

RESEARCH ARTICLE

Expression and Purification of Human Membrane Progesterin Receptor α (mPR α)

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Data Availability Statement: Nucleotide and amino acid sequences of human membrane progesterin receptor α (PAQR7) are available from the GenBank database (accession numbers BC034015, AY424285).

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Abstract

Membrane progesterin receptors (mPRs) are responsible for mediating the rapid, nongenomic activity of progestins and belong to the G protein-coupled receptor (GPCR) family. mPRs are also considered as attractive proteins to draw a new medicinal approach. In this study, we optimized a procedure for the expression and purification of recombinant human mPR α protein (hmPR α) by a methylotropic yeast, *Pichia pastoris*, expression system. The protein expressed in crude membrane fractions exhibited a binding affinity of $K_d = 3.8$ nM and $B_{max} = 288.8$ fmol/mg for progesterone. These results indicated that the hmPR α expressed in yeast was active. Solubilized hmPR α was purified through three column chromatography steps. A nickel-nitrilotriacetic acid (Ni-NTA) column was first used, and the mPR α proteins were then bound to cellulose resin with free amino groups (Cellufine Amino) and finally passed through an SP-Sepharose column. The optimization of expression and purification conditions resulted in a high yield of purified hmPR α (1.3–1.5 mg from 1 L culture). The purified hmPR α protein demonstrated progesterone binding ($K_d = 5.2$ nM and $B_{max} = 111.6$ fmol/mg). The results indicated that we succeeded in solubilizing and purifying hmPR α in an active form. Sufficient amount of active hmPR α protein will support the establishment of applications for the screening of ligands for mPR α .

Introduction

Progestins act as a key regulating factor in controlling the reproductive tissues. Progesterone was identified as a natural progestin in the human body [1]. Synthetic progestins have been produced and are frequently used for medical purposes. Progesterone is a well-known steroid that is produced by the ovary depending on the physiological conditions of the ovary and gonadotropin levels [2]. Progesterone generates a number of physiological effects in different tissues through various mediating mechanisms in each tissue. Although the physiological effects of progesterone have been known to be mediated by the regulation of gene expression associated with nuclear progesterone receptors [3], new insight on the activity of progesterone was provided by the identification of membrane progesterin receptors (mPRs) [4]. Certain nongenomic effects of progestins, such as oocyte maturation, are mediated by mPRs on the plasma

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membrane and induce rapid intracellular changes. Oocyte maturation-inducing steroids (MISs) are produced in response to luteinizing hormone (LH) in the follicular envelope in fish [5]. This progestin-induced nongenomic activity in oocytes mediated by mPR causes the cells to proceed through meiotic cell cycles [6–10]. Based on phylogenetic analysis, mPRs can be categorized into a new protein family of G protein-coupled receptors (GPCRs), the PAQR (progestin and adipoQ receptors) family [11]. This family contains three subordinate types termed mPR α , β and γ (corresponding to PAQR7, 8 and 5, respectively) [12]. Based on the analysis of proteins expressed in human breast cancer cells, PAQR6 and 9 were also categorized as new subtypes of mPR (mPR δ and ϵ , respectively) [13]. The expression of mPR mRNAs has been observed in reproductive tissues (ovary, testis, uterus and placenta) and in nonreproductive tissues (kidney, brain and intestinal tissues) in the human body [9]. The broad distribution of mPRs in different tissues suggests that mPRs perform various functions in a large range of target tissues. Additionally, mPRs could serve as a target for endocrine-disrupting chemicals (EDCs). Although the distinct role of mPRs remains under investigation, mPRs could be a target for a novel class of pharmaceuticals and EDCs. Thus, we aimed to produce a recombinant mPR protein.

The methylotrophic yeast *Pichia pastoris* is an efficient host for the expression of membrane proteins [14,15] and secretory proteins [16–19]. Recently, research has been conducted on human histamine H1 receptor and GPCR expression by *P. pastoris* [15]. Previously we reported the expression of mPR protein in human cancer cell lines and in *Escherichia coli* [20]. The large-scale culturing of *E. coli* is possible but did not produce an active form of recombinant mPR [20]. In addition, the mPR expression levels in mammalian cells were extremely low and did not generate a sufficient amount of protein for purification, structural and biochemical analysis. More than one hundred reports have emphasized the expression of GPCRs and their large-scale purification using *P. pastoris*. Natural ligand binding has been assessed by the expression of mammalian GPCRs in *P. pastoris* [21,22]. *P. pastoris* has been widely used for the expression of GPCRs. The structures of two human GPCRs (the histamine H1 and the adenosine A_{2a} receptor) were determined using recombinant protein expressed in *P. pastoris* [15,23]. Thus we selected *P. pastoris* for the expression of large amounts of mPR.

Previously, we established a procedure for producing and purifying recombinant goldfish mPR α , but this method generates very low amounts of protein [24]. In this study, we established a yeast strain for the expression of human mPR α (hmPR α) according to the method for goldfish mPR α and optimized the conditions to obtain large amounts of product. Through the optimization of culture conditions, homogenization protocol and conditions for column chromatography, we established a procedure to obtain relatively a large amount of hmPR α . This evolving new procedure could be used to produce sufficient amounts of hmPR α protein to develop a screening system for mPR α -affecting agents or to determine the structure of mPR α .

Results

1. Recombinant human mPR α protein expression

Wild yeast *P. pastoris* was used for the expression and production of recombinant hmPR α protein. For expression, the cDNA of hmPR α was fused to the secretion signal sequence of the α -factor from *S. cerevisiae* in the expression cassette (Fig 1A). The construct was inserted into the host yeast genome by homologous recombination. The successful insertion of the cassette, along with its promoter and terminator that control the transcription of the heterologous mPR α gene fusion, into the yeast cells was confirmed by PCR using AOX1 primer sets (Fig 1B).

The expressed fusion hmPR α protein carried a c-Myc epitope and a histidine tag (His-tag) on its C-terminal end. Expression was induced by the presence of 0.5% methanol in the

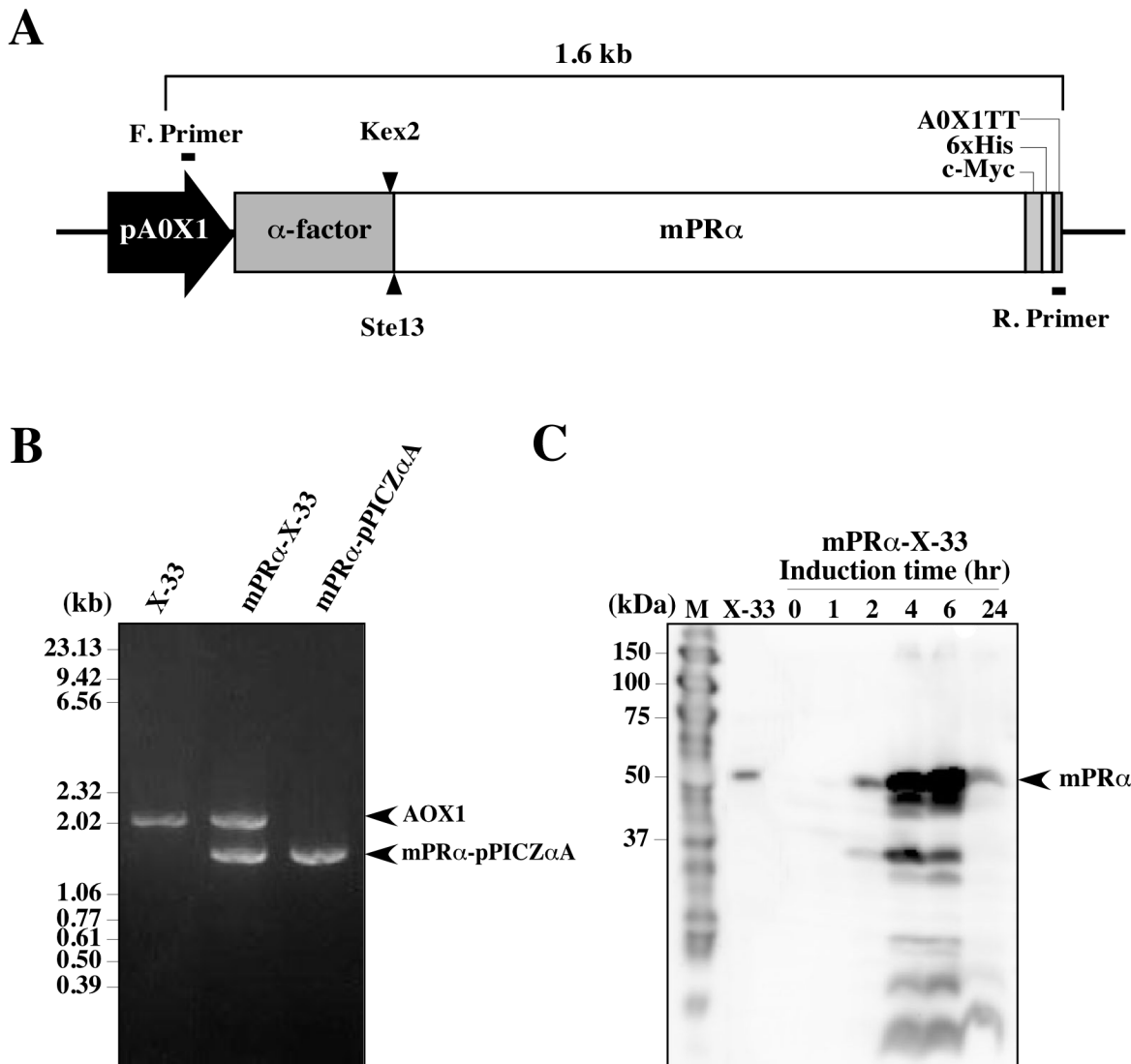


Fig 1. Expression of human mPR α in *Pichia pastoris*. (A) Schematic representation of the hmPR α expression cassette that was inserted into the yeast cells to produce mPR α protein. The fusion peptide consisted of hmPR α , a α -factor signal sequence, a C-terminal histidine (6x His), and a c-Myc epitope controlled by the methanol-inducible AOX1 promoter (pAOX1) and the AOX1 transcription termination region (AOX1 TT). The black bars above and below the cassette indicate the 5'AOX1 (F. primer) and 3'AOX1 (R. primer) primer binding sites, respectively. The AOX1 gene of the yeast cells remained within the expression cassette (2.1 kbp). (B) Gene insertion was verified by PCR. DNA fragments were amplified using genome DNA from untransformed yeast cells (X-33), genome DNA from hmPR α -transformed cells (mPR α -X-33) or transformed vector DNA (mPR α -pPICZ α A) as templates. (C) After protein expression induction in culture with methanol, samples were taken at 0, 1, 2, 4, 6 and 24 hours. Expression of hmPR α was determined by western blot analysis. A protein band of 50 kDa was reacted with anti-His-tag antibody in the extract prepared from hmPR α -transformed cells (mPR α -X-33).

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BMMY medium. The expression of hmPR α protein was confirmed by western blot analysis (Fig 1C). The protein band of approximately 50 kDa was detected. The theoretical molecular mass of hmPR α containing a α -factor signal peptide is approximately 52 kDa, which was consistent with the molecular mass of the detected band.

To determine the optimal conditions for the expression of hmPR α , 1 mL aliquots of the culture were collected after 0, 1, 2, 4, 6 and 24 hours, and the amount of expressed hmPR α was analyzed. The highest level of hmPR α expression was detected at 6 hours (Fig 1C). The optimal cell density before the initiation of induction was also examined. When the cell density

increased, the yield of hmPR α protein also increased. After several trials, we found that a cell density of OD₆₀₀ 21–23 during methanol induction was optimal, in contrast to previous conditions established for goldfish mPR α protein production at an OD₆₀₀ of 1.0–3.0 [24]. Therefore, we succeeded in producing the hmPR α protein at higher cell densities.

2. Specific binding of [³H]1,2,6,7-progesterone on plasma membranes prepared from hmPR α -expressed *P. pastoris*

To demonstrate specific binding of [³H]1,2,6,7-progesterone to the expressed hmPR α protein, digitonin was used for the preparation of the cell membrane fraction because this glycoside facilitates steroid receptor access [25,26]. Previously, a final concentration of 0.1% digitonin was reported to be optimal for facilitating steroid binding [24], which was measured using a filter-binding assay [25,26]. After the treatment of the crude cell membrane fractions with 0.1% digitonin, the specific [³H]1,2,6,7-progesterone-binding activity was significantly increased in membrane fractions from hmPR α -expressing cells. Under the same conditions, fractions from untransformed host cells exhibited lower binding activity (Fig 2A). Saturation analysis demonstrated that the progesterone binding to the cell membranes of hmPR α -expressing cells is saturable and of limited capacity (B_{max} = 288.8 fmol/mg). Scatchard analysis indicated the presence of a single site of high-affinity binding sites (K_d = 3.8 nM) in the cell membrane fraction of hmPR α (Fig 2B). Consequently, these results indicated that the heterologously produced recombinant hmPR α was active.

3. Solubilization and purification of hmPR α protein

To lyse large amount of yeast cells, a new technology was applied. We applied Ball Mill equipment (Ball Mill PM 100) that can disrupt the samples by rotating stainless steel balls in the

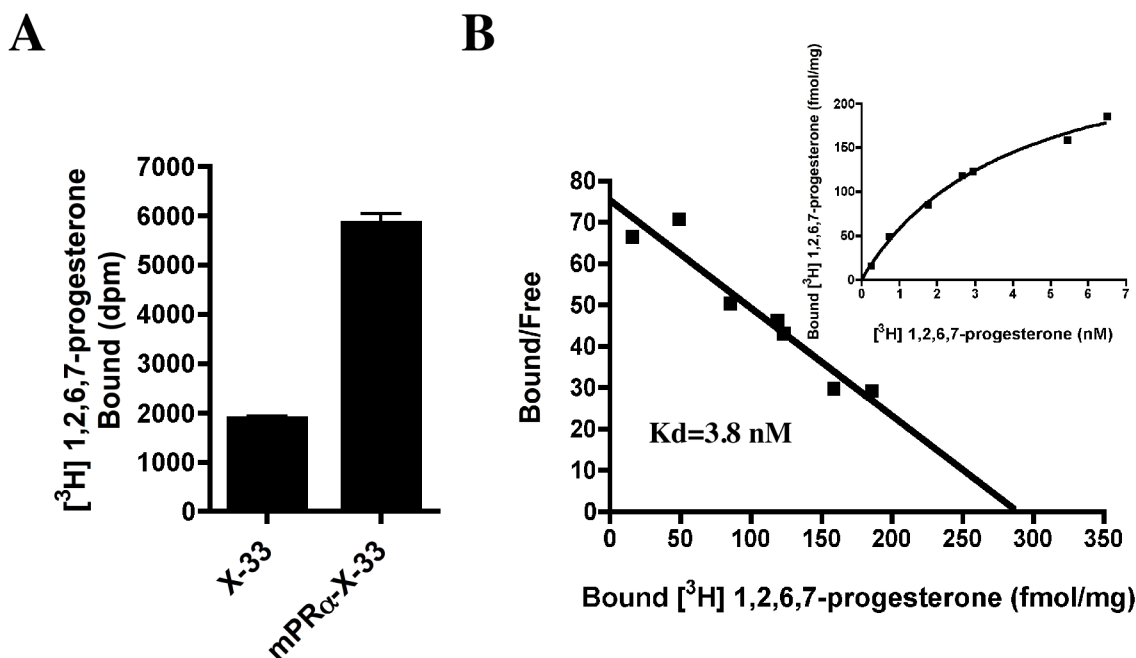


Fig 2. Characterization of binding activity in membrane fractions. (A) Specific binding activity of [³H]1,2,6,7-progesterone to membrane preparations from untransformed yeast cells (X-33) and hmPR α -producing cells (mPR α -X33). (B) Saturation curves and Scatchard plots of specific [³H]1,2,6,7-progesterone binding to membrane preparations from hmPR α -producing cells (mPR α -X33).

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chamber under freezing conditions [27,28]. By using this machine, we could homogenize cell precipitates from a 500 ml culture all at once. After the disruption of the yeast cells by the Ball Mill PM 100 instrument, the membrane proteins were solubilized using 0.1% n-dodecyl- β -D-maltoside (DDM), as described previously [24]. To optimize the conditions for Ni-NTA affinity chromatography, 16 lysis buffers (50 mM NaH₂PO₄, 300 mM NaCl, 1 mM PMSE, 10% glycerol, 0.1% DDM) with 4 different concentrations of imidazole (10, 20, 40 or 80 mM) and of pH 5.0, 6.0, 7.0 or 8.0 were tested (S1 Fig). Membrane preparations were incubated with one of the 16 different lysis buffers for 30 min on ice, and then the solubilized supernatant was separated from insoluble materials by centrifugation (20,000 \times g, 4°C, 20 min). The solubilized hmPR α fraction was applied to the Ni-NTA resin. Unbound materials in the Ni-NTA resin were subsequently separated by centrifugation, and bound proteins were eluted by elution buffer. The remaining materials were solubilized by denaturing buffer for SDS-PAGE. The hmPR α content in each fraction was analyzed by western blotting using anti-His-tag antibodies. Out of the 16 buffers tested, the 40 mM imidazole, pH 6.0, lysis buffer demonstrated the best separation of hmPR α from other proteins (S1 Fig). Thus, this buffer was selected as the lysis buffer and as the Ni-NTA chromatography running buffer.

In the first step of purification, the sample was separated on a Ni-NTA column. The protein content of the eluted fractions were analyzed by CBBR and immunoblotting with anti-His-tag antibodies. HmPR α protein was detected in fractions 11 to 16 (Fig 3A), which corresponded to 160 mM imidazole in the buffer. These fractions were pooled and applied to a Cellufine Amino column, which we selected as an effective resin for purification of the mPR α protein [24]. The proteins were eluted by linear gradient of sodium chloride (Fig 3B). In the third purification step, the hmPR α fractions were passed through a SP-Sepharose column. The purified hmPR α proteins were concentrated using Cellufine Amino resin. The SDS-PAGE and immunoblotting assay indicated that hmPR α was successfully purified with higher purity (Fig 4A).

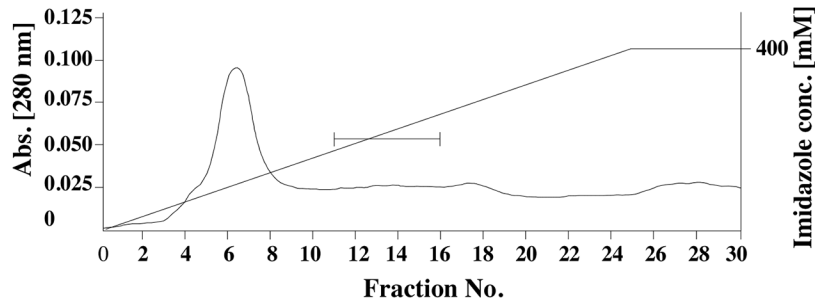
4. Characterization of purified recombinant hmPR α

The identity of the purified proteins was confirmed by MALDI-TOF/MS analysis. Peptide mass fingerprint analysis of the purified 50-kDa protein confirmed the presence of hmPR α (Fig 4B and 4C). From the results, the hmPR α protein was concluded to have been successfully expressed and purified. To examine the binding activity of the purified hmPR α , we modified the steroid binding assay for solubilized mPR proteins. When Ni-NTA was added into the reaction mixture, the steroid-binding activity of hmPR α could be detected (S2 Fig). Using this method, specific progesterone-binding activity was detected in the purified hmPR α fraction. Scatchard analysis indicated the presence of a single class of high-affinity binding site ($K_d = 5.2$ nM) with limited capacity ($B_{max} = 111.6$ fmol/mg) (Fig 5).

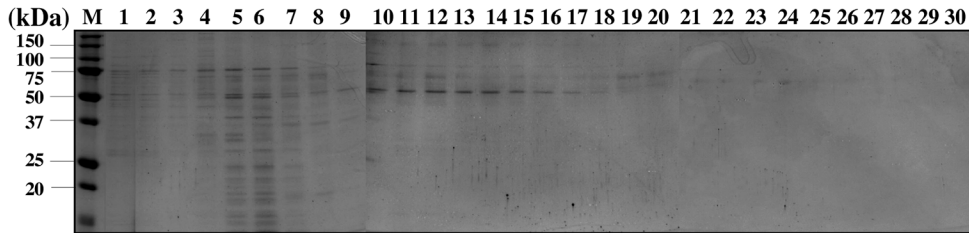
Discussion

Previously we reported the expression and purification of goldfish mPR α in a *P. pastoris* yeast host system [24]. Based on the procedure established for goldfish mPR α , we succeeded in producing and purifying a relatively large amount of recombinant human mPR α protein in this study. To obtain a large amount of product, we optimized the yeast culture and buffer conditions for Ni-NTA column chromatography. Furthermore, a new method of disrupting yeast cells using a ball mill was applied [27,28]. Using the ball mill, a membrane fraction with higher mPR α protein content could be obtained. The amount of expressed hmPR α was determined to be approximately 150 mg/L of culture by Western blot analysis. Of this amount of expressed protein, we could purify 1.2–1.5 mg of hmPR α with a yield of 0.8–1.0%. The yield of activity

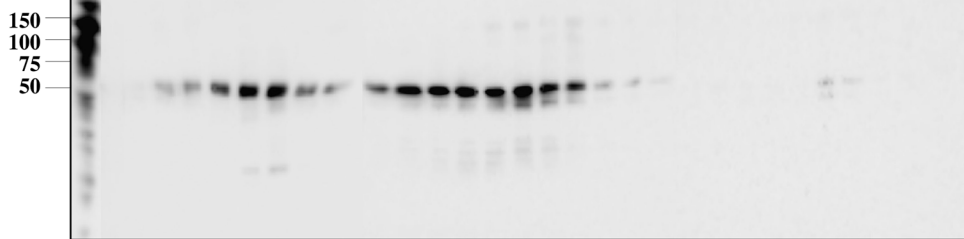
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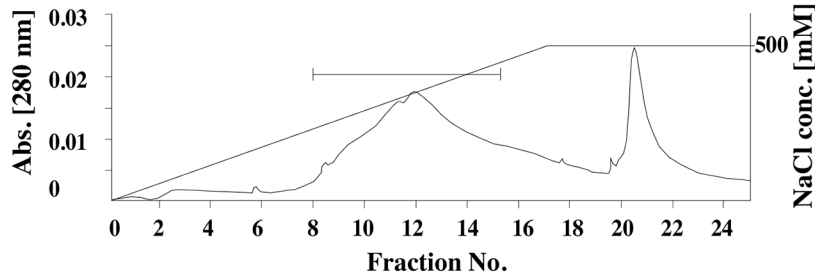
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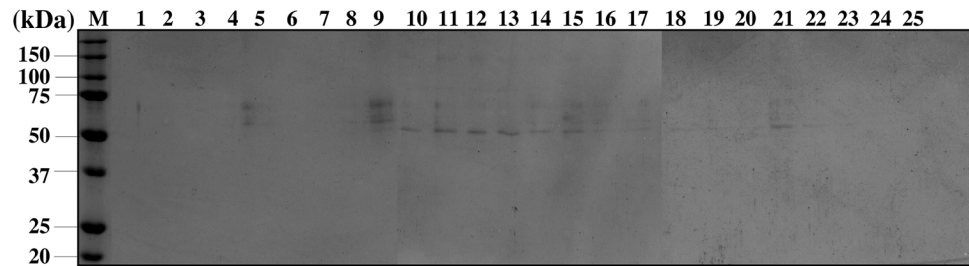
α -His



B



CBBR



α -His

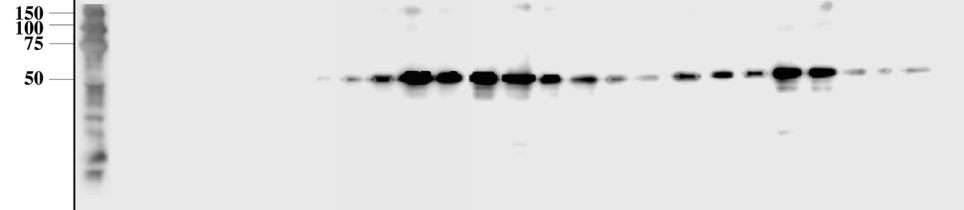


Fig 3. Purification of hmPR α protein by Ni-NTA and amino cellulose column chromatography. (A) Chromatogram and the SDS-PAGE and the western blot analysis results of the Ni-NTA column chromatography fractions obtained from the first purification step. (B) Chromatogram and the SDS-PAGE and western blot analysis results from the Cellufine Amino column chromatography conducted as the second purification step. The elution profile was monitored by absorbance at 280 nm. The horizontal bars in the chromatogram represent the fractions collected for further steps.

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was increased by more than 100 times compared to that of goldfish mPR α expression and purification (Table 1).

The progesterone-binding assay demonstrated that the hmPR α was expressed in an active form. The steroid-binding activity of membranes prepared from mPR α -expressing *P. pastoris* cells was detected in the presence of digitonin. The effect of digitonin was described in bovine membrane fractions [25]. In presence of digitonin, the binding of the membrane fraction of human sperm to progestin has also been detected [26]. A similar effect was confirmed in our previous study of goldfish mPR α [24]. The progesterone-binding assay using purified hmPR α demonstrated that the protein remained active after solubilization and purification (Fig 5). To measure the steroid-binding activity of solubilized hmPR α , we supplemented Ni-NTA resin into the reaction mixture, which trapped the protein on the filter (S2 Fig). Via this modification, we succeeded in measuring the steroid binding activity of purified hmPR α . The K_d value of purified hmPR α did not change significantly, compared with the value for the membrane fraction. Thus, we can conclude that we established a procedure to purify hmPR α protein in an active form.

mPRs have been identified in a wide variety of organisms ranging from fish to humans [29,30], and progestin-binding activity in these species have been reported in many species, including fish (goldfish, seatrout and zebrafish), frogs and mammals (cattle, rats, mice and humans) [8,31–33]. mPR α was found to be expressed in the reproductive tissues (ovary, uterus and testes), kidneys, brain and spinal cord in vertebrates [34–36]. The broad distribution of mPRs suggests that these proteins play a role in a wide variety of steroid-related functions in tissues.

Some studies regarding mPRs in the brain have been reported. Whole brain expression analysis of progestin receptors revealed that mPR α and β were expressed in thalamic nuclei [37]. The results suggested that mPRs are involved in the regulation of sensory and cognitive functions. The expression of 5 subtypes of mPRs, including two new subtypes of mPRs, δ and ϵ , was analyzed by q-PCR in human brain [13]. Among the mPR subtypes, mPR ϵ was the most abundant subtype in the brain and is a potential intermediary of the antiapoptotic effects of neurosteroids in the central nervous system. The roles of brain mPRs in the regulation of mammalian behavior have also been investigated [38,39].

Progesterone signaling through mPRs in human breast cancer cells has been investigated [40]. Zuo, L. et al. suggested that progesterone promotes epithelial-to-mesenchymal transition of breast cancer cells through mPRs [41]. The gene expression level of mPR α has been as a biomarker for breast cancer survival [42]. Recently, progesterone has been demonstrated to generate cancer stem cells through mPRs in mammary cells [43].

Progesterone may interact with mPR α , mPR β and mPR γ , which implies negative consequences on the proliferation of human T-cells that may attack fetuses during pregnancy, as indicated by changes in pH and Ca²⁺ levels inside T-cells [44]. Moreover, progesterone may be involved with bovine T-lymphocyte activation and proliferation through binding to mPRs in the corpus luteum [45]. Progesterone signaling in murine macrophages is associated with parturition that may be regulated by mPR α . This relationship may contribute to the functional withdrawal of progesterone associated with labor [46].

These studies have drawn attention to the discovery of novel drugs or treatment of diseases such as reproductive problems, cancers and encephalitis.

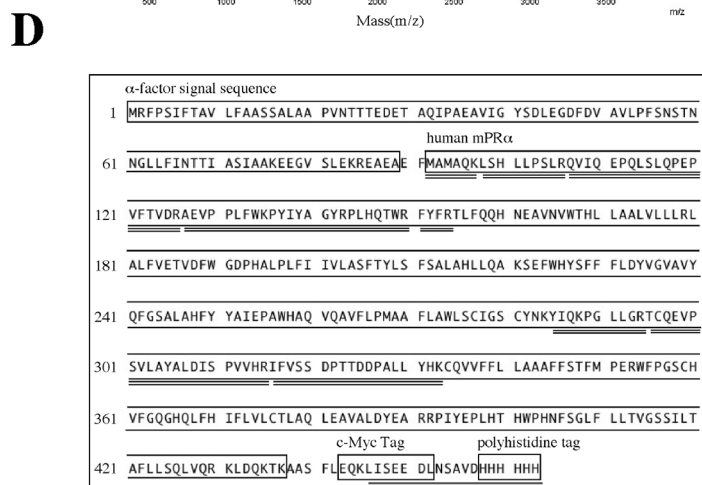
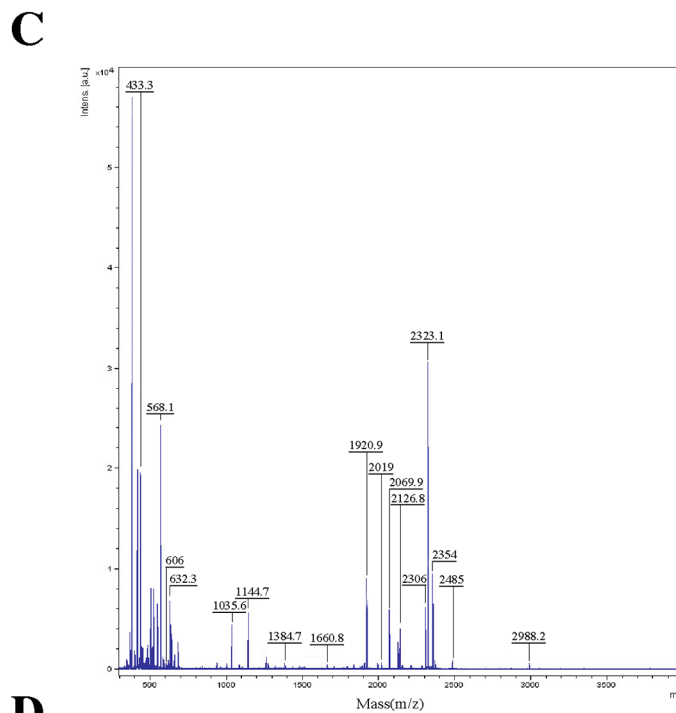
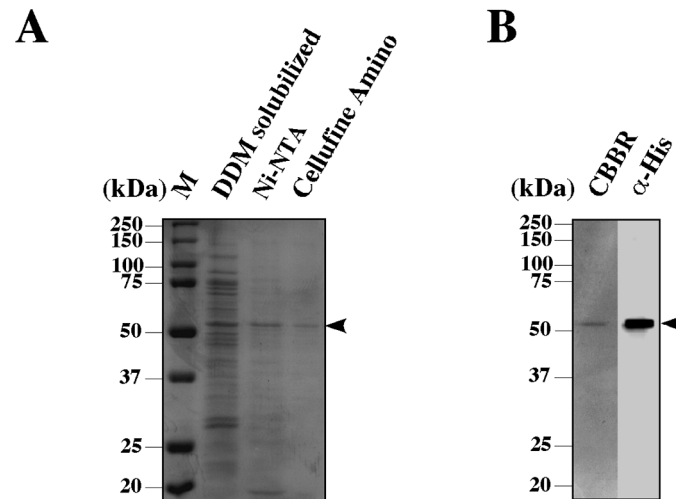


Fig 4. Identification of purified recombinant protein as mPR α . (A) SDS-PAGE analysis of representative fractions after solubilization of the membrane preparation (DDM solubilized), column chromatography over Ni-NTA, amino cellulose (Cellufine Amino). Protein bands were detected by CBBR staining. (B) SDS-PAGE analysis of purified hmPR α . Protein bands were detected by CBBR staining (CBBR) or were immunostained by anti-His-tag antibody (α -His). An arrow indicates hmPR α . (C) MALDI-TOF mass spectrum of purified hmPR α . (D) Amino acid sequence of recombinant hmPR α from produced in this study. The matching peptides from the peptide mass fingerprint analysis are underlined. The sequences of α -factor signal, c-Myc-tag and His-tag are boxed.

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In this study, we established an hmPR α -expressing yeast strain and a method to purify a large amount of active mPR α protein. The purified active hmPR α could be applicable for the screening of ligands for hmPR α . Additionally, endocrine-disrupting chemicals that interact with hmPR α could be identified by this screening. Very recently, the three-dimensional structure of PAQR1 was reported [47]. hmPR α belongs to a GPCR family, progesterin and adipoQ receptor family (PAQR family), which is composed of 11 genes. The three-dimensional structure of crystallized adipoQ receptor 1 (PAQR1) has been resolved. A novel class of proteins with a seven-transmembrane domain structure and a zinc-binding cavity was discovered. The strategy of structure resolution could be applied to mPRs. The recombinant protein expressed in this study will be useful for such an approach.

Recently, a new member of the membrane steroid receptor family was identified [48,49]. Through screening using monoclonal antibody for membrane proteins, a cDNA for a membrane androgen receptor (mAR) was identified as the previously reported zinc transporter ZIP9 subfamily (SLC39A9). Similar to mPRs, a seven-transmembrane domain structure was predicted from the amino acid sequence of ZIP9. ZIP9 is widely expressed in human tissues and upregulated in malignant breast and prostate tissues, suggesting that it is a potential therapeutic target for treating breast and prostate cancers. mAR has been found to be a seven-transmembrane domain receptor for three types of steroids (progesterin, estrogen and androgen). All of these receptors are conserved among vertebrates and are widely distributed in various tissues. Thus, these findings suggest that tissues are regulated by the nongenomic activity of

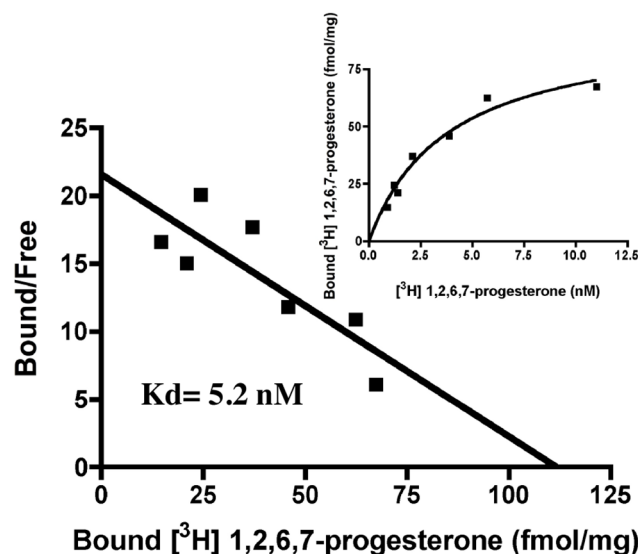


Fig 5. Scatchard plot analysis of purified hmPR α . Saturation curves and Scatchard plots of specific [³H] 1,2,6,7-progesterone binding to purified recombinant hmPR α .

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Table 1. Summary of the purification of recombinant human mPR α from *Pichia pastoris*.

	Protein (mg)	Total activity ^a Bound [³ H] 1,2,6,7 progesterone (nmol)	Specific activity (pmol/mg)	-fold	Yield ^b (%)
Crude extract	1264.5	0.534	0.42	1.0	100.0
DDM solubilization	197.2	0.232	1.18	2.8	43.4
Ni-NTA	68.4	0.104	1.52	3.6	19.5
Cellufine Amino	4.5	0.015	3.33	7.9	2.8
SP-Sepharose	1.2	0.012	10.00	23.8	2.2

^a The activities of each fraction were measured with 4 nM of [³H] 1,2,6,7 progesterone as described in Materials and Methods.

^b The yield of total progesterone binding activities of each fraction are presented as percentages of the crude extract, which was assumed to have a binding activity of as 100%.

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various types of steroids. The distinct roles of steroidal nongenomic effects in various tissues should be addressed.

Methods

Ethics Statement

The present study using human gene was approved by the Research Ethics Review Committee regarding Human Subjects of the Shizuoka University.

Materials

[³H]1,2,6,7-progesterone was purchased from PerkinElmer Inc. The modified trypsin (sequencing grade) was from Promega (Tokyo, Japan). The CHCA was obtained from Bruker Daltonics (Billerica, MA). Digitonin was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). The DNA polymerase and DNA Ligation Kit were from Takara Bio (Siga, Japan). The DNA fragment extraction kit from agarose gel was purchased from QIAGEN (Tokyo, Japan). The molecular weight marker for SDS-PAGE was from Bio-Rad (Hercules, CA). The anti-rabbit antibody conjugated with peroxidase and yeast nitrogen base without amino acids were obtained from Invitrogen (Carlsbad, CA). The anti-His-tag antibody was from Medical & Biological Laboratories (Nagoya, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Construction of recombinant pPICZ α A plasmid

Human mPR α protein was expressed in *P. pastoris* using the wild strain X33. The cDNA for hmPR α was prepared from human blood and amplified by polymerase (KOD plus neo, TOYOBO, Japan) using a primer set of Hs mPR α normal F, GTCACCTGGCTTTGCCTTTG, and Hs mPR α normal R, ATGCCATCCCCCTTCACTTG. Then, the amplified DNAs were inserted into a pBluescript II KS(+) plasmid and transformed into *E.coli* (XL1 Blue) for the cloning of the hmPR α gene. After the completion of cloning, the hmPR α fragment of the pBluescript II KS(+) plasmid was also amplified as a DNA template by polymerase (KOD plus neo, TOYOBO, Japan) using the primer set of Hs mPR α EcoRI, CGGAATTCATGGCCATGGCCCAGAACTCAGCCACCTCCTGCCGAG, and Hs mPR α -NotI, ATAAGAATGCGGCCGCCTTGGTCTTCTGATCAAGTTTGCCTGTACCAGC. For the expression in *P. pastoris*, the DNA was inserted into the *P. pastoris* expression vector pPICZ α A (Invitrogen). All of the ORF region DNA sequences of the expression vectors were verified by DNA sequencing.

The *P. pastoris* strain X-33 (Invitrogen) was transformed with the hmPR α -expression construct by electroporation, as previously described in detail [24]. The construct was linearized with PmeI digestion, and the linearized plasmid (168 μ g) was used to transform *P. pastoris* cells through electroporation. Electroporation was performed using a Gene Pulser instrument (Bio-Rad), following previously established protocols [24].

Yeast extract-peptone-dextrose medium (YPD) plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 500 μ g/mL Zeocin were selected for the culture of the recombinant colonies. The genomic amalgamation of hmPR α construct was verified by PCR using Ex Tag Polymerase (Takara Bio, Siga, Japan) and the primer set of 5' AOX1, GACTGGTTCCAA TTGACAAGC, and 3' AOX1, GCAAATGGCATTCTGACATCC, to amplify the sequence between the AOX1 promoter and terminator regions (Fig 1A). The production of recombinant protein was confirmed by analyzing several Zeocin-resistant clones. The clones with the highest expression levels were maintained and stored on MD plates contained 1.34% yeast nitrogen base, 4×10^{-5} % biotin, 2% dextrose, 1.5% agar [24] at 4°C.

Expression of hmPR α in *P. pastoris*

A single colony expressing hmPR α from the MD plate was inoculated in 100 ml of BMGY medium (1% yeast extract, 2% bactopectone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, 1% glycerol) and incubated for 21 hours at 30°C with shaking at 180 rpm. The volume was increased to 500 mL of BMGY medium in a 2 L baffled flask, and the yeast was incubated for 16.5 hours at 30°C with shaking at 180 rpm until the OD₆₀₀ nm reached 17–19. A 1 ml aliquot of the culture medium was used to determine the cell density. The remaining culture was harvested by centrifugation at 3,000 \times g for 5 min and was washed once using 300 ml BMMY medium. For the induction of mPR α protein expression, the cells were resuspended in 400 ml BMMY (1% yeast extract, 2% bactopectone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, 0.5% methanol) to an OD₆₀₀ of 21–23. The medium was placed in a 2 L baffled flask and incubated at 20°C for 6 hours with shaking at 180 rpm. After 6 hours, the cells were harvested by centrifugation at 3,000 \times g for 5 min, and the precipitate was frozen with liquid nitrogen and stored at -80°C.

Membrane preparation and solubilization of membrane proteins

Frozen cell pellets (\cong 20 g) that were harvested from 800 ml of culture were thawed and resuspended in 80 ml of ice-cold lysis buffer (50 mM sodium phosphate, 1 mM PMSF, 1 mM EDTA, 5% glycerol, pH 7.4). Then, re-frozen as shape of tubules in the stainless chamber for cell breaking with stainless ball. Consecutively, cells were broken by Retsch Ball Mill PM 100 (Verder Scientific Co., Ltd., Haan, Germany) with six rounds of shaking at fixed 400 rpm for 3 min with an interval of chilling with liquid nitrogen. Then, the disrupted cells were collected into centrifuge tubes. Nonhomogenized cells and debris were separated from the fractions containing the membranes by low-speed centrifugation (1,000 \times g, 4°C, 7 min). After the supernatant collection, the pellet was resuspended in 30 ml of ice-cold lysis buffer for a further round of supernatant collection. The supernatants were combined, and the membrane fractions were recovered by centrifugation at 20,000 \times g, 4°C, for 20 min. The precipitates were resuspended in buffers for the steroid binding assay or purification based on their intended use.

Purification

To purify the hmPR, the solubilized proteins were thawed on ice and loaded onto a 80 mL Ni-NTA Agarose (QIAGEN, Gaithersburg, MD, USA) column (ϕ 4.5 \times 5.0 cm) that was

equilibrated with lysis buffer containing 0.01% DDM and 1 mM PMSF. The proteins were eluted with a 500 mL gradient of 10–400 mM imidazole in the same buffer and washed with 100 ml of the same buffer containing 400 mM imidazole. The fractions that contained recombinant hmPR α were identified by western blot analysis and were collected and diluted for 4.5 times with DDW. Then samples were loaded onto a 5 mL of Cellufine Amino (JNC Corporation, Tokyo, Japan) column (ϕ 1.6 \times 10 cm) that was equilibrated with CA buffer (50 mM Tris-HCl buffer, pH 8.0, containing 0.01% DDM and 1 mM PMSF). The column was washed with 15 ml of the same buffer and eluted with a 120 mL gradient of 0–0.5 M NaCl in CA buffer. Fractions containing hmPR α were collected and were then passed through a SP-Sepharose column (1 ml) and applied to 1.5 ml of a Cellufine Amino column. The proteins were eluted with CA-buffer containing 0.5 M NaCl. The fractions that contained the hmPR α protein were collected and concentrated with Centriprep YM-3 filter units (Millipore, Billerica, MA).

SDS-PAGE and western blot analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel under denaturing conditions according to the method of Laemmli and were transferred to Immobilon membranes (Millipore, Billerica, MA). The membranes were blocked in 5% nonfat powdered milk in 20 mM Tris-buffered saline, pH 7.6 (TBS) containing 0.1% Tween 20 (TTBS) for 1–2 hours at room temperature. Then, the membranes were incubated with primary antibodies (1,000-fold dilution in TBS buffer) and with secondary antibodies (2,000-fold dilution in TBS buffer). The visualization of the target protein was performed by enhanced chemiluminescence using an ECL detection kit (PerkinElmer, Waltham, MA), a method based upon a chemiluminescent reaction mediated by peroxidase conjugated to a secondary antibody. The signals were digitized using a CCD camera system (Luminescent Image Analyzer LAS-4000 mini; Fujifilm, Tokyo, Japan).

Peptide mass fingerprint analysis by MALDI-TOF/MS

Purified recombinant mPR α proteins stained with CBBR in SDS-PAGE gel slices were trypsinized. Subsequently, the peptides were recovered using a ZipTip (Millipore) and were eluted through a solution (2 μ l) containing 60% acetonitrile, 0.1% TFA and 5 mg/ml of CHCA (Bruker Daltonics), as described previously for goldfish mPR α [24]. A 384-well plate was used for the loading of the samples, which was contained a double layer with CHCA and dissolved in acetone, after that air-dried. A MALDI-TOF/MS Autoflex (Bruker Daltonics, Billerica, USA) was used to detect the peptide mass spectrum in a positive ion mode. The spectra that were obtained from MALDI-TOF/MS were calibrated by a mixture of molecular weight standards (Bruker Daltonics). By using the MASCOT software (Matrix Science, London, UK), the peptide fingerprint was analyzed and was compared with peptides from human taxonomy using the NCBI database. Subsequent parameters used included cysteine modification by carbamidomethylation (C), a trypsin digest missed cleavage of zero and a peptide mass tolerance \pm 0.4 Da. The mPR α protein was identified from the molecular weight of peptide fragments using probability-based MOWSE scores.

Radiolabeled ligand binding assays

The plasma membrane pellet was obtained as described in the membrane preparation and solubilization section. Then, the pellet was resuspended in HEAD buffer (25 mM HEPES, 10 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, pH 7.6) containing 0.1% digitonin. Progesterin receptor binding to the membrane fractions was measured following previously established

procedures [20]. In the binding assay for the solubilized samples, Ni-NTA resin (100 μ l of 50% vol) was added. GF/B filters were presoaked in wash buffer without Tween 80.

Competition studies

One set of tubes contained 1.5 nM [3 H]1,2,6,7-progesterone alone (total binding); another set also contained cold progesterin competitor at a 100-fold greater concentration to measure nonspecific binding (NSB). After a 30 min incubation at 4°C with the membrane fractions, the reaction was stopped by filtration (Whatman GF/B filters, presoaked in wash buffer containing 2.5% Tween 80). The filters were washed three times with 5 mL of wash buffer (25 mM HEPES, 10 mM NaCl, 1 mM EDTA, pH 7.4) at 4°C, and the bound radioactivity were measured by scintillation counting.

Saturation analyses and Scatchard plots

Various concentrations (0.5–12.5 nM) of [3 H]1,2,6,7-progesterone (specific activity, 96.6 Ci/mmol) were added to the assay tubes with (nonspecific) or without (total) 100-fold molar excess cold progesterone. Linear and nonlinear regression analyses for all receptor binding assays and calculations of K_d and binding capacity (B_{max}) were conducted using GraphPad Prism for Macintosh (version 4.0c; GraphPad Software, San Diego, CA). The results are shown as Scatchard plots.

Supporting Information

S1 Fig. Optimization of conditions for Ni-NTA column chromatography. Binding of solubilized mPR α onto the Ni-NTA resin was examined with different concentrations of imidazole (10, 20, 40 or 80 mM) and pH values (pH 5.0, 6.0, 7.0, or 8.0) in Ni-NTA binding buffer (50 mM NaH₂PO₄, 300 mM NaCl). Samples for each lane are following; M, marker; S, solubilized mPR α protein fraction; T, flow-through protein after Ni-NTA binding; E, eluted proteins with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole pH 8.0); R, remained on Ni-NTA resin after elution. The proteins were detected by CBBR staining (upper panel in each set) or western blotting (lower panel in each set). The panels depict the results obtained using (A) 10 (B) 20 (C) 40 and (D) 80 mM imidazole-containing buffer of various pH levels.
(TIF)

S2 Fig. Optimization of the attachment of purified mPR α with Whatman UK GF/B filters for [3 H]1,2,6,7-progesterone-binding assay analysis. (A) The indicated amount of Ni-NTA resin (10, 20, 50 or 100 μ l) was supplemented into the reaction mixture of the steroid binding assay. After filtration, the mPR α protein content remaining on the filter or present in the flow-through was determined by Western blot analysis using α -His-tag antibody. (B) Specific binding activity of purified mPR α to [3 H]1,2,6,7-progesterone with 10 and 100 μ l Ni-NTA resin supplemented in the reaction mixture.
(TIF)

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Author Contributions

Conceived and designed the experiments: TT. Performed the experiments: MBH TO SH JW. Analyzed the data: MBH TO SH JW. Contributed reagents/materials/analysis tools: MBH TO SH JW. Wrote the paper: MBH TT.

References

1. Stanczyk FZ (2003) All progestins are not created equal. *Steroids* 68: 879–890. PMID: [14667980](#)
2. Ashley RL, Arreguin-Arevalo JA, Nett TM (2009) Binding characteristics of the ovine membrane progesterone receptor alpha and expression of the receptor during the estrous cycle. *Reprod Biol Endocrinol* 7: 42. doi: [10.1186/1477-7827-7-42](#) PMID: [19432978](#)
3. Boonyaratanakornkit V, Edwards DP (2004) Receptor mechanisms of rapid extranuclear signalling initiated by steroid hormones. *Essays Biochem* 40: 105–120. PMID: [15242342](#)
4. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P (2003) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A* 100: 2231–2236. PMID: [12574519](#)
5. Jalabert B, Finet B (1986) Regulation of oocyte maturation in the rainbow trout, *Salmo gairdneri*: role of cyclic AMP in the mechanism of action of the maturation inducing steroid (MIS), 17 α -hydroxy, 20 β -dihydroprogesterone. *Fish Physiol Biochem* 2: 65–74. doi: [10.1007/BF02264074](#) PMID: [24233168](#)
6. Hanna RN, Zhu Y (2009) Expression of membrane progestin receptors in zebrafish (*Danio rerio*) oocytes, testis and pituitary. *Gen Comp Endocrinol* 161: 153–157. doi: [10.1016/j.ygcen.2008.10.006](#) PMID: [18957293](#)
7. Mourot B, Nguyen T, Fostier A, Bobe J (2006) Two unrelated putative membrane-bound progestin receptors, progesterone membrane receptor component 1 (PGMRC1) and membrane progestin receptor (mPR) beta, are expressed in the rainbow trout oocyte and exhibit similar ovarian expression patterns. *Reprod Biol Endocrinol* 4: 6. PMID: [16457725](#)
8. Tokumoto M, Nagahama Y, Thomas P, Tokumoto T (2006) Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. *Gen Comp Endocrinol* 145: 101–108. PMID: [16139281](#)
9. Thomas P, Pang Y, Zhu Y, Detweiler C, Doughty K (2004) Multiple rapid progestin actions and progestin membrane receptor subtypes in fish. *Steroids* 69: 567–573. PMID: [15288771](#)
10. Tubbs C, Pace M, Thomas P (2010) Expression and gonadotropin regulation of membrane progestin receptor alpha in Atlantic croaker (*Micropogonias undulatus*) gonads: role in gamete maturation. *Gen Comp Endocrinol* 165: 144–154. doi: [10.1016/j.ygcen.2009.06.017](#) PMID: [19539624](#)
11. Tang YT, Hu T, Arterburn M, Boyle B, Bright JM, Emtage PC, et al. (2005) PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. *J Mol Evol* 61: 372–380. PMID: [16044242](#)
12. Thomas P, Pang Y, Dong J, Groenen P, Kelder J, de Vlieg J, et al. (2007) Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. *Endocrinology* 148: 705–718. PMID: [17082257](#)
13. Pang Y, Dong J, Thomas P (2013) Characterization, Neurosteroid Binding and Brain Distribution of Human Membrane Progesterone Receptors delta and {epsilon} (mPRdelta and mPR{epsilon}) and mPRdelta Involvement in Neurosteroid Inhibition of Apoptosis. *Endocrinology* 154: 283–295. doi: [10.1210/en.2012-1772](#) PMID: [23161870](#)
14. Asada H, Uemura T, Yurugi-Kobayashi T, Shiroishi M, Shimamura T, Tsujimoto H, et al. (2011) Evaluation of the *Pichia pastoris* expression system for the production of GPCRs for structural analysis. *Microb Cell Fact* 10: 24. doi: [10.1186/1475-2859-10-24](#) PMID: [21513509](#)
15. Shimamura T, Shiroishi M, Weyand S, Tsujimoto H, Winter G, Katritch V, et al. (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature* 475: 65–70. doi: [10.1038/nature10236](#) PMID: [21697825](#)
16. Cregg JM (1985) *Pichia pastoris* as a host system for transformations. *Mol Cell Biol* 5: 3376–3385. PMID: [3915774](#)
17. Cregg JM, Vedvick TS, Raschke WC (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology (N Y)* 11: 905–910.
18. Mizutani K, Hashimoto K, Takahashi N, Hirose M, Aibara S, Mikami B (2010) Structural and functional characterization of recombinant human serum transferrin secreted from *Pichia pastoris*. *Biosci Biotechnol Biochem* 74: 309–315. PMID: [20139607](#)

19. Mizutani K, Okamoto I, Fujita K, Yamamoto K, Hirose M (2004) Structural and functional characterization of ovotransferrin produced by *Pichia pastoris*. *Biosci Biotechnol Biochem* 68: 376–383. PMID: [14981301](#)
20. Tokumoto T, Tokumoto M, Thomas P (2007) Interactions of diethylstilbestrol (DES) and DES analogs with membrane progesterin receptor-alpha and the correlation with their nongenomic progesterin activities. *Endocrinology* 148: 3459–3467. PMID: [17446184](#)
21. Lundstrom K, Wagner R, Reinhart C, Desmyter A, Cherouati N, Magnin T, et al. (2006) Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. *J Struct Funct Genomics* 7: 77–91. PMID: [17120110](#)
22. Zeder-Lutz G, Cherouati N, Reinhart C, Pattus F, Wagner R (2006) Dot-blot immunodetection as a versatile and high-throughput assay to evaluate recombinant GPCRs produced in the yeast *Pichia pastoris*. *Protein Expr Purif* 50: 118–127. PMID: [16843005](#)
23. Hino T, Arakawa T, Iwanari H, Yurugi-Kobayashi T, Ikeda-Suno C, Nakada-Nakura Y, et al. (2012) G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature* 482: 237–240. doi: [10.1038/nature10750](#) PMID: [22286059](#)
24. Oshima T, Nakayama R, Roy SR, Tokumoto T (2014) Purification of the goldfish membrane progesterin receptor alpha (mPRalpha) expressed in yeast *Pichia pastoris*. *Biomed Res* 35: 47–59. PMID: [24573201](#)
25. Rae MT, Menzies GS, Bramley TA (1998) Bovine ovarian non-genomic progesterone binding sites: presence in follicular and luteal cell membranes. *J Endocrinol* 159: 413–427. PMID: [9834459](#)
26. Ambhaikar M, Puri C (1998) Cell surface binding sites for progesterone on human spermatozoa. *Mol Hum Reprod* 4: 413–421. PMID: [9665627](#)
27. Domanski M, Molloy K, Jiang H, Chait BT, Rout MP, Jensen TH, et al. (2012) Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels. *Biotechniques* 0: 1–6.
28. LaCava J, Molloy KR, Taylor MS, Domanski M, Chait BT, Rout MP (2015) Affinity proteomics to study endogenous protein complexes: Pointers, pitfalls, preferences and perspectives. *Biotechniques* 58: 103–119. doi: [10.2144/000114262](#) PMID: [25757543](#)
29. Thomas P (2008) Characteristics of membrane progesterin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progesterin actions. *Front Neuroendocrinol* 29: 292–312. doi: [10.1016/j.yfrne.2008.01.001](#) PMID: [18343488](#)
30. Tokumoto T, Tokumoto M, Oshima T, Shimizuguchi K, Fukuda T, Sugita E, et al. (2012) Characterization of multiple membrane progesterin receptor (mPR) subtypes from the goldfish ovary and their roles in the induction of oocyte maturation. *Gen Comp Endocrinol* 177: 168–176. doi: [10.1016/j.ygcen.2012.03.005](#) PMID: [22465781](#)
31. Smith JL, Kupchak BR, Garitaonandia I, Hoang LK, Maina AS, Regalla LM, et al. (2008) Heterologous expression of human mPRalpha, mPRbeta and mPRgamma in yeast confirms their ability to function as membrane progesterone receptors. *Steroids* 73: 1160–1173. doi: [10.1016/j.steroids.2008.05.003](#) PMID: [18603275](#)
32. Josefsberg Ben-Yehoshua L, Lewellyn AL, Thomas P, Maller JL (2007) The role of *Xenopus* membrane progesterone receptor beta in mediating the effect of progesterone on oocyte maturation. *Mol Endocrinol* 21: 664–673. PMID: [17185392](#)
33. Tubbs C, Thomas P (2009) Progesterin signaling through an olfactory G protein and membrane progesterin receptor-alpha in Atlantic croaker sperm: potential role in induction of sperm hypermotility. *Endocrinology* 150: 473–484. doi: [10.1210/en.2008-0512](#) PMID: [18801904](#)
34. Zhu Y, Bond J, Thomas P (2003) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progesterin receptor. *Proc Natl Acad Sci U S A* 100: 2237–2242. PMID: [12601167](#)
35. Hanna R, Pang Y, Thomas P, Zhu Y (2006) Cell-surface expression, progesterin binding, and rapid non-genomic signaling of zebrafish membrane progesterin receptors alpha and beta in transfected cells. *J Endocrinol* 190: 247–260. PMID: [16899559](#)
36. Labombarda F, Meffre D, Delespierre B, Krivokapic-Blondiaux S, Chastre A, Thomas P, et al. (2010) Membrane progesterone receptors localization in the mouse spinal cord. *Neuroscience* 166: 94–106. doi: [10.1016/j.neuroscience.2009.12.012](#) PMID: [20025939](#)
37. Intlekofer KA, Petersen SL (2011) Distribution of mRNAs encoding classical progesterin receptor, progesterone membrane components 1 and 2, serpine mRNA binding protein 1, and progesterin and ADI-POQ receptor family members 7 and 8 in rat forebrain. *Neuroscience* 172: 55–65. doi: [10.1016/j.neuroscience.2010.10.051](#) PMID: [20977928](#)

38. Frye CA, Koonce CJ, Walf AA (2013) Pregnane xenobiotic receptors and membrane progesterin receptors: role in neurosteroid-mediated motivated behaviours. *J Neuroendocrinol* 25: 1002–1011. doi: [10.1111/jne.12105](https://doi.org/10.1111/jne.12105) PMID: [24028379](https://pubmed.ncbi.nlm.nih.gov/24028379/)
39. Frye CA, Walf AA, Kohtz AS, Zhu Y (2014) Progesterone-facilitated lordosis of estradiol-primed mice is attenuated by knocking down expression of membrane progesterin receptors in the midbrain. *Steroids* 81: 17–25. doi: [10.1016/j.steroids.2013.11.009](https://doi.org/10.1016/j.steroids.2013.11.009) PMID: [24269738](https://pubmed.ncbi.nlm.nih.gov/24269738/)
40. Dressing GE, Alyea R, Pang Y, Thomas P (2012) Membrane progesterone receptors (mPRs) mediate progesterin induced antimorbidity in breast cancer cells and are expressed in human breast tumors. *Horm Cancer* 3: 101–112. doi: [10.1007/s12672-012-0106-x](https://doi.org/10.1007/s12672-012-0106-x) PMID: [22350867](https://pubmed.ncbi.nlm.nih.gov/22350867/)
41. Zuo L, Li W, You S (2010) Progesterone reverses the mesenchymal phenotypes of basal phenotype breast cancer cells via a membrane progesterone receptor mediated pathway. *Breast Cancer Res* 12: R34. doi: [10.1186/bcr2588](https://doi.org/10.1186/bcr2588) PMID: [20540763](https://pubmed.ncbi.nlm.nih.gov/20540763/)
42. Xie M, Zhu X, Liu Z, Shrubsole M, Varma V, Mayer IA, et al. (2012) Membrane progesterone receptor alpha as a potential prognostic biomarker for breast cancer survival: a retrospective study. *PLoS One* 7: e35198. doi: [10.1371/journal.pone.0035198](https://doi.org/10.1371/journal.pone.0035198) PMID: [22496908](https://pubmed.ncbi.nlm.nih.gov/22496908/)
43. Vares G, Sai S, Wang B, Fujimori A, Nenoï M, Nakajima T (2015) Progesterone generates cancer stem cells through membrane progesterone receptor-triggered signaling in basal-like human mammary cells. *Cancer Lett* 362: 167–173. doi: [10.1016/j.canlet.2015.03.030](https://doi.org/10.1016/j.canlet.2015.03.030) PMID: [25819032](https://pubmed.ncbi.nlm.nih.gov/25819032/)
44. Chien CH, Lai JN, Liao CF, Wang OY, Lu LM, Huang MI, et al. (2009) Mifepristone acts as progesterone antagonist of non-genomic responses but inhibits phytohemagglutinin-induced proliferation in human T cells. *Hum Reprod* 24: 1968–1975. doi: [10.1093/humrep/dep099](https://doi.org/10.1093/humrep/dep099) PMID: [19401324](https://pubmed.ncbi.nlm.nih.gov/19401324/)
45. Ndiaye K, Poole DH, Walusimbi S, Cannon MJ, Toyokawa K, Maalouf SW, et al. (2012) Progesterone effects on lymphocytes may be mediated by membrane progesterone receptors. *J Reprod Immunol* 95: 15–26. doi: [10.1016/j.jri.2012.04.004](https://doi.org/10.1016/j.jri.2012.04.004) PMID: [22709513](https://pubmed.ncbi.nlm.nih.gov/22709513/)
46. Lu J, Reese J, Zhou Y, Hirsch E (2015) Progesterone-induced activation of membrane-bound progesterone receptors in murine macrophage cells. *J Endocrinol* 224: 183–194. doi: [10.1530/JOE-14-0470](https://doi.org/10.1530/JOE-14-0470) PMID: [25472814](https://pubmed.ncbi.nlm.nih.gov/25472814/)
47. Tanabe H, Fujii Y, Okada-Iwabu M, Iwabu M, Nakamura Y, Hosaka T, et al. (2015) Crystal structures of the human adiponectin receptors. *Nature* 520: 312–316. doi: [10.1038/nature14301](https://doi.org/10.1038/nature14301) PMID: [25855295](https://pubmed.ncbi.nlm.nih.gov/25855295/)
48. Thomas P, Pang Y, Dong J, Berg AH (2014) Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis. *Endocrinology* 155: 4250–4265. doi: [10.1210/en.2014-1201](https://doi.org/10.1210/en.2014-1201) PMID: [25014355](https://pubmed.ncbi.nlm.nih.gov/25014355/)
49. Berg AH, Rice CD, Rahman MS, Dong J, Thomas P (2014) Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: I. Discovery in female atlantic croaker and evidence ZIP9 mediates testosterone-induced apoptosis of ovarian follicle cells. *Endocrinology* 155: 4237–4249. doi: [10.1210/en.2014-1198](https://doi.org/10.1210/en.2014-1198) PMID: [25014354](https://pubmed.ncbi.nlm.nih.gov/25014354/)