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Expression of Progesterone Receptor Membrane Component 1 and 2 in the Mouse Gonads and Embryos

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생쥐 생식소 및 배아의 프로게스테론 수용체 막성분 1과 2의 발현에 관한 연구

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ABSTRACT : Previously, we found progesterone receptor membrane component 2 (pgrmc2) was highly expressed in germinal vesicle (GV) stage oocytes. The present study was conducted to characterize the expression of pgrmc2, as well as pgrmc1, in the mouse gonads and embryos according to their developmental stages. We found that these membrane components were expressed in ovaries, testes, and embryos at various developmental stages in addition to oocytes. Progesterone-3-O-carboxymethyl oxime-BSA-fluorescein isothiocyanate (P4-BSA-FITC) was applied to visualize the presence of the progesterone receptor on mouse oocyte membrane, and we confirmed that immobilized progesterone is localized at surface of the oocyte. This is, at our knowledge, the first report regarding the expression of membrane component of progesterone receptor in the mouse oocytes, embryos, and gonads. The function and signal transduction pathway of progesterone receptor membrane components in oocytes requires further studies.
Key words : Progesterone receptor, Steroid receptor membrane component, P4-BSA-FITC, Mouse, Oocytes.

요 약 : 본 연구진은 선행 연구에서 progesterone receptor membrane component 2 (pgrmc2) 유전자가 미성숙 난자에서 높게 발현하는 것을 발견하였다. 본 연구는 pgrmc2와 pgrmc1 유전자가 쥐의 생식소와 배아의 발달 단계에 따라 어떻게 발현하는지 알아 보기 위해 수행하였다. 이들 유전자는 난소, 정소, 배아, 그리고 난자의 다양한 발달 단계에서 발현하는 것을 알 수 있었다. 쥐 난자 세포막에 프로게스테론 수용체가 존재하는 것은 progesterone-3-O-carboxymethyl oxime-BSA-fluorescein isothiocyanate (P4-BSA-FITC)의 결합을 이용하여 확인하였다. 본 결과는 프로게스테론 수용체의 세포막 구성 요소들이 생쥐의 난자, 배아, 그리고 생식소에서 발현함을 최초로 확인한 연구 결과다. 스테로이드 수용체의 세포막 구성 요소들의 기능, 특히 프로게스테론 수용체의 세포막 구성 요소들의 기능을 알아 보기 위해 추후 연구가 계속되어야 할 것이다.

INTRODUCTION

Progesterone is a key hormone in the regulation of growth, development, and function of female reproductive tissues. In the ovary, progesterone plays a central role in regulating ovulation (Rondell, 1974; Mori *et al.*, 1977; Kohda *et al.*, 1980) and lutenization (Park & Mayo, 1991; Natraj & Richards, 1993) via receptor-mediated pathways. Progesterone also regulates gonadotropin level and directs anti-apoptotic action, estrogen synthesis, and mitosis (Peluso, 2006; Peluso *et al.*, 2006).

Steroid hormones, unlike other hormones, have been well known for more than 30 years to bind to the intracellular receptors in the cytoplasm or nucleus, by entering the target cells. Rather than triggering a signal-transduction pathway, the receptor itself carries out the transduction of the hormonal signals (Campbell *et al.*, 2003). Progesterone induces a conformational change of its intracellular progesterone receptor (iPR) from a non DNA-

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binding, inactive form, into one that will bind DNA. This transformation is accompanied by a loss of associated heat shock proteins, and dimerization. The activated PR dimer then binds to specific DNA sequences within the promoter region of progesterone-responsive genes, referred to as progesterone response elements (PRE). The progesterone-bound PR is believed to activate transcription either by direct action on the general transcriptional machinery or by association with co-activators (Chabbert-Buffet *et al.*, 2005). This is the genomic activation pathway of progesterone. The iPR is expressed as two isoforms, PRA and PRB (Horwitz & Alexander, 1983; Savouret *et al.*, 1990); those are encoded by a single gene but controlled by separate promoters (Kastner *et al.*, 1990).

Recent findings suggest that some steroid hormone receptors are located on the membrane like other receptors of growth factors. In such cases, hormones bind to a specific receptor protein in the plasma membrane of the target cell, and this binding activates the receptor, which initiates a multistep signal-transduction pathway in the cell (Campbell *et al.*, 2003). Such findings characterize the other category of progesterone receptor, membrane progesterone receptor (mPR). Recently, it has been identified that mPR includes MPRα, MPRβ, and MPRγ, as well as progesterone receptor membrane component-1 and -2 (pgrmc1 and pgrmc2). This nongenomic pathway of progesterone induces a wide range of changes, including Ca^{2+} mobilization, opening of Na^{+} and Cl^{-} channels, and the activation of phospholipase C (leading to increased intracellular IP_3 and diacylglycerol generation), Pertussis toxin-insensitive G-protein-coupled receptors, protein kinase C, tyrosine kinase and MAP kinase pathways (Bramley, 2003; Falkenstein *et al.*, 2000a; Wehling, 1997).

Previously, we obtained the list of genes expressed during oocyte maturation by using annealing control primer -polymerase chain reaction (ACP-PCR) to investigate oocyte gene expression and changes during oocyte maturation. Among these genes, pgrmc2 was found as GV-selective gene that is expressed highly in the immature GV oocytes compared to the mature metaphase II (MII) oocytes.

Pgrmc1 has been detected in mouse granulosa (McRae *et al.*, 2005), lacteal cells (Cai & Stocco, 2005; Sasson *et al.*, 2004) and spontaneously immortalized granulosa cells (Peluso *et al.*, 2005), as a relatively small protein (28 kDa) that possesses a short N-terminal extracellular domain, a single transmembrane domain, and a cytoplasm domain (Selmin *et al.*, 1996). However, not much is known about the pgrmc2, as well as function of both pgrmc1 and pgrmc2. Therefore, the present study was conducted to evaluate the expression pattern of pgrmc1 and pgrmc2 in murine gonads, oocytes and embryos according to developmental stages.

MATERIALS AND METHODS

1. Isolation of Oocytes and Embryos

This study was approved by the Institutional Review Board (IRB) of the CHA Research Institute, CHA General Hospital, Seoul, Korea. For the isolation of germinal vesicle (GV) oocytes from preovulatory follicles, 4-week-old female ICR mice were injected with 5 IU of pregnant mare's serum gonadotropin (PMSG; Intervet, Holland). Mice were sacrificed 46 h after PMSG injection and the cumulus-enclosed oocyte complexes (COCs) were recovered from the ovaries by puncturing the surface of the ovary with 27-guage needles in M2 medium (Sigma, St. Louis, MO) containing 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) to inhibit germinal vesicle breakdown (GVBD). Cumulus cells (CC) were removed from oocytes by pipetting through a fine-bore pipette. Mural GC was recovered from the preovulatory follicles. Isolated oocytes, CC, and GC were snap-frozen and stored at −70℃ for RNA isolation. To obtain matured MII oocytes, we sequentially injected female mice with 5 IU of PMSG and human chorionic gonadotrophin (hCG; Intervet) at an internal of 46 h. Super-ovulated MII oocytes were obtained from oviduct 16 h after hCG injection. Cumulus cells surrounding MII oocytes were removed by treating COCs with hyaluronidase (300 U/ml, Sigma).

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surrounding MII oocytes were removed by treating COCs with hyaluronidase (300U/ml, Sigma). To collect embryos, female mice were super-ovulated and mated. Fertilized embryos were obtained post hCG injection as follows: pronucleus 1-cell embryo (PN) at 18~20 h, and 2-cell embryos (2C) at 44~46 h. Embryos at later developmental stages were obtained by culturing 2-cell embryos in M16 medium (Sigma) at 37℃ in a humidified atmosphere of 5% CO_2 in air. Embryos at 4-cell (4C), 8-cell (8C), morula (MO), and blastocyst (BL) stages were snap-frozen and stored at −70℃ until use.

2. Total RNA Isolation from Gonads

Ovaries (from post-natal day1, day5, day14, day21 and day28 ICR female mice) and testes (from post-natal day5, day14, day21 and day42 ICR male mice) were collected in Dulbecco's PBS (GIBCO-BRL). Ovaries and testes were homogenized on ice using a homogenizer in 1ml Trizol reagent and incubated for 5 min at room temperature. 0.2 ml chloroform was added to supernatant and mixed by vortexing and incubated for 15 min at room temperature. The tube was centrifuged at 12,000×g for 15 min at 4℃. After centrifugation, supernatant was transferred to a new tube containing 0.5 ml of isopropanol and precipitated. The pellet was washed with 75% ethanol, dried, dissolved in DEPC treated water, and stored at −70℃.

3. Messenger RNA Isolation

Messenger RNA was isolated from the same number of GV and MII oocytes using Dynabeads mRNA DIRECT kit (Dynal). Briefly, oocytes were resuspended in 300 μl lysis/binding buffer (100 mM Tris-HCl pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) for 5 min at room temperature. After vortexing, 20 μl of prewashed Dynabeads oligo(dT)$_{25}$ was mixed with the lysate and annealed by rotating for 5 min at room temperature. The beads were separated with a Dynal MPC-S magnetic particle concentrator and poly(A)$^+$ RNAs were eluted from the beads by incubation in 10 μl of Tris-HCl (10 mM Tris-HCl, pH 7.5) at 65℃ for 2 min, and the aliquot was used for reverse transcription.

4. Reverse Transcriptase Polymerase Chain Reaction

Complementary DNA was synthesized from total RNA using 0.5 mg oligo (dT) primer according to the SuperScript Preamplification System protocol (Gibco). Polymerase chain reactions (PCRs) were carried out in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 25 pM of each primer, 2.5 U of Taq DNA polymerase (Promega), and the rest filled with distilled water in a final volume of 20 μl. Single oocyte- and single embryo-equivalent cDNA was used as the template for PCR analysis. Primer sequences for pgrmc1, pgrmc2, and the glyceraldehydes-3-phosphate dehydrogenase (G3PDH) internal control, as well as their reverse transcriptase PCR conditions, are listed in Table 1. After PCR, 20 μl aliquots of each sample were electrophoresed on a 1.2% agarose gel and assessed by an Image Analyzer (Viber Lourmat). The relative gene expression levels were normalized to that of G3PDH. Experiments were repeated 3 times.

Table 1. Primer sequences and RT-PCR conditions

Gene	Access number	Primer sequence*	Annealing temperature	Product size
Pgrmc1	NM_016783	For - AGCCCATTTTACCCAACTGA Rev - CATACCTGTTCCTGCCCTGT	57℃	383 bp
Pgrmc2	BC044759	For - TGGAGAGTGTTCGAGAATGG Rev - GATGAAGCCCACCAGACATT	59℃	348 bp
Gdf9	NM_008110	For - GGTTCTATCTGATAGGCGAGG Rev - GGGGCTGAAGGAGGGAGG	65℃	446 bp
Gg3pdh	AK213421	For - TCCACCACCCTGTTGCTGTA Rev - ACCACAGTCCATGCCATCAC	60℃	451 bp

* For = Forward; Rev = Reverse.

5. Quantitative Real-time RT-PCR

To measure the amount of pgrmc1 and pgrmc2 mRNA in a single embryo, quantitative real-time RT-PCR analysis was performed using the iCycler apparatus (Bio-Rad, Hercules, CA). The iQ^{TM} SYBR Green supermix PCR Reagents (Bio-Rad) was used for monitoring amplification in real-time, and the results were evaluated by iCycler iQ real-time detection system software. The reaction mixture was consisted of cDNA, 20 pmol forward and reverse primers, and SYBR Green supermix 2 containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers. The template was amplified by 40 cycles of denaturation at 95℃ for 40 sec, annealing at 60℃ for 40 sec, and extension at 72℃ for 40 sec. At the completion of PCR, we monitored fluorescence continuously while slowly heating the samples from 60℃ to 95℃ at 0.5℃ intervals, producing melting curves to identify any non-specific products. Quantitation of gene amplification was made by determining the cycle threshold (C_T) based on the fluorescence detected within the geometric region of the semi-log amplification plot. Expression of each mRNA species was normalized to the level of rabbit-globin mRNA. Relative quantitation of target gene expression was evaluated using the comparative C_T method and the experiments were repeated at least three times using different sets of embryos.

6. Treatment of Oocytes with P4-BSA-FITC

To remove zona pellucida, denuded oocytes were placed into a 50 μl drop of prewarmed acidic Tyrode's solution (Sigma) under mineral oil for approximately 20 sec, followed by careful washing in five times with M16 medium (Sigma). Zona-free oocytes were incubated with or without 0.005 μg/μl progesterone for 2 h in M16 medium then incubated with 0.1 μg/μl of P4-BSA-FITC (Sigma) for 2 h in M16 medium. Oocytes were washed extensively with PBS including 0.1% PVA (Sigma). The localization of P4-BSA-FITC was detected from the emitted fluorescence by laser-scanning confocal microscope.

RESULTS

1. Expression of pgrmc1 and pgrmc2 according to the Gonadal Developmental Stages

In the alignment data, the nucleotide homology and the amino acid homology between pgrmc1 and pgrmc2 in mouse has been revealed about 25.6% and 6.6%, respectively (Fig. 1A, 1B).

Expression patterns of pgrmc1 and pgrmc2 were observed according to ovarian developmental stages by using RT-PCR analysis. Pgrmc1 was found equally expressed in all five developmental stages. Contrastingly, expression of pgrmc2 was at maximum expression at 2-week-old ovaries and decreased afterwards (Fig. 2A). Expression pattern of pgrmc1 and pgrmc2 according to testicular developmental stages is shown in Fig. 2B. Both pgrmc1 and pgrmc2 were equally expressed in all stages of testes (Fig. 2B).

2. Expression of pgrmc1 and pgrmc2 in Follicles and Embryos

Oocytes, cumulus cells, and granulosa cells, are all expressed pgrmc1 and 2 (Fig. 3). Expression of pgrmc1 and pgrmc2 during embryogenesis was evaluated by quantitative real-time RT-PCR. Both pgrmc1 and pgrmc2 were expressed in GV and MII stage oocyte then decreased as

A. Nucleotide sequence

B. Amino acid sequence

Fig. 1. Alignment of nucleotide (A) and amino acid (B) sequences of mouse pgrmc1 and pgrmc2. Identical residues are boxed, and numbers on the right indicate the amino acid position in each sequence. The GenBank accession numbers for mouse pgrmc1 and mouse pgrmc2 are NM_016738 and BC044759, respectively.

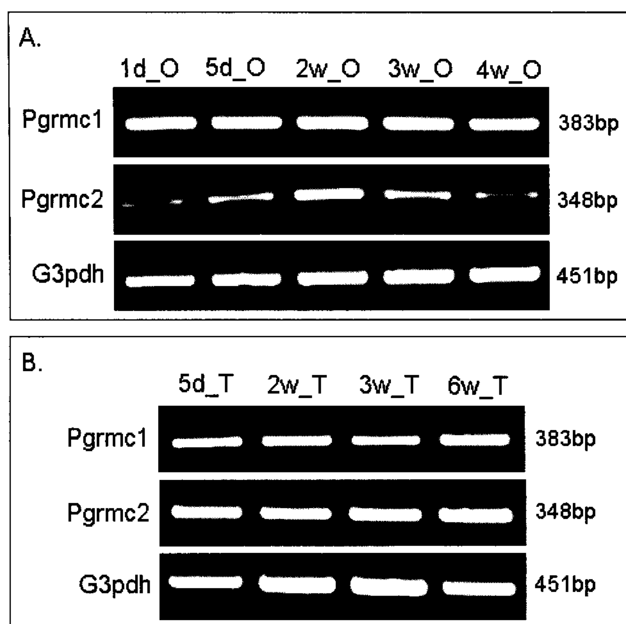

Fig. 2. RT-PCR analysis for pgrmc1 and pgrmc2. (A) Differential expression pattern of pgrmc1 and pgrmc2 according to ovarian developmental stages. 1d_O, neonatal 1-day ovary; 5d_O, 5-day ovary; 2w_O, 2-week-old ovary; 3w_O, 3-week-old ovary; 4w_O, 4-week-old ovary. (B) RT-PCR analysis for pgrmc1 and pgrmc2 according to testicular developmental stages. 5d_T, 5-day testis; 2w_T, 2-week-old testis; 3w_T, 3-week-old testis; 6w_T, 6-week-old testis. G3pdh was used as an internal control. Numbers on the right indicate the product size.

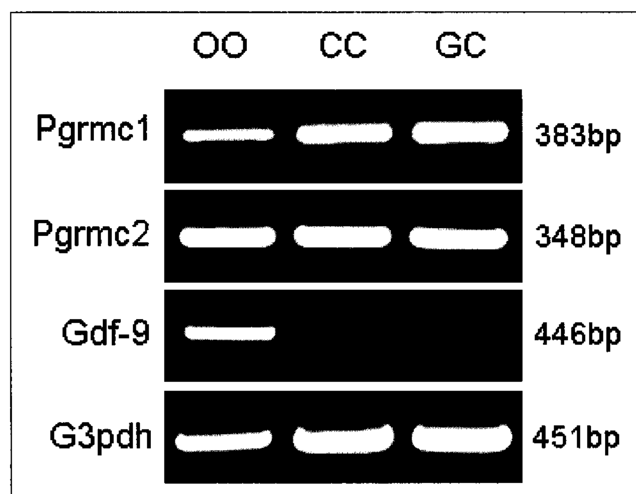

Fig. 3. RT-PCR analysis for pgrmc1 and pgrmc2 in oocyte and follicular cells. OO, germinal vesicle stage oocytes; CC, cumulus cells; GC, granulosa cells. Gdf-9 and G3pdh were used as internal controls. Gdf-9 is known as oocyte-specific and G3pdh is a housekeeping gene. Numbers on the right indicate the product size.

the embryo develops, reaching minimal expression at 2-cell stage. Their expression was increased again from 4-cell stage and later embryonic developmental stages (Fig. 4).

3. Localization of P4-BSA-FITC on the Plasma Membrane of Denuded Oocyte

When we measured the binding of P4-BSA-FITC to the plasma membrane of oocytes, fluorescence was visualized at the outside surface of the oocyte (Fig. 5C). In control group where zona-free oocytes were pretreated with cold progesterone at saturation level for 2 h, binding of P4-BSA-FITC was not detected (Fig. 5A).

4. Alignment of Amino Acid and Nucleotide Sequence for pgrmc2

Homology between mouse and human sequence is shown in Fig. 6. In the nucleotide alignment data, mouse pgrmc2 is homologous with human in 87.3% (Fig. 6A); while amino acid sequence is homologous with in 89.2% (Fig. 6B).

DISCUSSION

Progesterone has long been regarded as an important hormone regulating female fertility. Although usually associated with the maintenance of pregnancy, progesterone is also essential for ovulation via receptor- mediated pathways. The importance of ovarian iPR was demonstrated by the targeted interruption of the iPR gene; PR knockout female mice lacking PRA and PRB fail to ovulate, even in response to exogenous hormones, and are completely infertile (Gava *et al.*, 2004).

Steroid hormone receptors are a class of the nuclear receptor family whose mechanism of transcriptional regulation has been under intensive study for over 30 years. Several studies have suggested that nongenomic steroid-induced maturation may be mediated by receptors located in the plasma membrane (Watson & Gametchu, 1999; Wehling, 1997; Revelli *et al.*, 1998). The fact that action of progesterone is mediated by a membrane interaction was showed in *Xenopus* by direct injection of progesterone into oocytes is unable to promote oocyte maturation,

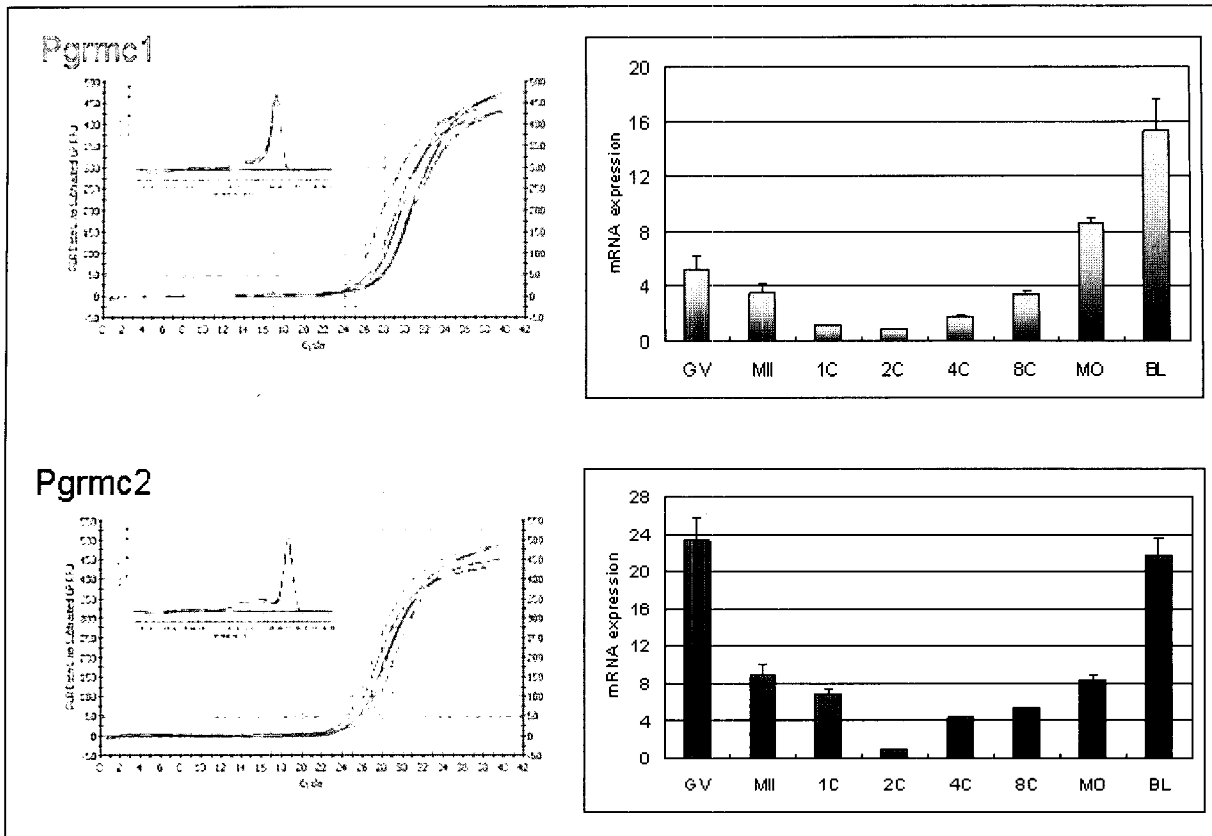

Fig. 4. Quantitative real-time RT-PCR analysis of pgrmc1 and pgrmc2. mRNA isolated from oocytes and various embryonic stages were reverse transcribed. For the PCR reaction, one oocyte- or one embryo-equivalent cDNA was used as a template for amplification. Expression amount was calculated from C_T values and the mRNA ratio was calculated against that of 2-cell embryo. Experiments were repeated at least three times and data were expressed as mean±SEM. GV, germinal vesicle oocyte; MII, metaphase II oocyte; 1C, pronucleus 1-cell zygote; 2C, 2-cell; 4C, 4-cell; 8C, 8-cell; MO, morula; BL, blastocyst embryo.

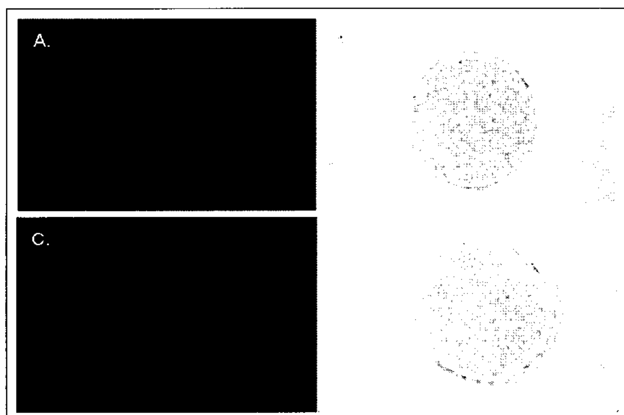

Fig. 5. Microphotographs of the zona-free oocyte after culture with P4-BSA-FITC. Zona-free oocytes were observed under a laser-scanning confocal microscope. (A, B) Control oocytes cultured 2 h with P4-BSA-FITC after progesterone pretreatment. (C, D) Oocytes cultured 2 h with FITC-labeled progesterone-BSA without progesterone treatment.

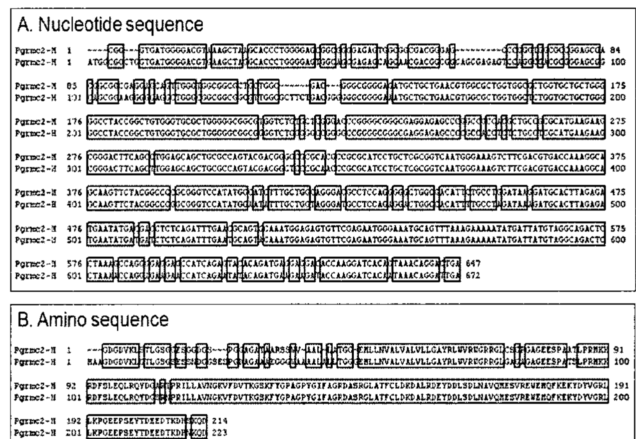

Fig. 6. Alignment of nucleotide (A) and amino acid (B) sequences for pgrmc2. Identical residues are boxed, and numbers on the right indicate the amino acid position in each sequence. The GenBank accession number for mouse and human are NM_006320 and BC044759, respectively.

whereas progesterone covalently bound to either BSA or polymer is able to cause oocyte maturation in *Xenopus* (Masui & Markert, 1971; Smith & Ecker, 1971). The presence of a progesterone receptor located at the outside of oocytes was indicated by radio receptor binding assays using total plasma membrane fraction in *Xenopus* and in *Rana pipens* (Kostellow *et al.*, 1993). However, no study was accomplished previously concerning the presence of progesterone membrane receptors in mouse oocytes, giving present study significant as a first report.

It has already been reported that progesterone receptor is localized on the membrane of the *Rana dybowskii* oocyte by using P4-BSA-FITC (Bandyopadhyay *et al.*, 1998). The binding of receptor on oocyte membrane with P4-BSA-FITC supports the existence of binding sites for progesterone on the outside of mouse oocyte. We observed that immobilized progesterone is localized only outside of the oocyte plasma membrane by using P4-BSA-FITC and the decreased binding of P4-BSA-FITC was observed after pretreatment with progesterone (Fig. 5A). We conclude that the progesterone receptor membrane component is present on the murine oocyte plasma membrane.

The preimplantation period of embryo development corresponds to a transcription between maternal and embryonic genes. This transition is not abrupt since both the accumulation of embryonic molecules resulting from the onset of embryonic transcription and the decay of maternal molecules were progressive processes. In mice, the onset of transcription of the embryonic genomes starts during the last steps of DNA replication at the end of the one-cell stage (Bouniol-Baly *et al.*, 1997). The mRNA degradation continues after fertilization and only 10～20% of these mRNAs remain detectable at the two-cell stages, so that the total content of mRNA is at a minimum when transcriptional activation of the genome occurs (Bachvarova & De Leon, 1980). Both pgrmc1 and pgrmc2 were found to decrease in expression as the embryo ages, reached minimum expression in 2-cell, and increased in later embryonic developmental stages. The expression of pgrmc1 and pgrmc2 were found to be dependent on the stage of embryos, so it is predictable that these genes may play a role in embryo development.

Communication between the oocyte and adjacent somatic cells (cumulus and granulosa cells) is essential for successful development of both follicular compartments. It is well documented that the oocytes orchestrate and coordinate the development of mammalian ovarian follicles (Eppig *et al.*, 2002). Also, the role of somatic cells around the oocyte is also crucial for follicular development and oocyte maturation (Braw-Tal, 2002). As seen in Fig. 3, neither pgrmc1 nor pgrmc2 were found to be oocyte-specific, for they were both expressed in cumulus and granulosa cells. Therefore, we assume that pgrmc1 and pgrmc2 may involve in development of follicles as well as oocyte maturation. Their function in oocyte and follicular cells requires further investigation.

In conclusion, results from this study demonstrated that progesterone receptor membrane components exist on mouse oocyte plasma membrane.

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