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Heterologous Gap Junctions between Granulosa Cells and Oocytes in Ayu (*Plecoglossus altivelis*): Formation and Role during Luteinizing Hormone-dependent Acquisition of Oocyte Maturation Competence

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Abstract. Gap junctions (GJs) are intercellular channels. Molecules with a molecular weight of 1 kDa or less can pass back and forth between adjacent cells through GJs. Communication between oocytes and the somatic cells that surround them via GJs is known to play key roles to initiate oocyte maturation in many vertebrates. However, little is known of the detailed functions of ovarian GJs during oocyte maturation in fish. The oocyte maturation of fish is induced by a maturation-inducing steroid (MIS). The sensitivity of oocytes to the MIS is known as oocyte maturational competence (OMC) and is induced by luteinizing hormone (LH). However, LH receptors are found on the surface of granulosa cells rather than oocytes. We therefore proposed that the LH signals received by granulosa cells were passed to oocytes via GJs. This review describes current knowledge of the role of GJs between granulosa cells and oocytes during the LH-induced acquisition of OMC in fish.

Key words: Ayu (*Plecoglossus altivelis*), Fish, Gap junction, Luteinizing hormone, Oocyte maturational competence, Signal transduction

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Mammalian oocytes are surrounded by cumulus cells, and their maturation is induced by luteinizing hormone (LH) stimulation. By contrast, fish oocytes are surrounded by a layer of granulosa cells, a basement membrane, and a further layer of theca cells (Fig. 1). LH stimulates these two layers to produce a maturation-inducing steroid (MIS). Oocyte maturation is induced by direct stimulation from the MIS. In most fish, the MIS is thought to be either 17,20 β -dihydroxy-4-pregnen-3-one (DHP) or 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S) [1].

Sensitivity of oocyte to MIS is known to be crucial for oocyte maturation in many teleost species including our model fish ayu (*Plecoglossus altivelis*). This sensitivity, which is known as oocyte maturational competence (OMC), is induced by LH [1, 2] (Fig. 2). LH therefore has two important functions in oocyte maturation: inducing the acquisition of OMC by stimulating the oocytes, and inducing MIS synthesis by the follicle cells. LH-induced acquisition of OMC and the subsequent MIS-induced oocyte maturation are separate steps both physiologically and mechanistically [1, 2]. We found that incubating ayu post-vitellogenic follicle-enclosed oocytes in a culture medium containing MIS did not induce oocyte maturation [3]. However, pre-treating the follicle-enclosed oocytes with a culture medium containing human chorionic gonadotropin (hCG), which is known to have an LH-like function, caused them to respond to MIS and induced oocyte maturation [3]. We therefore proposed that the ayu follicle-enclosed oocytes acquired OMC via LH stimulation. However, the LH receptors are localized on the surface of the follicle cells, and are not present on the oocytes themselves [4]. Therefore, the mechanism by which LH induces

the acquisition of OMC in cells without receptors has previously remained unclear.

Our research has focused on the channels known as gap junctions (GJs) that are formed between granulosa cells and oocytes. Ions and second-messenger molecules can pass freely through GJs by means of diffusion [5] (Fig. 1). In the Atlantic croaker (*Micro-pogonias undulatus*) [6, 7] and the red sea bream (*Pagrus major*) [8], an increase in the number of GJs between granulosa cells and oocytes has been observed by electron microscopy during the acquisition of OMC. Additionally, in the ayu, the presence of gap-junction-like structures between granulosa cells and oocytes, and between granulosa cells themselves, has been confirmed by electron microscopy [9–11]. However, the detailed function of the GJs between granulosa cells and oocytes in the LH-induced acquisition of OMC has previously remained unclear.

We speculated that the GJs between granulosa cells and oocytes might be the key to signal transduction during the LH-induced acquisition of OMC. Specifically, we proposed that LH signals are received by receptors on granulosa cells and converted into second messengers. The acquisition of OMC is then induced by the second-messenger molecules that pass through the GJs between granulosa cells and oocytes. This review reports on our research testing this hypothesis in an ayu model.

GJ Protein Expression in Ayu Ovaries

Each GJ is formed by an association of six molecules of connexin protein, with a molecular weight of 26–56 kDa, in the intercellular space [12] (Fig. 1).

Initially, we cloned connexin complementary DNAs (cDNAs)

Fig. 1. The structure of fish follicles. Oocytes are surrounded by a layer of granulosa cells, a basement membrane, and a further layer of theca cells. Gap junctions exist between granulosa cells and oocytes and allow the passage of molecules with a molecular weight of up to 1 kDa, including both second messengers and ions. Each gap junction channel is formed by hexamers of connexin (Cx) protein subunits.

Fig. 2. Schematic representation of LH-induced acquisition of oocyte maturational competence. Incompetent oocytes are unresponsive to MIS. The sensitivity of oocyte to MIS is acquired by LH stimulation. Maturationally competent oocyte can respond to MIS and germinal vesicle breakdown (GVBD) is induced by MIS.

Table 1. Temporal and spatial expression profiles of Cxs in the ayu ovary

Cxs	Localization	Vitellogenic stage	Competent stage	Mature stage	Ovulatory stage (egg)
Cx34.9	Follicle cells	△	○	○	×
	Oocyte	△	○	○	
Cx32.1	Follicle cells	○	△	△	×
	Oocyte	○	△	△	
Cx44.1	Follicle cells	×	×	×	△
	Oocyte	△	△	△	
Cx44.2	Follicle cells	×	×	×	△
	Oocyte	△	△	△	

Each mark indicates expression level of Cxs. ○, strong expression; △, weak expression; ×, no expression.

from ayu ovarian tissues. As a result, we isolated four connexin genes, Cx32.1, Cx34.9, Cx44.1 and Cx44.2, from samples that had already acquired OMC. Next, we determined the site and stage of expression of each connexin messenger RNA (mRNA) in the ayu ovarian tissue using *in situ* and northern hybridization. Our results showed that Cx34.9 was expressed in both the follicle cells and the oocytes (Table 1), and was strongly expressed from the maturationally-incompetent stage through to mature stage (Table 1) [13]. In addition, Cx32.1 was expressed in both the follicle cells and the oocytes, and was particularly strongly expressed during the vitellogenic stage (Table 1) [13]. By contrast, the expression of Cx44.1 and Cx44.2 was confirmed only in oocytes, and these connexins were present at constant levels during all stages, including after ovulation (Table 1) [13]. Our findings suggested that Cx34.9-present in both follicle cells and oocytes and strongly expressed from OMC acquisition through to oocyte maturation-formed the GJs between follicle cells and oocytes that functioned in OMC

acquisition. In addition, Cx32.1 appeared to form the GJs between follicle cells and oocytes during the vitellogenic stage. Cx44.1 and Cx44.2 accumulated as maternal factors in the oocytes; thus, we concluded that these connexins formed GJs that played an important role in early embryonic development after fertilization, providing channels between blastomeres and so on.

Are Functional GJs Present Between Granulosa Cells and Oocytes?

To clarify whether the connexin genes described above formed functional GJs between granulosa cells and oocytes, we carried out a dye-transfer assay. This method verifies the opening and closing of GJs using lucifer yellow (LY), which is a fluorescent dye capable of passing through these channels, to indicate movement of small molecules between cells [13, 14]. LY that is microinjected into oocytes can diffuse into granulosa cells, thereby confirming

Fig. 3. Effects of the GJ-specific inhibitor CBX on hCG-induced acquisition of OMC. Ovarian fragments containing maturationally incompetent follicle-enclosed oocytes were incubated with plain medium or 100 IU/ml hCG with or without CBX (1, 5, 25 or 50 μM) for 24 h (inhibitor was added to the medium 1 h prior to the hCG exposure). Following this incubation, ovarian fragments were exposed to 10 ng/ml DHP and GVBD was scored after 48 h. Each bar represents the mean (+ SEM) of three different fish. Bars marked with the same letter are not significantly different ($P < 0.05$).

the presence of functional GJs. We microinjected LY into maturationally competent follicle-enclosed oocytes, and then separated out the follicle cells and the oocytes under a binocular microscope after 2 h. Fluorescent microscopy confirmed the presence of a fluorescent signal in all of the follicle cells in this experiment. We also subjected ovarian fragments to pre-culture with the gap-junction-specific inhibitor carbenoxolone (CBX) before injecting the follicle-enclosed oocytes with LY, as described above, and separating out the follicle cells after 2 h. Fluorescent microscopy revealed that the LY diffusion was inhibited in 85.5% of the follicle cells [13]. Our findings that a low-molecular weight water-soluble substance such as LY diffused into the follicle cells from the oocytes, and that this process was significantly inhibited by CBX, confirmed the presence of functional GJs that allowed the movement of substances between granulosa cells and maturationally competent oocytes.

Requirement of GJs in OMC Acquisition

Although the presence of functional GJs between granulosa cells and oocytes was verified by the dye-transfer assay, it was unclear whether they were necessary for the LH-induced acquisition of OMC. We therefore cultured ovarian fragments that included maturationally incompetent follicle-enclosed oocytes with hCG in the presence of CBX, to clarify whether the hCG induced OMC when the GJs were closed. The results showed that CBX inhibited the acquisition of hCG-induced OMC in a concentration-dependent manner (Fig. 3). Similar results were obtained in experiments using 18α-glycyrrhetic acid (18α-GA), which is another gap-junction-specific inhibitor [3]. These findings suggest that GJs between granulosa cells and oocytes are necessary for the LH-

induced acquisition of OMC.

What Passes Through GJs between Granulosa Cells and Oocytes During OMC Acquisition?

The experiments described above demonstrated that GJs between oocytes and granulosa cells were necessary for the acquisition of OMC in the ayu. Thus, OMC appeared to be acquired as a result of an LH stimulus and the passage of a second messenger through GJs between granulosa cells and oocytes; however, the nature of the second messenger molecules was unknown. For LH to have this physiological function, the signals must be transferred into oocytes after binding with LH receptors on granulosa cells. The LH receptor is a G-protein-coupled receptor [15], and is therefore expected to activate an enzyme such as adenylate cyclase or phospholipase C which synthesizes second messengers through a trimeric G protein [15]. For example, adenylate cyclase synthesizes cyclic AMP (cAMP) by the hydrolysis of adenosine triphosphate (ATP), phospholipase C synthesizes inositol triphosphate (IP₃) and diacylglycerol (DG) using phosphatidylinositol diphosphate (PIP₂) as a substrate, and IP₃ accelerates the release of Ca²⁺ from the endoplasmic reticulum. The second messengers (cAMP, IP₃, DG, Ca²⁺ and so on) synthesized in these cells activate various types of protein kinase [15].

To identify the second messenger that passes through the GJs between granulosa cells and oocytes during the LH-induced acquisition of OMC, we focused on the signaling systems for protein kinase A (PKA) and protein kinase C (PKC), and verified whether they were activated during the process. Initially, maturationally incompetent ovarian fragments were cultured with hCG in the presence of a PKA or PKC inhibitor, and we verified whether the hCG could induce the acquisition of OMC under these conditions. The results showed no effect of the PKC inhibitor GF109203X (GF), whereas the PKA inhibitor H8-dihydrochloride (H8) substantially inhibited the hCG-induced acquisition of OMC in a concentration-dependent manner (Fig. 4A) [3]. Next, ovarian samples were cultured with the PKA activator forskolin and the PKC activator phorbol-12-myristate 13-acetate (PMA) to verify whether they could independently induce the acquisition of OMC. Our results showed that forskolin induced the acquisition of OMC in a concentration-dependent manner (Fig. 4B) [3]. However, no effect was found for PMA at any of the concentrations tested [3]. These findings suggest that the signal-transduction pathway activated during the LH-induced acquisition of OMC is PKA-based.

PKA is activated by the second messenger cAMP. Therefore, we investigated whether the cAMP concentration increased in oocytes during the acquisition of LH-induced OMC. Maturationally incompetent ovarian fragments were cultured for 8, 16, 24 or 48 h with hCG, and the amounts of cAMP in the follicle-enclosed oocytes were measured using an enzyme-linked immunosorbent assay (ELISA). The results showed significantly increased levels of cAMP in the follicle-enclosed oocytes from 8 h after the start of the culture onwards compared with the controls [3]. Therefore, cAMP appeared to pass through the GJs between granulosa cells and oocytes during the LH-induced acquisition of OMC. cAMP is thus a strong candidate for the second messenger molecule that

Fig. 4. Effects of PKA inhibitor and activator on acquisition of OMC. (A), Ovarian fragments containing maturationally incompetent follicle-enclosed oocytes were incubated in plain medium or 100 IU/ml hCG with or without the PKA inhibitor H8 (A; 1, 10 or 100 μ M) for 24 h (inhibitors were added to the medium 1 h prior to the hCG exposure). Following this incubation, ovarian fragments were exposed to 10 ng/ml DHP in fresh medium and GVBD was scored after 48 h. (B), Ovarian fragments containing maturationally incompetent follicle-enclosed oocytes were incubated with or without the PKA activator forskolin (0.01, 0.1 or 1 μ M) for 24 h. Following this incubation, ovarian fragments were exposed to 10 ng/ml DHP in fresh medium and GVBD was scored after 48 h. Each bar represents the mean (+ SEM) of three different fish. Bars marked with the same letter were not significantly different ($P < 0.05$).

transmits the LH signal from granulosa cells to oocytes.

Summary and Conclusion

LH receptors are localized on the surface of granulosa cells, and are not present on oocytes themselves. It has previously been unclear how LH signals induce the acquisition of OMC. Our findings suggest a novel hypothesis, as follows (Fig. 5). First, LH binds to its receptors on granulosa cells and stimulates cAMP production (Fig. 5A). Second, as the cAMP molecules cannot permeate the cell membranes, they are transported via GJs formed by Cx34.9 between granulosa cells and oocytes (Fig. 5B). Third, in response to cAMP, PKA is activated and induces the acquisition of OMC (Fig. 5C). The LH-dependent increase in MIS receptor activity is probably responsible, at least in part, for the enhanced oocyte sensitivity to MIS that defines the acquisition of OMC [2]. Therefore, PKA, which is stimulated by LH, might activate MIS receptor or induce the synthesis of the receptor protein. MIS is not believed to act via the classical intracellular mechanism of steroid action, involving the activation of nuclear steroid receptors; instead, it is thought to initiate its actions at the surface of oocytes by binding to specific membrane receptors [2, 16]. Further studies will be needed to identify the MIS membrane receptors in the ayu, and to measure their activity during the LH-induced acquisition of OMC.

Fish such as the ayu that spawn more than once per breeding cycle are known as multiple spawners [17] and have asynchronous-type ovaries; these contain multiple clusters of follicles, which are at different stages of development. In such species, LH induces the

Fig. 5. Schematic representation of a novel hypothesis of LH-induced acquisition of OMC. (A), LH binds to its receptor on the granulosa cells and stimulates the cAMP production. (B), cAMP are transferred from granulosa cells to oocyte via gap junctions formed by Connexin 34.9. (C), In response to cAMP, PKA is activated and induces the acquisition of OMC.

acquisition of maturational competence selectively in the most advanced cluster of follicles that has completed vitellogenesis. The other clusters do not mature, even though the MIS diffuses throughout the ovary as the cell membrane permeability is high. These clusters are not sensitive to the MIS, and continue growing in the ovary until the next episode of spawning. Conversely, fish that spawn only once per breeding cycle, such as salmon, are known as single spawners and have synchronous-type ovaries. The ovaries of these species contain only one cluster of oocytes at a single stage of development. In ovaries of this type, the MIS is not required to selectively influence oocytes according to their stage of development. The control of the acquisition of maturational competence in single spawners is thus not as precise as that in multiple spawners. *In vitro* cultures of salmonid ovarian fragments at the post-vitellogenic stage with MIS revealed the presence of numerous oocytes that had already acquired the sensitivity to MIS [18]. This led us to suggest an overlap between the late vitellogenic stage and the acquisition of OMC. The variation in the timing of the acquisition of OMC according to spawning type is an interesting phenomenon. The mechanism for the LH-induced acquisition of OMC for multiple spawners with asynchronous-type ovaries might be considered superior, as it selects only the most advanced cluster of follicles for the acquisition of maturational competence. Further, ovarian GJs between granulosa cells and oocytes are one of the key structures for this LH-induced process.

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