Expression of *Gas6* Receptors, *Tyro3*, *Axl*, and *Mertk*, in Oocytes and Embryos and Effects of *Mertk* RNAi on the Oocyte Maturation

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ABSTRACT : Recently, we reported growth arrest-specific gene 6 (*Gas6*) as a new maternal effect gene (MEG), that expressed in the oocytes but functioned principally during embryogenesis. *Gas6* RNAi-treated oocytes developed to metaphase II (MII) stage but they have affected M-phase promoting factor (MPF) activity and incurred abnormal pronuclear (PN) formation during fertilization. *Gas6* is a ligand of TAM family members (*Tyro3*, *Axl* and *Mertk*) of receptor tyrosine kinase (RTK). Purpose of the present study was to evaluate the expression of *Tyro3*, *Axl* and *Mertk* transcripts in oocytes and early embryos. Expression of *Gas6* and *Mertk* mRNA was detectable in oocytes and follicular cells, while Tyro3 and Axl mRNA was expressed only in follicular cells. Expression of *Mertk* mRNA was relatively constant during oocytes maturation and embryogenesis, but the other receptors, *Tyro3* and *Axl*, were not expressed in oocytes and PN stage of embryos at all. Knockdown of *Mertk* mRNA and protein by using sequence-specific *Mertk* double strand RNA (dsRNA) did not affect oocytes maturation. In this case, however, contrary to the ligand Gas6 RNA interference (RNAi), MPF activity had not been changed by *Mertk* RNAi. Therefore, we concluded that the *Gas6-Mertk* signaling is not directly related to the oocyte maturation. It is still required to study further regarding the function of *Mertk* as the receptor of *Gas6* during preimplantational early embryogenesis.

Key words : Gas6 receptors, *Tyro3*, *Axl*, *Mertk*, RNA interference

INTRODUCTION

Oocytes accumulate maternal mRNA, protein, and regulatory molecules for preparing the completion of meiosis, fertilization, and early embryogenesis (Eppig and Wigglesworth, 2000). Maternal accumulation is principally controlled by post-transcriptional regulatory mechanisms, such as RNA poly-adenylation, localization, sorting and masking, and protein phosphorylation (Gandolfi and Gandolfi, 2001). For that reason, we have been accomplished functional analysis of maternally expressed genes to reveal molecular regulatory mechanisms of oocyte nuclear and cytoplasmic maturation as well as fertilization and early embryogenesis (Kim et al., 2008; Yoon et al., 2009).

Previously, we found that *Gas6* is highly expressed in germinal vesicle (GV) oocytes by using annealing control primer (ACP)-PCR (Yoon et al., 2005) and it functions in regulating activity of M-phase promoting factor (MPF) by using RNAi (Kim et al., 2011). *Gas6* is a ligand for TAM family members (*Tyro3*, *Axl*, and *Mertk*) of receptor tyrosine kinase (RTK) (Nagata et al., 1996). Cell type-specific functions of Gas6-mediated signaling is implicated in cell survival, growth arrest, proliferation, and differentiation (Goruppi et al., 1996; Li et al., 1996; Manfioletti et al., 1993; Nakano et al., 1995). The role of Gas6 and its receptor signaling has been studied in thrombosis, spermatogenesis and myelination of oligodendrocyte (Lu et al., 1999; Manfioletti et al., 1993; Binder et al., 2011), but not in oocytes.

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By using RNA interference (RNAi), we previously have shown that *Gas6* RNAi changed M-phase promoting factor (MPF) activity (Kim et al., 2011). After parthenogenetic activation, *Gas6*-silenced metaphase II (MII) oocytes had not developed further and arrested at MII (90.0%). In the same study, we found that the *Gas6*-silenced MII oocytes showed markedly decreased normal Ca^{2+} oscillation and no exocytosis of cortical granules after Sr^{2+}-induced stimulation. In these MII oocytes with normal shape but changed cytoplasm, sperm penetration into the oocyte cytoplasm was observed after fertilization, but not PN formation. These results strongly suggest that *Gas6* signaling is involved in fertilization and early embryo development.

Therefore, the objective of the present study was, in order to study the action mechanism of *Gas6*, to investigate the expression of *Tyro3*, *Axl* and *Mertk* in oocytes and embryos, and to evaluate whether *Mertk* plays a role in oocyte maturation.

MATERIALS AND METHODS

1. Animals

ICR mice were obtained from Koatech (Pyengtaek, Korea) and maintained at the animal facility of the CHA Stem Cell Institute of CHA University. All procedures described were reviewed and approved by the University Institutional Animal Care and Use Committee (IACUC), and were performed in accordance with the guiding principles for the Care and Use of Laboratory Animals.

2. Reagents

Chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless noted otherwise.

3. Isolation of Oocytes and Embryos

For the isolation of GV oocytes from preovulatory follicles, 3-week-old female ICR mice injected with 5 IU pregnant mare serum gonadotrophin (PMSG) and sacrificed 46 h later. Cumulus-enclosed oocyte complexes (COCs) were recovered from ovaries by puncturing the preovulatory follicles with 27-gauge needles. M2 medium containing 0.2 mM 3-isobutyl-methyl-xanthine (IBMX) was used to inhibit GVBD while handling oocytes. Isolated oocytes were snap-frozen and stored at −80℃ prior to RNA or protein extraction.

To obtain MII oocytes, female mice were injected with 5 IU of PMSG injection, followed by 5 IU hCG after 46 h. Super-ovulated MII oocytes were obtained from the oviduct 16 h later of hCG injection. CCs surrounding MII oocytes were removed by treating COCs with 300 U/ml hyaluronidase. Female mice were super-ovulated and then mated. Next morning, embryos were harvested at specific time points post hCG injection as follows: pronucleus 1-cell embryos (PN) at 18-20 h, 2-cell embryos (2C) at 44-46 h, 4-cell embryos (4C) at 56-58 h, 8-cell embryos (8C) at 68-70 h, morula (MO) stage at 80-85 h, and blastocyst (BL) stage at 96-98 h.

4. mRNA Isolation and Reverse Transcriptase (RT)-PCR

mRNA of oocytes and fertilized eggs was isolated using the Dynabeads mRNA DIRECT kit (Invitrogen Dynal As, Oslo, Norway) as previously described (Kim et al., 2011). The isolated mRNA was utilized as a template for reverse transcription and cDNA was synthesized with oligo dT_{25} primers according to the MMLV (Promega, Madison, WI) protocol. PCR was carried out with single-oocyte equivalent amount of cDNA and primers (Table 1). PCR products were separated on 1.5% agarose gels and quantified using Image J software.

5. Real-Time RT-PCR

To analyze mRNA expression of TAM receptor tyrosine kinases in oocytes and embryos, quantitative real-time RT-PCR analysis was performed using the iCycler (Bio-Rad, Hercules, CA) as described previously (Kim et al., 2008). Relative gene expression levels were normalized with *Hlfoo*. All experiments were repeated in triplicate.

Table 1. Sequence of oligonucleotide primers used in this study, their annealing temperature, and expected PCR product size

Gene	Accession numbers	Primer sequence * (5'→3')	Annealing temperature	Product Size
Mertk-A**	NM_008587	For- TTCTGGTTCTCGGCAAAGTT Rev- TCCACTTCACAGGCATTTTG	60℃	543 bp
Mertk-B**	NM_008587	For- AACAACACGGGGAATGACTC Rev- CACGAGAACAACCTTCGTGA	60℃	399 bp
Tyro3	NM_019392	For- AATCGGGGAGAACCCTTTTA Rev- TCATGATCTCCCACATGGTC	60℃	323 bp
Axl	NM_009465	For- TCATTGGCGTCTGTTTTCAG Rev- TCCACTTGACTGGCATCTT	60℃	348 bp
Gas6	BC005444	For-AAAGGGCCAGAGTGAAGTGA Rev-TTTTCCCGTTTACCTCCAGA	60℃	175bp
Gdf9	NM_008110	For- GGTTCTATCTGATAGGCGAGG Rev- GGGGCTGAAGGAGGGAGG	65℃	446 bp
Plat	NM_008872	For- CATGGGCAAGAGTTACACAG Rev- CAGAGAAGAATGGAGACGAT	60℃	631 bp
Mos	NM_020021	For- TGGCTGTTCCTACTCATTTC Rev- CTTTATACACCGAGCCAAAC	60℃	273 bp
H1foo	NM_138311	For- GCGAAACCGAAAGAGGTCAGAA Rev- TGGAGGAGGTCTTGGGAAGTAA	60℃	378 bp
GFP	EU056363	For- ATGGTGAGCAAGGGCGAG Rev- CTTGTACAGCTCGTCCAT	60℃	717 bp
Gapdh	BC092294	For- ACCACAGTCCATGCCATCAC Rev- TCCACCACCCTGTTGCTGTA	60℃	451 bp

* For = Forward; Rev = Reverse

** Primer set-A was used for preparation of dsRNA, while set-B was used to confirm the gene-specific knockdown after RNAi.

6. Oocyte Dot Blot Analysis

Oocyte lysate was made as previously described (Kim et al., 2008), and then loaded onto a Hybond-P PVDF membrane (GE healthcare, Buckinghamshire, UK). The membrane was blocked for 1 h in blocking buffer (Tris-buffered saline-Tween [TBST; 0.15 M NaCl, 0.1% Tween-20, and 10 mM Tris (pH 7.4)], containing 5% non-fat dry milk). The blocked membranes were then incubated with goat polyclonal anti-MERTK antibody (1:1000; sc-6873, Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal anti-α-Tubulin antibody (1:1000; sc-8035, Santa Cruz Biotechnology) in blocking buffer. After washing primary antibody, the membrane was incubated with horseradish-peroxidase-conjugated anti-goat IgG (1:2000; A5420, Sigma) or anti-mouse IgG (1:2000; A-2554, Sigma) in TBST for 1 h at room temperature. After each step, the membranes were washed several times with TBST and bound antibody was detected using an enhanced chemiluminescence detection system (GE healthcare) according to the manufacturer's instruction.

7. Preparation of *Mertk* dsRNA

To synthesize dsRNA for *Mertk* (543 bp), primer set-A was used for dsRNA preparation and set-B for the confirmation of knockdown of endogenous transcripts (Table 1). *Mertk* cDNAs was cloned into pGEM®-T Easy (Promega) and linearized with SpeI. Insert orientation was confirmed by PCR amplification using the T7 primer with each

Set-A primer. Single stranded (ss) RNA for each orientation was synthesized using the MEGAscript RNAi Kit (Ambion, Austin, TX) and T7 RNA polymerase. Complementary RNAs were mixed and incubated for annealing at 75℃ for 5 min, then cooled to room temperature. To confirm the formation of dsRNA, its mobility was compared with ssRNA by 1% agarose gel electrophoresis. The concentration of RNA was adjusted to 2 μg/μl for microinjection.

8. Microinjection and *In Vitro* Maturation of Oocytes

GV oocytes were microinjected with *Mertk* dsRNA in M2 medium containing 0.2 mM IBMX. The injection pipette containing dsRNA solution was inserted into the cytoplasm of an oocyte and 10 pl dsRNA was microinjected using a constant flow system (Transjector; Eppendorf, Hamburg, Germany). To assess injection damage, oocytes were injected with elution buffer alone and used as sham controls. To determine the rate of maturation *in vitro*, oocytes were cultured in 0.3% BSA + M16 medium containing 0.2 mM IBMX for 8 h, and then replaced the M16 medium without IBMX for 16 h in 5% CO_2 at 37℃. After RNAi experiments, *in vitro* maturation rates and morphological changes were recorded as previously described (Kim et al., 2008). Briefly, the maturation stage of oocytes was scored by the presence of a germinal vesicle (GV oocyte), a polar body (MII oocyte), or neither a germinal vesicle nor a polar body (MI oocyte).

9. Dual Kinase Activity Assay

Oocytes were washed in Dulbecco PBS containing 0.1% polyvinyl alcohol (PBS-PVA), and then each oocyte was placed in an 1.5 ml tube with 1 μl 0.1% PBS-PVA and 4 μl ice-cold extraction buffer (80 mM β-glycerophosphate, 25 mM HEPES [pH 7.2], 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM APMSF, 0.1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Samples were frozen at −80℃ until the assay. After thawing, oocytes were centrifuged at 13,000×g for 3 min, followed by the addition of 5 μl kinase buffer and 5 μl substrates, and then incubated for 20 min at 37℃. The kinase buffer comprised 75 mM HEPES (pH 7.2), 75 mM β-glycerophosphate, 75 mM $MgCl_2$, 6 mM DTT, 10 mM EGTA, 60 μM ATP, 15 μM cAMP-dependent protein kinase inhibitor peptide and 0.3 μCi/μl [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, Buckinghamshire, UK). The substrate solution for MPF and MAPK double kinase assay contained 4.5 μl histone H1 (5 mg/ml, calf thymus) and 0.5 μl myelin basic protein (MBP; 5 mg/ml, bovine brain). The reaction was terminated by the addition of 5 μl 4 × SDS sample buffer and boiling for 5 min. Samples were separated by 15% PAGE, and labeled MBP and histone H1 were analyzed by autoradiogram.

10. Statistical Analysis

Statistical analysis of real-time PCR data was evaluated using one-way analysis of variance (ANOVA) and a log linear model. Data were presented as means ± SEM, derived from at least three to five separate and independent experiments, and a value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Previously, we reported that *Gas6* transcript expressed highly in GV and MII oocytes, gradually decreased to an undetectable level at 2C and 4C stages, and slightly increased again between 8C and blastocyst stage embryos (Kim et al., 2011). *Gas6* is known as the soluble ligand of RTK of TAM family members (*Tyro3*, *Axl*, and *Mertk*) (Korshunov, 2012). TAM family members share high sequence homology with each other. The binding affinities of *Gas6* for the three types of receptor are different: *Axl* > *Tyro3* > *Mertk* (Nagata et al., 1996). *Axl* is ubiquitously expressed in various tissues, just like *Gas6*, and it appears that *Axl* is a major receptor for *Gas6* (Cavet et al., 2010). *Tyro3*, on the other hand, is mainly expressed in the brain; therefore, *Gas6* may play a tissue-specific role in neurons that is mediated by *Gas6-Tyro3* signaling (Ohashi

et al., 1994; Ohashi et al., 1995; Prieto et al., 2000). Although the binding affinity of *Gas6* to *Mertk* is relatively low, *Gas6-Mertk* signaling may play an essential role in particular cells or tissues under certain physiological conditions (Graham et al., 1995; Shao et al., 2009).

At first, we evaluated the expression of mRNA of *Gas6* and its three receptors in follicular components. As shown in Figure 1A, mRNA expression of *Gas6* and *Mertk* was detected in oocytes (OO) as well as follicular cells (cumulus cells; CC and granulosa cells; GC), whereas *Tyro3* and *Axl* were not expressed in oocytes at all. Expression of *Mertk* mRNA was relatively constitutive during oocyte maturation (GV, GVBD, and MI) with slight decrease in MII (Fig. 1B) and was persistent during early embryogenesis (Fig. 1C). Meanwhile the expression of *Axl* was not detected at all in oocytes and embryos (Fig. 1C). Interestingly, *Tyro3* mRNA level was not detected from GV oocytes to 2C stage embryos, but suddenly began to increase at 4C until blastocyst stages (Fig. 1C). Based on the expression pattern, we speculated that the *Gas6-Mertk* signaling presumably functions in oocytes and early stage embryos, while *Gas6-Tyro3* signaling works after 4C or thereafter. Oocyte maturation in the follicle requires the cumulus and granulosa cells in nature. Our data disclosed that mRNA of *Gas6* and all three receptors are expressed in the follicular cells. Therefore, the role of *Gas6* and its signaling with other receptors in the follicular cells on the oocyte maturation requires further studies in the near future.

Next, we examined effects of *Mertk* downregulation by microinjecting sequence-specific dsRNA into GV oocytes and its consequent cellular changes. Despite the significant decrease of mRNA and protein, 86.8% of oocytes treated with *Mertk* RNAi matured to the MII stage as similar to the result of *Gas6* (Table 2). In the case of control and buffer-injected oocytes, 95.7% and 90.1% matured to the MII stage, respectively (Table 2). In addition, MII oocytes

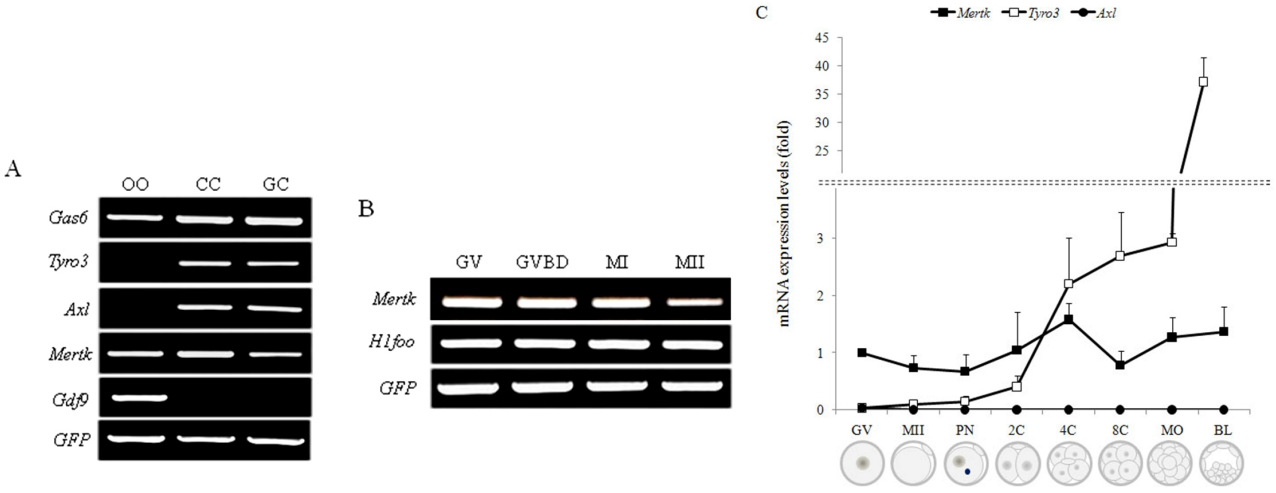

Fig. 1. Differential expression of *Gas6* and TAM family members (*Tyro3*, *Axl*, *Mertk*) of RTK in oocytes and follicular cells. (A) RT-PCR analysis of *Gas6* and its receptor expressions in follicular components. OO, GV oocyte; CC, cumulus cells; GC, granulosa cells. *Gdf9* was used as an oocyte-specific marker. (B) Expression pattern of *Mertk* during oocyte maturation. The mRNA equivalent to a single oocyte taken after culture for 0, 2, 8, and 16 h, corresponding to GV, GVBD, MI, and MII stages, respectively, was used for each lane. *H1foo* was used as an internal control. *GFP* was used as an external control to measure equal recovery. (C) Expression of *Tyro3*, *Axl*, and *Mertk* during early embryogenesis. Relative expression levels of TAM family members were calculated from C_T values, normalized to added GFP synthetic RNA, and the relative expression fold was calculated against *Mertk* level of GV oocyte. Experiments were repeated at least three times, and data were expressed as mean ± SEM. Closed squares, Mertk; Open squares, Tyro3; Closed circles, Axl.

Table 2. *In vitro* maturation of mouse oocytes after injection of *Mertk* dsRNA into GV oocytes

	Number of oocytes (%)			
	Total	Germinal vesicle (GV)	Metaphase I (MI)	Metaphase II (MII)
Control	92	0 (0) [a]	4 (4.3)	88 (95.7) [a]
Buffer	90	2 (2.2) [a]	7 (7.7)	81 (90.1) [b]
Mertk RNAi	107	7 (6.6) [b]	7 (6.6)	93 (86.8) [b]

[a,b] Different letters mean significant difference at $p<0.05$.

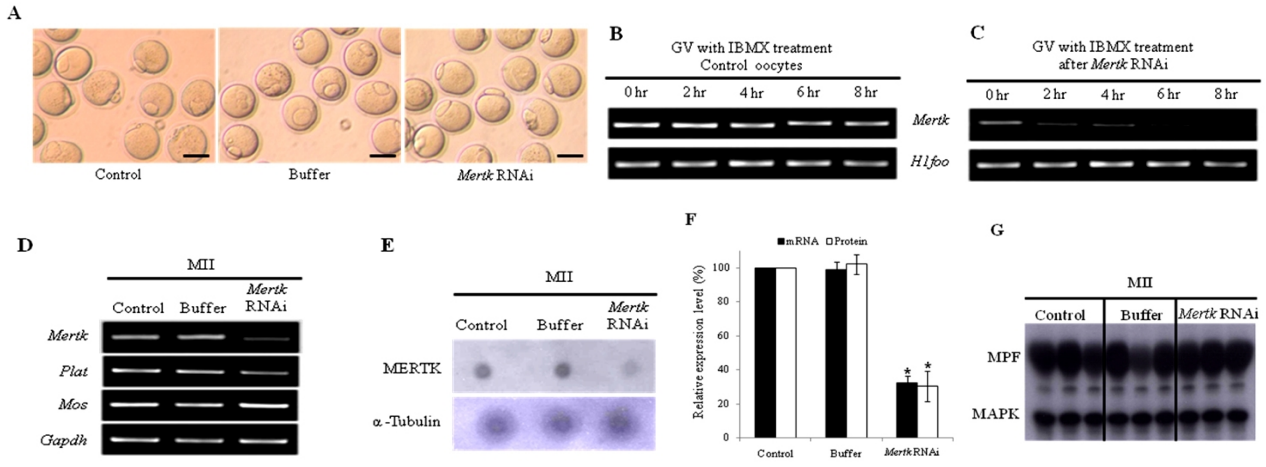

Fig. 2. *Mertk* knockdown oocytes matured normally to the MII stage. (A) Microphotographs of MII oocytes with or without *Mertk* RNAi. Scale bars indicate 100 μm. (B, C) Determination of the critical time point for complete knockdown of *Mertk* after RNAi. Oocytes were collected every 2 h after RNAi, and *Mertk* mRNA was assessed by RT-PCR. (D) RT-PCR analysis of gene expression profiles of targeted and untargeted, unrelated genes in *Mertk* dsRNA-injected MII oocytes. Targeted *Mertk* transcript was suppressed by *Mertk* RNAi without affecting untargeted mRNAs. (E) Reduced *Mertk* protein expression in MII oocytes following microinjection of *Mertk* dsRNA. *Mertk* Protein was analysed by oocyte dot blot, modified from Western Blot. Each dot contains proteins extracted from three MII oocytes. (F) Graphic presentation of results from B and C showing decreased *Mertk* mRNA and protein expression after *Mertk* RNAi in GV oocytes. Data are presented as mean ± SEM. Asterisks represent statistical significance at $p<0.05$. Black bars indicate the amount of *Mertk* mRNA and white bars indicate that of *Mertk* protein. Control, uninjected oocytes; Buffer, buffer-injected for sham control oocytes; Mertk RNAi, Mertk dsRNA-injected oocytes. (G) *Mertk* RNAi does not have the effect on the activity of MPF and MAPK. Phosphorylation of Histone H1 and MBP substrates reflecting the kinase activities of MPF and MAPK, respectively, was determined. Each lane contained one MII stage oocyte. Control, uninjected oocytes; Buffer, buffer-injected control oocytes; *Mertk* RNAi, Mertk dsRNA-injected oocytes.

with RNAi-mediated knockdown of *Mertk* showed normal morphology and maturation rate, quite similar to that of the control groups (Fig. 2A). Complete knockdown of *Mertk* mRNA was evaluated at 2h intervals in the IBMX supplemented medium (Fig. 2B-C). It took around 8 h to completely knock down the *Mertk* mRNA expression transiently. The reduction of *Mertk* mRNA (~68%) and protein (~70%) level as compared to the control (nothing injected) and buffer-injected groups was confirmed in MII oocytes (Fig. 2D-F). Therefore, it seemed likely that neither *Mertk* nor *Gas6* is involved in the nuclear maturation of oocyte.

M-phase promoting factor (MPF) consists of a catalytic subunit $p34^{cdc2}$ and a regulatory subunit cyclin B. During oocyte maturation, MPF activity is low in the G2 phase and high in the first and second metaphase oocytes, with a transient decrease at the first polar body emission (Masui, 2001). After polar body emission, cyclin B degradation stops and anaphase promoting complex/cyclosome (ACP/C) is reactivated upon entry into the second meiotic M phase by high levels of MPF. The equilibrium between slow degradation and continuous synthesis of cyclin B depends on cytostatic factor (CSF), and MPF activity is maintained at high levels in MII oocytes (Brunet and Maro, 2005; Ledan et al., 2001). When MPF activation was inhibited in oocytes, meiotic resumption was prevented and oocytes arrested at the GV stage (Goudet et al., 1998). Blocking MPF activity in oocytes before or immediately after first polar body extrusion also prevented entry into the second metaphase and led the oocytes into interphase, as manifested by the presence of a well-defined nucleus and the de-condensed chromosome. These findings provide evidence that MPF reactivation is absolutely necessary for maintenance of interkinesis (Josefsberg et al., 2003).

We have revealed in the previous study that although *Gas6*-silenced oocytes exhibited decreased MPF activity, those oocytes progressed into the MII stage (Kim et al., 2011). The activity of MPF in MII oocytes was dramatically lowered by *Gas6* RNAi compared to the control group, whereas the activity of MAPK in MII oocytes was maintained after *Gas6* RNAi at a level similar to that of the controls (Kim et al, 2011). On the contrary, *Mertk* RNAi showed no change in MPF as well as MAPK activities (Fig. 2G).

It has been reported that MAPK ($p42^{MAPK}$/Erk2 and $p44^{MAPK}$/Erk1) pathways are mainly involved in downstream of *Gas6* signaling through its RTKs in cell culture studies with recombinant human GAS6 protein (Goruppi et al., 1999; Sainaghi et al., 2005). For example, *Gas6-Axl* signaling in GnRH protects neuronal cells from apoptosis via phosphorylation of ERK1 and ERK2 (Allen et al., 1999). This signaling also involves migration of GnRH neurons mediated by p38 MAPK activation (Allen et al., 2002). In mouse osteoclasts, *Gas6-Tyro3* signaling up-regulated the activation of ERK1 and ERK2, but not p38 MAPK, resulting in stimulation of osteoclastic bone resorption (Katagiri et al., 2001). *Gas6-Mertk* signaling also transiently activated Erk2 in 293 cells (Chen et al., 1997). These results suggest that *Gas6* is a ligand for Tyro3, Axl, Mertk among RTKs, and its interactions with these receptors have been implicated in cell-specific features under specific conditions. The activity of MAPK did not alter in either *Gas6* or *Mertk*-deficient MII oocytes. These results indicate that *Gas6* and *Mertk* are not associated with meiotic nuclear maturation, events of GVBD, and progression to MII, with maintained ERK1 and ERK2 activity.

When *Gas6* knockdown MII oocytes were exposed to $SrCl_2$ for parthenogenetic activation, the oocytes did not extrude the second polar body and remained in the MII stage, suggesting that *Gas6* plays a critical role in the initiation for embryo development (Kim et al., 2011). When cytoplasmic maturation is not completed, oocytes fail to undergo fertilization and early embryo development (Hegele-Hartung et al., 1999). The loss of MPF activity induced by *Gas6*-RNAi is associated with the failure of cytoplasmic maturation, fertilization and embryogenesis.

Gas6-Mertk signaling is involved in various biological functions such as inflammation (Zizzo et al., 2012), thrombus formation (Cosemans et al., 2010), myelination (Binder et al., 2011), and cancer-mediated endothelial recruitment (Png et al., 2011). For instance, Karl et al. (2008) reported that endogenous *Gas6* and L-type Ca^{2+}-channel activation modulate phagocytosis through the stimulation of *Mertk* in retinal pigment epithelium. They suggested that L-type Ca^{2+}-channel could regulate downstream of both *Mertk* and $\alpha\beta 5$-*integrin*. However, no details about *Gas6-Mertk* function has been reported in the oocyte. In the present study, we revealed that *Mertk*-RNAi is not affected MPF activity and thus the *Gas6-Mertk* signaling is not related with the oocytes maturation. Nevertheless, the possibility

should not be eliminated that *Gas6-Mertk* signaling may play a role in early embryogenesis.

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