

Expression of Membrane Fusion Related Genes in Mouse Ovary

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마우스 난소에서 막융합 관련 유전자의 발현

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Granulosa cells surround the oocyte within the ovarian follicle and play an essential role in creating conditions required for oocyte as well as follicular development. The current study was conducted to examine the gene expression profile of mouse ovaries during the primordial to primary follicle transition process. Total RNAs from mouse ovaries on day 5 and day 12 were synthesized cDNA using annealing control primers. The DEGs were cloned and their identities were analyzed by BLAST search. The *Plekha5* and *Anxa11* were highly expressed in primary follicle stage. By contrast, their expression was increased in granulosa cells at the primary follicle stage. We have successfully discovered a list of genes expressed in day 5 and day 12 ovaries and confirmed that some of them are differentially expressed in PMF and/or PRI. This is a spatial-temporal regulatory mechanism on the ovarian folliculogenesis through membrane fusion. The gene expression profile from the current study would provide insight for future study on the mechanism(s) involved in primordial-primary follicular transition. This will provide information for identification of the mechanism of ovarian dysfunction.

Keywords: In situ hybridization, Membrane fusion, Ovarian development

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Introduction

Mammalian folliculogenesis is a complex process regulated by various extraovarian and intraovarian factors [1]. Despite many studies on the growth and maturation of antral follicles, relatively small numbers of studies have been conducted on early folliculogenesis. Mechanisms responsible for the initiation of follicular growth, also known as primordial follicle

activation, are completely unknown.

When ovarian follicles are formed, they enter the resting, primordial stage and the mechanisms of the arrest and initiation of follicular growth of these primordial follicles, also known as primordial follicle activation, are completely unknown [2,3].

At birth, the majority of follicles in mammalian ovaries are arrested at the primordial stage of follicles, and oocytes inside

follicles are arrested at the diplotene stage of the first prophase.

In vertebrates, the development and function of the ovary is mainly controlled by gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary [4]. However, it is well known that various local factors from the ovary also play important roles in regulating follicle growth and maturation in endocrine, paracrine or autocrine manners [5].

Recently, in the sex determination phase, which relies on the transcription factor GATA-4 and its cofactor Friend of GATA (FOG2), the subsequent regulation of ovarian differentiation is dependent upon GATA-4 but not on FOG2. The depletion of the follicular pool in a GATA-4 deficient ovary results in the formation of ovarian cysts and sterility [6].

Therefore, if researchers could compile list of genes and profiles of their expression during early folliculogenesis, particularly at the primordial-primary follicle transition period, it would give insight in order to study the regulating mechanism of this specific process.

Also, the goal for the present study was to investigate differentially expressed genes (DEGs) and their expression pattern that may play important roles in the arrest and/or initiation of primordial follicle growth.

The identification of differential gene expression is possible with the use of powerful tools, such as polymerase chain reaction (PCR) technology controlled by annealing control primer (ACP; Seegene, Seoul, Korea). This primer has a unique tripartite structure with a polydeoxyinosine [poly(dI)] linker between the 3' end target core sequence and the 5' end nontarget universal sequence [7].

The ACP linker prevents annealing of the 5' end nontarget sequence to the template and facilitates primer hybridization at the 3' end to the target sequence at specific temperatures, resulting in a dramatic improvement in annealing specificity.

This primer with annealing specificity and sensitivity to the template allows only real products to be amplified, such that it enables the researchers to find only real products. We describe the identification of DEGs in day 5- and 12- ovary by using the ACP-PCR method.

The day 1-ovary consists mainly of primordial follicles,

while the day 5-ovary consists of primordial and primary follicles, and is devoid of more advanced follicles [8]. Following ACP-PCR, several genes were chosen to confirm their differential expression between day 5 and day 12 ovaries.

To identify differentially expressed genes as well as discover novel genes that may play important roles in primordial to primary follicle transition, we carried out ACP-PCR and to investigate follicular gene expression and the changes during early folliculogenesis.

Materials and Methods

1. Source of tissues

Mice were purchased by Santako Bio Korea (Osan, Korea). Ovaries were collected from day 5 and day 12 neonates for RNA isolation and histological preparation. Animal care and handling was conducted according to the guidelines of the Yong-In University Institutional Animal Care and Use Committee.

2. mRNA isolation and first-strand cDNA synthesis

Isolation of total RNA from day 5 and day 12 ovary were carried out using Trizol Reagent (Invitrogen, Frederick, MD). The procedure for Total RNA extraction was essentially the same as described previously [9].

The RNA pellet was then washed by adding 75% ethanol, and centrifuged at 7,500 g for 5 min at 4°C. The RNA was dissolved in 0.1% Diethylpyrocarbonate (DEPC)-water, and the amount was determined by spectrophotometry and then stored at -70°C until use. For genomic DNA removal the total RNA (3 µg) were treated DNase I (Gibco BRL, Grand Island, NY) for 15 min at 37°C. For first-strand cDNA synthesis, the reaction was carried out by dT-ACP1, wherein the 3'-end core portion comprises a hybridizing sequence complementary to a poly A region of mRNA transcripts.

Treated total RNA was incubated with 2 µL of dT-ACP1 at 80°C for 3 min, then reverse transcription (RT) reaction was carried out in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM Dithiothreitol (DTT), 0.5 mM of each dNTPs, 20 U of RNase inhibitor, and 200 U of Superscript III (Invit-

rogen, Carlsbad, CA). The reaction mixture was incubated at 42°C for 90 min, then 94°C for 2 min. For PCR amplification, synthesized cDNA was diluted before PCR.

3. Amplification of cDNA target sequence and cloning

PCR was conducted using arbitrary ACPs to synthesize second-strand cDNAs under conditions in which the 3'-end core portion of the dT-ACP2 is prevented from annealing to the first-strand cDNAs and only the 3'-end core portion of the arbitrary ACP comprising a hybridizing sequence sufficiently complementary to a region of the first-strand cDNAs is involved in annealing to the first-strand cDNAs. A two-ovary amplification reaction was conducted in 20 µL reaction mixture comprising 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM of each dNTPs, 1 µL of arbitrary ACP (5 µM), 0.5 µL of dT-ACP2 (10 µM), and 2.5 U of Taq DNA polymerase (Promega, Madison, WI). For the PCR reaction, 1 µL of diluted cDNA (1:5) were used as template for each amplification using arbitrary ACP. We used the following PCR conditions: one cycle of 94°C for 5 min, 50°C for 3 min, and 72°C for 1 min; followed by 40 cycles of 94°C for 40 sec, 65°C for 40 sec, 72°C for 40 sec, and post-extension was done at 72°C for 5 min. After PCR, the amplified products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The differentially expressed bands were extracted with a gel extraction kit (Qiagen, Valencia, CA) and cloned into a TOPO TA cloning vector (Invitrogen), and the cloned DNA was analyzed by sequencing and BLAST search.

4. Tissue preparation and *in situ* hybridization

For histology, ovaries were fixed in 10% neutral-buffered formalin solution (NBF) for 24h at 4°C, paraffin-embedded, sectioned at 5 µm thickness, and mounted on slides. The sections were deparaffinized by two 5 min washes in xylenes and then rehydrated through a graded ethanol series. Next these samples were stained with hematoxylin and eosin for histological observation. Ovaries for *in situ* hybridization were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Paraffin-embedded tissues were cut into 5 µm sections, mounted on positively charged slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA), and analyzed by *in situ* hybridi-

zation as follows.

Digoxigenin (DIG)-labeled riboprobes of genes were synthesized with the *in vitro* transcription kit (Promega); the antisense orientation employed SP6 RNA polymerase, and the sense orientation used T7 RNA polymerase along with a DIG RNA labeling mix (Roche, Indianapolis, IN). After incubation at 37°C for 6h, the probes were purified with G-50 columns (Amersham Pharmacia Biotech Ltd., Piscataway, NJ) and diluted with Harland hybridization buffer (50% formamide, 5X SSC, 1 mg/mL Torula yeast RNA, 100 µg/mL heparin, 1X Denhardt's solution, 0.1% Tween-20, 0.1% 3-(β-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 0.5 mM EDTA).

Tissue sections were deparaffinized, rehydrated with an ethanol series, and refixed in 4% paraformaldehyde in PBS for 10 min to ensure firm attachment of the sections to the slides. Sections were rinsed in 100 mM triethanolamine (TEA) for 5 min, transferred to 100 mM TEA containing 0.25% acetic anhydride for 10 min, followed by dehydration with an ethanol series and allowed to air dry. Hybridization with the appropriate probe (1:100) was performed overnight at 65°C. After washing in a 50% formamide, 2X SSC, 10 mM EDTA solution for 30 min at 65°C, sections were blocked for 1h in blocking buffer (20% sheep serum, 2% nucleic acid) hybridization blocking reagent (Roche) in maleic acid buffer (MAB) (100 mM maleic acid, 150 mM NaCl, pH 7.5), then were incubated with anti-DIG alkaline phosphatase-conjugated Fab antibody fragments (anti-DIG-AP, Roche; 1:1000 dilution) for 2 h at RT. Sections were washed thoroughly with MAB, color produced by incubation for 1 h at RT with nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP) (Sigma-Aldrich Co., St. Louis, MO), then counterstained with Nuclear Fast Red (Dako, Carpinteria, CA). The primer sequence and condition were as follows (Table 1).

5. Immunohistochemistry

Ovaries and testis were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 µm thickness, and mounted on positively charged slides (ProbeOn Plus, Fisher Scientific). Immunohistochemistry was performed as described

Table 1. Primers sequence and real-time PCR conditions

Gene	GI No	Primer sequence	Annealing temp.°C	Product size, bp
<i>Anxa11</i>	160707920	For-AGGTACCATCACTGCTGCTT Rev-CTCGTTGAAAAGTCCACCA	60	619
<i>Plekha5</i>	110835705	For-AAGCATCCGGTTACAGGACA Rev-GCACCAGAGCAGCATCTAAC	60	556
<i>G3PDH</i>	20828350	For-TCCACCACCCTGTTGCTGTA Rev-ACCACAGTCCATGCCATCAC	60	451

[10]. Tissue sections were deparaffinized and treated with 3% H₂O₂ in methanol for 20 min to quench endogenous peroxidase activity, preincubated with blocking buffer (Dako, Carpinteria, CA) for 15 min at room temperature and followed by incubation with a primary antibody (ABIN1037368; Biocompare Inc., CA) against each target.

After incubation for 1 h at room temperature, the sections were rinsed in PBS, treated for 20 min at room temperature with a biotinylated secondary antibody (rabbit), then for 20 min at room temperature with a streptavidin-biotin-peroxidase complex. Peroxidase activity was developed with AEC⁺ and hematoxylin was used for counterstaining. In each experiment, control sections were incubated with a dilution buffer lacking the primary antibody.

Results

1. Histological examination

Prior to activation, these immature follicles contain nongrowing oocytes and nondividing, flattened (squamous) pregranulosa cells surrounded by a basal lamina. Following activation, follicle growth begins with the transformation of squamous pregranulosa cells into a single layer of cuboidal granulosa cells surrounding a growing oocyte. The granulosa cells proliferate to form multiple layers, requiring expansion of the basal lamina, and leading to two-layered and multi-layered follicle stages. A fluid-filled antral cavity, or antrum, forms in the granulosa cell compartment of the follicle later in folliculogenesis [11].

2. Typical expression of DEGs, list up by ACP-PCR

Identifying DEGs exclusively in primordial, primary and secondary follicles may play an important role in the arrest of primordial follicles and/or initiation of their growth into

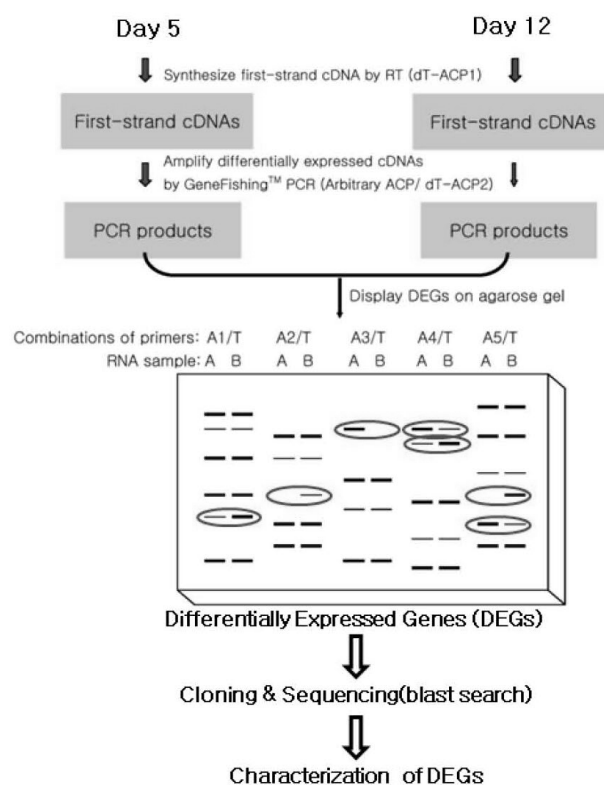


Fig. 1. Schematic representation of processing in this study. Typical expression patterns of DEGs on day 5 and day 12 mouse ovaries by ACP-PCR. RNA samples from day 5 and day 12 ovary were analyzed by using ACPs. Discovered DEGs were processed in cloning, sequencing, characterizing.

primary follicles. The day 5 and day 12 of mouse ovaries acquire cDNA by the same quantity and use ACP to carry out PCR (Fig. 1). Using ACP technology, we isolated differentially expressed bands between day 5 and day 12 ovaries (Fig. 2). Among the DEGs, acquired gene list numbers were between day 5 and day 12 (Table 2).

3. RNA localization of DEGs by *in situ* hybridization

Anxa11 were expressed in oocyte cytoplasm with developmental stage ovaries, and decreases expressed in growing granulosa cells. *Plekha5* decreases were expressed in oocyte

Table 2. Lists of DEGs on the day 5 and day 12 ovaries

Expression	Gene name	Gi No
Day 5 > Day 12	Annexin A11 (<i>Anxa11</i>)	160707920
	Pleckstrin homology domain containing, family A member 5 (<i>Plekha5</i>)	110835705
	RING zinc finger protein homolog	12856802
Day 5 < Day 12	Indian hedgehog (<i>Ihh</i>)	31981670
	Transducer of ERBB2, 2 (<i>Tob2</i>),	108796647
	F-box and WD-40 domain protein 7 (<i>Fbxw7</i>)	295293104
	Centrin 2 (<i>Cetn2</i>)	142368991
	Hepatoma derived growth factor-like 1 (<i>Hdgfl1</i>)	254553322

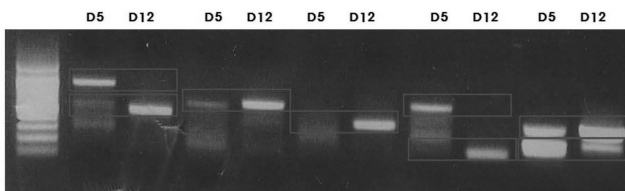


Fig. 2. Typical expression patterns with DEGs in ovary by ACP-PCR. RNA samples from day 5 (D5), The day 12 (D12) were analyzed by using ACPs. The 3 DEGs were highly expressed in day 5 ovary and highly expressed 5 DEGs in day 12 ovary (arrow; 400 bp).

nucleolus in developmental growing follicle stages and granulosa cells were totally expressed in developmental stages.

Using *in situ* hybridization, the *Anxa11* mRNA expression is oocyte nucleolus with primordial follicle in the mouse ovary. *Plekha5* mRNA weak expression is oocyte cytoplasmic in the primordial follicle. Therefore, there was increased expression in growing follicles. Further, Granulosa cells increasingly expressed both *Anxa11* and *Plekha5* in developing follicles (Fig. 3).

4. Expression of PLEKHA5 protein by immunohistochemistry analysis

Using the immunohistochemistry analysis, PLEKHA5 protein expressed in oocyte cytoplasm with mouse ovaries at postnatal various stages (Fig. 4). PLEKHA5 protein were expressed in oocyte cytoplasm at all stages from primordial to preovulatory follicles.

Discussion

This study was a day 5 and day 12 DEGs transcript list obtained by ACP-PCR, respectively. We used day 5 and day 12 whole mouse ovaries for ACP-PCR rather than isolating each

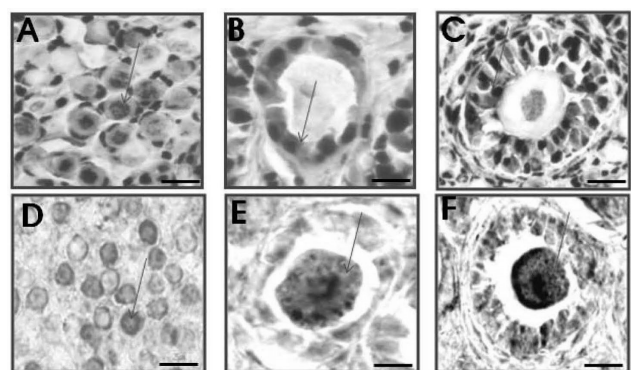


Fig. 3. RNA localization of DEGs in primordial follicle as well as in primary and secondary follicle using the in situ hybridization. A, B, C represented on *Plekha5*; D, E, F represented on *Anxa11*. A and D were consisted primordial follicle, B and E were consisted primary follicle, C and F were consisted antral follicle. *Plekha5* expressed in primordial oocyte and theca cell. But, *Anxa11* not expressed in theca cell primordial follicle stage (A, D), Their expression in the granulosa cells increased in the developing follicles (B, C, E, F). $\times 400$, Scale bar; 25 μm .

follicular stage, since follicles are very small in size and difficult to isolate homogeneous and enough mRNA for ACP-PCR. Of those, they characterized that spatio-temporal localization pattern during the folliculogenesis.

Previously, Cyclin dependent kinase (Cdks) associate with cyclins to form heterodimers that are sequentially activated during the cell cycle [11]. Metazoan cells have multiple Cdks and cyclins that are temporally regulated. In normal cell cycles, Cdk4 and Cdk6 pair with D-type cyclins during G1, Cdk2 pairs with E- and A-type cyclins during S and G2, and Cdk1 pairs with A- and B-type cyclins during G2 and M.

Annexins involved random gene duplications, speciation, and molecular drift in gene structures [12]. Annexin 11 is a Ca^{2+} / phospholipid - binding protein that interacts with a member of the S100 protein family, calyculin (S100A6), in a Ca^{2+} -dependent manner [13]. Calyculin may be involved in

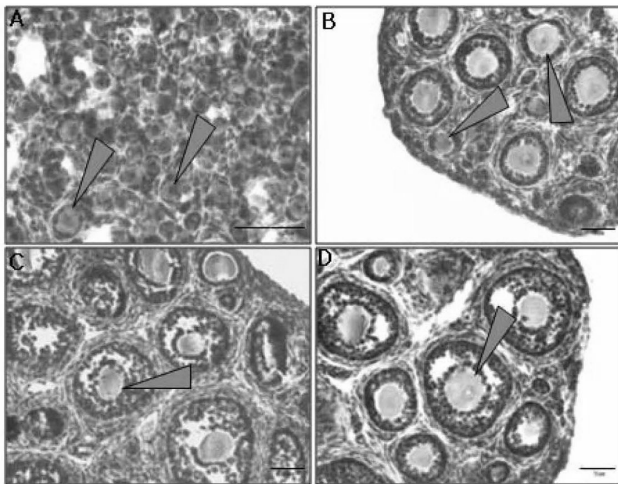


Fig. 4. Immunohistochemistry analysis of PLEKHA5 in the mouse ovaries at postnatal various stages. PLEKHA5 is highly expressed in every oocyte cytoplasm and relatively less in the granulosa cells of the developing follicles. $\times 400$, Scale bar: 50 μm .

signal transduction of the Ca^{2+} induced release of insulin [14,15].

The fork head domain-containing gene family (Fox) comprises over 20 members in mammals and is defined by a conserved 110 amino-acid motif containing a winged helix structure DNA-binding domain. The members of this gene family have been implicated as key regulators of embryogenesis, cell cycling, cell lineage restriction and cancer. The *Foxn2* gene is expressed in postgastrulation embryos in multiple tissues that serve as important signaling centers as well as end-stage-differentiated cell types that arise from different germ layers of the developing embryo [16].

Plekha5 (pleckstrin homology domain containing, family A) was gene synonym (*Ayu21-9*, *Gt(pU21)9*, *Pepp2*).

The diffuse B-cell lymphoma (*Dbl*) family guanine nucleotide exchange factors (GEFs) contain a region of sequence similarity consisting of a catalytic Dbl homology (DH) domain in tandem with a pleckstrin homology (PH) domain. The pleckstrin homology domains are protein motifs of approximately 100 amino acids that have been identified in more than 100 molecules involved in signal transduction, including serine/threonine and tyrosine kinases, phospholipases, cytoskeletal proteins, and regulators of small GTPases.

Annexin 11 is one of the 12 vertebrate subfamilies in the annexin superfamily of calcium/phospholipid-binding proteins, distinguishable by long, non-homologous N-termini rich in

proline, glycine and tyrosine residues. Several functions have been described for annexins in vitro, including anti-coagulatory and anti-inflammatory activities and involvement in signal transduction, membrane fusion, endo- and exocytosis as well as calcium-channel regulation. However, little is known about their physiological role in vivo.

The *annexin* possesses an extensive N-terminal region, rich in proline, glycine and tyrosine residues. The N-terminal region of *annexin 11* has been proposed to be responsible for its autoantigenicity, nuclear localization and tyrosine phosphorylation. ACP-PCR analysis provides a powerful and rapid means of reconstructing the transcriptome of specific tissues and cell types and for identification of differentially expressed genes.

This study compiled a list of genes specifically expressed during primordial, primary and secondary follicular development. *Anxa11* and *Plekha5* were one of the stimulators for primordial follicle growth and further development. The characterization and functional analysis of genes in the lists may provide a basis for future studies on the molecular mechanisms of primordial-primary follicle transition, and the development of secondary follicles in mice, as well as in humans.

요약

포유류의 난소에서 과립막 세포는 난포내 난자를 둘러싸고 난자 뿐만 아니라 난포의 발달에 필요한 상태를 만드는데 중요한 역할을 한다. 그리고 혈막세포 등의 성장과 분화를 조절하는 매우 복잡한 과정이다. 본 연구의 목적은 성장이 정지되어 있는 원시난포에서 성장이 개시되는 1차난포, 2차난포로 전환하는 동안에 관여하여 발현하는 유전자를 조사하는 것이다. 본 연구에서는 5일자, 12일자 난소로부터 총 리보핵산을 추출하여 동량의 RNA로부터 cDNA를 합성하여 ACP-PCR을 수행하였다. 서로 다르게 발현하는 유전자들 (DEGs)을 클로닝과 BLAST를 통한 염기서열 분석하였다. *Anxa11*과 *Plekha5*는 5일자 난소에서 높게 나타났으며 특히 난자핵과 세포질에서 높게 발현하였다. 이에 반해 과립막 세포에서는 난포발달단계에 따라 증가하는 양상을 보였다. 본 연구에서 성공적으로 5일, 12일 난소에서 서로 다르게 발현하는 발현유전자 목록을 일부 찾아 확인하였다. 이들은 막융합을 통해 난소의 난포발달과정에서 시간적-공간적인 조절 기전에 의해 이루어 질 것으로

보인다. 이 유전자 발현 정보는 원시난포의 개시와 성장을 위한 전환에 관여하는 기전을 이해하는 기초정보와 난소기능부전의 기전을 규명하는데 정보를 제공할 것으로 기대된다.

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