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Proline-rich tyrosine kinase2 is involved in F-actin organization during *in vitro* maturation of rat oocyte

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Abstract

Microfilaments (actin filaments) regulate various dynamic events during meiotic maturation. Relatively, little is known about the regulation of microfilament organization in mammalian oocytes. Proline-rich tyrosine kinase2 (Pyk2), a protein tyrosine kinase related to focal adhesion kinase (FAK) is essential in actin filaments organization. The present study was to examine the expression and localization of Pyk2, and in particular, its function during rat oocyte maturation. For the first time, by using Western blot and confocal laser scanning microscopy, we detected the expression of Pyk2 in rat oocytes and found that Pyk2 and Try402 phospho-Pyk2 were localized uniformly at the cell cortex and surrounded the germinal vesicle (GV) or the condensed chromosomes at the GV stage or after GV breakdown. At the metaphase and the beginning of anaphase, Pyk2 distributed asymmetrically both in the ooplasm and the cortex with a marked staining associated with the chromosomes and the region overlying the meiotic spindle. At telophase, Pyk2 was observed in the cleavage furrows in addition to its cortex and cytoplasm localization. The dynamics of Pyk2 were similar to that of F-actin, and this kinase was found to co-localize with microfilaments in several developmental stages during rat oocyte maturation. Microinjection of Pyk2 antibody demolished the microfilaments assembly and also inhibited the first polar body (PB1) emission. These findings suggest an important role of Pyk2 for rat oocyte maturation by regulating the organization of actin filaments.

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Introduction

Completely grown mammalian oocyte is arrested at the diplotene stage of first meiotic prophase, which is also termed germinal vesicle (GV) stage. The GV stage-arrested oocyte can resume meiosis under the stimulation of hormones or after release from the inhibitory environment of follicles *in vitro*. The resumption of meiotic maturation is manifested by germinal vesicle breakdown (GVBD), followed by chromatin condensation and microtubule reorganization. These events lead to the formation of the metaphase spindle, separation of the homologous chromosomes, emission of the first polar body (PB1), and subsequent completion of the first meiotic division, after which the oocyte enters meiosis II and becomes arrested at metaphase II (MII) until fertilization or parthenogenetic activation.

Protein kinases have been shown to play an important role in this maturation process, serving to regulate key signal transduction pathways common to many other cell types. Protein tyrosine kinases (PTKs) are essential in extracellular signaling and actin cytoskeleton organization. A small group of PTKs have been identified in eggs of invertebrates, lower vertebrates and some mammals, and inhibitor studies have suggested that they are involved in egg activation (Sato *et al.* 2000, Runft *et al.* 2002, Talmor-Cohen *et al.* 2004). It has been suggested that protein tyrosine phosphorylation may be implicated in the regulation of mouse oocyte maturation. Genistein, an inhibitor of PTKs, inhibited GVBD in a dose-dependent manner (Kimura 1996). A recent study shows that Xyk, an egg Src-related protein tyrosine kinase, may be involved in accelerating

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meiotic maturation in *Xenopus* oocytes and its activity increases at the time of GVBD and remains elevated till the completion of maturation (Tokmakov *et al.* 2005). These data suggest that PTKs may have some functions during meiotic maturation process. However, little is known about the subcellular distribution and specific function of any of these PTKs during mammalian oocyte maturation. Proline-rich tyrosine kinase2 (Pyk2) (Lev *et al.* 1995), also known as cell adhesion kinaseβ (CAKβ) (Sasaki *et al.* 1995) or related adhesion focal tyrosine kinase (RAFTK) (Avraham *et al.* 1995), is a nonreceptor tyrosine kinase related to focal adhesion kinase (FAK). Pyk2, activated by multiple stimuli including extracellular matrix, hormones, growth factors, inflammatory cytokines, TNF-α, angiotensin, depolarization, u.v. irradiation, changes in osmolarity, stress-related signals, and increase in intracellular calcium concentration, is involved in the control of cell adhesion, migration and survival. Pyk2 has an autophosphorylation site at tyrosine 402 and this phosphorylated residue creates a binging site for the SH2 domain of Src, which in turn phosphorylates and activates Pyk2 (Dikic *et al.* 1996). Pyk2 also binds to proteins that interact with the cytoskeleton, such as paxillin (Salgia *et al.* 1996), p130cas, the Rho-GAP protein Graf (Ohba *et al.* 1998), and a LIM domain containing protein (Lipsky *et al.* 1998, Matsuya *et al.* 1998), suggesting its role in regulation of the cytoskeleton and cellular morphology in response to extracellular stimuli. Overexpression of Pyk2 in fibroblastic cells leads to reorganization of actin-associated cytoskeleton structures and cell rounding (Du *et al.* 2001). Co-localization of Pyk2 and actin filaments has been demonstrated in fibroblastic cell periphery and growing neurites (Wang *et al.* 2003, Haglund *et al.* 2004). Studies of cells derived from Pyk2-deficient mice indicate that Pyk2 is important for F-actin organization (Okigaki *et al.* 2003). Pyk2 exerts its effect on microfilaments by regulating other proteins, such as Cb1, ArgBP2, Src (Haglund *et al.* 2004), and gelsolin (Wang *et al.* 2003), which are implicated in cytoskeleton regulation. Pyk2 is also shown to regulate the activity of Rho family (Hall 1998) and the deficiency of Pyk2 leads to impaired Rho and PI-3 kinase activation in macrophages (Okigaki *et al.* 2003).

The aim of this study was to investigate the expression and localization of Pyk2 in rat oocyte and to analyze the functional significance of the interactions between Pyk2 and actin filaments, and in particular, the role of Pyk2 during rat oocyte maturation. By using Western-blot analysis, confocal microscopy, and antibody microinjection, we revealed the expression and localization of Pyk2 and its co-localization with actin filaments as well as its regulation on actin organization during rat oocyte maturation.

Materials and Methods

Chemicals

All the chemicals used in this experiment were purchased from Sigma Chemical Company unless otherwise mentioned.

Oocyte collection

Twenty-one-day-old female Wister rat was injected i.p. with 20 IU pregnant mare's serum gonadotrophin (PMSG). Completely grown germinal vesicle-intact oocytes were collected approximately 48–50 h later from the ovaries and mechanically denuded of their cumulus cells with a thin-bore glass pipette and cultured in M2 medium supplemented with 60 μg/ml penicillin and 50 μg/ml streptomycin. All the cultures were carried out at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. Mature MII oocytes were obtained from female rat of the same strain. After i.p. injections of 20 IU of PMSG and hCG 54 h apart, the rats were killed and oviducts were removed at 15 h after hCG injection. Using a pair of fine forceps to tear the oviducts, cumulus masses were collected in M2 medium. To remove the cumulus cells, eggs were briefly exposed to 300 IU/ml hyaluronidase followed by three washes in M2 medium. The oocytes at different stages were collected for confocal microscopy.

Western-blot analysis

For the detection of Pyk2 expression in rat oocytes, proteins from a total of 300 GV or MII stage oocytes were extracted with SDS sample buffer and heated to 100 °C for 5 min. After cooling on ice and centrifuging at 10 000 ***g*** for 1 min, the total proteins were separated by SDS-PAGE with a 5% (w/v) stacking gel and a 6% (w/v) separating gel for 30 min at 70 V and for 1.5 h at 110 V respectively and then transferred onto PVDF transfer membrane for 2.5 h at 200 mA and 4 °C. The membrane was blocked for 2 h at room temperature in Tris-buffered saline (pH 7.4, 20 mM Tris, 137 mM NaCl) with 0.1% (v/v) Tween 20 (TBST) containing 5% (w/v) skimmed milk. The membrane was then incubated overnight at 4 °C with polyclonal Pyk2 antibody diluted 1:2000 in TBST containing 0.5% (w/v) skimmed milk. After three washes of 10 min each in TBST, the membrane was incubated for 1 h at 37 °C with goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2000. The membrane was washed three times in TBST and then processed using the electrochemiluminescence (ECL) detection system

Confocal microscopy

Oocytes at the desired stages were fixed in 4% (w/v) paraformaldehyde in PBS for 30 min and permeabilized

for 30 min in the incubation buffer (0.5% Triton X-100 in 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose and 0.02% NaN_3) at room temperature. After blocking in PBS containing 1% (w/v) BSA for 1 h and incubated with polyclonal Pyk2 antibody or polyclonal Tyr 402 phospho-Pyk2 (PY402-Pyk2) antibody (Cell signaling technology, Inc., Hong Kong) diluted 1:200 for 1 h, the oocytes were washed in PBS with 0.1% (v/v) Tween 20 three times and incubated for 1 h with 1:200 fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), followed by three washes and staining with 10 μg/ml propidium iodide. Finally, the oocytes were mounted on glass slides in 1,4-diazabicyclo (2.2.2) octane hydrochloride-containing (DABCO) medium, and examined using a laser scanning confocal microscope (Zeiss LSM510, Zeiss, Oberkochen, Germany). As a negative control, the first antibody was replaced with 1% BSA.

When oocytes were double labeled for microfilaments and Pyk2 or PY402-Pyk2, they were incubated with 1:200 TRITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h after incubation in rabbit anti-Pyk2 or PY402-Pyk2 antibody and three washes as described earlier. After another three washes, the eggs were incubated with 1:200 FITC-conjugated phalloidin for 1 h. Finally, after three washes, the eggs were mounted on glass slides and examined. The first antibody was replaced with 1% BSA in PBS as a negative control.

The actin filaments dynamics during the oocyte maturation as well as the F-actin and tubulin organization after antibody microinjection were examined by incubating the oocytes in 1:200 diluted FITC-conjugated phalloidin or FITC-conjugated α-tubulin antibody for 1 h after blocking as described above. Then the chromosomes were marked using 10 μg/ml propidium iodide after washing thrice. Finally, the samples were added on glass slides and examined.

Antibody microinjection

In order to observe the role of Pyk2 during the maturation of rat oocyte, Pyk2 antibody (0.5 mg/ml in PBS without Ca^{2+}, pH 7.4) was microinjected into the cytoplasm of GV oocytes as described by Dai *et al.* (2000). Each experiment was repeated three times, and 30–40 oocytes per group per experiment were used. To minimize the damage to oocyte, microinjection needle diameter was smaller than 1 μm. Hypoxanthine (HX, 4 mM) was added to the medium when GV stage oocytes were operated to prevent GVBD. A microinjection volume of about 7 pl per oocyte was used in all the experiments. The same amount of rabbit IgG (0.5 mg/ml) diluted in PBS was injected as control. After microinjection, the oocytes were incubated in 4 mM HX M2 medium for 30 min at 37 °C and then washed thoroughly with M2 medium. The oocytes were then cultured in M2 medium for the evaluation of GVBD and PB1 extrusion after 3 and 12 h respectively. The oocytes were collected for confocal microscopy at the same time.

Statistical analysis

All data on the rate of GVBD and the extrusion of the PB1 after microinjection were evaluated by χ^2-test. If $P<0.05$, the difference was considered as statistically significant.

Results

Expression of PYK2 in GV and MII oocytes

As shown in Fig. 1, Pyk2 was detected by Western-blot analysis in rat oocytes. A polyclonal Pyk2 antibody recognized a single band at about 116 kDa both in GV stage and MII stage oocytes.

Subcellular localization of Pyk2, Tyr402 phospho-Pyk2 and F-actin during rat oocyte meiotic maturation

We examined the Pyk2 and Tyr402 phospho-Pyk2 localization during the maturation of rat oocytes by using polyclonal anti-Pyk2 and PY402-Pyk2 antibody staining. The specimens were stained with propidium iodide to visualize the DNA and confirm the stage of meiotic maturation. Aggregation of both Pyk2 and phospho-Pyk2 in the cell cortex was a common feature of oocytes at different maturation stages (Figs 2 and 3). In completely grown GV stage oocytes, Pyk2 and phospho-Pyk2 distributed around the germinal vesicles (Figs 2A and 3A). Pyk2 was also found in the germinal vesicle (Fig. 2A). After GVBD, when chromatin assembled to form chromosome clusters, both Pyk2 and phospho-Pyk2 were associated with chromosomes and in the centre of the cytoplasm (Figs 2B and 3B). At

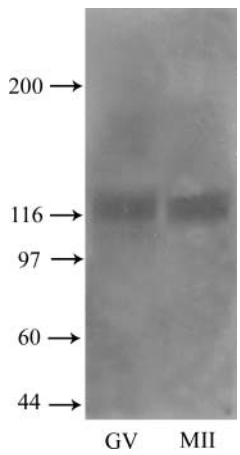

Figure 1 Western-blot analysis of Pyk2 in rat oocytes at GV and MII stages. Proteins from 300 rat oocytes were resolved by SDS-PAGE, transferred to PVDF membranes and then incubated with antibody raised against Pyk2 protein. A specific band was observed at about 116 kDa by comparison with co-migrating size marker.

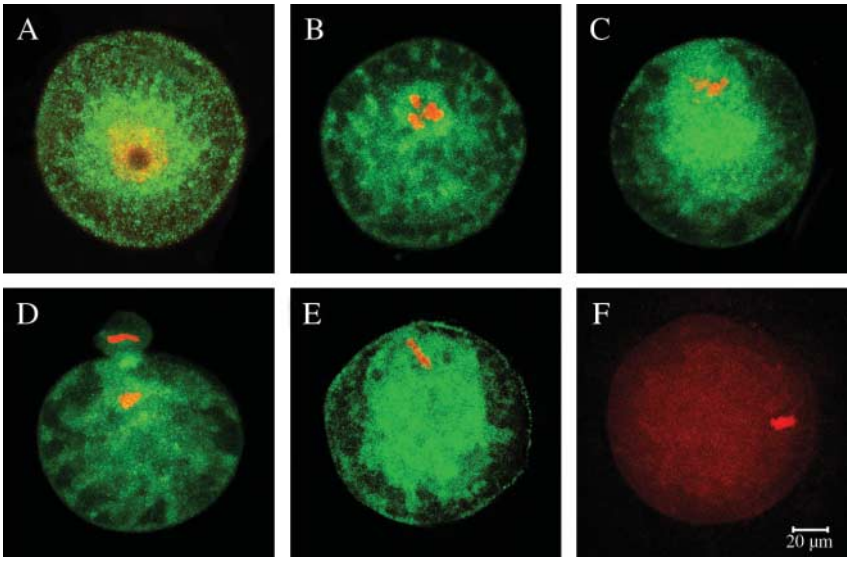

Figure 2 Subcellular localization of Pyk2 during rat oocyte maturation. Pyk2 was visualized after staining by FITC-labeled goat anti-rabbit serum after incubation with rabbit polyclonal Pyk2 antibody. The associated chromosomes were stained with propidium iodide. Bar represented 20 μm (F). All images represented a projection of multiple sections. Pyk2 was commonly localized in the cortex and the nucleus-surrounding area ((A)–(E)). Pyk2 surrounded the GV and the condensed chromosomes at the GV stage and after GVBD respectively ((A) and (B)). Abundant Pyk2 was aggregated in the cortex overlying the spindle (C). At anaphase/telophase, Pyk2 staining was associated with the separated chromosomes and in the cleavage furrow area between the extruding first polar body and the oocyte (D). In MII oocyte, Pyk2 evidently distributed around the chromosomes and the cortex associated with the spindle (E). Pyk2 staining could not be observed in the MII oocyte without Pyk2 antibody treatment (F).

the beginning of the anaphase I, staining was localized right around the chromosomes and at the cortex, prominently in the area overlying the meiotic spindle (Figs 2C and 3C). In telophase I, both Pyk2 and its activated isoform concentrated in the PB1 cleavage furrow as well as the central cytoplasm (Figs 2D and 3D). At MII (Figs 2E and 3E), with the organization of chromosomes to the equatorial plate of second meiotic

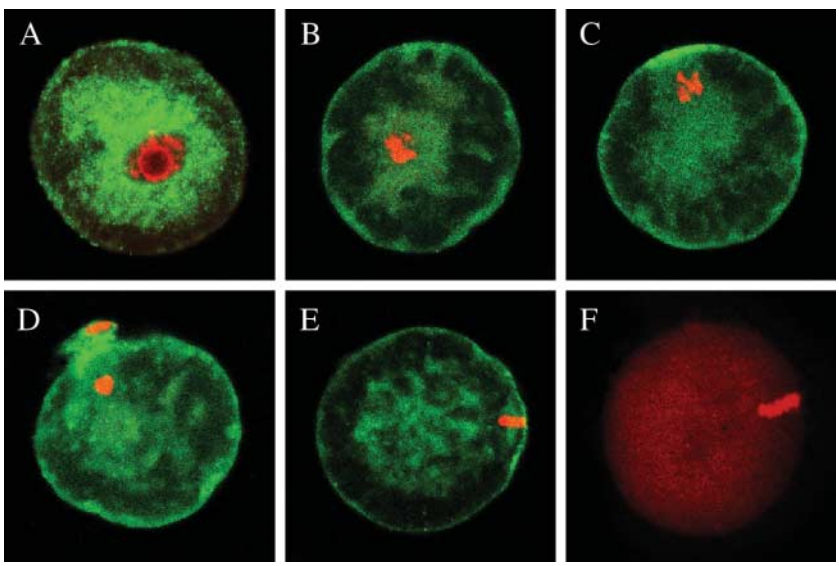

Figure 3 Subcellular localization of PY402 phospho-Pyk2 during maturation of rat oocytes. Immunofluorescence staining was performed using rabbit polyclonal PY402 phospho-Pyk2 antibody and p-Pyk2 was visualized by FITC-labeled goat anti-rabbit antibody. All images represented a projection of multiple sections. In GV stage oocyte, p-Pyk2 mainly surrounded the germinal vesicle and some weak staining was observed in the cortex (A). After GVBD, the cortex signal was increased and the cytoplasm localization was associated with the condensed chromosomes (B). At the beginning of anaphase, bright staining of p-Pyk2 was detected in the cortical region overlying the meiotic spindle despite cytoplasm localization (C). P-Pyk2 was mainly distributed in the cleavage furrow and the cell cortex in telophase (D). At metaphase II, p-Pyk2 was associated with the spindle and in the center of the cytoplasm. A thick staining was observed overlying the metaphase II spindle (E). An MII oocyte without staining by first antibody was used as a negative control for p-Pyk2 (F).

spindle, the immunoreactivity was observed to surround the spindle and in the ooplasm. A marked staining of phospho-Pyk2 was observed in the region overlying the metaphase spindle (Fig. 3E). The MII oocytes without primary antibody treatment were used as negative controls for Pyk2 and phospho-Pyk2 staining (Figs 2F and 3F).

To test whether there exists the distribution correlation between Pyk2 and actin filaments, we further localized F-actin during rat oocyte maturation by using FITC-labeled phalloidin. F-actin was distributed throughout the oocyte with a concentration in the cortex and around the GV (Fig. 4A). After GVBD, a dense uniform F-actin layer was observed at the cell cortex in addition to its staining around the condensed chromosomes (Fig. 4B). In anaphase I and telophase I, in addition to the cytoplasm localization, brightening staining was observed around the cortex especially the area overlying the spindle and in the cleavage furrow respectively (Fig. 4C–E). In MII oocyte, F-actin was detected on the spindle, around the cortex, and in the centre of ooplasm (Fig. 4F).

Co-localization of Pyk2/Tyr402 phospho-Pyk2 and actin filaments during oocyte maturation

We investigated the co-localization between Pyk2 and F-actin during *in vitro* maturation of the rat oocytes. As shown in Fig. 5, the staining pattern of Pyk2 or phospho-Pyk2 was identical with that of phalloidin conjugated F-actin staining except some specific microdomains. In GV-stage oocytes, Pyk2/ p–Pyk2 co-localized with F-actin both in the oocyte cortex and the periphery of the germinal vesicle (Fig. 5A and *a*, B and *b*). Pyk2 was also found in the GV (Fig. 5A), but phospho-Pyk2 (Fig. 5B) and F-actin (Fig. 5*a* and *b*) did not exist in the GV. After GVBD, F-actin was detected in the centre and the cortex of oocytes and some microfilament arrays radiated from central cytoplasm to the cortex (Fig. 5*c* and *d*). The localization of Pyk2 and activated Pyk2 was consistent with F-actin as shown in Fig. 5C and D. At MII stage, Pyk2 and phospho-Pyk2 were distributed asymmetrically and evident staining was found to aggregate on one side of the cytoplasm and the cortex (Fig. 5E and F). F-actin was co-localized with Pyk2 in the cortex and cytoplasm (Fig. 5*e* and *f*). MII oocytes without primary antibody staining were used as a negative control for Pyk2 (Fig. 5G and *g*) and phospho-Pyk2 (Fig. 5H and *h*).

Effects of Pyk2 antibody injection on oocyte maturation and F-actin organization

The GVBD rate of oocytes was not evidently inhibited by microinjection with Pyk2 antibody (63.0%, 85/135) compared with the control group (68.1%, 94/138). However, the F-actin organization around the chromosomes was severely disrupted by Pyk2 antibody injection (Fig. 6A). Actin filaments

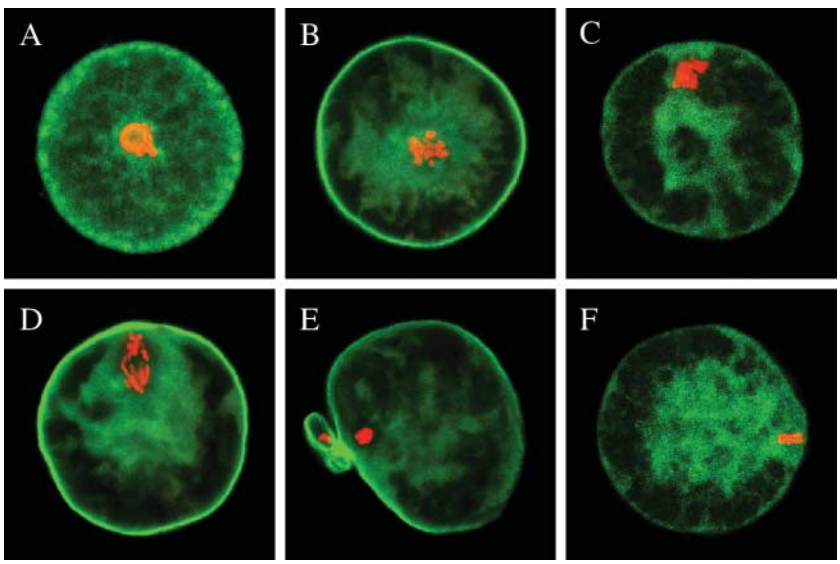

Figure 4 Subcellular localization of F-actin during oocyte maturation. F-actin was stained by FITC-conjugated phalloidin. All images represented a projection of multiple sections. A cortex localization of F-actin was observed in all stages of oocyte maturation ((A)–(E)). A thick layer of F-actin was distributed in the GV oocyte cortex and around the germinal vesicle (A). After GVBD, condensed F-actin surrounded the chromosome and distributed uniformly in the cortex (B). In anaphase/telophase, F-actin was in the periphery of the chromosome and in the center of the ooplasm. The cortical F-actin distribution became asymmetrical with a relatively thick layer covering the spindle ((C) and (D)). F-actin aggregated in the cleavage furrow and the nearby cortex (E). In metaphase II, actin staining was detected around the metaphase II spindle and in the center of the cytoplasm (F).

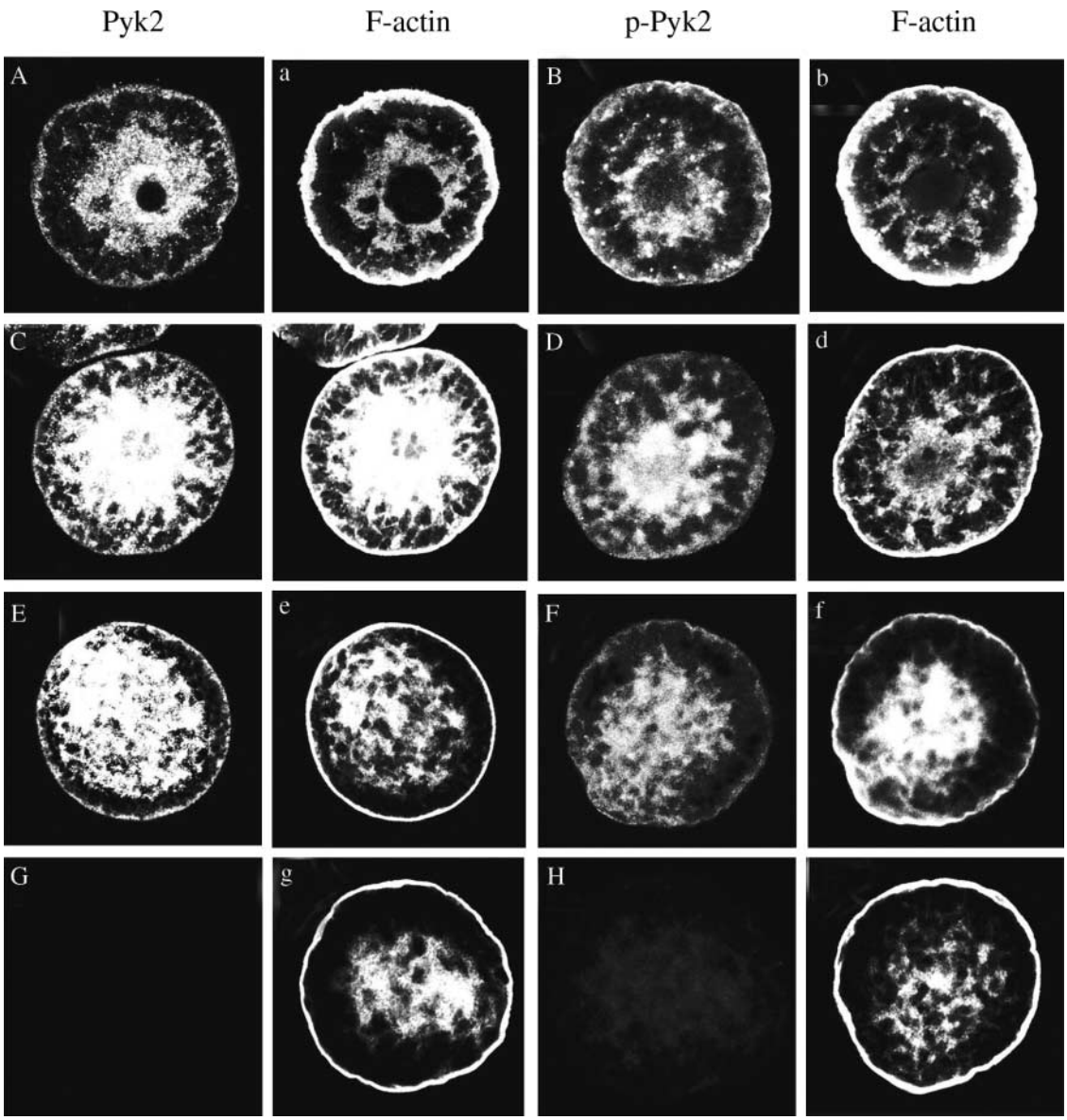

Figure 5 Co-localization of Pyk2/p-Pyk2 and F-actin during oocyte maturation. Pyk2 ((A), (C) and (E)) and p-Pyk2 ((B), (D) and (F)) were stained with goat anti-rabbit TRITC-conjugated antibody followed by Pyk2/p-Pyk2 antibody treatment, while F-actin (a–h)) was stained with FITC-phalloidin. All images represented a projection of multiple sections. The distribution of Pyk2 and p-Pyk2 were coincident with that of F-actin in rat eggs except some specific areas ((A)–(F)). Both Pyk2 (A)/p-Pyk2 (B) and F-actin (a and b) were localized in the oocyte cortex and the center of the cytoplasm around the germinal vesicle. Pyk2 was also detected in GV (A). Pyk2 (C)/p-Pyk2 (D) and F-actin (c and d) were observed in the center and the cortex of the cell after GVBD. Many filament arrays radiated from the cytoplasm to the cortex. At MII, Pyk2 (E)/p-Pyk2 (F) and F-actin (e and f) distributed asymmetrically in the cytoplasm and in the cortex. An MII oocyte without primary antibody treatment was used as a negative control for Pyk2 ((G) and g) and p-Pyk2 ((H) and h) staining.

were detected around the chromosomes in the control group oocytes (Fig. 6B). At 12 h, the antibody- and sham-injected oocytes were scored for the emission of PB1. The rate of PB1 emission was decreased from 56.1% (32/57) in the control group to 24.7% (20/81) in the antibody microinjected group ($P<0.05$). In the control group, more than half of the oocytes (56.1%) formed a typical telophase spindle and extruded the PB1 (Fig. 7A–C). F-actins were detected in the cytoplasm and in the cleavage furrow (Fig. 7C). While the antibody microinjected oocytes either did not form meiosis I spindle (Fig. 7D) or was blocked at MI stage (Fig. 7E). Some oocytes were at the anaphase stage, while the chromosomes separated in an abnormal direction, which was parallel instead of vertical to the plasma membrane (Fig. 7F). The cytoplasm microfilaments in the experimental oocytes were destroyed, while the uniform cortex F-actins still could be detected (Fig. 7F).

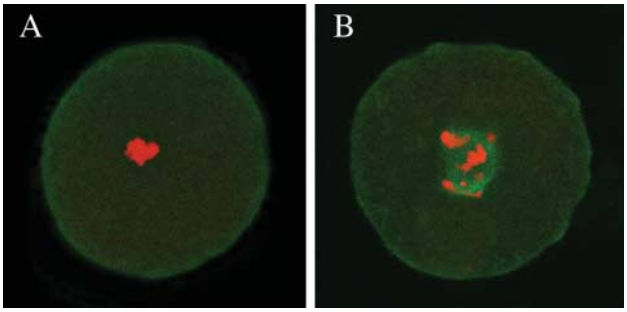

Figure 6 Microfilament localization for 3 h after microinjection. F-actin was stained with FIFC-conjugated phalloidin. Both images represented a projection of multiple sections. F-actin was disrupted both in the cytoplasm and the cortex of the oocyte after Pyk2 antibody microinjection (A), while some F-actins were observed associated with the condensed chromosome and in the cortex in control oocyte (B).

Discussion

The aim of our study was to examine the expression and localization of Pyk2, and its potential role during rat oocyte maturation.

Pyk2 is expressed at a high level in the brain, hematopoietic cells, osteoclasts and to a lesser extent in certain epithelial cells, smooth muscle cells, and fibroblasts (Avraham *et al.* 2000). Recent data show that Pyk2 is also expressed in prostate (Picascia *et al.* 2002) and testis (Chieffi *et al.* 2003). However, its expression and localization in oocyte is not known. Expression of Fyn, a member of Src kinase family (SFKs), is demonstrated and its activity is detected in rat eggs, which represents the first demonstration of a PTK in mammalian eggs (Talmor *et al.* 1998). Furthermore, other members of SFKs, including Src and Yes, were detected in rat eggs (Talmor-Cohen *et al.* 2004). In the present study, for the first time, we detected Pyk2 expression in mammalian eggs.

We have found that Pyk2 is co-localized with F-actin at various stages of oocyte maturation, suggesting that Pyk2 may be involved in regulating the polymerization and/or the organization of actin filaments during the oocyte maturation. The co-localization of Pyk2 and cortical F-actin is a common feature during rat oocyte maturation. The actin filaments that are associated with plasma membrane are important for generating cell-surface specialization areas and also provide the driving force for remodeling cell structure. This is achieved through the actin-binding proteins (Sun & Schatten 2006). Dmoesin, a member of ezrin-radixin-moesin (ERM) family, is required during oogenesis to anchor microfilaments to oocyte cortex in *Drosophila* (Polesello *et al.* 2002). Little is known about the proteins interacting with F-actin in the mammalian egg cortex. Immunostaining of Rho proteins shows that Rac1 and RhoB are present in the cortical ooplasm in mouse (Kumakiri *et al.* 2003). According to our results, the cortical localization of Pyk2 may imply that Pyk2 connects with F-actin and regulates its functions in the rat oocyte cortex. However, F-actin staining becomes weak but does not disappear after antibody microinjection and this may be due to the substitute pathways that regulate F-actin assembly which exist during oocyte maturation.

In GV stage oocyte, although expressed, Pyk2 is distributed in both cell periphery and cytoplasm including in the GV, activated Pyk2 is not located in the germinal vesicle. The staining pattern of activated Pyk2 is in close proximity with that of F-actin, suggesting that activated Pyk2 may contribute to the actin polymerization in GV oocyte. After GVBD, both Pyk2 and F-actin are aggregated around the condensed chromosomes. When GV oocytes are microinjected

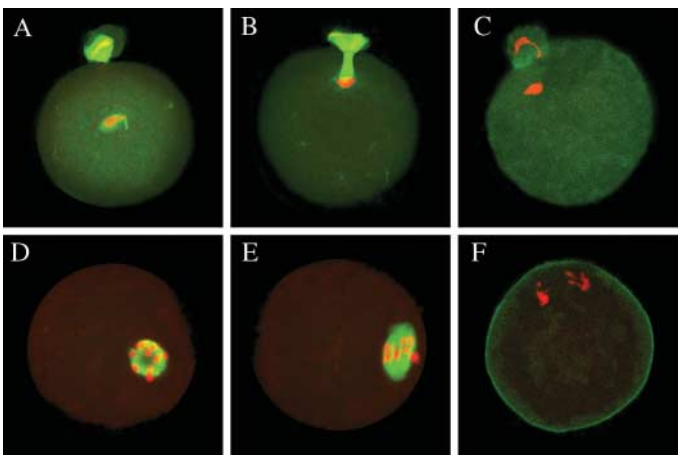

Figure 7 Tubulin and microfilament localization 12 h after microinjection. F-actin and tubulin were stained with FIFC-conjugated phalloidin and α-tubulin antibody respectively. All images represented a projection of multiple sections. In the control group, the oocytes extruded first polar body ((A)–(C)), and form a correct telophase spindle (B). F-actin was observed in the cleavage furrows and the cytoplasm in sham injected oocyte (C). After Pyk2 antibody injection, oocytes could not properly organize a metaphase I spindle (D) or stayed at MI (E). The cytoplasmic microfilaments were destroyed while the cortex staining was present uniformly after antibody injection and the chromosome separated parallel to the plasma membrane (F).

with Pyk2 antibody, the microfilaments are disrupted while GVBD happened. These results suggest that actin filaments have no effect on the GVBD and Pyk2 plays an important role in F-actin polymerization during the process of GVBD. Although F-actin is still detected co-localized with the chromosomes in the control group oocyte, its staining pattern is different from the normal culture oocyte. This may be due to the damage of the microinjection and the effect of the injected IgG. After GVBD, the condensed chromosomes organize to align at the metaphase plate and the meiotic spindle and then move peripherally. Coincident with the cortical localization of the chromosome is the formation of an area in the cortex rich in microfilaments and the polarization of the oocyte, which is required for asymmetric division. In our study, in both metaphase and the beginning of the anaphase, Pyk2 is asymmetrically associated with the chromosomes in ooplasm and the oocyte cortical area overlying the metaphase and the anaphase spindle. The distribution asymmetry and similarity with F-actin suggest that Pyk2 may contribute to polarity establishment in rat oocytes. Confocal microscopy results show that oocytes are arrested before MI stage or at anaphase after antibody injection. Some eggs separate the chromosomes with the spindle axis parallel instead of vertical to the plasma membrane. This may be due to the rotation of the spindle which is blocked. Although the cortical F-actin still exists after antibody injection, the polarity of the microfilament distribution disappears. They distribute uniformly in the cortex, which may contribute to the disability of the spindle rotation. However, how Pyk2 participates in polarity establishment is not known. A recent report indicates that PAR-3, a member of evolutionarily partitioning defective (PAR) proteins, is located within a central subdomain of the polarized actin cap, which overlies the spindle, prior to emission of the first and the second polar bodies. PAR-3 is phosphorylated during maturation. These results suggest a role for PAR-3 in establishing polarity in the egg and in defining the future site of polar body emission in mouse oocyte (Duncan *et al.* 2005). Our study also shows abundant distribution of phosphorylated Pyk2 in the cortical domain overlying the meiotic spindle, indicating that tyrosine phosphorylation may be involved in this process. We propose that Pyk2 may regulate the reorganization of F-actin in the spindle-associated cortical area by phosphorylating some microfilament regulatory proteins to form the polarity of the egg. In telophase, bundles of F-actin form a contractile ring and create a cleavage furrow to partition the oocyte into two daughter cells. Myosin II proteins also concentrate in the second polar body cleavage furrows of mouse egg (Simerly *et al.* 1998). Distinct Pyk2 staining is observed in meiosis I cleavage furrows in our study. Microinjecting Pyk2 antibody into the oocyte at GV stage results in an inhibition of the PB1 emission in our experiment. This may indicate that Pyk2 functions as a regulator of the proteins participating in contractile ring formation such as F-actin and/or myosin or a part of the contractile apparatus during the PB1 emission. However, the PB1 emission is lower in the IgG injection group (control group, 56.1%) compared with the normal cultured oocytes (about 75%) and the pattern of F-actin staining is quite different between the microinjected oocytes and the normal culture oocytes. It suggests that microinjection of IgG may injure the oocyte and have some effects on oocyte maturation. In our experiments, the PB1 emission is not totally inhibited by Pyk2 antibody microinjection, this is because Pyk2 is not a key regulator for F-actin or some other proteins or kinases participate in regulating F-actin together with Pyk2. Recently, p-MARCK (myristoylated alanine-rich C-kinase substrate), a major substrate for protein kinase C, is found to enrich in the periphery of the actin cap overlying the MI or MII spindle to form a ring-shaped subdomain (Michaut *et al.* 2005). In mitotic cells, contractile ring assembly is directed by the RhoA guanosine triphosphatase (GTPase), which activates myosin and actin filaments assembly (Glotzer 2005). RhoA and Cdc42 are found located in the cleavage furrow, and inhibition of RhoA and Cdc42 activation blocks polar body formation (Zhong *et al.* 2005, Ma *et al.* 2006). The mechanism of actin filament regulation during the polar body emission needs to be further elucidated in the mammalian eggs.

PY402 and its kinase activity are crucial for Pyk2 function on regulating polymerization of actin filaments (Wang *et al.* 2003). Our results reveal a consistent distribution of activated Pyk2 (Tyr 402) and F-actin during the process of oocyte maturation, which suggests an important role of tyrosine phosphorylation in regulating function of F-actin in oocyte. Phosphorylation of tyrosine 402 (tyr402), an auto-phosphorylation site in Pyk2 creates a binding site for the SH2 domains of Src family kinase. Src binding to tyr402 leads to activation of Src, which in turn phosphorylates and activates Pyk2 (Dikic *et al.* 1996). We have examined the immunoactivity of SFKs in rat oocyte, which are also associated with the chromosomes and localized in the cytoplasm and the cell cortex (data not shown), so it is intriguing to speculate that Pyk2 may regulate functions of RhoA or other proteins important for actin polymerization by the activation of Src.

Taken together, in this study, for the first time we show the expression and localization of Pyk2 during *in vitro* maturation of rat oocyte. We also detect the co-localization of Pyk2 and F-actin in the oocytes at various maturational stages. Furthermore, we find that the microinjection of Pyk2 antibody inhibits the organization of F-actin after GVBD and blocks the PB1 emission. These results lead us to propose that Pyk2 may regulate actin filaments polymerization and organization during rat oocyte maturation.

References

Avraham S, London R, Fu Y, Ota S, Hiregowdara D, Li J, Jiang S, Pasztor LM, White RA, Groopman JE & Avraham H 1995 Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *Journal of Biological Chemistry* **270** 27742–27751.

Avraham H, Park SY, Schinkmann K & Avraham S 2000 RAFTK/Pyk2 mediated cellular signaling. *Cellular Signalling* **12** 123–133.

Chieffi P, Barchi M, Agostino SD, Rossi P, Tramontano D & Geremia R 2003 Prolin-rich tyrosine kinase2 (PYK2) expression and localization in mouse testis. *Molecular Reproduction and Development* **65** 330–335.

Dai Y, Lee C, Hutchings A, Sun Y & Moor R 2000 Selective requirement for Cdc25C protein synthesis during meiotic progression in porcine oocytes. *Biology of Reproduction* **62** 519–532.

Dikic I, Tokiwa G, Lev S, Courtneidge SA & Schlessinger J 1996 A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383** 547–550.

Du QS, Ren XR, Xie Y, Wang Q, Mei L & Xiong WC 2001 Inhibition of PYK2-induced actin cytoskeleton reorganization, PYK2 autophosphorylation and focal adhesion targeting by FAK. *Journal of Cell Science* **114** 2977–2987.

Duncan FE, Moss SB, Schultz RM & Williams CJ 2005 PAR-3 defines a central subdomain of the cortical actin cap in mouse eggs. *Developmental Biology* **280** 38–47.

Glotzer M 2005 The molecular requirements for cytokinesis. *Science* **307** 1735–1739.

Haglund K, Ivankovic-Dikic I, Shimokawa N, Kruh GD & Dikic I 2004 Recruitment of Pyk2 and Cb1 to lipid rafts mediates signals important for actin reorganization in growing neurites. *Journal of Cell Science* **117** 2557–2568.

Hall A 1998 Rho GTPases and the actin cytoskeleton. *Science* **279** 509–514.

Kimura H 1996 Studies on protein tyrosine phosphorylation in mouse oocyte maturation. *Nippon Sanka Fujinka Gakkai Zasshi* **48** 1149–1156.

Kumakiri J, Oda S, Kinoshita K & Miyazaki S 2003 Involvement of Rho family G protein in the cell signaling for sperm incorporation during fertilization of mouse eggs: inhibition by *Clostridium difficile* toxin B. *Developmental Biology* **260** 522–535.

Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, Plowman GD, Rudy B & Schlessinger J 1995 Protein tyrosine kinase PYK2 involved in Ca2+-induced regulation of ion channel and MAP kinase functions. *Nature* **376** 737–745.

Lipsky BP, Beals CR & Staunton DE 1998 Leupaxin is a novel LIM domain protein that forms a complex with PYK2. *Journal of Biological Chemistry* **273** 11709–11713.

Ma C, Benink HA, Cheng D, Montplaisir V, Wang L, Xi Y, Zheng PP, Bement WM & Liu XJ 2006 Cdc42 activation couples spindle positioning to first polar body formation in oocyte maturation. *Current Biology* **16** 214–220.

Matsuya M, Sasaki H, Aoto H, Mitaka T, Nagura K, Ohba T, Ishino M, Takahashi S, Suzuki R & Sasaki T 1998 Cell adhesion kinaseβ forms a complex with a new member, Hic-5, of proteins localized at focal adhesions. *Journal of Biological Chemistry* **273** 1003–1014.

Michaut MA, Williams CJ & Schultz RM 2005 Phosphorylated MARCKS: a novel centrosome component that also defines a peripheral subdomain of the cortical actin cap in mouse eggs. *Developmental Biology* **280** 26–37.

Ohba T, Ishino M, Aoto H & Sasaki T 1998 Interaction of two proline-rich sequences of cell adhesion kinase beta with SH3 domains of p130Cas-related proteins and a GTPase-activating protein, Graf. *Biochemical Journal* **330** 1249–1254.

Okigaki M, Davis C, Falasca M, Harroch S, Felsenfeld DP, Sheetz MP & Schlessinger J 2003 Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *PNAS* **100** 10740–10745.

Picascia A, Stanzione R, Chieffi P, Kisslinger A, Dikic I & Tramontano D 2002 Pyk2 regulates proliferation and differentiation of prostate cells. *Molecular and Cellular Endocrinology* **186** 81–87.

Polesello C, Delon I, Valenti P, Ferrer P & Payre F 2002 Dmoesin controls actin-based cell shape and polarity during *Drosophila melanogaster* oogenesis. *Nature Cell Biology* **4** 782–789.

Runft LL, Jaffe LA & Mehlmann LM 2002 Egg activation at fertilization: where it all begins. *Developmental Biology* **245** 237–254.

Salgia R, Pisick E, Sattler M, Li JL, Uemura N, Wong WK, Burky SA, Hirai H & Chen LB 1996 P130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene. *Journal of Biological Chemistry* **271** 25198–25203.

Sasaki H, Nagura K, Ishino M, Tobioka H, Kotani K & Sasaki T 1995 Cloning and characterization of cell adhesion kinase β, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *Journal of Biological Chemistry* **270** 21206–21219.

Sato K, Tokmakov AA & Fukami Y 2000 Fertilization signaling and protein-tyrosine kinases. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **126** 129–148.

Simerly C, Nowak G, de Lanerolle P & Schatten G 1998 Differential expression and functions of cortical myosin IIA and IIB isotypes during meiotic maturation, fertilization, and mitosis in mouse oocytes and embryos. *Molecular Biology of the Cell* **9** 2509–2525.

Sun QY & Schatten H 2006 Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction* **131** 193–205.

Talmor A, Kinsey WH & Shalgi R 1998 Expression and immunolocalization of $p59^{c\text{-}fyn}$ tyrosine kinase in rat eggs. *Developmental Biology* **194** 38–46.

Talmor-Cohen A, Tomashov-Matar R, Eliyahu E, Shapiro R & Shalgi R 2004 Are Src family kinases involved in cell cycle resumption in rat eggs?. *Reproduction* **127** 455–463.

Tokmakov A, Iwasaki T, Itakura S, Sato K, Shirouzu M, Fukami Y & Yokoyama S 2005 Regulation of Src kinase activity during *Xenopus* oocyte maturation. *Developmental Biology* **278** 289–300.

Wang Q, Xie Y, Du QS, Wu XJ, Feng X, Mei L, Mcdonald JM & Xiong WC 2003 Regulation of the formation of osteoclastic actin rings by proline-rich tyrosine kinase 2 interacting with gelsolin. *Journal of Cell Biology* **160** 565–575.

Zhong ZS, Huo LJ, Liang CG, Chen DY & Sun QY 2005 Small GTPase RhoA is required for ooplasmic segregation and spindle rotation, but not for spindle organization and chromosome separation during mouse oocyte maturation, fertilization, and early cleavage. *Molecular Reproduction and Development* **71** 256–261.

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