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Endo-siRNA deficiency results in oocyte maturation failure and apoptosis in porcine oocytes

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Abstract. Both microRNAs (miRNAs) and endogenous small interfering RNAs (endo-siRNAs) play key regulatory roles in gene expression. Some studies have demonstrated that the function of miRNA is suppressed in mouse oocytes, suggesting that endo-siRNA, not miRNA, is essential for female meiosis. This finding has yet to be confirmed in other species. In this study, by knockdown of *DICER1*, *DROSHA* and its cofactor *DiGeorge syndrome critical region 8* (*DGCR8*) in porcine oocytes, we found that the proportion of oocytes with *DICER1* deficiency that developed to meiosis II (MII) stage was significantly lower than oocytes with *DROSHA* and *DGCR8* deficiency (39.23 versus 68.71 and 71.25% respectively; $P < 0.05$). Oocytes lacking *DROSHA* and *DGCR8* formed a barrel-shaped metaphase I spindle, with chromosomes tightly aligned at the metaphase plate whereas most oocytes (87%) lacking *DICER1* showed spindle abnormalities during oocyte *in vitro* maturation. Furthermore, *DICER1* deficiency also resulted in oocyte apoptosis. These results indicate that endo-siRNAs are essential for oocyte maturation in pigs.

Additional keywords: gene regulation, *in vitro* maturation, livestock.

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Introduction

Small RNAs are involved in RNA interference (RNAi) and include three classes: microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi interacting RNAs (piRNAs; García-López *et al.* 2014). There are both endogenous siRNAs (endo-siRNAs) and exogenous siRNAs (exo-siRNAs). Gene expression can be artificially repressed by siRNAs (Saito and Siomi 2010; Claycomb 2014). PiRNAs (24–31 nucleotides (nt) in length) associated with Piwi-subfamily proteins are essential for male fertility (Ghildiyal and Zamore 2009; Kim *et al.* 2009; Reuter *et al.* 2011; Stein *et al.* 2015). The other two classes, miRNAs and endo-siRNAs, are both important for oocyte maturation. There are many similarities between miRNAs and endo-siRNAs including their length, both being about 21–23 nt (Hamilton *et al.* 2002; Ambros *et al.* 2003). All of these similarities make it hard to distinguish the function of endo-siRNAs from miRNAs. Therefore, the functions of miRNAs and endo-siRNAs need to be further clarified.

Dicer is involved in biogenesis of both miRNAs and endo-siRNAs while *Drosha* and *DiGeorge syndrome critical region 8* (*DGCR8*) are only needed in miRNA biogenesis (Bernstein *et al.* 2001; Murchison *et al.* 2007; Kim *et al.* 2009). In previous studies, researchers studied small RNA function in *Dicer* knockout (KO) oocytes and zygotes and suggested the abnormalities were caused by a miRNA deficiency (Murchison *et al.* 2007; Tang *et al.* 2007). Recently, scientists knocked out *Drosha* and *DGCR8* and neither of the KO oocytes was a phenocopy of the *Dicer* KOs, indicating that endo-siRNAs may play an important role (Ma *et al.* 2010; Suh *et al.* 2010; Yuan *et al.* 2014). In these studies of mouse, neither *Drosha* nor *DGCR8* deficiency resulted in inaccurate oocyte maturation while *Dicer* deficiency caused oocyte maturation failure. Moreover, *Argonaute* (*AGO*) proteins play an indispensible role in target regulation of both endo-siRNAs and miRNAs. However, miRNAs bind to partially complementary sites in target mRNA 3′ untranslated regions (UTRS) and cause translational repression and mRNA decay through association with any of the four *AGO* proteins (*AGO1–4*), whereas endo-siRNAs bind to *Argonaute 2* (*AGO2*), the only mammalian *AGO* protein thought to possess endonucleolytic activity, and mediate endonucleolytic cleavage of target mRNA (Watanabe *et al.* 2006; Ghildiyal and Zamore 2009). Endo-siRNAs regulate gene expression through

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their duplexes with *AGO2*. Only one of the two strands, the 'guide' strand, is incorporated into the multi-protein RNA-induced silencing complex (RISC); the other ('passenger') strand is discarded (Tam *et al.* 2008; Czech and Hannon 2011; Nejepinska *et al.* 2012). The guide strand recognises a target mRNA by Watson–Crick base pairing and based on the degree of sequence complementarity between the siRNAs and target mRNA, either endonucleolytic cleavage or translational repression of the target mRNA follows (Carthew and Sontheimer 2009). Thus, it is clear that *AGO2* is necessary for endo-siRNA function. Inactivation of *AGO2* led to the same results as *Dicer* inactivation, which further confirmed that endo-siRNAs were indispensible during oocyte maturation (Kaneda *et al.* 2009; Stein *et al.* 2015). However, the results have only been obtained in the mouse model, so further studies will be required to understand the function of endo-siRNAs in other species.

Pigs (*Sus scrofa*) are an important species for disease modelling, biomedical research and food production. Pigs are important not only in agriculture but also in biomedicine. In the field of biomedicine, pigs are more anatomically and physiologically analogous to humans than mice (Pratt *et al.* 2006; Whyte and Prather 2011). Alterations of porcine key genes in the reproductive pathway provide model animals to improve our understanding of the causes and potential treatments of many human reproductive disorders. Therefore, in this study, we examined the functions of miRNAs and endo-siRNAs in porcine oocytes by knockdown of oocyte *DICER1*, *DROSHA* and *DGCR8*. We found that the absence of *DICER1*, but not of *DROSHA* or *DGCR8*, resulted in spindle abnormalities and oocyte apoptosis during oocyte *in vitro* maturation in pig, indicating that endo-siRNA rather than miRNA is essential for oocyte maturation.

Materials and methods

Oocyte collection and in vitro *maturation (IVM)*

Porcine ovaries were collected from a local slaughter house and kept in 0.9% saline with antibiotics at 37°C. Antral follicles whose diameter was between 3 and 5 mm were aspirated with an appropriate needle. Aspirated oocytes with an evenly granulated cytoplasm and at least three uniform layers of compact cumulus cells were selected and washed three times with maturation medium (TCM199 (Invitrogen) plus 0.05 mg mL^{-1} epidermal growth factor, 0.5 mg mL^{-1} LH and 0.5 mg mL^{-1} FSH (all Sigma-Aldrich)). The oocytes with or without microinjection were cultured in 24-well plates (Corning) containing 500 mL of maturation medium at 39°Cin 5% CO_2 in air and saturated humidity (Kong *et al.* 2014).

Microinjection

To perform *DICER1*, *DROSHA* and *DGCR8* knockdown experiments, the granulosa cells of oocytes at germinal vesicle (GV) stage were denuded. Then locked nucleic acid (LNA)-siRNA microinjections were carried out with an Eppendorf FemtoJet microinjector and Narishige NT-88NE micromanipulators. For injection, a glass capillary Femtotip II (Eppendorf) was loaded with 10 pL of 10 μM LNA-*DICER1*, LNA-*DROSHA* or LNA-*DGCR8* (Exiqon) by microloader (Eppendorf) and the solution was injected into the cytoplasm of GV oocytes in a 200-μL drop of manipulation medium (TCM-199 (Invitrogen) plus 30 mg mL^{-1} bovine serum albumin (BSA)) supplemented with 7.5 μg mL^{-1} cytochalasin B. The injection conditions consisted of 250 hPa injection pressure, 60 hPa comensation pressure and 0.7 s injection time. Immediately after microinjection, oocytes were washed and co-cultured with mural granulosa cells in maturation medium. A scrambled LNA-siRNA (Exiqon) was used as a negative control (NC; Wei *et al.* 2011).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using the PureLink Micro-to-Midi System (Invitrogen) according to the manufacturer's instructions and reverse transcription was used to generate cDNAs using the PrimeScript RT Reagent kit (TaKaRa). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) and the 7500 Real-Time PCR System (Applied Biosystems). The reaction parameters were 95°C for 30 s followed by 40 two-step cycles of 95°C for 5 s and 60°C for 34 s. All the primer pairs used for PCR amplification are shown in Table S1, available as Supplementary Material to this paper. Ct values were calculated using Sequence Detection System software (Applied Biosystems) and the amount of target sequence normalised to the reference sequence was calculated by the $^{\Delta\Delta}$CT method. The 18SrRNA was used as control. The oocytes without injection were used as a reference sample. Each pool contained 50 oocytes. All data are based on biological triplicates.

Western blot

Proteins from 100 oocytes at the appropriate stage of maturation were collected in sodium dodecyl sulfate (SDS) sample buffer and heated for 10 min at 100°C. After cooling on ice and centrifugation at 10 000*g* for 3 min at room temperature, samples were frozen at −80°C until use. The total proteins were separated by polyacrylamide gel electrophoresis with SDS (SDS-PAGE) with a 4% stacking gel and a 9% separating gel at 90 V, 0.5 h and 110 V, 2.5 h respectively and were then electrophoretically transferred onto a polyvinylidene difluoride membrane for 1 h, 350 mA at 4°C. Membranes were blocked in phosphate-buffered saline-Tris (PBST) buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% BSA (blocking solution) for 1 h at room temperature and then incubated with antibody against *DICER1* (ab14601; Abcam) or *p53* (sc-65226; Santa Cruz Biotechnology) diluted 1 : 2000 in blocking solution overnight at 4°C. After three 10-min washes in PBST, the membrane was incubated with a secondary antibody against mouse IgG (A9044; Sigma) diluted 1 : 10 000 in PBST for 1 h at 37°C. After being washed three times for 10 min each, the membrane was processed using the enhanced chemiluminescence (ECL) detection system (Amersham). Equal protein loading was confirmed by detection of *β-actin* (A1978; Sigma; Kong *et al.* 2014).

Immunofluorescence and confocal microscopy

After removing the zona pellucida in acidic Tyrode's solution (pH 2.5), oocytes were fixed with 4% paraformaldehyde in PBS

(pH 7.4) for at least 30 min at room temperature. Oocytes were permeabilised with 1% Triton X-100 overnight at 37°C, followed by blocking in PBS containing 1% BSA (blocking solution) for 1 h and incubation overnight at 4°C with antibody against *β-tubulin* (T5201; Sigma) diluted 1 : 500 in blocking solution. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 (washing solution) for 5 min each, the oocytes were labelled with antibody against *alpha-fetoprotein* (*AFP*) (H00000174-M01; Abnova) diluted 1 : 500 in washing solution for 1 h at room temperature. After one wash in washing solution, the nuclear status of the oocytes was evaluated by staining with Hoechst 33342 (5 μg mL^{-1} in washing solution; Sigma-Aldrich) for 2 min. After another three washes in washing solution for 8 min each, oocytes were mounted on glass slides in ProLong Diamond Antifade Mountant reagent (Life Technologies). Cells were observed under a confocal laser-scanning microscope (Leica TCS SP2 AOBS) as soon as possible after preparation. Each experiment was repeated three times and at least 30 oocytes were examined each time. In addition, the same instrument settings were used for each replicate (Xu *et al.* 2009; Kong *et al.* 2014).

Annexin-V assay

An annexin-V conjugate (Molecular Probes) was used to identify phosphatidylserine exteriorisation in apoptotic cells. According to the manufacturer's instructions, oocytes without zonae pellucidae were washed twice in PBS at 4°C and then washed three times in binding buffer. They were then incubated with annexin-V for 15 min before being transferred to a 0.25 mg mL^{-1} solution of propidium iodide (Sigma-Aldrich) to allow recognition of necrotic cells. Oocytes were washed three times in binding buffer before mounting in ProLong Diamond Antifade Mountant reagent (Life Technologies) and analysing via fluorescence microscopy.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for MicroSoft Windows (IBM). Data are shown as the mean ± s.e.m. One-way analysis of variance was used to assess any differences between groups. The Duncan method was used for pairwise comparisons followed by a Bonferroni correction. $P < 0.05$ (two-tailed) was considered to be statistically significant.

Results

Efficient knockdown of DICER1, DROSHA and DGCR8

To obtain *DICER1*, *DROSHA* and *DGCR8* knockdown porcine oocytes, we synthesised two locked nucleic acids (LNAs) for each gene and microinjected the LNAs into oocytes at the GV stage. Quantitative PCR was performed after 24 h of microinjection to test the knockdown efficiencies; efficient knockdown of *DICER1*, *DROSHA* and *DGCR8* in porcine oocytes was confirmed (Fig. 1*a*). Moreover, a dramatic decrease in *DICER1* protein was observed by western blot analysis (Fig. 1*b*). Therefore, we were successful in knockdown of *DICER1*, *DROSHA* and *DGCR8* in porcine oocytes.

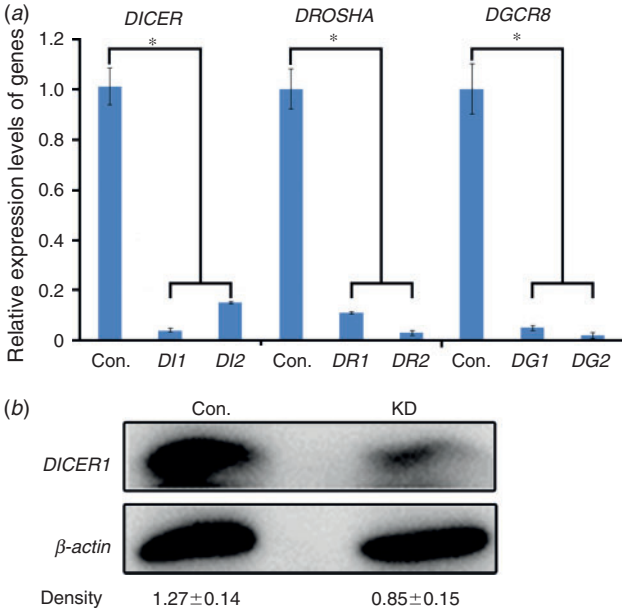

Fig. 1. Efficient knockdown of *DICER1*, *DROSHA* and *DGCR8* in porcine oocytes. (*a*) Effective knockdown of *DICER1*, *DROSHA*, *DGCR8* mRNA checked by quantitative PCR. The oocytes without LNA-siRNA injection (Con.) were used as a reference sample. Each pool contained 50 oocytes. All data are based on biological triplicates. (*b*) Effective knockdown of Dicer1 protein checked by western blot with *β-actin* as a control. Error bars represent s.e.m. ($n = 3$). *$P < 0.05$.

Table 1. Effect of *DICER1*, *DROSHA* and *DGCR8* knockdown on PB1 extrusion of porcine oocytes

Values with different superscripts in the same column differ significantly ($P < 0.05$)

Group	No. of oocytes (repeats)	No. of MII oocytes (% ± s.e.m.)
Control	125 (3)	94 (75.2 ± 3.76)[a]
Negative control	119 (3)	83 (69.75 ± 5.86)[a]
DICER1	130 (3)	51 (39.23 ± 4.62)[b]
DROSHA	129 (3)	88 (68.71 ± 6.36)[a]
DGCR8	130 (3)	91 (71.25 ± 5.83)[a]

Knockdown of DICER1 results in nuclear maturation failure and abnormal chromosome alignment

We examined the effect of *DICER1*, *DROSHA* and *DGCR8* knockdown on nuclear maturation of porcine oocytes by LNA microinjection. The results showed that the rates of first polar body (PB1) extrusion showed no significant difference between control, scrambled (negative control) LNA, *DROSHA*-LNA and *DGCR8*-LNA groups (75.2 and 69.75 versus 68.71 and 71.25% respectively; $P > 0.05$; Table 1), but the proportion of oocytes that developed to metaphase II (MII) stage in the *DICER1*-LNA group was significantly lower than in the control and scrambled

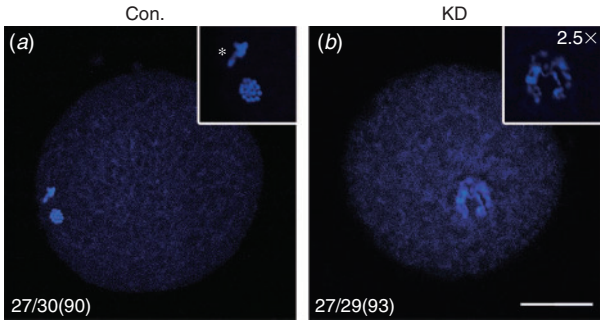

Fig. 2. Chromosome morphology in *DICER1* knockdown oocytes. (*a*) Normal MII oocytes. (*b*) *DICER1* knockdown oocytes. The inset of each image shows the nucleus enlarged 2.5 times. Figures at bottom left indicate the number of cells exhibiting the morphology shown (percentages shown in parentheses). *First polar body. Chromosomes were stained with Hoechst33342 (blue). Scale bar = 50 μm.

LNA groups (39.23 versus 75.2 and 69.75% respectively; $P < 0.05$; Table 1). Considering that *DICER* knockdown oocytes cannot extrude the PB1, we decided to observe the nuclear morphology by Hochest33342 staining. The PB1 and well-organised chromosomes were found in the oocytes without injection ($n = 27$; 90%; Fig. 2*a*) whereas the *DICER1* knockdown oocytes displayed unaligned chromosomes ($n = 27$; 93%; Fig. 2*b*). Given that *DICER1* is important for endo-siRNA biogenesis while *DROSHA* and *DGCR8* are dispensable, we believed that endo-siRNA not miRNA deficiency led to the abnormal PB1 extrusion and chromosome alignment.

Oocyte DICER1 knockdown leads to disordered spindle morphology

Earlier studies showed that *Dicer* rather than *Drosha* or *DGCR8* knockdown can regulate the formation of the spindle in mouse oocytes. In the study, immunofluorescence analysis of the spindle using *β-tubulin* antibody was performed to determine the progression of meiosis in porcine oocytes. Results revealed that 83%, 80%, 73% and 93% of oocytes arrived at the metaphase I (MI), anaphase I (AI), telophase I (TI) and MII stages at 18 h, 20 h, 22 h and 42 h during IVM respectively ($n = 25$, 24, 22 and 28 respectively; see Fig. S1, available as Supplementary Material to this paper). We found that oocytes from the *DROSHA*-LNA, *DGCR8*-LNA and scrambled LNA groups proceeded to form a barrel-shaped metaphase I spindle, with chromosomes tightly aligned at the metaphase plate (Fig. 3*b–b″*, c–c″, d–d″), and, by the extrusion of PB1, completed meiosis I and arrested at the metaphase stage of meiosis II (Fig. 3*f–f″*, g–g″, h–h″). In contrast, in most oocytes of the *DICER1*-LNA group, the chromosomes remained dispersed and never aligned and the spindle was extraordinarily disorganised (Fig. 3*a–a″*, e–e″). Considering that *DICER1* is indispensible for endo-siRNAs while *DROSHA* and *DGCR8* are not, these results suggest that endo-siRNA is indispensible for chromosome alignment and spindle formation of porcine oocytes. In other words, endo-siRNA is necessary for meiosis of porcine oocytes.

Oocyte DICER1 deficiency results in apoptosis

We have shown that the absence of *DICER1* leads to immature oocytes in pigs. However, it is unknown whether the knockout of *DICER1* causes oocyte apoptosis. To detect phosphatidylserine (PS) exteriorisation (a signal of early apoptosis) in MII-stage oocytes, we use fluorescent annexin-V that can only combine with PS outside of the membrane with Ca^{2+} (Andree *et al.* 1990; van Engeland *et al.* 1998). Results showed 75% of oocytes in the *DICER1*-LNA group were annexin-V-positive ($n = 15$; Fig. 4*b*) whereas annexin-V-positive oocytes were significantly fewer in the control group (10%; $n = 2$; Fig. 4*a*). Further, western blotting of MII-stage oocytes showed that the level of *p53* protein in the *DICER1*-LNA group was dramatically higher than in the control group (Fig. 4*c*). Together, these results indicate that endo-siRNA deficiency leads to oocyte apoptosis.

Discussion

In this study, the lack of *DICER1* but not of *DROSHA* or *DGCR8* resulted in spindle abnormalities and oocyte apoptosis during oocyte *in vitro* maturation in the pig, indicating that endo-siRNA rather than miRNA is indispensible for oocyte maturation.

It has been reported that a lack of mouse endo-siRNAs results in some female reproductive diseases such as premature ovarian failure and infertility (Yuan *et al.* 2014). However, the phenomenon was not demonstrated in other species. In this study, we examined whether porcine endo-siRNA deficiency resulted in any defects like in the mouse. We knocked down porcine oocyte *DICER1*, *DROSHA* and *DGCR8* by microinjection of their LNAs which can knockdown genes efficiently (Kanwar *et al.* 2015; Jolly *et al.* 2016). By quantitative real-time PCR we confirmed that the LNAs for these three genes were effective. Considering that *DICER1* knockdown caused oocyte maturation failure (rather than *DROSHA* or *DGCR8* as in the mouse), we further checked the porcine *DICER1* knockdown effect by western blot (Fig. 1). So far, we believe that this is the first time that *DICER1*, *DROSHA* and *DGCR8* have been knocked down in porcine oocytes.

The extrusion of the first polar body is an important mark for oocyte maturation (Evans and Robinson 2011; Schmerler and Wessel 2011). It has been found that *Dicer* deficiency causes improper oocyte maturation due to failure of PB1 extrusion in the mouse (Ma *et al.* 2010; Suh *et al.* 2010). We knocked down *DICER1*, *DROSHA* and *DGCR8* in porcine oocytes and found that *DICER1* deficiency caused PB1 extrusion failure whereas *DROSHA* and *DGCR8* deficiency did not. To ensure this phenotype was a result of *DICER* deficiency rather than microinjection, we microinjected the scrambled LNA into oocytes and found normal PB1 extrusion (Table 1). Furthermore, the staining results showed that *DICER1*-deficient oocytes exhibited abnormal chromosome morphology and did not complete meiosis I (Fig. 2). Some studies demonstrated that *Dicer* deficiency led to spindle formation failure in mouse oocytes, which could be a cause of chromosome disorder. Thus, we performed immunofluorescence to check the chromosome and spindle morphology. The *DROSHA* and *DGCR8* knockdown oocytes showed exactly the same chromosome and spindle morphology as both control and negative control groups

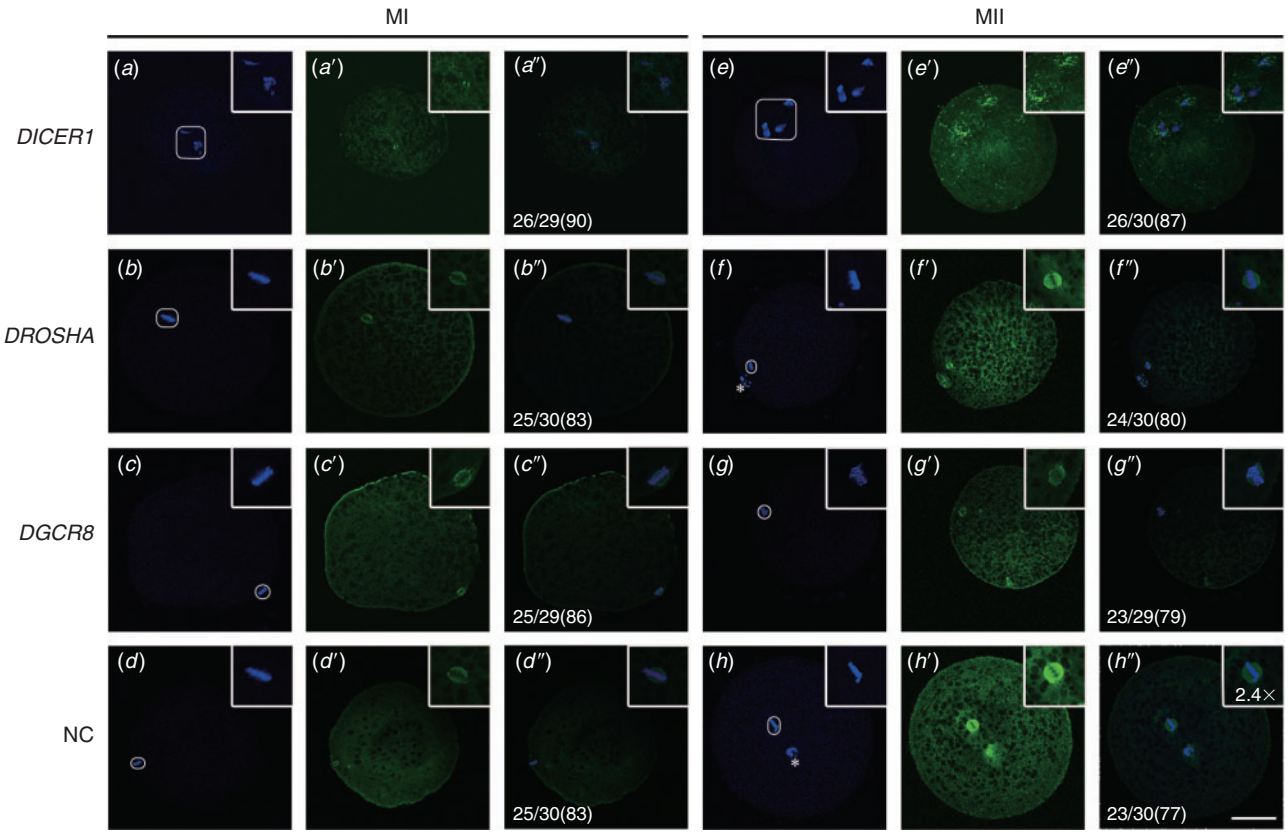

Fig. 3. Effect of *DICER1*, *DROSHA* and *DGCR8* knockdown on chromosome and spindle morphology of porcine oocytes. Oocytes with microinjection were *in vitro* matured to MI and MII stage. Immunofluorescence was performed as described in Materials and methods. The spindle was stained with *β-tubulin* antibody (green) and DNA was counterstained with Hoechst33342 (blue). (*a*–*a*″) Oocytes with *DICER1* knockdown at MI stage. (*b*–*b*″) Oocytes with *DROSHA* knockdown at MI stage. (*c*–*c*″) Oocytes with *DGCR8* knockdown at MI stage. (*d*–*d*″) Control oocytes at MI stage. (*e*–*e*″) Oocytes with *DICER1* knockdown at MII stage. (*f*–*f*″) Oocytes with *DROSHA* knockdown at MII stage. (*g*–*g*″) Oocytes with *DGCR8* knockdown at MII stage. (*h*–*h*″) Control oocytes at MII stage. White circles indicate chromosomes. *First polar body. The inset of each image shows the area from the white circle enlarged 2.4 times. Figures at bottom left indicate the number of cells exhibiting the morphology shown (percentages shown in parentheses). Scale bar = 50 μm.

(Fig. 3). It is believed that *Dicer* is involved in both endo-siRNA and miRNA biogenesis whereas *Drosha* and *DGCR8* only function in miRNA biogenesis, thus we concluded that a lack of endo-siRNA and not miRNA was contributing to the abnormalities exhibited. Why does endo-siRNA deficiency cause disordered chromosomes and spindle morphology? The explanation could be that centromeres are composed of repetitive sequences and endo-siRNAs are involved in silencing of repetitive sequences (Bagasra and Prilliman 2004; Ekwall 2007; Banisch *et al.* 2012; Fukagawa and Earnshaw 2014). The loss of endo-siRNAs may activate heterochromatin repetitive sequences and prevent centromeres from connecting to microtubules (Kanellopoulou *et al.* 2005; Ekwall 2007; Saito and Siomi 2010; Castel and Martienssen 2013; Li 2014). As a result, the spindle cannot form properly during oocyte maturation.

It has been shown in some studies that abnormal meiosis causes oocyte apoptosis (Ene *et al.* 2013; Tripathi and Chaube 2015). Therefore, we examined apoptosis in the *DICER1*-deficient oocytes. Under knockdown of *DICER1*, we showed apoptosis in porcine oocytes by testing PS exteriorisation and *p53* expression (Fig. 4), suggesting that endo-siRNA deficiency leads to oocyte apoptosis. Endo-siRNAs are so important in RISC that they are involved in regulation of gene expression. As a result, endo-siRNA deficiency may lead to abnormal expression of the gene network during oocyte maturation and result in oocyte apoptosis (Hussein *et al.* 2006; Coticchio *et al.* 2015).

Our findings indicate that the function of endo-siRNAs in porcine oocyte maturation is more significant than in the mouse. Pigs are more anatomically and physiologically analogous to humans than are mice, so further study of the reproductive diseases in humans is aided by studies in pigs. In summary, our findings provide two major insights into the roles of small RNAs in porcine oocytes by *DICER1* knockdown. First, endo-siRNAs instead of miRNAs are essential for nuclear maturation of

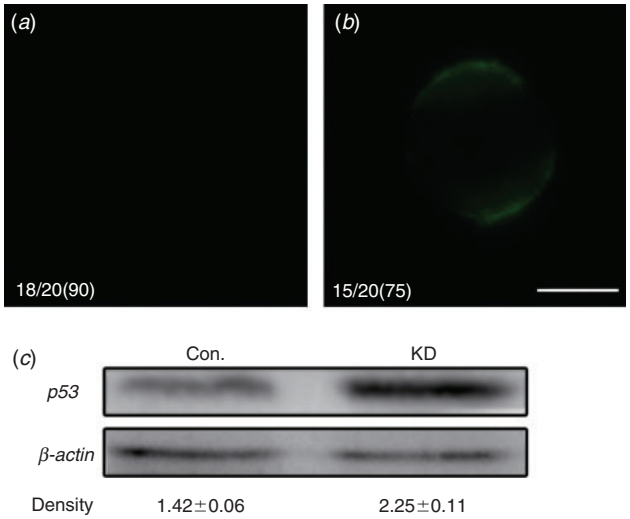

Fig. 4. Apoptosis in *DICER1* knockdown oocytes. (*a–b*) Annexin-V assay was performed as described in Materials and methods. (*a*) Normal oocytes. (*b*) Oocytes with *DICER1* knockdown. (*c*) Western blot of oocytes was performed with antibodies against *p53*, with *β-actin* as control. Figures at bottom left indicate the number of cells exhibiting the morphology shown (percentages shown in parentheses). Scale bar = 50 μm.

porcine oocytes. Second, the loss of endo-siRNAs leads to oocyte apoptosis.

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