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Successful Piglet Production in a Chemically Defined System for *In-vitro* Production of Porcine Embryos: Dibutyryl Cyclic AMP and Epidermal Growth Factor-family Peptides Support *In-vitro* Maturation of Oocytes in the Absence of Gonadotropins

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Abstract. To induce meiotic resumption of porcine oocytes, it is thought to be necessary to expose the cumulus-oocyte complexes (COCs) to gonadotropins during *in-vitro* maturation (IVM). However, the detailed mechanism of meiotic resumption by gonadotropins is still unknown, and successful piglet production has not been reported by using oocytes matured in gonadotropin-free media and fertilized *in vitro*. The present study was undertaken to examine the combinational effects of epidermal growth factor (EGF)-family members and dibutyryl cyclic AMP (cAMP) in a chemically defined medium on IVM of porcine oocytes and the developmental competence following *in vitro* fertilization (IVF). The basic IVM medium was a chemically defined medium, modified porcine oocyte medium (mPOM). Supplementation of the IVM medium with 10 or 1000 ng/ml EGF, amphiregulin and betacellulin during the whole IVM period, except for 10 ng/ml amphiregulin, increased the percentage of oocytes maturing to the metaphase-II stage. When COCs were exposed to both dibutyryl cAMP and EGF-family members during the first 20-h of IVM and then culture was continued in the absence of EGF-family members and dibutyryl cAMP, the incidence of metaphase-II oocytes was significantly increased and was not different from that of oocytes cultured in a standard IVM system with gonadotropins. The developmental competence of the oocytes to the blastocyst stage following IVF was no different from that of control oocytes matured with gonadotropins. When these blastocysts were transferred into the uterine horn of three recipients, all of gilts became pregnant and delivered a total of 11 piglets. These observations indicate that supplementation of a chemically defined maturation medium with EGF-family members and dibutyryl cAMP during the first 20 h of IVM can support well the meiotic progress and developmental competence of porcine oocytes.

Key words: Cyclic AMP (cAMP), Epidermal growth factor (EGF), *In vitro* maturation, Oocyte, Pig
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Successful *in-vitro* production of porcine embryos has the potential to provide an effective system for mass production of embryos for better understanding of the physiology of embryonic development and animal reproductive technologies, including production of transgenic and/or cloned animals [1, 2]. Relatively efficient systems for *in-vitro* production of porcine embryos have already been developed, but they contain materials derived from organisms, such as porcine follicular fluid and bovine serum albumin, in the culture media [3, 4]. Chemically defined media, which only known chemicals, but none derived from animals, yeast or plants, should be useful in clarifying the mechanism of interactive communication between the oocyte and the surrounding cumulus cells during meiosis, the oocyte reaction following sperm penetration and early development. Furthermore, application of a defined system for *in-vitro* embryo production would be good for a quality control against the risk of viral contamination during culture. Recently, defined media for *in vitro* production of porcine embryos using a single basic medium have been developed that enable suc-

cessful blastocyst formation [5]. These chemically defined media include a porcine oocyte medium (POM) for *in-vitro* maturation (IVM) [5], porcine gamete medium (PGM) for *in-vitro* fertilization (IVF) [6, 7] and porcine zygote medium (PZM) for *in vitro* culture (IVC) of zygotes based on the composition of porcine oviductal fluid [8]. Even in this system, however, gonadotropins derived from organisms were included in the maturation medium [5]. Although recombinant gonadotropins are commercially available, the downstream mechanism of gonadotropins to induce meiotic resumption and development to the metaphase-II stage is still unknown in chemically defined media. Furthermore, successful piglet production has not been reported using oocytes matured in gonadotropin-free media, whereas, very recently, it has been reported that porcine oocytes can undergo meiosis under gonadotropin-free conditions [9, 10].

Unlike bovine, porcine COCs need to be exposed to gonadotropins for resumption and processing of meiosis [11]. FSH stimulation induces the expression of LH receptor in cumulus cells, and binding of LH to the receptor induces an increase in the cyclic AMP (cAMP) level and acceleration of meiotic resumption in oocytes [12]. LH stimulation induces a transient and sequential

expression of epidermal growth factor (EGF) family members, amphiregulin, epiregulin and betacellulin, in rodents [13], horses [14] and humans [15]. These EGF family peptides seem to mediate LH stimulation of oocyte maturation in mice [13, 16] and rats [17]. Therefore, although supplementation with EGF during IVM in the presence of porcine follicular fluid and hormonal supplements does not affect the cumulus expansion, nuclear maturation or developmental competence of oocytes [18], it may stimulate the meiotic resumption of oocytes. Furthermore, exposure of COC to dibutyryl cAMP and gonadotropins only for the first 20 h of culture for IVM accelerate meiotic resumption of oocytes and improves developmental competence compared with COCs exposed to only gonadotropins [19, 20]. Recently, when exposure of COCs in the presence of fetal bovine serum to an inhibitor of phosphodiesterase (PDE), 3-isobutyl-1-methylxanthine, only for the first 20 h of culture well supported meiotic maturation to the metaphase-II stage [10]. Therefore, application of chemicals affecting the intracellular cAMP level and/or EGF signaling pathway may successfully support IVM of porcine oocytes in a gonadotropin-free medium.

In the present study, we examined the effects of EGF-family peptides alone or in combination with dibutyryl cAMP in maturation medium on meiotic maturation of porcine oocytes. Furthermore, we examined the developmental competence of the oocytes and successfully produced, for the first time, piglets from oocytes matured in a gonadotropin-free medium and fertilized and developed to the blastocyst stage in chemically defined media.

Materials and Methods

Chemicals and culture media

Potassium chloride, KH_2PO_4 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, sodium citrate and citric acid were purchased from Ishizu Pharmaceutical (Osaka, Japan). Sodium chloride and paraffin liquid were obtained from Nacalai Tesque (Kyoto, Japan). Unless specified, other chemicals were purchased from Sigma Aldrich Japan (Tokyo, Japan).

The medium used for collecting and washing COCs 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 Na-lactate, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 $\mu\text{g}/\text{ml}$ gentamicin and 65 $\mu\text{g}/\text{ml}$ potassium penicillin G. The basic IVM medium used was a BSA-free chemically defined medium, POM (Research Institute for the Functional Peptides, Yamagata, Japan), supplemented with 50 μM beta-mercaptoethanol (mPOM). This IVM medium well supports blastocyst development following IVF if gonadotropins are added during IVM [5]. The chemically defined media for IVF and *in vitro* development to the blastocyst stage were PGM-tac4 (Research Institute for the Functional Peptides, Yamagata, Japan) and PZM-5 (Research Institute for the Functional Peptides, Yamagata, Japan), respectively. All media (except for modified TL-HEPES-PVA) were equilibrated at 39 C in an atmosphere of 5% CO_2 in air overnight prior to use (only PGM-tac4 was under paraffin liquid).

Preparation and culture of cumulus-oocyte complexes

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl containing 75 $\mu\text{g}/\text{ml}$ potassium penicillin G and 50 $\mu\text{g}/\text{ml}$ streptomycin sulphate. Using an 18-gauge needle and a disposable 10-ml syringe, COCs were aspirated from antral follicles (3 to 6 mm in diameter) on the surface of the ovaries and washed three times with modified TL-HEPES-PVA medium at room temperature (25 C) [19]. Forty to fifty COCs with uniform ooplasm and a compact cumulus cell mass were washed three times with IVM medium. These complexes were subsequently cultured in 500 μl of IVM medium for a total of 44 h at 39 C in an atmosphere of 5% CO_2 in air. After IVM culture, the oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and evaluated for nuclear maturation. Some of the oocytes were washed with modified TL-HEPES-PVA three times, mounted, fixed for 48 h or more in 25% (v/v) acetic acid-alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and then examined under a phase-contrast microscope at 400 $\times$ magnification.

Preparation of fresh boar spermatozoa and in vitro fertilization

Semen-rich fractions (30 to 50 ml) were collected from boars, three Berkshire and one Duroc, by the glove-hand method at a local experimental station and were diluted 4 times with modified Modena solution [21]. The diluted semen samples were transported to the laboratory within 2 h of collection. After washing once by centrifugation at 750 g for 3 min, the spermatozoa were resuspended at a concentration of 1×10^8 cells/ml in modified Modena solution containing 5 mM cysteine and 20% (v/v) boar seminal plasma. The diluted spermatozoa were used for IVF immediately or after storage overnight at 15 C. When a diluted sperm specimen was stored overnight, the sample was cooled down from room temperature to 15 C for 4 h and then kept at the same temperature. Just before use, the stored spermatozoa were placed at room temperature for 15 to 20 min, washed three times by centrifugation at 750 g for 3 min with modified TL-HEPES-PVA solution and then resuspended at a concentration of 1×10^8 cells/ml in PGM-tac4.

After dilution to 1×10^6 cells/ml with PGM-tac4, fifty microliters of diluted sperm suspension was inseminated in the same volume of PGM-tac4. Thirty denuded oocytes were co-cultured with spermatozoa in a 100- μl droplet of PGM-tac4 under paraffin oil for a total of 8 h at 39 C in an atmosphere of 5% CO_2 in air. At 8 h after insemination, some of the cultured eggs were fixed, stained with 1% (w/v) orcein and examined at 200 $\times$ and 400 $\times$ magnification. Oocytes were designated as penetrated when they had at least one sperm head, a decondensed sperm nucleus or a male pronucleus and corresponding sperm tail in the vitellus.

Just after co-culture with spermatozoa, some of the oocytes were incubated in 500 μl of PZM-5 for 7 days at 39 C in an atmosphere of 5% CO_2 in air or for 5 days at 39 C in an atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 (in the embryo transfer experiment). Cleavage and morula/blastocyst formation of the oocytes were examined on Day 2 and Days 5 or 7 of culture, respectively.

Surgical embryo transfer of IVM-IVF blastocysts

After culture in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ for 5 days, IVF embryos that developed to the blastocyst stage were washed with PXM-Hepes medium [5] three times and then transferred with the same medium into the uteri of recipients. According to a previous report [6], luteolysis of recipient cross bred gilts ([Landrace × Large White] × Duroc, 7 mo old, 96–112 kg body weight) was induced by intramuscular injections of prostaglandin F2 α , as 15 mg dinoprost (Panacelan Hi; Meiji Seika, Tokyo, Japan) twice daily for 3 days during the functional luteal phase. Ovulation was induced by intramuscular injections 750 IU of hCG (Puberogen; Novartis Animal Health, Tokyo, Japan) 5 days after start of the prostaglandin F2 α treatments. Day-5 embryos that developed to blastocysts were surgically transferred into the uteri of 3 recipients (18–19 blastocysts/recipient) 5 days after hCG injection. Pregnancy diagnosis was carried out by ultrasonography 21 days following hCG injection. Gilts considered to be pregnant were allowed to continue to full term, and the gestation lengths, litter sizes and body weights of the piglets at birth were examined. All animal-related procedures followed in this study were approved by the Institutional Care and Use Committee of the Laboratory Animals of the National Institute of Animal Health (protocol No. 08-003).

Experimental design

Experiment 1: To determine the effect of EGF and EGF-family peptides on resumption of oocyte meiosis, COCs were cultured for 44 h in gonadotropin-free mPOM with or without EGF, amphiregulin or betacellulin at 10 or 1000 ng/ml, denuded and then fixed to observe meiotic status.

Experiment 2: To examine whether the effect of EGF on the resumption of oocyte meiosis is amplified by a combination with amphiregulin and/or betacellulin, COCs were cultured for 44 h in IVM medium with EGF alone or with EGF plus amphiregulin and/or betacellulin. The meiotic status of the oocytes was examined at the end of IVM culture.

Experiment 3: The combination effect of dibutyryl cAMP with EGF alone or EGF plus amphiregulin on meiotic resumption in oocytes was evaluated. COCs were cultured for 20 h in gonadotropin-free mPOM with dibutyryl cAMP alone or with dibutyryl cAMP plus EGF or plus EGF and amphiregulin and then cultured for 24 h in gonadotropin-free mPOM without dibutyryl cAMP,

EGF and amphiregulin. As positive controls, some COCs were cultured in IVM medium supplemented with gonadotropins (10 iu/ml eCG and 10 iu/ml hCG) and 1 mM dibutyryl cAMP for 20 h and then in unsupplemented medium for an additional 24 h [19, 22]. The meiotic status of the oocytes was examined at the end of IVM culture.

Experiment 4: To examine the effect of dibutyryl cAMP plus EGF and amphiregulin on the meiotic resumption of cumulus-free oocytes, denuded oocytes were exposed to dibutyryl cAMP plus EGF and amphiregulin for the first 20 h of IVM and then culture was continued in fresh mPOM for a further 24 h. At the end of IVM culture, the meiotic status of the oocytes was examined.

Experiment 5: The developmental competence of oocytes exposed to dibutyryl cAMP plus EGF and amphiregulin for the first 20 h of IVM to the blastocyst stage was examined following IVF and culture in PZM-5 for 7 days at 39 C in an atmosphere of 5% CO₂ in air. Furthermore, the developmental competence of the oocytes to full term was also examined following IVF. IVF embryos were cultured in PZM-5 for 5 days at 39 C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Only embryos that developed to the blastocyst stage were surgically transferred to the uteri of recipient gilts (18–19 blastocysts/recipient).

Statistical analysis

All oocytes were randomly distributed within each experimental group, and each experiment was repeated at least 3 times. All percentage data were subjected to arcsine transformation before statistical analysis. Statistical analyses of results from 3–7 replicates were used for treatment comparisons and carried out by analysis of variance (ANOVA) using the JMP 5.0 (SAS Institute, Cary, NC, USA) program. If the P value was smaller than 0.05 in ANOVA, the Tukey-Kramer HSD test was performed using the same program. All data were expressed as means $\pm$ S.E.M. $P \leq 0.05$ was considered to be statistically significant.

Results

Effect of EGF, amphiregulin and betacellulin on resumption of meiosis (Exp. 1)

As shown in Table 1, with the exception of 10 ng/ml amphiregulin, the presence of EGF, amphiregulin and betacellulin at 10 ng/ml and 1000 ng/ml in maturation medium increased the incidence of

Table 1. Effect of EGF-family peptides in a gonadotropin-free medium on the resumption of meiosis in oocytes*

EGF-family peptides	Concentration (ng/ml)	No. of oocytes examined*	%** oocytes completing GVBD	%** Pro M-I oocytes	%** M-I oocytes	%** A-I/T-I oocytes	%** M-II oocytes
None (control)	0	115	13.9 $\pm$ 1.4 ^a	1.8 $\pm$ 1.0	2.6 $\pm$ 1.7	0 $\pm$ 0	9.6 $\pm$ 2.2 ^a
EGF	10	114	37.9 $\pm$ 5.1 ^b	0.8 $\pm$ 0.8	7.1 $\pm$ 1.5	0.9 $\pm$ 0.9	29.1 $\pm$ 5.3 ^b
	1000	112	47.3 $\pm$ 3.9 ^b	0 $\pm$ 0	7.2 $\pm$ 2.1	3.5 $\pm$ 2.0	36.7 $\pm$ 5.8 ^b
Amphiregulin	10	118	16.1 $\pm$ 1.7 ^a	1.7 $\pm$ 1.7	4.2 $\pm$ 2.1	1.7 $\pm$ 1.7	8.5 $\pm$ 2.2 ^a
	1000	113	42.5 $\pm$ 8.1 ^b	0.9 $\pm$ 0.9	8.9 $\pm$ 2.3	2.6 $\pm$ 1.7	30.1 $\pm$ 6.6 ^b
Betacellulin	10	113	48.3 $\pm$ 5.0 ^b	0 $\pm$ 0	4.6 $\pm$ 1.8	4.4 $\pm$ 0.9	39.3 $\pm$ 7.2 ^b
	1000	114	37.7 $\pm$ 4.9 ^b	0.9 $\pm$ 0.9	4.4 $\pm$ 0.8	1.8 $\pm$ 1.8	30.7 $\pm$ 6.7 ^b

* COCs were cultured for 44 h in gonadotropin-free mPOM. EGF-family peptides (EGF, amphiregulin, betacellulin) were added at 0, 10 or 1000 ng/ml for the whole culture period. **Percentage based on the total number of oocytes examined. Data are presented as means $\pm$ S.E.M. of four replicated experiments. Values with different superscripts within the same column are significantly different ($P < 0.05$).

Table 2. Effect of combination of EGF-family peptides in a gonadotropin-free medium on *in-vitro* maturation of oocytes*

EGF-family peptides	No. of oocytes examined*	%** oocytes completing GVBD	%** Pro M-I oocytes	%** M-I oocytes	%** A-I/T-I oocytes	%** M-II oocytes
None (control)	115	14.8 ± 1.6 ^a	1.8 ± 1.0	5.2 ± 2.2	0 ± 0	7.9 ± 1.7 ^a
EGF	114	39.6 ± 3.6 ^b	0.8 ± 0.8	7.1 ± 1.5	0 ± 0	31.7 ± 3.0 ^b
EGF + amphiregulin	116	52.1 ± 3.9 ^b	1.9 ± 1.2	3.5 ± 1.6	1.3 ± 1.3	45.8 ± 3.3 ^c
EGF + betacellulin	103	50.6 ± 7.0 ^b	0.7 ± 0.7	1.4 ± 0.9	1.2 ± 1.2	46.1 ± 7.9 ^c
EGF + amphiregulin + betacellulin	120	53.3 ± 2.6 ^b	0.9 ± 0.9	9.0 ± 2.6	2.6 ± 1.7	40.8 ± 1.4 ^{bc}

* COCs were cultured for 44 h in gonadotropin-free mPOM. EGF-family peptides (EGF, 10 ng/ml; amphiregulin, 1000 ng/ml; betacellulin, 10 ng/ml) were added for the whole culture period. **Percentage based on the total number of oocytes examined. Data are presented as means ± S.E.M. of four replicated experiments. Values with different superscripts within the same column are significantly different ($P < 0.05$).

Table 3. Effect of combination of EGF-family peptides with dibutyl cAMP in a gonadotropin-free medium on *in-vitro* maturation of oocytes*

Supplements	No. of oocytes examined*	%** oocytes completing GVBD	%** Pro M-I oocytes	%** M-I oocytes	%** A-I/T-I oocytes	%** M-II oocytes
None (negative control)	163	26.6 ± 5.9 ^a	1.8 ± 1.8	5.1 ± 1.4	0 ± 0	19.8 ± 5.4 ^a
EGF	176	46.6 ± 2.9 ^b	1.1 ± 0.7	5.9 ± 2.6	1.6 ± 0.7	38.0 ± 4.1 ^b
Amphiregulin	175	41.9 ± 4.3 ^b	2.1 ± 1.4	2.2 ± 1.1	0.5 ± 0.5	37.1 ± 2.7 ^b
Dibutyl cAMP	147	64.2 ± 5.2 ^c	4.2 ± 2.4	10.4 ± 3.5	3.5 ± 1.2	46.1 ± 4.9 ^b
EGF + dibutyl cAMP	180	83.9 ± 3.1 ^d	0.6 ± 0.6	7.2 ± 1.5	3.8 ± 1.8	71.2 ± 3.8 ^c
EGF + amphiregulin + dibutyl cAMP	182	87.8 ± 2.0 ^{de}	0.6 ± 0.6	6.0 ± 2.0	2.8 ± 1.4	78.4 ± 3.5 ^c
Positive control (Gns + dibutyl cAMP)	194	96.7 ± 0.9 ^e	2.7 ± 1.4	10.9 ± 1.8	1.6 ± 1.2	81.4 ± 2.8 ^c

* COCs were cultured for 44 h in gonadotropin-free mPOM. EGF-family peptides (10 ng/ml EGF, 1000 ng/ml amphiregulin) and dibutyl cAMP (1 mM) were added only for the first 20 h of culture. As a positive control, gonadotropins (Gns; 10 U/ml eCG and 10 U/ml hCG) and dibutyl cAMP (1 mM) were added only for the first 20 h of culture. **Percentage based on the total number of oocytes examined. Data are presented as means ± S.E.M. of six replicated experiments. Values with different superscripts within the same column are significantly different ($P < 0.05$).

oocytes with induced meiotic resumption and development to the metaphase-II stage compared with the controls. However, there were no differences in the incidences of oocytes that completed germinal vesicle breakdown (GVBD) and of oocytes that matured to the metaphase-II stage among the groups supplemented EGF (10 ng/ml and 1000 ng/ml), amphiregulin (1000 ng/ml) and betacellulin (10 ng/ml and 1000 ng/ml).

Effect of combination of EGF with amphiregulin and/or betacellulin on resumption of meiosis (Exp. 2)

Addition of amphiregulin and/or betacellulin to maturation medium containing EGF supplement increased the percentage of oocytes completing GVBD, although there were no difference among the combined groups (Table 2). The incidence of oocytes matured to the metaphase-II stage also increased when amphiregulin or betacellulin was added to maturation medium containing EGF supplement. When both amphiregulin and betacellulin were added to maturation medium containing EGF, however, the percentage of oocytes at the metaphase-II stage did not differ from those of oocytes cultured in the presence of EGF alone and EGF plus amphiregulin or betacellulin. Since the variation in the incidence of oocytes matured to the metaphase-II stage was greater in the presence of EGF and betacellulin, rather than EGF and amphiregulin, the latter combination was utilized in the following experiments.

Effect of combination of EGF-family peptides with dibutyl cAMP on resumption of meiosis (Exp. 3)

Exposure of COCs to dibutyl cAMP alone during the first 20 h of IVM culture stimulated the incidences of oocytes completing GVBD and of oocytes maturing to the metaphase-II stage to the same level stimulated by EGF during the first 20 h of IVM culture (Table 3). However, addition of dibutyl cAMP to maturation medium containing EGF supplement(s) increased the percentages of oocytes completing GVBD and of oocytes maturing to the metaphase-II stage. When COCs were exposed to dibutyl cAMP and EGF during the first 20 h of IVM culture, the percentage of oocytes maturing to the metaphase-II stage was relatively similar to that of oocytes cultured in a standard IVM system with gonadotropins and dibutyl cAMP ($P = 0.07$). The presence of dibutyl cAMP, EGF and amphiregulin during the first 20 h of IVM supports the percentages of oocytes completing GVBD and of oocytes maturing to the metaphase-II stage to the same level as oocytes cultured in a standard IVM system with gonadotropins and dibutyl cAMP ($P = 0.57$, Table 3). Thus, this combination of dibutyl cAMP, EGF and amphiregulin was utilized in the following experiments.

In vitro maturation of denuded oocytes exposed to EGF-family peptides and/or dibutyl cAMP (Exp. 4)

Exposure of cumulus-free oocytes to dibutyl cAMP alone or in combination with EGF-family members, even in a standard IVM

Table 4. *In vitro* maturation of denuded oocytes cultured with EGF-family peptides and/or dibutyryl cAMP in a gonadotropin-free medium*

Supplements	No. of oocytes examined*	%** oocytes completing GVBD	%** Pro M-I oocytes	%** M-I oocytes	%** A-I/T-I oocytes	%** M-II oocytes
None (negative control)	77	36.1 ± 5.9	1.3 ± 1.3	6.4 ± 3.4	0	27.1 ± 8.7
Dibutyryl cAMP	109	57.1 ± 8.8	4.5 ± 2.6	7.3 ± 3.0	3.8 ± 1.8	41.5 ± 8.8
EGF + amphiregulin	77	50.5 ± 8.1	9.3 ± 9.3	1.1 ± 1.1	0	40.1 ± 13.7
EGF + amphiregulin + dibutyryl cAMP	106	49.0 ± 2.7	1.9 ± 1.1	10.9 ± 6.1	0	36.2 ± 6.4
Positive control (Gns + dibutyryl cAMP)	93	52.0 ± 13.7	3.5 ± 2.1	11.9 ± 4.4	3.5 ± 2.1	33.1 ± 7.8

*Denuded oocytes were cultured for 44 h in gonadotropin-free mPOM. EGF-family peptides (10 ng/ml EGF, 1000 ng/ml amphiregulin) and dibutyryl cAMP (1 mM), or gonadotropins (Gns; 10 U/ml eCG and 10 U/ml hCG) and dibutyryl cAMP (1 mM) were added only for the first 20 h of culture. **Percentage based on the total number of oocytes examined. Data are presented as means ± S.E.M. of three or four replicated experiments.

Table 5. *In vitro* development of oocytes matured in a gonadotropin-free chemically defined system following IVF

Experimental group	No. of oocytes inseminated*	%** oocytes cleaved	%** oocytes developing beyond the morula stage	%** oocytes developing to the blastocyst
Control (Gns + dibutyryl cAMP)	92	74.9 ± 16.0	24.1 ± 3.3	17.3 ± 4.2
EGF + amphiregulin + dibutyryl cAMP	161	72.1 ± 14.6	33.9 ± 6.0	25.0 ± 4.0

*COC were cultured in mPOM containing Gns (10 U/ml eCG and 10 U/ml hCG) ± 1 mM dibutyryl cAMP or 10 ng/ml EGF ± 1000 ng/ml amphiregulin ± 1 mM dibutyryl cAMP for 20 h and then without these supplements for 24 h. Following *in-vitro* culture for maturation, the denuded oocytes were co-cultured with fresh spermatozoa for 8 h in PGM-tac4 and then cultured in PZM-5 for 7 days. **Percentage based on the number of oocytes inseminated. Data are presented as means ± S.E.M. of three and six replicated experiments.

Fig. 1. a) Blastocysts transferred. The bar shows 200 micrometers. b) Piglets (one day old on December 31, 2008) obtained by transfer of blastocysts derived from oocytes matured in a gonadotropin-free IVM medium and fertilized and developed in chemically defined media.

system with gonadotropins and dibutyryl cAMP, did not affect the percentage of oocytes completing GVBD or the percentage of oocytes maturing to the metaphase-II stage (Table 4). However, there was a relatively higher tendency in the percentages of oocytes completing GVBD and maturing to the metaphase-II stage in the control denuded oocytes, as compared with the control COCs (Tables 1–3).

Developmental competence of oocytes matured in a gonadotropin-free IVM system (Exp. 5)

As shown in Table 5, there was no difference in developmental competence to the blastocyst stage following IVF between oocytes matured in a gonadotropin-free chemically defined system and those matured in a standard IVM system with gonadotropins. Following embryo transfer of blastocysts (Fig. 1a) produced in a gonadotropin-free chemically defined IVM-IVF-IVC system to three recipients, all of the recipients achieved pregnancy and farrowed a total of 11 piglets; of these, 10 were alive (Table 6, Fig. 1b). No clinical abnormalities were observed in the piglets that were born alive. The gestation lengths and body weights of the piglets were within the normal range (Table 6).

Table 6. Result of transfer of blastocysts derived from IVF oocytes matured in a gonadotropin-free chemically defined system

Recipient	No. of embryos transferred*	Pregnancy	Gestation length (days)*	Total no. of piglets born (No. of live piglets)	Average body weight of piglets at birth (kg)
Z128	19	Yes	115	1 (1)	1.40
Z129	19	Yes	112	6 (5)	1.59 ± 0.09
Z130	18	Yes	114	4 (4)	1.26 ± 0.17
Overall	56		113.7 ± 1.1	11 (10)	1.44 ± 0.09

* Day 0=2 days after hCG injection. Data are presented as means ± S.E.M.

Discussion

This is the first report to document the birth of piglets from oocytes matured in a gonadotropin-free medium and fertilized and developed to the blastocyst stage in chemically defined media. Several studies have reported piglet production by surgical embryo transfer using IVM-IVF-IVC blastocysts [6, 23, 24]. In these reports, however, porcine oocytes were matured in media containing materials derived from other organisms, such as follicular fluid and/or gonadotropins. Recently, Yoshioka *et al.* have also produced blastocysts in the chemically defined IVF and IVC system from oocytes matured in a simple chemically defined medium supplemented with gonadotropins [5]. In the current study, we succeeded in improving the IVM system by making it gonadotropin-free. The efficiency of piglet production (the number of piglets produced per number of blastocysts transferred) was relatively high. This evidence indicates that the quality of the embryos produced by the current IVM-IVF-IVC system is enough to enable development to full term, suggesting the possibility of useful application of this gonadotropin-free IVM and chemically defined IVF-IVC system for safe production of porcine embryos *in vitro*.

Potential factors that play roles in triggering oocyte maturation have been reported to include gonadotropins (e.g., FSH and LH), growth factors (e.g., amphiregulin and epiregulin) and sterols (e.g., follicular fluid-derived meiosis-activating sterol) [25]. In this study, we also found that EGF alone and in cooperation with dibutyryl cAMP has critical roles in resumption of meiosis and support of the nuclear and cytoplasmic maturation of porcine oocytes in gonadotropin-free chemically defined medium. In the present study, EGF and betacellulin affected stimulation of meiotic resumption and progression. EGF-family members, such as amphiregulin, epiregulin and betacellulin, are likely paracrine mediators of the LH signal in the horse [14] and rat [17]. In mice, deficit in either amphiregulin or epiregulin, two EGF receptor ligands, delays or reduces oocyte maturation and cumulus expansion [16]. LH stimulation induces the transient and sequential expression of EGF family members amphiregulin, epiregulin and betacellulin, and incubation of follicles with these growth factors recapitulates the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation [13]. The current observations confirm that the potential role of EGF-family members in oocyte maturation, as previously observed in other mammalian species, is also true in pigs. In the present study, EGF and betacellulin worked well at 10 ng/ml, whereas amphiregulin was not

effective at 10 ng/ml, but was effective at 1000 ng/ml. This difference in the effective concentration may be due to the difference in binding affinity, since the affinity of amphiregulin is several orders of magnitude less than that of EGF [26, 27].

Furthermore, it is very interesting that a combination of EGF with another EGF-family member (amphiregulin or betacellulin), as compared with EGF alone, improved the percentage of oocytes developing to the metaphase-II stage. A similar synergistic effect on oocyte maturation has been observed in buffalo COCs exposed to EGF plus IGF-I [28]. The EGF receptor family consists of four members: ErbB1, ErbB2, ErbB3 and ErbB4. The receptor of amphiregulin, as well as EGF, is ErbB1, whereas betacellulin binds to ErbB1 and ErbB4 [29]. Therefore, the common receptor of these EGF-family members should be ErbB1. However, the structure of the ectodomain fragment of ErbB1 resembles the corresponding domain of the insulin-like growth factor-1 (IGF-1) receptor [30]. Supplementation of IVM medium with IGF-1 is also well known to promote the incidence of porcine oocytes reaching the metaphase-II stage [31, 32], whereas the stimulating effect has been reported to be masked in the presence of EGF [33]. Furthermore, there is a positive relationship between the mean circulating concentrations of IGF-I during the follicular phase and the proportion of metaphase-II oocytes in pigs [34]. Therefore, the combination effect of EGF and amphiregulin or betacellulin in the current study may be due to a cross-linkage to the IGF-1 receptors, consequently increasing the percentage of oocytes progressing to the metaphase-II stage. Further experiments will be required to clarify this combination effect.

In the present study, we demonstrated that supplementation of a gonadotropin-free chemically defined medium with dibutyryl cAMP during the first 20 h of IVM significantly induced the meiotic resumption of oocytes. The incidence of oocytes completing GVBD was higher than that of oocytes cultured in a gonadotropin-free chemically defined IVM medium with EGF or amphiregulin alone, whereas the incidence of oocytes developed to the metaphase-II stage did not differ between these oocytes. In IVM medium containing gonadotropins, the effects of dibutyryl cAMP have been well documented. Treatment of prepubertal COCs with dibutyryl cAMP during the first half of IVM improves the synchronicity of meiotic resumption and progression [19] and the developmental competence of porcine oocytes following IVF [19, 20, 35] by increasing the mitochondrial membrane potential and decreasing the incidence of apoptosis in preimplantation-stage porcine embryos [36]. This evidence shows that control of meiotic

resumption in porcine oocytes is highly regulated by cAMP [37]. In the present study, we confirmed, in a chemically defined gonadotropin-free medium, that dibutyryl cAMP could regulate the meiotic resumption of porcine oocytes more effectively than EGF-family peptides alone when COCs are exposed to dibutyryl cAMP or EGF-family peptides during the first 20 h of IVM. Intracellular cAMP appears to regulate meiotic resumption of oocytes through the signaling pathways involved in maturation promoting factor (MPF) activation [38].

In the current study, we also found a synergistic effect of dibutyryl cAMP and EGF-family peptides not only on meiotic resumption, but also on the regulation of meiotic progression to the metaphase-II stage in porcine oocytes. In particular, exposure of COCs to a combination of dibutyryl cAMP with EGF plus amphiregulin during the first 20 h of IVM improved both the incidences of meiotic resumption and progression to the metaphase-II stage to the same levels as oocytes treated with gonadotropins and dibutyryl cAMP. Therefore, our current observations indicate that both the cAMP and EGF pathways need to be stimulated for successful meiotic resumption of porcine oocytes in a chemically defined gonadotropin-free medium. The promotional activity of EGF on porcine oocyte maturation is independent of the cAMP pathway and probably mediated by the protein kinase C (PKC) pathway [39]. In fact, it has been reported that an EGF-family peptide, amphiregulin, dose-dependently stimulates meiotic resumption of porcine COCs maintained in meiotic arrest with dibutyryl cAMP [40]. Further research will be required to clarify whether stimulation of both the PKA and PKC pathways induces successful meiotic maturation of porcine oocytes and to clarify the regulating mechanisms of meiotic resumption and progression to the metaphase-II stage.

Furthermore, increased cAMP resulting from inhibition of PDE3 in oocytes blocks GVBD, whereas increased cAMP resulting from inhibition of PDE4 activates the mitogen-activated protein kinase (MAPK) pathway in cumulus cells, which is essential for GVBD induction [41]. Recently, meiotic resumption in porcine COCs has also been shown to be transiently blocked by adenosine monophosphate-activated kinase in cumulus cells [42]. Under the current culture conditions, we demonstrated that treatment of denuded oocytes with dibutyryl cAMP for the first 20 h of IVM did not stimulate meiotic resumption and progression to the metaphase-II stage, whereas there was a tendency for relatively higher GVBD and maturation incidences in denuded oocytes. This evidence suggests that the positive synergistic effect of dibutyryl cAMP and EGF-family peptides on meiotic resumption and progression to the metaphase-II stage in porcine oocytes, as well as factors to maintain meiotic arrest, act via communication within the COC. Shimada *et al.* reported that cAMP appears to play an important role not only in meiotic resumption, but also in the regulation of meiotic progression beyond the metaphase-I stage in porcine oocytes when transported into oocytes from the cumulus cells via gap junctions [43]. Recently, it also has been reported that phosphatidylinositol 3-kinase-induced mitogen-activated protein kinase activation in cumulus cells mediates EDF-induced meiotic resumption in porcine COCs [44].

In conclusion, the current results indicate that meiotic resump-

tion and progression to the metaphase-II stage could be induced in porcine oocytes when COCs were exposed to a combination of dibutyryl cAMP and EGF-family peptides during the first 20 h of IVM in a chemically defined gonadotropin-free medium. The developmental competence of the oocytes was normal since embryo transfer of the oocytes following IVF could produce piglets with a relatively high efficiency. Therefore, in a chemically defined gonadotropin-free medium, we would recommend addition of a combination of dibutyryl cAMP and EGF-family peptides during the first 20 h of porcine IVM.

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