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# Expression of Focal Adhesion Kinase in Mouse Cumulus-Oocyte Complexes, and Effect of Phosphorylation at Tyr397 on Cumulus Expansion

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## SUMMARY

We investigated the expression of focal adhesion kinase (FAK) in mouse cumulus–oocyte complexes (COCs), as well as the role of FAK phosphorylation at Tyr397 during oocyte maturation. The effect of inhibiting FAK phosphorylation at Tyr397 during in vitro maturation (IVM) on subsequent fertilization and preimplantation embryo development was also examined. Western blotting analyses revealed that total and Tyr397-phosphorylated FAK were expressed in vivo in both cumulus cells and oocytes. Immunocytochemical studies localized this kinase throughout the cytoplasm of cumulus cells and oocytes; in particular, Tyr397-phosphorylated FAK tended to accumulate in regions where cumulus cells contact each other. Interestingly, the in vivo level of Tyr397 phosphorylation in cumulus cells was significantly lower after compared to before cumulus expansion. Addition of FAK inhibitor 14, which specifically blocks phosphorylation at Tyr397, stimulated oocyte meiotic maturation and cumulus expansion during IVM in the absence of follicle-stimulating hormone (FSH). Reverse-transcriptase PCR showed that the mRNA expression of hyaluronan synthase 2 (*Has2*), a marker of cumulus expansion, was significantly induced in cumulus cells. Subsequent in vitro fertilization and culture showed that more oocytes developed to the blastocyst stage when they were treated with FAK inhibitor 14 during IVM, although the blastocyst total cell number was lower than in oocytes stimulated with FSH. These results indicate that FAK is involved in the maturation of COCs; specifically, phosphorylation at Tyr397 may regulate cumulus expansion via the expression of *Has2* mRNA in cumulus cells, which could affect the developmental competence of oocytes.


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## INTRODUCTION

The final events of oogenesis occur in large antral follicles, where the oocyte is intimately surrounded by supporting cumulus cells, forming cumulus -oocyte complexes (COCs). In this special niche-cumulus cells are tightly connected to each other and to the oocyte via cell adhesion complexes and gap junctions (Eppig, 1991; Kidder and Mhawi, 2002). The oocyte depends on these associated

**Abbreviations:** COCs, cumulus-oocyte complexes; EGF[R], epidermal growth factor [receptor]; ERK1/2, extracellular signaling-regulated kinase 1/2; FAK, focal adhesion kinase; FSH, follicle-stimulating hormone; GV, germinal vesicle; Has2, hyaluronan synthase 2; hCG, human chorionic gonadotropin; IVM, in vitro maturation; LH, luteinizing hormone; MI/II, metaphase I/II; PMSG, pregnant mare serum gonadotropin; SFK, SRC-family tyrosine kinase

cumulus cells for glucose metabolism and supplying pyruvate for energy production (Biggers et al., 1967; Gardner et al., 1996; Preis et al., 2005). Oocytes are arrested for an exceptionally long time in prophase I of meiosis, which is referred to as the germinal vesicle (GV) stage. Meiotic resumption is manifested by germinal vesicle breakdown (GVBD), which is followed by chromosome condensation and metaphasel (MI) spindle assembly; homologous chromosome segregation; and completion of the first meiotic division. Meiosis is arrested again at metaphase II (MII) until fertilization.

COCs expand dramatically during meiotic maturation in oocytes. This phenomenon, termed "cumulus expansion", occurs after the pre-ovulatory surge of gonadotropins, and is believed to support COC dissociation from the follicle wall and its expulsion through the ruptured follicle wall during ovulation (Chen et al., 1993). Cumulus expansion is essential for fertilization and the development of early embryos (Chen et al., 1993; Hess et al., 1999), and failure of these processes causes infertility (Russell and Robker 2007).

Compelling evidence from mouse studies has shown that the ovulatory surge of luteinizing hormone (LH) induces a rapid secondary cascade of epidermal growth factor (EGF)-like peptides (such as amphiregulin, epiregulin, β-cellulin, etc.) in the somatic cells of the follicle, leading to cumulus expansion, oocyte maturation, and ovulation (Park et al., 2004; Hsieh et al., 2007). These peptides act on granulosa and cumulus cells via EGF receptor (EGFR) (Park et al., 2004; Shimada et al., 2006; Noma et al., 2011). Binding of EGF stimulates EGFR tyrosine kinase activity, resulting in EGFR autophosphorylation and subsequent tyrosine phosphorylation of numerous substrates within the cell (Carpenter and Cohen, 1990). Insulin-like growth factor I, which binds to a tetrameric transmembrane receptor tyrosine kinase, efficiently reduces the apoptosis of cumulus cells cultured in vitro (Sirotkin et al., 2002) while stimulating the proliferation and terminal differentiation of cumulus cells during the expansion process (Khamsi and Armstrong, 1997). Nerve growth factor, whose tyrosine kinase receptor, TRKA, is also located in cumulus cells, induces marked cumulus expansion and progressive cumulus-oocyte uncoupling (Barboni et al., 2002).

In oocytes, on the other hand, SRC-family tyrosine kinases (SFKs), which are closely related both structurally and functionally (Thomas and Brugge, 1997), are expressed at much higher levels than in other cell types. *Fyn* and *Yes* transcripts, in particular, are more abundant in oocytes than in most other mammalian cell types (Luo et al., 2009). Antibodies specific for the catalytic domains of SFKs revealed that both inactive and active SFKs are closely associated with the meiotic spindle in mouse oocytes (McGinnis et al., 2007; Zheng et al., 2007). Therefore, tyrosine kinase activity is important for regulating oocyte meiotic maturation and cumulus expansion.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that plays a critical role in a variety of biological functions, including cell proliferation, spreading, motility, and survival or apoptosis (Schaller et al., 1992; Schwartz et al., 1995; Hanks and Polte, 1997; Chan et al., 1999; Sonoda et al., 2000; Wang et al., 2001; Goel and Dey, 2002). FAK is phosphorylated on tyrosine residues in response to integrin engagement, G protein-coupled receptor activation, and growth-factor receptor stimulation (Zachary and Rozengurt, 1992; Girault et al., 1999). Integrin-stimulated FAK phosphorylation, for example, is complex, and can occur at six or more sites in vivo: two sites within the FAK amino-terminal domain (Tyr397 and 407); two sites within the kinase domain activation loop (Tyr576 and 577); and two sites within carboxy-terminal domain (Tyr861 and 925) (Calalb et al., 1995; Schlaepfer and Hunter, 1996). Phosphorylation of Tyr397 generally occurs first, which allows binding of SH2 domains of SRC (Schaller et al., 1994), FYN (Cobb et al., 1994), or phosphatidylinositol 3-kinase (PI3K) (Chen et al., 1996). Recruitment of these SFKs stimulates phosphorylation of additional tyrosine residues (Schlaepfer et al., 1994; Calalb et al., 1995; Calalb et al., 1996). Phosphorylation at Tyr576 and 577 in the catalytic domain increases FAK activity (Calalb et al., 1995; Owen et al., 1999), while phosphorylation at Tyr925 can activate the RAS pathway and extracellular signaling-regulated kinase 1 and 2 (ERK1/2) (Schlaepfer et al., 1994). Binding of PI3K, on the other hand, activates the anti-apoptotic protein kinase B (PKB or AKT) pathway (Chen et al., 1996; Sonoda et al., 2000). In some cells, the SH2 domain of GRB2 binds to phosphorylated Tyr927 and triggers RAS-dependent activation of the mitogen-activated protein kinase (MAPK) pathway (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996). Adding to the complexity, FAK can be phosphorylated by SRC at Tyr407 and Tyr861 (Calalb et al., 1996). FAK could also play a scaffolding role mediating crosstalk between signaling pathways; for example, by inducing anchorage-dependent JNK activation in a kinase-independent fashion by interacting with paxillin (Igishi et al., 1999). Therefore, FAK phosphorylation on Tyr397 appears to be critical for both its activation and scaffolding function in vitro, the latter of which can trigger assembly of the multi-molecular complexes responsible for its cellular effects.

FAK is overexpressed in ovarian cancer, and its overabundance is predictive of poor clinical outcome (Judson et al., 1999; Sood et al., 2004). Yet, the role of FAK and its phosphorylation at Tyr397 in the normal ovary was not fully understood until recently. We demonstrated that total FAK and Tyr397-phosphorylated FAK are expressed in normal mouse ovaries, particularly enriched in granulosa cells of growing follicles where phosphorylation at Tyr397 is implicated in cellular survival (Sakurai et al., 2012). FAK protein abundance has been quantified in porcine oocytes (Pelech et al., 2008), and has been histologically proposed to function in pro-survival events (Okamura et al., 2001). Recently, FAK was shown to be expressed in mouse oocytes at the MII stage, and Tyr861-phosphorylated FAK was concentrated in the oocyte cortex following sperm incorporation and during anaphase, together implicating FAK in fertilization (McGinnis et al., 2013).

To our knowledge, the sequence of events from the LH surge to FAK phosphorylation has been revealed only in

mouse Leydig tumor cells: addition of human chorionic gonadotropin (hCG) to MA-10 cells expressing the endogenous LH receptor results in the phosphorylation of FAK at Tyr576 and Tyr577 and of paxillin, which is a downstream target of the FAK–SFK complexes (Mizutani et al., 2006). Detailed analyses of the expression and function of FAK, especially Tyr397-phosphorylated FAK, in COCs during oocyte maturation have not been conducted. Therefore, we examined the expression of FAK and the importance of phosphorylation at Tyr397 during oocyte maturation in mice. In addition, the developmental capability of oocytes treated with an FAK phosphorylation inhibitor during in vitro maturation (IVM) was evaluated by in vitro fertilization and subsequent in vitro culture of the resulting embryos.

## RESULTS

### In Vivo Expression and Distribution of Total FAK and Tyr397-Phosphorylated FAK in Cumulus Cells

We first examined the in vivo expression levels of total FAK and Tyr397-phosphorylated FAK in mouse cumulus cells before and after cumulus expansion at 0 or 12 hr after hCG treatment, respectively, following pregnant mare serum gonadotropin (PMSG) treatment. Western-blot analyses demonstrated that total FAK and Tyr397-phosphorylated FAK were present in cumulus cells (Fig. 1A; Supplemental Fig. S1). Interestingly, although the expression of total FAK before and after expansion did not differ, the abundance of Tyr397-phosphorylated FAK was significantly lower after cumulus expansion than before (Fig. 1B).

The distribution of total FAK and Tyr397-phosphorylated FAK in cumulus cells was analyzed by immunofluorescence staining of mouse COCs. During COC maturation, total FAK and Tyr397-phosphorylated FAK localized throughout the cell, with intense expression of Tyr397-phosphorylated FAK observed in regions of cell-cell contact (Fig. 1C). Lower FAK phosphorylation levels were similarly observed after cumulus expansion compared to before (Fig. 1C).

### In Vivo Expression and Distribution of Total FAK and Tyr397-Phosphorylated FAK in Oocytes

The expression levels of total FAK and Tyr397-phosphorylated FAK were examined in vivo in GV- and MII-stage mouse oocytes, respectively 0 or 12 hr after hCG treatment. Western blot analyses demonstrated that total FAK and Tyr397-phosphorylated FAK were present in oocytes (Fig. 2A), and their expression levels did not differ between the two stages (Fig. 2B). Total and Tyr397-phosphorylated FAK resided in the cytoplasm, forming small punctate structures. This distribution pattern was the same when the oocytes were classified according to nuclear status (Fig. 2C), and total FAK distribution at MII is consistent with that in a previous report (McGinnis et al., 2013).

### Inhibition of FAK Phosphorylation at Tyr397 During IVM: Effect on Oocyte Meiotic Maturation

To determine the function of FAK during oocyte meiotic maturation, we blocked FAK phosphorylation at Tyr397 during IVM using FAK inhibitor 14, which specifically and directly blocks phosphorylation at Tyr397 in a dose- and time-dependent manner. As FAK inhibitor 14 is known to induce non-apoptotic cell death by necrosis at 100 μM, thus decreasing the viability of cells (Golubovskaya et al., 2008), we first tested for any adverse effects it might have on oocytes by assessing the normality of nuclear status using α-tubulin immunostaining. Oocytes possessed degenerated nuclei after 18 hr of IVM in the presence of FSH and the inhibitor (Fig 3A). Compared to oocytes with normal nuclei and meiotic spindles (Fig 3A, upper panel), some oocytes treated with the inhibitor exhibited deformed GVs, abnormally shaped spindles, and nuclear condensation (Fig 3A, lower panel). Immunostaining for α-tubulin revealed that 92.6% of the untreated oocytes had typical MII spindles, and that treatment with 5 μM FAK inhibitor 14 during IVM did not adversely affect the oocytes, that is, 90.5% were at MII stage (Fig. 3B, white bar). In contrast, only 31.1 or 36.9% of oocytes treated with 10 or 25 μM inhibitor, respectively, had normal nuclei; but arrested between GV and MI stages (Fig. 3B, diagonal-line and dotted bars). Moreover, 17.6 or 63.3% of oocytes treated with 10 or 25 μM inhibitor, respectively, had degenerated nuclei (Fig. 3B, gray, cross-hatched, and black bars).

Detailed analysis of phenotypes at each dose of inhibitor revealed that increasing inhibitor concentration caused more adverse effects—that is, meiotic arrest with normal-shaped nuclei or nuclear degeneration—at earlier stages (Fig. 3B, non-white bars). Therefore, the number of oocytes that formed a normal-shaped MII spindle was significantly lower in oocytes treated with 10 and 25 μM inhibitor than in those treated with 0 and 5 μM inhibitor (Fig. 3B).

### Effect of Inhibiting FAK Phosphorylation at Tyr397 During IVM on Cumulus Expansion

In general, the addition of FSH to IVM medium induces oocyte meiotic maturation and cumulus expansion. To analyze the effect of FAK phosphorylation at Tyr397 on cumulus expansion, we therefore added FAK inhibitor 14 to IVM medium that did not contain FSH. The diameter of the COCs was then measured by digital imaging at the end of the maturation period. Despite the absence of FSH, remarkable cumulus expansion was observed when FAK inhibitor 14 was added (Fig 4, upper panel); those cumulus cells that had fallen off during culture remained alive and adhered to the dish (Supplemental Fig. S2). Quantitative morphometric analyses (Fig. 4, graph) demonstrated that COC diameter was significantly larger in the presence of 5 or 10 μM FAK inhibitor 14 than in the absence of inhibitor, indicating that blocking FAK phosphorylation at Tyr397 can induce cumulus expansion. Indeed, 0.5 μM PF-562271, a different FAK inhibitor that blocks phosphorylation of

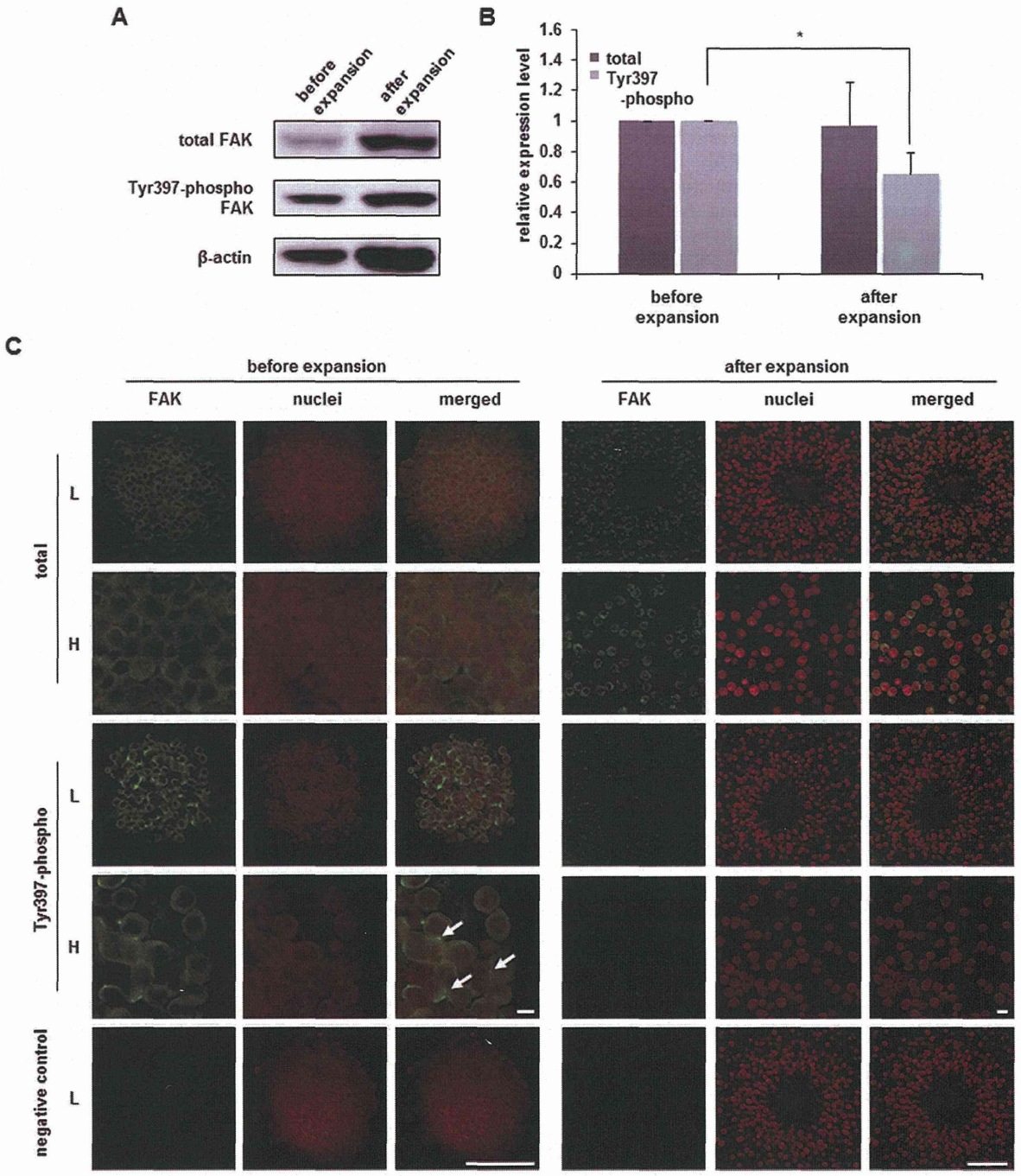


**Figure 1. A:** Expression of total FAK and Tyr397-phosphorylated FAK in cumulus cells before and after in vivo cumulus expansion. **B:** Relative expression of total and Tyr397-phosphorylated FAK protein. Values shown are the means ± standard deviations. A significant difference ($P < 0.01$) in Tyr397 phosphorylated FAK is indicated by an asterisk. **C:** Distribution of total and Tyr397-phosphorylated FAK in cumulus cells. COCs were immunostained for total and Tyr397-phosphorylated FAK with Alexa Fluor 488 (green) before (hCG 0 hr) and after (hCG 12 hr) cumulus expansion. Nuclei are shown in red. Arrows indicate the strong expression in the cell–cell contact regions. Scale bars, 50 μm/L. low-magnification; H, high-magnification.

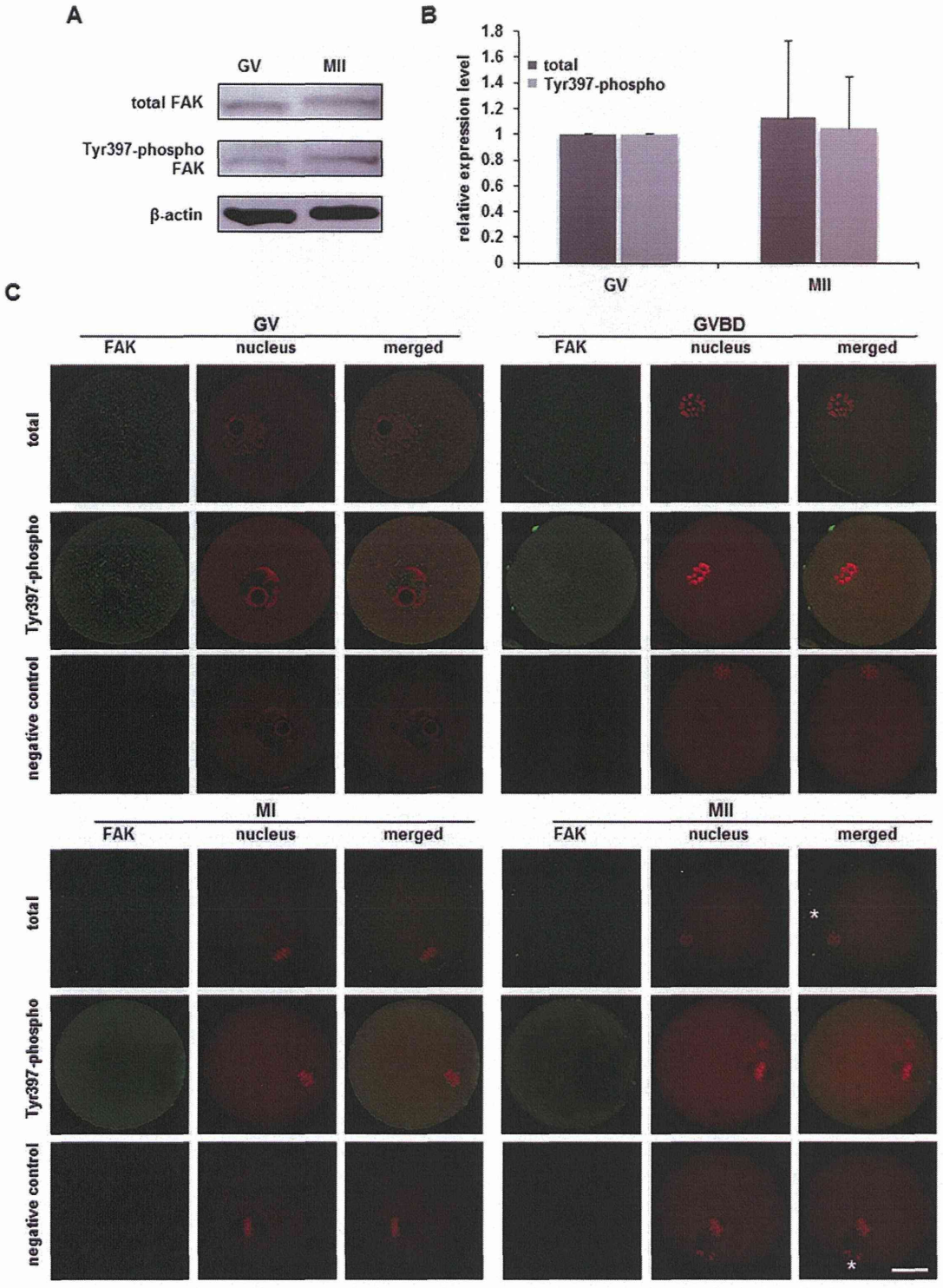


**Figure 2.** **A:** Expression of total FAK and Tyr397-phosphorylated FAK in in vivo-matured GV and MII oocytes. **B:** Relative expression of total and Tyr397-phosphorylated FAK. Values shown are the means ± standard deviations. **C:** Distribution of total and Tyr397-phosphorylated FAK in oocytes during meiotic maturation. Oocytes were immunostained for total and Tyr397-phosphorylated FAK with Alexa Fluor 488 (green). Nuclei are shown in red. Scale bars, 20 μm. COCs were collected at 0, 6, 10, and 18 hr after hCG for GV, GVBD, MI, and MII, respectively. Asterisks indicate the first polar body.

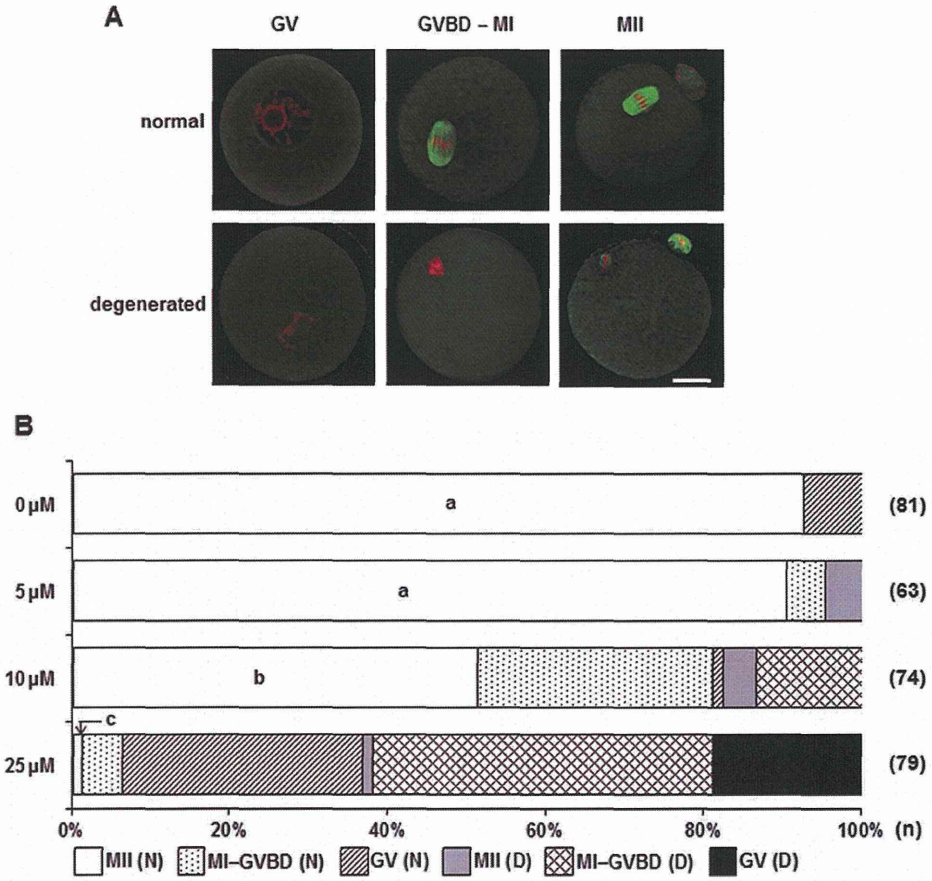


**Figure 3.** Effect of an FAK Tyr397 phosphorylation inhibitor on nuclear status. COCs at 48 hr after PMSG were matured in Waymouth's MB752/1 medium with FSH and increasing doses of FAK inhibitor 14 for 18 hr to specifically inhibit FAK phosphorylation at Tyr397, then immediately fixed for α-tubulin immunostaining. **A:** Normal or degenerated nucleus and the distribution of α-tubulin in mouse oocytes. Oocytes were immunostained for α-tubulin with Alexa Fluor 488 (green). Nuclei are shown in red. Scale bar, 20 μm. **B:** The percentage of oocytes at each meiotic stage with a normal (N) or degenerated (D) nucleus, according to FAK inhibitor concentration. Nuclei with normal morphology, but arrested between the GV and MI stage, were defined as abnormal (D). Values with different superscripts (a–c) are significantly different ($P < 0.05$) in the frequency of MII oocytes. Numbers in parentheses indicate the number of oocytes tested per group.

Tyr397, also induced cumulus expansion when added to the IVM medium, (Supplemental Fig. S3). Moreover, both inhibitors decreased levels of phospho-Tyr397 FAK prior to cumulus expansion (Supplemental Fig. S4).

### Effect of Inhibiting FAK Phosphorylation at Tyr397 During IVM on *Has2* Transcription

Cumulus expansion is induced by the synthesis and secretion of hyaluronan-enriched extracellular matrix by cumulus cells, which is regulated by hyaluronan synthase 2 (HAS2) (Fulop et al., 1997). We therefore measured the abundance of *Has2* mRNA in cumulus cells to determine if the effect of FAK phosphorylation at Tyr397 on cumulus expansion was related to *Has2* expression. Semi-quantitative analysis indicated that *Has2* mRNA abundance was significantly higher in cumulus cells cultured in IVM medium containing 5 μM FAK inhibitor 14 than in cells cultured in IVM medium without inhibitor (Fig. 5; Supplemental Fig. S5), implying a positive effect of lower Tyr397 phosphorylation.

### FAK Phosphorylation at Tyr397 Regulates the Maturation and Development of Oocytes

Finally, we investigated the maturation of oocytes in COCs expanded by FAK inhibitor 14, and their developmental competence, by performing in vitro fertilization followed by culturing. Based on the titration curve shown above, we determined that the optimum concentration of inhibitor 14 to be 5 μM; at this concentration, cumulus expansion was effectively induced with no adverse effects on oocyte meiotic maturation. As expected, the omission of FSH decreased the rate of oocytes reaching MII, although some oocytes spontaneously matured. In contrast, the addition of 0–5 μM FAK inhibitor 14 induced oocyte maturation without FSH in a

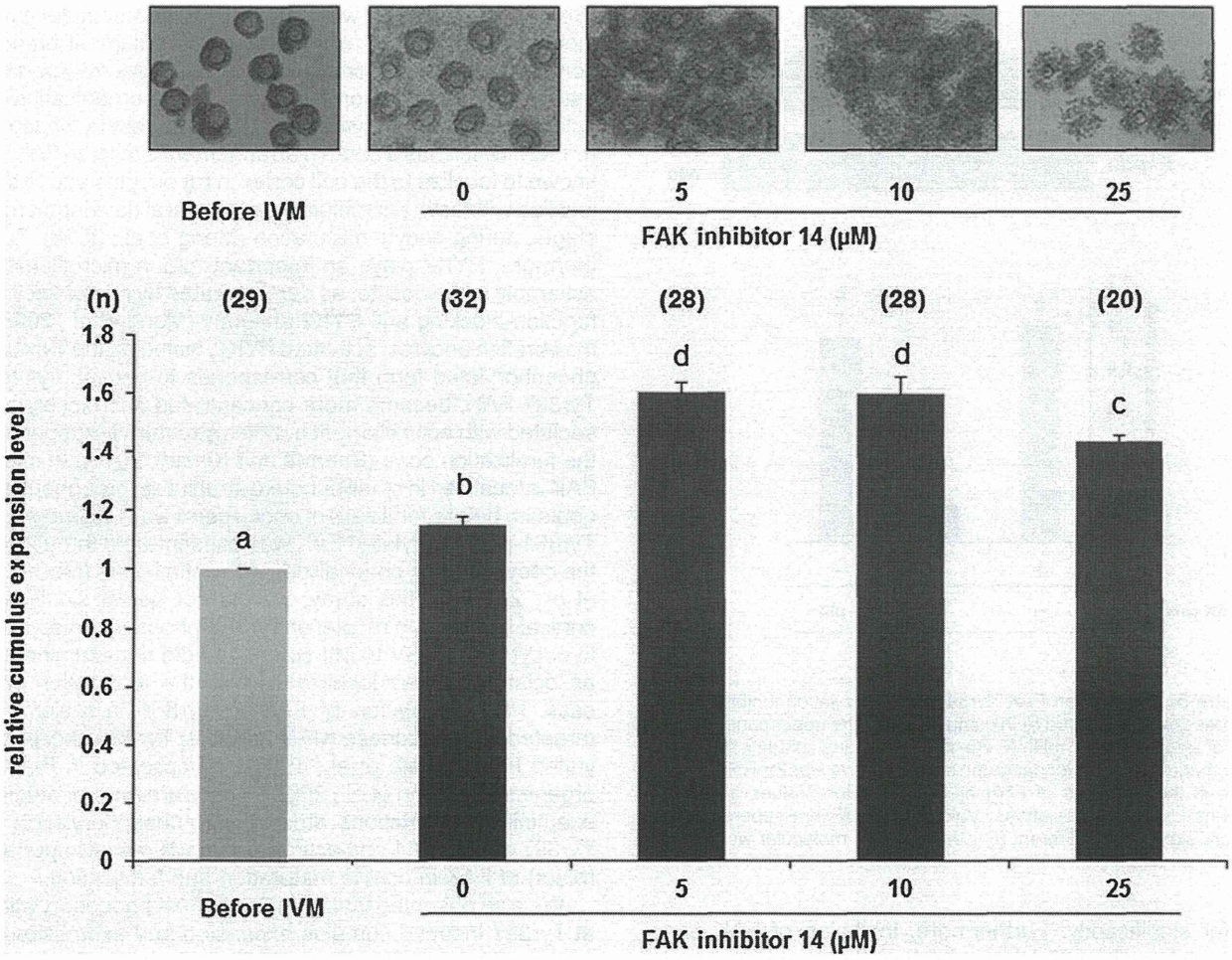


**Figure 4.** Effect of a FAK Tyr397 phosphorylation inhibitor on cumulus expansion. The upper panel shows COCs cultured with increasing doses of FAK inhibitor 14 without FSH for 18 hr. The lower bar graph shows the relative cumulus-expansion level of COCs. Values shown are the means ± standard deviations. Values with different superscripts (a–d) are significantly different ($P < 0.01$). Numbers in parentheses indicate the number of COCs tested per group.

dose-dependent manner, with the rate of MII oocytes significantly higher at 5 μM than in the absence of inhibitor (Fig. 6A). The addition of FAK inhibitor 14 to the IVM medium had no significant effect on fertilization rate after in vitro fertilization (Fig. 6B). In contrast, the rate of blastocyst formation of oocytes cultured with 5 μM FAK inhibitor 14 was significantly higher than that of oocytes cultured without inhibitor, but lower than those cultured with FSH (Fig. 6C). Total cell numbers in the blastocysts were lower in the blastocysts treated with FAK inhibitor 14 during IVM than in the blastocysts stimulated with FSH (Fig. 6D).

## DISCUSSION

FAK is highly enriched in focal adhesions (Schaller et al., 1992; Hanks and Polte, 1997), and can regulate several cellular processes, such as cell proliferation and migration (Mitra et al., 2005), in response to extracellular stimuli (e.g., signals from the extracellularl matrix). FAK is required for the efficient assembly and disassembly of adhesions (Owen et al., 2007). Its dephosphorylation at Tyr397 is rapidly observed during integrin endocytosis and focal adhesion turnover, which occur during cellular detachment from an extracellular matrix (Nagano et al., 2012). In this study, strong expression of Tyr397-phosphorylated FAK was detected at cell-contact regions of cumulus cells, which suggest its possible function in the intercellular adhesion of the cumulus layer. This specific localization suggested that decreased phosphorylation of FAK at Tyr397, but not total FAK abundance, may participate in the disassembly of cumulus cells during cumulus expansion.

Localization and abundance of total FAK and Try397-phosphorylated FAK in the oocyte cytoplasm during meiotic maturation, specifically at the GV and MII stages, did not

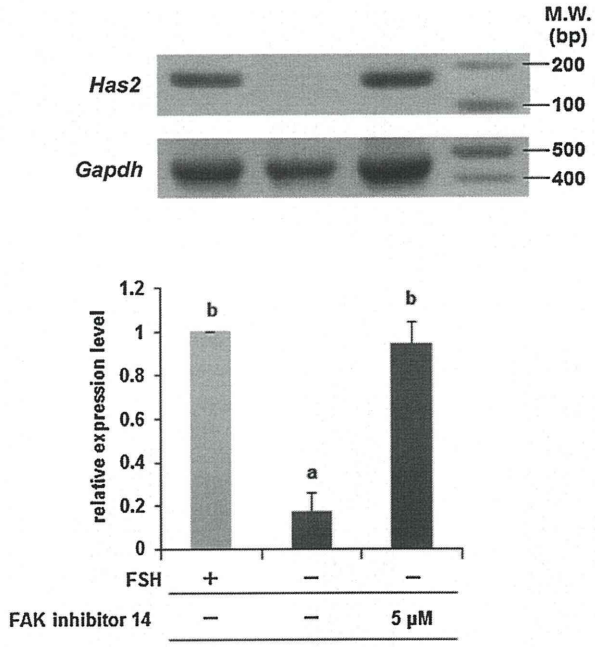


**Figure 5.** Effect of an FAK Tyr397 phosphorylation inhibitor on the expression of *Has2* mRNA in cumulus cells. The upper panel shows a gel of amplified *Has2* mRNA. The right-most lane contains the molecular markers. The lower bar graph shows relative *Has2* mRNA expression in the presence of FSH or FAK inhibitor. Values shown are means ± standard deviations. Values with different superscripts (a, b) are significantly different ($P < 0.01$). M.W., molecular weight.

differ significantly. Furthermore, inhibition of FAK phosphorylation at Tyr397 during IVM resulted in meiotic arrest, formation of abnormal nuclei and nuclear condensation. Okamura et al. (2001) showed that FAK was expressed in the oocytes of porcine antral follicles, and suggested that the role of FAK may be to prevent apoptosis in oocytes. Consistent with this observation, the FAK/PI3K-mediated cell survival pathway may target PKB (or Akt) activity (Vivanco and Sawyers, 2002), whose signal is transduced into changes in expression of BCL-2-associated X protein (BAX) and BCL-2-like protein 1 (BCL2L1), which are important players in oocyte apoptotic pathways (Jin et al., 2005). In this study, we frequently observed meiotic arrest or nuclear condensation in the oocytes treated with FAK inhibitor 14 at less than 100 μM, a concentration at which non-apoptotic cell death was induced (Golubovskaya et al., 2008); such nuclear condensation is well-associated with cellular apoptosis (Enari et al., 1998; Susin et al., 2000). Taken together, these results implicate FAK and its Tyr397-phosphorylated form in retaining the nuclear status of oocytes.

FAK functions in a variety of cellular events. For example, spindle translocation depends on the cytoplasmic meshwork of F-actin filaments (Longo and Chen, 1985). FAK is known to directly bind to actin-related protein (ARP) 3 and to enhance ARP2/3-dependent actin polymerization (Serrels et al., 2007), whereby the ARP2/3 complex promotes the addition of actin monomers at filament branch points (Svitkina and Borisy, 1999). Thus, FAK may participate in spindle migration by regulating the organization of actin filaments in oocytes. Proline-rich tyrosine kinase 2 (PYK2), which has a domain arrangement similar to FAK, is known to localize to the cell cortex in rat oocytes and to co-localize with actin microfilaments at several developmental stages during oocyte maturation (Meng et al., 2006). Furthermore, PYK2 plays an important role in microfilament assembly in the oocyte, as demonstrated by microinjecting function-blocking anti-PYK2 antibody (Meng et al., 2006). In zebrafish oocytes, activated PYK2, including the Tyr402-phosphorylated form that corresponds to phosphorylated Tyr397 FAK, became more concentrated and closely associated with actin filament bundles structurally supporting the fertilization cone (Sharma and Kinsey, 2013). In mice, FAK is localized in small punctate structures throughout the ooplasm before fertilization; once sperm was incorporated, Tyr861-phosphorylated FAK was concentrated throughout the oocyte cortex, co-localizing with actin layers (McGinnis et al., 2013). In this study, we did not detect significant cortical localization of total or Tyr397-phosphorylated FAK in oocytes from GV to MII stage, and did not examine the association of their localization with the fertilization process. Phosphorylation of FAK at Tyr861, a major site targeted by SRC kinase when bound to Tyr397-phosphorylated FAK (Calalb et al., 1996), is implicated in F-actin organization (Lunn et al., 2007). Therefore, further studies examining the relations among FAK phosphorylation at Tyr397 and Tyr861 and actin filament will reveal important role(s) of FAK in oocyte maturation and fertilization.

We also observed that inhibition of FAK phosphorylation at Tyr397 induced cumulus expansion and expression of *Has2* mRNA, even though FSH was absent from IVM medium. Cumulus expansion can be induced in vitro by FSH (Eppig 1979; Salustri et al., 1992) and by EGF in mouse, pig, cow, and rabbit (Downs 1989; Singh et al., 1993; Boland and Gosden, 1994; Lorenzo et al., 1994; Lorenzo et al., 1996). FSH stimulates elevated adenosine-3′,5′-cyclic monophosphate (cAMP) levels in cumulus cells (Dekel et al., 1979; Eppig, 1979), followed by the increased synthesis of key enzymes involved in the production of hyaluronan (i.e., HAS2) and the binding of hyaluronan to its receptors, such as prostaglandin-endoperoxidase synthase 2 (PTGS2) and tumor necrosis factor α-induced protein 6 (TNFAIP6) (Richards et al., 2002; Vanderhyden, 2002). cAMP is also known to activate protein kinases, including p38 mitogen-activated protein kinase (MAPK), and protein kinase A (PKA), which induce the expression of EGF-like peptides in cumulus cells and thereby indirectly activate ERK1/2 (Shimada et al., 2006; Yamashita et al., 2007). Activated ERK1/2 was recently shown to promote the dephosphorylation of FAK at Tyr397 by inducing the interaction between FAK and protein tyrosine phosphatase (PTP)-PEST (Zheng et al., 2011). ERK1/2, stimulated indirectly by FSH, could therefore induce the dephosphorylation of FAK at Tyr397 and the subsequent detachment of cumulus cells, thus explaining the surprising cumulus

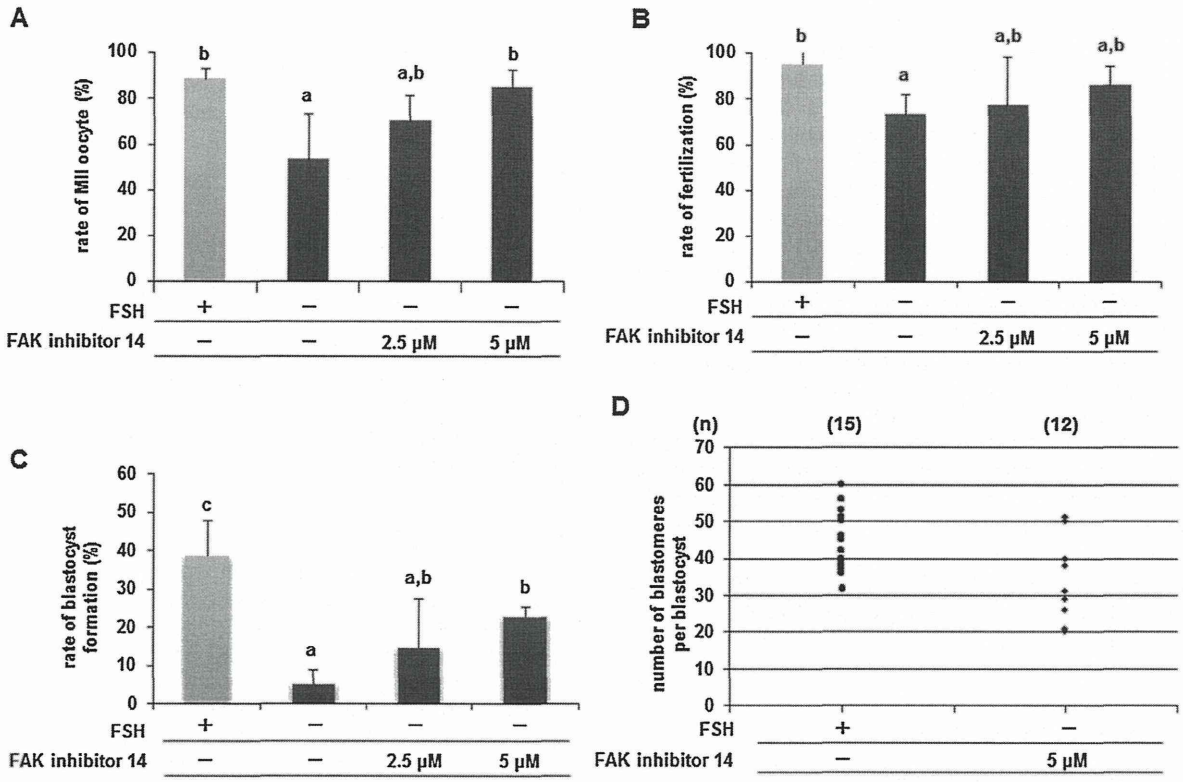


**Figure 6.** Maturation, fertilization, and development of oocytes treated with a FAK Tyr397 phosphorylation inhibitor. **A:** Rate of maturation to MII oocytes following treatmen with FAK inhibitor 14. **B:** Fertilization rate after IVM. **C:** Rate of blastocyst formation after in vitro fertilization. Bars represent oocytes matured in Waymouth's MB752/1 medium with FSH (white) or FAK inhibitor 14 (black). Values shown are means ± standard deviations. Values with different superscripts (a–c) are significantly different ($P < 0.05$). A total of 77, 54, 58, or 82 oocytes were used in IVM with FSH or 0, 2.5, or 5 μM of FAK inhibitor 14, respectively. **D:** Total number of blastomeres per blastocyst. Numbers in parentheses indicate the number of blastocysts tested per group.

expansion we observed in the presence of FAK inhibitor 14 and the absence of FSH during IVM. On the other hand, ERK1/2 can be regulated by FAK activation when presented with different matrix components (Schlaepfer and Hunter, 1997; Sanders and Basson, 2000; Harnois et al., 2004). In growing fibroblasts, for example, FAK contains phosphotyrosines (Calalb et al., 1995; Schlaepfer and Hunter 1996), but becomes completely dephosphorylated upon cell detachment (Calalb et al., 1995). Considering that contacts among cumulus cells are lost by the accumulation of hyaluronan, it is reasonable to assume that decreased FAK phosphorylation parallels the secretion and accumulation of this matrix protein. Although the molecular relationship between FAK and other factors, including ERK1/2 and HAS2, in cumulus cells is not entirely known, our study provided meaningful insights, including the role of FAK in the induction of cumulus expansion and the regulation of *Has2* mRNA expression by phosphorylation at Tyr397 in mouse cumulus cells.

Treatment of COCs with 5 μM FAK inhibitor 14 during IVM significantly increased the rates of maturation and blastocyst formation compared to those in COCs not treated with FAK inhibitor 14. Bidirectional communication between mammalian oocytes and cumulus cells is essential for the development of both cell types: while fully grown oocytes regulate the proliferation, gene expression, and function of cumulus cells, nutritional support from cumulus cells is essential for the growth and development of these oocytes (Eppig 2001). We found that levels of Tyr397-phosphorylated FAK after in vivo COC maturation are lower in cumulus cells, but not in oocytes, than before maturation. Therefore, we predicted that addition of FAK inhibitor 14, to decrease FAK phosphorylation levels at Tyr397 in cumulus cells during IVM, would have a positive effect on the maturation of cumulus-enclosed oocytes and subsequent embryo development after in vitro fertilization. While there was some benefit, the rate of blastocyst formation remained significantly lower in the presence of 5 μM FAK inhibitor 14 than in the presence of FSH. Thus, cumulus-enclosed oocytes treated with this inhibitor did not achieve the full developmental competence needed to reach the blastocyst stage, which may be why these inhibitor/IVM-derived blastocysts contained fewer blastomeres. The apparent contradiction presented in this study—that FAK

inhibitor decreases oocyte maturation when FSH is present but increases oocyte maturation when FSH is absent —may be explained as follows: FSH induces a low level of Tyr397 FAK phosphorylation that is compatible with oocyte meiotic maturation, and FAK inhibitor 14 can partly mimic this process in the absence of FSH. Combined treatments of FSH and FAK inhibitor 14, however, are too strong for the oocyte, resulting in a decreased maturation rate. Further investigations exploring the relationship between FAK phosphorylation at Tyr397 and other molecules, especially those implicated in FSH-induced signaling pathways, may reveal the function of FAK in oocytes, cumulus cells, and preimplantation embryos. This knowledge should help improve the developmental competence of oocytes by regulating the functions of FAK.

In this study, we demonstrated that FAK expressed in oocytes plays an important role in maintaining nuclear status during meiotic maturation, and that Tyr397-phosphorylated FAK regulates cumulus expansion via *Has2* mRNA expression. Our results also suggest that inhibition of Tyr397 phosphorylation during IVM has a similar effect on the signaling pathways that are induced by FSH during oocyte maturation and preimplantation embryo development.

## MATERIALS AND METHODS

### Animals and Ethics Statement

All ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and bred in our laboratory. To minimize suffering, the principles of laboratory animal care were followed. All procedures were conducted in accordance with the guidelines of the Committee for the Care and Use of Laboratory Animals for Research of the Graduate School of Agricultural Science (Tohoku University, Japan). Animal protocols used in this study were approved by the Ethics Committee (Permit Number: 2012-001).

### Maturation of Cocs In Vivo and In Vitro

To obtain in vivo-matured COCs, immature, 20- to 23-day-old female mice were injected with 5 IU of PMSG (Teikoku Hormone MFG, Tokyo, Japan), followed by 5 IU of hCG (Teikoku Hormone MFG) 48 hr later. Mice were killed by cervical dislocation, and the oviducts were removed at 12 hr post-hCG injection. COCs were collected in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) containing 0.1% (w/v) polyvinyl alcohol (PVA) (Sigma, St. Louis, MO), when oocytes in the COCs were at the MII stage.

IVM was performed as described previously (Sakai et al., 2011). Briefly, ovaries were removed 48 hr after the PMSG injection, and COCs were isolated by puncturing the large antral follicles with a 26-gauge needle and then collecting them in Leibovitz's L-15 medium containing 0.1% PVA and 4 mM hypoxanthine (Sigma) when the oocytes in the COCs were at the GV stage. COCs were cultured in Waymouth's MB752/1 medium (Invitrogen) containing 5% (v/v) fetal calf serum (Gemini Bio, West Sacramento, CA), 4 mM hypoxanthine, 100 IU/l FSH (Sigma), 0.23 mM pyruvic acid (Sigma), 75 mg/l penicillin G (Meiji Seika, Tokyo, Japan), and 50 mg/l streptomycin sulfate (Meiji Seika), for 18 hr in a humidified atmosphere containing 5% (v/v) $CO_2$ at 37 °C.

### Collection of COCs, Oocytes, and Cumulus Cells

Oocyte stages were defined as GV (48 hr after PMSG, i.e., hCG 0 hr) and MII (18 hr after hCG in vivo or 18 hr of IVM). The COC and cumulus-cell stages were defined as before cumulus expansion (hCG 0 hr) and after expansion (12 hr after hCG in vivo or 12–18 hr of IVM). Cumulus cells were separated from their oocytes before and after cumulus expansion by pipetting and by treatment with 0.1% (w/v) hyaluronidase (Sigma) at room temperature. Oocytes that had extruded their first polar body were defined as mature MII-stage oocytes.

### Western Blotting

Levels of total and Tyr397-phosphorylated FAK in cumulus cells and oocytes were analyzed as described previously (Sakurai et al., 2012). Briefly, lysates of cumulus cells isolated from 30 COCs or 60 denuded oocytes were loaded into each lane and separated by SDS–PAGE. Separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4 °C with antibodies against total FAK (1:2000) (Abcam, Cambridge, UK), Tyr397-phosphorylated FAK (1:2000) (Invitrogen) or β-actin (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Probes were detected using the ECL Plus Western blot detection system (GE Healthcare, Little Chalfont, UK). The density of the bands representing total FAK and Tyr397-phosphorylated FAK was normalized to that of β-actin; these relative expression values were presented and used for statistical analysis. Experiments were replicated five or four times for cumulus cells or oocytes, respectively.

### Immunocytochemistry

Immunolocalization of total and Tyr397-phosphorylated FAK in COCs and denuded oocytes was performed as described previously (Kogasaka et al., 2013). Briefly, collected COCs or oocytes were fixed and permeabilized with 2% (w/v) paraformaldehyde (Sigma) in phosphate-buffered saline (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.1% PVA and 0.2% (v/v) Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 30 min. They were incubated overnight at 4 °C with rabbit anti-total FAK or rabbit anti-Tyr397-phosphorylated FAK, or for 1 hr at room temperature with mouse anti-α-tubulin (for oocytes; Sigma) antibodies. Then, samples were probed with Alexa Fluor 488-labeled IgG (Molecular probes, Eugene, OR) and counterstained with 10 μg/ml propidium iodide (Sigma). Stained COCs and oocytes were observed using a LSM 700 confocal microscope system