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Guanosine and its downstream signaling mediate meiotic arrest in mouse oocytes

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Guanosine and its downstream signaling mediate meiotic arrest in mouse oocyte

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ABSTRACT

Guanosine and its downstream signaling mediate meiotic arrest in mouse oocytes

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Follicle-enclosed oocytes are arrested at first meiotic prophase until puberty in mammals characterized with germinal vesicle (GV). The ovulatory surge of gonadotropins like Luteinizing hormone (LH) triggers resumption of oocyte meiosis. This is defined as the oocyte maturation. GV break down (GVB) indicates the resumption of nuclear maturation in the oocyte. Cumulus originated factors have the ability to inhibit meiotic resumption. In addition, it is suggested that there are some molecules which have the inhibitory effect on meiosis in antral follicles because oocytes of antral follicle underwent oocyte maturation spontaneously *in vitro*. cAMP is a representative molecule regulating meiotic arrest. When cAMP level is high, protein kinase A (PKA) is activated and phosphorylates its major downstream proteins, Wee1B and Myt1 kinases in mice. Phosphorylated Wee1B/Myt1 phosphorylates Thr14, Tyr15 residues of CDK1. PKA also phosphorylates and inactivates cdc25B which dephosphorylates the same

residues of CDK1, thereby inactivating Maturation promoting factor (MPF) and maintaining the meiotic arrest. Meanwhile, it is known that ERK1/2 are required during oocyte maturation after GVB in mice and GVB initiation in *Xenopus* oocytes. One of the laboratory date (not published) showed that guanosine have inhibitory roles in GVB of follicle free oocytes. It is suggested that some of the effects of extracellular guanosine could be transduced via its specific binding site on cell membrane in rat brain. On the other hand, proteomics analysis suggested that ERK1 level was increased by guanosine treatment. So far, it is not established that the mechanism of purines, especially guanosine during oocyte maturation. To evaluate the signaling molecules which are involved in putative guanosine receptor signaling, cAMP and ERK1/2 were analyzed with western blot, chemicals, and inhibitors. 3-4 weeks old female CD-1 mice were primed with pregnant mare serum gonadotropin (PMSG) (5 IU). The mice were sacrificed after 46hr of injection and oocytes were collected by ovarian puncture. Cumulus cells were mechanically removed. Guanosine and Gpp[NH]p, its competitive inhibitory molecule, were treated to GV intact oocytes. The high level of ERK1 expression was detected using proteomics analysis, western blot analysis. ERK1/2 levels were high in GV oocytes. Phosphorylated ERK1/2 were detected in GV stage oocytes in antral follicles. Cotreatment of guanosine and Gpp[NH]p released the oocyte from GV arrest. Guanosine increased the level of cAMP after incubation but cotreatment with Gpp[NH]p decreased its levels. Microinjection of the ERK phosphorylation inhibitor caused release from GV arrest by guanosine and antral follicle. It is shown that ERK1 and 2 mediate the meiotic arrest induced by guanosine. Based on these results, it is suggested that guanosine regulates GV arrest through cAMP and ERK1/2, eventually oocyte maturation along with the putative guanosine membrane receptor.

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INTRODUCTION

Mammalian oocytes are arrested at the dictyate stage of the first meiotic prophase and display a characteristic nucleus commonly known as the germinal vesicle (GV). The oocytes at the GV stage acquire the capacity to resume meiosis during growth of the ovarian follicle from the primary to the preovulatory stage (Canipari et al., 1984). After acquisition of meiotic competence, the ovulatory surge of gonadotropins, such as LH, triggers oocyte maturation and rupture of the ovarian follicle (Richard, 2014). The resumption of maturation in the oocyte after the meiotic arrest is characterized by GV break down and occurs spontaneously after removed from their follicular environment *in vitro* (Pincus et al., 1935).

cAMP is a key regulator to meiotic arrest which is supplied from cumulus cells and entered via gap junctions between cumulus cells and oocytes (Aktas et al., 1995; Norris et al., 2009; Yijing et al., 2015). When cAMP level is high, PKA (protein kinase A) is activated and phosphorylates its major downstream proteins, Wee1B and Myt1 kinases in mice (Han et al., 2005; Han et al., 2006). Phosphorylated Wee1B/Myt1 phosphorylates Thr14, Tyr15 residues of CDK1. PKA also phosphorylates and inactivates cdc25B which dephosphorylates the same residues of CDK1, thereby inactivating maturation promoting factor (MPF) and maintaining the meiotic arrest (Oh et al., 2009; Pirino et al., 2009) .

On the other hand, the meiotic arrest in mouse oocytes can be maintained without cumulus cells according to other studies using transgenic mice. Adenylyl cyclase3 (AC3) produces cAMP and is stimulated by Gs subunit of GPR3 in mouse oocytes (Mehlmann et al., 2004). And PDE3A is the essential isoform which hydrolyze cAMP causing resumption of meiosis in oocytes.

PDE3A-deficient oocytes do not resume meiosis after an LH surge or spontaneously *in vitro* (Masciarelli et al, 2004, Petr et al., 2010). These results indicate that the oocyte has the ability to maintain the first meiotic arrest itself.

Previously in the laboratory, inhibitory roles of adenosine and guanosine in meiotic resumption were elucidated. Guanosine suppressed meiotic resumption in follicle free condition (Cheon et al., 1997; Cheon, 2004). So far, the suggested mechanisms could use guanosine as a substrate for the second messenger. It is known that there are receptors for purine like adenosine in various cell type (Bertil et al., 2001). On the other hand, guanosine receptor is not identified. But the effects of extracellular guanosine could be transduced via its specific binding site on cell membrane in rat brain (Rosaria et al., 2011, Ugo et al., 2002). Furthermore, guanosine induced a dose-dependent increase in intracellular cAMP in rat brain cortex (Ugo et al., 2003).

Various signaling molecules are phosphorylated and/or dephosphorylated during meiosis (Aritro et al., 2013; Swathi, 2017). ERK1/2 are also required for the maturation of the mouse COC (Su et al., 2003; You-Qiang et al., 2013) and denuded oocytes (Yin-Li et al., 2015). But, it is not proved that ERK is involved in meiotic arrest. In the previous data (not published), the high level of ERK1 expression was appeared using proteomics protocol and there is a result of western blot analysis that phosphorylated ERK1/2 existed in GV stage mouse oocytes. In accordance with an experiment using recombinant proteins, Wee1B phosphorylated by ERK2 (Sarah et at., 2000).

Although the possible roles of guanosine are suggested, the mechanisms are not clear. In this study, the possible mechanisms of guanosine in GV arrest were examined in CD-1 mouse using proteomics, western blot, chemicals, and specific inhibitors. The molecules involved in signaling were analyzed such as cAMP and

ERK1/2 in denuded mouse oocytes.

MATERIALS AND METHODS

Experimental animals

All experiment involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. CD-1 female mice were maintained under standard condition at Sungshin University diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 hr and clean room system. Animals were fed a standard rodent diet and water *ad libitum* from weaning at 21 days after birth.

Oocytes collection

3-4 weeks old female CD-1 mice were primed with PMSG (5 IU). The mice were sacrificed after 46hr of PMSG injection. Ovaries were transferred to BWW medium containing 0.4% bovine serum albumin (BSA). Cumulus-enclosed oocytes were isolated from antral follicles using a 30-gauge needle in the medium and the cumulus cells were removed mechanically. The denuded oocytes were frozen in liquid nitrogen and kept at -80°C.

Chemical treatment

Oocytes were cultured in BWW medium containing 0.4% BSA with or without guanosine and Gpp[NH]p which is a competitive antagonist of guanosine for 6hr. Table 1 indicates the concentration of these agents and time for observation of the inhibition effect on the meiosis resumption. And the time point for oocytes collection during oocytes maturation are in Table 2.

Proteomics analysis of oocytes

Denuded oocytes were collected with the ovarian puncture method. With collected oocytes were treated with guanosine or not (GV intact) in 0.4% BSA BWW medium. These oocytes were analyzed by proteomics protocol using Q-TOF (Agilent, 6520).

Measurements of cAMP in mouse oocytes

3-4 weeks old female CD-1 mice were primed with PMSG (5 IU). The mice were sacrificed after 46-48hr of PMSG injection. Ovaries were transferred to BWW medium containing 0.4% bovine serum albumin (BSA). Cumulus-enclosed oocytes were isolated from antral follicles using a 30-gauge needle in the medium and the cumulus cells were removed mechanically. The denuded oocytes were frozen in liquid nitrogen. The intraoocyte cAMP level was measured using the Mouse/Rat cAMP parameter assay kit (R&D system).

Western blotting

Oocytes were washed using 0.1% PVA in PBS and mixed with y-PBS (0.7mM PMSF, 1 mM Benzamidine-HCl, 4㎍/ml Leupeptin, 2㎍/ml Aprotinin, and 2 mM EDTA). Oocytes in tube became lysate by freezing and thawing. 60ea oocytes were boiled in SDS/β-mercaptoethanol sample buffer (10% glycerol, 2% SDS, 80mM pH6.8 Tris-Hcl, 0.02% phenolbromo blue, 0.1% β-mercaptoethanol), and loaded onto each lane of 10% SDS-PAGE (Bro-Rad, TGX stain-free fastcast

acrylamide kit, 10%, USA). The proteins were separated by electrophoresis and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Merck, Germany) in transfer buffer (25 mM Tris base, 192 mM Glycine, 0.1% SDS, and 20% Methanol, pH 8.3). The membranes were blocked in 5% skimmed dry milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) for 1 hr at RT, and washed three times with TBST. The membranes were incubated for 1 hr with mouse monoclonal ERK1/2 antibody (dilution 1:1000), mouse monoclonal phosphorylated ERK1/2 antibody (dilution 1:500). After incubation, membranes were washed three times and incubated for 1hr with horseradish peroxidase conjugated goat anti-mouse IgG (dilution 1:1000). The bands were developed with Clarity Max ECL solution (Bio-Rad, cat. 1705062, Italy) and detected by ChemiDoc MP (Bio-Rad, cat.17001402, USA). The intensity of each band was normalized with total protein using ChemiDoc Image lab software.

Oocyte microinjection

3-4 weeks old female CD-1 mice were primed with PMSG (5 IU). After 46 hr of injection, the mice were sacrificed after 46 hr of PMSG injection. Ovaries were transferred to BWW medium containing 0.4% bovine serum albumin (BSA). Cumulus-enclosed oocytes were isolated from antral follicles using a 30-gauge needle in the medium and the cumulus cells were removed mechanically. Denuded oocytes were transferred to 0.4% BSA BWW medium with 750uM guanosine. 10pL of 0.5, 1 and 2pM ERK1/2 phosphorylation inhibitor SCH772984 (Selleckchem, USA) was injected to GV intact oocytes. 0.1% DMSO in BWW was injected as control oocytes. After incubation for 1.5 or 3 hr, maturation state of oocytes were examined under a dissecting microscope and collected. The

collected oocytes were frozen in liquid nitrogen and kept at -80°C.

Follicle microinjection

3-4 weeks old female CD-1 mice were primed with PMSG (5 IU). The mice were sacrificed after 36hr of PMSG injection. Ovaries were transferred to BWW medium containing 0.4% bovine serum albumin (BSA). Ovarian follicles were separated using a 30-gauge needle and forceps in the medium. 10pL of 1, 2pM ERK1/2 inhibitor SCH772984 (Selleckchem, USA) was injected to follicle-enclosed oocytes. 0.1% DMSO in BWW was injected as control oocytes. These follicles were incubated for 1.5 or 3 hr in inverted state 96-well plate with alpha-MEM medium. The maturation state of oocytes were examined under a dissecting microscope and collected. The collected oocytes were frozen in liquid nitrogen and kept at -80°C.

Statistics

The chi-square test and student's *t*-test were performed to evaluate the statistical significance between control and experiment group. Results were presented as mean ± SEM. Values of $P < 0.05$ were considered to be significantly different.

Table 1. Putative guanosine receptor antagonist released meiotic arrest of guanosine

	Step Ⅰ (Pre-treatment)	Step Ⅱ (Co-treatment)	
Group	Gpp[NH]p	Guanosine	Gpp[NH]p
Control	0	0	0
1	7.5 mM	0.75 mM	7.5 mM
2	3.75 mM	0.75 mM	3.75 mM
3	0.75 mM	0.75 mM	0.75 mM
4	0	0.75 mM	0

Pre-treatment was performed for 10 min before transfer to step Ⅱ.

Table 2. Schedule of oocytes collection during culture

	Control	Guanosine (G)			G + Gpp[NH]p			Control		
Time point (hr)	0	1.5	3	6	1.5	3	6	1.5	3	6

Table 3. Antibodies information

Name		Company
ERK1/2 - Mouse monoclonal	sc514302	Santa cruz
p-ERK1/2 - Mouse monoclonal	sc7383	Santa cruz

RESULTS

Putative guanosine receptor antagonist increased the spontaneous meiotic resumption.

Gpp[NH]p, non-hydrolysable analogue of GTP, is a competitive inhibitor for guanosine binding site and makes the affinity of guanosine decrease (Ugo et al., 2003). But, high-dose Gpp[NH]p was required to compete with guanosine. Thus, Gpp[NH]p was pretreated as described in Table 1. More than 80% of the oocytes degraded when they were cultured with 7.5mM Gpp[NH]p (data not shown).

Most of the oocytes underwent GVB when they were cultured for 6hr in control medium. But, oocytes cultured in 750uM guanosine treated medium were arrested at GV stage (Fig. 1). The pretreatment of 3.75mM Gpp[NH]p has effect on GVB rate and was significantly different. 750uM Gpp[NH]p-pretreated group has no significant difference but an increasing trend of GVB rate was shown during culture. These results indicate that Gpp[NH]p can suppress the effect of guanosine and the effect on meiosis of guanosine is reversible.

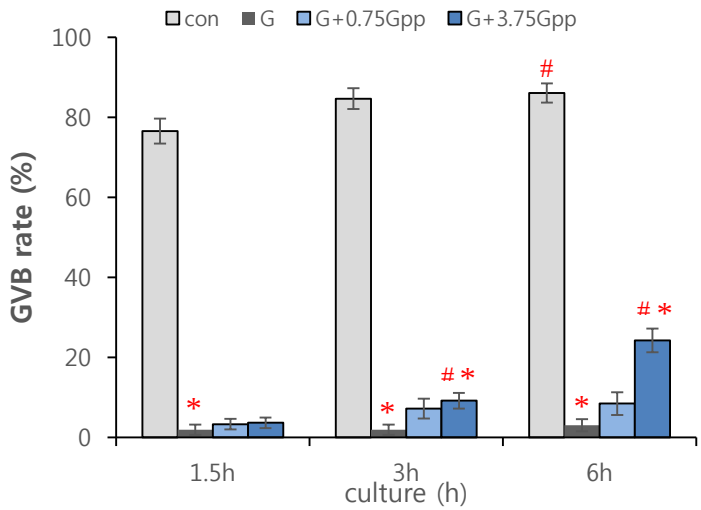
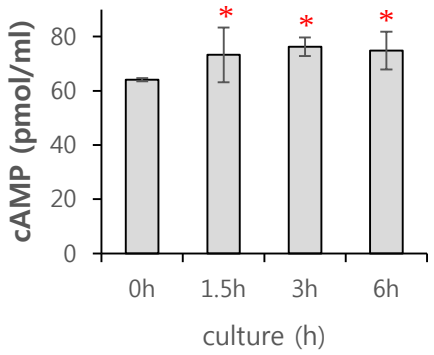


Fig. 1. Effects of guanosine and/or Gpp[NH]p on the meiotic arrest of immature oocytes *in vitro* in mouse. GVB rate (%) after culture. Statistical analysis by Student's *t*-test (*: p<0.05 vs control of each time point, #: p<0.05 vs within the group.).

cAMP levels in guanosine or Gpp[NH]p treated oocytes by the incubation time

The cAMP level was increased significantly 1.5hr after incubation in control group and other experimental groups (Fig. 2). In control groups, the level of cAMP was maintainged the higher level than the control as expected. In previous reports (Aktas et al., 1995), the level of cAMP is decreased just before GVB and increased during maturation processes. In guanosine treated groups, the patterns were similar with the control. However, the cAMP levels showed fluctuation by cotreatment of Gpp[NH]p. It was similar to the GVB rate in cotreatment group in Fig. 1. It was decreased at 3 hr, whereas it was increased the rate of GVB in cotreatment group (Fig. 2C).

(A)



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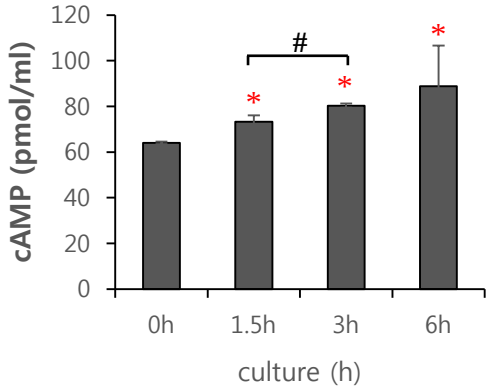


(C)

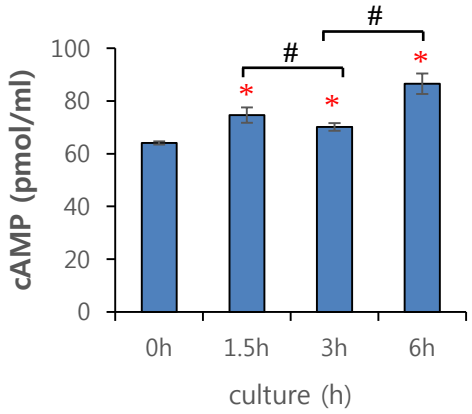


Fig. 2. The level of cAMP in guanosine or Gpp[NH]p treated oocytes *in vitro*. cAMP level from 120ea oocytes cultured in control medium (A), 750uM guanosine treated medium (B), 750uM guanosine and 3.75mM Gpp[NH]p treated medium (C). Statistical analysis by Student's *t*-test (*: $p<0.05$ vs 0h control).

Proteomic analysis of oocytes

Quantitative proteomics analysis was performed as mentioned in materials and methods. Interestingly, the levels of ERK1 and Gαs subunit of GPCR were increased more than 2-fold in guanosine treated oocytes. The insilco analysis showed that ERK can work at downstream of cAMP (Fig. 3).

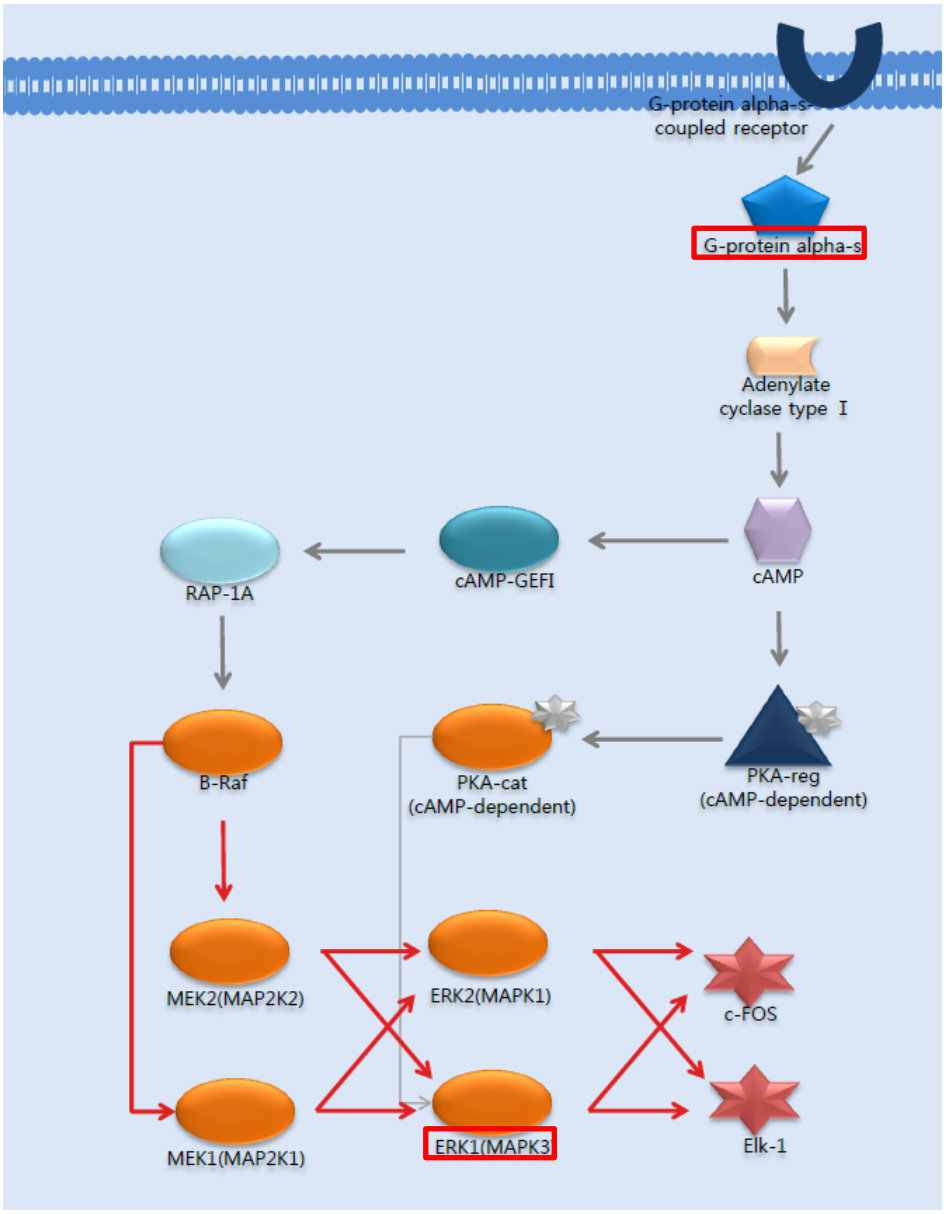


Fig. 3. Proteomics map of GV stage and cultured oocytes.

ERK1/2 levels and activation were modified by guanosine

ERK1/2 detected from GV intact oocytes to GVB oocytes. Guanosine and Gpp[NH]p had no effect on ERK1/2 expression (Fig. 4A). In addition, the activated, phosphorylated ERK1/2 were also detected in GV intact oocytes (Fig. 4B). Activation of ERK1/2 is high corresponding to the proteomics result (Fig. 4A,C,D). At the same time point, both ERK1 and ERK2 were more phosphorylated in guanosine-treated groups than control groups (Fig 4C,D).

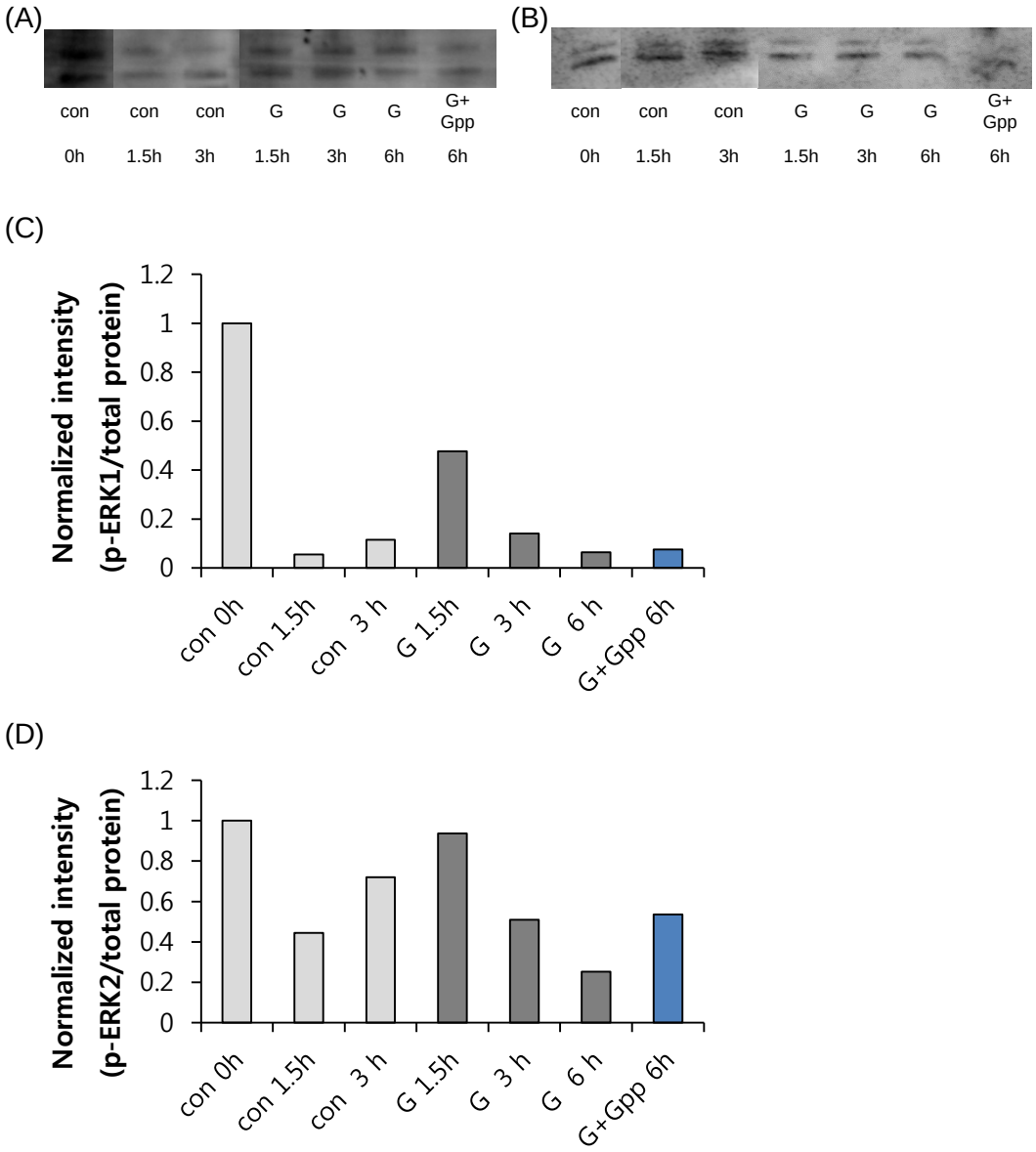
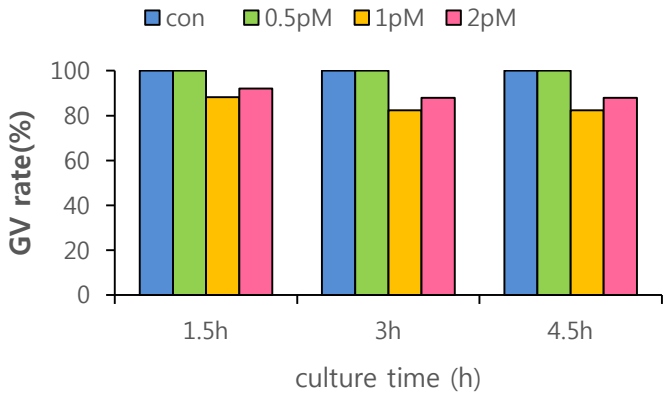


Fig. 4. Expression profiles of ERK1/2 and phosphorylated ERK1/2 in mouse oocytes. (A) ERK1/2 expression, (B) phosphorylated ERK1/2, (C) phosphorylated ERK1, (D) phosphorylated ERK2 band intensity normalized whit total protein in mouse oocytes (60ea/group).

Inhibition of ERK1/2 released GV arrest in guanosine treated oocytes

SCH772984 is a specific inhibitor of ERK1/2 phosphorylation. 0.5pM ERK phosphorylation inhibitor had no effect on the first meiotic arrest induced by 750uM guanosine. On the other hand, some of oocytes injected with 1 and 2pM SCH772984 underwent GVB (Fig. 5A,B). Control group was injected with 0.1% DMSO and meiotic arrested by guanosine.

(A)



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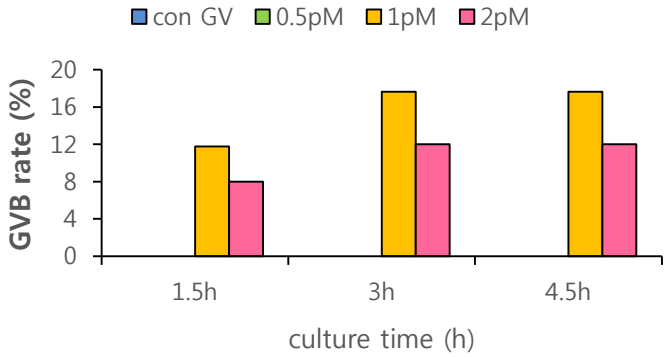

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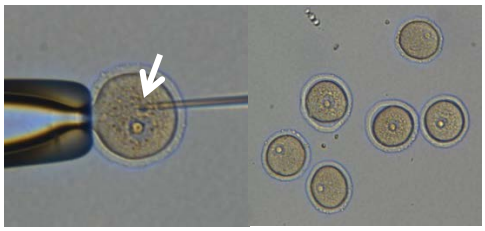
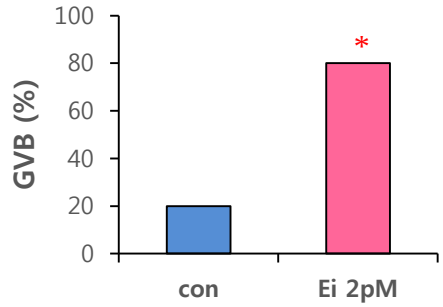


Fig. 5. Effects of ERK phosphorylation inhibitor SCH772984 on meiotic arrest induced by guanosine *in vitro*. GV rate, (B) GVB rate after ERK phosphorylation inhibitor injection and culture in BWW control medium with 750uM guanosine. (C) Microinjected oocytes.

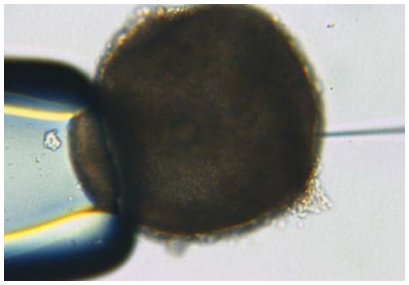
Inhibition of ERK1/2 released GV arrest in follicle-enclosed oocytes

2pM of SCH772984 was injected to follicle-enclosed oocytes. 0.1% DMSO was injected as a control. After 6hr culture, oocytes were punctured and checked the maturation state under a dissecting microscope (Fig. 6A,C). The oocytes injected 2pM of SCH772984 underwent GVB but not in the control group.

(A)



(B)



(C)

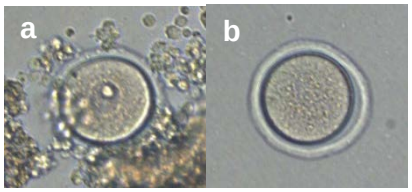


Fig. 6. Effects of ERK phosphorylation inhibitor SCH772984 on meiotic arrest within antral follicle. (A) GVB rate after injection and 6hr culture (5ea/group). (B) A microinjected follicle-enclosed oocyte and (C) the maturation states of punctured oocyte after 6hr culture. a: control injection, b: SCH772984 injection.

DISCUSSION

Most of the oocytes underwent GVB when they were released from antral follicle and cultured for 6hr in control medium whereas oocytes cultured in 750uM guanosine treated medium were arrested at GV stage. These results correspond with previous studies (Cheon et al., 1997; Cheon, 2004). On the other hand, guanosine level is increased by the follicle growth (Downs et al., 1989). These mean that guanosine is a mediator for GV arrest of oocytes in pre-ovulatory follicles.

The level of cAMP is fluctuated during spontaneous maturation. Just before GVB, the level of cAMP is decreased and then increased to the certain levels (Aktas et al., 1995). Guanosine treatment increased cAMP levels during *in vitro* culture with similar patterns to control. However, cotreatment of putative guanosine receptor antagonist modulated the patterns. It was decreased after 3hr culture and then increased as expected. At 3 hr of culture, the GVB rate were significantly increased in cotreatment groups. Such results mean that guanosine suppresses the GVB through its putative membrane receptor.

It is well established that cell cycle regulatory molecules such as Wee1B/Myt1 kinases, Cdc25B, MPF, APC/C are controlled by variation of cAMP during mammalian oocyte maturation (Petros et al., 2007; Teng et al., 2017; Tripathi et al., 2010). The distribution and cooperation of Wee1B kinase and Cdc25B within oocyte are important to meiotic reentry before GVB. At GV stage, Wee1B is localized in the nucleus and Cdc25B is in the cytoplasm. Just before GVB, Wee1B is exported from and Cdc25B is entered the nucleus. And this translocalization is significant that induces GVB and MPF activation (Oh et al., 2009; Pirino et al.,

2009). From the results of proteomics analysis, it was suggested that ERK1/2 could work as a downstream molecules in signaling pathway.

In *Xenopus* egg extract and mammalian somatic cells, p42 MAPK (the ortholog of ERK2) regulates Cdc25C during the G2/M transition. In addition, it is suggested that mammalian ERKs could be involved in the regulatory system of Cdc25C activation during mitosis and there is a control mechanism that ERKs phosphorylate Cdc25C in mammalian cells (Ruoning et al., 2007). Therefore, ERK1/2 are key proteins for GV arrest and important in GV arrest of *Xenopus* oocytes.

The possible role of ERK is controversial in mammals. Thr and Tyr residues of Wee1B are phosphorylated, thereby activated by p42 MAPK (ERK2 of *Xenopus*) with condition exists a constitutive active version of MEK which is the upstream of ERK1/2 (Sarah et al., 2000). Thus, it is shown that p42 MAPK directly phosphorylates Wee1B resulting in delayed Cdc2 activation and extension of G2 phase duration.

Eppig group (1989) showed that guanosine level is high in antral follicular fluid. In addition, phosphorylated ERK1/2 could be detected at GV intact stage with western blotting methods in this study. Besides the microinjection of the phosphorylation inhibitor for ERK1/2 blocked the effects on GV arrest of guanosine or antral follicles. These are suggested that guanosine suppresses germinal vesicle breakdown of the oocyte in antral follicle through ERK1/2 activation.

In summary, phosphorylated ERK1/2 were detected in GV intact mouse oocytes with western blot methods and proteomics analysis. Guanosine maintained GV arrest and it caused cAMP accumulation and activation of ERK1/2. Microinjection of the inhibitor for phosphorylation of ERK1/2 released the oocytes from the GV

arrest both in guanosine-treated denuded oocytes and antral follicular oocytes. As depicted in Fig.7, it is proposed that the guanosine is a regulator of meiotic arrest of the oocyte in preovulatory follicle via its putative membrane receptor mediating cAMP, ERK1 and ERK2.

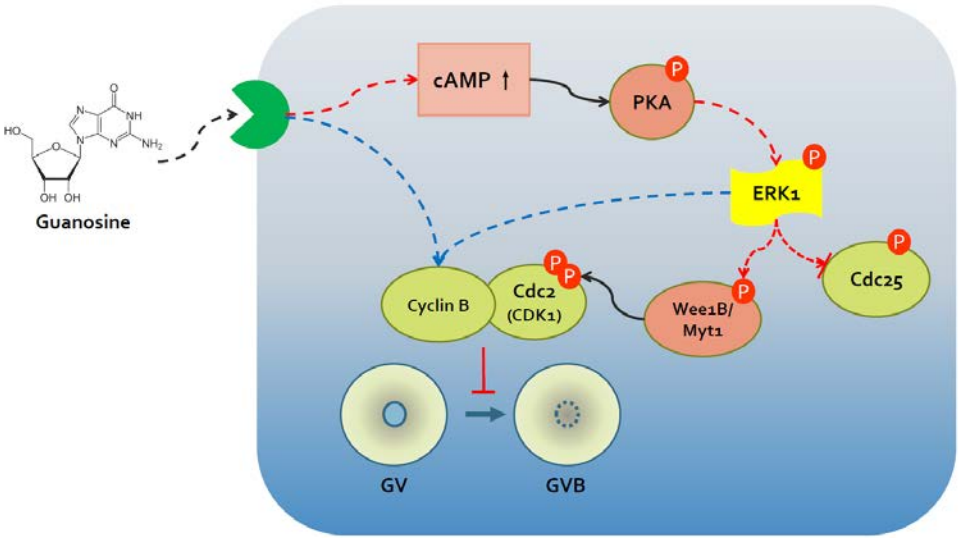


Fig. 7. Schematic map summarizing potential pathway of molecules in meiotic arrest.

REFERENCE

Aktas H, Wheeler MB, First NL, Leibfried-Rutledge ML. 1995. Maintenance of meiotic arrest by increasing [cAMP]i may have physiological relevance in bovine oocytes. J Repord Fertil 105:237-245.

Aritro S, Francesco C. 2013. Oocyte maturation: A story of arrest and release. Front Biosci 5:451-77

Bertil B, Adriaan P, Kenneth A, Karl-norbert K, Joel L. 2001. International union of pharmacology.XXV.Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527-552.

Canipari R, Palombi F, Riminucci M, Mangia F. 1984. Early Programming of Maturation Competence in Mouse Oogenesis. Dev Biol 102:519-524.

Cheon YP, Kim CH, Mik JE, Kim MK. Effects of adenosine, guanosine nad azaserine on maturation of mouse oocytes in vitro. Korean J Animal Reprod. 1997;21:123–130.

Cheon YP. 2004. Guanosine Regulates Germinal Vesicle Breakdown (GVBD) in Mouse Oocytes. Dev Biol 28:267-273.

Downs SM, Daniel SA, Bornslaeger EA, Hoppe PC, Eppig JJ. 1989. Maintaenance of meiotic arrest in mouse oocytes by purines: modulation of cAMP levels and cAMP phosphodiesterase activity. Gamete Res 23:323-334.

Han SJ, Chen R, Paronetto MP, Conti M. 2005. Wee1B Is an Oocyte-Specific Kinase Involved in the Control of Meiotic Arrest in the Mouse. Curr Biol 15:1670-1676.

Han SJ, Conti M. 2006. New pathways from PKA the Cdc2/cyclin B Complex in Oocytes: Wee1B as a Potential PKA Substrate. Cell Cycle 5:227-231.

Norris R, Ratzan W, Freudzon M, Mehlmann L, Krall J, Movsesian M, Wang H , Hengming K, Nikolaev V, Jaffe L. 2009 Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development 136:1869-187

Oh JS, Han SJ, Conti M. 2009. Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption. J Cell Biol 188:199-207.

Petr S, Richard M, Jan M. 2010. Prophase I arrest and progression to metaphase I in mouse oocytes: comparison of resumption of meiosis and recovery from G2-arrest in somatic cells. Mol Hum Reprod 16:654-664.

Petros M, Emmy WV, Ruby C, Peter KJ, John C. 2007. Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC/Cdh1. JCB. 176: 65-75.

Pincus G, Enzmann E. 1935. The comparative behavior of mammalian eggs in vivo and in vitro: The activation of ovarian eggs. J Ecp Med 62:665-675.

Pirino G, Wescott MP, Donovan PJ. 2009. Protein Kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes. Cell Cycle 8:665-670.

Ruoning W, Guangan H, Mayra N, Cheryl L, Gary E, P.Todd S, Marc W, Jian K. 2007. Regulation of Cdc25C by ERK-MAP kinases during the G2/M transition. Cell 128:1119-1132.

Su YQ, Denegre JM, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ. 2003.

Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte-cumulus cell complex. Dev Biol 263:126-138.

Swathi A. 2017. Signaling-mediated regulation of meiotic prophase I and transition during oogenesis. Results Probl Cell Differ 59:101-123.

Ugo T, Giulia B, Patrizia DI, Renata C, Eva S. W, Michel P. R. 2002. Specific [^{3}H]-guanosine binding sites in rat brain membranes. Br J Pharmacol 135:969-976.

Tripathi A, Kumar KV, Chaube SK. 2010. Meiotic Cell Cycle Arrest in Mammalian Oocytes. J Cell Physiol 223:592-600.

Ugo T, Giulia B, Emidio C, Antonio M, Gabriele C, Clara P, Francesco C, Roberto P. 2003. Rat brain guanosine binding site: biological studies and pseudo-receptor construction. Bioorg Med Chem. 11:5417-5425.

Rosaria V, Gabriella M, Michela B, Diego DB, Catia L, Carmen L, Ram C. M, Ajiroghene T, Gloria C. 2011. Evidence for the Existence of a Specific G Protein-Coupled Receptor Activated by Guanosine. ChemMedChem 6:1074-1080.

Sarah A. W, Sarah N. G, James E. F Jr. 2000. Activation of Wee1 by p42 MAPK In Vitro and in Cycling *Xenopus* Egg Extracts. Mol Biol Cell 11:887-896.

Yijing W, Zhen T, Ge L, Xinyi M, Zhengpin W, Lizhao F, Wanbao N, Kun H, Xi X, Chao W, Hua Z, Guoliang X. 2015. Cyclic AMP in oocytes controls meiotic prophase I and primordial folliculogenesis in the perinatal mouse ovary. Development 142:343-351.

You-Qiang S, James M, Keren W, Frank L, Marylin J, John J. 2013. Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in

cumulus cells is required for the maturation of the mouse oocyte-cumulus cell complex. Dev Biol 263:236-138.

Yin-Li Zhang, Xiao-Man Liu , Shu-Yan Ji, Qian-Qian Sha, Jue Zhang, Heng-Yu Fan. 2015. ERK1/3 activities are dispensable for oocyte growth but are required for meiotic maturation and pronuclear formation in mouse. JGG 42:477-485.

Teng Z, Yang Z, Li L, Zhen-Bo W, Wei S, Heide S, Qing-Yuan S. 2017. CenpH regulates meiotic G2/M transition by modulating the APC/Ccdh1-cyclinB1 pathway in oocytes. Development 144:305-312.

논 문 개 요

포유동물의 난자는 난포 안에서 사춘기까지 제 1 차 감수분열 전기의 망상기(dictiate stage)에 머물러있다. 이때의 난자는 난핵포(germinal vesicle)를 특징으로 한다. 배란 직전에 황체형성호르몬(LH)과 같은 성선자극호르몬(gonadotrophin)이 분비되면 난자의 감수분열이 다시 시작되면서 핵막붕괴(germinal vesicle breakdown)가 나타나며 이러한 감수분열의 재개를 난자의 성숙이라고 한다. 따라서 핵막붕괴는 난자성숙의 지표라고 할 수 있다. *In vitro* 에서, 난포로부터 유리된 미성숙 난자가 자발적으로 성숙이 진행되는 것으로 보아 난포에 감수분열을 억제시키는 물질이 있다는 것을 알 수 있다. 또한 유전자변형 생쥐(mouse)를 통한 실험에서 난자가 자체적으로 감수분열 재개 억제를 유지할 수 있는 능력이 있다고 제시되고 있다. cAMP 는 가장 대표적인 감수분열 억제를 조절하는 물질이다. cAMP 의 농도가 높을 때 활성화된 PKA 는 MPF 억제 단백질인 Wee1B, Myt1 kinase 를 인산화한다. 이들 kinase 는 CDK1 의 Thr14, Tyr15 잔기를 인산화하며, 이때 같은 잔기를 탈인산화하는 Cdc25B 는 PKA 에 의해 인산화되어 불활성화 상태가 된다. 인산화된 CDK1 은 결과적으로 MPF 의 불활성 상태를 유지하게 하고 감수분열 재개를 억제한다. 선행연구에서, 유리된 난자에 아데노신, 구아노신과 같은 퓨린 계열의 분자를 처리하면 감수분열 억제효과가 있음을 밝힌 바 있다. 또한 rat 의 brain 에서, 구아노신은 세포 표면에서 작용하여 그 효과를 나타내며 cAMP level 을 증가시켰다는 연구결과가 보고되었다. 그러나 아직 퓨린계 물질, 특히 구아노신의 난자성숙에서의 작용기작은 잘 알려지지 않았다. 한편, ERK 는

난자의 성숙과정에 관여한다고 알려져 있으며, Wee1B kinase 에 ERK2 가 인산화하는 site 가 있다고 보고된 바 있다. 우리 연구실의 선행 data 에 의하면, GV 단계의 난자에서 ERK1 의 발현이 많고 인산화된 ERK1/2 가 확인되었다. 따라서 본 연구에서는 구아노신이 난자 표면에 존재하는 구아노신 수용기와 인산화된 ERK1/2 를 통하여 난자성숙을 억제하는가를 알아보고자 하였다. 미성숙 난자는 CD-1 mice (3-4 주령)에 5IU 의 PMSG 를 복강주사하여 난포성장을 유도한 후, 46 시간에 난포를 터뜨려 회수하였다. 난자를 둘러싼 과립세포는 pipet 을 이용하여 기계적으로 제거하였다. 건강한 난자만을 선택하여 실험에 사용하였다. 난자의 자발적인 성숙을 억제하기 위해 배양액 100ul 를 사용하여 난포액의 효과를 보도록 하였다. 유리된 난자에 구아노신 및 구아노신의 길항제를 함께 처리하였는데 GVB 가 진행되었다. 한편, 이들은 cAMP level 에 영향을 주었는지 경쟁적 효소 면역학적 검정을 진행하였다. ERK1/2 의 발현과 인산화된 정도를 western blot 을 통해 확인하였다. 그 결과, GV 상태의 난자에서 ERK1/2 의 활성이 있으며, 시간에 따른 증감이 있었다. 난포로부터 유리된 난자에 구아노신을 처리한 후 ERK 활성 억제제를 미량주사(microinjection)하여 *in vitro* 에서 구아노신에 의한 감수분열의 억제가 ERK 를 매개로 한다는 것을 확인하였다. 그 결과, 1pM 의 ERK 활성 억제제를 미량주사한 그룹에서 감수분열 재개가 진행됨을 보았다. 또한 난포에 둘러싸인 난자에 ERK 활성 억제제를 미량주사하여 ex vivo 에서의 결과를 확인하였다.

이러한 결과를 종합해보면, oolemma 에 구아노신 수용기가 있어 이를 통하여 cAMP, ERK 활성을 조절하고 난자성숙을 조절한다고 제안할 수 있다.

감사의 글

먼저 이 논문을 심사해주시고 아낌없는 조언을 주신 김해권 교수님, 이동률 교수님, 그리고 학부에서 이어진 인연으로 석사 과정을 무사히 마치기에 이른 지금까지 열심히 지도하고 이끌어주시고 아껴주신 전용필 지도교수님께 마음 깊이 감사 드립니다. 또한 인연이 되어 여러 가르침을 주시고 조언해주신 교수님들, 힘이 되어주셔서 감사합니다.

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사소한 것 하나도 성심성의 배우고 따라준 후배 우리, 희선, 민영, 주혜 그리고 민진, 상은이에게 항상 고마웠습니다. 배움을 계속하여 똑똑하고 당찬 미래의 과학자로 성장하길 바라고, 저를 포함한 선배들을 좋은 방향으로 이끌어주길 기대합니다.

지금까지 지치지 않게 격려해주고 마음고생을 함께 나누느라 저보다 더 고생했을지도 모를 부모님과 친척, 친구들, 연인에게... 앞으로 남은 시간 동안 받았던 것보다 더 크게 보답할 수 있도록 노력하겠습니다. 저에게 무한한 지원과 응원을 주시고 항상 사랑해주시는 분들께 이 자리를 빌려 감사하다고 전할 수 있어서 행복합니다.

많은 분들이 베풀어주신 은혜에 언제나 감사하는 마음으로 매일 더 나아지는 사람이 되도록 노력하겠습니다.

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