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

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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

INTRODUCTION

In most mammals ovary, meiosis of oocytes is initiated during fetal life but stops prenatally in the dictyotene (diplotene) stage of the prophase I. The meiotic arrest is maintained until follicular development to an 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 neurotropic 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 (DIT), 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% parafomaldehyde 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 xylene 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'-indoly-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

| Gene name                                      | GI No.    | Identity (%) |
|------------------------------------------------|-----------|--------------|
| solute carrier family 39 (zinc transporter), member 10 (SLC39A10) | 40254227  | 99           |
| apoptosis inhibitor 5 (Ap5), mRNA              | 6671566   | 98           |
| S-phase kinase-associated protein 2 (p45) (Skp2), mRNA | 7949134   | 100          |
| solute carrier family 23 (nucleobase transporters), member 3 (SLC23A3), 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 (Hmg2), mRNA | 31980676  | 100          |

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The experiment was repeated 3~4 times.

5. Immunohistochemistry

Ovaries 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 [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 the 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.

Table 2. Primers sequence and real-time PCR conditions

| Gene   | GI No.   | Primer sequence                      | Annealing temp (°C) | Product size (bp) |
|--------|----------|--------------------------------------|---------------------|-------------------|
| SLC39A10 | 40254227 | For-TTCTCGGGGGACATGACC<br>Rev-AGGAGGGGATTTGTTGGG | 60                  | 394               |
| SLC23A3 | 124244078| For-TGCCAAGCTTCCACCTGCC<br>Rev-AGGAAAGGCAGCCCCATTGG | 60                  | 374               |

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).
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  |
| SLC39A10 mRNA    | +      | ++  | +   | ±   |     |     |
| SLC39A10 Protein | +++    | +++ | ++  | +   |     |     |
| SLC23A3 mRNA     | −      | ±   | −   | −   |     | ++  |
| Abbreviation: PMF, primordial follicle; PF, primary follicle; GF, growing follicle; N, nucleus; C, cytoplasm.

Development (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 Brdt 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 aboutly 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-knowned 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.

요 약

난소 내 난자를 둘러싼 고등 실험동물의 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요한다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전자는 성장 제어 및 난포의 발달에 중요하다. Solute carrier family 유전

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