The Expression of *Solute carrier family members* Genes in Mouse Ovarian Developments

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생쥐의 난소 발달과정에서 *Solute carrier family* 유전자들의 발현양상

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Granulosa cells, which surround the oocyte within the ovarian follicle, play an essential role in creating conditions required for the development of oocytes and follicles. The *solute carrier family* (SLC) is comprised of influx transporters of steroidal hormones, various drugs, and several other substrates. The differential expression of selected DEGs was confirmed using in situ hybridization analysis. *SLC23A3* and *SLC39A10* were highly expressed in the ovary. The *SLC39A10* gene was expressed in the primordial follicle stage, but *SLC23A3* was expressed in the growing follicle stage. Contrastingly, the expression of *SLC23A3* was increased in granulosa cells at the growing follicle stage. The differential expressions of *SLC23A3* and *SLC39A10* between the primordial and primary follicles were additionally confirmed by using follicle isolations. The gene expression profile from the present study may provide insight for future studies on the mechanism(s) involved in primordial-primary follicular transition and suggestions to promote follicular development in ovarian dysfunction.

Key words: Folliculogenesis, in situ hybridization, Ovarian development, *Solute carrier family members*, Zinc transporter

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INTRODUCTION

In most mammals ovary, meiosis of oocytes is initiated during fetal life but stops prenatally in the diplotene (diplotene) stage of the prophase I. The meiotic arrest is maintained until follicular development to antral follicle [1].

The numbers of primordial follicles that have the potential to mature into a fertilizable oocyte are one

component of the ovarian reserve [2]. The transition from primordial to primary follicle involves oocyte growth, granulosa cell differentiation from flat to cuboidal, and theca cell recruitment. It is very difficult to study primordial follicle formation and activation in humans, and animal models have been essential to better understand the genetics of folliculogenesis [3].

Ovarian follicles, the functional units within the female

gonad, are comprised of three cell types: oocytes, surrounding granulosa cells, and an external thecal cell layer. Follicles serve to nurture maturation of the oocyte, a process that is critical for successful reproduction. Folliculogenesis starts with the assembly of primordial follicles [4].

In ovary, myriad network of factors that regulate the initial stages of folliculogenesis, and this network includes several TGF- β family proteins. Activin may play a role in primordial follicle formation. Bone morphogenetic protein 7 (BMP7) and BMP4 promote the transition of early folliculogenesis, whereas anti-müllerian hormone (AMH) inhibits initial follicle growth. Growth differentiation factor 9 (GDF9) is critical for follicles to advance to the secondary stage. In primary follicles, inhibin may be suppressed by GDF9, which allows for granulosa cell proliferation by activin. Recently, adding to this growing list of factors, a distant TGF- β ligand, glial cell-derived neurotrophic factor (GDNF), was recently found to promote the primordial to primary follicle transition [5].

Recently, the post-transcriptional gene regulatory mechanisms have been well studied in both the male and female germ cells, as it was recognized that germ cell transcription and protein synthesis were uncoupled at different periods during gamete development [6]. Various other miRNA are highly abundant in ovarian tissues, its suggested that the role of miRNA and regulation of ovarian development and function [7].

In extraovarian, the protein of follicular fluid were modulate oocyte maturation and ovulation. Also, the identified protein biomarkers serve as a useful resource for *in vitro* fertilization and oocyte quality [8].

The autocrine and paracrine factors involved in follicle growth and differentiation include proteins and hormones [9].

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

ACP-PCR (annealing control primer-polymerase chain

reaction) techniques were highly accurate and sensitive PCR technology controlled by annealing control primer (ACP; Seegene, Seoul, Korea). This primer has unique tripartite structure with a polydeoxyinosine [poly(dI)] linker between the 3' end target core sequence and the 5' end non target universal sequence [10]. 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 of annealing specificity [10]. ACP-PCR analysis provides a powerful and rapid means of reconstructing the transcriptome of specific tissues for identification of differentially expressed genes.

Given the increased understanding of the roles of transporters in normal physiology and disease. Due to the known roles of many SLC family members in drug disposition, transporters are now being evaluated as a routine part of the drug development process.

The day 1-ovary consists mainly of primordial follicles, while the day 5-ovary consists of primordial and primary follicles [11], and day 12-ovary are more growing secondary follicles. Following ACP-PCR, several genes were chosen to confirm their differential expression between day 5 and day 12 ovaries.

To identify and list up DEGs exclusively in primordial or primary follicles that may play important roles in the arrest of primordial follicles and/or initiation of their growth into primary follicles. The objective of the investigation was to use a ACP-PCR experimental approach to identify gene expression profiles involved in regulating primordial follicle assembly and regulatory factors were identified that correlate with folliculogenesis.

MATERIALS AND METHODS

1. Source of tissues

Pregnant ICR mice were housed under temperature-controlled conditions (12 h light/12 h dark cycle). Mice were maintained as use of Laboratory Animals. Ovaries were collected from day 5 and day 12 neonates for RNA isolation and slide 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, USA). The procedure for Total RNA extraction was essentially the same as described previously [11]. For genomic DNA removal the total RNA (3 µg) were treated DNase I (Gibco BRL, Grand Island, NY, USA) 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 (Invitrogen, Carlsbad, CA, USA). 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. The procedure for cDNAs were

essentially the same as described previously [12]. The differentially expressed bands were extracted with a gel extraction kit (Qiagen, Valencia, CA, USA) and cloned into a TOPO TA cloning vector (Invitrogen), and the cloned DNA was analyzed by sequencing and BLAST search (Table 1).

4. Tissue preparation and *in situ* hybridization

Ovaries for *in situ* hybridization were fixed in 4% paraformaldehyde in PBS overnight at 4°C. paraffin-embedded, sectioned at 5 µm thickness, and mounted on charged slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA, USA). The sections were deparaffinized by two 5 min washes in xylenes and then rehydrated through a graded ethanol series. and to analyzed by *in situ* hybridization as follows. Digoxigenin (DIG)-labeled riboprobes of genes were synthesized with the *in vitro* transcription kit (Promega) and 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. For hybridization with the appropriate probe (1:100) was performed overnight at 65°C. and the color produced by incubation for 1h at RT with nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3'-indolyl-phosphate p-toluidine salt (BCIP) (Sigma-Aldrich, MO, USA), then counterstained with Nuclear Fast Red (Dako, Carpinteria, CA, USA). The procedure for *in situ* hybridization was essentially the same as described previously [12]. The primer sequence and condition were as follows (Table 2).

Table 1. Lists of DEGs on the day 5 and day 12 ovaries

Expression	Gene name	GI No.	Identity (%)
Day 5 > Day 12	solute carrier family 39 (zinc transporter), member 10 (<i>SLC39A10</i>)	40254227	99
	apoptosis inhibitor 5 (Api5), mRNA	6671566	98
	S-phase kinase-associated protein 2 (p45) (Skp2), mRNA	7949134	100
Day 5 < Day 12	solute carrier family 23 (nucleobase transporters), member 3 (<i>SLC23A3</i>), mRNA	124244078	99
	anaphase promoting complex subunit 2 (Anapc2), mRNA	31341553	98
	bromodomain containing 3 (Brd3), mRNA	31981063	99
	gap junction membrane channel protein beta 5 (Gjb5), mRNA	31981752	99
	high mobility group nucleosomal binding domain 2 (Hmgn2), mRNA	31980676	100

The experiment was repeated 3~4 times.

5. Immunohistochemistry

Ovaries were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μ m thickness, and mounted on positively charged slides (ProbeOn Plus, Fisher Scientific). Immunohistochemistry was performed as described [13]. 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, USA) for 15 min at room temperature and followed by incubation with a primary antibody (*SLC39A10*; AB117556; Abcam, MA, USA) 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. The experiment was repeated 3~4 times.

RESULTS

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

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

2. RNA localization of *SLC39A10*, *SLC23A3* by *in situ* hybridization

Using *in situ* hybridization, the *SLC39A10* mRNA expression is oocyte cytoplasm with primordial follicle in the mouse ovary. *SLC23A3* mRNA very weakly expression is oocyte cytoplasm in primordial follicle. In granulosa cells, *SLC39A10* were expressed in theca cell and developing follicle oocyte. *SLC23A3* were expressed granulosa cells during folliculogenesis (Figure 2). The primer sequence and condition as followed (Table 2). *SLC23A3* were weakly expressed in oocyte nucleus with early follicular stage and granulosa cells were totally expressed during follicular development.

Figure 1. Typical expression patterns with DEGs in ovary by ACP-PCR. RNA samples from day 5 (D5) and 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; 500 bp).

Table 2. Primers sequence and real-time PCR conditions

Gene	GI No.	Primer sequence	Annealing temp (°C)	Product size (bp)
<i>SLC39A10</i>	40254227	For-TTCTCAGGGTGGACATGACC Rev-AGGAGGGGATTCTTGTGGG	60	394
<i>SLC23A3</i>	124244078	For-TGCCAACTTCTTTCACTGCC Rev-AGGAAAGGCAGCCCATTTTG	60	374

Figure 2. RNA localization of solute carrier family members genes in primordial follicle as well as in primary and secondary follicle using the in situ hybridization. (A, B) represented on *SLC39A10*; (C, D) represented on *SLC23A3*. (A, C) were consisted primordial or primary follicle. (B, D) were consisted secondary follicle. *SLC39A10* expressed in primordial oocyte and some theca cell (A). Growing follicle oocyte (B). But, *SLC23A3* expressed in theca primordial follicle stage (C) and growing follicle granulosa cells (D). ×400, Scale bar; 25 μm.

Figure 3. Immunohistochemistry analysis of *SLC39A10* in the mouse ovaries at postnatal various stages. The *SLC39A10* is highly expressed in oocyte nucleus and cytoplasm at primordial follicle (A). ×100, scale bar; 50 μm. Oocyte cytoplasm more relatively expressed than granulosa cells of the developing follicles (B). ×400, Scale bar; 25 μm.

3. Expression of *SLC39A10* protein by immunohistochemistry analysis

Using the immunohistochemistry analysis, The *SLC39A10*

protein differs from that expressed pattern in the cytoplasm of oocytes in both primordial follicles and growing follicles (Figure 3). In totally, summarized mRNA and protein localization pattern during early follicular de-

Table 3. Summarized RNA and protein localization patterns of *solute carrier family members* genes expression during early folliculogenesis

	Oocyte						Granulosa cell					
	PMF		PF		GF		PMF		PF		GF	
	N	C	N	C	N	C	N	C	N	C	N	—
<i>SLC39A10</i> mRNA	+	+++	+	+	+	±	—	+	—	—	—	—
<i>SLC39A10</i> Protein	+++	+++	++	++	+	+	—	+	—	—	—	++
<i>SLC23A3</i> mRNA	—	±	—	—	—	—	—	++	—	++	—	—

Abbreviation: PMF, primordial follicle; PF, primary follicle; GF, growing follicle; N, nucleus; C, cytoplasm.

velopment (Table 3). The *SLC39A10* were highly expressed in oocyte cytoplasm with early developmental stage ovary. And decrease expressed in growing granulosa cells.

DISCUSSION

This study were day 5 and day 12 DEGs transcript lists obtained by ACP-PCR, respectively. We used to whole mouse ovaries of day 5 and day 12 for ACP-PCR rather than isolating each follicular stage, because follicles are very small in size and difficult to isolate homogeneous. Therefore, the *SLC23A3* and *SLC39A10* were characterized by *in situ* hybridization and immunohistochemistry according to the ovarian developmental stage. Ovarian folliculogenesis were regulated by differentiation, cell death, and cell-cell communication. Also, the oocytes were secreted factors that are important for the development and maintenance of oocyte-granulosa cell complexes. This study showed that cell-cell communication of signaling in growing mouse ovaries leads to the early follicular development. Therefore, the *SLC23A3* and *SLC39A10* were selected and characterized.

The *SLC23A1* was required for transport of ascorbic acid into many tissues and across the placenta. Deficiency of the transporter is lethal in newborn mice [14], The *SLC39A10* protein was previously localized to oocytes and not somatic follicular cells [15]. The ZIP7 [solute carrier family 39 (zinc transporter) member 7, also known as *SLC39A7*] were enhanced response to exogenous zinc, and increased growth factor receptor activation, leading to increased growth and invasion. which zinc-induced activation of growth factor receptors may be suppressed multiple growth factor pathways [16].

The granulosa cells and theca cells cooperate to produce the steroids essential for proper oocyte production. Granulosa cells are the primary site of estradiol production, which is essential for proper antral follicle formation and maintenance, as well as ovulation. At birth, the ovary contains a finite number of oocytes available for folliculogenesis. This finite number of available oocytes is termed “the ovarian reserve” The determination of ovarian reserve is important in the assessment and treatment of infertility [17].

The paracrine signaling in the cumulus-oocyte complex (COC) controls both oocyte and cumulus cell functions. Zinc increases in the oocyte during maturation and is required for progression and completion of meiosis [18]. The specific transporter(s) that regulate zinc transport within the oocyte or from the extracellular environment are unknown. Given the importance of zinc for many cellular processes it is not surprising that multiple transporters are present in the oocyte. Exactly where these are localized and how each transporter is involved in modulating zinc flux into and out of the oocyte as well as between different intracellular compartments are important [19].

The *survivin* depletion in GCs led to abnormal follicle growth, ovulation failure (follicular atresia) and impaired luteinization, which were caused by massive GCs apoptosis. In the absence of healthy GCs, ovarian follicles are destined to undergo atresia because of loss of the main supporting signals [20].

S-phase kinase associated protein-2 (Skp2) ubiquitin ligase p45 (SKP2) is important in the degradation of p27kip1 (a cyclin dependent kinase inhibitor) and progression through the G1-S cell-cycle checkpoint. Cyto-

plasmic expression of Skp2 defines a subset of aggressive melanomas and could represent another pathway of deregulation of the cell cycle [21].

The anaphase-promoting complex/cyclosome (APC/C) plays a critical role in mitosis, but much remains to be understood about its function in meiosis. Its mechanism for developmental regulation of an APC/C activator and suggest it is one strategy for control of the female meiotic cell cycle in a multicellular organism [22].

Among the four mammalian BET family members, Brd2 and Brd3 show greater similarity to one another than to Brd1 or Brd4. The bromodomain-containing genes exist in mouse and human and encode proteins involved in broad cellular processes, including replication, transcription, splicing, gene silencing, and chromatin remodeling [23].

Gap junction channels are made of integral membrane proteins called connexins. The connexin genes family consists of about 20 members in humans. Cx43 as a SUMOylation target protein and represent the first evidence that gap junctions are regulated by the SUMO system [24].

In mammalian cells, the nucleosome-binding protein HMGN1 (high mobility group N1) affects the structure and function of chromatin and plays a role in repair of damaged DNA [25].

This study successfully discovered of cell cycle and cell-cell communication related gene and RNA localization was highly expressed oocyte in early follicular stage. Therefore, the follicular growth during the preantral early antral transition is mainly regulated by intraovarian oocyte-granulosa-theca cell interactions and regulators, such as growth factors, cytokines, and steroids [13,26].

In conclusion, This study were compiled a list of well-known genes and discovered the specifically localization during follicular development. The *SLC23A3* and *SLC39A10* were localized in primordial follicle initiation and follicular development. The characterization and functional analysis of their genes may conducted the basis for future researches on the molecular mechanisms of primordial-primary follicle transition and the development of secondary follicles in mouse and humans.

요약

난포 내 난자를 둘러싸고 있는 과립세포는 난자를 위한 성장 상태 및 난포의 발달에 중요하다. *Solute carrier family* 유전자는 스테로이드 호르몬, 다양한 약물, 몇몇 다른 기질을 유입시킨다. 연구에서 획득한 서로 다르게 발현하는 유전자들 (DEGs) 중 일부를 *in situ* hybridization을 통해 분석하였다. 분석한 결과 *SLC23A3*과 *SLC39A10*이 난소에서 높게 발현하였다. *SLC39A10* 유전자는 원시난포에서 높게 발현하였고, *SLC23A3*은 일차, 이차 난포에서 높게 발현하였다. 특히 성장하는 난포의 과립막 세포에서 발현하였다. *SLC23A3*과 *SLC39A10*은 원시난포와 일차난포에서 다르게 발현하는 것은 각 난포의 분리를 통해 좀 더 확인해야 할 것이다. 본 연구에서는 유전자 발현 정보를 통해 원시난포의 개시와 성장을 위한 전환에 관여하는 기전을 이해하는데 기초정보와 난포발달 촉진을 위한 난소기능부전의 기전을 규명하는데 정보를 제공할 것으로 기대된다.

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