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Interaction between Transforming Growth Factor β and Cumulus Cells during *In Vitro* Maturation in Porcine Oocytes

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돼지난자의 체외성숙시 Transforming Growth Factorβ와 난구세포의 상호작용

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요 약

본 연구는 돼지난자의 체외성숙에 미치는 transforming growth factor β(TGF β)와 난구세포의 역할에 대하여 검토하였다. 난자의 체외성숙시 TGF β를 여러 농도에서 첨가한 경우 metaphase-Ⅱ로 성숙한 난자의 비율은 52~69%로 유의적인 차이는 나타내지 않았다. 성숙배양 24시간에서 난구세포의 유무에 관계없이 TGF β가 무첨가된 배양액 내에서는 성숙된 난자가 관찰되지 않았으나 TGF β첨가(5 및 4%)의 경우 성숙난자가 관찰되었다. 그러나, 성숙배양 48시간후 난자의 성숙율은 난구세포가 부착되어 있는 경우 TGF β첨가(70%)가 무첨가(52%)에 비해 높았으며, 난구세포를 세포를 제거한 경우(35 및 26%)에 비해 유의적으로 높은 성숙율을 나타냈다($P < 0.05$). 한편, 난구세포가 부착된 난자의 성숙배양시 TGF β의 첨가시기에 의한 성숙율(54~71%)에는 큰 차이가 없었으나, 난구세포를 제거한 경우 전반기(59%) 또는 후반기(57%) 24시간 동안 TGF β를 첨가한 경우 48시간 동안 계속하여 첨가(27%) 또는 무첨가(38%)에 비하여 유의적으로 높은 성숙율을 나타냈다($P < 0.05$). 이와 같은 결과는 난구세포가 돼지난자의 체외성숙시 필수적이지만, TGF β는 난구세포를 제거한 경우 난자의 성숙에 계속적인 역할을 하지 않는 것으로 추측된다.

(Key words : Porcine oocytes, *In vitro*, Maturation, TGF β, Cumulus cells)

Ⅰ. INTRODUCTION

The maturation of mammalian oocytes is initiated during fetal life and arrested at the diplotene stage of prophase-Ⅰ until shortly before ovulation(Wassarman, 1988). In this arrested stage, the oocyte is incapable of being fertilized. Fertilization can occur only after resumption of meiosis to metaphase-Ⅱ and formation of the polar body. Fully grown mammalian oocytes, surrounded by a compact mass of somatic cumulus cells, are maintained in the mature, germinal vesicle stage *in vivo* until a preovulatory gonadotropin surge provokes a dramatic physiological response. In the hours following the ovulation stimulus, the oocyte resumes nuclear maturation, manifested initially by germinal vesicle breakdown(GVBD), while the cumulus oophorus undergoes mucification

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and becomes embedded in a glycosaminoglycan matrix, a process termed cumulus expansion. By the time of ovulation, the cumulus cells have fully expanded and encompass an oocyte that has progressed meiotically to metaphase-Ⅱ. It is generally accepted that gonadotrophins, specifically luteinizing hormone(LH), provide the stimulus *in vivo* that brings about the resumptiom of meiosis and expansion of the cumulus oophorus (Tsafriri et al., 1982 ; Eppig and Downs, 1984). However, the possibility exists that other types of molecules participate in the mechanism controlling these physiological changes during the preovulatory period.

Recent studies using growth factors have shown that meiotic resumption in cumulus-oocyte complexes or follicle enclosed oocytes in several species can be induced by epidermal growth factor(EGF)(Sanbuissho et al., 1990 ; Das et al., 1991 ; Reed et al., 1993), transforming growth factor α(TGF α)(Brucker et al., 1991 ; Tsafri et al., 1991) and transforming growth factor β (TGF β)(Feng et al., 1988). The major site of action of growth factors that regulate oocyte maturation is the cumulus cells(Coskun and Lin, 1993). It is reported that somatic cells supply nutrients and other substances to the oocytes and communicate with other and the oocyte via gap junction(Dekel et al., 1981). Communication through these junctions is important for cellular interaction, especially those regulated by endocrine and paracrine signaling(Eppig, 1991). Therefore, the objectives of the present studies were examined effects of TGF β and cumulus cells on nuclear maturation of porcine oocytes in our culture system.

Ⅱ. MATERIALS AND METHODS

1. Oocyte preparation

Porcine ovaries were collected from a local slaughter-house and kept in saline(NaCl, 0.9% W/V ; Penicillin 100,000 IU/L ; Streptomycin 100mg/L and Amphotericine B 250μg/L ; Sigma Chemical, St-Louis, MO, USA) at 30 to 32 ℃. Cumulus-oocytes complexes were aspirated from 2 to 6 mm follicles with a 10-ml syringe with an 18-G needle. The collected oocytes were washed three times in Hepes-buffered Tyrode's medium (TLH) and once in maturation mediun, oocytes with a compact and complete cumulus cells were introduced to droplets of maturation medium(10 oocytes/50μl droplet), covered with mineral oil and were cultured under an atmosphere of 5% CO_2 in air at 39℃ for 42~44 h. The maturation medium consisted of TCM-199 with Earle's salts(Gibco Lab., NY, USA) supplemented with 3.05mM glucose, 0.32mM Ca-lactate, 2.5mM Hepes(Sigma), 10% fetal calf serum(FCS), 0.2mM Na-pyruvate(Sigma), 50μg/ml gentamycin(Sigma), 1μg/FSH(Sigma), 5μg/ml LH(Sigma), 1μg/ml estradiol 17β(Sigma) and 10%(v/v) porcine follicular fluid (PFF).

2. Experimental design

In the first series of experiments, the effect of TGF β concentrations(0, 1, 5 and 10ng/ml) during *in vitro* maturation was determined using the oocyte culture system described above.

In the second serious of experiments, porcine oocytes with or without cumulus cells were cultured in presence or absence of TGF β(1ng/ml). At 24 and 48 h after culture, the oocytes were examined for maturation status.

In the third serious of experiment, oocytes with or without cumulus were cultured in medium with or without TGF β(1ng/ml). TGF β were added for various periods(0~24, 24~48 or 0~48 h after culture) during *in vitro* maturation.

3. Evaluation of oocyte maturation

At the end of examination, the oocytes were mounted, fixed(acetic acid : ethanol 1:3) for 2~3 days and stained with 1% acetic-orcein in 40% acetic water solution, and examined under a phase-contrast microscope at magnification of 200× and 400×.

The stages were classified as germinal vesicle(GV), prophase-I (P-I), metaphase-I (M-I), anaphase-I (A-I), telophase-I (T-I) and metaphase-II (M-II).

4. Statistical analyses

Chi-square analysis with the Yates correction was used to test the significance of individual comparisons for rates of matutation status.

Ⅲ. RESULTS

In the first experiment, when complexes oocytes cumulus cultured with different concentrations of TGF β, the proportions of oocytes matured to metaphase-II were 53(42/79), 69(61/88), 64(57/89) and 52%(31/60) for 0, 1, 5 and 10ng/ml of TGF β, respectively(Table 1).

Table 1. Effect of concentrations of TGF β on *in vitro* maturation in porcine oocytes

Concentrations of TGF β(ng/ml)	No. of oocytes examined	No. of oocytes		
		GV	P-I~T-I	M-II(%)
0	79	0	37	42(53)
1	88	0	27	61(69)
5	89	0	32	57(64)
10	60	0	29	31(52)

GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II

Table 2. Effect of TGF β(1 ng/ml) on *in vitro* maturation in oocytes with or without cumulus cells at 24 h after culture

Presence of cumulus cells	Addition of TGF β	No. of oocytes examined	No.(%) of oocytes		
			GV	P-I~T-I	M-II
+	+	96	8(8)	83(86)	5(5)
	−	104	13(13)	91(88)	0(0)
−	+	74	11(15)	60(81)	3(4)
	−	97	17(18)	80(82)	0(0)

GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II

Table 3. Effect of TGF β(1 ng/ml) on *in vitro* maturation in oocytes with or without cumulus cells at 48 h after culture

Presence of cumulus cells	Addition of TGF β	No. of oocytes examined	No.(%) of oocytes		
			GV	P-I~T-I	M-II
+	+	96	1(1)	28(29)	67(70)a
	−	106	11(10)	40(38)	55(52)ab
−	+	69	6(9)	39(57)	24(35)b
	−	62	6(10)	40(65)	16(26)b

GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II
a-b, $P < 0.05$

To examine the effect of TGFβ and cumulus cells during *in vitro* maturation, oocytes with or without cumulus cells were cultured in medium with various addition times of TGF β. At the 24 h after culture, the maturation rates were higher in oocytes cultured with(5 and 4%) than without(0 and 0%) TGFβ despite of presence of cumulus cells(Table 2). However, the proportions of oocytes matured to metaphase-II at 48 h after culture were significantly($P < 0.05$) higher in medium with(70 and 52% for with and without TGF β) than without(35 and 26% for with and without TGF β) cumulus cells(Table 3).

In the third experiment(Table 4), oocytes were cultured in medium with or without TGF β (1ng /ml) for various durations after culture. In oocytes with cumulus, there were not significant differences in the maturation rates(60, 65, 71 and 54%) in oocytes exposured with various durations during the *in vitro* maturation. However, the proportions of oocytes matured to metaphase-II were significantly($P < 0.05$) higher in medium with TGF β for 0~24(59%) h or 24~48(57%) h than in medium with(27%) or without(38%) TGF β for 48 h after culture.

Ⅳ. DISCUSSION

In preparation of the oocyte for fertilization, not only must meiotic maturation occur, but the cytoplasm of the oocyte must undergo critical changes in order to achieve competency to support sperm chromatin decondation and subsequent male pronuclear formation. Although nuclear maturation of oocytes could be achieved spontaneously *in vitro*, these *in vitro* matured oocytes may lack the ability to decondense sperm chromatin and subsequently to form male pronuclei. Cumulus cells play a very important role in the cytoplasmic maturation(Critser et al., 1986). Yoshida et al.(1992) and Zheng and Sirard(1993) showed enhancement of cytoplasmic maturation when enclosed-oocytes were cultured in medium supplemented with follicular fluid or follicular shells. These results strongly indicate that follicular cells secrete factors regulating cytoplasmic maturation of oocyte via paracrine and autocrine mechanism.

Growth factors such as transforming growth factors(TGF β, Feng et al., 1988 ; TGF α, Br-

Table 4. Effect of exposure time of TGFβ during *in vitro* maturation in oocytes with or without cumulus cells

Presence of	Duration of the presence TGFβ(h. after culture)		No. of oocytes	No. of oocytes		
cumulus cells	0~24	24~48	examined	GV	P-I~T-I	M-II(%)
+	+	+	58	5	18	35(60)a
	+	−	60	3	18	39(65)a
	−	+	63	3	15	45(71)a
	−	−	63	3	26	34(54)a
−	+	+	67	6	43	18(27)b
	+	−	63	1	25	37(59)a
	−	+	69	5	35	39(57)a
	−	−	79	6	43	30(38)b

GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II
a-b, $P < 0.05$

ucker et al., 1991) have been demonstrated to stimulate or enhance nuclear maturation in oocytes. The present study were examined effect of TGF β in the stimulation of both oocyte maturation and cumulus cells. However, it was not different at various concentrations of TGF β during *in-vitro* maturation(Table 1). In some study, TGF was previously shown to stimulate oocyte maturation in rats and mice(Brucker et al., 1991 ; Tsafriri et al., 1991). TGF is structurally and functionally related to EFG(Marquardt et al., 1983), a factor show to stimulate porcine oocyte maturation *in vitro*(Singh and Armstrong, 1992 ; Arellano et al., 1993 ; Coskun and Lin, 1993 ; Reed et al., 1993).

TGF β can stimulate or inhibit aspects of cellular growth and differentiation depending on species, culture conditions, and the steroid being evaluated(Mulheron and Schomberg, 1993). It is believed that the ability of TGF β to inhibit cell growth allows for a more differentiated state of the cell(Skinner and Parrot, 1994). Failure of TGF β to alter morphological and steroidogenic responses, as well as cell cycle distribution of bovine granulosa cell function *in vitro*, may reflect the absence of TGF β recepter on bovine granulosa cells after 4 days in these culture conditions or the insensitivity of these granulosa cells to TGF β. Difference in amino acid sequence between porcine and bovine TGF β can not account for the lack of response, as these sequences are identical in human, bovine and porcine TGF β(Ackland et al., 1992).

In this study, TGF β did not signigicantly affect maturation of porcine oocytes with or without cumulus at 24 h after culture(Table 2). However, it has showed that TGF β is a potent stimulator of meiotic resumption in both cumulus-intact and cumulus-free oocytes at 24 h after culture. In contrast, Mullerian inhibiting substance, which has close homology to TGF β (Pfeilschifter, 1990), inhibited spontaneous rat oocyte maturation(Uneo et al., 1988). Although TGF β alone had no effect on spontaneous rat oocyte maturation, it suppressed LH-induced rat oocyte maturation *in vitro*(Tsafriri et al., 1989).

Cumulus-oocytes complexes are comprised of cumulus cells and oocytes that are morphologically and functionally associated with each other through heterologous gap junctions(Albertini and Anderson, 1974). It has been reported that communication via gap junctions between cumulus cells and oocytes provide avenues for the bidirectional transfer of information(Downs, 1993). Two theories concerning the relationship of gap junctions to meiotic maturation have been proposed. The first theory is that gap junctions may mediate the transfer of inhibitory substances from cumulus cells to the oocyte(Sherizly et al., 1988). The second theory suggests that a positive stimulus is generated by cumulus cells in response to hormones, and this stimulus is then transferred into oocytes via gap junctions to stimulate maturation(Downs et al., 1988). Since TGF β was unable to enhance spontaneous maturation of denuded oocytes alone or in coculture with cumulus cells, this data seem to support the latter hypothesis.

The present study confirmed that TGF could stimulate nuclear maturation of porcine oocytes *in vitro* in an apparently exposure time-dependent manner in oocytes without cumulus cells (Table 4). These results suggests that because of the interaction between TGF β and cumulus cells, TGF β was required for 24 h only during *in vitro* maturation in porcine oocytes. Immunohistochemical studies have confirmed that TGF α and TGF β are present in the thecal /interstitial cells of rat and bovine ovaries(Campbell et al., 1996 ; Kudlow et al., 1987) and granulosa cells of rat preovulatory follicles(Teerds and Dorrington, 1995).

In summary, the present study shows that TGF β can stimulate nuclear maturation in porcine oocytes. These results indicated that cumulus cells is essential for *in vitro* maturation in porcine oocytes but TGF β can promote oocytes maturation in cumulus-free oocytes.

V. SUMMARY

This study was undertaken to evaluate the interaction between cumulus cells and TGF β1 on *in vitro* maturation in porcine oocytes. No differences were found in maturation rates when follicular oocytes were cultured in medium with various concentrations of TGF β. At 24 h after maturation, the oocytes matured to metaphase-II were found in medium with TGF β regardless of cumulus cells. On the other hand, the maturation rates were significantly($P < 0.01$) higher cumulus-enclosed(70 and 52%) than cumulus-denuded oocytes(35 and 26%) in medium with or without TGF β at 48 h after culture. In another experiment, the same maturation rates (54-71%) were observed when cumulus-enclosed oocytes were cultured with various addition time of TGF β. However, the maturation rates in cumulus-denuded oocytes were significantly ($P < 0.05$) higher in medium added at 0~24 h (59%) or 24~48 h(57%) after culture than in medium with(27%) and without(38%) TGF β for 48 h. These results indicated that cumulus cells is essential for *in vitro* maturation in porcine oocytes but TGF β can promote oocytes maturation in cumulus-free oocytes.

VI. REFERENCES

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