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—Original Article—

Expression and function of exportin 6 in full-grown and growing porcine oocytes

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Abstract. Exportin 6, which functions specifically in the nuclear export of actin family proteins, has been reported to be absent in immature *Xenopus* oocytes, which have a huge nucleus containing a large amount of actin. In mammalian oocytes, however, the presence and the function of exportin 6 remain uninvestigated. In this study, we assessed the expression and effects of exportin 6 on meiotic resumption in porcine oocytes after cloning porcine exportin 6 cDNA and carrying out overexpression and expression inhibition by mRNA and antisense RNA injection, respectively. We found for the first time that exportin 6 was expressed in mammalian full-grown germinal-vesicle-stage oocytes and was involved in the nuclear export of actin. In contrast, exportin 6 was absent from the growing oocytes, which are meiotically incompetent and maintain the germinal-vesicle structure in the long term; the regulatory mechanism appeared to be active degradation. We examined the effects of exportin 6 on meiotic resumption of porcine oocytes and noted that its expression did not affect the onset time but increased the rate of germinal vesicle breakdown at 24 h via regulation of the nuclear actin level, which directly influences the physical strength of the germinal-vesicle membrane. Our results suggest that exportin 6 affects the nuclear transport of actin and meiotic resumption in mammalian oocytes.

Key words: Actin, Exportin 6, Meiotic resumption, Nuclear export, Pig oocyte

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Nuclear-cytoplasmic transport of molecules > 50 kDa, such as proteins and nucleic acids, generally requires the binding of these molecules to nuclear-transport receptors, such as importins and exportins, which interact with the nuclear-pore complexes (NPCs) [1]. In mammals, 20 types of nuclear-transport receptors have been reported to be expressed and to recognize different cargos, leading to complex regulation of nuclear transport systems [2, 3]. Exportin 1 (XPO1), a typical exportin, is involved in the nuclear export of various proteins [4–7], whereas other exportins are responsible for transporting a specific cargo [2, 3]. Exportin 6 (XPO6) specifically plays a pivotal role in the nuclear export of actin family proteins due to its high affinity toward profilin-binding G-actin [8]. Actin exhibits various functions inside as well as outside the nucleus [9, 10]. Although actin can pass through NPCs due to its relatively small size (about 42 kDa), a majority of it exists in the cytoplasm in general somatic cells, suggesting the stable involvement of XPO6 [8, 10].

In contrast, actin has been widely known to localize in the nuclei of immature *Xenopus* oocytes [11]. These oocytes have a large nucleus, called the germinal vesicle (GV), that is preserved in the prophase of the first meiosis (the so-called GV-stage). It has been reported that actin forms a meshwork on the inner surface of the nuclear membrane, suggesting its contribution in GV maintenance by supporting the mechanical strength of the membrane [12]. This report also revealed that XPO6 protein did not exist in *Xenopus* GV-stage oocytes but was expressed around the meiotic resumption, and that the expression of exogenous XPO6 made the GV membrane fragile [12]. These findings indicate that the existence of XPO6 in the GV-stage oocytes has a significant adverse effect on the maintenance of the GV structure.

Recently, we analyzed the expression and effect of other exportin, that is XPO1, in porcine GV-stage oocytes. We reported its quite-stable existence during the GV-stage without detectable degradation and synthesis, and its positive function on meiotic resumption [13]. These results lead to investigation about the presence of other nuclear-transport receptors, including XPO6, and their functions on meiotic resumption in porcine oocytes; however, XPO6 has never been analyzed in the oocytes other than that of *Xenopus*, despite its probable important influence on GV maintenance. In the present study, we examined the expression and nuclear-export function of XPO6 in porcine GV-stage oocytes. The majority of GV-stage oocytes in the mammalian ovary are in a nongrowing or growing stage; they are meiotically incompetent and maintain the GV structure for until they acquire meiotic competence via oocyte growth [14]. Therefore, we investigated not only the expression and effects of XPO6 on the meiotic resumption in full-grown oocytes (FGOs) but also XPO6 expression in the growing oocytes (GOs) to explore the influence of XPO6 on GV maintenance.

Materials and Methods

Collection and in vitro maturation of porcine oocytes

Full-grown oocytes (115–125 μm in diameter) and GOs (95–105 μm

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in diameter) were collected from 2–5 mm and 0.4–1.0 mm follicles, respectively, present in gilt ovaries obtained from a commercial slaughterhouse, as described previously [13, 15]. Collected cumulus-oocyte complexes (COCs) were cultured as mentioned previously [13]. Some COCs were injected with RNAs before culturing, as described below. For the inhibition of meiotic resumption and protein synthesis, 100 μM roscovitine (Ros; Cell Signaling Technology, Danvers, MA, USA) and 35 μM cycloheximide (CHX; Wako Pure Chemical Industries, Osaka, Japan) were added to the maturation medium, respectively. After culture, the oocytes were removed surrounding cumulus-cells by repeated pipetting and subjected to immunoblotting or immunocytochemistry as explained below, or nuclear-status examination after fixation and staining, as described previously [13].

RT-PCR

The total RNA was extracted from porcine uncultured oocytes using TRIzol Reagent (Thermo Fisher Scientific, Tokyo, Japan), followed by reverse transcription using SuperScript First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific), according to the manufacturer's instructions. Expression of *XPO6* mRNA was examined via PCR (35 cycles) of the total RNA using a thermal cycler (Bio-Rad, Hercules, CA, USA) with a primer set (forward: 5′-GGATCCACATGGCCTCTGAAGAAGC and reverse: 5′-GGCGAGCAGGCCGTGCCTAG) designed according to the sequence found in the NCBI nucleotide database (accession no.: XM 003124525). The PCR condition was as follows: 95°C for 4 min heat denaturation at first, then 95°C for 30 sec (heat denaturation) + 57°C for 30 sec (annealing) + 72°C for 1 min/kb (elongation reaction), which was repeated in 35 cycles; eventually, 72°C for 7 min elongation reaction before cooling to 4°C. Ribosomal protein L19 (*RPL19*) was detected as an endogenous control by the same method with a primer set (forward: 5′-ATGAAATCGCCAACGCCAAC and reverse: 5′-GGTGTTTTTCCGGCATCGAG; accession no.: XM_003131509). The PCR product was detected via electrophoresis and ethidium-bromide staining.

Cloning of XPO6 and vector construction

For cloning of porcine *XPO6* cDNA, a whole porcine *XPO6* ORF obtained by RT-PCR as mentioned earlier, was cloned into the pGEM-T Easy vector (Promega, Fitchburg, WI, USA). We also obtained a partial porcine *XPO6* cDNA comprising 1519 bp of the 3′-side of the ORF by RT-PCR with a primer set (forward: 5′-GCATGCTCAGTCCCTGGCT and reverse: 5′-GGCGAGCAGGCCGTGCCTAG), and the partial cDNA was cloned into the pGEM-T easy vector for the synthesis of antisense RNA of XPO6 (*asXPO6*). These vectors were sequenced using a commercial sequencing kit (Applied Biosystems) and a DNA sequencer (Applied Biosystems), according to the manufacturer's instructions.

In vitro transcription of RNAs

For *in vitro* synthesis of *XPO6* mRNA and *asXPO6*, the vectors of whole *XPO6* and partial *XPO6* were linearized by appropriate restriction enzymes and transcribed *in vitro* in the presence of m7G(5′)ppp(5′)G to synthesize capped RNA transcripts with either T7 or SP6 RNA polymerase (Promega). Messenger RNAs of enhanced green fluorescent protein (*EGFP*) and *XPO1* were prepared by the same procedure from each coding vector, which were then constructed using the same vector used in the present study, as reported previously [13, 16]. The RNA transcripts were precipitated with absolute ethanol, washed, and resuspended in RNase-free water. The RNA solutions were then stored at −80°C until further use.

Microinjection

Approximately, 50 pl of RNA solution was injected into each ooplasm of uncultured porcine COCs, as described previously [13]. The final concentration of *XPO6* mRNA, *asXPO6,* and *XPO1* mRNA was 500 ng/μl. Each solution was added to 50 ng/μl *EGFP* mRNA, indicating successful injection. Only oocytes expressing EGFPs were subjected to the following analyses. In the experiment of nuclear localization analysis, 25 ng/μl Texas-red-dextran (Molecular Probes, Eugene, OR, USA) was used instead of *EGFP* mRNA.

Immunoblotting

The antibodies used were anti-XPO6 polyclonal antibody (SAB2700674; Sigma-Aldrich, St. Louis, Missouri, MO, USA) and anti-beta actin polyclonal antibody (GTX109639; Gene Tex, Los Angeles, California, CA, USA). The procedure for visualization of the protein-bound antibodies and detection of XPO1 was the same, as described previously [13].

Immunocytochemistry

Texas-red-dextran-injected and *XPO6* mRNA-injected COCs were cultured with Ros (100 μM) for 24 h to maintain GV stage. After the culture, denuded oocytes were fixed and permeabilized as mentioned earlier [13], and were then blocked in 1% bovine serum albumin for 20 min. The first antibody reaction was performed over-night with anti-beta actin polyclonal antibody (1:1000 dilution; GTX109639; Gene Tex). The antibody-mounted oocytes were washed, treated with fluorescein-isothiocyanate-conjugated anti-rabbit IgG (1:500 dilution; ab6717; Abcam, Cambridge, UK) for 45 min, and the DNA was stained with 0.4 μg/ml 4',6-diamidino-2-phenylindole for 15 min. All reagents were prepared with phosphate buffered saline comprising 0.1% polyvinylpyrrolidone. The oocytes were mounted on a glass slide and then examined under a confocal laser scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany). The fluorescence intensity of each oocyte was analyzed using ImageJ software. The ratio of fluorescence intensity in the nucleus to that in the cytoplasm was calculated as the index of the cargo's nuclear localization (details are presented in Fig. 2B).

Statistical analysis

Each experiment was repeated more than three times. The results were evaluated with either a Mann-Whitney U test, a Chi-square test, or a Student's *t*-test. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Expression of endogenous XPO6 in porcine oocytes

First, the protein expression of endogenous XPO6 was examined via immunoblotting with a XPO6-specific antibody, and ~130-kDa

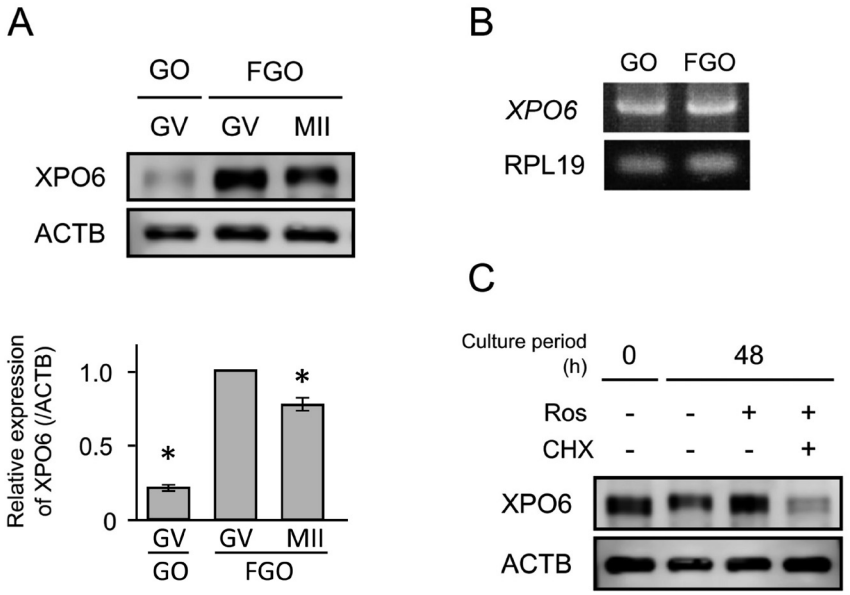

Fig. 1. Expression and turnover of endogenous XPO6 in porcine growing (GO) or full-grown (FGO) oocytes. (A) The XPO6 protein expression was examined via immunoblotting. Fifteen oocytes were used without culture (GV) and 15 were used after 48 h of maturation culture (MII). A representative result is presented in the upper panel. Beta-actin (ACTB) was used as a loading control. The XPO6 expression levels are presented as the ratio to ACTB in the lower panel (mean ± SD, * $P < 0.05$ compared with GV of FGOs). (B) The mRNA expression of XPO6 was analyzed via RT-PCR. RPL19 was used as a loading control. (C) The turnover of endogenous XPO6 in FGOs was analyzed via immunoblotting. Meiotic resumption and protein synthesis were inhibited by roscovitine (Ros) and cycloheximide (CHX), respectively. Fifteen oocytes were loaded in each lane.

bands were detected in FGOs at the GV stage and at the second meiotic metaphase (MII), and in GOs. The protein level of the MII oocytes was slightly but significantly less than that in the GV-stage oocytes, suggesting a gradual decrease in the XPO6 level with oocyte maturation (Fig. 1A, upper panel, also see Fig. 1C). In comparison to FGOs, the XPO6 level in GOs was extremely small, about one-fifth of the level of FGOs even in the same GV stage (Fig. 1A, lower panel). The presence of *XPO6* mRNA was confirmed by RT-PCR in both GOs and FGOs (Fig. 1B).

Second, the turnover of XPO6 protein was analyzed in the GV-stage FGOs by inhibiting protein synthesis. A decrease in the XPO6 level in the 48 h-cultured oocytes (MII oocytes) was not detected when the meiotic resumption was inhibited by Ros, an inhibitor of maturation-promoting factor (MPF), and the level of the uncultured oocytes was maintained for 48 h (Fig. 1C). In contrast, the amount of XPO6 protein significantly decreased on culturing with cycloheximide (CHX), an inhibitor of protein synthesis (Fig. 1C). These results suggest that XPO6 protein is synthesized in porcine FGOs and that the XPO6 level is kept constant during the GV stage by balancing its synthesis and degradation.

Overexpression and nuclear-export function of XPO6 in porcine oocytes

Expression of exogenous XPO6 was attempted by the microinjection of *in vitro*-synthesized *XPO6* mRNA into GV-stage oocytes. As presented in Figure 2A (left panel), XPO6 overexpression was detected in FGOs 24 h after the injection, and the XPO6 protein level was about 2-fold of the noninjected FGOs (2.01 ± 0.53). A significant overexpression of XPO6 was detected also in GOs; however, the level was still minute and incomparable even with that of the noninjected FGOs (0.36 ± 0.06). This result was in sharp contrast to the accumulation of XPO1 in GOs injected with *XPO1* mRNA (Fig. 2A, right panel), which had the same 3'-UTR and 5'-UTR structures as *XPO6* mRNA.

The nuclear-transport function of XPO6 in porcine oocytes was analyzed by the localization of beta-actin (ACTB), which is one of the actin family members known to be XPO6 cargo and is the predominant form of actin in porcine oocytes [17]. Localization of ACTB was detected by the immunocytochemistry of GV-arrested oocytes after 24 h of *XPO6* mRNA injection, and the nuclear localization index was calculated for each oocyte as presented in Fig. 2B. The nuclear localization index in uninjected FGOs was about 1.4, whereas it was significantly reduced to 0.9 by XPO6 overexpression (Fig. 2C, D), indicating that porcine XPO6 functions as the nuclear-export receptor. In contrast with FGOs, ACTB in GOs was uniformly distributed in whole oocytes, and the nuclear localization index remained unaltered by injecting *XPO6* mRNA (Fig. 2C, D), consistent with the relatively low expression of XPO6 in GOs.

Effects of XPO6 on meiotic resumption in FGOs

The aforementioned results suggested that XPO6 effects are not exerted during the growing phase but become prominent after growth completion in porcine oocytes. As the most prominent difference between FGOs and GOs is the meiotic competence, we further examined whether XPO6 affects the meiotic resumption of porcine FGOs by overexpression and expression inhibition of XPO6. The GV

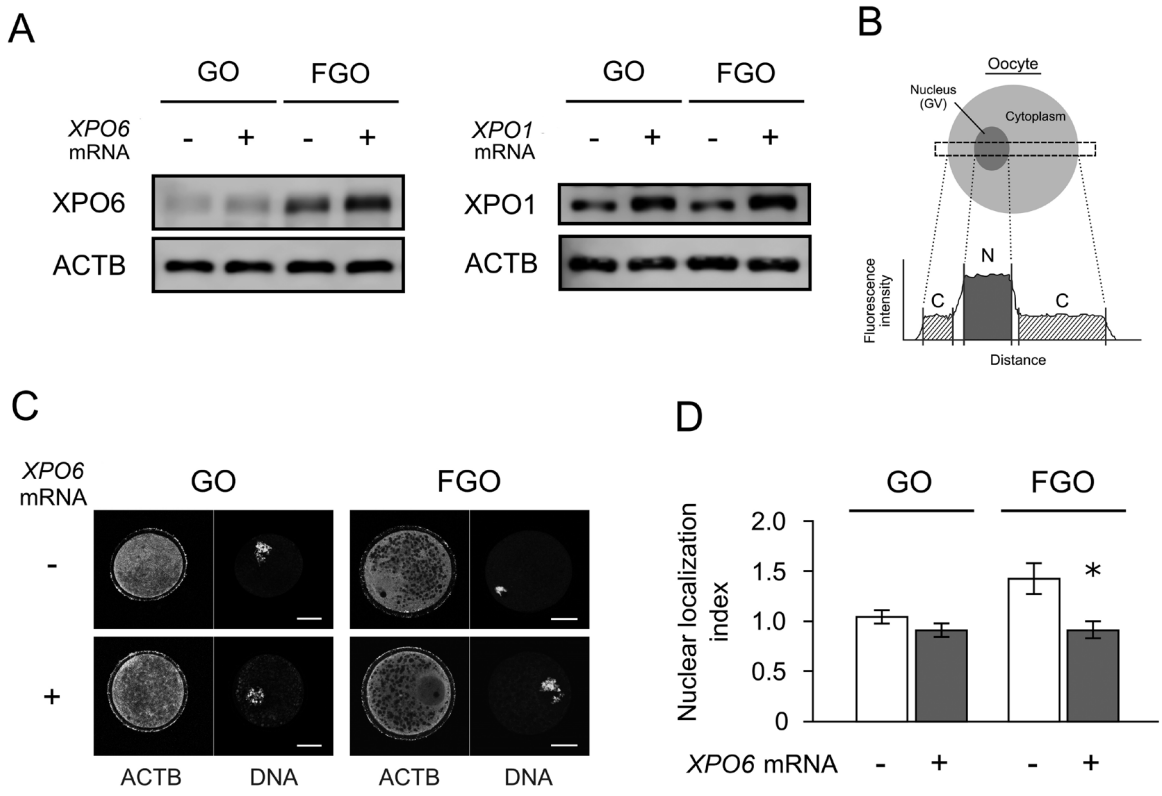

Fig. 2. Overexpression and nuclear-export function of XPO6 in porcine growing (GO) and full-grown (FGO) oocytes. (A) Oocytes with or without *XPO6* or *XPO1* mRNA injection were cultured for 24 h and the expression of XPO6 (left panel) or XPO1 (right panel) was examined via immunoblotting. Fifteen oocytes were used for each lane. Beta-actin (ACTB) was used as a loading control. (B) Calculation method for the nuclear localization index of ACTB. A rectangle (broken line) was drawn on the oocyte diameter including the nucleus with the fixed lengthwise using ImageJ software as presented in the upper part of the image. The obtained plot represented in the lower part indicates the average fluorescence intensity of the lengthwise dimension of the rectangle. The nuclear and cytoplasmic averages of the fluorescence intensity were calculated as the quotient of their area (gray, nucleus (N); stripe, cytoplasm (C)) divided by their width. The part of the nucleolus was excluded for calculating the nuclear average. The ratio of fluorescence intensity in the nucleus to that in the cytoplasm was obtained from each oocyte and used as the nuclear localization index. (C) Representative localization of ACTB with and without XPO6 overexpression was examined via immunocytochemistry. DNA was stained by 4',6-diamidino-2-phenylindole. Scale bar, 50 µm. (D) The nuclear localization index of ACTB was calculated as the ratio of fluorescence intensity in the nucleus to that in the cytoplasm as described in (B). More than eight oocytes were analyzed in each group (mean ± SEM, * $P < 0.05$).

breakdown (GVBD) rates in *EGFP* mRNA-injected oocytes (control; 18%, 46%, and 51% at 18, 21, and 24 h of culture, respectively) were equivalent to those in our previous reports [13, 18, 19]. In *XPO6* mRNA-injected oocytes, the GVBD rate at 18 h (25%) did not differ from that in the control oocytes, indicating that the onset time of meiotic resumption was unaffected by the XPO6 overexpression; however, the rates at 21 and 24 h (70% and 81%, respectively) were significantly higher than those in control oocytes (Fig. 3A). Eventually, we tried to suppress XPO6 expression using *XPO6* antisense RNA (*asXPO6*) in porcine oocytes. The reduction of XPO6 protein was confirmed 48 h after the injection of *asXPO6* in GV-arrested FGOs (58% of noninjected oocytes: Fig. 3B). In control oocytes, which were injected with *EGFP* mRNA and maintained at GV-stage for 48 h, 52% and 71% of oocytes revealed meiotic resumption in 6 and 8 h after the release from GV arrest, respectively (Fig. 3C), whereas the GVBD rate in *asXPO6*-injected oocytes (26% and 47% at 6 and 8 h after the release, respectively) was significantly lower than that in the control oocytes (Fig. 3C). These results suggest that the nuclear-membrane breakdown was delayed by the decreased XPO6 protein level in porcine FGOs.

Discussion

XPO6 plays a pivotal role in regulating the nuclear export of actin in somatic cells [8, 10]; however, it has never been analyzed in mammalian oocytes, and its expression and effects remain unclear. The expression of XPO6 in oocytes has been examined only in *Xenopus* to date, wherein it was found to be absent in GV-stage oocytes and appeared only after GVBD, suggesting the necessity of actin localization in the GV for maintaining enormous nuclear structure in *Xenopus* oocytes [12]. In the present study, we examined the expression of endogenous XPO6 in porcine oocytes, and artificially regulated the XPO6 expression to analyze the nuclear-transport function of XPO6 and its effect on meiotic resumption. Our results revealed that XPO6 existed and functioned as the nuclear-export receptor of actin in porcine FGOs. The present study is the first to report the presence

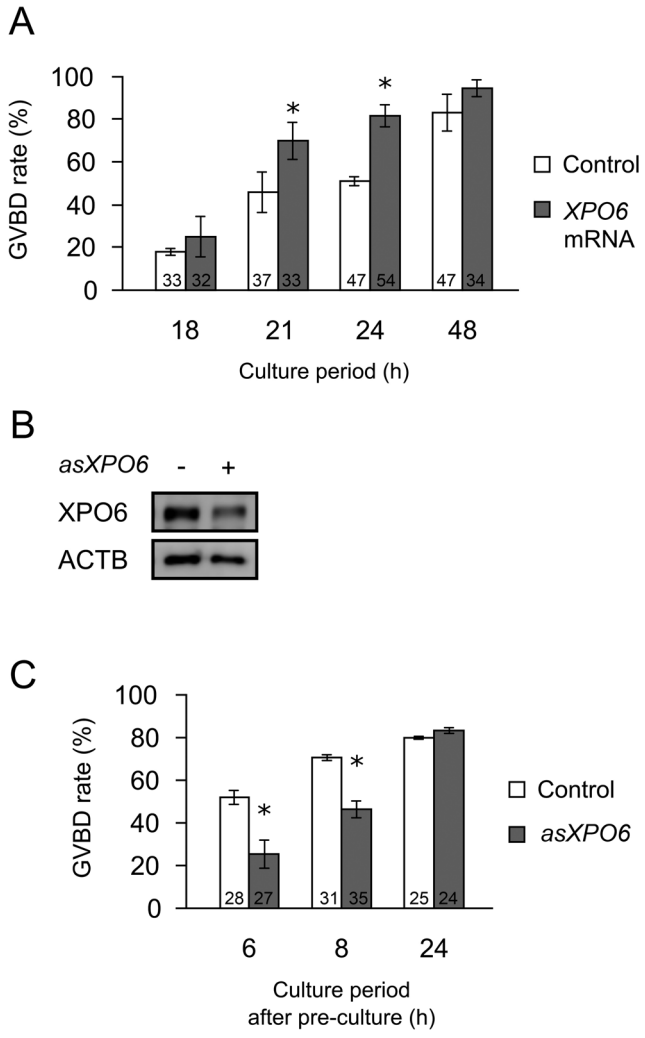

Fig. 3. Effect of XPO6 on meiotic resumption of porcine full-grown oocytes. (A) The GVBD rate of the *XPO6* mRNA-injected oocytes was examined at the indicated culture periods (* $P < 0.05$). The oocytes into which only *EGFP* mRNA was injected were used as a control. The number of oocytes examined in every experimental group was indicated in each bar. Results were shown as mean ± SEM. (B) The XPO6 expression in oocytes with or without *XPO6* antisense RNA (*asXPO6*)-injection was analyzed via immunoblotting. The oocytes were cultured for 48 h with roscovitine (Ros) for GV-stage maintenance. Fifteen oocytes were used for each lane. (C) The *asXPO6*-injected oocytes were precultured for 48 h with Ros to decrease endogenous XPO6. After the preculture, the oocytes were cultured without Ros and the GVBD rate was examined at the indicated culture periods (* $P < 0.05$). The oocytes into which only *EGFP* mRNA was injected were used as the control. The number of oocytes examined in every experimental group was indicated in each bar. Results are presented as mean ± SEM.

of XPO6 in vertebrate GV-stage oocytes. Although the necessity of nuclear localization of actin for maintaining GV structure in porcine oocytes is presently unclear, the fact that the nuclear volume of porcine oocytes is considerably smaller than that of *Xenopus* oocytes and that the actin localization in the GV was relatively weak in porcine oocytes might indicate that actin is not particularly important for maintaining the GV structure in porcine FGOs.

The present study suggested that the XPO6 amount is kept constant during the GV stage by balancing active synthesis and degradation, unlike XPO1, which is stably present in the GV-stage oocytes without synthesis and degradation [13]. It was also revealed that the XPO6 amount decreased slightly after GVBD when nuclear transport became dispensable. In contrast, the level of endogenous XPO6 protein was considerably low in GOs. Moreover, the accumulation of exogenous XPO6 after *XPO6* mRNA injection was relatively low, and consequently its nuclear-export function was not confirmed in GOs. XPO6 was overexpressed in FGOs after *XPO6* mRNA injection, and XPO1 was sufficiently translated even in GOs injected with *XPO1* mRNA, which had the same 5'-UTR and 3'-UTR structures that as the *XPO6* mRNA. Therefore, presumably, XPO6 protein is translated from the injected *XPO6* mRNA in GOs as well as FGOs, but is degraded to maintain low levels and to prevent its activity. This situation of porcine GOs is in accordance to that of *Xenopus* immature FGOs, in which XPO6 is absent. Recently, nuclear actin has been reported to support DNA repair at DNA breaks and then to preserve genome integrity [20–22]. The low XPO6 level in nucleus might contribute to genome protection by suppressing the nuclear export of actin in GOs, which is meiotically arrested for a long period. Nongrowing or growing oocytes cannot be induced into GVBD by hormonal stimulation or *in vitro* culture, unlike FGOs [15, 23–25], and this inability is generally attributed to a deficiency of GVBD-inducing maturation-promoting-factor (MPF) and the suppression of MPF activity by high protein-kinase-A activity [15]. In addition, the low level of XPO6 protein in GOs may also maintain the GV structure over a long period by suppressing the nuclear export of actin, which might contribute to the protection of the nuclear membrane from accidental breakdown even in mammals.

Actin is highly localized in the GV in *Xenopus* oocytes [12], whereas ACTB was present uniformly in whole oocytes without nuclear accumulation in porcine GOs. This result is in accordance with the previous report [17]. Actin can passively diffuse into and out of the nucleus by passing through NPCs alone due to its size (about 42 kDa). Therefore, without active nuclear transport, the concentrations of actin in the nucleus and cytoplasm are roughly equivalent. Thus, importin 9, a nuclear-import receptor of actin [26, 27], could be actively involved in actin distribution in *Xenopus* oocytes; conversely, the stable involvement of XPO6 is suggested in general somatic cells, which mostly contain cytoplasmic actin [8, 10]. In porcine GOs, importin 9 might not have been expressed as highly as XPO6, because ACTB was equally present in the nucleus and cytoplasm and the nuclear transport of actin is considered to be inactive. In contrast, porcine FGOs might express not only XPO6 but also importin 9, because the nuclear localization index of actin was higher in FGOs than in GOs. Further studies are required to improve our understanding of the nuclear transport of actin and the reason for actin accumulation in the nucleus of FGOs.

As the present study reported an increase in the XPO6 expression in porcine FGOs, the involvement of XPO6 in the process of meiotic resumption was analyzed by its overexpression and expression inhibition. The results revealed that the GVBD rate in XPO6-overexpressing oocytes was comparable with that in the control oocytes until 18

h of culture but was significantly increased after 21 and 24 h of culture. This result suggests that the increased nuclear export of actin or subsequent decrease of nuclear actin did not accelerate the regulation mechanism of meiotic resumption or the MPF activation mechanism itself, but rather shortened the period required for the full-activation of MPF, the signal transduction downstream of MPF, or the breakdown of nuclear membrane. The decreased GVBD rate observed in the XPO6-inhibited oocytes as a result of *asXPO6* injection further supported these ideas. GVBD is induced via phosphorylation and depolymerization of nuclear lamin caused by fully-activated MPF [28, 29]. In *Xenopus* oocytes, nuclear-localized actin appears to play a pivotal role in supporting nuclear lamin [12]. Considering these reports and the present results, it is possible that it is difficult to maintain the GV membrane in XPO6-overexpressing oocytes because of the decreased actin support of the nuclear lamina. Thus, the period for GVBD was shortened; however, the time required for the onset of MPF activation remained unaffected. The nuclear actin level might be one of the key factors in determining the time required for GV membrane disintegration.

In conclusion, this is the first study to indicate that XPO6 is expressed in full-grown GV-stage mammalian oocytes, it functions in nuclear export of actin, it might be preferentially degraded by the GOs, and that XPO6 might be involved in the progress of GVBD through the regulation of the nuclear actin level affecting the physical maintenance of the GV membrane. Nevertheless, its influence on GV-stage maintenance might not be so crucial in porcine oocytes due to the smaller size of the nucleus and lower localization of actin in the nucleus compared to *Xenopus* oocytes. Further studies on importin 9 [26, 27], cofilin [27], and profilin [8], as well as XPO6 will be beneficial to achieve more information on the effects of XPO6 during nuclear transport of actin in mammalian oocytes.

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