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Stage-specific Expression of Lanosterol 14α-Demethylase in Mouse Oocytes in Relation to Fertilization and Embryo Development Competence*

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ABSTRACT : Follicular fluid meiosis-activating sterol (FF-MAS) has been suggested as a positive factor which could improve the oocyte quality and subsequent embryo development after *in vitro* fertilization. However, FF-MAS is a highly lipophilic substance and is hard to detect in studying the relationship between MAS and quality of oocyte maturation. The present study focused on the expression of lanosterol 14α-demethylase (LDM), a key enzyme that converts lanosterol to FF-MAS, on mouse oocyte maturation and its potency on development. LDM expression was strong in gonadotropin-primed germinal vesicle stage oocytes, weak after germinal vesicle breakdown (GVBD), and then strong in MII stage oocytes. The LDM-specific inhibitor azalanstat significantly inhibited oocyte fertilization (from 79.4% to 68.3%, $p<0.05$). Also, azalanstat (5 to 50 μM) decreased the percentage of blastocyst development dose-dependently (from 78.7% to 23.4%, $p<0.05$). The specific inhibition of sterol Δ14-reductase and Δ7-reductase by AY9944 accumulates FF-MAS and could increase blastocyst development rates. Additionally, in the AY9944 group, the rate of inner cell mass (ICM)/ total cells was similar to that of *in vivo* development, but the rate was significantly decreased in azalanstat treatment. In conclusion, LDM, the key enzyme of FF-MAS production, may play an important role in fertilization and early development of the mouse embryo, especially *in vitro*. (**Key Words :** Mouse Oocyte, Lanosterol 14α-Demethylase, *In vitro* Fertilization, Early Embryo Development)

INTRODUCTION

To complete successful fertilization and embryo development, the nucleus and cytoplasm maturation of oocyte must be coordinated with intercellular events, including growth, meiotic resumption, and ovulation. Many studies indicated that early embryos produced by *in vitro* fertilization (IVF) can not achieve satisfactory implantation and embryo development (Abeydeera et al., 2001; Hamamah et al., 2005; Jang et al., 2008). To improve the quality of these early embryos, it is necessary to notice the *in vivo* fertilization and early embryo development environment.

Follicular fluid meiosis-activating sterols (FF-MAS) is a lipophilic molecule (4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol), which was found in high concentrations in the follicular fluid of mammals including humans and proved to be stimulatory to oocyte meiotic resumption (Byskov et al., 1995; Byskov et al., 2002). Subsequent data showed that the synthesization of MAS is significantly increased with response to gonadotropins (Grondahl et al., 2003; Xie et al., 2004; Yang et al., 2008). Meanwhile, recent studies indicated that FF-MAS dramatically promotes the later stages of meiotic maturation, namely the progression of MI to MII. The treatment of FF-MAS on mouse oocytes during meiotic maturation also increased their subsequent competence to complete early embryos development (Marin Bivens et al., 2004a). However, the positive effect of FF-MAS was still controversial with the following results. FF-MAS increased the rate of chromosomal abnormality and had detrimental effects on cleavage and human early embryos development (Loft et al., 2004; 2005).

In trying to understand the effect of FF-MAS during oocyte maturation, FF-MAS synthesis is often taken into

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consideration. Involved in the cholesterol biosynthesis, cytochrome P450 lanosterol 14α-Demethylase (LDM) is the crucial and rate-limiting enzyme in the synthesis of FF-MAS by removing the methyl group from lanosterol (Rozman et al., 2002). A 2.5-fold increase in LDM mRNA and protein in rat ovaries and follicles has been previously described after hCG stimulation (Vaknin et al., 2001). Otherwise, the activity of LDM is supported to be gonadotropin dependent (Wang et al., 2006). Therefore, up-regulation of LDM induced by gonadotropin maybe directly lead to MAS production.Yet, the expression profile of LDM in mouse oocytes during meiotic maturation is unknown.

In present study, two highly specific inhibitors (azalanstat and AY9944-A-7) related to the metabolism of FF-MAS were introduced. Azalanstat could specifically inhibit LDM function and therefore decrease the production of FF-MAS of cumulus enclosed oocytes (CEOs) (Burton et al., 1995); whereas AY9944 could specifically inhibit activities of the sterol Δ14-reductase and Δ7-reductase (Kim et al., 1995) to decrease the transform of T-MAS from FF-MAS, which leads to the accumulation of FF-MAS in CEOs (Leonardsen et al., 2000).

Our objectives were to: i) investigate the expression profile of LDM during mouse oocyte meiotic maturation from GV stage to MII stage and to validate the involvement of FF-MAS accumulation in fertilization and development of early embryos; ii) use specific inhibitors (azalanstat and AY9944-A-7) related to the metabolism of FF-MAS to determine the role of FF-MAS on mouse oocyte competence, this may validate the effect of endogenous FF-MAS on the quality of oocyte maturation.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this experiment were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) except for those specifically mentioned. Azalanstat (also named RS-21607. gift from Dr. D. C. Swinney, Roche Bioscience, USA; (Swinney et al., 1994)) was prepared as 10 mM stock solution by dissolving in ethanol, 10 mM AY9944 (gift from Dr. Wyeth-Ayerst, Princeton, NJ, USA) stock solution was dissolved in M2 medium, both stock solutions stored in a dark box at -20°C.

Animals, superovulation and oocyte recovery

Kunming White female mice, 6-8 weeks after birth, were kept in a mouse keeping room with 14 h/10 h light-dark cycles, the dark starting from 8 PM. Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethical committee of the China Agricultural University. To obtain *in vivo* maturated oocytes, mice were induced to superovulation by injection of eCG (10 IU/mouse, i.p.), 48 h later followed by injection of hCG (10 IU/mouse, i.p.). The superovulated mice were killed 11 or 15 h after hCG injection according to experimental design, and the mature follicles in the ovary or the oviductal ampullae were ruptured in M2 to release the CEOs. To analyze the expression of LDM during mouse oocytes meiotic maturation *in vivo*, CEOs were collected at each time point after hCG injection, and removed cumulus cells with 0.1% hyaluronidase in M2 medium.

Staining of LDM and confocal microscopy

After removing the zonae pellucidae in acid Tyrode solution (pH 2.1) (Nicolson et al., 1975), oocytes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. To compare LDM expression of different development stages in the same immunostaining experiment, the samples were processed in parallel. Cells were permeabilized with 1% (v/v) Triton-X100 for 30 min at 37°C, followed by blocking in 1% (w/v) BSA for 1 h at room temperature, and then incubated with 1:300 rabbit anti-human LDM antibody (gift from Dr. M. R. Waterman, Vanderbilt University School of Medicine, Nashville, USA) overnight at 4°C. The oocytes were rinsed three times and incubated with 1:200 FITC-conjugated goat anti-rabbit IgG for 1 h. Following three washes, the nuclear status of oocytes was evaluated by staining with 10 mg/ml propidium iodide in PBS for 10 min. In negative control groups, the polyclonal rabbit anti-human LDM antibody was replaced with rabbit IgG Following extensive washing, samples were mounted between a coverslip and a glass slide supported by four columns of a mixture of vaseline and paraffin (9:1). Cells were observed under a laser scanning confocal microscope (Leica Microsystems, Leica, Wetzlar, Germeny); all samples were visualized or pictured using the same laser power. A total of approximately 30 oocytes were collected at each time point after hCG injection, the same group was repeated for at least 3 times.

In vitro fertilization and embryos culture

Sperm were collected from the cauda epididymis of fertile male mice in T6 medium (Quinn et al., 1982) supplemented with 10 mg/ml BSA, and capacitated in the same medium under mineral oil at 37.5°C for 1.5 h. CEOs collected at 11 h after hCG injection were washed in the fertilization medium (T6 containing 20 mg/ml BSA) and were placed in fertilization drops (20 oocytes per 40 μl drop). Capacitated sperm were added to the fertilization drops to give a final sperm concentration of about 1×10^6 cells/ml. After 6 h of incubation, the presumed fertilized oocytes were denuded of cumulus cells by pipetting in M2 containing 0.1% hyaluronidase and cultured in CZB medium to obtain *in vitro* fertilization blastocysts. *In vivo*

fertilized zygotes as a positive control. Zygotes were collected 16 h post-hCG from the oviduct ampullae of superovulated females that had been mated with the same strain of males. After removing cumulus cells with 0.1% hyaluronidase in M2 medium, zygotes were cultured in CZB medium for 96 h to obtain *in vitro* cultured blastocysts. Some females with vaginal plugs were killed 100 h post hCG and the uterine horns were flushed with M2 medium to obtain *in vivo* developed blastocysts.

Cell counting of blastocysts

Differential staining of inner cell mass (ICM) and trophoblastic ectoderm (TE) cells of blastocysts was performed as described previously (Ma et al., 2005) with minor modifications. Briefly, zonae pellucidae of blastocysts were removed by exposing the embryos in groups of ten to fifteen to acid Tyrode solution, until the zonae pellucidae became wrinkly and dissolved (after about 3 to 5 second). After rinsing in M2 medium with 1 mg/ml polyvinyl alcohol (PVA-M2), the zona-free embryos were exposed for 1 h to a 1:5 dilution of rabbit anti-mouse whole serum (Sigma, M-5774), rinsed three times (5 min each) in PVA-M2, and placed in a 1:5 diluted mouse complement serum (Sigma, S-3269) containing both 5 μg/ml propidium iodide (PI) and 5 μg/ml bisbenzimide (Hoechst 33342) for 1 h. After briefly rinsing in PVA-M2, the embryos were mounted between slide and a coverslip and examined with ultraviolet light using an epifluorescent microscope or a laser scanning confocal microscope.

Statistical analysis

All experiments were replicated at least three times. And the values were given as mean±SEM. Statistical analyses of all data were done by t-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (SigmaStat; Systat Software, Inc., Richmond, CA, USA). All percentage data were subjected to arc-sine transformation before statistical analysis. $p<0.05$ was considered to indicate a significant difference. In all groups, percentage of fertilized zygotes is a fraction of total survived oocytes, as opposed to total oocytes used; in the same way, percentage of blastocyst development is a fraction of fertilized zygotes.

RESULTS

The time points of meiotic progression of mouse oocytes during *in vivo* maturation

For studying LDM protein expression in mouse oocytes during meiotic maturation, firstly, we identified the time points of meiotic progression of mouse oocytes during *in vivo* maturation by following experiment. The mouse

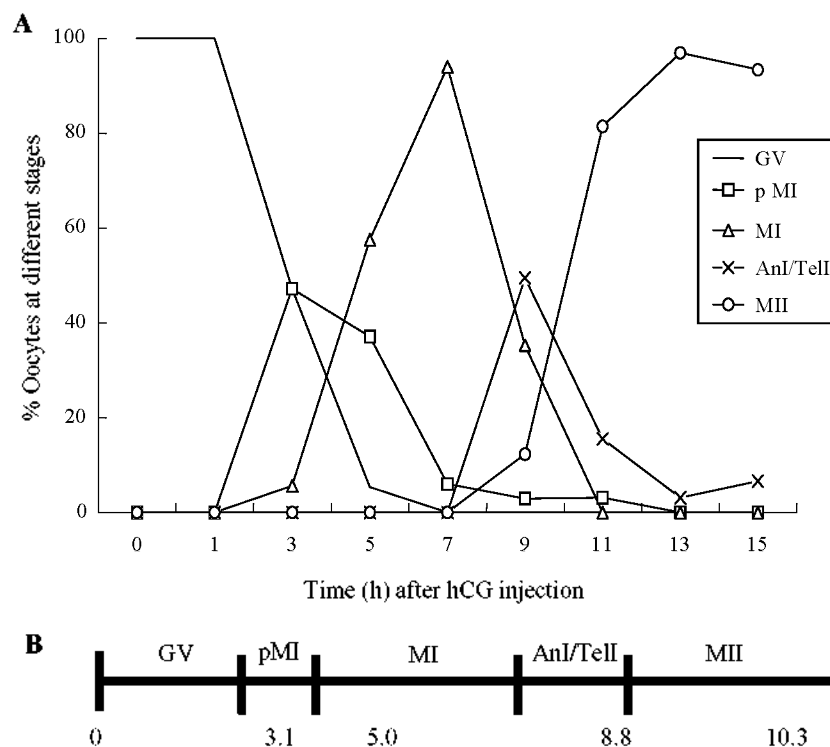

Figure 1. Nuclear progression during maturation of mouse oocytes *in vivo*. (A) Percentages of oocytes at different stages of nuclear maturation while collected at different time after hCG injection (GV, germinal vesicle; pMI, pro-metaphase I; MI, metaphase I; AnI/TelI, anaphase I and telophase I; MII, metaphase II stages). (B) The mean time (h) that an oocyte spent at each stage of nuclear progression during *in vivo* maturation after hCG injection.

oocytes were recovered at different time after hCG injection (Figure 1A), and then were classified, under a confocal microscope, as germinal vesicle (GV, Figure 2B), pro-metaphase I (pMI, Figure 2C), metaphase I (MI, Figure 2D), anaphase I (AnI, Figure 2E), telophase I (TelI, Figure 2F) and metaphase II (MII, Figure 2G and H) stages. All the oocytes were at GV stage at 0 h post hCG. More than half of oocytes reached pMI or MI at 3 h post hCG and >90% oocytes entered MI stage at 7 h post hCG. By 11 h or 15 h after hCG treatment, 81.4% of the oocytes extruded first polar bodies (PB1). After 15 h, 93.4% of the oocytes had entered MII stage. To prepare oocytes at proper time point during meiotic maturation *in vivo* for immunostaining experiment, we computed the average time of each stage of nuclear progression during *in vivo* maturation after hCG injection. The calculation was performed according to the method reported by Sirard et al. (Sirard et al., 1989) and was based on the data of Figure 1A. As shown in Figure 1B, oocytes collected at 0, 4, 7, 9 and 11 h post hCG treatment were at GV stage, pro-metaphase I, metaphase I, anaphase I/telophase I and metaphase II, respectively.

Expression of LDM during oocyte meiotic maturation

In order to detect LDM protein expression in mouse oocytes during meiotic maturation, samples were taken from GV stage to 15 h after fertilization. Intracellular distribution of LDM in the oocytes was detected using confocal microscopy. As negative control, the polyclonal rabbit anti-human LDM antibody was replaced with rabbit IgG and no staining was found in the embryos virtually (Figure 2A). The results showed that LDM mainly exhibited a sub-membrane distribution pattern in oocytes at all meiotic maturation stages (Figure 2). Strong LDM expression distributed evenly in the GV oocyte (Figure 2B), while, LDM immunostaining was at a basal level at pro-metaphase I, metaphase I, anaphase I and telophase I stage (Figure 2C, D, E and F). The LDM immunostaining signal was slightly increased at the metaphase II stage collected at 11 h after hCG injection (Figure 2G) and was significantly increased at the metaphase II stage collected at 15 h after hCG injection (Figure 2H).

LDM inhibitor significantly decreased fertilization and blastocyst development of mouse CEOs

To verify whether the stage-specific expression profile of LDM in the oocytes is related to oocyte fertilization, the CEOs collected at 11 h post hCG injection were pre-incubated 4 h in medium containing increasing concentrations of azalanstat (a specific inhibitor of LDM) from 0 to 50 μM and then were inseminated; CEOs in the control group which collected at 15 h post hCG injection were inseminated directly without any pre-incubation. After *in vitro* fertilization, all groups were assessed for fertilization and blastocyst development after 3.5 days culture.

As shown in Figure 3A, 6 h after insemination, 79.4%

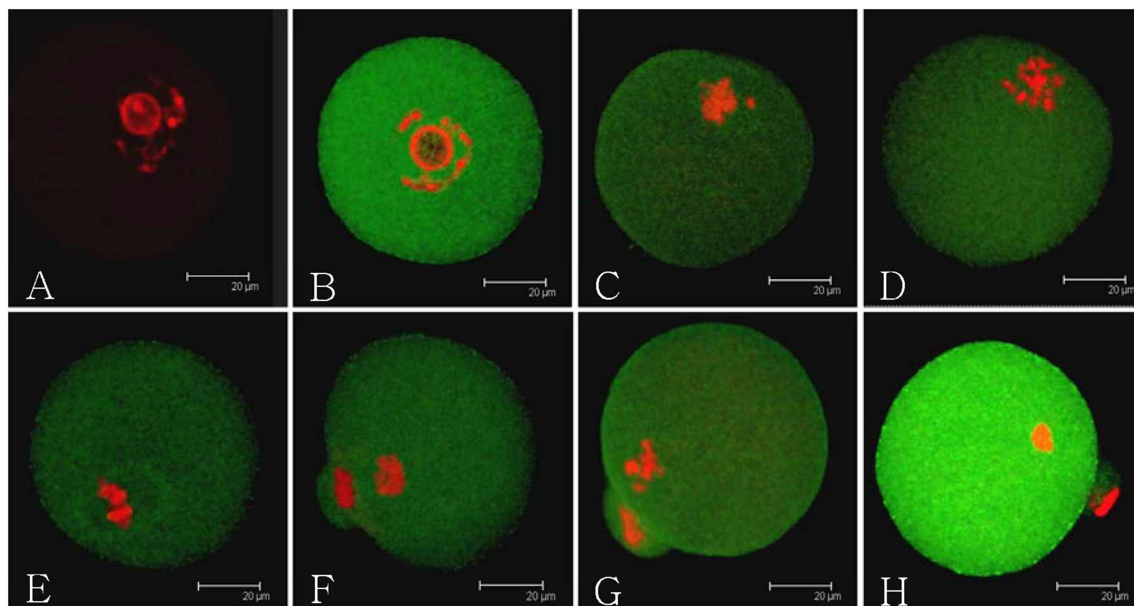

Figure 2. Immunofluorescent localization of LDM during oocyte meiotic maturation *in vivo*. Green, LDM; red, chromatin. LDM distributed evenly in the GV oocyte (B), after GVBD occurred, basal level of LDM immunostaining in the oocytes respectively at pro-metaphase I, metaphase I, anaphase I and telophase I stage (C, D, E and F); at the metaphase II stage collected at 11 h after hCG injection, a slightly increased LDM immunostaining in the whole oocyte (G); at the metaphase II stage collected at 15 h after hCG injection, a high level LDM expressed in the oocyte (H). A GV stage oocyte was used as a negative control for the LDM confocal microscopy, in which no first antibody was used but the fluorescent second antibody was used just as the experimental group (A).

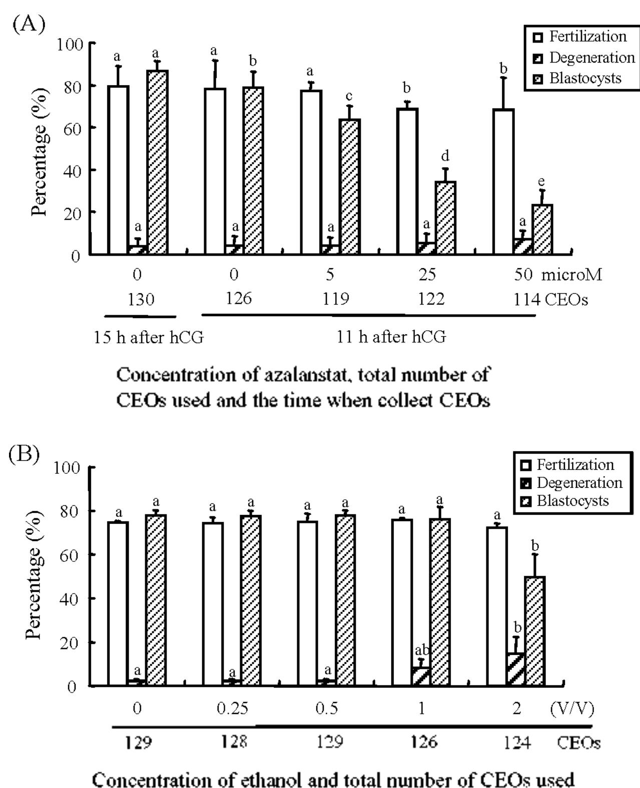

Figure 3. Dose response of azalanstat and ethanol on mouse cumulus-enclosed oocytes (CEOs) fertilization and blastocyst development. (A) Mouse CEOs which collected at 11 h post hCG injection were treated with azalanstat in different concentration for 4 h before inseminated, while CEOs which collected at 15 h post hCG injection were inseminated directly without azalanstat treatment as positive control. (B) Mouse CEOs which collected at 11 h post hCG injection were cultured in medium plus increasing concentrations of ethanol for 4 h before inseminated. Groups with no identical letters above the bars are significantly different ($p<0.05$).

of the oocytes had formed pronuclei, and 86.8% of the fertilized oocytes developed into blastocysts in the control group where CEOs were collected at 15 h post hCG injection.. This result indicated that the *in vitro* fertilization system we established was well performed and suitable to further experiments. When CEOs were collected at 11 h post hCG injection and then treated with increasing concentrations of azalanstat for 4 h pre-incubate, the percentage of blastocyst development dose-dependently decreased (from 78.7% to 23.4%). Azalanstat pre-incubation inhibited fertilization of oocytes and reduced blastocyst development competence, while it had no difference between the fertilization of oocytes in low concentration (0 and 5 μM) azalanstat treated groups and the control group.

To examine the effect of the azalanstat vehicle on oocyte culture, CEOs collected at 11 h post hCG injection were exposed to increasing concentrations of ethanol from 0 to 2% (V/V). Azalanstat dilutions were prepared from a 10 mM stock solution, 0.5% ethanol corresponds to final azalanstat concentration of 50 μM. As shown in Figure 3B, ethanol dose-dependently inhibited blastocyst development in CEOs (by 49.6% at 2% ethanol), but had no effect on fertilization even at concentrations as high as 2%.

Effect of AY9944 on fertilization of mouse CEOs

In order to validate whether endogenous FF-MAS could stimulate fertilization of oocytes and improve blastocyst development competence, AY9944 (an inhibitor of Δ7-reductase and Δ14 reductase), which could accumulate FF-MAS, was employed to examine the function on oocytes fertilization and blastocyst development. The CEOs collected at 11 h post hCG injection were pre-incubated 4 h in medium containing increasing concentrations of AY9944 from 0 to 50 μM and then inseminated. In the control group, CEOs which collected at 15 h post hCG were inseminated directly without any pre-incubation.

As shown in Figure 4, 6 h after insemination, in the control group, 77.4% of the oocytes had formed pronuclei, and 87.8% fertilized oocytes developed into blastocysts. Similar to the control group, more than 80% fertilization oocytes in 5 μM AY9944 pre-incubated group developed into blastocysts. Meanwhile, the blastocysts development rates of other pre-incubated groups (0, 25 and 50 μM) were significantly lower than 5 μM AY9944 pre-incubated group ($p<0.05$).

Cell numbers in pre-incubated oocytes derived blastocysts

When mouse blastocysts were examined under a laser scanning confocal microscope after differential staining with Hoechst 33342 and PI, all cells were stained blue with Hoechst 33342. Trophoblast cells were stained red with PI. The trophoblast cells appeared pink in the merged picture and ICM cells appeared blue. Total and ICM cell numbers and ICM/total ratio were compared using well-expanded blastocysts which derived from azalanstat or AY9944 pre-incubated oocytes. As shown in Figure 5, both the total and ICM cell numbers of the blastocysts derived from 25 μM azalanstat pre-incubated oocytes were significantly lower than those blastocysts of *in vivo* developed (IVO), fertilized zygotes cultured *in vitro* (IVC) and *in vitro* fertilization (IVF) ($p<0.05$), which were not exposed to azalanstat. The total cell numbers of the blastocyst derived from 5 μM AY9944 pre-incubated oocytes was similar to the IVC and IVF groups; whereas the ICM cell numbers was significantly lower ($p<0.05$) than those of IVC and IVF blastocysts. The ICM/total ratio of 5 μM AY9944 pre-incubated oocytes derived blastocysts was more similar to the IVO blastocysts.

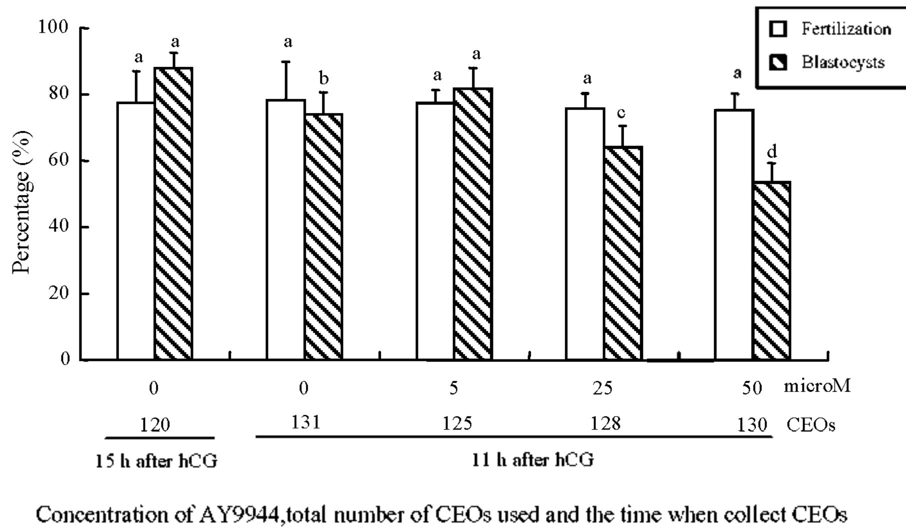

Figure 4. Effect of AY9944 on fertilization and blastocyst development of mouse cumulus-enclosed oocytes (CEOs). Mouse CEOs which collected at 11 h post hCG injection were treated with different concentration azalanstat for 4 h before inseminated, while CEOs which collected at 15 h post hCG injection were inseminated directly without AY9944 treatment as positive control. Groups with no identical letters above the bars are significantly different ($p<0.05$).

DISCUSSION

In this study, we first evaluated the protein stage-specific expression of LDM in mouse oocytes during meiotic maturation in detail. The results demonstrated that the expression of LDM during mouse oocyte meiotic maturation *in vivo* firmly rely on the meiotic phase. As has been proposed, FF-MAS has the ability to trigger resumption of mouse oocytes meiosis, which can promote not only the nuclear but also plasma maturation (Marin Bivens et al., 2004b). The high level expression of LDM at the GV stage may play a role to induce oocyte to undergo germinal vesicle breakdown by accumulating FF-MAS. Interestingly, the level of LDM immunostaining was significantly increased at the metaphase II stage in the whole oocyte collected at 15 h after hCG injection than the level at 11 h after hCG injection (Figure 2H andG). This secondary increase of LDM expression gives a hint that FF-

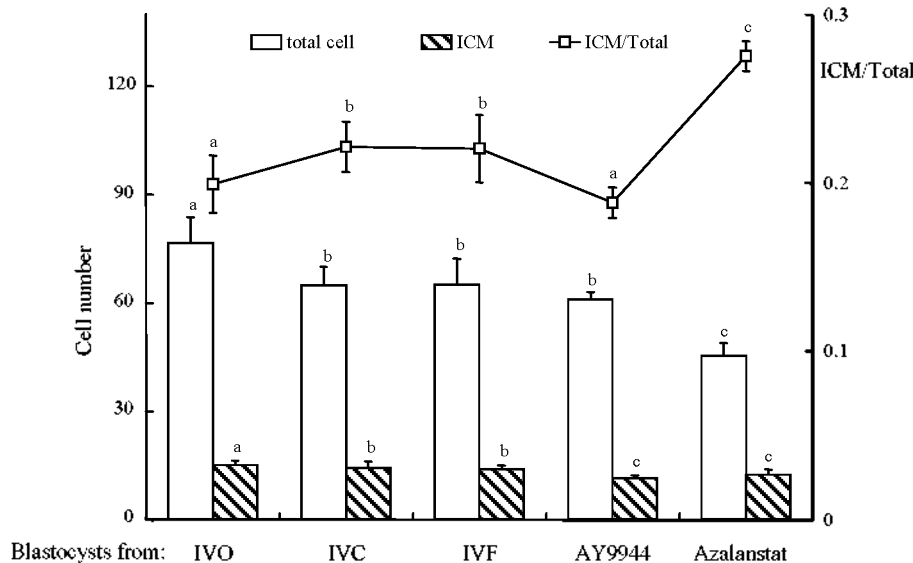

Figure 5. Cell numbers of blastocysts. IVO: *in vivo* developed, IVC: fertilized zygotes cultured *in vitro*, IVF: oocytes collected 15 h post-hCG were cultured *in vitro* after *in vitro* fertilization; AY9944: oocytes collected 11 h post-hCG were pre-incubated with 5 μM AY9944 for 4 h, and then cultured *in vitro* after *in vitro* fertilization; Azalanstat: oocytes collected 11 h post-hCG were pre-incubated with 25 μM azalanstat for 4 h, and then cultured *in vitro* after IVF. Groups with no identical letters above the bars are significantly different ($p<0.05$).

MAS may be beneficial for the fertilization.

It is reported that lanosterol 14alpha-demethylase (CYP51, LDM) is an immediate early response gene (Fink et al., 2005); exposure of JEG-3 cells to forskolin, a mediator of the cAMP-dependent signaling pathway, provokes an immediate early response of *CYP51*, and the *CYP51* mRNA increases up to 4-fold in 2 h and drops to basal level after 4 h. In our present study, during 11 h to 15 h after hCG injection, oocytes may have enough time to further promote the plasma maturation for fertilization and early embryo development by accumulation of MAS under the activity of LDM. It is known that during normal fertilization *in vivo*, oocytes are not fertilized until a few hours after being released from the ovary into the oviduct (Ma et al., 2005). In addition, at the time of ovulation *in vivo*, oocytes are expelled together with the follicular fluid which contains high concentration of FF-MAS (Byskov et al., 1995; Byskov et al., 2002) and presumably still exposed to FF-MAS during the first part of its passage through the oviduct. All these suggest that FF-MAS which derived from lanosterol by the LDM may play some important role on fertilization.

To prove this suggestion, azalanstat, a specific inhibitor of LDM, was used in this study. It has been confirmed that azalanstat could inhibit synthesis of FF-MAS specifically and decrease the production of FF-MAS both *in vivo* and *in vitro* (Burton et al., 1995). Our results showed that azalanstat pre-culture dose-dependently inhibited fertilization of oocytes and reduced blastocyst development competence. Since the azalanstat was dissolved in ethanol as a stock solutions according to previous studies (Burton et al., 1995; Wang et al., 2006). Actually, studies on bovine oocytes have indicated that ethanol could influence both nuclear and cytoplasmic maturation (Avery and Greve, 2000). Plasma membrane outward currents and hyperpolarization of membrane potential were generated by treating oocytes with ethanol (Tosti et al., 2002); these changes may be harmful to embryos development. It is therefore important that caution be exercised when using ethanol as a vehicle in oocyte culture experiments. In addition, mouse oocytes are able to oxidize ethanol to acetaldehyde in the presence of NAD^+ and alcohol dehydrogenase (ADH); the analysis of differentiation and cell number of mouse morulae and blastocysts shows that acetaldehyde is three orders of magnitude more toxic than ethanol, which indicate that the metabolite is responsible for the embryo toxicity of ethanol in preimplantation embryos (Lau et al., 1991). It is important to point out that although higher concentrations of ethanol (2%, V/V) significantly inhibited blastocyst development in our results (Figure 3B), the final concentration of ethanol in this 25 μM azalanstat preparation was only 0.25%, a concentration alone that has no any effect on fertilization and further blastocyst development. Thus, the action of azalanstat on oocyte fertilization and early embryo development is specific for inhibiting lanosterol 14α-demethylase activity. Our results suggest that 5, 25 and 50 μM azalanstat prevent oocyte development to the blastocyst stage, and the doses have been shown to effectively suppress FF-MAS synthesis (Xie et al., 2004).

AY9944 can specifically inhibit activities of the sterol Δ14-reductase and Δ7-reductase to decrease the transform of T-MAS from FF-MAS (Kim et al., 1995), which leads to the accumulation of FF-MAS in mouse CEOs (Leonardsen et al., 2000). In our results, similar to the positive control, more than 80% of fertilization oocytes in 5 μM AY9944 pre-cultured group developed into blastocysts (Figure 4). However, blastocyst development in groups pre-cultured were significantly lower with 0, 25 or 50 μM AY9944. Meanwhile, the oocytes degeneration after *in vitro* fertilization among all AY9944 pre-culture groups was dose-dependently increased (data not shown). Previous studies have shown that addition of AY-9944 to rat follicle-enclosed oocytes (FEOs) in culture increased FF-MAS within 8 h of culture and caused the resumption of meiosis, and further extension of the culture period led to an increase in the number of degenerating oocytes (Cao et al., 2004). As an inhibitor of cholesterol biosynthesis, high concentration of AY9944 caused the oocyte degeneration. This result indicates that cholesterol is necessary for oocytes to mature and get full fertilization and early embryos development competence, since high concentration of AY9944 may inhibit the production of cholesterol deeply.

Improvement of outcome after *in vitro* fertilization is a constant challenge. The introduction of new stimulation protocols and drugs may increase the proportion of mature oocytes, fertilization, and cleavage rates together with better early embryo quality and thereby higher implantation rates. Previous studies have shown that addition of FF-MAS to culture medium had a positive effect on the cytoplasmic and nuclear maturation of the oocytes (Hegele-Hartung et al., 1999; Donnay et al., 2004). We compared the cell numbers among azalanstat or AY9944 pre-cultured oocytes derived blastocysts with normal *in vivo* developed blastocysts. In 5 μM AY9944 pre-cultured oocytes derived blastocysts, the total cell number was similar to the control group in which collected CEOs were inseminated directly without any pre-culture, and the ICM/total ratio was more similar to the *in vivo* developed blastocysts. However, the blastocysts derived from 25 μM azalanstat pre-cultured oocytes has a significant low total cell number and more abnormal ICM/total ratio than *in vivo* developed blastocysts. The results show that possible accumulation of FF-MAS in mouse CEOs by a proper concentration of AY9944 pre-

culture may increase blastocyst development and improve embryo quality in cell number level.

In summary, our results suggest that LDM may have a positive effect on the oocyte plasma maturation for fertilization and early embryo development in mouse, the possible mechanism of this event remain to be established.

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