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Membrane progestin receptor beta (mPR-β): A protein related to cumulus expansion that is involved in *in vitro* maturation of pig cumulus–oocyte complexes

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ABSTRACT

A new group of putative membrane receptors have now been isolated from fish and other vertebrates, including human. These proteins are classified into three groups known as membrane progestin receptor alpha, beta and gamma (mPR-α, -β and -γ). In the present study we have investigated the role of mPR-β in regulating *in vitro* maturation (IVM) of pig cumulus–oocyte complexes (COCs). RT-PCR and Western blot analysis indicated that COCs contain transcripts and proteins for mPR-β. The levels of both transcripts and proteins increased between 0 and 20 h IVM, but then decreased between 20 and 44 h. The luteinizing hormone (LH) and follicle-stimulating hormone (FSH) did not affect mPR-β expression during IVM. Immunofluorescence analysis indicated that the mPR-β was localized in the plasma membrane of cumulus cell. However, in mouse embryonic fibroblasts (MEFs), mPR-β was detected at the endoplasmic reticulum (ER) rather than the plasma membrane. Cumulus expansion was impaired significantly ($P < 0.05$) when COCs were incubated in maturation medium containing 10% (v/v) anti-mPR-β serum during IVM. Bioinformatics analysis predicted that mPR-β had an ER retention motif and an endocytosis internalization motif. These results suggest that the mPR-β is a molecule related to cumulus expansion and it might function by regulation of exocytosis. In conclusion, this is the first description of the expression patterns and subcellular localization of mPR-β in COCs and might shed light on the function of the protein.

Although the non-genomic or rapid action of progesterone was found over 20 years ago [1], efforts to determine the structures of non-genomic progesterone receptors were unsuccessful until recently [2–6]. In 2003, Zhu et al. [5,6] isolated a group of proteins from fish and other vertebrates including humans that they called membrane progestin receptors (mPRs), because they were localized at the plasma membrane and bound progestin with high affinity [5]. These proteins were classified into three groups called membrane progestin receptor alpha, beta and gamma (mPR-α, -β and -γ) [5]. All of these mPRs have seven deduced trans-membrane domains, which is the characteristic of G-protein coupled receptors (GPCRs) [5]. Furthermore, the N-terminus regions of mPRs are extracellular and C-terminus regions are intracellular [5], which are also characteristics of GPCRs. Some reports suggest that the mPRs are physiological progesterone receptors involved in oocyte maturation of fish [5,6] and *Xenopus* [7].

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The problem of non-genomic progesterone was thought to be resolved [8], but several subsequent studies failed to support some of the results mentioned above [9]. For example, Krietsch et al. [9] reported that mPRs are localized in the endoplasmic reticulum (ER) rather than plasma membrane, mPRs do not induce mobilization of calcium ions in response to progesterone, *etc.* Subsequently, researchers have found that the mPRs belong to the family of progestin and AdipoQ receptors (PAQR), which are a conserved family of proteins spanning the bacterial, plant and animal kingdoms [10]. However, the N-terminus of PAQR1 is intracellular and C-terminus is extracellular [10]. It was also suggested that the N-terminus of mPRs is not extracellular [9]. Therefore, further studies are required to elucidate the functions of mPRs.

The previous studies supported the attitude that all the three mPR isoforms shared the similar subcellular localization and function in vertebrates and most studies explored two or more isoforms at term. The accumulated data suggests that the mPR-β was not regulated by gonadotrophin in both fish [5] and rat [11] ovaries. However, it play role in mediating maturation of *Xenopus* oocytes [7] and regulating human myometrium [12] in a progesterone dependent way. As mPR-α and -γ, many functions and subcellular localization of mPR-β is still controversial now [7,9,12]. Furthermore, there are currently no data relating to the role of mPRs in mammalian oocyte maturation.

In order to explore if mPR-β play roles in mammalian oocyte maturation, we use pig oocyte in vitro maturation system as a model and herein we have investigated the role played by mPR-β in pig cumulus–oocyte complexes (COCs) matured *in vitro*, including the dynamics, subcellular localization, and putative function mechanism. For the first time, we described the expression and dynamics of mPR-β in pig COCs matured *in vitro* and demonstrated that the subcellular localization of mPR-β varied in different tissues. Furthermore, we found that mPR-β participated in cumulus cell expansion, which might shed light on the function of the protein and accelerate the elucidation of the molecular mechanism underlining mammalian cumulus cell expansion.

1. Materials and methods

1.1. *Oocyte collection and in vitro maturation*

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory at approximate 37 °C in saline solution. COCs were aspirated from follicles (diameter, 2–6 mm), and oocytes surrounded by at least two layers of compact cumulus cells were used for IVM. Groups of 25–30 COCs were transferred into 100 μl drops of mTCM-199 (supplemented with 0.5 μg/ml FSH + LH, 10 ng/ml EGF, 0.1 μg/ml cysteine and 10% porcine follicle fluid) and cultured for 44 h in an incubator with 5% CO_2 and saturated humidity at 39 °C. The drops were covered with paraffin oil before culture.

1.2. *Experiment design*

Experiment (i) was conducted to investigate the effects of cell maturation. 150–200 COCs were cultured *in vitro* for 0, 12, 20, 36 and 44 h, respectively and then used for RNA isolation. Western blot analyses used 150 COCs cultured *in vitro* for 0, 20 or 44 h.

Experiment (ii) was conducted to determine if LH or FSH affect the expression of mPR-β during IVM. Treatments were performed with either LH or FSH absent from the normal maturation medium, which was used as a control.

1.3. *Primer design, RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)*

PCR primers of mPR-β (sense, GAAGGACTCACAGAACTAGC; antisense, AACCCCACACATCTCTCAAC) were designed based on the pig putative membrane progestin receptor beta (NCBI accession no. AF313615), and primers of glyceraldehyde-3-phosphatedehydrogenase (G3PDH) (sense, ATTGCCCTCAACGACCACTT; antisense, ACATGACGAGGCAGGTCTCC) were designed according to Ref. [13].

Based on this experimental design, 150–200 COCs were used for RNA isolation. The first strand of cDNA was synthesized by using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (Takara Co., Dalian, China). The PCR protocol included two steps. At the first step, the sense and anti-sense primers for mPR-β were added into the PCR mixture. The initial denaturation was conducted at 95 °C for 4 min, followed by 9 cycles of 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C. Final elongation was performed at 72 °C for 3 min. Then the sense and anti-sense primers for G3PDH and 0.15 μl Ex Taq polymerase (hot start) were added into the PCR mixture. At the second step, the initial denaturation was conducted at 95 °C for 4 min, followed by 9 cycles of 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C. Final elongation was performed at 72 °C for 3 min.

Reaction products were separated on 2% agarose gels and stained with ethidium bromide. The samples were quantified by a GeneGenius gel documention system (Gene Co., USA).

1.4. *Preparation of rabbit anti-pig mPR-β antibodies*

The amino acid sequence of mPR-β was based on the putative membrane steroid receptor [Sus scrofa] (NCBI accession no. NP_998905). Epitopes of mPR-β were predicted using online software: http://bio.dfci.harvard.edu/Tools/antigenic.html. An epitope near the N-terminus of mPR-β was selected for preparation of the antibody [14]. Its amino acid sequence was PETDVPQLFREPYIRAGYRPIG.

In order to ensure the antibody was special for mPR-β, we aligned the amino acid sequence through: http://www.ncbi.nlm.nih.gov/blast/ [15]. The BLAST result was shown in Table 1. The result showed that the amino acid sequence selected was much conserved among the mammalians. Compared with pig mPR-β, the mouse mPR-β just have an (R versus H) amino acid difference, which were two alkaline amino acids and share the similar characters. Furthermore, it was not conserved between pig mPR-α and -β at this site. There was not any matched site between pig mPR-α and -β including more than four amino acid residues. Generally speaking, an epitope including five or more amino acid residue [16], which excluded the possibility of cross action between the anti-mPR-β antibody and pig mPR-α.

This peptide was synthesized and subsequently used to vaccinate the rabbits for preparation of the antibody. Both