BIOLOGY OF REPRODUCTION **75,** 547–554 (2006)
Published online before print 28 June 2006.
DOI 10.1095/biolreprod.106.051300

A Growth-Maturation System That Enhances the Meiotic and Developmental Competence of Porcine Oocytes Isolated from Small Follicles[1]

De Wu, Queenie Ching-Kwan Cheung, Lihua Wen, and Julang Li[2]

Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

ABSTRACT

In livestock, most of the follicles on the ovarian surface are small follicles. A procedure that supports the in vitro growth and maturation of these small follicle-derived oocytes may offer a new source of useable oocytes for both biotechnological and fundamental research purposes. The objective of the current study was to test the hypothesis that providing a more growth-supporting and less maturation-promoting environment during the first phase of small follicle-derived oocyte maturation may improve oocyte competence for meiosis and embryo development upon activation. In our small follicle-derived oocyte growth-maturation system (SGM group), cumulus-oocyte complexes (COCs) from small follicles (1–3 mm) were first cultured in oocyte growth medium for 24 h, then in oocyte maturation medium for 20 h. As controls, COCs from small (SM group) and large (LM group) follicles were cultured using a conventional in vitro maturation (IVM) approach in which they were directly cultured in oocyte maturation medium. At 24 h of culture, the percentage of small follicle-derived oocytes that underwent germinal vesicle breakdown (GVBD) in the SGM group was comparable to that of large follicle-derived oocytes (LM group) but was significantly higher than that of the SM group ($P < 0.05$). At 44 h of culture, compared to 36% in the SM group, 55% of the SGM group oocytes reached metaphase II (MII; $P < 0.05$). In addition, the level of cyclin B in oocytes of the SGM group was comparable to that of oocytes from LM group and was significantly higher than that of oocytes from the SM group ($P < 0.05$). When activated and in vitro fertilized (IVF), 7.3 and 9.0 times more parthenogenetic and IVF embryos developed to blastocyst stage in the SGM group than in the SM group ($P < 0.05$). The mRNA expression levels of three developmentally important genes—DNA-methyltransferase 1, Pou domain class 5 transcription factor 1, and Fibroblast growth factor receptor 2—in embryos of the SGM group were comparable to those of embryos developed from the LM group, whereas they were significantly lower in those of the SM group ($P < 0.05$). Our data suggest that the oocyte growth-maturation system facilitates the final stage of oocyte growth and thus resulted in better oocyte nuclear, cytoplasmic maturation, and developmental competency compared with the conventional direct oocyte maturation system.

cytoplasmic maturation, early development, embryo, embryo development, gamete biology, gene expression, gene regulation, oocyte development, oocyte maturation

[1]Supported by Natural Sciences and Engineering Research Council (NSERC), Agriculture and Agri-Food Canada, Ontario Ministry of Agriculture and Food, Ontario Pork, and Canadian Institutes of Health Research (CIHR). D.W. is a visiting scientist from Institute of Animal Nutrition, Sichuan Agricultural University, supported by National Natural Science Foundation and China Scholarship Council.
[2]Correspondence. FAX: 519 836 9873; e-mail: jli@uoguelph.ca

Received: 31 January 2006.
First decision: 19 February 2006.
Accepted: 27 June 2006.
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ISSN: 0006-3363. http://www.biolreprod.org

INTRODUCTION

Unlike the mouse, in which oocytes are fully grown when the follicle develops to early antral follicle stage [1], large animal oocytes grow until follicles reach late antral stage [2–4]. However, most follicles on the ovarian surface are small, less than 2 mm in diameter [5]. A system that supports the in vitro growth and maturation of these oocytes would offer a new source of useable oocytes for both biotechnological and fundamental research purposes. The system supporting the development of primordial follicles to the mature stage has been developed, and it resulted in live birth in mice [6, 7]. However, due to species differences, a system supporting the entire process of follicular development in vitro for large animals is to be established.

Using a conventional in vitro maturation (IVM) system, oocytes isolated from porcine follicles that are <3 mm in diameter are not fully competent for meiosis and are enormously incapable of supporting early embryonic development. For instance, previous in vitro studies revealed that a significantly lower percentage of the small follicle-derived oocytes reached metaphase II (MII) stage and developed to blastocyst stage after in vitro fertilization compared with those derived from large follicles [8–11]. It is well known that a grown oocyte contains numerous transcripts and proteins necessary to complete meiosis and survive the first few cell cycles of embryonic development. We reasoned that oocytes isolated from small follicles are still at their last stage of growth phase, and induction of IVM of these oocytes upon isolation via conventional IVM strategy may force them to complete meiosis when their intracellular environment is not completely ready for the process. This premature meiosis may attribute to a low developmental competence of porcine oocytes. In an in vivo physiologic environment, the oocyte is arrested at the germinal vesicle stage in an intact follicle. Only the fully grown oocytes can respond to the gonadotropin surge by completing the first meiotic division and being ovulated. However, following isolation from the intact follicles and culture in IVM medium, oocytes spontaneously re-enter the meiotic process [12]. Previous studies have attempted to prevent germinal vesicle breakdown (GVBD) using inhibitors at the early stage of in vitro culture to improve porcine oocyte cytoplasmic maturation. Although the effect of the inhibitors on meiosis was reversible, the developmental competence of the oocytes was not improved [13, 14]. We hypothesized that providing a more growth-supporting and less maturation-promoting environment during the first phase of small follicle-derived oocyte maturation may improve the competence of these oocytes to complete meiosis and embryo development upon activation.

MATERIALS AND METHODS

Oocyte Collection and IVM

Porcine ovaries were collected from prepubertal gilts at local slaughterhouses and were transported to the laboratory in a thermal container containing

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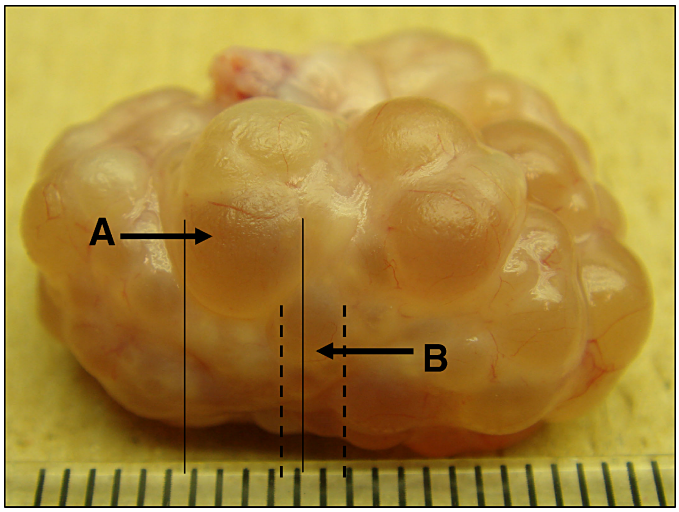

FIG. 1. An example of porcine ovarian follicles used in the experiment. Arrows indicate large follicle (**A**) (~4.5 mm, delineated in solid lines) and small follicles (**B**) (~2 mm; delineated in dashed lines).

sterile 1× PBS at 37°C within 2 h postslaughter. Oocyte cumulus complexes (COCs) were aspirated from small (1–3 mm in diameter), and large (4–6 mm) follicles with a 10-ml syringe equipped with an 18-gauge needle. The follicle diameters were measured with a ruler when the COC was aspirated for the first three isolations. After that we estimated follicle diameter based on experience. Figure 1 shows a representative image of large and small follicles used in the experiments. Oocytes with a uniform ooplasm and granulosa cell mass were placed in tissue culture medium TCM199 (GibcoBRL) with HEPES (Sigma) by a narrow-bore pipette. Under a dissection microscope, COCs were washed three times with culture medium. The COCs isolated were divided into three groups of 50 COCs each: COCs from large follicles, and COCs from small follicles that were randomly divided into two groups. One GOC group from small follicles was first cultured in oocyte growth medium: TCM-199 supplemented with 0.08 IU/ml FSH (Sigma), 0.08 IU/ml LH (Sigma), 5 μl/ml ITS (insulin [5 μg/ml], transferrin [3 μg/ml], selenium [3 ng/ml]; Invitrogen, catalogue number 41400–045), 100 μg/ml L-ascorbic acid (Sigma), 0.57 mM cysteine (Sigma), 10 ng/ml EGF (Sigma), 0.1% polyvinyl alcohol (PVA; Sigma), 3.05 mM D-glucose (Sigma), 0.91 mM sodium pyruvate (Sigma), 10 μg/ml streptomycin sulfate, 75 μg/ml penicillin G, and 10% (v/v) porcine follicular fluid (aspirated from large follicles [4–6 mm], centrifuged twice [5000 × *g* for 15 min each], and stored at −80°C until used) at 38.5°C with 5% CO_2 in air for 24 h. They then were cultured in the maturation medium (TCM-199 supplemented with 20 IU/ml FSH, 20 IU/ml LH, 0.57 mM cysteine, 10 ng/ml EGF, 0.1% polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 10 μg/ml streptomycin sulfate, 75 μg/ml penicillin G, and 10% porcine follicular fluid) for another 20 h. This group is denoted as small follicle-derived oocyte growth and maturation group (SGM). The other group of COCs from small follicles and the COCs from large follicles, denoted SM and LM, respectively, were cultured directly in the maturation medium after isolation at 38.5°C with 5%CO_2 in air for 44 h. After maturation, cumulus cells were removed from COCs by gentle vortexing in 0.1% hyaluronidase (Fisher) in TCM 199.

Assessment of Oocyte Nuclear Maturation

To determine the rate of oocytes that underwent GVBD, COCs were collected after 24 h in the maturation or growth medium. After cumulus cells were removed and washed, oocytes were mounted on slides, fixed in an acetic-ethanol solution (1:3 v/v; Fisher), and stained with 1% aceto-orcein (Fisher). GVBD was classified as described by Motlik and Fulka [15]. Maturation of oocytes to MII at 44 h of culture was scored based on the presence of the first polar body. Each treatment was repeated five times.

Parthenogenetic Embryo Generation and Culture

After 44 h of IVM oocytes were washed three times in TCM199 supplemented with HEPES and were activated electrically as described previously [16]. Briefly, oocytes were placed between two platinum electrodes (1 mm apart) and covered with 1 ml fusion medium consisting of 0.001 mM $CaCl_2$ (Sigma), 297 mM mannitol (Calbiochem), 0.5 mM $MgCl_2$ (Sigma), and 0.1% BSA (fraction V; Sigma). The oocytes then were stimulated with direct current pulses at 2.6 kV generated by a BTX ElectroCell 2001 manipulator (BTX Instrument Division, Holliston, MA). After activation, embryos were washed twice with North Carolina State University 23 (NCSU-23) medium and were cultured in NCSU-23 medium supplemented with 4 mg/ml BSA (Fisher) and overlaid with light mineral oil (Sigma) for 7 days to observe preimplantation embryo development. A total of 750 embryos were analyzed (250 per treatment group) in five independent experiments.

In Vitro Fertilization and Embryo Culture

Denuded MII oocytes were washed three times in TCM199 containing HEPES and once in fertilization medium containing 60 μM glucose, 34 μM sodium citrate, 12.4 μM EDTA, 17 μM citric acid, 54 μM trizma base, and 1 mM caffeine-sodium benzoate (Sigma). Semen was collected from a boar and washed twice in fertilization medium. Approximately 50 oocytes per group were incubated in fertilization medium with sperm at 38.5°C for 5–6 h. Then, oocytes were washed three times in NCSU-23 and transferred to NCSU-23 supplemented with 4 mg/ml BSA and covered with light mineral oil. Embryos were cultured for 7 days in vitro. Cleavage rate was scored 48 h after in vitro fertilization (IVF) and is expressed as embryos with ≥2 cells/MII oocytes × 100. Eight-cell embryo rate is expressed as embryos with 8 cells/MII oocytes × 100 at 72 h after IVF. Blastocyst rate is expressed as blastocysts/cleavage embryos × 100 at 7 days after IVF. A total of 600 embryos were analyzed (200 per treatment group) in four independent experiments.

Western Blotting

A total of 200 oocytes from each group posttreatment were lysed in lysis buffer containing 50 mM Tris (pH 7.5; Invitrogen); 150 mM NaCl (Fisher); 1 mM EDTA (Fisher); 0.5% Nonidet P-40 (Sigma); 1 mM sodium orthovanadate (Sigma); 1 mM NaF (Sigma); 0.75 mm phenylmethylsulfonylfluoride (Fisher); 15% glycerol (Sigma); and 10 μg/ml each of aprotinin (Sigma), pepstatin (Sigma), and leupeptin (Sigma), as described previously [17]. Lysed samples were boiled for 3 min and centrifuged for 3 min at 13 000 rpm. The supernatant containing the soluble protein was subjected to 10% SDS-polyacrylamine gel electrophoresis (SDS-PAGE). The proteins then were transferred to polyvinylidine difluoride (PVDF) membranes that were subsequently blocked overnight in 5% skim milk powder at 4°C before antibody detection. Membranes were incubated with primary antibodies (mouse anti-cyclin B1, monoclonal; BD PharMingen, San Diego, CA, clone GNS-11) diluted in 1× PBS, 0.1% Tween 20, and 5% skim milk powder for 1 h at room temperature, followed by incubation with secondary antibody (anti-mouse IgG horseradish peroxidase; Cell Signaling Technology) for 1 h at room temperature. Proteins were detected by the enhanced chemiluminescence (ECL) Advance Western Blotting Detection Kit (Amersham). For GAPDH detection, membranes were stripped in a stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 30 min. Membrane blocking and antibody incubation were repeated as described above with mouse anti-GAPDH (1:20 000; ABCAM) for normalization of protein loading. Densitometry analysis of band intensities was performed using GeneTools (Syngene, Frederick, MD). The experiment was repeated three times.

Reverse Transcription and Real-Time PCR

Individual embryos at the eight-cell stage were lysed in 8 μl lysis buffer containing 2 U/μl porcine RNase Inhibitor (Amersham,Piscataway, NJ) and 5 mM dithiothreitol (Invitrogen) by boiling for 1 min, followed by vortexing for 2 min each. Lysed embryos were stored at −80°C until use. DNase I treatment then was performed according to the manufacturer's instructions (Invitrogen). Reverse transcription (RT) was carried out in a total volume of 20 μl using 2.5 μM random hexamer primers (Applied Biosystems) to obtain the widest array of cDNA. The reaction mixture consisted of 1× First Strand Buffer (Invitrogen), 0.2 mM dNTP (Invitrogen), 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen), and the entire DNase-treated lysate. The RT reaction was performed at 65°C for 10 min and at 37°C for 50 min, followed by a 15-min step at 70°C to inactivate the MMLV. A negative control was included in which reverse transcriptase was omitted during the RT reaction (data not shown).

To analyze mRNA expression from these embryos, real-time PCR was performed following the RT reactions as described in Zhu et al. [18]. Complementary DNA was amplified using SYBR Green PCR Mix (Qiagen), which contained $MgCl_2$, dNTP, and HotStar Taq polymerase, and the Smart Cycler (Cepheid). In each of the reaction, 2.5 μl of each of the DNase I-treated cDNA prepared from the previous RT step was added to a total volume of 25 μl mastermix, which included 0.3 μM of each sequence-specific forward and reverse primer, 12.5 μl SYBR Green mix, and 8.5 μl RNase-free water

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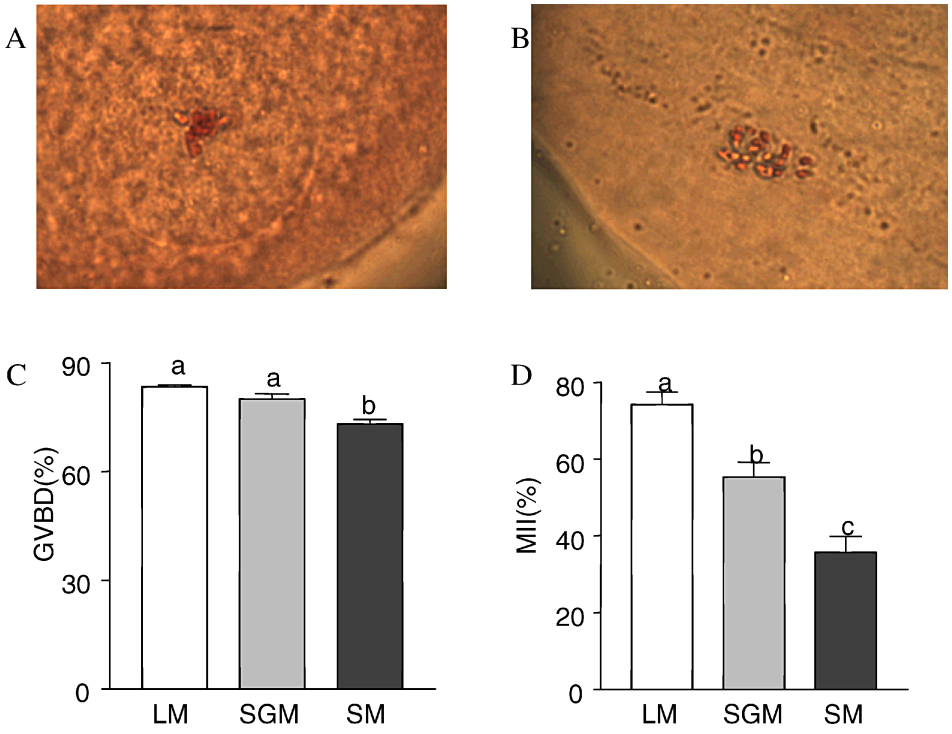

FIG. 2. Growth-maturation system improved the nuclear maturation competence of oocytes isolated from small follicles in vitro. Typical morphology of GV (**A**) and GVBD (**B**) stage oocytes. **C**) Percentage of GVBD oocytes derived from different follicle sizes in the different culture systems after 24 h of in vitro culture. **D**) Percentage of MII oocytes derived from different follicle sizes in the different culture systems after 44 h of in vitro culture. Data represent the mean ± SEM of five independent experiments; different superscripts indicate significant difference ($P < 0.05$). Original magnification ×400 (**A** and **B**).

(Invitrogen). The sequences for the forward and reverse primers were described previously [18] and were: histone 2 H2aa (*HIST2H2AA*), previously known as H2A histone family, member O (*H2AF0*), F: 5′-gtggcaaacaaggaggaaag-3′, R: 5′-atgcgggtcttcttgttgtc-3′; DNA-methyltransferase 1 (*DNMT1*), F: 5′-aggtgaggacatgcagcttt-3′, R: 5′-aacttgttgtcctccgttgg-3′; fibroblast growth factor receptor 2 (*FGFR2*), F: 5′-attctgtgccggatgaagac-3′, R: 5′-ggtgttggagttcatggagg-3′; Pou domain class 5 transcription factor 1 (*POU5F1*), previously known as *OCT4*, F: 5′-cgaagctggacaag- gagaag-3′, R: 5′-aaagtgagccccacatcg-3′; and tumor susceptibility gene 101 (*TSG101*), F: 5′-aatatgcctgtggtgctg-3′, R: 5′-tgacgtgaataagccccaac-3′. To ensure the absence of contamination, an additional negative control in which cDNA was replaced with water was included upon each preparation of real-time mastermix. To confirm the specificity of the product amplified, melt curve analysis was performed, and the RT real-time PCR products were subjected to electrophoresis to verify the size of the product. PCR amplification products were sequenced to confirm identity.

Gene expression was quantified by a comparative Ct method [17]. *HIST2H2AA* was amplified for each embryo to confirm the presence of RNA and was used as an internal control to determine the relative transcriptional levels of the target genes in each sample. Real-time PCR data were analyzed using the $2^{-[\text{delta}][\text{delta}]\text{Ct}}$ method [19]. The Ct value for each gene was determined at a threshold of 30 fluorescence units [20]. The real-time PCR efficiencies were comparable between *HIST2H2AA* and the genes of interest ($R = 0.9889$). The experiment was repeated three times, with each replicate consisting of 6–8 individual embryos from each treatment group.

Statistical Analysis

The percentages of oocytes reaching the GVBD, MII, cleavage, eight-cell, and blastocyst stages, as well as Western blot results were analyzed by one-way ANOVA. Significant results from ANOVA were further analyzed by Tukey test. Results were considered significant at $P < 0.05$ for all tests. Normalized Ct values from real-time PCR were analyzed by Kruskal-Wallis test, and Dunn multiple comparison tests were performed to compare the values of LM, SGM, and SM groups with each other. Each experiment was repeated at least three times, and data represent the mean ± SEM of all repeats.

RESULTS

Comparison of the Growth-Maturation and Conventional Direct- Maturation Systems on Oocyte Nuclear Maturation

To determine whether the growth-maturation system is capable of improving the nuclear maturation competence of oocytes isolated from small follicles in vitro, the following experiments were performed. COCs from small follicles were cultured first in oocyte growth medium for 24 h and then in oocyte maturation medium for 20 h (growth-maturation system; SGM group). In the conventional direct maturation system, COCs from small follicles (SM group) were cultured directly in oocyte maturation medium for 44 h as described previously [16, 18]. As a control, COCs from large follicles were cultured using the conventional system to reach mature stage (LM group). At 24 h of culture, typical morphologies of different GV and GVBD stages are shown in Figure 2, A and B, respectively. As shown in Figure 2C, a slightly but significantly higher percentage of small follicle-derived oocytes in the SGM group reached GVBD stages compared with those from the SM group (80% vs. 73%, $P < 0.01$), whereas no significant difference was observed between the percentages of oocytes from the LM and SGM groups.

At 44 h of culture, further progress of nuclear maturation to the MII was examined using extrusion of the first polar body as an index. While the percentage of oocytes that progressed to MII in both the SM and SGM groups was lower than that from the LM group, a significantly higher percentage (55%) of the SGM group oocytes progressed to the MII stage compared with those from the SM group (36%; $P < 0.01$; Fig. 2D).

Growth-Maturation System Enhanced Cyclin B1 Expression in Ooctyes Derived from Small Follicles

The expression of cyclin B1 has been described as a marker to reflect cytoplasmic maturation [9, 16]. To investigate whether the growth-maturation system also influences the cytoplasmic maturation of oocytes from small follicles, cyclin B1 protein levels were analyzed using Western blotting after 44 h of culture. As shown in Figure 3A, the 62-kDa cyclin B1 protein was detectable in both large follicle- and small follicle-derived oocytes of both culture systems. Densitometry analysis indicated that the level of cyclin B1 was 2.04-fold higher in small oocytes from the SGM group compared with the SM group ($P < 0.05$; Fig. 3B) and was comparable to that of oocytes from the LM group.

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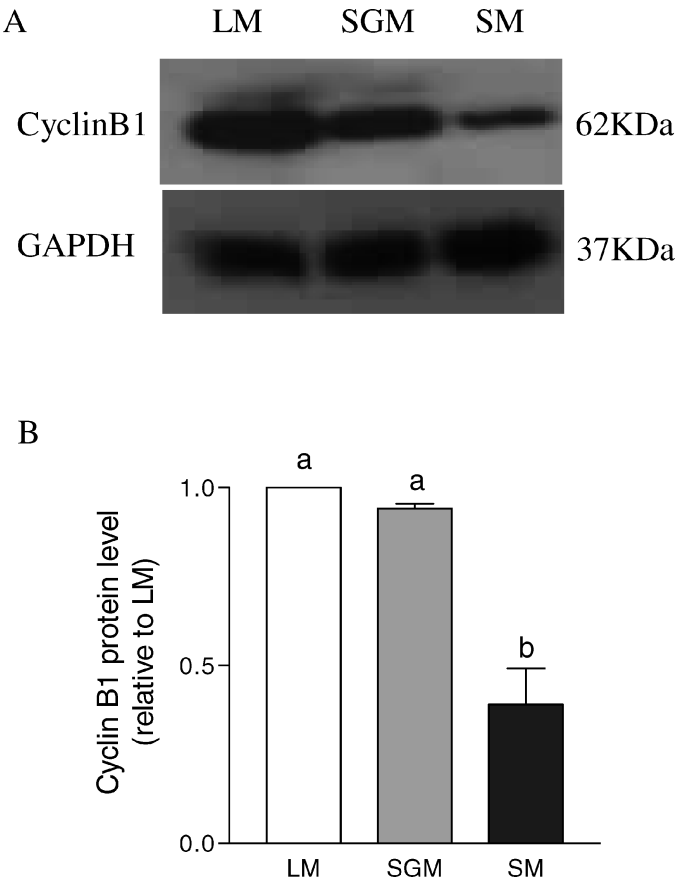

FIG. 3. Influences of in vitro systems on cyclin B1 protein expression revealed by Western blot. **A**) A representative immunoblot showing the detection of cyclin B1 and GAPDH in LM, SGM, and SM group oocytes. **B**) Relative expression level of cyclin B1 normalized by GAPDH. Data are the mean ± SEM of three experiments; different superscripts indicate significant difference ($P < 0.05$).

Small Follicle-Derived Oocyte Developmental Potential Was Improved by the Growth-Maturation System

Cytoplasmic maturation and, thus, the quality of the oocytes are known to play a key role in determining the developmental potential of preimplantation embryos. To compare the capabilities of the growth-maturation system and the conventional direct maturation system in supporting oocyte cytoplasmic maturation of small follicle-derived oocytes, the preimplantation development of parthenogenetic and in vitro fertilization embryos was monitored. Cleavage rates (two-cell stage) and eight-cell stages were assessed at 24 h and 72 h after activation and fertilization, and the percentage of embryos that developed to the blastocyst stage was determined on Day 7 of culture. As shown in Figure 4A and Figure 5A, no statistical difference in cleavage rate (cleavage/MII oocyte) between the SGM and SM groups was observed**.** The percentages of embryos reaching the eight-cell (eight-cell/MII) and blastocyst (blastocyst/MII) stages were significantly higher in the SGM group compared with the SM group (Fig. 4, B and C, and Fig. 5, B and C; $P < 0.05$). Although there were increases of 7.3- and 9.0-fold in the blastocyst rate of parthenogenetic and in vitro-fertilized embryos over the conventional direct maturation system, the developmental potential of small follicle-derived oocytes from growth-maturation system culture was still significantly lower than that of large follicle-derived oocytes (Fig. 4C and Fig. 5C; $P < 0.05$).

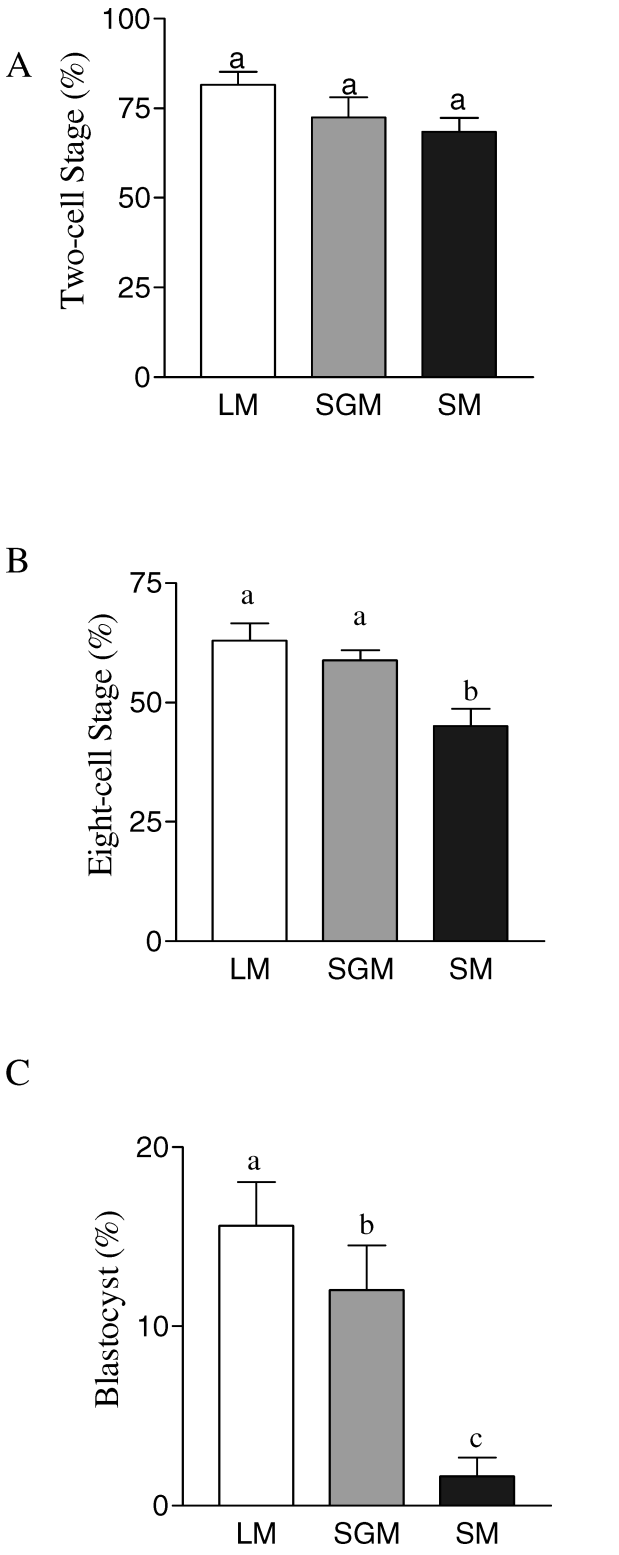

FIG. 4. Small follicle-derived oocyte developmental potential of parthenogenetic embryos was improved by the growth-maturation system. **A**) Cleavage rate (cleavage/MII oocyte) at 24 h. **B**) Percentage of eight-cell-stage embryos (eight-cell embryos/MII) at 72 h. **C**) Percentage of embryos that developed to the blastocyst (blastocyst/MII) stage at 7 days after activation. A total of 750 embryos were analyzed (250 per treatment group). Data are the mean ± SEM of five experiments; different superscripts indicate significant difference ($P < 0.05$).

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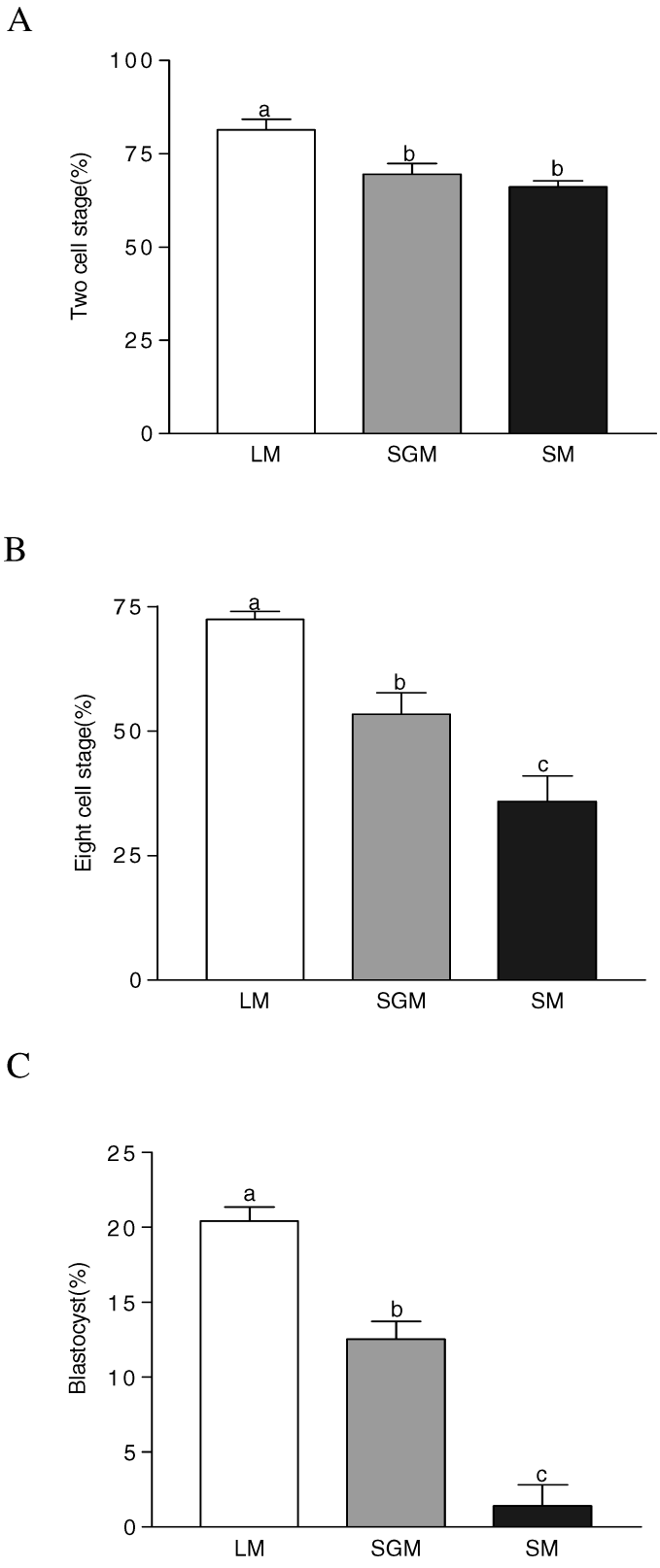

FIG. 5. Small follicle-derived oocyte developmental potential of IVF embryos was improved by the growth-maturation system. **A**) Cleavage rate (cleavage/MII oocyte) at Day 2. **B**) Percentage of eight-cell-stage embryos (eight-cell embryos/cleavage) at Day 4. **C**) Percentage of embryos that developed to the blastocyst (blastocyst/cleavage) stage at 7 days after fertilization. A total of 600 embryos were analyzed (200 per treatment group). Data are the mean ± SEM of four experiments; different superscripts indicate significant difference ($P < 0.05$).

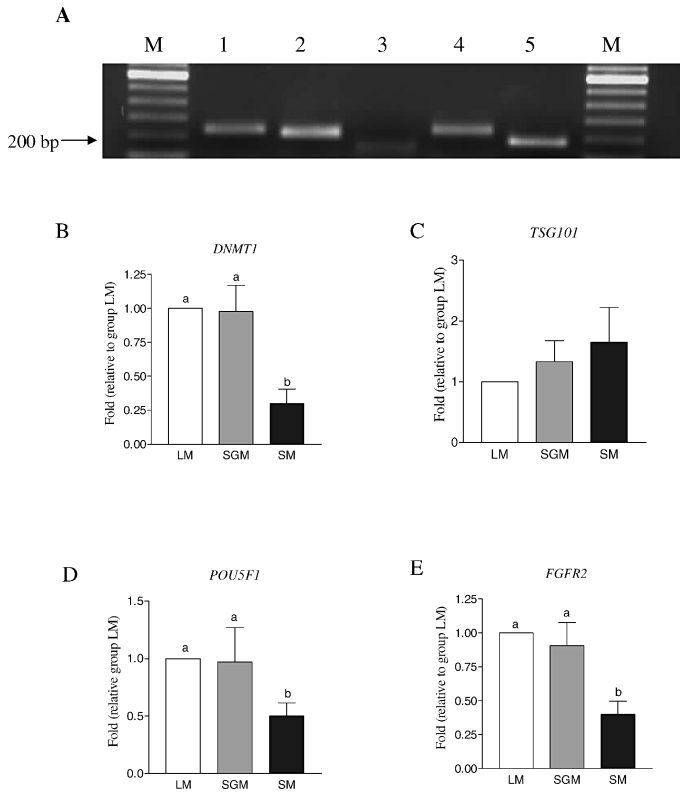

FIG. 6. Expression of *DNMT1*, *FGFR2*, *POU5F1,* and *TSG101* in SGM and SM groups relative to LM group. **A**) Representative agarose gel image of real time RT-PCR amplification of *HIST2H2AA* (226 bp; lane 1), *DNMT1* (213 bp; lane 2), *FGFR2* (121 bp; lane 3), *POU5F1* (230 bp; lane 4), and *TSG101* (179 bp; lane 5) from an individual eight-cell embryo**.** Relative expression of *DNMT1* (**B**), *TSG101* (**C**), *POU5F1* (**D**), and *FGFR2* (**E**). Data are the mean ± SEM of three experiments with 6–8 embryos per group per experiment. Different letter subscripts indicate statistical differences ($P < 0.01$).

Differential Expression Pattern of Developmental Important Genes from SGM and SM Groups

The expression of four developmentally important genes was investigated in in vitro-produced eight-cell embryos. A similar gene expression pattern was observed in *DNMT1*, *POU5F1*, and *FGFR2* (Fig. 6, B, D, and E, respectively), for which the transcript expression levels of these three genes in the SGM group resembled those in the LM group. The expression levels of these three genes in the SM group were significantly lower than those in both the LM group and the SGM group ($P < 0.05$). However, the level of *TSG101* transcript in the LM, SGM, and SM groups was not statistically different ($P > 0.05$; Fig. 6C).

DISCUSSION

O'Brien et al. demonstrated the in vitro development of oocyte of primordial follicles to the fully competent stage, which resulted in live offspring using the two-step (ovarian organ and then oocyte granulosa cell complex) culture system [6, 7]. Their protocol laid the foundation for developing an in vitro culture system to support follicle development. Our protocol was designed using a strategy similar to their second-step culture, with modifications made according to the species and follicle stage differences. Porcine follicles that are <3 mm in diameter are early antral follicles. The majority of the oocytes isolated from these follicles were capable of re-

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entering meiosis, such that over 70% of them reached GVBD stage after 24 h of in vitro culture (Fig. 2C). However, they were still at their last phase of growth. At this stage, FSH supported further growth of follicles and induced the expression of LH receptor (for review, see Gougeon [21] and Zeleznik [22]). Using hypophysectomy and GnRH antagonist, it was suggested that FSH is essential for the growth of porcine follicles with diameters ranging from 1.1 to 2 mm, whereas LH is important for the growth of follicles that are >2 mm of porcine ovaries [23]. When follicles are developed from the early to the late antral stage, FSH and LH stimulate steroid production. Steroids such as estradiol can promote oocyte nuclear maturation [24]. One mechanism was suggested recently by which LH can stimulate the activity of metalloprotein-mediated cleavage of membrane-bound EGF moieties, allowing soluble EGF to bind to granulosa cells to stimulate steroid production [25]. Due to the dual roles of FSH (which facilitates follicle growth and granulosa cell steroidogenesis) and the oocyte nuclear maturation-promoting role of LH, a low concentration of the gonadotropins (1/250 of the concentrations of FSH and LH used in the IVM medium) was included in our growth medium. Our data demonstrated that placing the small follicle-derived COCs in this more growth-supporting and less maturation-promoting environment in the first phase of in vitro culture allows better oocyte growth compared with the direct maturation-stimulating system. In a rodent system it was shown that a combination of FSH and insulin negatively influenced the potential of oocytes to complete preimplantation development. When FSH, LH, and EGF were removed from the last phase of in vitro oocyte–granulosa cell complex culture before IVM, the developmental competence was improved significantly [7]. Our earlier experiments showed that exclusion of the gonadotropins and EGF in the oocyte growth medium resulted in only 1% of embryos being capable of developing to the blastocyst stage (data not shown). This difference may reflect species-specific requirements for the last phase of oocyte growth in vitro.

It is well known that oocyte growth is transcription and protein synthesis dependent, in which factors that support meiosis, fertilization, and early embryo development are produced and stored. Insulin thus was included in the medium in our growth-promoting phase of in vitro culture because of its known role in stimulating transcription and protein synthesis in general [26]. Compared with the IVM medium, another component added to the growth medium was the L-ascorbic acid. It is generally understood that oxygen level is higher in an in vitro culture environment compared with an in vivo one. Oxidative stress is detrimental to granulosa cells and oocytes. As an important antioxidant, L-ascorbic acid has been shown to suppress apoptosis in rat and mouse follicles in vitro [27, 28]. Moreover, inclusion of ascorbic acid 2-*O*-alpha glucoside, a stable ascorbate derivative in IVM medium, improved porcine oocyte cytoplasmic maturation and enhanced developmental potential after in vitro fertilization [29]. L-ascorbic acid in our growth medium may have helped to protect cumulus cells from apoptosis and thus support oocyte cytoplasmic maturation.

Oocyte maturation involves the activation of various signal transduction pathways that converge to activate maturation promoting factor (MPF), which is composed of cyclin B and Cdc2 kinase [30]. The amount of cyclin B is the principal factor of MPF activity [31]. In the pig, cyclin B protein accumulates during interphase, reaches the highest level during M phase, and is subsequently degraded, which results in the inactivation of MPF and the exit of the oocyte from metaphase [32]. Microinjection of cyclin B1 antisense RNA into porcine oocytes prevented arrest at the second meiotic metaphase, whereas antisense cyclin B2 RNA microinjection had almost no effect on oocyte maturation. It thus was suggested that of the two subforms of cyclin B, cyclin B1 is the principal molecule involved in the regulation of mammalian oocyte maturation [33]. The importance of cyclin B levels in oocyte maturation also was shown in a study in which the inhibition of porcine oocyte maturation by dexamethasone was found to be associated with decreased cyclin B but not with p34cdc2 levels [34]. Cyclin B1 levels have been suggested as important markers for evaluating cytoplasmic maturation [9]. Moreover, oocyte cytoplasmic maturation also is reflected by the potential of early embryo development, especially to blastocyst stage. Studies have shown that the complete growth of oocytes is critical in developing competence to complete the two cells to blastocyst progression. It was reported previously that the majority of the oocytes isolated from small antral follicles and matured in vitro were not capable of completing preimplantation development upon fertilization, even though they were able to complete nuclear maturation and cleavage [8, 9, 35]. In the present study, cyclin B1 was detected at a higher level and the blastocyst rate increased by 7-fold in the SGM group compared with the SM group. This observation indicated that the oocyte growth-maturation system facilitated the final stage of oocyte growth and, thus, resulted in better oocyte cytoplasmic maturation compared with the conventional direct oocyte maturation system.

The functions of *DMNT1*, *TSG101*, *POU5F1,* and *FGFR2* are essential to the survival of the preimplantation embryo, as disruption of their expression results in severe early developmental defects and lethality [36–39]. Proper temporal and spatial expression of these genes may reflect the developmental potential of the embryos. In the current study we monitored the expression of these gene transcripts in early embryos from SGM, SM, and LM groups. Gene expression was analyzed at the eight-cell stage, the earliest stage to detect zygotic gene expression in the pig, with the maternal to zygotic transition occurring at the four-cell stage. At first glance, the finding that zygotic gene expression pattern was influenced by the in vitro oocyte culture environment was somewhat surprising. However, it is conceivable that the methylation status of the maternal genome and the factors governing this process or the subsequence reprogramming process upon activation are affected by oocyte cytoplasmic maturation. It was suggested previously that an inadequate culture could result in both poor oocyte quality and decreased development of embryos (for review, see Wrenzychi et al. [40]). The fact that embryos from the SGM group expressed *DNMT1, FGFR,* and *POU5F1* at the same levels as those from the LM group and significantly higher than those of the SM group suggests that the growth-maturation system may have provided higher quality oocytes to allow the expression of developmentally important genes at the early embryo stage. On the other hand, no statistical difference was observed between the SGM and SM groups for the *TSG101* transcript level.

Although substantial improvements were achieved in generating more competent small follicle-derived oocytes with the current growth-maturation system, the developmental potential of these embryos is still significantly lower compared with that of large follicle-derived oocytes. Future studies in identifying endocrine/paracrine/autocrine factors and the time window for these factors to impact oocyte growth and maturation may further promote optimization of a system that fully supports in vitro oocyte development in large animals.

Oocyte maturation in vitro in clinical IVF and possibly future therapeutic cloning is gaining more attention due to its advantages in providing more oocytes and bypassing the need

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for exogenous gonadotropin-induced ovulation in vivo. The feasibility of IVM of oocytes from small antral follicles is being explored [52]. As a placental livestock species, pigs have developed numerous evolutionary similarities with humans [51]. For example, the timing of meiosis in porcine oocytes resembles meiosis in the human oocyte more closely than in the mouse. In the human and the pig GVBD occurs approximately 20 h following LH stimulation, and oocytes further progress to MII in another 20 h. In contrast, in the mouse GVBD occurs 2–5 h after the LH peak, and oocytes reach MII 7–8 h after this point. Our oocyte growth-maturation system may provide another starting point for further development of an in vitro system that would produce more usable oocytes from small follicles for agriculture, human assisted reproduction, and therapeutic cloning.

ACKNOWLEDGMENTS

We would like to thank Paul Dyce for his excellent technical assistance and discussions. We also thank the staff at the Conestoga Farm Fresh Pork for assistance in collecting porcine ovaries.

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