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Changes of Ganglioside GM3 Expression in Porcine Oocyte Maturation and Early Embryonic Development *In Vitro*

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ABSTRACT

Gangliosides exist in glycosphingolipid-enriched domains on the cell membrane and regulate various functions such as adhesion, differentiation, and receptor signaling. Ganglioside GM3 by ST3GAL5 enzyme provides an essential function in the biosynthesis of more complex ganglio-series gangliosides. However, the role of gangliosides GM3 in porcine oocytes during *in vitro* maturation and early embryo development stage has not yet understood clear. Therefore, we examined ganglioside GM3 expression patterns under apoptosis stress during maturation and preimplantation development of porcine oocytes and embryos. First, porcine oocytes cultured in the NCSU-23 medium for 44 h after H_2O_2 treated groups (0.01, 0.1, 1 mM). After completion of meiotic maturation, the proportion MII (44 h) was significantly different among control and the H_2O_2 treated groups (76.8±0.3 vs 69.1±0.4; 0.01 mM, 55.7±1.0; 0.1 mM, 38.2±1.6%; 1 mM, $P<0.05$). The expressions of ST3GAL5 in H_2O_2 treated groups were gradually decreased compared with control group. Next, changes of ST3GAL5 expression patterns were detected by using immunofluorescene (IF) staining during preimplantation development until blastocyst. As a result, we confirmed that the expressions of ST3GAL5 in cleaving embryos were gradually decreased ($P<0.05$) according to the early embryo development progress. Based on these results, we suggest that the ganglioside GM3 was used to the marker as pro-apoptotic factor in porcine oocyte of maturation and early embryo production *in vitro,* respectively. Furthermore, our findings will be helpful for better understanding the basic mechanism of gangliosides GM3 regulating in oocyte maturation and early embryonic development of porcine *in vitro*.

(Key words : ganglioside, ST3GAL5, apoptosis, oocyte maturation, porcine)

INTRODUCTION

In vitro culture system for embryo production including to the *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* production (IVP) until blastocyst are very important for studying the physiology of early embryo and pregnancy, biomedical research purposes, as well as the producing cloning animals (Day, 2000). Although remarkable advances in improvement efficiency of embryo development have been reported in a variety of mammals, production of commercial animals is still low due to the developmental defects of *in vitro* culture system and conditions (Popova *et al.*, 2011).

The glycocalyx is composed of glycolipids, glycosaminoglycans and glycosphingolipids (GSLs), and its covers eukaryotic cell surfaces (Kolter, 2012). And, the gangliosides are sialic acid containing glycosphingolipids found widely in the plasma membrane of most mammalian tissues and cells. They are particularly abundant in the central nervous system, and are in high abundance in neural cells. GSLs are lipids that contain one or more sugar residues. In addition, gangliosides play important roles in various cellular mechanisms such as cell apoptosis, differentiation, growth control and membrane related signaling (Kwak *et al.*, 2011).

Gangliosides regulate the activity of epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGF) and fibroblast growth factor receptor (FGFR), to effect in the absence of serum and growth factor-like in cancer mechanisms (Posse de Chaves and Sipione, 2010). Among them, particularly the ST3GAL5 catalyzes for the formation of ganglioside GM3, first produced the ganglioside (Yoshikawa *et al.*, 2015). Gang-

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lioside GM3 has the simplest carbohydrate structure and is known to be involved in induction of cell differentiation, cellular apoptotic response, modulation of cellular proliferation in embryogenesis and stem cell differentiation (Kwak *et al.*, 2011). From these functions of GM3, the importance of surface membrane components is clear. However, it is unknown which gangliosides GM3 are directly affected during the *in vitro* maturation of oocytes and early embryonic development in pigs.

Recent studies has detected that gangliosides is expressed in the matured oocytes and preimplantation stage embryos (Hwang *et al.*, 2015; Kwak *et al.*, 2003). Also, GM3 regulates follicular development and ovulation during the estrous cycle. And, as published studies, cultured granulosa cells derived from immature rat ovaries expressed GM3 in response to insulin and follicle stimulation hormone (FSH) *in vitro* (Choo, 1999). However, the activation and expression of gangliosides GM3 did not investigate in porcine oocytes and preimplantation stage embryos.

Based on this concepts, we investigated that the changes of ganglioside GM3 expression in porcine oocytes and embryos during *in vitro* production progression by immunofluorescence staining. And, our study examined whether expression pattern of ganglioside GM3 is implicated in porcine oocyte maturation and early embryonic development.

MATERIALS AND METHODS

1. Chemicals

Unless noted otherwise, all chemicals used in the present study were purchased from Sigma Aldrich Korea (St. Louis, MO, USA).

2. *In Vitro* Maturation (IVM)

Porcine ovaries were collected from at a local slaughterhouse and transported to the laboratory at 30～35℃ in 0.9% saline supplement with 75 mg/ml potassium penicillin G. Immature cumulus-oocyte complexes (COCs) were aspirated from follicles between 3 and 6 mm in diameter using an 18-gauge needle into a throwaway 10 ml syringe. After, Undamaged COCs with the same quality cytoplasm and surrounded by cumulus cells were selected using mouth pipettes and then washed three times in TL-HEPES medium, approximately 50～60 COCs were matured in 500 ml of IVM medium in 4-well multi-dish (Nunc, Roskilde, Denmark) at 38.5℃ and under 5% CO_2 in air. At this stage, NCSU-23 medium for 44 h between control and H_2O_2 (0.01, 0.1, 1 mM) treated groups was used. The medium used for oocyte maturation was Carolina State University (NCSU) 23 medium with 0.57 mM cystein, 10% follicular fluid, 10 ng/ml epidermal growth factor (EGF), 10 ng/ml β-mercaptoethanol, 10 IU/ml pregnant mare serum gonadotropin (PMSG) and 10 IU/ml human chorionic gonadotropin (hCG) (Petters and Wells, 1993) was used for oocyte maturation. After culturing for 22 h COCs were washed three times and then further cultured in oocyte maturation medium without PMSG and hCG for 22 h. During the maturation periods, H_2O_2 was added to the maturation medium. Upon completion of IVM, the oocytes were subjected to *in vitro* fertilization.

3. *In Vitro* Fertilization (IVF) and Culture (IVC)

Porcine IVF was performed as descried by Abeydeera and Day (1997). This medium, designated as modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$ 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA. Fresh semen was kindly supplied once a week by an artificial insemination company (Darby Porcine AI Center, Anseong, Korea) and kept at 17℃ for 5 days. Semen was then washed three times by centrifugation with Dulbecco's phosphate buffered saline (DBPS, Gibco BRL, Grand Island, NY) supplemented with 1 mg/ml BSA (Fraction V), 100 mg/ml penicillin G., and 75 mg/ml streptomycin sulfate. At the end of washing, the spermatozoa were resuspended in mTBM at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA (fatty acid free), after which they were placed into 48 μl of mTBM under mineral oil. Then 2 μl of diluted spermatozoa were co-incubated with oocytes for 6 h at 38.5℃ in an atmosphere of 5% CO_2 in air. Next experiments, embryos were cultured in 50 μl drops of PZM-3 medium with 3 mg/ml BSA at 38.5℃ under 5% CO_2. After 48 h of culture, 25～30 cleaved embryos were further cultured in 50 μl drops of PZM-3 medium supplemented with 3 mg/ml BSA at 38.5℃ under 5% CO_2 for 4 days. Blastocyst formation was evaluated after 6 days of culture.

4. Assessment of Meiotic Maturation

At the end of each IVM experiment a representative sample of oocytes was denuded by gently pipetting in NCSU-23 me-

dium containing 0.1% hyaluronidase, washed in PBS-PVA medium and mounted on microscope slides. The samples were fixed for 3 days in acetic acid : ethanol (1:3, v/v) solution and stained with 0.1 % acetic orcein (v/v) solution for 5 min. The samples were destained in glycerol : acetic acid : water (1:1:3, v/v/v) solution and the meiotic stage was evaluated under a microscope (Leica, Solms, Germany).

5. Immunofluorescence Staining

Oocytes and embryos were washed with 0.3% PVA-PBS and fixed in 4% (v/v) paraformaldehyde/PBS with 2.5% glutaraldehyde solution for 1 h at room temperature. Oocytes were made permeable with 0.2 % Triton X-100 at room temperature for 40 min and then incubated in 0.3% PVA-PBS overnight at 4℃. The oocytes were incubated with antibodies: anti-GM3 synthase (Santa Cruz Biotechnology, CA, USA) dilution of 500:1 in 0.3% PVA-PBS overnight at 4℃, and washed with 0.3% PVA-PBS. Oocytes were reacted with the secondary antibodies, FITC-conjugated goat anti-mouse IgG (Santa Cruz) at dilutions of 1000:1 in 0.3% PVA-PBS. DAPI reagent (2 mg/ml) was used to stain the nuclei. Finally, immunoreactivity was then observed under an epifluorescence microscope (IX 51, Olympus, Tokyo, Japan).

6. Statistical Analysis

All percentage data obtained in this study are presented as the mean ± standard deviation (SD). The results were analyzed using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test and using *t*-tests. All data were performed using the GraphPad Prism 5.0 software package (San Diego, CA, USA). A probability of $P<0.05$ was considered significant.

RESULTS

1. Changes of ST3GAL5 Expression and Oocyte Maturation Investigated in Porcine Oocyte after H_2O_2 Treatment

To investigate the expression pattern of ST3GAL5 as ganglioside GM3 synthesize enzyme, we performed the investigation of expression pattern ST3GAL5 in oocyte *in vitro* maturation. Recent study has reported that GM3 was expressed under inducing apoptotic stress and conditions, we used to the H_2O_2 as inducer of apoptotic stress in oocyte maturing *in vitro*(Vandaele *et al.*, 2010). First, under the inducing of apoptotic stress, we treated the H_2O_2 of various concentrations in IVM medium supplemented with H_2O_2 (0.01, 0.1, 1 mM). Then, we observed the fluorescence expression level of ST3GAL5 immunofluorescence staining at 22 h and 44 h, respectively (Fig. 1A and 1B). Next, we confirmed the porcine oocyte maturation and meiotic maturation as different concentration. As expected, oocyte maturation significantly decreased ($p<0.05$; 38.2±1.6%) in H_2O_2 high concentration (0.1 or 1 mM) treated groups compared to control group (76.8±0.3%) at 44 h of IVM progression (Fig. 2). These results indicate that the maturated oocyte of porcine normally has a high expression level of ST3GAL5 via inducing apoptotic condition though H_2O_2 treatment.

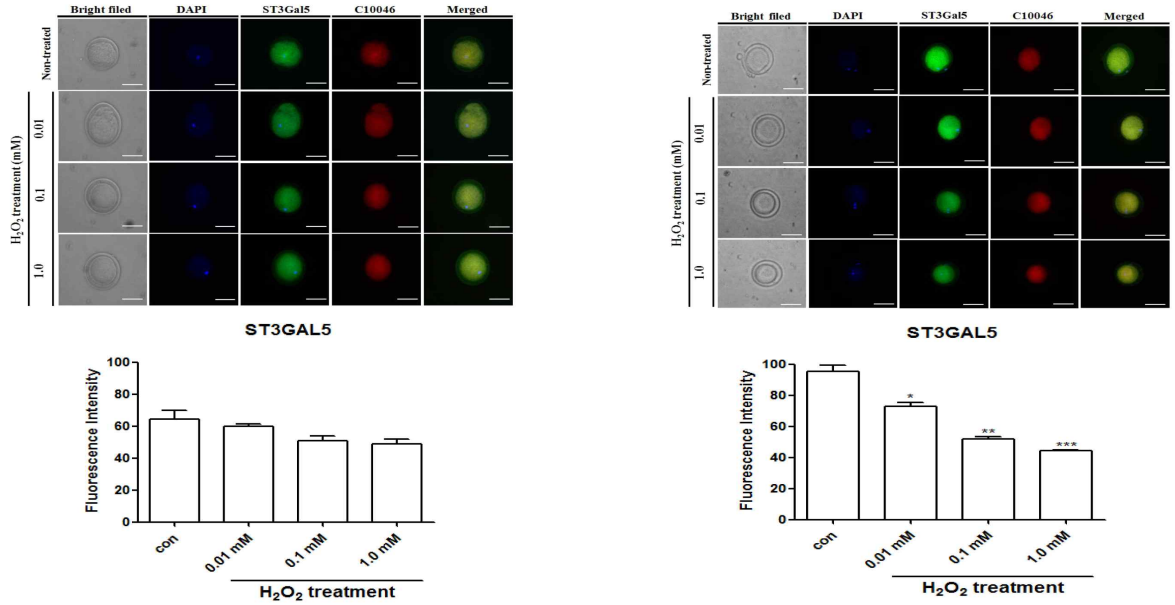
2. Detection of ST3GAL5 Expression by Using Immunofluorescence Staining in Porcine Early Embryo Development

To observe the ST3GAL5 expression in cleaved porcine embryo after *in vitro* fertilization, we performed the immunofluorescence staining (Fig. 3). Changes of ST3GAL5 expression pattern were continuously decreased in porcine embryo during early embryonic development until blastocyst.

These results demonstrated that there was a significant difference in ST3GAL5 expression decreased during embryonic development in porcine embryo.

DISCUSSION

In present study, we investigated that ST3GAL5 as ganglioside GM3 synthesizing enzyme was expressed in porcine oocyte maturation and early embryo development of *in vitro*. During maturing *in vitro*, the expression level of ST3GAL5 decreased in matured porcine oocytes according to H_2O_2 concentration-dependent (0.01, 0.1, 1.0 mM) treatment. And, meiotic maturation significantly decreased after H_2O_2 treatment. Also, the expression level of ST3GAL5 decreased in early embryo development until blastocyst. Entire *in vitro* production stage of porcine oocyte and embryo were showed the same expression pattern of ST3GAL5. Although the expression pattern of ganglioside GM3 has been reported for apoptotic stress condition and DNA damage in mouse oocyte maturation *in vitro*, the direct expression of ST3GAL5 as ganglioside GM synthesizing enzyme in porcine oocyte and embryo is unclear. The observations documented herein provided the novel evidence supporting the relationship of ganglioside GM3 or ST3GAL5 as ganglioside GM3 synthesizing enzyme between maturing *in vitro* and preimplantation development.

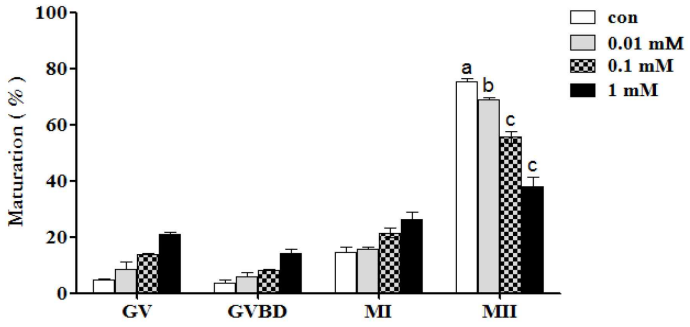


(A) IVM I : 22 h (B) IVM II : 44 h

Fig. 1. Changes of ST3GAL5 fluorescence expression patterns were observed in maturing porcine oocyte by using immunofluorescence staining. Expression of ST3GAL5 in *in vitro* maturation oocyte of porcine (A) 22 h, (B) 44 h, ST3GAL5 expression was analyzed by fluorescence microscope. DAPI: blue, ST3Gal5: GM3 synthase shown in green, C10046: Cell membrane stained dye shown in red. The fluorescence intensity of analysis was performed using Image J software (NIH). Bar graph data represent the least-squares means±SD of three independent experiments. * P<0.05, ** P<0.01, and *** P<0.001; Dunnett's Multiple Comparison test. Scale bars = 100 μM.

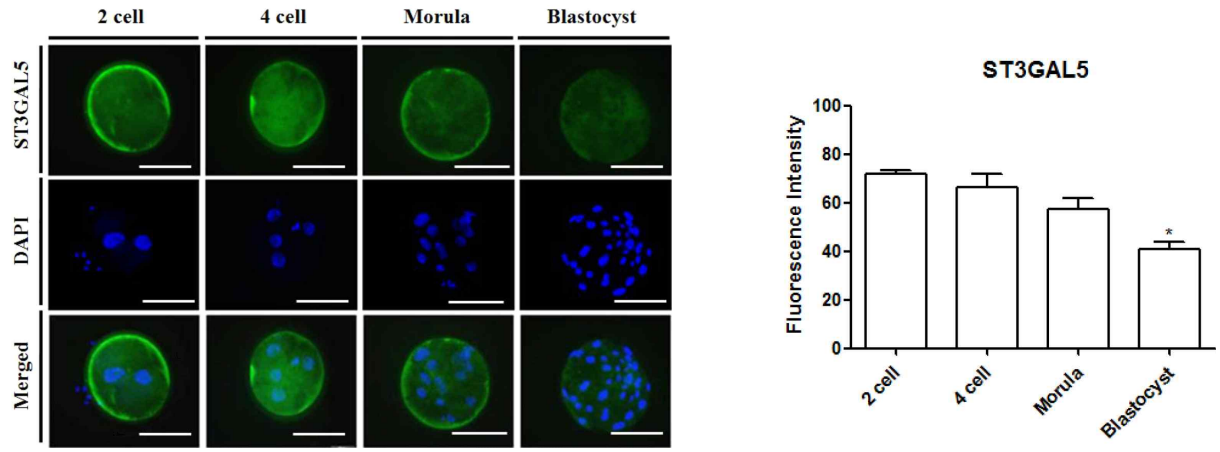
H_2O_2 (mM)	Ho. of oocytes examined	% of oocytes (n)			
		GV	GVBD	MI	MII
0	82	4.9±0.5 (4)	3.7±1.8 (3)	14.6±2.0 (12)	76.8±0.3 (63)[a]
0.01	81	8.7±1.2 (7)	6.1±0.6 (5)	16.1±0.1 (13)	69.1±0.4 (56)[b]
0.1	84	14.3±0.1 (12)	8.4±0.1 (7)	21.6±0.8 (18)	55.7±1.0 (47)[c]
1	76	21.1±0.1 (16)	14.4±0.6 (11)	26.3±1.3 (20)	38.2±1.6 (29)[c]

(A)



(B)

Fig. 2. Effects of H_2O_2 on nuclear maturation in porcine oocyte. (A) Maturation rate of porcine oocyte, (B) Percentage of MII stage observed in porcine oocyte matured in the presence or absence (control) of H_2O_2 after 0 and 44 h culture in the experiment. H_2O_2, an apoptosis stress inducer. This experiment was repeated three times. Bar graph data represent the least-squares means±SD of three independent experiments. $^{a\sim d}$ Values with different superscript letters within a column differ significantly (P<0.05). Scale bars = 100 μM.



(A) (B)

Fig. 3. Comparison of ST3GAL5 expression early developmental embryo stage by using immunofluorescence staining. Fluorescence microscopy imaging of ST3GAL5 expression (A) and fluorescence intensity (B) in 2 cell, 4 cell, morula and blastocyst stages. The chromatin content is stained by DAPI (blue). The fluorescence intensity of analysis was performed using Image J software (NIH). Bar graph data represent the least-squares means±SD of three independent experiments. * $P<0.05$; Dunnett's Multiple Comparison test. Scale bars = 100 μM.

Some ganglioside in oocytes and embryos of vertebrates have been shown to exist in the cytoplasm by immunocytochemical (IHC) studies, suggesting that cytoplasmic distribution and location of gangliosides may be a general phenomenon, at least in oocytes and embryos (Choo *et al.*, 1995). As shown in Fig. 1, the expression of ST3GAL5 as ganglioside GM3 synthesizing enzyme detected the in cytoplasm of denuded oocyte at 22 h and 44 h of in *in vitro* maturation, respectively. Moreover, the ST3GAL5 expression levels showed the cyto- plasm of embryo in 2 cell, 4 cell, morula and blastocyst.

Apoptosis in response to improper culture conditions and stress is a common physiological process that occurs in embryo development *in vitro*. Apoptosis is also crucially involved in the development and differentiation of embryos (Suzanne and Steller, 2013). Accordingly, increased apoptosis is an important indicator of improper conditions of mammalian embryos. Apoptotic cell death in preimplantation mammalian embryos has been well described. Apoptosis plays an important role in embryo development (Brill *et al.*, 1999). Therefore, we used the H_2O_2 as an inducer of apoptotic stress in oocyte maturing *in vitro*. As shown in Fig. 1A, the expression of ST3GAL5 rapidly reduced in the IVM medium supplemented with H_2O_2 (0.01, 0.1, 1 mM) at 22 h and 44 h of in *in vitro* maturation, respectively. Based on these results, we speculated the increase of ganglioside GM3 in maturing *in vitro* oocyte under apoptotic stress or inducing oxidative stress conditions. Therefore, we thought that ganglioside GM3 is very important in apoptosis response and/or oxidative stress conditions of *in vitro* cultured oocytes and embryo.

Previously many reports showed oxygen toxicity and its harmful effects on preimplantation embryos *in vitro*. Apoptosis, a type of programmed cell death, is a physiological process occurred spontaneously during normal preimplantation embryo development. Moreover, *in vitro* embryo culture process is exposed to oxidative stress, different from the vivo environment and increased production of ROS in the embryos (Dalvit *et al.*, 2005). ROS can cause irreversible damage to cellular macromolecules such as DNA, RNA, protein, and lipid membranes, which may result in altered cellular function and cellular death. Oxidative stress (OS) affects multiple physiological processes, from oocyte maturation to fertilization to embryo development and pregnancy (Agarwal *et al.*, 2005). However, apoptosis and oxidative stress also has a beneficial role in the cellular response to suboptimal developmental conditions and stress. In addition, it is not clear whether GM3 treatment or gangliosides affect the oocyte maturation and preimplantation in porcine.

Recently, it was reported that GM3 expression was increased in diabetic db/db mice during ovarian maturation in primary and Graafian follicle (Kwak *et al.*, 2003). The significant increase in the expression of ganglioside GM3 synthase mRNA

was observed in the blastocyst embryonic cells treated with the three apoptosis inducers, which was closely accorded with the elevated level of ganglioside GM3 expression in apoptotic embryos. Based on these studies, we speculated the ganglioside GM3 in embryo plays an important role in cellular apoptotic response.

In this study, we first examined the expressional changes of ST3GAL5 during oocyte maturation and early embryonic development (Fig. 4). The results obtained here show that as the apoptotic stress, the expression of ganglioside GM3 synthesizing enzyme ST3GAL was decreased in porcine oocyte and embryo during *in vitro* production progression. It can be concluded that expression pattern of ganglioside GM3 synthesizing enzyme ST3GAL were found to be involved in *in vitro* production oocyte and embryo in porcine. In addition, these findings suggest a relationship between the expression of ST3GAL5 synthesizing enzyme and apoptotic response in embryo genesis mechanisms during porcine *in vitro* embryo production. Therefore, it is important to improve the blastocyst efficiency for analysis of ganglioside mechanism during early development in *in vitro* culture of embryos.

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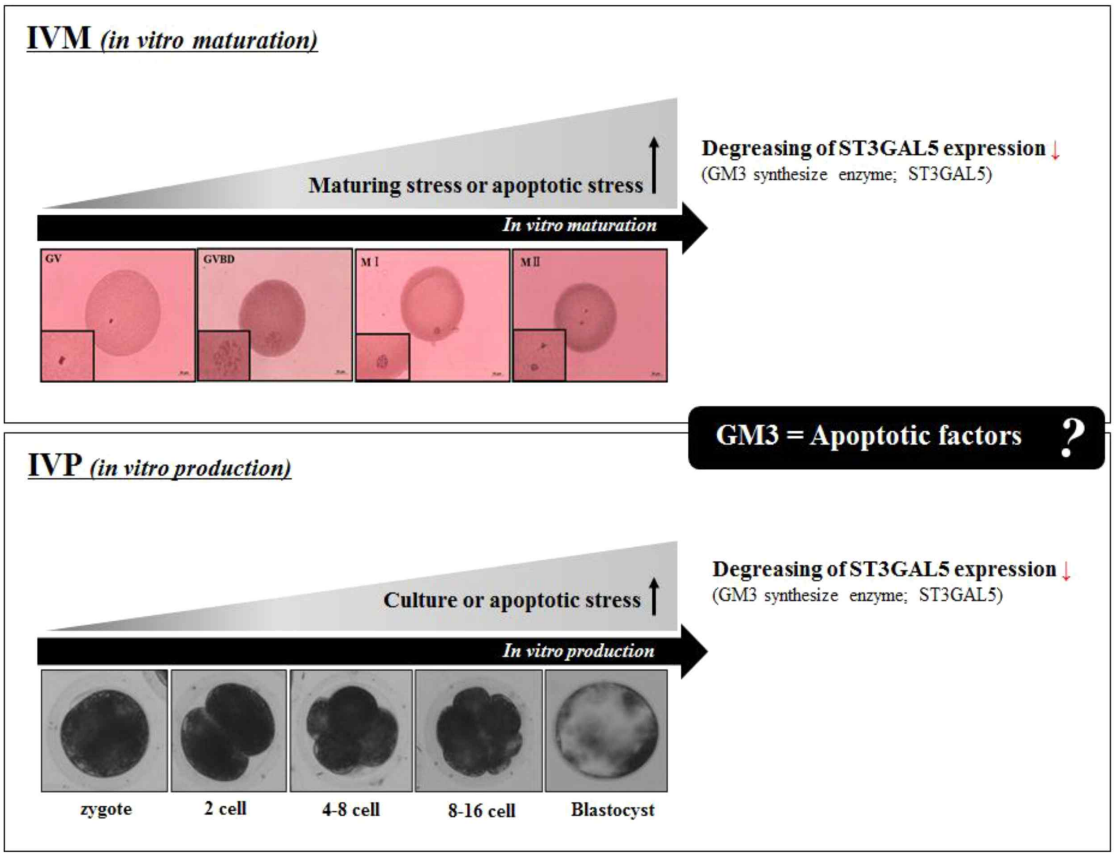

Fig. 4. Graphical summarized between expression pattern of ST3GAL5 and relationship of GM3 in porcine matured oocytes and early development embryos during induced apoptosis stress. Top panel; expression patterns of ST3GAL5 in *in vitro* maturing porcine oocytes. Bottom panel; expression patterns of ST3GAL5 in the preimplantation porcine embryos.

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