Involvement of peroxiredoxin 2 in cumulus expansion and oocyte maturation in mice

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Abstract.   Peroxiredoxin 2 (Prdx2), an antioxidant enzyme, is expressed in the ovary during the ovulatory process. The aim of the present study was to examine the physiological role of Prdx2 during ovulation using *Prdx2*-knockout mice and mouse cumulus–oocyte complex (COC) from WT mice. Two days of treatment of immature mice (21–23 days old) with equine chorionic gonadotrophin and followed by treatment with human chorionic gonadotrophin greatly impaired cumulus expansion and oocyte maturation in *Prdx2*-knockout but not wild-type mice. Treatment of COCs in culture with conoidin A (50 μM), a 2-cys Prdx inhibitor, abolished epiregulin (EPI)-induced cumulus expansion. Conoidin A treatment also inhibited EPI-stimulated signal molecules, including signal transducer and activator of transcription-3, AKT and mitogen-activated protein kinase 1/2. Conoidin A treatment also reduced the gene expression of EPI-stimulated expansion-inducing factors (hyaluronan synthase 2 (*Has2*), pentraxin 3 (*Ptx3*), TNF-α induced protein 6 (*Tnfaip6*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*)) and oocyte-derived factors (growth differentiation factor 9 (*Gdf9*) and bone morphogenetic protein 15 (*Bmp15*)). Furthermore, conoidin A inhibited EPI-induced oocyte maturation and the activity of connexins 43 and 37. Together, these results demonstrate that Prdx2 plays a role in regulating cumulus expansion and oocyte maturation during the ovulatory process in mice, probably by modulating epidermal growth factor receptor signalling.

Additional keywords: cumulus cell, Graafian follicle, ovary, ovulation.

Introduction

Reactive oxygen species (ROS) play an important role in ovulation. Administration of antioxidant agents to rodents reduces the ovulation rate and cumulus expansion (Shkolnik *et al.* 2011; Park *et al.* 2012). A moderate increase in ROS in preovulatory follicles triggers oocyte maturation in rats. However, the excess generation of ROS causes oxidative stress, affecting ovarian physiology (Sugino 2005; Tsai-Turton and Luderer 2006).

Excess ROS are eliminated by antioxidant enzymes, such as superoxide dismutase (SOD), catalase and peroxiredoxins (PRDXs). The six isoforms of mammalian PRDXs are classified into three subtypes with four typical two-cysteine (2-Cys) residues (PRDX1–4), one atypical 2-Cys residue (PRDX5) and one 1-Cys residue (PRDX6; Wood *et al.* 2003). PRDX2 has been implicated in several cellular functions, such as cell proliferation, apoptosis and intracellular signalling (Latimer and Veal 2016; Rhee 2016). PRDX2 is also associated with cancer,

cardiovascular dysfunction and neurodegeneration (Rhee 2016; Nicolussi *et al.* 2017). However, *Prdx2*-deficient mice appear normal and fertile because of a compensation mechanism (Lee *et al.* 2003).

To prevent the detrimental effects of excess ROS, the ovary possesses antioxidant defences. Ascorbic acid, a water-soluble antioxidant vitamin, accumulates during ovulation (Guarnaccia *et al.* 2000). The antioxidant tripeptide glutathione prevents the apoptosis of preovulatory rat follicles (Tsai-Turton and Luderer 2006; Tsai-Turton *et al.* 2007). The major ROS scavenging enzymes SOD1 and SOD2 are expressed in the ovaries of several species, including humans and rodents (Sasaki *et al.* 1994; Tilly and Tilly 1995; Nomura *et al.* 1996). SOD2-deficient female mice have reduced fertility (Ho *et al.* 1998; Matzuk *et al.* 1998) and impaired progesterone secretion (Noda *et al.* 2012). PRDX1 and PRDX2 are the major PRDXs expressed in the ovary during ovulation (Park *et al.* 2012).

PRDX2 is expressed in cumulus cells and oocytes, as well as in the granulosa cells of preovulatory follicles. Prdx2 plays a vital role in inhibiting ovarian apoptosis through the clearance of H_2O_2 in mice (Yang *et al.* 2011). In addition, Prdx2 is required for spindle assembly and chromosome organisation during meiotic maturation in mice (Jeon *et al.* 2017).

Although the expression of PRDX2 has been demonstrated in the ovary, its physiological role during ovulation remains to be determined. In the present study, the function of Prdx2 was examined *in vivo* using *Prdx2*-null mice and *in vitro* using culture of cumulus–oocyte complexes (COCs). We assumed that the expression of Prdx2 in preovulatory follicles of *Prdx2*-null mice may be gradually replaced by other Prdxs during follicular growth from prepubertal to adult age. To shorten this compensation period, the growth of preovulatory follicles was artificially induced by administration of gonadotrophins to prepubertal Prdx2-null mice. In addition to the *in vivo* study in mice, the function of Prdx1/2 was examined in an *in vitro* culture of COCs using conoidin A, an inhibitor of typical 2-Cys PRDX1–4. Conoidin A has been shown to inhibit PRDX1 and PRDX2 by blocking their hyperoxidation activity (Haraldsen *et al.* 2009). The results of the present study demonstrate the regulation of cumulus expansion and oocyte maturation by Prdx2 in mice.

Materials and methods

Animals

Immature female C57BL/6 mice were purchased from Samtako. Prdx2$^{-/-}$ (knockout) and Prdx2$^{+/-}$ (heterozygous) mice with the C57BL6/J background were kindly provided by DY Yu (Korea Research Institute of Bioscience and Biotechnology; Lee *et al.* 2003). Animals were housed in groups under a 14-h light : 10-h dark regimen and provided food and water *ad libitum*. All animals were maintained and treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council 2011), as approved by the Institution Animal Care and Use Committee at Chonnam National University.

Examination of ovulation in Prdx2$^{-/-}$ mice in vivo

To induce superovulation, wild-type, Prdx2$^{+/-}$ and Prdx2$^{-/-}$ mice (21–23 days old) were injected subcutaneously with 5 IU equine chorionic gonadotrophin (eCG, also referred to as pregnant mare's serum gonadotrophin (PMSG); Sigma) to stimulate the growth of multiple follicles. Two days later, some eCG-primed mice were injected intraperitoneally with 5 IU human chorionic gonadotrophin (hCG; Sigma).

To examine ovulation rate, mice were killed, the oviducts were excised and flushed and the oocytes were counted under a dissecting microscope 24 h after hCG administration.

To examine cumulus expansion, ovaries were isolated 6 h after hCG injection and placed in α-minimum essential medium (MEM; Invitrogen) supplemented with 25 mM HEPES, 0.25 mM sodium pyruvate, 3 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Preovulatory follicles were then punctured using 26-gauge needles to release COCs. Cumulus expansion was assessed by microscopic examination using a previously described scoring system (Vanderhyden *et al.* 1990). Scores of 0–1 indicate no expansion or minimum expansion; a score of 2 indicates that cells in the outer two layers have expanded; a score of 3 indicates expansion of all layers of the cumulus except corona radiata cells; and a score of 4 indicates expansion of the whole cumulus, including corona radiata cells. Using these scores, a mean cumulus expansion index (CEI; range 0.0–4.0) was calculated for each group.

To examine oocyte maturation, ovaries were isolated 6 h after hCG injection and placed in α-MEM. COCs released from preovulatory follicles were denuded of cumulus cells by gentle pipetting. Denuded oocytes were examined under a stereomicroscope for evidence of the dissolution of the oocyte nuclear membrane (a process called germinal vesicle breakdown (GVBD)), a hallmark of oocyte meiotic resumption. The experimental design is outlined in Fig. S1, available as Supplementary Material to this paper.

Cumulus expansion and oocyte maturation in the culture of COCs

Unexpanded COCs were collected in complete α-MEM from the ovaries of immature wild-type mice primed with eCG for 48 h. For *in vitro* COC expansion, 30–35 COCs were plated in separate wells of Nunclon four-well dishes (Nunc) in 150 μL α-MEM with 3 mg mL^{-1} bovine serum albumin and 5% fetal bovine serum under a cover of mineral oil. The COCs treated with 100 nM epiregulin (EPI) and/or 1–100 μM conoidin A (Cayman), an inhibitor of typical 2-Cys PRDX1–4, and were incubated at 37°C in a modular incubation chamber (Billups Rothenberg) infused with 5% O_2, 5% CO_2 and 90% N_2. COCs were pretreated with conoidin A for 1 h prior to EPI treatment and then cultured in the presence of conoidin A for the duration of the culture period (14 h). The degree of cumulus expansion was then assessed by microscopic examination using the previously described scoring system (Vanderhyden *et al.* 1990).

To assess oocyte maturation, 30 COCs were cultured in 150 μL α-MEM containing 10 μM 3-isobutyl-1-methylxanthine (IBMX) to block spontaneous GVBD in Nunclon four-well dishes in the presence of 100nM EPI with or without 1–100 μM conoidin A at 37°C for 4 h in a humidified atmosphere of 5% CO_2 in air. After removal of cumulus cells by pipetting, oocyte maturation was assessed by scoring GVBD. Experiments were repeated three times with three different mice.

RNA isolation and real-time polymerase chain reaction analysis

Total RNA was isolated using a Total RNA mini kit (Favourgen Biotech) according to the manufacturer's instructions. Total RNA was reverse transcribed, and real-time polymerase chain reaction (PCR) was performed on a Rotor-Gene Q 5plex (QIAGEN), located at the Korea Basic Science Institute, using a QuantiTect SYBR Green PCR Kit (QIAGEN). Primers were designed using Primer3 software (http://primer3.ut.ee/, accessed 6 March 2020) and are listed in Table S1. The mean C_t value of three determinations for each gene was divided by the linear C_t of the β-actin (*Actb*) gene to obtain the relative abundance of the transcript. Mean values were obtained from three or four
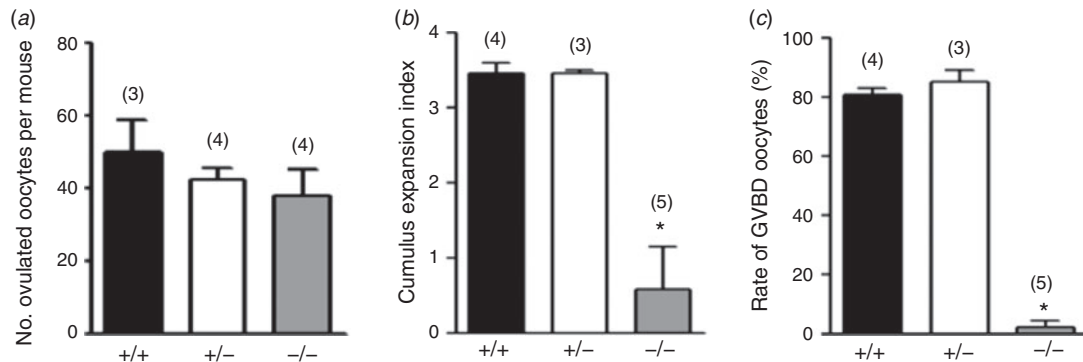
Fig. 1. Impairment of the ovulatory process in peroxiredoxin 2 (*Prdx2*)-null mice. Immature wild-type (+/+), heterozygous (+/−) and *Prdx2*-knockout (−/−) mice primed with equine chorionic gonadotrophin for 48 h were injected with human chorionic gonadotrophin (hCG) to induce ovulation. (*a*) Ovulated oocytes in the oviductal ampullae were counted 24 h after hCG administration, whereas (*b*) cumulus expansion and (*c*) oocyte maturation (examined as the presence of germinal vesicle breakdown (GVBD)) were monitored 6 h after hCG injection. The degree of cumulus expansion was assessed using a subjective scoring system from 0 (no expansion) to +4 (maximum expansion), with the cumulus expansion index calculated as microscopic examination of expanded condition of cumulus cells. The number of mice examined is indicated above each column. Values are the mean ± s.e.m. *$P < 0.05$ compared with wild-type mice.

separate experiments. *Actb* was used as an internal control for all measurements.

Western blot analysis

To evaluate the effects of conoidin A on the downstream signalling pathway for EPI activation, 100 COCs were cultured in 500 μL α-MEM without serum in four-well dishes in the presence of EPI (100 nM) with or without conoidin A (50 μM) for 15 min at 37°C. To assess the expression of connexin (Cx) 43 and Cx37, 200 COCs were cultured in a four-well dish for up to 2 h, in 500 μL α-MEM at 37°C in a humidified atmosphere of 5% CO_2 in air. Lysates of COCs were resolved by 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen) and transferred to nitrocellulose membranes (Amersham Bioscience), per the procedure and incubation conditions as described by Park *et al.* 2012 and Jeon *et al.* 2017. Membranes were blocked with 3% skim milk before immunoblotting using a primary antibody (1 : 500 final dilution) and a horseradish peroxidase-conjugated secondary IgG (1 : 1000 final dilution). The anti-Cx37 (ab185820) and anti-Cx43 (ab11370) antibodies were purchased from Abcam. The antibody against phosphorylated (at Ser[368]) Cx43 (3511S) and all the antibodies used for the signalling study were purchased from Cell Signaling. After washing in 1× Tris-buffered saline Tween-20 (TBST), signals were visualised with enhanced chemiluminescence. Band intensities were quantified using UNSCAN-IT Gel 6.1 software (Silk Scientific) after subtraction of background signal and normalised against glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Santa Cruz Biotechnology).

Statistical analysis

Data are presented as the mean ± s.e.m. One-way analysis of variance (ANOVA) followed by the Dunnett test was used for comparisons among multiple groups. Comparisons between any two points were evaluated using Student's two-tailed *t*-test. $P < 0.05$ was considered significant.

Results

Cumulus expansion and oocyte meiotic resumption in immature Prdx2-deficient mice

Mice deficient in the *Prdx2* gene exhibit normal postnatal development and fertility, perhaps due to a compensatory mechanism from other Prdxs (Lee *et al.* 2003). To circumvent this compensation even partially, prepubertal mice were treated with gonadotrophins in the present study to induce superovulation. The ovulation rate in hCG-treated *Prdx2*-null mice (38 ± 17 oocytes) was not significantly different to that in wild-type or heterozygous mice (50 ± 17 and 42.3 ± 7.3 oocytes respectively; Fig. 1*a*).

Cumulus expansion and oocyte maturation were examined in COCs collected from ovarian preovulatory follicles of mice 6 h after hCG administration. Only 14.3 ± 24.8% of COCs in *Prdx2*-null mice were expanded, compared with 85% and 87% of COCs in the wild-type and heterozygous mice respectively, in which cumulus cells reached almost full expansion (i.e. CEI 3–4; Fig. 1*b*). Interestingly, the rate of oocyte meiotic resumption, scored as percentage GVBD, was markedly reduced in *Prdx2*-null mice (2.2 ± 3.8%), whereas more than 80% of oocytes exhibited GVBD in wild-type and heterozygous mice (Fig. 1*c*).

Regulation of cumulus expansion by conoidin A in mouse COC culture

Complementary to the *in vivo* analysis, we examined *in vitro* cumulus expansion using conoidin A, a 2-Cys Prdx inhibitor. Unexpanded COCs isolated from eCG-primed mice were pretreated with conoidin A for 1 h. When COCs were cultured with 100 nM EPI for 14 h in the absence of conoidin A, a maximum degree of expansion was observed (Fig. 2*a*). The addition of higher concentrations of conoidin A (50–100 μM) to the EPI-containing medium reduced expansion up to 66%, whereas lower concentrations (1–30 μM) had no effect (Fig. 2*b*).
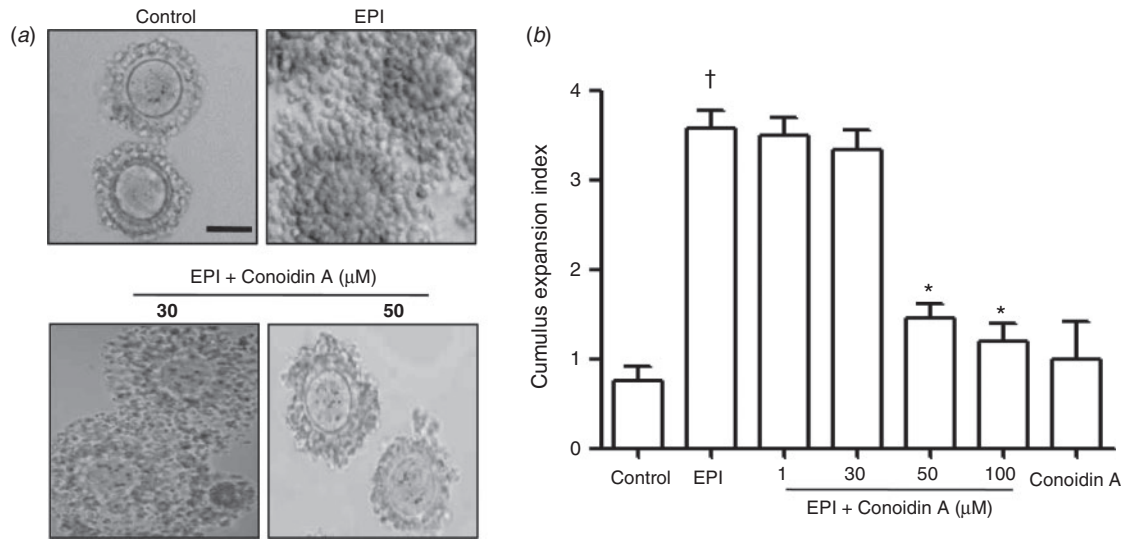
Fig. 2.   Effects of conoidin A, a 2-Cys peroxiredoxin 2 (Prdx2) inhibitor, on cumulus expansion in cultured cumulus–oocyte complexes (COCs). Unexpanded COCs collected from equine chorionic gonadotrophin-primed mice were cultured in the presence of epiregulin (EPI; 100 nM), with or without different concentrations of conoidin A (1–100 μM), for 14 h. (*a*) Representative micrographs of COCs cultured for 14 h. Scale bar = 100 μm. (*b*) The degree of cumulus expansion was assessed using a subjective scoring system from 0 (no expansion) to +4 (maximum expansion), with the cumulus expansion index calculated as microscopic examination of expanded condition of culumus cells. Data are the mean ± s.e.m. of 30–35 COCs examined from three different experiments. $^{\dagger}P < 0.05$ compared with control; $^{*}P < 0.05$ compared with EPI alone.
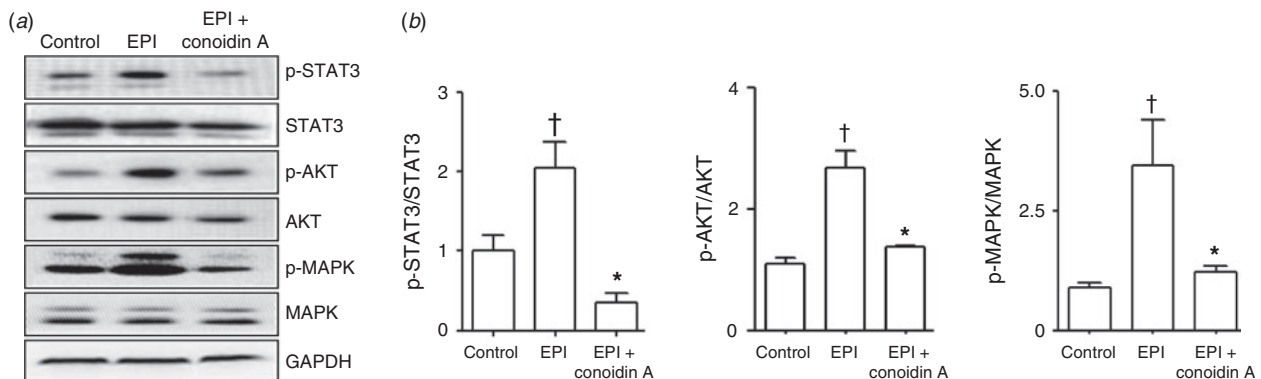

Fig. 3.   Suppression of epiregulin (EPI)-activated signalling pathways by conoidin A in cultured cumulus–oocyte complexes (COCs). COCs isolated from equine chorionic gonadotrophin-primed mice were cultured in the absence (control) or presence of 100 nM EPI, with or without 50 μM conoidin A, in serum-free medium for 15 min. (*a*) Representative western blot analysis; 100 COCs were used for each sample treatment. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control. p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; MAPK, mitogen-activated protein kinase. (*b*) Mean (±s.e.m.) band intensities of phosphorylated to total protein ratios for three individual experiments. $^{\dagger}P < 0.05$ compared with control; $^{*}P < 0.05$ compared with EPI alone.

Exposure to and culture of unexpanded COCs in the presence of conoidin A alone resulted in a similar degree of cumulus expansion to that seen in the control group.

To examine the effect of Prdx2 on epidermal growth factor (EGF) receptor signalling, unexpanded COCs were treated with EPI and/or conoidin A for 15 min in serum-free medium. EPI increased the phosphorylation of signal transducer and activator of transcription (STAT) 3, AKT and mitogen-activated protein kinase (MAPK) 1/2 (Fig. 3*a*), whereas the addition of conoidin A suppressed EPI-stimulated STAT3,

AKT and MAPK 1/2 phosphorylation by 85%, 48% and 65% respectively (Fig. 3*b*).

We next examined the effect of conoidin A on the regulation of genes known to affect expansion during COC expansion. The addition of conoidin A suppressed EPI-stimulated expression of hyaluronan synthase 2 (*Has2*), pentraxin 3 (*Ptx3*), TNF-α induced protein 6 (*Tnfaip6*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*) by 60–72% (Fig. 4*a*). Interestingly, the expression of the oocyte-derived factors growth differentiation factor 9 (*Gdf9*) and bone morphogenetic protein 15 (*Bmp15*) was
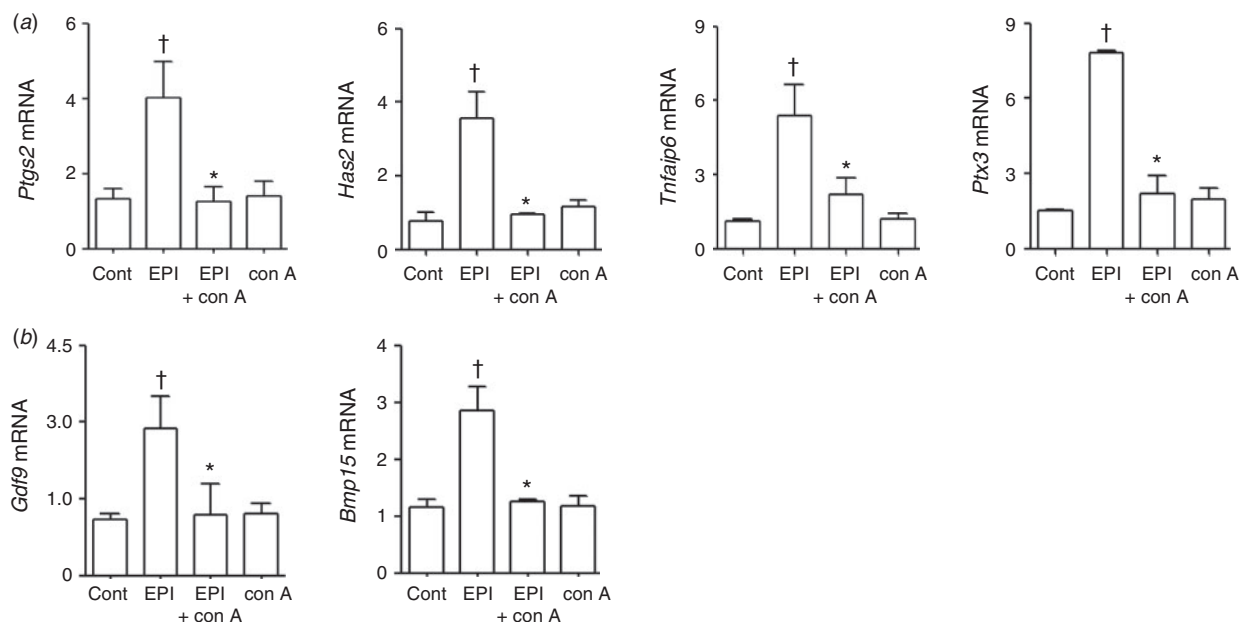
Fig. 4. Effects of conoidin A on gene expression of (*a*) cumulus expansion-related transcripts and (*b*) oocyte-derived factors. Mouse cumulus–oocyte complexes (COCs; 300 for each treatment group) were cultured in the presence of 100 nM epiregulin (EPI), with or without 50 μM conoidin A (con A) for (*a*) 6 or (*b*) 2 h. Total RNA in COCs was assayed using real-time reverse transcription–polymerase chain reaction. Transcript levels are shown as fold activity relative to control value. Data are the mean ± s.e.m. of four experiments. $^{†}P < 0.05$ compared with control; $^{*}P < 0.05$ compared with EPI alone. *Bmp15*, bone morphogenetic protein 15; *Gdf9*, growth differentiation factor 9; *Has2*, hyaluronan synthase 2; *Ptgs2*, prostaglandin-endoperoxide synthase 2; *Ptx3*, pentraxin 3; *Tnfaip6*, TNF-α induced protein 6.

stimulated by EPI, and the addition of conoidin A abolished this effect (Fig. 4*b*).

Regulation of oocyte maturation by conoidin A in COC culture

To demonstrate the *in vitro* function of 2-Cys Prdxs in oocyte maturation, unexpanded COCs were cultured for 4 h in the presence of 10 μM IBMX (to block spontaneous maturation) with 100 nM EPI and/or 1–100 μM conoidin A. In EPI-treated COCs, release from meiotic arrest was significantly higher (75% GVBD) than in COCs cultured with IBMX alone (control; Fig. 5). The addition of conoidin A inhibited EPI-induced GVBD in a dose-dependent manner, with maximum inhibition at 50 μM conoidin A (52% GVBD). Treatment with conoidin A alone resulted in a similar level of GVBD as that in the control.

Cx43 and Cx37 play an important role in oocyte maturation (Richard and Baltz 2014). Treatment of COCs with EPI for 1 h resulted in a twofold increase in Cx43 phosphorylation (Fig. 6*a*). The addition of conoidin A for 1 h inhibited this EPI-induced increase in Cx43 phosphorylation by 46.3%. Treatment with EPI and/or conoidin A for 2 h had no significant effect on Cx43 phosphorylation (Fig. 6*b*). Expression of Cx37 was markedly increased by EPI treatment for 1 and 2 h (approximately twofold), and the addition of conoidin A completely abolished the effects of EPI.

Discussion

Although Prdx2 is expressed in preovulatory follicles (Park *et al*. 2012), its physiological function has not been reported. To
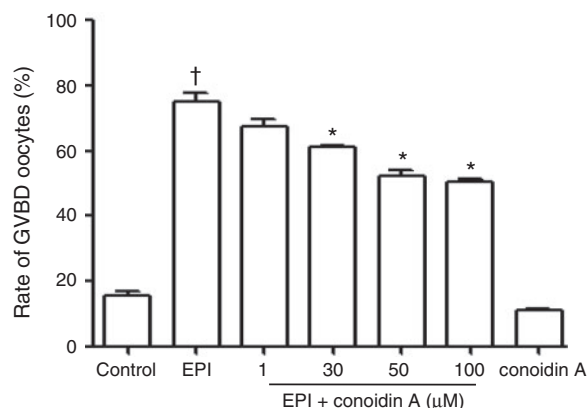

Fig. 5. Effects of conoidin A on oocyte maturation in cultured cumulus–oocyte complexes (COCs). Mouse COCs (30 for each treatment group) were cultured in medium containing 10 μM 3-isobutyl-1-methylxanthine (IBMX) to prevent spontaneous maturation in the presence of 100 nM epiregulin (EPI), with or without 100 μM conoidin A, for 4 h. Oocyte maturation was assessed as the presence of germinal vesicle breakdown (GVBD). Results are the mean ± s.e.m. of three different experiments. $^{†}P < 0.05$ compared with control; $^{*}P < 0.05$ compared with EPI alone.

the best of our knowledge, the present study demonstrated, for the first time, Prdx2 regulation of cumulus expansion and oocyte maturation during ovulation in mice. *Prdx2*-deficient mice showed less cumulus expansion and oocyte maturation, but no difference in ovulation rate, than Prdx2 wild-type mice after
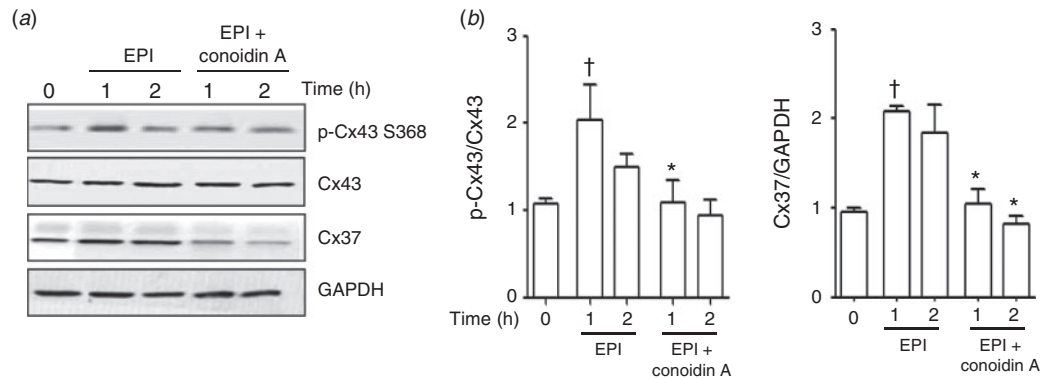
Fig. 6. Effects of conoidin A on expression levels of connexin (Cx)43 and Cx37 in cultured cumulus–oocyte complexes (COCs). Mouse COCs (200 for each treatment group) were cultured in the presence of 100 nM epiregulin (EPI) with or without 50 μM conoidin A. (*a*) Representative blot. Protein lysates of COCs were assayed by western blot analysis using specific antibodies against phosphorylated (p-) Cx43, Cx43 and Cx 47. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control. (*b*) Mean (±s.e.m.) band intensities from three individual experiments. [†]$P < 0.05$ compared with control; *$P < 0.05$ compared with EPI alone.

administration of eCG and hCG to prepubertal mice. The role of Prdx1/2 in cumulus expansion and oocyte maturation was demonstrated in the *in vitro* culture of COCs using conoidin A, an inhibitor of 2-Cys Prdxs. Conoidin A inhibited EPI-induced cumulus expansion by suppressing the expansion-inducing transcripts *Has2*, *Ptx3*, *Tnfaip6* and *Ptgs2* and the oocyte-derived factors *Gdf9* and *Bmp15*. Conoidin A also inhibited oocyte maturation by suppressing the expression of gap junction proteins Cx37 and Cx43. Prdx2 plays an important role in the meiotic maturation of mouse oocytes (Jeon *et al.* 2017).

The fact that *Prdx2*-deficient mice exhibit normal fertility (Lee *et al.* 2003) indicates the existence of a redundant pathway in the ovary. The structure and function of Prdx1 and Prdx2 are similar (Kang *et al.* 2005), and both are expressed in the ovary (Leyens *et al.* 2003; Park *et al.* 2012). In preovulatory follicles, the expression of Prdx2 is higher than that of Prdx1 (Park *et al.* 2012), indicating that Prdx2 is a major form of Prdxs expressed during ovulation. Therefore, when Prdx2 expression is absent in *Prdx2*-deficient mice, Prdx1 may be highly expressed up to the levels of Prdx2 for functional compensation, which may explain the normal fertility reported in *Prdx2*-deficient mice. The present results regarding reduced cumulus expansion and oocyte maturation may indicate that injection of *Prdx2*-deficent mice with gonadotrophin at an early age (prepubertal) partially eliminated this redundant pathway. The ovary of adult mice contains many preovulatory follicles, whereas the ovary of prepubertal mice primarily contains immature preantral follicles (Komatsu and Masubuchi 2018). In *Prdx2*-deficent mice, the expression of Prdx2 in preovulatory follicles may be compensated for by other Prdxs, especially by Prdx1, during growth from preantral follicles in prepubertal mice to preovulatory follicles in adult mice. Therefore, the induction of growth from preantral follicles to preovulatory follicles by gonadotrophins within 2 days in prepubertal *Prdx2*-deficient mice may shorten the time for compensation by other Prdxs, and thus Prdx2 may be temporally expressed in preovulatory follicles. Unfortunately, however, the detailed mechanism by which Prdx2

regulates cumulus expansion and oocyte maturation could not be examined using *Prdx2*-deficient mice because the breeding of these mice was stopped due to a viral infection. The ovulation rate was not altered in *Prdx2*-deficient mice, suggesting that Prdx2 may not be involved in regulating factors important for follicle rupture, such as the progesterone receptor and its downstream molecules (Richards *et al.* 2002*a*).

The present data obtained from superovulated *Prdx2*-deficient mice suggest that Prdx2 may be a major antioxidant enzyme regulating the generation of ROS during the ovulatory process. Because large amounts of ROS are generated during the inflammatory process (Johnson *et al.* 1986), the analogy of ovulation to acute inflammation (Richards *et al.* 2002*b*) indicates a role for ROS in this process (Shkolnik *et al.* 2011). ROS have both deleterious and beneficial effects depending on their concentration. Such dual effects of ROS can be modulated by PRDX2 antioxidant enzyme during ovulation, which acts as a floodgate to maintain low resting levels of ROS and permit higher levels during signal transduction (Laloraya *et al.* 1988).

Although conoidin A inhibits 2-Cys PRDX1–4 (Haraldsen *et al.* 2009), the effect of conoidin A on cumulus expansion and oocyte maturation observed in the present *in vitro* study may be mediated through the inhibition of Prdx2 and/or Prdx1, because Prdx1/2 are the major Prdxs expressed in granulosa cells, cumulus cells and oocytes of preovulatory follicles. In rats, *Prdx2*, but not *Prdx1*, is expressed in the oocytes of preovulatory follicles (Park *et al.* 2012). Granulosa cells and corpora lutea express both *PRDX1* and *PRDX2* in mice. However, Prdx1 and Prdx2 protein is expressed in mouse oocytes (Jeon *et al.* 2017). Nevertheless, there is limited ability to come to any conclusion regarding the effects of Prdx2 on cumulus expansion and oocyte maturation in the present study because of the use of the non-specific PRDX inhibitor conoidin A.

The inhibitory effect of conoidin A in COC culture suggests that Prdx1/2 may regulate the EGF receptor (EGFR) signalling pathway to stimulate cumulus expansion-inducing transcripts, resulting in the expansion of cumulus cells. The most prominent

EGFR signalling pathway is the MAPK cascade for cumulus expansion, which is activated by EGF-like factors such as EPI (Richards and Ascoli 2018). Although PRDXs inhibit activation of H_2O_2-activated signalling pathways, PRDXs have also been found to be important for the activation of EGFR signalling, including MAPK (Latimer and Veal 2016). PRDX2 facilitates protein tyrosine phosphatases 1B (PTPIB) inactivation (Dagnell *et al.* 2017; Kim *et al.* 2018), a key protein tyrosine phosphatase in the regulation of EGFR-dependent singling (DeYulia and Carcamo 2005). Therefore, PRDX2 may potentiate EGFR signalling by inactivating PTPIB, and thus contribute to cumulus expansion.

Gonadotrophin treatment of prepubertal *Prdx2*-deficient mice resulted in a defect in cumulus expansion without affecting the ovulation rate. Defects in cumulus expansion often reduce ovulation efficiency (Zhuo and Kimata 2001). Cumulus expansion is severely impaired in mice deficient in the prostanoid Ep_2 receptor, one of the receptors for prostaglandin E_2, with ovulation efficiency reduced by 28.9% (Kennedy *et al.* 1999). The ovulated COCs in Ep_2-null mice show an irregularly aggregated cumulus oophorus leading to severely impaired fertilisation. Mice deficient in bikunin, which is necessary for the formation of the cumulus hyaluronan-rich matrix, show reduced ovulation efficiency (by 57%) with rarely fertilised oocytes (Zhuo *et al.* 2001). Mice deficient in natriuretic peptide receptor 2 (Npr2) show a defect in cumulus expansion leading to severe female infertility but not affecting ovulation efficiency (Kiyosu *et al.* 2012). Therefore, it seems that in knockout mice the magnitude of the effect of the defect in cumulus expansion on ovulation efficiency may depend on the gene deleted. In the present study, deletion of *Prdx2* impaired cumulus expansion without affecting ovulation rate. Another possibility is that the defect in cumulus expansion observed in *Prdx2*-deficient mice was due simply to delayed cumulus expansion, rather than to a complete block. It is possible that, in early time of LH/hCG injection to induce ovulation, Prdx1 was weakly expressed but near the time of follicle rupture Prdx1 was highly expressed to compensate for the lack of Prdx2 function on cumulus expansion and GVBD in preovulatory follicles of superovulated *Prdx2*-deficient mice. In fact, based on dissecting microscope observations, the cumulus cells seemed to be intact without denuded oocytes in ovulated COCs (data not shown). Further elaboration of the state of cumulus cells in ovulated COCs and examination of fertilisation efficiency after superovulation of *Prdx2*-null mice is needed. In addition, the generation of ovulation-specific *Prdx2*-deficient mice, and mice deficient in both *Prdx1* and *Prdx2* is necessary.

The inhibitory effect of conoidin A on cumulus expansion may be mediated by regulation of phosphatase and tensin homologue (PTEN) activity to modulate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway for cumulus expansion. Targeted depletion of the *Pten* gene in mouse granulosa cells increases ovulation rates by enhancing proliferation (Fan *et al.* 2012). EGFR and FSH activate the PI3K/AKT signalling pathway to regulate granulosa cell differentiation (Wayne *et al.* 2007; Richards and Ascoli 2018). The binding of PRDX1/2 to PTEN alters its downstream signalling to inhibit tumourigenesis (Cao *et al.* 2009; Verrastro *et al.* 2016). However, activation of the PI3K/AKT pathway of EGFR signalling

in cumulus cells has not been clearly demonstrated. It is also likely that PRDX2 regulates the PTEN/AKT signalling pathway of cumulus expansion-inducing cytokines, such as interleukin (IL)-6 and interferon (IFN)-α which were generated by EGF-like factors. IL-6 (Liu *et al.* 2009) and IFN-α (Jang *et al.* 2015) induce cumulus expansion in mice by activating the MAPK and AKT signalling pathways. It is also possible that PRDX2 may contribute to the activation of STAT3 of IL-6 or IFN-α singling. PRDX2 participates directly in the activation of STAT3 (Sobotta *et al.* 2015).

Another way that PRDX may contribute to cumulus expansion is by regulating the oocyte-derived factors GDF9 and BMP15. The expression and activation of EGFR in cumulus cells are dependent on GDF9 and BMP15 (Fan *et al.* 2012). GDF9 promotes cumulus expansion by stimulating *Ptgs2* and *Has2* expression (Elvin *et al.* 1999) and BMP15 regulates cumulus expansion via a mechanism requiring EGFR signalling (Yoshino *et al.* 2006).

PRDX regulates oocyte maturation by modulating the disruption of cumulus cell–oocyte gap junctions mediated via EGF-like factors. One of the pathways for the maintenance of oocyte meiotic arrest is via the supply of cGMP generated by the NPR2–C-type natriuretic peptide (CNP) system in granulosa and cumulus cells (Zhang *et al.* 2010; Conti *et al.* 2012) through gap junction Cx43 (granulosa–cumulus cell junction) and Cx37 (cumulus–oocyte junction) to the oocyte, which increases the activity of phosphodiesterase (PDE) 3A, the predominant cAMP PDE in the oocyte (Masciarelli *et al.* 2004). In addition to downregulating Cx43 and Cx37 expression, LH and hCG stimulate the phosphorylation of Cx43 protein (Gershon *et al.* 2007). EGFR activation causes gap junction closure by MAPK-dependent Cx phosphorylation, which blocks cGMP supply to the oocyte to induce meiotic resumption (Norris *et al.* 2010; Conti *et al.* 2012). Thus, it is likely that PRDX regulates the MAPK activity of EGFR signalling and thus stimulates the phosphorylation of Cxs.

In summary, the present study demonstrated that Prdx2 plays an important role in cumulus expansion and oocyte maturation during the ovulatory process, probably by modulating the EGFR signalling pathway. Further studies are needed to determine the mechanism underlying this role of Prdx2 by inducing the tissue-specific knockdown of Prdx2 during ovulation. Because ROS have been suggested to be associated with female infertility (Fujii *et al.* 2005), it would be of interest to investigate the clinical relevance of PRDX2 in women undergoing IVF.

Conflicts of interest

The authors declare no conflicts of interest.

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