Effects of Cumulus Cells and Follicular Fluid on Plasminogen Activator Activity during *In Vitro* Maturation of Porcine Oocytes

Ji-Young Ann[1], Soo-Jin Sa[1], Yang Cao[1,4], Sang-Young Lee[3], Hee-Tae Cheong[2], Boo-Keun Yang[1] and Choon-Keun Park[1,†]

[1]*College of Animal Life Science and* [2]*School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea*
[3]*Biotechnology Division, Gyeongsangnam Province Advanced Swine Research Institute, Sancheong 666-692, Korea*
[4]*College of Bioengineering, Dalian University, Dalian 116622, P.R. China*

ABSTRACT

The present study was conducted to investigate the effects of cumulus cells and porcine follicular fluid (pFF) on plasminogen activator (PA) activity and oocytes maturation *in vitro* in the pig. The cumulus-oocyte complexes (COCs) and denuded oocytes (DOs) were incubated in NCSU-23 medium with or without 10% pFF for 0, 24, or 48 hr. In the presence of cumulus cells, the proportions of oocytes matured to metaphase-II stage were significantly ($P<0.05$) higher in medium with pFF than without pFF (69.8 vs. 37.7%, respectively). When COCs and DOs were cultured in the presence of pFF, tissue-type PA (tPA), urokinase-type PA (uPA), and tPA-PA inhibitor (tPA-PAI) were observed in COCs, and PA activities were higher at 48 hr than 24 hr. When COCs and DOs were cultured in the absence of pFF, tPA and tPA-PAI were observed in COCs, and PA activities were increased as duration of culture increased. No PA activities were detected in DOs regardless of pFF supplementation. When porcine oocytes were cultured in the presence of pFF for 24 and 48 hrs, the activities of tPA-PAI, tPA, and uPA were observed in both COCs and DOs. In medium of absence of pFF, PA activities were observed in oocytes with cumulus cells only. On the other hand, three plasminogen-dependent lytic bands (tPA-PAI, tPA, and uPA) were observed in pFF cultures. Particularly uPA activity was higher than the other kinds of PA activity. When oocytes and cumulus cells were separated from porcine COCs at 0 hr of culture, tPA-PAI, tPA, and uPA were detected in cumulus cells at 48 hr of culture, but no PA activities were in DOs. The presence of pFF and cumulus cells in maturation medium stimulated not only nuclear and cytoplasmic maturation in porcine COCs, but also PA production by cumulus cells and COCs. It is possible that PAs produced by cumulus cells migrated through the gap junction between oocyte and cumulus cells. These results suggest that porcine oocytes have no ability to produce PA themselves.

(Key words : Cumulus cells, Porcine follicular fluid (pFF), Plasminogen activator, Porcine oocytes, *In vitro* maturation)

INTRODUCTION

Although maturation of porcine oocytes *in vitro* was inhibited during culture in medium with porcine follicular fluid (pFF) from small and medium- sized follicles, the addition of pFF to the media improved maturation, penetration, and normal fertilization rates (Yoshida *et al.*, 1992). Similar contradictory effects of follicular fluid have been reported for bovine oocytes (Lonergan *et al.*, 1994). To induce normal *in vitro* maturation (IVM) of oocytes, researches have been performed on the factors such as collection and culture times of oocyte (Bousquet *et al.*, 1994), ovarian form and follicular diameter (Nagai, 1994), and gonadotropin, serum, growth factor and so on (Racowsky, 1985).

The processes of cytoplasmic maturation as well as nuclear maturation in mammalian oocytes are prerequisites for normal fertilization and subsequent embryonic development. The mammalian oocytes and its surrounding cumulus cells are metabolically coupled through gap junctions, that provide a unique means of entry into the ooplasm for several metabolites (Larsen and Wert, 1988). Cumulus cells have a close connection with oocytes during the course of maturation in mammals. Gonadotropins, steroids, and other factors from the follicle cells also interact with oocytes to provide essential support for *in vivo* maturation of oocytes (Warnes *et al.*, 1977). It is generally accepted

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† Corresponding author : Phone: +82-33-260-8627, E-mail: parkck@kangwon.ac.kr

that cumulus cells support the maturation of oocytes to the metaphase-Ⅱ stage and greatly enhance cytoplasmic maturation, which is responsible for the capacity to undergo normal fertilization and subsequent embryonic development. Several studies have indicated that cumulus-denuded oocytes can undergo meiotic maturation *in vitro* in rats (Magnusson, 1980), sheep (Staigmiller and Moor, 1984), and cattle (Chian *et al.*, 1994).

Plasminogen is a ubiquitous zymogen, and abundant in plasma and in most extracellular fluids including uterine fluid (Bruse *et al.*, 1998), ovarian follicular fluid (Beers, 1975; Colgin and Murdoch, 1997) and seminal plasma (Liu *et al.*, 1996). Plasminogen activators (PAs) are serine proteases, known to be secreted by a large number of cell types. PAs are proteolytic enzymes, which convert the abundant extracellular proenzyme plasminogen into plasmin. Plasmin is a trypsin like proteolytic enzyme with broad substrate specificity, which also has the ability to activate latent forms of some other proteinases, such as procollagenase and proelastase (Dano *et al.*, 1985). PAs are claimed to play a role in a variety of physiologic processes, including fibrinolysis, ovulation, mammary involution, implantation (Dano *et al.*, 1985), and fertilization (Huarte *et al.*, 1993). Two types of PA have been characterized by molecular mass: tissue-type PA (tPA) and urokinase-type PA (uPA) (Kim and Menino, 1995). The two PAs are inhibited specifically by plasminogen activator inhibitor-1 (PAI-1). PAI-1, an important regulator of both fibrinolysis and PAs.

PA activity has been shown in follicular fluid (Smokovitis *et al.*, 1989), COCs (Liu *et al.*, 1986; Liu and Hsueh, 1987; Yamada *et al.*, 1996), oocytes (Huarte *et al.*, 1985, 1993) and cumulus cell cultures (Liu and Hsueh, 1987), while tPA and its mRNA have been detected in oocyte cytoplasm (Strickland *et al.*, 1988).

PA is secreted from cumulus cell, thecal cell, endothelial cell, oocyte, and early embryo, is known that the activation is increased, which due to the stimulus with protein kinase A and C of oocyte by various supplemented materials during IVM. However, these studies in the case of the pig is very insufficient actual circumstances. Therefore, this study investigated the effects of cumulus cells and pFF on IVM and change of PA activity in porcine oocytes.

MATERIALS AND METHODS

Collection and Culture of Oocytes

Porcine ovaries were collected from a local slaughterhouse and kept in physical saline (NaCl, 0.9% w/v ; Penicillin 100,000 IU/l ; Streptomycine 100 mg/l and Amphotericin B 250 μ g/l ; Sigma, St. Louis, MO, USA) at 35 to 37℃. Cumulus-oocyte complexes (COCs) were aspirated from 2 to 8 mm follicles with a 10 ml syringe with 18-G needle. COCs with uniform ooplasm and a compact cumulus cell mass were prepared in Hepes-buffered Tyrode's (TLH) containing 0.1% (v/v) polyvinylalchol (TLH-PVA). The collected oocytes were washed three times with a specified maturation medium. Depending on the experiments, some COCs were freed from cumulus cells by 0.1% (w:v) hyaluronidase from bovine testis (Sigma). Each group of 100 oocytes (COCs and denuded oocytes, DOs) were matured in 500 μ l drop of BSA-free NCSU-23 (NCSU-23; Petters and Wells, 1993) supplemented with 50 mM hypotaurin, 0.57 mM cysteine, 10 IU/ml hCG (Sigma), PMSG (Sigma) and/or 10% (v/v) porcine follicular fluid (pFF) under paraffin oil. The pFF was aspirated from follicles (2 to 8 mm in diameter) at estrus with a syringe with 18-G needle, and centrifuged at 3,850 ×g for 20 min. After culture for 22 hr, COCs or DOs were washed three times and then cultured in maturation medium without hormones for another 22 hr at 39℃, 5% CO_2, in air. After culture of oocytes, COCs, DOs, freed cumulus cells and the conditioned medium removed all cells were separately put into microtubes containing 20 μ l of sample buffer (5.0% [w:v] SDS, 20% [v:v] glycerol, and 0.0025% [w:v] bromophenol blue in 0.125 M Tris-HCl buffer) and frozen at −70℃ until used for zymographic analysis.

Electrophoresis and Zymogram

SDS-PAGE and zymogram were carried out by the procedures described by Dyk and Menino (1991), which were modified from Granelli-Piperno and Reich (1978). Frozen samples were thawed and homogenized with a sonicator. As a standard of tPA, stock solution of 0.5 ng/ml tPA from human melanoma cell culture (Sigma) was prepared in sample buffer. Each homogenized sample (15 μ l) being compared in each experiment, a stock solution of human tPA (5 μ l), and molecular mass markers (10 μ l; Bio-Rad Lab., Hercules, CA) were placed in a castellated well in a 4.5% stacking gel with a 10.0% separating gel. Electrophoresis was conducted at 20 mA for 2 hr.

After electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 for 45 min, rinsed with distilled water three times, and incubated for 30 min at 39℃ in phosphate-buffered saline (PBS). Each gel was carefully laid on a casein-agar gel (zymogram) containing purified human plasminogen (Sigma) supported in a plastic chamber. For preparing zymograms, 4% of nonfat dry milk was dissolved in 10 ml of buffer containing 0.0013 M $CaCl_2 \cdot 2H_2O$, 0.1 M glycine, 0.038 M Tris, and 0.005 M sodium azide. The nonfat dry milk mixture were heated to 55℃ and combined with 7.5 ml of 2% (v:v) melted agarose dissolved in distilled water and maintained at 55℃. Purified human plasminogen stock solution was added to 17.5

ml of the warmed mixture to yield a final plasminogen concentration of 50 μ g/ml and this mixture were cast into a warmed 100 × 100 mm plastic plate and allowed to cool. Zymograms containing no plasminogen were used for detection of any nonspecific proteolytic activity. Zymograms and polyacrylamide gels were incubated at 39℃ for 24~48 hr. PA migration was determined during the incubation period and after fixing the gel by measuring the distance from the edge of the separating gel to the center of the lytic bands in each lane. Incubation of zymograms was terminated by separating the gels. All experiments measuring PAs activity were repeated at least three times.

Experimental Design

To determine the effects of pFF and cumulus cells on PAs activity in porcine oocytes and conditioned medium during IVM, the COCs and DOs were incubated in NCSU-23 medium with or without 10% pFF for 24 or 48 hr. On the other hand, at 24 or 48 hr after start of culture, COCs, DOs, pFF or conditioned medium were sampled for determination of PA activity.

Statistical Analysis

Differences in data were evaluated by Duncan's multiple-range test using the Gerneral Linear Models procedure in the Statistical Analysis System. Difference with values of $P<0.05$ were considered to be statistically significant.

RESULTS

The effects of cumulus cells on IVM of porcine oocytes were examined by culturing for 48 hr in medium with (+) or without (−) pFF (Table 1). In the presence of cumulus cells, the proportions of oocytes matured to the metaphase-II stage were significantly ($P<0.05$) higher in medium with pFF than without pFF (69.8 vs. 37.7%).

Table 1. Effects of cumulus cells on *in vitro* maturation of porcine oocytes cultured for 48 hr in medium with or without pFF

Presence of cumulus cells	Presence of pFF	No. of oocytes examined	No. (%) of oocytes matured
COCs	+	368	257(69.8)[a]
	−	323	122(37.7)[b]
DOs	+	384	72(18.7)[bc]
	−	483	63(13.0)[c]

a~c Values with different superscripts differ ($P<0.05$).

As shown in Fig. 1, when COCs and DOs were cultured in the presence of pFF, activities of tPA-PAI, tPA and uPA were observed in COCs, and were higher at 48 hr than 24 hr. However, no PA activity was detected in DOs. When COCs and DOs were cultured in the absence of pFF, the activities of tPA-PAI and tPA were observed in COCs, and were higher at 48 hr than 24 hr. But, no PA activity was detected in DOs.

When conditioned medium with COCs and DOs were cultured in the presence or absence of pFF, the activities of tPA-PAI, tPA and uPA were observed in conditioned medium with COCs and DOs cultured for 24 and 48 hrs in the presence of pFF. On the other hand, the activity of tPA-PAI was observed only in conditioned medium with COCs cultured for 24 hr in the absence of pFF, and no PA activity was detected in

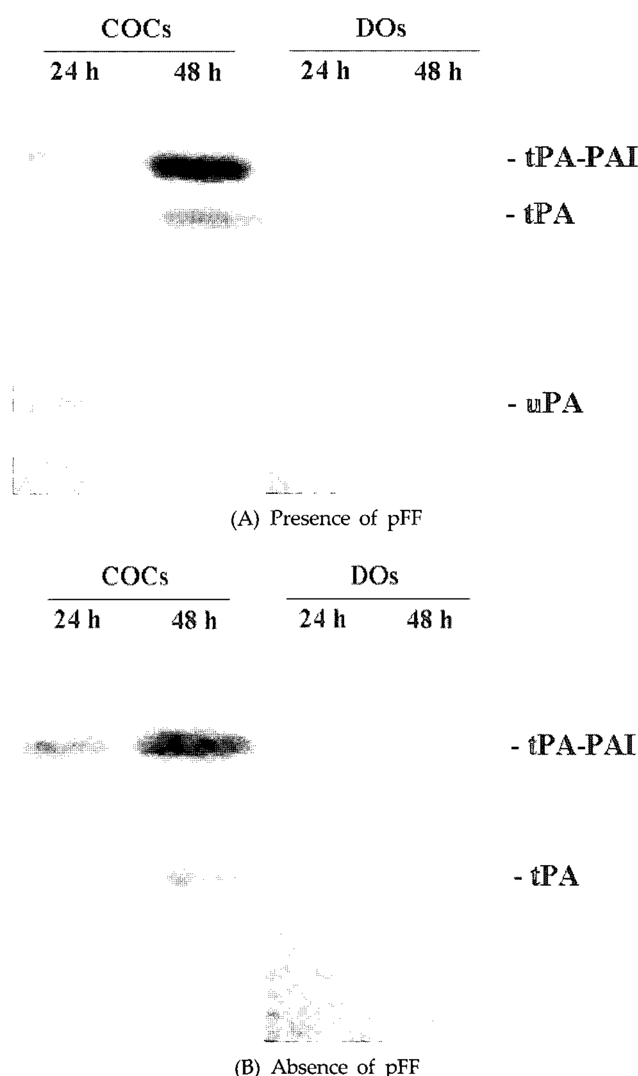
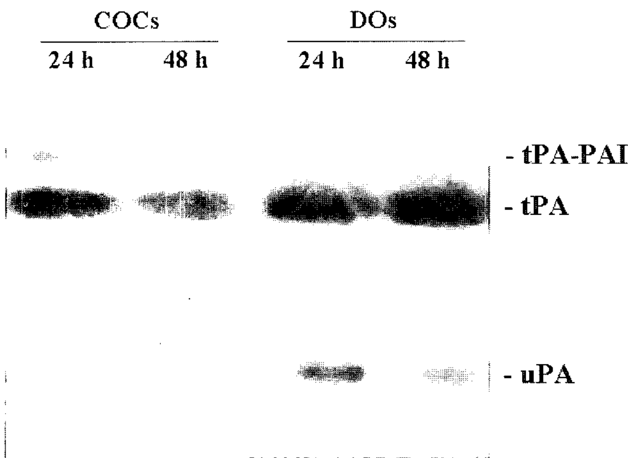
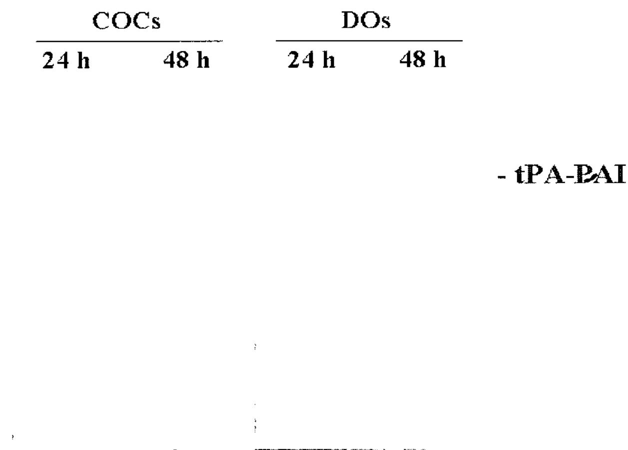


Fig. 1. Zymographic analysis of porcine COCs and DOs cultured for 24 and 48 hrs in the presence (A) or absence (B) of pFF. DOs were separated from porcine COCs at 0 hr of culture.



(a) Presence of pFF



(b) Absence of pFF

Fig. 2. Zymographic analysis of conditioned medium with COCs and DOs cultured for 24 and 48 hrs in the presence (a) or absence (b) of pFF. DOs were separated from porcine COCs at 0 hr of culture.

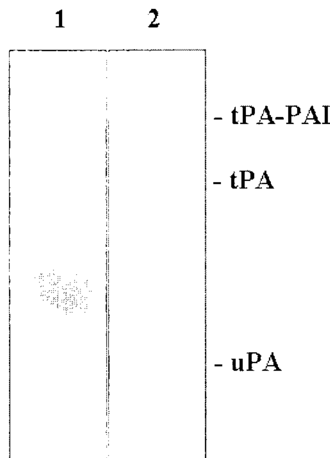


Fig. 3. Zymographic analysis of pFF obtained from 2~8 mm follicle. Lines 1 and 2 contained human tPA standard and pFF, respectively.

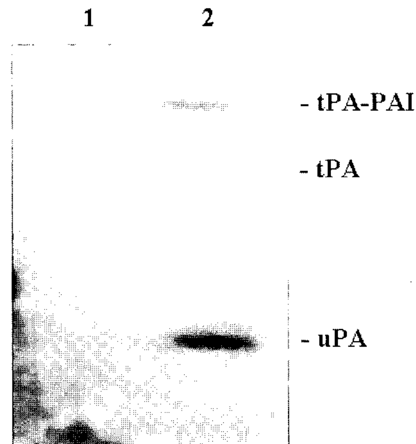


Fig. 4. Zymographic analysis of DOs (lane 1) and cumulus cells (lane 2) cultured for 48 hr. Cumulus cells were separated from porcine COCs at 0 hr of culture.

conditioned medium with DOs (Fig. 2).

In Fig. 3, three plasminogen-dependent lytic bands (tPA-PAI, tPA, and uPA) were observed in pFF cultures. Particularly uPA activity was higher than the other kinds of PA activity. When oocytes and cumulus cells were separated from porcine COCs at 0 hr of culture, tPA- PAI, tPA, and uPA were detected in cumulus cells at 48 hr of culture, but no PA activities were in DOs (Fig. 4).

DISCUSSION

In the present study, three plasminogen-dependent proteases are produced from porcine COCs during IVM. The molecular mass suggested that the 114 kDa, 70 kDa, and 54 kDa species that were detected in COCs are tPA-PAI, tPA and uPA, respectively. Tissue-type PA (tPA) is synthesized as a proenzyme with a molecular mass of ~70 kDa and composed of two polypeptide chains, a heavy chain (40 kDa) and light chain (30 kDa), linked by a single disulfide bond (Degen *et al.*, 1986). uPA exists in one- and two-polypeptide chain forms of approximately ~50 kDa. The one-chain molecule is a proenzyme. The bioactive two-chain form is composed of a heavy B-chain (approximately 30 M_r) and a lighter A-chain (approximately 20 M_r) linked by a disulfide bond. An active form of uPA with a M_r of approximately 30 kDa also has been reported in some species. It appears that the low M_r uPA, similar to that detected in our studies, is derived form the two-chain form by proteolytic cleavage (Dano *et al.*, 1985). Both uPA and tPA can form complexes with PAI, a family of specific inhibitors for PA, that are resistant to dissociation by SDS and retain PA activity in the zymograph (Rehemtulla *et al.*, 1990). Levels

of endogenous proteinase inhibitors dramatically influence activity of the plasminogen activators and plasmin in the extracellular milieu (Dow *et al.*, 2002). Multiple bands of plasmin activity (of similar M_r) have been observed in mouse ovarian homogenates (Ny *et al.*, 1997) and human blood plasma (Roche *et al.*, 1983). The gonadotropin surge-induced increase in plasmin activity in follicular fluid can most likely be attributed to the observed increase in follicular fluid levels of uPA and enhanced activation of ubiquitous plasminogen in follicular fluid (Dow *et al.*, 2002). Plasmin has been detected previously in the follicular fluid of cattle (Beers, 1975) and other species including the rabbit, horse and pig (Yamada *et al.*, 1996). Plasmin in follicular fluid may help degrade high molecular weight proteoglycans, causing a decrease in follicular fluid viscosity that facilitates oocyte escape. It is reported that supplementing maturation medium of sow COCs with large follicular fluid enhanced induction of cumulus expansion, and increased nuclear maturation and competence to develop into blastocyst compared to COCs matured in the presence of small follicular fluid (Algriany *et al.*, 2004). Tao *et al.* (1995) found no difference in nuclear maturation of COCs matured in pFF collected from small or large follicles. On the other hand, it has been reported that more oocytes reach MII stage when cultured with pFF from the same sized follicles (Vatzias and Hagen, 1999). Sun *et al.* (1994) reported that follicular fluid from small or large ovine follicles or human follicular fluid enhances maturation, fertilization, and further development of sheep oocytes compared to treatment with FCS. Moreover, bovine follicular fluid large follicles enhanced maturation and further development of bovine oocytes *in vitro* (Elmileik *et al.*, 1995). It agrees with our finding in the present study. Liu *et al.* (1986) proposed that plamsin may assist in cumulus expansion by terminating oocyte-cumulus cell communication. Before the time of ovulation, the number of cumulus cell processes to the oocyte decrease (Kraicer *et al.*, 1976), and there is a hormonally induced elevated concentration of plasminogen activator in the follicular fluid that is produced by the granulosa cells (Beers *et al.*, 1975).

It has been reported that cumulus cells are involved in the cytoplasmic maturation of oocytes followed by the acquisition of developmental competence (Larsen and Wert, 1988; Chian *et al.*, 1994). Chian *et al.* (1994) demonstrated that although the presence of cumulus cells coupled to bovine oocytes was not necessary for nuclear maturation, the developmental competence of DOs after *in vitro* fertilization (IVF) was prominently lower than that of COCs. These findings indicate that the poor developmental competence of DOs might be caused by the lack of cytoplasmic maturation of oocytes (Tatemoto *et al.*, 2000). It also agrees with our results in the present study. The aberrant patterns of microfilament organization in porcine oocytes during IVM were distinct from those in oocytes matured *in vivo*, suggesting that this inadequate microfilament organization impairs the function of cytoplasmic organelles controlling pronuclear formation and polar body formation in the oocyte (Kim *et al.*, 1996).

Yamada *et al.* (1996) reported that bovine oocytes, denuded from the cumulus cell layer and cultured, did not induce lysis on fibrin plates, and concluded that bovine oocytes are not capable of producing and secreting plasminogen activators. This conclusion is based on the finding that cultured denuded oocytes do not release plasminogen activators (Rekkas *et al.*, 2002), and it is in accordance with our results in the present study. tPA has been detected in the ooplasm of rat (Liu *et al.*, 1986; Liu and Hsueh, 1987; Bicsak *et al.*, 1989), mouse (Huarte *et al.*, 1985) and pig (Kim and Menino, 1995) oocytes. Fibrinolytic activity has been observed only in cumulus cells of bovine COCs before and after maturation (Yamada *et al.*, 1996). The increase of PA activity in COCs during maturation *in vitro* or *in vivo* is also reported in rats (Liu *et al.*, 1986; Liu and Hsueh, 1987), and pigs (Kim and Menino, 1995). However, the type of PAs detected in COCs seems to be different according to the different species: in rats, low amounts of tPA are detected in freshly obtained COCs, but both tPA and uPA activity increases during maturation *in vivo* (Liu and Hsueh, 1987) and *in vitro* (Liu *et al.*, 1986). Whereas in pigs, uPA activity is not detected in COCs before and after maturation, but both tPA and tPA-PAI activity increases during IVM (Kim and Menino, 1995). In the present study, it is considered that COCs do not contain uPA activity or contain low amounts. In the COCs, the level of uPA activity was detected significantly difference with or without pFF. Regardless of addition of pFF, however, no uPA activity was detected in DOs. It is reported that rat denuded oocytes freed from cumulus cells just after collection from follicles do not contain tPA activity (Huarte *et al.*, 1985) or contain (Liu *et al.*, 1986; Liu and Hsueh, 1987; Bicsak *et al.*, 1989) low amounts, but the activity was time-dependently increased during maturation *in vivo* (Liu and Hsueh, 1987; Bicsak *et al.*, 1989) or *in vitro* without cumulus cells (Huarte *et al.*, 1985; Liu *et al.*, 1986). An increase of tPA activity during IVM is also reported in cumulus-free mouse oocytes (Huarte *et al.*, 1985). Rat and mouse oocytes produce only tPA during spontaneous *in vitro* meiotic maturation (Huarte *et al.*, 1985). However, cultured COCs produce both tPA and uPA (Liu *et al.*, 1986). Similarly, uPA activity increased in COCs just before cumulus cells expansion. It has been also demonstrated rat oocytes contain mRNA for tPA, suggesting that oocytes synthesize tPA themselves and do not simply take up tPA from the extracellular space (Bicsak *et al.*, 1989). It is reported that mouse

COCs synthesize low levels of PAs throughout the COC expansion process, when matrix deposition occurs, but rapidly increase PA synthesis thereafter, when matrix disassembly and cumulus dispersion begin (D'alessandris *et al.*, 2001). D'alessandris *et al.* (2001) also provide evidence indicating that modulation of uPA activity by cumulus cells mainly depends on changes in cumulus cells-oocyte interaction. However, these result are not consistent with those of the present study using porcine oocytes. In the present study, addition of pFF in medium increased not only rate of IVM of porcine oocytes but also PA production by oocytes and cumulus cells, and it showed significantly difference in DOs with or without pFF. No PA activity was detected either in oocytes or in cumulus cells just after aspiration of COCs from follicles, and no PA activity was also detected in DOs during IVM. Also, the cumulus cells produced the tPA for oneself. These suggested that tPA production in porcine oocytes may not be derived from oocyte but is obtained from cumulus cells, which produce tPA during IVM. It is not clear how oocytes obtained the ability to produce tPA from cumulus cells, but it is possible that tPA produced by cumulus cells is transported into ooplasm through gap junctions between oocyte and cumulus cells.

In conclusion, it is suggested that the pFF and cumulus cells in maturation medium stimulated not only nuclear and cytoplasmic maturation in porcine COCs but also PA production by cumulus cells and COCs. Also, it is possible that PAs produced by cumulus cells coupled through gap junction between oocyte and cumulus cells. These results suggest that porcine denuded oocytes have no ability to produce PA themselves or its production is limited.

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