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The Role of Hyaluronan in Porcine Granulosa Cells and Its Apoptosis Inhibitory Mechanisms

著者	Woro Anindito Sri Tunjung
号	45
学位授与機関	Tohoku University
学位授与番号	農博第958号
URL	http://hdl.handle.net/10097/60296

	ウォロ アニンディト スリ トゥンジュン
氏名（本籍地）	Woro Anindito Sri Tunjung
学位の種類	博士（農学）
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論文題目	The Role of Hyaluronan in Porcine Granulosa Cells and Its Apoptosis Inhibitory Mechanisms（ブタ顆粒層細胞のアポトーシス抑制メカニズムにおけるヒアルロン酸の役割）
博士論文審査委員	（主査）教授 佐藤英明 教授 加藤和雄 教授 西森克彦

論 文 内 容 要 旨

Introduction

During this decade porcine has become one of the main industrial animals. Besides food production, in fact, research using porcine is remarkably increasing, genetic engineering to create excellent domestic animals, somatic clone porcine, production of transgenic animals and female inheritance related researches are only few representative examples showing the high potentiality of the porcine model. Intriguing biotechnological applications are the use of spare parts for xenotransplantation (transplanting organs/tissues from other species to humans) of degenerative diseases such as diabetes mellitus, heart disease etc. Porcine becomes the most popular choice because of physiological reasons (such as compatibility of blood vessel, blood type, size etc) and practical reasons in porcine production.

To sustain all these technologies a continuous supply of high quality oocytes should be warranted. Oocyte growths and develops inside the ovarian follicle before its ovulation. Several millions of follicles at varying stages are present in each ovary, but the majority of them (> 99%) does not complete their developmental process, and undergo to a degenerative process called atresia. The consequence of such a big reduction from the resting pool of growing follicles is that, during animal lifetime, less than 1% of oocytes will reach ovulation. Hence, atresia limits the number of oocyte available for fertilization and embryonic development.

Atresia is initiated by the death of granulosa cells via apoptotic mechanism followed by oocyte degeneration. In order to rescue follicles from atresia and, indeed, to increase the number of ovulatory follicles, it is of great interest to elucidate the mechanism of induction of follicular atresia in porcine granulosa cells. Atresia mainly occurs in follicles that have reached the multilayered–small preantral stage, when granulosa cells start to express gonadotropin receptors. Because of this fact, gonadotropin surge is thought to be an important factor in controlling atresia. The two principal gonadotropins are follicle stimulating hormone (FSH) and luteinizing hormone (LH). Consistent with *in vivo* studies, FSH is well known to function as apoptotic suppressor in granulosa cells under *in vitro* condition in many species. However, the mechanism by which FSH inhibits apoptosis in granulosa cells is still unclear.

Our previous study (Kimura N *et al*, 2002) revealed that FSH stimulates the mRNA expression of hyaluronan synthase (*has*), the enzyme necessary for hyaluronan (HA) synthesis, in porcine cumulus cells. This result evidenced the close correlation between FSH and hyaluronan synthesis. Hyaluronan (HA) is a glycosaminoglycan widely distributed in the extra cellular matrix of most mammalian tissues. In the ovary, HA is mainly detected during cumulus expansion, a process important to sustain a successful ovulation. We have previously reported that HA naturally produced by cumulus cells prevents fragmentation of oocytes in culture (Sato E *et al*, 1987). Recently, we have

detected HA in the expanded cumulus cell during cumulus expansion and oocyte maturation (Yokoo M *et al*, 2004), thus indicating that HA is an important factor for oocyte survival and maturation. However, the function of HA in granulosa cells remains unclear. Combined with these finding, HA could be a good candidate for inhibiting apoptosis in porcine granulosa cells. Furthermore we hypothesized that FSH possibly inhibits apoptosis in granulosa cells through HA.

Therefore, the objective of this study was to elucidate the ability and the possible mechanisms by which HA could inhibit apoptosis in porcine granulosa cells.

Study 1
Inhibitory action of follicle stimulating hormone on apoptosis of granulosa cells in porcine COCG culture involves hyaluronan synthesis

We established an *in vitro* culture system using oocyte cumulus cells complex anchored to granulosa layer (COGC) (Fig.1). It is well known that in *in vivo*, bi-directional interactions between oocyte and their somatic neighbor cells (cumulus and granulosa cells) are a crucial factor from follicle formation to ovulation. Since follicle atresia is initiated by apoptosis of granulosa cells followed by oocyte degeneration, the COCG culture system could be a valid model to study follicle apoptosis *in vivo*.

Porcine antral follicles having a diameter of 3-5 mm were isolated from the ovaries and were punctured using a scissors. COCG was mechanically dissected from the healthy follicles using fine forceps. Granulosa layers were then cultured for 48 h in basic medium (DMEM/F12) alone (control group) or were supplemented with either 50 mU FSH; 50 mU FSH and 0.1, 0.5, and 1 mM 4-methylumbeliferone sodium salt (4-MU, a HAS inhibitor which selectively inhibites only HAS without inhibiting other glycosaminoglycans.); or 50, 100, and 200 μg/ml HA (Molecular weight: 100.000 – 150.000 Da).

Mammalian cells express 3 different isoforms of HAS: HAS1, 2 and 3, all able to catalyze the HA synthesis. Fig. 2 showed the expression of *has*1, 2 mRNA in COCG cells by RT-PCR. After 2 h of culture, *has*1, 2 mRNA was expressed in granulosa layers, reached a maximum after 6 h but decreased after 24 h. Since no expression was detected in the medium alone (control), *has* mRNA expression was FSH dependent. HAS produces HA at the inner side of the plasma membrane and extrude it through the membrane into the extra cellular space. Hence, the synthesized HA might not be retained in granulosa cells but directly flowed into the culture medium. To ascertain this, we analyzed, by means of a HA measurement kit, the content of secreted HA in the culture medium. Fig. 3 showed a significant difference in HA concentrations in the culture medium between granulosa cells cultured without or with FSH supplementation. Furthermore, higher concentration of the HAS inhibitor 4-MU (0.5 and 1 mM) significantly decreased the HA concentration. These

results indicate that HA can be secreted by FSH stimulation and when HAS function is inhibited by 4-MU, the HA synthesis by granulosa cells is perturbed as evidenced by a significant decrease in its concentration.

In general, there are 2 major upstreams of apoptosis signaling pathways in cells: an extrinsic and an intrinsic pathway. Activation of the extrinsic pathway (caspase-8) through cell surface death receptors or activation of the intrinsic/mitochondrial pathway (caspase-9) triggered the initiation of the caspases cascade. Accordingly, our result (Fig. 4A) showed a decreased expression of procaspase-8, -9 protein (i.e. the precursor of caspase) in progressing atretic follicles, in which most cells were apoptosis. This finding makes us to suppose that those procaspases are already activated. Moreover, presented results also indicated that porcine atresia in follicle apoptosis involved both extrinsic and intrinsic pathways. The expression of procaspase-3, the executer of apoptosis, decreased in progressing atretic follicles suggests that procaspase-3 was already activated into cleaved caspase-3. Our present study showed that the activation of procaspase-8, -9, the upstream activator, and caspase-3, the downstream effector of the caspase cascade, may trigger an initiation of apoptosis.

As shown at Fig. 4B, procaspase-3, -8, -9 expression was high in granulosa cells cultured in a medium supplemented with FSH alone and FSH + 0.1 mM 4-MU, whereas low expression was detected when they were cultured with FSH + 0.5 or 1 mM of 4-MU. Moreover, a high expression of procaspase-3, -8, -9, detected under supplementation with different concentrations of exogenous HA, suggesting a dose-dependent suppression of procaspase-3, -8, -9 activation by exogenous HA. In order to obtain more evidence, we supplemented the medium simultaneously with FSH, 4-MU, and HA. Interestingly, as shown at Fig. 5, addition of higher concentrations of 4-MU decreased the expression of procaspase-3, -8, and -9 and very faint bands were observed; in particular, after addition of 1 mM the bands were very faint and almost undetectable. However, simultaneous addition of 200 μg/ml of HA produced stronger bands as compared to those without HA addition. This phenomenon indicated that addition of exogenous HA into a medium lacking of HA derived from granulosa cells could suppress procaspase-3, -8, -9 activation. In conclusion, it became evident that the HA endogenously produced by granulosa cells as well as that exogenously added, control the activation of both initiator and executor of the caspase cascade.

Once caspase-3 has been activated by other caspases, it cleaves a variety of proteins which contributes to the condensation and fragmentation of DNA, a hallmark of apoptosis. To deeply investigate the caspase cascade in porcine granulosa cells, we analyzed the DNA condensation (apoptotic rate) using hoescht staining. We cultured granulosa cells taken from healthy follicles, in which the apoptosis rate was almost 0% (Fig. 6A). Our result as shown at Fig. 6B showed that apoptosis rate of granulosa cells after cultured for 48 h without FSH addition was very high, whereas it significantly decreased

after FSH treatment. Moreover, the apoptosis rate in granulosa cells increased when the secretion of HA was inhibited. In addition, exogenous HA suppressed the apoptosis rate in granulosa cells in a dose-dependent manner.

Fig. 7 is a panel of pictures representing the morphology of COCG before and after culture for 48 h in presence of FSH (Fig. 7C), FSH plus 4-MU at increasing concentrations (0.1, 0.5 and 1 mM, Fig. 7D-F) and exogenous HA in various concentrations (50, 100, 200 μg/ml; Fig. 7G-I). After 48 h of culture in the control medium, granulosa cells became shrunken, with a condensed COCG layer, rich of black and unclear figure. The connection between COC and granulosa cells was very weak. This phenomenon is a sign of apoptosis. In the contrary, granulosa cells in the COCG cultured for 48 h with FSH 50 mU were not shrunken, and the connection between COC and granulosa cells was tighter (Fig. 7B). The morphological condition of granulosa cells evidenced that FSH inhibit apoptosis in granulosa cells. The 4-MU and HA treatments induced similar morphology with FSH treatment.

In summary, study 1 provided evidences that FSH stimulated the synthesis of HA in porcine granulosa cells. Furthermore, FSH, HA derived from granulosa cells and exogenous HA inhibited procaspase activation and suppressed apoptosis rate, thus suggesting that HA has the ability to inhibit apoptosis in granulosa cells. It should be noted that one of inhibitory mechanism of FSH might be mediated through stimulation of HA synthesis.

Study 2
The binding of hyaluronan to CD44 involves apoptosis inhibition of granulosa cells in porcine COCG culture

Since HA is located in the extracellular matrix, it needs hyaluronan binding proteins to organize the hyaluronan-rich matrix and function within the cell. CD44 is well known as a principal cell surface receptor of HA. The present study was aimed to elucidate the binding of HA with CD44 and its function on apoptosis inhibition.

We analyzed the expression of CD44 in granulosa cells and the main result of this study, as shown in Fig.8, is that CD44 was not expressed in granulosa cells from healthy, early atretic and progressing atretic follicle. On the other hand, after supplementation with FSH, granulosa cells began to express CD44. However, when HA synthesis by granulosa cells was inhibited using 4-MU, the expression of CD44 by granulosa cells decreased, suggesting that when the content of secreted HA in culture medium decreased the expression of CD44 also decreased. Interestingly, the expression of CD44 gradually increased with the culture time: in fact, no band detected at 0 and 2 h, slight expression was evident after 24 h and strong expression at 48 h. No bands were detected without FSH addition. Moreover, when granulosa cells did not synthesize HA, they mostly did not

express CD44. These results indicate that when the amount of secreted HA increased, the CD44 expression also increased. Our data became a new finding that the amount of synthesized HA could be detected by the expression of CD44. Accordingly with the results of *has* mRNA expression in study 1, it is possibly that granulosa cell synthesize HA in the presence of *has* mRNA. Then the secreted HA binds cell surface receptors such as CD44 that transduces intracellular signals, thus influencing the cellular form and function directly inside the cell. Because of this reason, expression of CD44 is delayed if compared to *has* mRNA expression. Meanwhile, these became the first evidence that porcine granulosa cells also synthesized HA and expressed CD44 in *in vitro* condition, being comprehensive with the evidences obtained about oocyte and cumulus cells. Also, FSH treatment stimulated both HA synthesis and its receptor in granulosa cells.

Furthermore addition of IM7, anti-CD44 antibody that reduces the HA binding ability to CD44, inside the culture medium containing FSH decreased the expression of procaspase-3, -8, -9 (Fig. 9) compared to FSH alone or normal rat IgG. We found that although granulosa cell synthesized HA, separation of HA-CD44 binding led to the activation of caspase-8, -9 (the initiator of apoptosis) and caspase-3 (the executer of apoptosis). These results suggested that HA binds cell surface receptors such as CD44 that transduces intracellular signals, thus directly functioning.

In summary, study 2 provided evidences that there is a close relation between the HA synthesis and CD44 expression. Moreover, sufficient interaction between HA and its receptor is necessary for cell survival signaling. Hence, HA-CD44 interaction was implicated in apoptosis inhibition of granulosa cells.

Study 3
Hyaluronan inhibit apoptosis of granulosa cells in porcine COCG culture through PI3K/Akt pathway

Recent studies revealed that survival and growth factors inhibit apoptosis in many cells through phosphoinositide 3-kinase (PI3K)/Akt pathway. PI3K is an enzyme implicated in growth factor signal transduction by associating with receptor and non receptor tyrosine kinases. Akt is a serine/threonine protein kinase that mediates the downstream effects of PI3K. The present study was aimed to elucidate the apoptosis inhibitory action of HA in porcine granulosa cells by analyzing the relation between HA and PI3K/Akt pathway.

As shown in Fig. 10, we found that PI3K and Akt were detected in granulosa cells from healthy and early atretic follicle, but decreased their expression from very low to undectable level in progressing atretic follicle. Since most cells in progressing atresia are apoptotic cells, the decreased expression of PI3K and Akt in progressing atresia indicated that PI3K/Akt pathway is involved in the apoptosis mechanism of porcine granulosa cells.

Accordingly, Fig. 11 showed that PI3K and Akt were also expressed in granulosa cells cultured with FSH or HA, but not in medium alone (control). Furthermore, when HA synthesis was inhibited using 4-MU, the band of PI3K and Akt also decreased, indicating that HA possibly functions through PI3K/Akt pathway.

Meanwhile we examined the relation between HA-CD44 and PI3K/Akt pathway by analyzing the PI3K expression after culturing COCG cells with IM7, an anti-CD44 antibody (Fig. 12). The expression of PI3K decreased after IM7 treatment, compare with FSH alone or FSH + normal rat IgG. This finding revealed that the binding of HA to CD44 activated the PI3K signaling pathway inside the granulosa cells, and perturbation of the binding of HA-CD44 led to a decreased of PI3K expression.

We finally analyzed the relations between PI3K/Akt and procaspases activation by adding PI3K or Akt inhibitor inside the culture medium containing FSH or HA. Firstly, we confirmed that addition of PI3K or Akt inhibitor effectively inhibited PI3K or Akt function in COCG cells (Fig. 13). Secondly, we found that procaspase-3, -8, -9 expressions decreased after addition of PI3K or Akt inhibitor, thus indicating that inhibition of PI3K or Akt function led an activation of procaspases (Fig. 14).

In summary, study 3 provided evidences that HA inhibit apoptosis in porcine granulosa cells through PI3K/Akt pathway.

Summary and conclusion

The main findings obtained from the present studies are as follows (Fig. 15):

1. HA synthesis in granulosa cells was stimulated by FSH addition.
2. HA binds CD44 and transduces the signals directly into the cell.
3. The binding of HA to CD44 activated the PI3K/Akt pathway.
4. PI3K/Akt inhibited procaspase-8, -9 (the initiator of apoptosis) and procaspase-3 (the executer of apoptosis) activation.

In conclusion, HA is FSH-dependently synthesized and a survival factor for preovulatory follicle by inhibiting apoptosis in granulosa cells.

References

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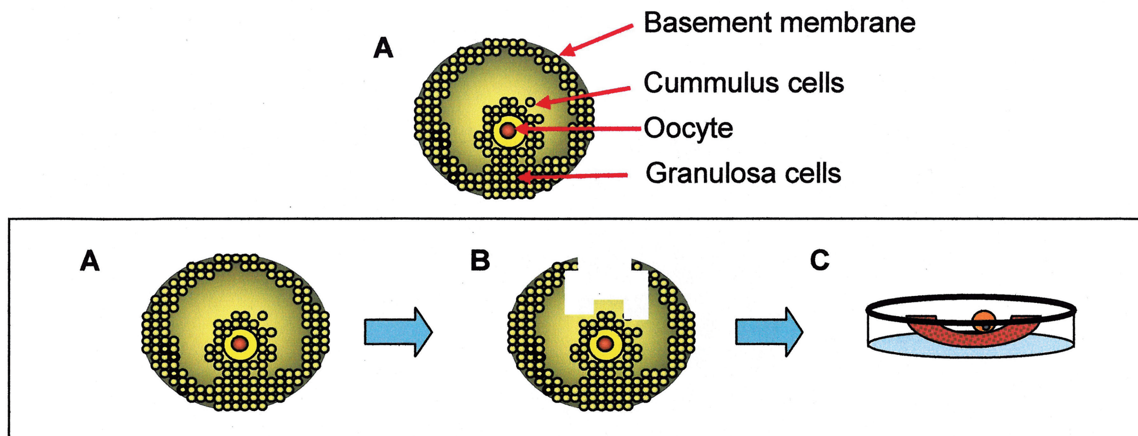


Fig. 1. The schematic of COCG culture system. A. Inside the ovarian follicle. B. Removal of basement membrane. C. Establish an in vitro culture system using oocyte cummulus cell complex anchored to granulosa layer (COCG).

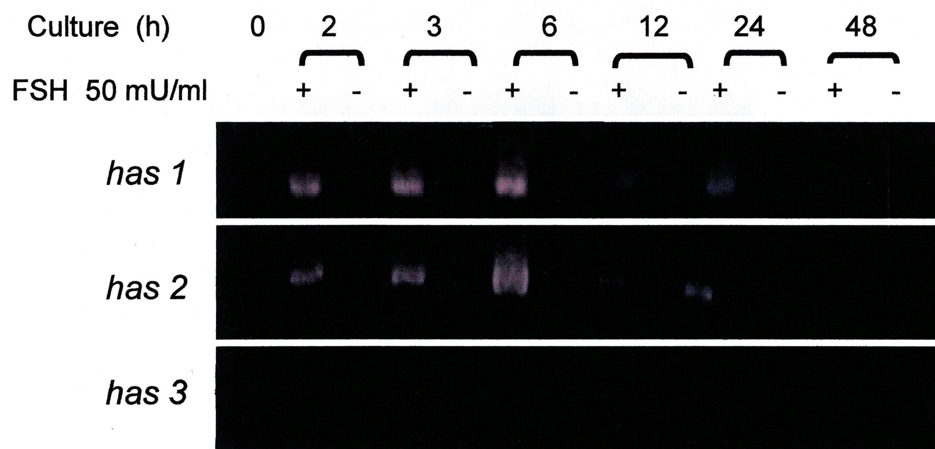


Fig. 2. Effect of FSH on *hyaluronan synthase* (*has*) mRNA expression in COCG cells.
COCG was cultured in several time course in DMEM/F12 supplemented with or without 50 mU FSH.

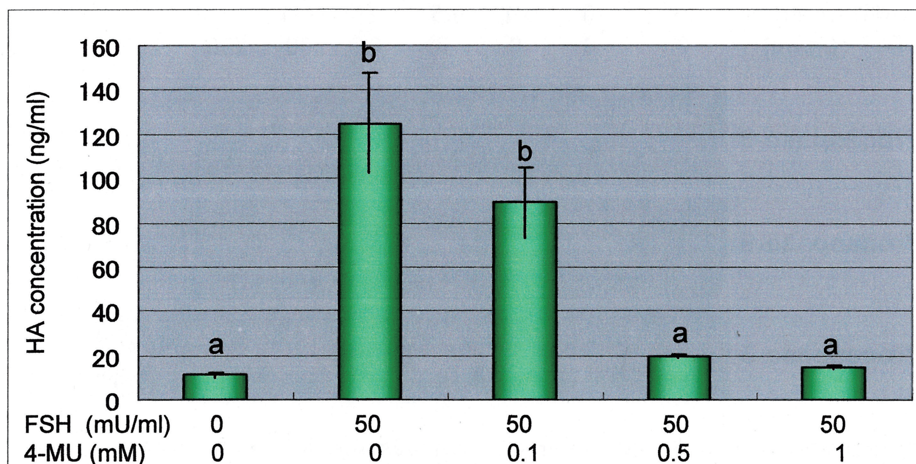


Fig 3. Effect of FSH on HA synthesis in COCG cells.
COCG was cultured for 48 h in DMEM/F12 supplemented with 50 mU FSH or 50 mU FSH + 0.1, 0.5,1 mM 4-MU and HA secretion in the culture medium was detected by using the HA measuring kit.
Values are mean ± SEM of 3 replicates. Data were analyzed by one-way ANOVA followed by Bonferroni/Dunn test. Different letters indicate significant differences ($P < 0.05$).

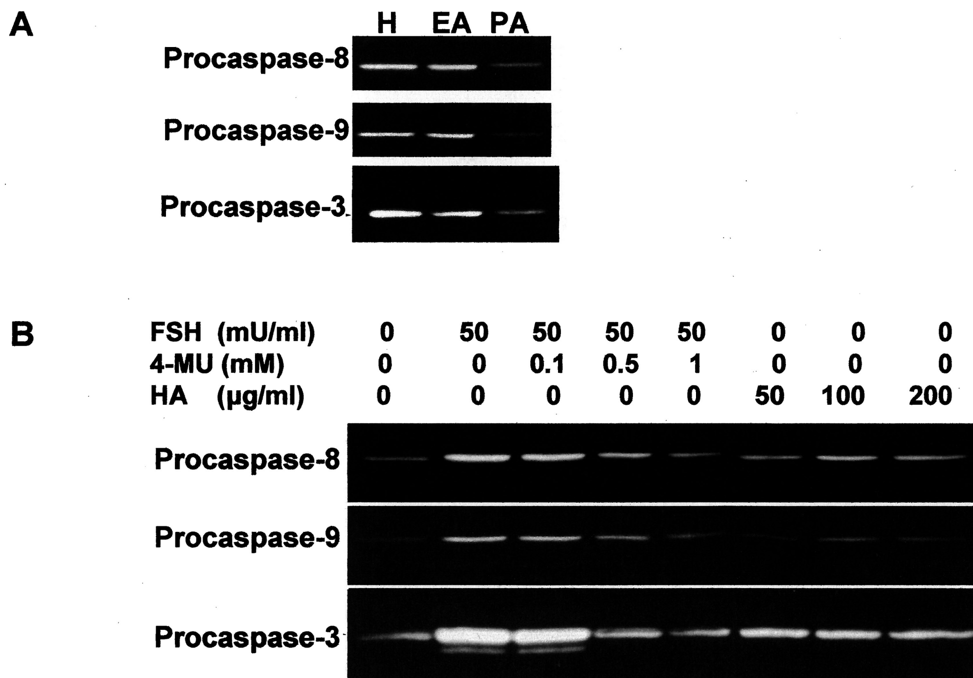


Fig. 4. Expression of procaspase-3, -8, -9 in granulosa cells.
COCG 0 h (A) and after 48 h culture (B). H: healthy follicle, EA: early atretic follicle, PA: progressing atretic follicle. COCG cells after 48 h culture with the addition of 50 mU FSH or 50 mU FSH + 0.1, 0.5, 1 mM 4-MU or 50, 100, 200 µg/ml HA (B). Experiments were repeated 3 times and similar results were obtained.

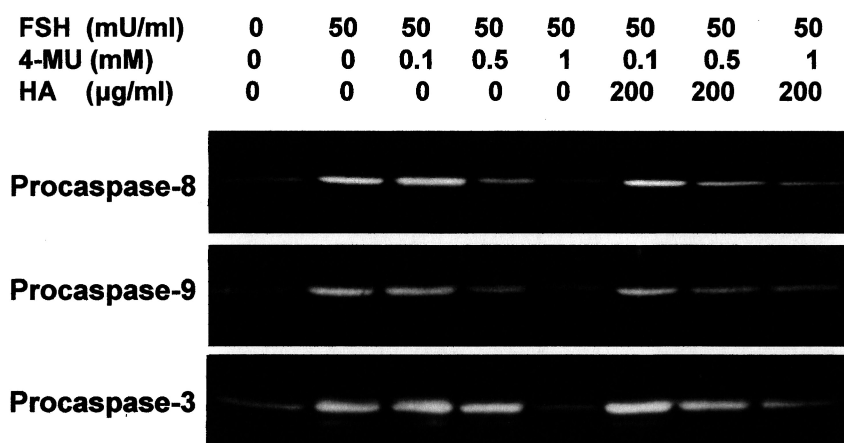


Fig. 5. Expression of procaspase-3, -8, and-9 after simultaneous addition of FSH, 4-MU, and HA.
COCG was cultured for 48 h in DMEM/F12 supplemented with FSH 50 mU + 4-MU (0.1, 0.5, 1 mM) + HA 200 µg/ml. By simultaneous addition with 200 µg/ml l of HA, the band of procaspase-3, -8, -9 became stronger compared to without HA addition.

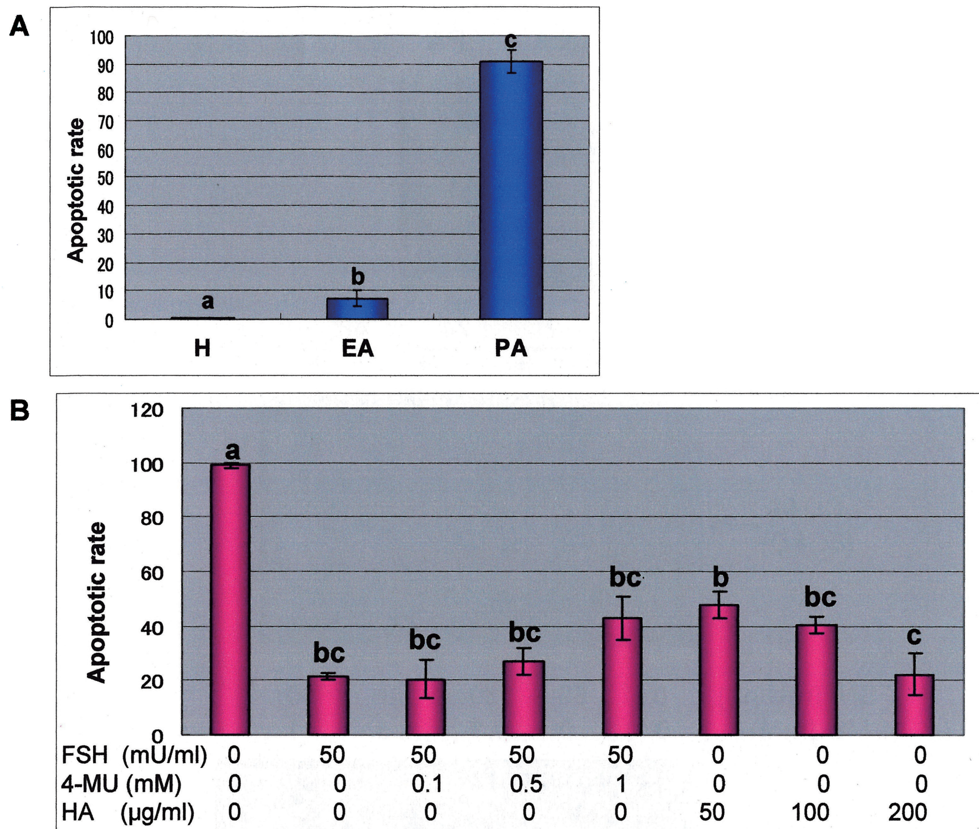


Fig. 6. The apoptotic rate of granulosa cells 0 h (A) and after 48 h culture (B). H: healthy follicle, EA: early atretic follicle, PA: progressing atretic follicle. COCG cells after 48 h culture with the addition of 50 mU FSH or 50 mU FSH + 0.1, 0.5, 1 mM 4-MU or 50, 100, 200 μg/ml HA (B).
Values are mean ±SEM of 3 trials. Different letters indicate significant differences ($P<0.05$)

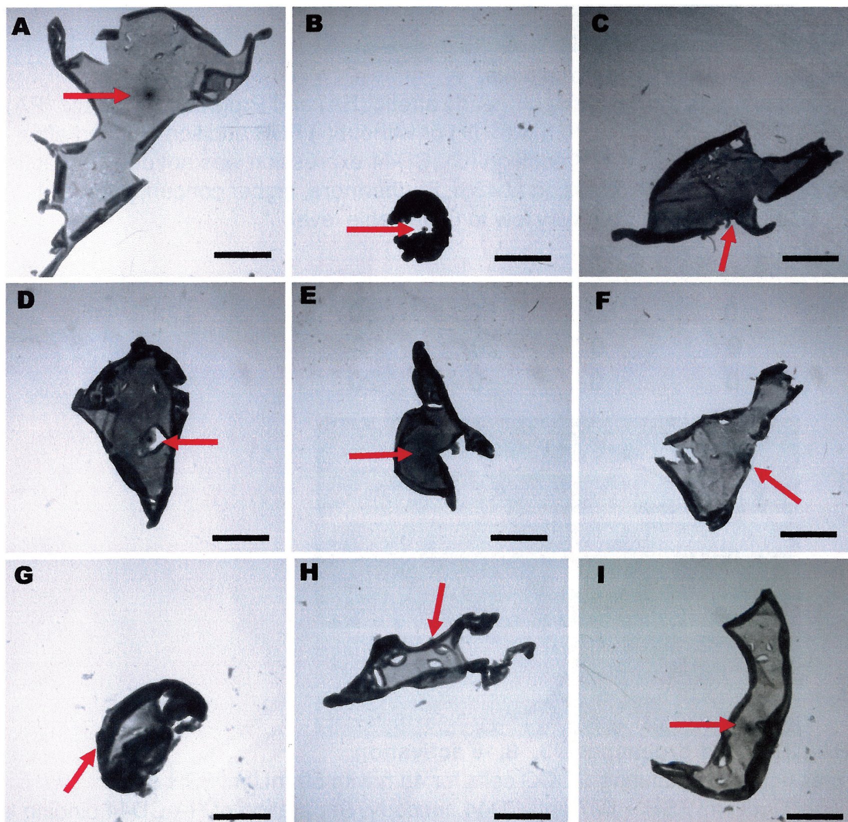


Fig. 7. Morphology of COCG after cultured for 48 h.
COCG 0 h (A);
COCG 48 h, medium only (B);
50 mU FSH (C);
50 mU FSH + 0.1 mM 4-MU (D);
50 mU FSH + 0.5 mM 4-MU (E);
50 mU FSH + 1 mM 4-MU (F);
50 μg/ml HA (G);
100 μg/ml HA (H);
200 μg/ml HA (I).
Granulosa cells without FSH and HA treatment (Panel B) show apoptosis.
The arrows indicate COCs.
Bar = 1 mm.

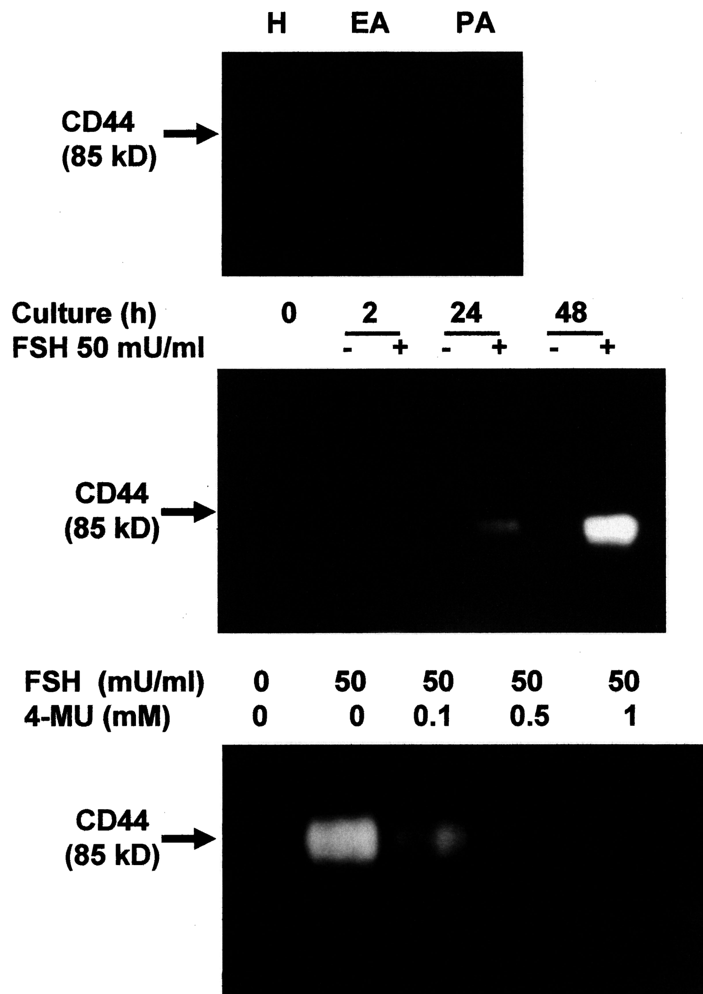


Fig. 8: Effect of FSH on CD44 expression in granulosa cells.
Study of CD44 expression in granulosa cells from healthy (H), early atretic (EA) and Progressing atretic (PA) follicle (A); after culturing COCG cells for 0, 2, 24 and 48 h with (+) or without (-) FSH addition (B); and after culturing COCG cells for 48 h with FSH or FSH + 4-MU addition (C). CD44 expression was not detected at 0 and 2 h, slight expression after 24 h, and strong expression at 48 h. Furthermore, higher concentration of 4-MU (0.5 and 1 mM) decreased CD44 expression from very low to undectable level.

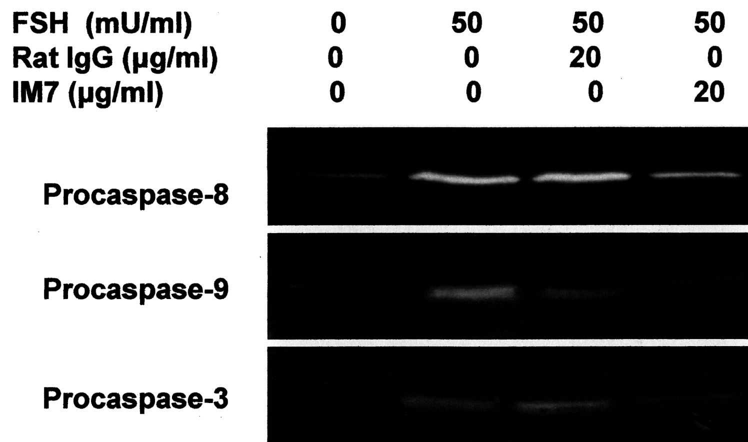


Fig. 9. The relation between HA-CD44 and procaspase-3, -8, -9 activation
Study of procaspase-3,-8,-9 expression after culturing COCG cells for 48 h with 50 mU/ml FSH or 50 mU/ml FSH + normal rat IgG or 50 mU/ml FSH + IM7,anti-CD44 antibody. Separation of HA-CD44 binding led to the activation of procaspase-3,-8,-9.

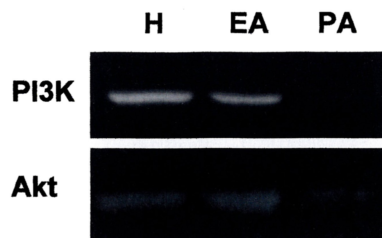


Fig. 10. The relation between PI3K/Akt and apoptosis in porcine granulosa cells.
Study of PI3K or Akt expression using western blot in healthy (H), early atretic (EA) and progressing atretic (PA) follicle. Decreased expression of PI3K and Akt in progressing atretic follicle indicated that PI3K/Akt pathway involved in apoptosis of porcine granulosa cells.

FSH (mU/ml)	**0**	**50**	**50**	**50**	**50**	**0**	**0**	**0**
4-MU (mM)	**0**	**0**	**0.1**	**0.5**	**1**	**0**	**0**	**0**
HA (μg/ml)	**0**	**0**	**0**	**0**	**0**	**50**	**100**	**200**

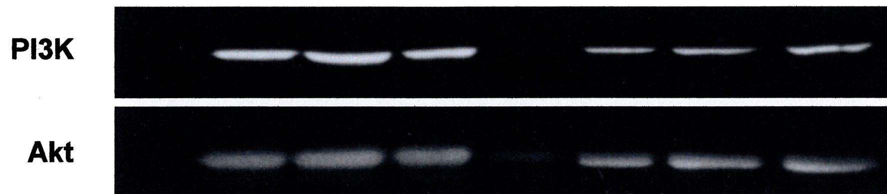


Fig. 11. The relation between PI3K/Akt and HA in porcine COCG cells
Study of PI3K or Akt expression after culturing COCG cell with FSH, FSH + 4 MU or HA.
The expression of PI3K and Akt decreased when the HA synthesis is inhibited using 4-MU.

FSH (mU/ml)	**0**	**50**	**50**	**50**
Rat IgG (μg/ml)	**0**	**0**	**20**	**0**
IM7 (μg/ml)	**0**	**0**	**0**	**20**

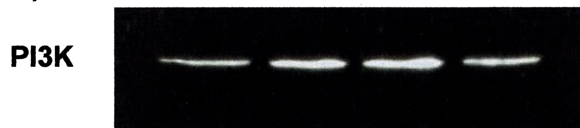
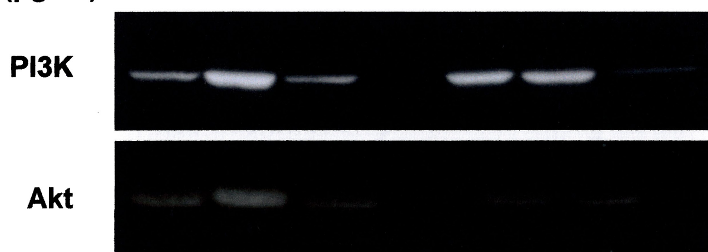


Fig. 12. The relation between PI3K and the binding of HA-CD44
Study of PI3K expression after culturing COCG cell with FSH, FSH + normal IgG, FSH + IM7, anti-CD44 antibody. Perturbation of HA-CD44 binding led to a decreased PI3K expression.

A

FSH (mU/ml)	**0**	**50**	**50**	**50**	**0**	**0**	**0**
HA (μg/ml)	**0**	**0**	**0**	**0**	**200**	**200**	**200**
LY294002 (μg/ml)	**0**	**0**	**40**	**50**	**0**	**40**	**50**



B

FSH (mU/ml)	**0**	**50**	**50**	**50**	**0**	**0**	**0**
HA (μg/ml)	**0**	**0**	**0**	**0**	**200**	**200**	**200**
Akt Inhibitor (μg/ml)	**0**	**0**	**50**	**100**	**0**	**50**	**100**

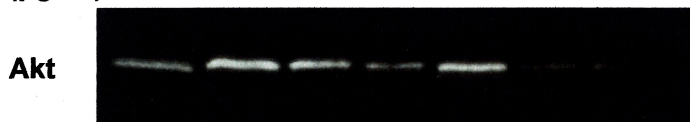


Fig. 13. The expression of PI3K/Akt after addition of PI3K or Akt inhibitor.
Study of PI3K or Akt expression after culturing COCG cell with LY294002 (PI3K inhibitor) (A) or Akt inhibitor (B). Decreased expression of PI3K and Akt confirmed that PI3K or Akt inhibitor effectively inhibited PI3K or Akt function in porcine granulosa cells.

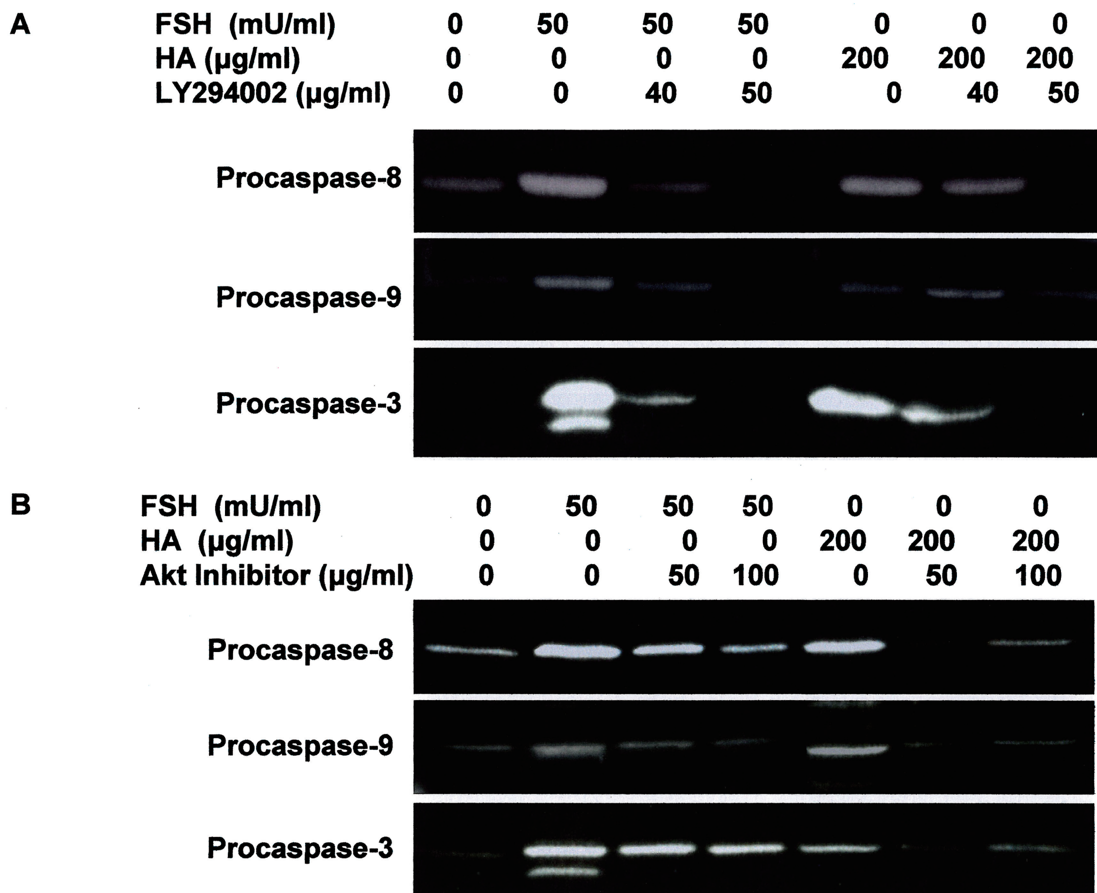


Fig. 14. The relation between PI3K/Akt and Procaspase-3, -8, -9 activation.
Study of procaspase-3,-8,-9 expression after culturing COCG cells for 48 h with LY294002 (PI3K Inhibitor) (A) or Akt inhibitor (B). Decreased expression of procaspases indicate that inhibition of PI3K or Akt function led to an activation of procaspases.

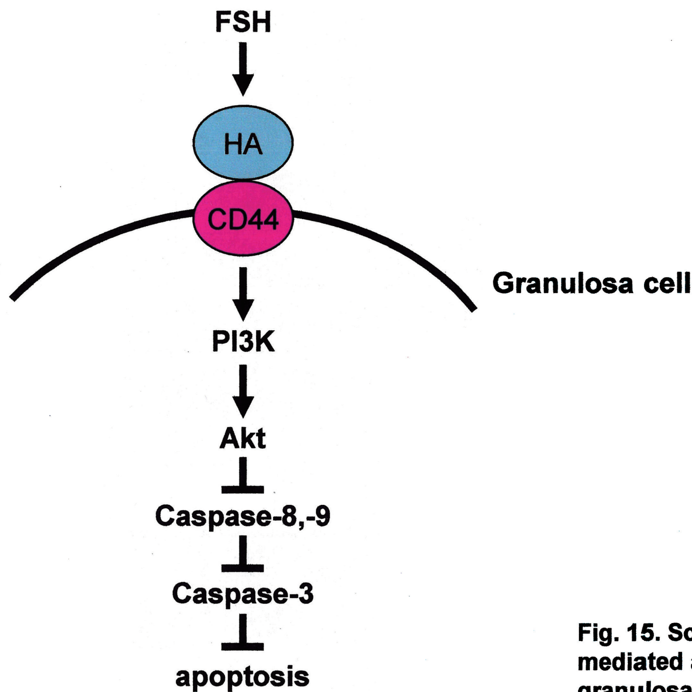


Fig. 15. Schematic representation of HA mediated apoptosis mechanism in porcine granulosa cell.

論文審査結果要旨

哺乳類卵巣では多くの卵子が卵胞の発育とともに成長するが、発育の途中で卵胞閉鎖が起こり最終的な排卵卵子は1-10数個と少なく、99%以上卵子が死滅する。卵胞閉鎖は顆粒層細胞のアポトーシスから開始するが、卵胞刺激ホールモン(FSH)が顆粒層細胞のアポトーシス抑制作用があること、FSH刺激により卵丘細胞でヒアルロン酸合成酵素（HAS）のmRNA転写レベルが高まること、ヒアルロン酸が卵子のアポトーシスを抑制することが明らかにされてきたが、顆粒層細胞におけるヒアルロン酸の役割は未だ不明な点が多い。本研究では、ブタ顆粒層細胞のアポトーシスにおけるヒアルロン酸の役割を調べ、その作用メカニズムを明らかにすることを目的とした。

ブタ卵巣内卵胞（直径3-5mm）から卵子-卵丘細胞複合体を含む顆粒層細胞を単離し、FSH、またはヒアルロン酸を含むDMEM/F12培地で48時間培養し、各種発現解析に供した。FSH添加により顆粒層細胞におけるHAS発現が高まり、培地中に分泌されたヒアルロン酸濃度も上昇した。また、FSH添加により顆粒層細胞のカスパーゼ活性が抑制され、アポトーシスが減少することが明らかになった。一方、HAS抑制剤を添加するとヒアルロン酸分泌が抑制され、顆粒層細胞のカスパーゼが活性化し、アポトーシスが増加した。また、HA分泌はFSHの刺激により高まることを明らかにした。

ヒアルロン酸分泌が抑制されるとそのレセプター（CD44）の発現も低下することやヒアルロン酸分泌とCD44の発現には相関があることを明らかにした。また、ヒアルロン酸はCD44と結合することによりカスパーゼ活性を抑制し、アポトーシスを抑制することを明らかにした。

ヒアルロン酸-CD44の下流のメカニズムを解析するため、PI3K/Akt経路に着目した。ヒアルロン酸の刺激により、PI3K/Aktの発現は高まるが、HASまたはCD44の結合を阻害すると発現は低下した。また、PI3K/Aktの発現を阻害するとプロカスパーゼ-3, -8, -9の発現は低下した。以上の結果から、ヒアルロン酸はPI3K/Aktを介してブタ顆粒層細胞のアポトーシスを抑制すると推察した。本研究はブタをモデルとして顆粒膜細胞のアポトーシス制御系を明らかにしたものであり、応用動物学分野において高く評価できる。よって博士（農学）の学位を授与できるものと判断した。