MINI REVIEW

Transcripts Encoding the Enzymes that Convert Acetyl-CoA to Cholesterol are Induced in Cumulus Cells and are Essential for Progesterone Biosynthesis and Meiotic Resumption of Porcine Oocytes

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Introduction

The surge of luteinizing hormone (LH) induces marked functional (endocrine, biochemical and molecular) changes in the preovulatory follicle. Estrogen concentrations decline in follicular fluid as a consequence of reduced transcription of aromatase (*CYP19*) and 17 α HSD (*CYP17* α) genes in granulosa and theca cells, respectively, whereas progesterone concentrations rise in association with induction of P450scc (*CYP11A1)* in granulosa cells [1,2]. The LH surge also initiates ovulation, a process that is inhibited in rats by treatment with either anti-progesterone antiserum [3] or epostane [4], a compound that blocks the synthesis of progesterone. Female mice null for progesterone receptor (PR) (PRKO) fail to ovulate, even in response to exogenous hormones [5], suggesting that the LH-induced increase of progesterone is essential for ovulation.

In association with *in vitro* meiotic maturation of cumulus enclosed oocytes in human [6], rat [7], cattle [8] and pig [9-11], progesterone production by cumulus cells is increased in response to FSH and LH stimulation. In our previous studies [12], we showed that the high concentrations of progesterone secreted by porcine COCs accelerated meiotic resumption of oocytes and improved the rate of early embryonic development to the blastocyst stage after *in vitro* fertilization. We also reported that progesterone produced by porcine COCs bound PR, another FSH- and LH-induced factor in cumulus cells. LH also reduced proliferative activity of cumulus cells and closed the gap junctional communication within cumulus cells [11, 13]. Conversely, when COCs were cultured with FSH, LH and PR antagonist RU486, prolilferation and gap junctional communication was restored [13,14]. These results suggest that progesterone produced by cumulus cells acts within cumulus cells and/or at the oocyte cell surface to facilitate *in vitro* meiotic resumption of porcine oocytes. Thus, in order to obtain oocytes with a high developmental competence to blastocyst stage *in vitro*, it is essential to analyze the factors controlling progesterone biosynthesis in cumulus cells.

Serum supplementation is not required for progesterone production by cumulus cells during meiotic resumption of porcine oocytes

In our *in vitro* oocyte maturation system, COCs cultured with both 0.02 μg/ml FSH and 1.0 μg/ml LH produced more progesterone than was detected in cultures using either gonadotropin alone [15]. Since progesterone is synthesized from free cholesterol by cytochrme P450 cholesterol side-chain cleavage (*CYP11A1*) [16, 17] and 3β-hydrosteroid dehydorogenase /isomerase (*3*β*HSD*) enzymes [18], we analyzed levels of mRNA encoding these proteins in FSH- and LH-stimulated COCs. The data showed that mRNA levels were up-regulated in cumulus cells cultured with FSH and LH for 10 or 20-hr cultivation (Fig. 1A). In follicular cells, it has been shown that lipoprotein-bound choles-

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This work was supported in part, by Grant-in-Aid for Scientific Research (M.S., No.14760179, 15380222, 16780195) and a Research Fellowship for Young Scientist (Y.Y., No.08254) from the Japan Society for the Promotion of Science (JSPS).

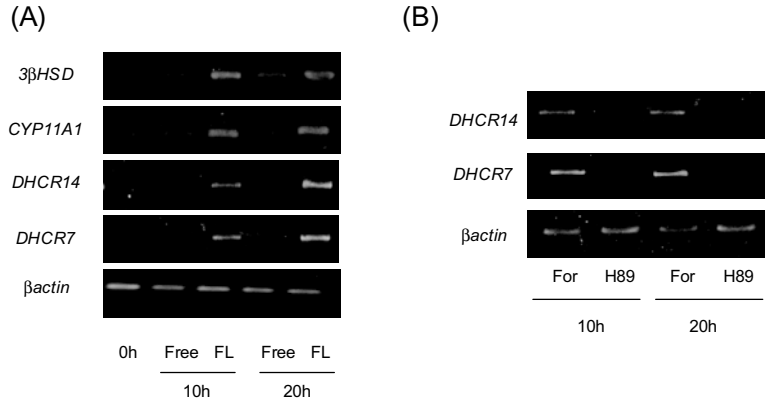

Fig. 1. Levels of mRNA encoding *3βHSD*, *CYP51*, *DHCR14* and *DHCR7* in cumulus cells of porcine COCs cultured with FSH and LH.
(A)Porcine COCs were cultured without (control) or with FSH (F) and LH (L) (FL) for 10 or 20 hr. (B) Porcine COCs were cultured with 10μM forskolin (For) and/or 10 μM of PKA inhibitor H89 for 10 or 20 hr.z 0h: COCs were collected from small antral follicles.

terolester and stored cholesterolester comprise the major source for the cholesterolester used for steroid hormone biosynthesis [19-21]. In contrast, luteinized follicular cells contain numerous lipid droplets, and the stored cholesterolesters are hydrolyzed by cholesterol esterase to provide the substrate for progesterone biosynthesis [22]. Furthermore, Armstrong [23] and Armstrong et al. [24] reported that the increased production of progesterone in rabbit ovary and bovine corpus luteum was exerted at a point subsequent to the biosynthesis of cholesterol. However, in cumulus cells just after collection from the antral follicles, the level of cholesterolesters was much lower than that in luteinized cells (our unpublished data). Therefore, we hypothesized that exogenous cholesterol might be used as the substrate for progesterone biosynthesis in cumulus cells. When COCs were cultured in medium without serum for 20 hr, the level of progesterone was lower than that in serum-containing medium (Fig. 2A). Unexpectedly, the addition of cholesterolester to the serum free medium did not increase the progesterone production, whereas the level was increased by exogenous essential amino acids (Fig. 2A). These results indicated that exogenous cholesterol did not serve as an immediate substrate. Rather, the addition of specific inhibitors to enzymes in the cholesterol biosynthetic pathway (shown Fig. 2B) significantly suppressed progesterone production (Fig. 2C), suggesting that cholesterol was derived by *de novo* synthesis from acetate. These divergent results suggest that gonadotropins increase the amounts and/or activities of enzymes that involve in cholesterol biosynthesis, the major source of progesterone is delivered from *de-novo* synthesized cholesterol during meiotic resumption of porcine oocytes in vitro.

Gonadotropins induce the expression of enzymes that convert acetyl-CoA to cholesterol as well as *CYP11A1* and *3βHSD*

The cholesterol biosynthetic pathway is regulated by over 30 cytochrome P450 superfamily enzymes that require NADPH as functional coactivator (Fig. 2B) [25]. The most important reaction is catalyzed by HMG-CoA reductase (HMGR), the rate limiting enzyme of the cholesterol biosynthetic pathway [26]. The enzyme activity is regulated by a negative feedback mechanism mediated by sterols and non-sterol metabolites derived from mevalonate, the product of the reaction catalyzed by HMGR [27-30]. When levels of the pathway endproducts such a cholesterol are low, activity of HMGR is high. Conversely when the levels of endproducts are low, enzyme activity increases. This feedback mechanism involves sterol regulation of the HMGR gene (*Hmgcr*) promoter [31]. Specifically, when sterol binds a sterol regulatory enhancer element (SRE), transcription of this gene is repressed whereas in the absence of sterols transcription of the promoter is activated [31]. Thus, sterols including cholesterol regulate both gene expression and serve as substrate for enzyme activity of HMGR.

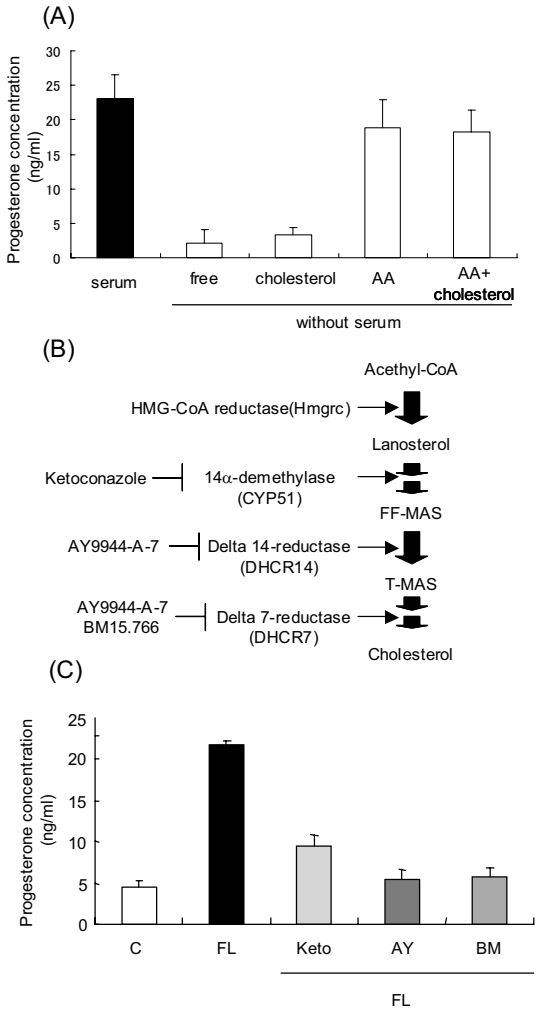

Fig. 2. (A) Progesterone production by COCs cultured with FSH and LH (FL) for 20 hr. Ser μM: COCs were cultured with 10% fetal calf, contorl: COCs were cultured without ser μM, Cholesterol: COCs were cultured with 1% cholesterol ester solution (Gibco), AA: COCs were cultured with non-essential and essential amino acid (Gibco), AA+cholesterol: COCs were cultured with cholesterol ester and amino acid. Values are mean +/- SEM of 3 replicates.
(B) Simplified outline of the cholesterol biosynthetic pathway Arrows denote enzymatic conversions, either a single step (one arrow) or multienzymatic steps (2 arrows). Ketoconazole blocks the activity of 14α-demethylase, AY9944-A-7 blocks the activity of both the delta 14-reductase and delta 7-reductase, and BM15.766 only blocks the activity of delta 7-reductase.
(C) Effects of Ketoconazole, AY9944-A-7 or BM15.766 on the progesterone production by COCs cultured for 20 hr. Values are mean +/- SEM of 3 replicates. Control: COCs were cultured without FSH and LH for 20 h. FL: COCs were cultured with FSH and LH for 20 h. Keto: COCs were cultured with FSH, LH, and Ketoconazole (1mM) for 20 h. AY: COCs were cultured with FSH, LH, and AY9944-A-7 (1 μM) for 20 h. BM: COCs were cultured with FSH, LH, and BM15.766 (20 μM) for 20 h.

Thaddeus and Strauss [31] have shown that exposure of cultured granulosa cells to 8-bromoadenosine cyclic 3', 5'-phosphate (8-bromo-cAMP) results in a rapid increase in the content of the message for *Hmgcr*. 14α-sterol demethylase (*CYP51*) which catalyzes the oxidative removal of 14 alpha-methyl group (C32) of lanosterol (Fig. 2B) was also expressed in rat ovary within 48 h after equine chorionic gonadotropin (eCG) priming [32]. The promoter regions of both genes contain a cAMP-responsive element (CRE) as well as SRE, suggesting that in follicular cells cAMP signaling stimulates transcription of the *Hmgcr* and *CYP51*. Our study showed that in response to FSH and LH or forskolin, transcription of two additional genes in the biosynthesis of cholesterol is induced in cumulus cells: delta 14-reductase (*DHCR14*) and delta 7-reductase (*DHCR7*) (Fig. 1A). Furthermore, agonist induced expression of these two genes suppressed by the PKA inhibitor H89 (Fig. 1B). Serum used as a source of cholesterol and other sterols did not suppress expression of these genes [33], suggesting that *DHCR7* and *DHCR14* promoters are regulated by cAMP-dependent pathway, but not negative feedback via a SRE mechanism. Thus, the gonadotropin stimulation induces transcription of several genes regulating sterol and steroid biosynthesis in cumulus cells: *Hmgcr*, *CYP51*, *DHCR14*, *DHCR7*, *CYP11A1* and *3βHSD*. Importantly, the induced expression of these genes leads to increased local concentrations of progesterone in cumulus cells that impact meiotic resumption of porcine oocytes (Fig. 3).

The expression of *DHCR14* and *DHCR7* plays an important role in meiotic resumption of porcine cumulus-enclosed oocytes

Meiosis-activating sterol, FF-MAS, first reported to be purified from human follicular fluid (4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol), was synthesized by COCs in response to FSH stimulation [34]. This type of sterol is an intermediate in the cholesterol biosynthetic pathway produced by demethylation of lanosterol by 14α-demethylase (Fig. 2B). Byskov et al. [34] showed that FF-MAS can stimulate in a dose-dependent manner *in vitro* meiotic maturation of mouse denuded oocytes. Addition of an inhibitor of FF-MAS synthesis, ketoconazole, resulted in the inhibition of GVBD of mouse oocytes in a dose-dependent fashion [35]. Our results also showed that GVBD in porcine oo-

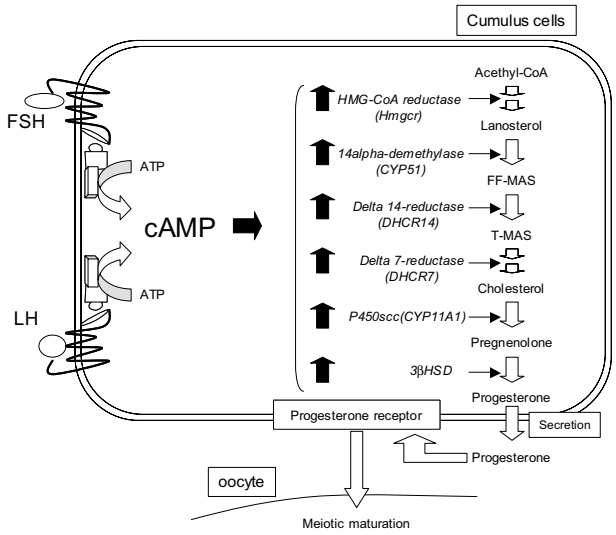

Fig. 3. Simplified outline of the progesterone biosynthesis pathway and its role in oocyte meiotic maturation.
Three sources of cholesterol can be used as substrate: 1) exogenous cholesterol (HDL or LDL); 2) hydrolysis of stored cholesterol ester by cholesterol esterase; 3) *de-novo* synthesized cholesterol from acetate.

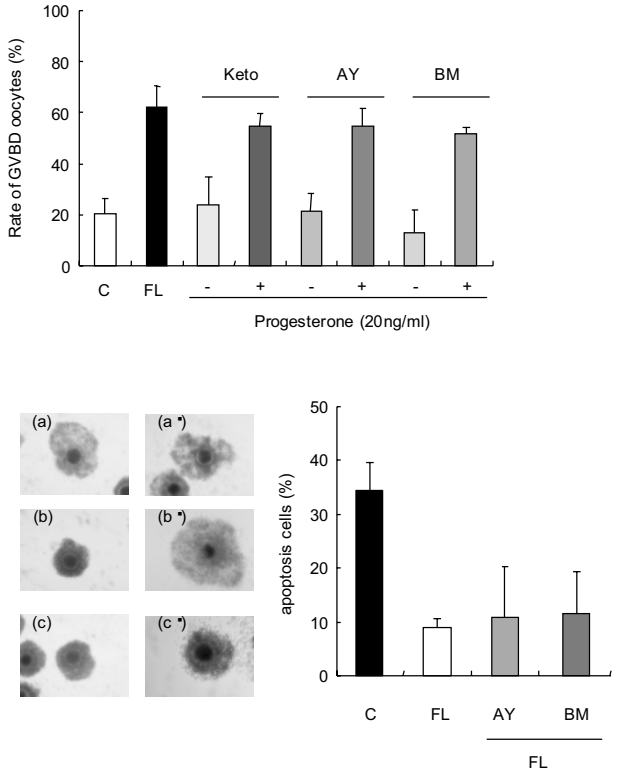

Fig. 4. (A) The effects of Ketoconazole, AY9944-A-7 or BM15.766, and progesterone on GVBD in oocytes cultured for 20 hr. Values are mean +/- SEM of 3 replicates. (B) The effects of AY9944-A-7 or BM15.766 on the cumulus cells expansion.
(a) COCs were cultured with FSH and LH for 28 h (a') COCs were cultured with FSH, LH, and progesterone (20ng/ml) for 28 h (b) COCs were cultured with FSH, LH, and AY9944-A-7 (1 μM) for 28 h (b') COCs were cultured with FSH, LH, AY9944-A-7 (1 μM), and progesterone (20ng/ml) for 28 h (c) COCs were cultured with FSH, LH, and BM15.766 (20 μM) for 28 h (c') COCs were cultured with FSH, LH, BM15.766 (20 μM), and progesterone (29ng/ml) for 28 h.
(C) The effects of AY9944-A-7 or BM15.766 on the apoptosis of cumulus cells cultured for 28 h. Control: COCs were cultured without FSH and LH for 28 hr. FL: COCs were cultured with FSH and LH for 28 h. AY: COCs were cultured with FSH, LH, and AY9944-A-7 (1uM) for 28 h. BM: COCs were cultured with FSH, LH, and BM15.766 (20 μM) for 28 h. Values are mean +/- SEM of 3 replicates.

cytes cultured with ketoconazole for 20 hr was significantly lower than that of oocytes cultured without this drug (Fig. 4A). Although these data suggest that sterol biosynthesis from lanosterol in cumulus cells is a factor involved in the acceleration of GVBD in mammalian oocytes, metabolism of FF-MAS was also required for meiotic progression of cumulus-enclosed oocytes. Fig. 4A showed that the addition of 1 μM AY9944-A-7 or 20 μM BM15.766 to FSH+LH- medium significantly decreased GVBD as well as progesterone production. Furthermore, we showed that progesterone could reverse the negative effects of ketoconazole, AY9944-A-7 or BM15.766 on GVBD (Fig. 4A). Judging from these results, we propose that FF-MAS is synthesized in cumulus cells but does not accumulate. Rather it is metabolized to cholesterol, which is then converted into progesterone. The secreted progesterone accelerates ongoing GVBD of porcine oocytes *in vitro* (Fig. 3). Since the secreted progesterone also impacts the process of cumulus expansion of porcine COCs [36], we analyzed cumulus expansion of porcine COCs which were cultured with cholesterol biosynthesis inhibitor. The cumulus expansion of COCs was also significantly suppressed when COCs were cultured with FSH, LH and either 1 μ M AY9944-A-7 or 20 μ M BM15.766 (Fig. 4B). The negative effects by both inhibitors were not mediated by cumulus cell death by toxicity (Fig. 4B), since the rate of apoptosis was as same as in control. Furthermore, cumulus expansion was restored by addition of 20 ng/ml progesterone. As such, we propose that the expression and functions of *DHCR14* and *DHCR7* in cumulus cells are required for gonadotropin-induced cumulus cells differentiation and oocyte maturation via a progesterone- and PR-dependent pathway.

Conclusion

Gonadotropins induce transcription of several genes of the *Cyp* superfamily that encode the enzymes essential for the conversion acetyl-CoA to cholesterol and cholesterol to progesterone. Progesterone, in turn, acts via receptor (nuclear and membrane) mediated mechanisms to support nuclear and cytoplasmic maturation of cumulus-enclosed oocytes, as well as COC expansion, not only in pig but also in rodent oocytes [6, 10, 11, 37, 38]. Thus, the up-regulation of the gene expression in COCs is required for proper meiotic maturation of oocytes with high developmental competence *in vitro*.

Acknowledgment

Thanks to Dr. JoAnne S. Richards, Professor of Baylor College of Medicine for insightful comments and suggestions concerning the manuscript. Mr. Tetsuji Okazaki and Mrs. Kaori Matsuo gave us numerous helps. We thank the staff of the Meat Inspection Office in Hiroshima City for supplying the porcine ovaries.

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