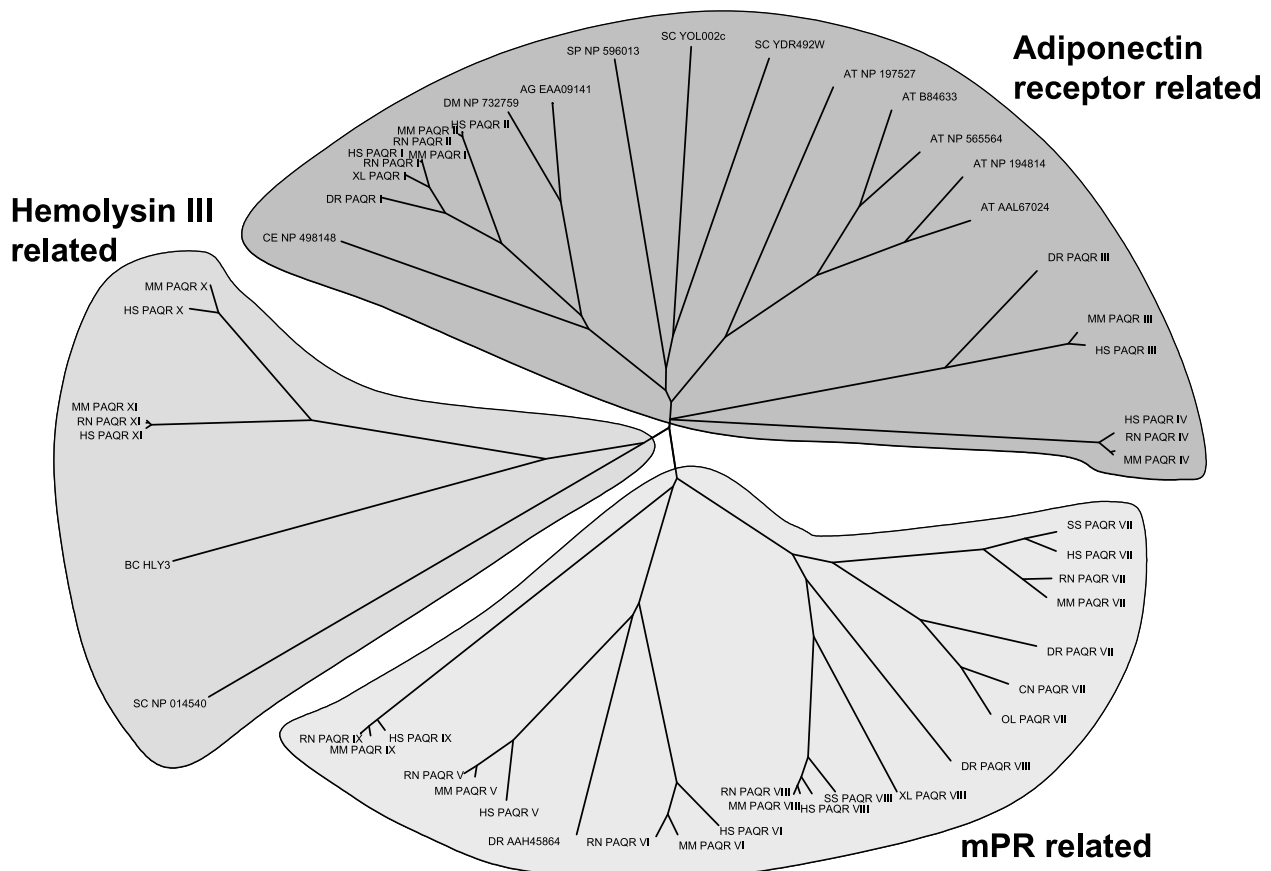


Figure 7 An unrooted phylogram showing sequence relationships between members of the PAQR family of proteins. The protein name prefixes relate to source species of the sequence: HS, *Homo sapiens*; MM, *Mus musculus*; RN, *Rattus norvegicus*; SC, *Saccharomyces cerevisiae*; SP, *Schizosaccharomyces pombe*; CE, *Caenorhabditis elegans*; DR, *Danio rerio*; XL, *Xenopus laevis*; DM, *Drosophila melanogaster*; BC, *Bacillus cereus*; AG, *Anopheles gambiae*; AT, *Arabidopsis thaliana*; CN, *Cynoscion nebulosus*; OL, *Oryzias latipes*; SS, *Sus scrofa*.

family members bind ligands and transduce signals are unknown. Although they are believed to be membrane proteins, conflicting evidence exists as to their membrane topology. The mPRs are thought to have extracellular N-termini, akin to 'classical' GPCRs (Zhu *et al.* 2003b), whereas the adiponectin receptors PAQR I and PAQR II are thought to have intracellular N-termini (Yamauchi *et al.* 2003). Similarly, the mPRs are thought to signal via a G protein pathway, whereas adiponectin receptor signalling does not appear to be coupled to G proteins (Yamauchi *et al.* 2003, Zhu *et al.* 2003b).

Phylogenetic analysis shows the PAQR family to fall into three main subgroups: the adiponectin-related receptors, the membrane progesterin-related receptors and the haemolysin III-related receptors (Fig. 7). The adiponectin-related receptors include PAQR I, PAQR II, PAQR III, PAQR IV, and YOL002c and other yeast proteins. Functionally, these receptors have been implicated in regulating fatty acid, phosphate and zinc metabolism, and, in the

case of PAQR I and PAQR II, in binding the adipokine adiponectin. The membrane progesterin-related receptors include PAQR V (mPR γ), PAQR VI, PAQR VII (mPR α), PAQR VIII (mPR β) and PAQR IX. The haemolysin III-related receptors include the mammalian PAQR X and PAQR XI receptors. In bacteria, haemolysin III may function as a pore-forming membrane protein (Baida & Kuzmin 1996), but there are currently no data on the potential function of the mammalian homologues.

Expression profiling of the various PAQR transcripts in cycling endometrium and pregnancy tissues yielded a number of interesting observations. First, among all the transcripts examined, only PAQR III expression did not alter significantly in either cycling endometrium or upon labour in any of the tissues examined. Combined with our phylogenetic analysis, this expression profile suggests that PAQR III is likely to have a homeostatic function and, notably, its *Drosophila* orthologue CG7530 has been identified in a screen for genes that confer resistance to

oxidative stress (Monnier *et al.* 2002). Conversely, mPR α was the only family member whose expression at mRNA level was significantly upregulated in the endometrium upon the postovulatory rise in progesterone levels, as well as downregulated in the myometrium upon 'functional' progesterone withdrawal associated with the onset of labour. Furthermore, the apparent gradient in the abundance of mPR α transcripts in gestational tissues, which declined from the placenta toward the myometrium, supports the notion that this isoform is regulated in a paracrine fashion by its putative ligand. Interestingly, the ovary, which, like the placenta, produces large quantities of progesterone, has been shown to contain several specific, membrane-associated progesterone-binding sites, although the identity of these proteins remains to be established (Bramley *et al.* 2002, Peluso 2004). The abundance of mPR α transcripts in placenta and fetal membranes was several times higher than those of mPR β or γ , underscoring the importance of this isoform in pregnancy tissues. In pooled endometrial samples, mPR β transcripts were relatively more abundant than mPR α . However, expression of both isoforms was very low when compared with gestational tissues. Nevertheless, immunohistochemistry demonstrated expression of mPR α protein in human endometrium, predominantly in the glandular compartment during the secretory phase of the cycle. However, mPR α immunoreactivity was markedly lower in endometrial tissues than in trophoblast of term villi (Fig. 6). Unfortunately, our anti-peptide antibody failed to detect endogenous or transfected mPR α on Western blot analysis. We are currently generating additional antibodies to validate the expression profiles of the mPR family members at protein level.

Interestingly, the relative tissue expression profiles of PAQRX and mPR α transcripts were almost indistinguishable and correlated inversely with the expression of nuclear PR in gestational tissues. It is, however, likely that both genes are differentially regulated as, in contrast to mPR α , PAQRX mRNA expression was significantly downregulated in secretory phase endometrium, and there was a trend, albeit non-significant, to higher expression levels in the myometrium after the onset of labour. The distinct expression profile of PAQRX, and the fact that, phylogenetically, it falls into the mPR subgroup of PAQR proteins, could indicate that it functions as a membrane steroid receptor.

In conclusion, through phylogenetic and structural analysis, we have characterized for the first time the mPR subgroup of the PAQR family of proteins. We also demonstrated that cDNAs encoding for various mPR isoforms are differentially expressed in pregnancy tissues. Furthermore, the data point to an important role of mPR α , and possibly PAQRX, in mediating progesterone actions in those tissues that express low levels of nuclear PR. A major challenge now is to elucidate the physiological consequences of mPR signalling in these target tissues.

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