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# Synchronization of Meiosis in Porcine Oocytes by Exposure to Dibutyryl Cyclic Adenosine Monophosphate Improves Developmental Competence Following In Vitro Fertilization[1]

**Hiroaki Funahashi,[3] Thomas C. Cantley, and Billy N. Day[2]**

*Department of Animal Sciences, University of Missouri-Columbia, Columbia, Missouri 65211*



## ABSTRACT

**The effect of stage of maturation of the germinal vesicle of porcine oocytes at the time of in vitro maturation on subsequent developmental competence was examined. A large variation exists in the germinal vesicle morphology of oocytes at the time of collection of cumulus-oocyte complexes (COCs) and after culture in the absence of dibutyryl cAMP (dbcAMP) for 20 h. However, the morphology of the germinal vesicle was synchronized to a specific stage after culture in the presence of 1 mM dbcAMP for 20 h. There was no difference in germinal vesicle breakdown rate (total mean, 75.0 ± 5.4%) or in maturation rate (total mean, 82.1 ± 2.1%) at 28 and 44 h of culture, respectively. However, differences in meiotic progress of oocytes were observed ($p < 0.05$) at 36 h of culture when COCs were exposed to dbcAMP for the first 20 h of maturation, as compared to controls. The incidence of embryos that developed to the blastocyst stage after in vitro fertilization was higher ($p < 0.05$) when COCs were exposed to dbcAMP (21.5 ± 2.5%) as compared to controls (9.2 ± 1.6%). After transfer of experimental embryos to four recipient gilts, the three pregnant recipients delivered 19 live piglets. These results indicate that exposure of COCs to dbcAMP for the first 20 h of culture for maturation increases the homogeneity of oocyte nuclear maturation and improves the efficiency of in vitro production of swine embryos.**

## INTRODUCTION

For porcine embryo production in vitro, extensive use has been made of cumulus-oocyte complexes (COCs) with uniform ooplasm and a compact cumulus cell mass that have been collected from antral follicles of slaughtered prepubertal gilts. However, although we have collected COCs from antral follicles 3–6 mm in diameter on the surface of ovaries from an abattoir for in vitro production of porcine embryos, the size of follicles that are selected for collection of COCs has varied both between and within investigations [1]. Further, oocyte maturation has been assessed by only the integrated number of oocytes that reached the metaphase II (MII) stage against the total number of those examined, whereas there appears to be a large variation in the rate of meiotic progression of porcine oocytes in vitro [1, 2]. In normal mature gilts, there are dramatic changes in the number and size of follicles during the period from Day 16 to 21 of the estrous cycle [3–5]. The nuclear morphology of oocytes in developed antral follicles was drastically changed soon after the injection of hCG at 72 h after an injection of eCG [6]. Recently, a transient increase in cAMP concentrations of in vivo oocytes was observed around 12 h after hCG injection and was also induced by LH during the first 12 h of in vitro maturation (IVM) in the presence of follicular cells [7]. Since dibutyryl cAMP (dbcAMP) inhibits meiotic resumption reversibly [7, 8], exposure of COCs to dbcAMP for the early period of IVM may synchronize the oocytes to a specific germinal vesicle (GV) stage and improve early embryonic development following in vitro fertilization (IVF). The present studies were designed to determine whether exposure of COCs to dbcAMP for the first 20 h of culture for maturation would synchronize nuclear morphology of porcine oocytes during IVM and improve early embryonic development following IVF.

## MATERIALS AND METHODS

### *Culture Media*

The medium used for COC collection and washing was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM $KH_2PO_4$, 10 mM sodium lactate, 0.5 mM $MgCl_2/6H_2O$, 2 mM $CaCl_2/2H_2O$, 10 mM HEPES, 0.2 mM sodium pyruvate, 12 mM sorbitol, 25 mg/ml gentamicin, 65 mg/ml penicillin G, and 0.1% polyvinyl alcohol. The maturation medium (OMM37) used was BSA-free North Carolina State University 37 medium [9] supplemented with 50 μM β-mercaptoethanol, 0.6 mM cysteine, 5 mg/ml insulin, 10% (v:v) porcine follicular fluid, and hormonal supplements (10 IU/ml eCG and 10 IU/ml hCG). The basic medium used for IVF was modified Medium 199 (mM199) [10]. All media were equilibrated at 39°C in an atmosphere of 5% $CO_2$ in air for a minimum of 3 h before incubation of oocytes. Porcine follicular fluid was prepared as described previously [10].

### *Preparation and Culture of COCs*

Ovaries from prepubertal gilts were collected at a local abattoir. Transportation of ovaries to the laboratory was carried out at 23–27°C in physiological salt solution (0.9% NaCl) supplemented with antibiotic-antimycotic solution (GIBCO BRL, Life Technologies Inc., Grand Island, NY). COCs were aspirated through an 18-gauge needle into a disposable 10-ml syringe from antral follicles (3–6 mm in diameter) on the surface of ovaries, washed three times with modified TL-HEPES-PVA medium, and then collected in a droplet (3 ml) of fresh modified TL-HEPES-PVA medium. Fifty COCs with uniform ooplasm and a compact cumulus cell mass were washed three times with OMM37 and then cultured in 500 μl of OMM37 covered with paraffin oil (light mineral oil; Fisher Scientific, Fair Lawn, NJ) for 20 h at 39°C in an atmosphere of 5% $CO_2$ in air. The complexes were then transferred to 500 μl of OMM37 without hormonal supplements, and cultured for an addi-

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[2]Correspondence: Billy N. Day, University of Missouri-Columbia, Department of Animal Sciences, 159 Animal Sciences Research Center, Columbia, MO 65211. FAX: (573) 884-7827;
e-mail: bill_day@muccmail.missouri.edu

[3]Current address: Okayama University, Faculty of Agriculture, 1-1-1 Tsushima-naka, Okayama 700, Japan.

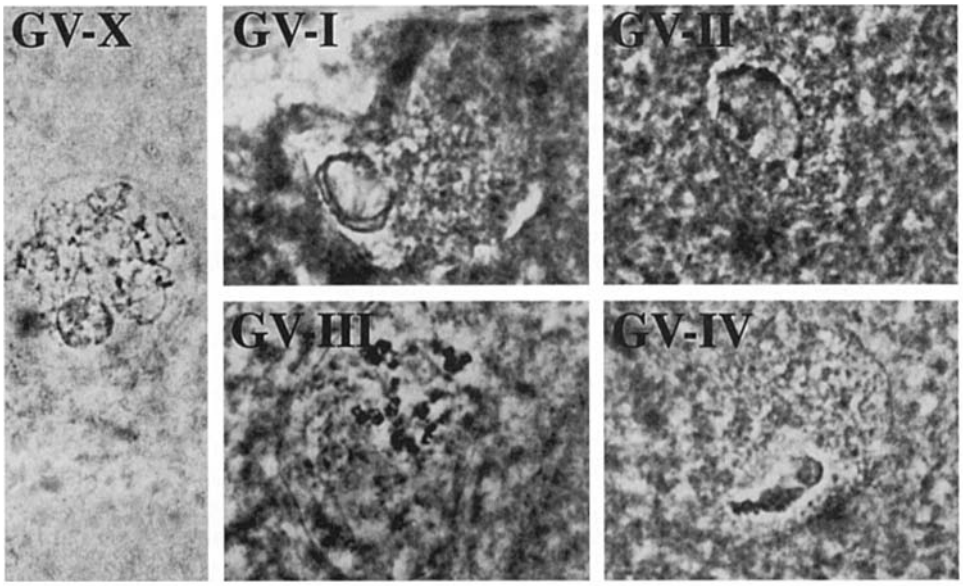


FIG. 1. Categories of the nuclear morphology of porcine oocytes at the GV stage: GV-X, characterized by a nucleolus and filamentous chromatin located throughout the whole area of the GV; GV-I, characterized by a nucleolus and chromatin in the form of a ring or horseshoe around the nucleolus; GV-II, characterized by a nucleolus and chromatin as a ring or horseshoe around the nucleolus but with some well-stained clumps localized mainly around the ring- or horseshoe-like chromatin; GV-III, characterized by an irregular network of filamentous bivalents in the whole area of the GV and no nucleolus; and GV-IV, characterized by diakinesis chromatin. These categories were modified from those by Motlik and Fulka [13].

tional 24 h [11, 12]. After culture, oocytes were pipetted with 0.1% (w:v) hyaluronidase in OMM37 without hormonal supplements and stripped of cumulus cells.

### *IVF and Culture for Early Embryonic Development*

Sperm-rich fractions of an ejaculate were collected from a boar, kept at 20°C for 16 h, and washed three times with physiological salt solution (0.9% NaCl, pH 7.2) supplemented with 1 mg/ml BSA, 63 mg/L penicillin-G, and 50 mg/L streptomycin sulfate. Spermatozoa were preincubated at $2 \times 10^8$ cells/ml in mM199 supplemented with 1% (v:v) porcine follicular fluid and 4 mg/ml BSA at pH 7.8 for 90 min. Fifty denuded oocytes were cocultured with spermatozoa at a final concentration of $1 \times 10^6$ cells/ml in a 100-μl droplet of mM199 supplemented with 5 mM caffeine sodium benzoate and 4 mg/ml BSA for 6 h at 39°C in an atmosphere of 5% $CO_2$ in air. The eggs were then transferred to a 500-μl droplet of NCSU23 medium [9] supplemented with 5 mg/ml insulin, and cultured for 6 days at 39°C in an atmosphere of 5% $CO_2$ in air. At 10 h after insemination, some oocytes were fixed and stained with 1% (v:v) orcein to examine the incidence of oocytes penetrated normally by assessment of monospermy and male pronuclear formation.

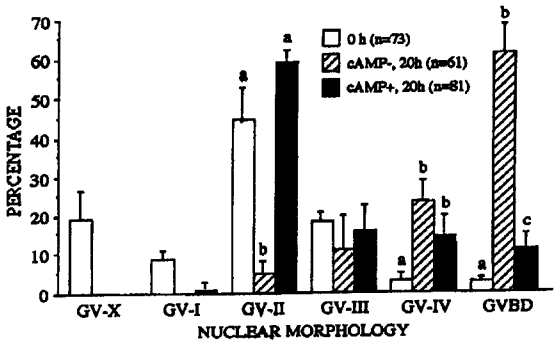


FIG. 2. Distribution (mean ± SEM) of the nuclear morphology of porcine oocytes before (0 h) and after culture in the absence (cAMP−) or presence (cAMP+) of 1 mM dibutyryl cAMP in the maturation medium for 20 h. The number of oocytes examined in each group is given in parentheses. Different letters above the bars denote statistically significant differences ($p < 0.05$).

### *Experimental Design*

*Experiment 1.* COCs were cultured in the absence or presence of 1 mM dbcAMP in OMM37 for 20 h. At 0 and 20 h of culture for maturation, nuclear morphology of oocytes at the GV stage was classified into five categories (Fig. 1) modified from those of Motlik and Fulka [13].

*Experiment 2.* After culture in the absence or presence of 1 mM dbcAMP in OMM37 for 20 h, COCs were continued in culture in the absence of dbcAMP in OMM37 without gonadotropins for 24 h. Meiotic progress of oocytes was examined at 28, 36, and 44 h of culture for maturation.

*Experiment 3.* After COCs were cultured for maturation for a total of 44 h, as described in experiment 2, denuded oocytes were cocultured with spermatozoa for 6 h and then cultured in modified NCSU37 for 6 days. In vitro embryonic development to the 2-cell, morula, and blastocyst stages was assessed at 48 h, 5 days, and 6 days after insemination, respectively. In addition, 24–36 h after insemination, 40 cleaved embryos were transferred surgically to the ampullar section of an oviduct of four recipient gilts on Days 2–4 (one on Day 2, two on Day 3, and one on Day 4).

### *Statistical Analysis*

Statistical analyses of samples from three replicate trials for treatment comparisons were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program. All percentage data were subjected to arc sine transformation before statistical analysis. Data were expressed as mean ± SEM. Probability of $p < 0.05$ was considered to be statistically significant.

## RESULTS

### *Experiment 1*

The distribution of nuclear morphology of oocytes at 0 h and at 20 h when cultured in the absence or presence of 1 mM dbcAMP in OMM37 is shown in Figure 2. At the start (0 h) of culture, the majority (44.8 ± 7.9%) of the oocytes aspirated were at the GV-II stage followed by the GV-X (18.7 ± 7.4%), GV-III (17.9 ± 2.5%), GV-I (8.1 ± 2.0%), GV-IV (3.1 ± 1.6%), and GVBD (2.2 ± 1.2%)

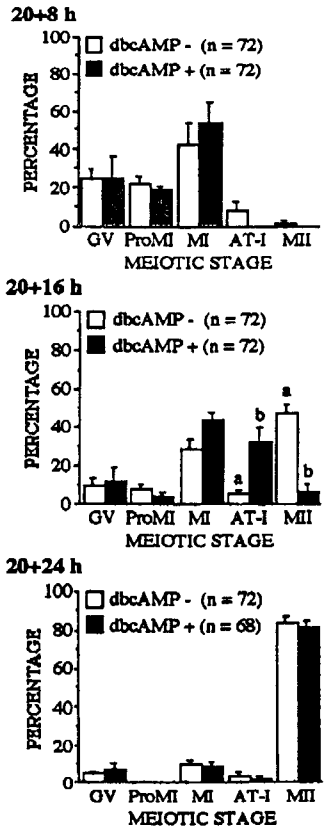


FIG. 3. Distribution (mean ± SEM) of meiotic-stage porcine oocytes at 8-h periods during 24 h of culture after COCs had been cultured in the absence (cAMP−) or presence (cAMP+) of 1 mM dibutyryl cAMP for 20 h. The number of oocytes examined in each group is given in parentheses. Different letters above the bars denote significant differences ($p < 0.05$).

stages. After 20 h of culture, most (58.9 ± 2.7%) of the oocytes cultured in the presence of dbcAMP were arrested at the GV-II stage, but GVBD was observed in more than 61 ± 7.3% of oocytes cultured in the absence of dbcAMP.

### *Experiment 2*

Meiotic progression of oocytes cultured in the absence or presence of dbcAMP for 20 h and then in the control medium for an additional 8, 16, and 24 h is shown in Figure 3. There was no difference in GVBD rate (75.0 ± 4.8% in the absence of dbcAMP vs. 75.0 ± 11.0 in the presence of dbcAMP) or maturation rate (83.6 ± 2.2% in the absence of dbcAMP vs. 80.6 ± 3.7 in the presence of dbcAMP) at 28 and 44 h of culture, respectively. However, the incidence of oocytes at the anaphase-I to telophase-I stages was higher ($p < 0.05$) when cultured in the presence of dbcAMP,

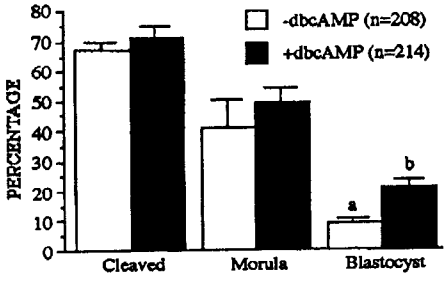


FIG. 4. Early development in vitro of porcine oocytes that were matured in the absence (cAMP−) or presence (cAMP+) of 1 mM dibutyryl cAMP for the first 20 h of maturation culture and then fertilized in vitro. The number of oocytes examined in each group is given in parentheses. Different letters above the bars denote significant differences ($p < 0.05$).

and more oocytes ($p < 0.05$) cultured in the absence of dbcAMP had matured to the MII stage at 36 h of culture (Fig. 3).

### *Experiment 3*

At 10 h after insemination, as shown in Table 1, there was no difference in the incidence of maturation, penetration, monospermy, and male pronuclear formation between oocytes cultured in the absence and those cultured in the presence of dbcAMP for the first 20 h of IVM. However, as shown in Figure 4, the development of IVM eggs to the blastocyst stage after IVF was higher ($p < 0.05$) when COCs had been exposed to dbcAMP (21.5 ± 2.5%) as compared to controls (9.2 ± 1.6). When embryos that were exposed to dbcAMP during the first 20 h of IVM were transferred to four recipient gilts, three recipients, which were on Day 2 and Day 3 of the estrous cycle at embryo transfer, became pregnant, and they farrowed a total of 19 live piglets (mean birth weight: 996.8 ± 99.8 g).

## DISCUSSION

In the present studies, the concentration of dbcAMP was at 1 mM, and the length of exposure of COCs to dbcAMP was 20 h from the start of culture. This protocol was based on the report by Mattioli et al. [7] that the presence of 1 mM dbcAMP for 20 h completely inhibited the meiotic resumption of cumulus-enclosed porcine oocytes which had also been maintained in connection with the everted follicle wall, but that the presence of LH did not inhibit the meiotic resumption. Our objective for the first experiment was to determine whether dbcAMP would cause nuclear activated oocytes to arrest at a specific phase. The results indicate that the presence of 1 mM dbcAMP in medium for the first 20 h of IVM arrested a majority (59%) of the oocytes at the GV-II stage. Since 23% of the oocytes were at stages

TABLE 1. Sperm penetration of porcine oocytes matured in the absence and presence of 1 mM dibutyryl cAMP during the first 20 h of culture for maturation and then fertilized in vitro.*

| | No. of oocytes | | | | | |
|---|---|---|---|---|---|---|
| dbcAMP | Examined | Matured (%) | Penetrated (%)[a] | Monospermy (%)[b] | MPN formed (%)[b] | Fertilized normally (%)[a] |
| − | 72 | 63 (87.5) | 58 (92.1) | 39 (67.2) | 52 (89.7) | 36 (50.0) |
| + | 72 | 67 (93.1) | 65 (97.0) | 41 (63.1) | 57 (87.7) | 37 (51.4) |

[a] Percentage of oocytes matured.
[b] Percentage of oocytes penetrated.
* There were no significant differences between groups ($p > 0.05$).

beyond the GV-II stage at the start of culture for maturation, most of the oocytes before the GV-II stage appeared to be arrested at the dictyate stage of prophase (GV-II) by dbcAMP treatment. However, oocytes at stages beyond the GV-II stage did not appear to be blocked by dbcAMP since the incidence of oocytes at the GV-IV and GVBD stages increased during dbcAMP treatment. It has been reported that exposure of porcine COCs to dbcAMP for 48 h decreased the incidence of GVBD in some but not all oocytes [14]. This observation appears to be consistent with our results. Meiotic maturation of porcine oocytes is also reversibly prevented when dbcAMP is added in combination with follicular cells [7] or testosterone [8, 14]. Further, we recently observed that nuclear morphology of porcine oocytes in vivo matured to the GV-II stage after hCG injection [6]. Similar results have been observed in a previous study by Motlik and Fulka [13]. As reported by Mattioli et al. [7], a transient increase in cAMP concentration of in vivo porcine oocytes follows hCG injection. Therefore, increased intracellular cAMP concentration appears to be associated with the transition of nuclear morphology in porcine oocytes to the GV-II stage or the arrest at the GV-II stage. Decreasing the cAMP concentration would permit oocytes to resume meiosis.

In the second experiment, we demonstrated that meiotic progression of porcine oocytes was more synchronized when exposed to dbcAMP during the first 20 h of IVM. Distribution of oocytes at various meiotic stages did not differ between dbcAMP-treated COCs and controls at 28 h of IVM, but did differ at 36 h. It should be noted that in porcine oocytes the first metaphase stage is relatively longer than later stages [15]. Our observation also confirms that the effects of dbcAMP on meiotic arrest at the dictyate stage of prophase are reversible [7, 8] and do not prevent subsequent meiotic maturation [7]. Changes in the meiotic distribution of oocytes between 20 h and 28 h of culture suggests that treatment with dbcAMP may stimulate subsequent meiotic progression, and consequently the incidence of matured oocytes after dbcAMP treatment was not different from that in controls at the end of culture for maturation. Asynchronous meiotic progression of porcine oocytes has been repeatedly observed during IVM [2, 13, 16]. The present studies demonstrate that this asynchrony, which is partially due to heterogeneity in the morphology of the GV at oocyte collection, can be reduced during IVM by dbcAMP treatment.

In the present studies, we demonstrated that the incidence of IVM-IVF porcine embryos that developed to the blastocyst stage was almost double when COCs were exposed to dbcAMP during the first 20 h of IVM as compared to controls. Kim and Menino [17] have reported that culture in the presence of dbcAMP stimulates plasminogen activator activity in porcine COCs. It has been suggested that in the rat a possible role of the tissue-type plasminogen activator released during the cortical granule exocytosis is to contribute to the block to polyspermy [18]. We hypothesized that porcine mature oocytes exposed to dbcAMP would have a lower incidence of polyspermic penetration. However, there was no difference in the normal sperm penetration rate between dbcAMP-treated oocytes and controls. Therefore, increased productivity of blastocysts in these studies did not appear to be due to improvement in normal fertilization of oocytes. In the second experiment, we found that about 47% of oocytes in the control group had already developed to the MII stage after 36 h of maturation culture. Since sperm penetration takes place about 3 h after insemination in our culture system [19], this suggests that almost half of the control oocytes had aged at least 11 h before fertilization. We have demonstrated that histone H1 kinase activity, which is associated with cell cycle [20], is lower in aged porcine oocytes as compared to freshly matured oocytes [21]. Low histone H1 kinase activity may lead to a decreased incidence of oocytes that develop to the blastocyst stage after IVF. Naito et al. [22] have reported lower histone H1 kinase activity in porcine oocytes matured in a medium known to result in lower embryonic development of oocytes to blastocysts after IVF [23] than in oocytes matured in a more effective maturation medium.

Recently, we have also observed that nuclear morphology of oocytes in mature follicles is synchronized to a specific stage, GV-I, after eCG injection, and then the nuclear morphology changes to another stage, GV-II, after hCG injection [6]. Motlik and Fulka [13] have also observed that more than 90% of oocytes collected from eCG-treated gilts were at the GV-I stage. Therefore, there appears to be a mechanism for synchronization of the nuclear morphology of oocytes to the GV-I stage in vivo. In the present studies, however, there was a large variation in the nuclear morphology of porcine oocytes just after aspiration of COCs from follicles 3–6 mm in diameter, and about 45% of the oocytes were at the GV-II stage. For in vitro embryo production, many investigators are collecting COCs from antral follicles on the surface of slaughterhouse ovaries, which are derived from prepubertal gilts. Therefore, our observation indicates that the meiotic stage of porcine oocytes that are being used for in vitro embryo production differs from that present during the follicular phase of the estrous cycle.

In summary, we have demonstrated in these studies that there is a large variation in the nuclear morphology of porcine oocytes used for in vitro embryo production. Further, the presence of dbcAMP during the first 20 h of culture for maturation induces a more synchronous meiotic progression of porcine oocytes and improves the rate of early embryonic development to the blastocyst stage after IVF.

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## REFERENCES

1. Day BN, Funahashi H. In vitro maturation and fertilization of pig oocytes. In: Miller RH, Pursel VG, Norman HD (eds.), Beltsville Symposia in Agricultural Research XX. Biotechnology's Role in the Genetic Improvement of Farm Animals. Savoy, IL: American Society of Animal Science; 1996: 125–144.
2. Funahashi H, Day BN. Effects of different serum supplements in maturation medium on meiotic and cytoplasmic maturation of pig oocytes. Theriogenology 1993; 39:965–973.
3. Grant SA, Hunter MG, Foxcroft GR. Morphological and biochemical characteristics during ovarian follicular development in the pig. J Reprod Fertil 1989; 86:171–183.
4. Hunter MG, Wiesak T. Evidence for and implications of follicular heterogeneity in pigs. J Reprod Fertil Suppl 1990; 40:163–177.
5. Hunter RHF, Baker TG. Development and fate of porcine graafian follicles identified at different stages of the oestrous cycle. J Reprod Fertil 1975; 43:193–196.
6. Funahashi H, Tatemoto H, Cantley TC, Day BN. Nuclear morphology of swine oocytes during follicular development following stimulation by eCG injection. Biol Reprod 1996; 54(suppl 1):156.
7. Mattioli M, Galeati G, Barboni B, Seren E. Concentration of cyclic AMP during the maturation of pig oocytes *in vivo* and *in vitro*. J Reprod Fertil 1994; 100:403–409.

8. Petr J, Tepia C, Rozinek J, Jilak F. Effect of testosterone and dibutyryl c-AMP on the meiotic competence in pig oocytes of various size categories. Theriogenology 1996; 46:97–108.
9. Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993; 48:61–73.
10. Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN. *In vitro* development of *in vitro* matured porcine oocytes following chemical activation or *in vitro* fertilization. Biol Reprod 1994; 50:1072–1077.
11. Funahashi H, Cantley TC, Day BN. Different hormonal requirement of porcine oocyte-complexes during maturation *in vitro*. J Reprod Fertil 1994; 101:159–165.
12. Funahashi H, Day BN. Effects of the duration of exposure to supplemental hormones on cytoplasmic maturation of pig oocytes *in vitro*. J Reprod Fertil 1993; 98:179–185.
13. Motlik J, Fulka J. Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. J Exp Zool 1976; 198:155–162.
14. Rice C, McGaughey RW. Effect of testosterone and dibutyryl cAMP on the spontaneous maturation of pig oocytes. J Reprod Fertil 1981; 62:245–256.
15. Hunter RHF, Polge C. Maturation of follicular oocytes in the pig after injection of human chorionic gonadotrophin. J Reprod Fertil 1966; 12:525–531.
16. Yoshida M, Bamba K, Kojima Y. Effects of gonadotropins and estradiol-17β on the timing of nuclear maturation and cumulus mass expansion in pig oocytes cultured *in vitro*. Jpn J Anim Reprod 1989; 35:86–91.
17. Kim N-H, Menino ARJ. Effects of stimulators of protein kinase A and C and modulators of phosphorylation on plasminogen activator activity in porcine oocyte-cumulus cell complexes during in vitro maturation. Mol Reprod Dev 1995; 40:364–370.
18. Zhang X, Rutledge J, Khamsi F, Armstrong DT. Release of tissue-type plasminogen activator by activated rat eggs and its possible role in the zona reaction. Mol Reprod Dev 1992; 32:28–32.
19. Funahashi H, Stumpf TT, Cantley TC, Kim N-H, Day BN. Pronuclear formation and intracellular glutathione content of in vitro-matured porcine oocytes following in vitro fertilization and/or electrical activation. Zygote 1995; 3:273–281.
20. Jacob T. Control of cell cycle. Dev Biol 1992; 153:1–15.
21. Stumpf TT, Funahashi H, Terlouw SL, Day BN. Histone H1 kinase activity after electrical activation of *in vitro* matured porcine oocytes. J Anim Sci 1994; 72(suppl 1):74.
22. Naito K, Dean FP, Toyoda Y. Comparison of histone H1 kinase activity during meiotic maturation between two types of porcine oocytes matured in different media in vitro. Biol Reprod 1992; 47:43–47.
23. Naito K, Fukuda Y, Ishibashi I. Developmental ability of porcine ova matured in porcine follicular fluid in vitro and fertilized in vitro. Theriogenology 1989; 31:1049–1057.