Cytosolic DNA sensor-initiated innate immune responses in mouse ovarian granulosa cells

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Abstract

Viral infections of the ovary may perturb ovarian functions. However, the mechanisms underlying innate immune responses in the ovary are poorly understood. The present study demonstrates that cytosolic viral DNA sensor signaling initiates the innate immune response in mouse ovarian granulosa cells and affects endocrine function. The cytosolic DNA sensors p204 and cGAS and their common signaling adaptor stimulator of interferon (IFN) genes (STING) were constitutively expressed in granulosa cells. Transfection with VACV70, a synthetic vaccinia virus (VACV) DNA analog, induced the expression of type I interferons (IFNA/B) and major inflammatory cytokines (TNFA and IL6) through IRF3 and NF- κ B activation respectively. Moreover, several IFN-inducible antiviral proteins, including 2',5'-oligoadenylate synthetase, IFN-stimulating gene 15 and Mx GTPase 1, were also induced by VACV70 transfection. The innate immune responses in granulosa cells were significantly reduced by the transfection of specific small-interfering RNAs targeting p204, cGas or Sting. Notably, the VACV70-triggered innate immune responses affected steroidogenesis *in vivo* and *in vitro*. The data presented in this study describe the mechanism underlying ovarian immune responses to viral infection.

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Introduction

Various virus types can infect the ovary and impair ovarian functions. Vaccinia virus exhibits high tropism for the ovary and can cause ovary pathology and sterility (Zhao *et al.* 2011). Immunohistochemistry studies have revealed that vaccinia virus can penetrate through the theca and granulosa layers *in vivo* (McCart *et al.* 2001). Mumps virus can infect granulosa cells and induce cell apoptosis, which is associated with premature ovarian failure (Morrison *et al.* 1975, Wang *et al.* 2016). Human immunodeficiency viruses frequently infect the ovary and cause ovarian dysfunction (Cejtin *et al.* 2006, Ohi *et al.* 2010). Hepatitis B viruses can infect human oocytes and ovarian granulosa cells, which may lead to maternal–fetal transmission (Hu *et al.* 2011, Jin *et al.* 2011). However, the mechanisms underlying innate immune responses in ovarian cells and their detrimental effects on ovarian functions remain largely unknown.

Pattern recognition receptors (PRRs) recognize conserved pathogen-associated molecular patterns (PAMPs) of pathogens and initiate the innate immune response, which is the frontline of defense against invading pathogens (Kumar *et al.* 2011). Several subfamilies of PRRs have been identified thus far: toll-like receptors (TLRs) (Li *et al.* 2009), retinoic acid-inducible

gene I (RIG-I)-like receptors (RLRs) (Yoneyama & Fujita 2007), nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Martinon *et al.* 2009) and cytosolic DNA sensors (Keating *et al.* 2011, Orzalli & Knipe 2014). These PRRs can be recognized by PAMPs from diverse pathogens, such as bacteria, viruses, fungi and parasites (Kumar *et al.* 2011). Various PRRs, including TLRs, RLRs and DNA sensors, can recognize viral nucleic acids and initiate the innate immune response (Barbalat *et al.* 2011). We recently demonstrated that TLR3, RIG-I and melanoma differentiation-associated gene 5 (MDA5) initiate innate immune responses in ovarian cells and perturb ovarian functions (Yan *et al.* 2013, 2014, 2015). However, the cytosolic viral DNA sensor-initiated innate immune response in the ovary has yet to be investigated. Several cytosolic DNA sensors, including DNA-dependent activator of interferon regulatory factors (DAI), RNA polymerase III (Pol III), cGMP–AMP synthase (cGAS) and interferon (IFN)-inducible protein 16 (p204 in mouse), initiate innate immune responses to viral DNA challenge. DAI was initially recognized as a cytosolic DNA sensor that initiates innate immune responses (Takaoka *et al.* 2007). Pol III can be considered a DNA sensor because it can transcribe DNA to RNA and activate retinoic acid-inducible gene I-like receptors

(Chiu *et al.* 2009). IFI16 is a DNA-binding protein and mediates DNA virus-triggered innate immune responses (Veeranki & Choubey 2012). cGAS has been identified as a cytosolic DNA sensor that can trigger innate immune response signaling (Sun *et al.* 2013). The stimulator of IFN genes (STING) is a common adaptor of DNA sensor-mediated signaling (Ishikawa *et al.* 2009).

Cytosolic DNA sensor signaling can be initiated by viral DNA and result in the activation of nuclear factor kappa B (NF- κ B) and interferon regulatory factor 3 (IRF3), thereby inducing the expression of pro-inflammatory cytokines and type I interferons (IFNA/B) respectively (Wang *et al.* 2008, Unterholzner *et al.* 2010). Type I IFN production is the primary antiviral response and subsequently induces a broad spectrum of antiviral proteins in the infected cells and facilitates antiviral adaptive immunity against invading viruses (Randall & Goodbourn 2008, Desmet & Ishii 2012). The antiviral proteins IFN-stimulated gene 15 (ISG15), 2',5'-oligoadenylate synthetase (OAS1), double-stranded RNA (dsRNA)-activated protein kinase (PKR) and Mx GTPase 1 (MX1) can amplify antiviral signaling, degrade viral RNA, inhibit viral protein translation and block viral mRNA transcription respectively (Medzhitov 2007, Sadler & Williams 2008).

The ovarian follicle, in which numerous granulosa cells encompass an oocyte, is devoid of immune cells (Petrovska *et al.* 1996). It is reasonable to speculate that granulosa cells possess innate defense capabilities against invading pathogens (Sheldon & Bromfield 2011). Increasing evidence has shown that various PRRs are expressed and functional in ovarian cells. TLR4 is expressed in human granulosa cells from *in vitro* fertilization (Serke *et al.* 2009). TLR signaling pathways have recently been analyzed in human granulosa cell lines and in bovine and hen primary granulosa cells (Woods *et al.* 2009, Bromfield & Sheldon 2011, Price *et al.* 2012). TLR1–9 is expressed in normal human ovaries and in ovarian cancer (Zhou *et al.* 2009). Lipopolysaccharides initiate innate immune responses in bovine granulosa cells, perturbing estradiol synthesis and oocyte meiotic progression through TLR4 signaling (Herath *et al.* 2007, Bromfield & Sheldon 2011). TLR2 and TLR4 regulate ovulation and fertilization in mice (Shimada *et al.* 2006, 2008, Liu *et al.* 2008). A recent study showed that virus infection initiated innate immune responses through the activation of RIG-I and MDA5 in the ovaries of shelducks (Fu *et al.* 2016). We recently demonstrated that viral dsRNA sensors, including TLR3, MDA5 and RIG-I, initiated innate immune responses in mouse ovarian granulosa and stromal cells after challenge with dsRNA, which inhibited the steroidogenesis of the ovary (Yan *et al.* 2013, 2014).

Although DNA viruses, such as vaccinia virus and hepatitis B virus, can infect the ovary, DNA sensor-initiated innate immune responses in ovarian cells are yet to be investigated. The present study aims to

elucidate the roles of cytosolic DNA sensors in initiating innate immune responses in mouse ovarian granulosa cells to vaccinia viral DNA challenge. VACV70 is a double-stranded 70bp oligonucleotide containing vaccinia virus DNA motifs, which can potentially trigger cytosolic DNA sensor signaling mimicking vaccinia virus (Unterholzner *et al.* 2010). We demonstrate that VACV70 triggers innate immune responses by the activation of p204 and cGAS in mouse ovarian granulosa cells.

Materials and methods

Animals

C57BL/6J mice were obtained from the Laboratory Animal Center, Chinese Academy of Medical Sciences (Beijing, China). The mice were bred under specific pathogen-free conditions with standard nutrition (food and water *ad libitum*) and light cycles (12h light; 12h darkness). Three- to five-week-old female mice were used in this study. The mice were handled in compliance with the Guidelines for the Care and Use of Laboratory Animals established by the Chinese Council on Animal Care. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (the permit number: SCXK (jing) 2007-0001).

Antibodies and major reagents

The TNFA-neutralizing antibody (ab176489) was purchased from Abcam. The other antibodies used in this study are listed in Table 1. VACV70 (tlrl-vav70n), BX795 (tlrl-bx7) and BAY11-7082 (tlrl-b82) were purchased from InvivoGen (San Diego, CA, USA). Small-interfering RNAs (siRNAs) targeting mouse *p204* (sc-40700), *cGas* (sc-95512) and *Sting* (sc-154411) and the control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology.

Isolation and culture of granulosa cells

Mouse ovarian granulosa cells were isolated based on previously described procedures with modifications (Vanderhyden *et al.* 1992). Briefly, three-week-old female mice were intraperitoneally injected with 5 IU pregnant mare serum gonadotropin (Beijing Chief-East Tech Co., Beijing, China). At 45h after injection, the follicles on the surface of the ovaries were punctured with a 25-gauge needle to release oocyte-cumulus cell complexes and clumps of mural granulosa cells. After the removal of oocytes using a mouth-operated glass fine pipette, the granulosa cells were collected and cultured in F12/DMEM (Life Technologies) supplemented with 10% fetal calf serum (FCS, Life Technologies) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin).

Isolation of macrophages

Mouse macrophages were collected from peritoneal cavities based on a previously described procedure (Chong *et al.* 2005). Briefly, the peritoneal cavities were lavaged with 5 mL

Table 1 Antibodies used in this study.

Vendor	Antibody	Host species	Catalog number	Use	Working dilution	Conjugation	
Sigma-Aldrich	Anti-β-actin	Mouse	A5316	WB	1:4000		
	Anti-cGAS	Rabbit	SAB3500110	WB	1:500		
				IF	1:100		
				IHC	1:100		
Abcam	Anti-p204	Rabbit	ab104409	WB	1:1000		
				IF	1:200		
				IHC	1:200		
	Anti-STING	Rabbit	ab179775	WB	1:1000		
	Anti-DAI	Rabbit	ab81526	WB	1:1000		
	Anti-Pol III	Rabbit	ab22236	WB	1:1000		
	Anti-OAS1	Rabbit	ab86343	WB	1:1000		
	Anti-ISG15	Rabbit	ab131119	WB	1:500		
	Anti-P450arom	Rabbit	ab18995	WB	1:500		
	Anti-P450SCC	Rabbit	ab175408	WB	1:1000		
	Anti-HSD3B	Rabbit	ab150384	WB	1:1000		
	Anti-STAR	Rabbit	ab58013	WB	1:500		
	Santa Cruz Biotechnology	Anti-IRF3	Rabbit	sc-9082	WB	1:1000	
					IF	1:200	
Anti-MX1		Rabbit	sc-50509	WB	1:500		
Cell Signaling	Anti-FSHR	Goat	sc-7798	IF	1:200		
	Anti-p65	Rabbit	4764	WB	1:1000		
				IF	1:200		
	Anti-p-p65	Rabbit	3031	WB	1:1000		
Zhongshan Biotechnology Co., Beijing, China	Anti-p-IRF3	Rabbit	4947	WB	1:1000		
	Mouse IgG	Goat	ZB-2305	WB	1:4000	HRP	
	Rabbit IgG	Goat	ZB-2301	WB	1:4000	HRP	
				IHC	1:200		
	Rabbit IgG	Goat	ZF-0311	IF	1:200	FITC	

FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IF, immunofluorescence staining; IHC, immunohistochemistry; WB, Western blot.

of ice-cold $1\times$ phosphate-buffered saline (PBS). The lavaged cells were collected by centrifugation at 250g for 5 min at room temperature. The cells were subsequently re-suspended in RPMI-1640 medium (Life Technologies) supplemented with 10% FCS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and incubated in a humidified atmosphere with 5% CO₂ at 37°C. After 24 h, non-adherent cells were removed by washing with PBS. The adherent cells were determined to be macrophages with a purity of more than 95% based on immunostaining for F4/80, a marker of macrophages (Hume *et al.* 1984).

Transfection

Ovarian granulosa cells were seeded in 6-well plates at a density of 5×10^5 cells/well and cultured for 24 h. The medium was then replaced with serum-free F12/DMEM. Two hours later, the cells were transfected with 2 μ g/mL VACV70 using 2 μ L of Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. For gene silencing with siRNA, 2×10^5 granulosa cells per well were seeded in 6-well plates. At 24 h after seeding, the cells were transfected with 100 nM siRNA using the method described previously. After 24 h, the cells were transfected with VACV70.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After treatment

with RNase-free DNase I (Invitrogen) to remove genomic DNA contamination, the RNA (1 μ g) was reverse transcribed into cDNA in a 20 μ L reaction mixture containing 2.5 μ M random hexamers, 2 μ M dNTP and 200 U Moloney murine leukemia virus reverse transcriptase (Promega). PCR was performed in a 20 μ L reaction system containing 0.2 μ L of cDNA, 0.5 μ M each forward and reverse primers and 10 μ L of 2 $\times$ Power SYBR Green PCR Master Mix (Applied Biosystems) using an ABI PRISM 7300 real-time cycler (Applied Biosystems). The mRNA levels of the target genes were normalized to β -actin using the comparative threshold cycle method described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). The primer pairs used for PCR are listed in Table 2.

Western blot analysis

Cells or tissues were lysed using a lysis buffer (Appligen Technologies Inc., Beijing, China). The protein concentration of the lysates was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology). Equal amount of proteins (20 μ g) were separated on 10% SDS-PAGE gels and subsequently electrotransferred onto polyvinylidene difluoride membranes (Millipore). After blocking in Tris-buffered saline (TBS, pH 7.4) containing 5% nonfat milk for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. Then, the membranes were washed twice with TBS containing 0.1% Tween20 and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Zhongshan Biotechnology

Table 2 Primers used for real-time quantitative RT-PCR.

Target genes	Primer pairs (5'–3')	
	Forward	Reverse
<i>β-Actin</i>	GAAATAGTGGCTGACATCAAAG	TGTAGTTTCATGGATGCCACAG
<i>p204</i>	TCAGTTTCAGTAGCCACGGTAGCA	TGGTCCCAAACAAGTGATGGTGC
<i>Dai</i>	GGAAGATCTACCACTCACGTC	CCTTGTTGGCAGATCATGTTG
<i>cGas</i>	ACGAGAGCCGTTTTATCTCGTACCC	TGTCCGGAAGATTCACAGCATGTT
<i>Pol III</i>	TTTCCAGCAGATGCCTCTTT	ACGAGCTGCATCCTAGTGCT
<i>Sting</i>	CCTAGCCTCGCACGAACCTG	CGCACAGCCTTCCAGTAGC
<i>Tnfa</i>	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
<i>Il6</i>	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
<i>Ifna</i>	GACCTCCACCAGCAGCTCAA	ACCCCCACCTGCTGCAT
<i>Ifnb</i>	GACGTGGGAGATGTCCTCAAC	GGTACCTTTGCACCCTCCAGTA
<i>Mx1</i>	GACCATAGGGGTCTTGACCAA	AGACTTGCTCTTTCTGAAAAGCC
<i>Oas1</i>	ATTACCTCCTTCCCGACACC	CAAACCTCCACCTCCTGATGC
<i>Isg15</i>	CCAGTCTCTGACTGTGAGAGC	GCATCACTGTGCTGCTGGGAC
<i>P450arom</i>	CGGAGGAATGCACAGGCTCGAG	CGATGACTTCCAGCACAGC
<i>P450scc</i>	AGGTCCTTCAATGAGATCCCTT	TCCCTGTAAATGGGGCCATAC
<i>Hsd3b</i>	TATTCTCGGTTGTACGGGCAA	GTGCTACCTGTCACTGTGACC
<i>Star</i>	ATGTTCTCTGTCACGTTCAAG	CCCAGTGCTCTCCAGTTGAG
<i>Fshr</i>	GGCGGCAAACCTCTGAAC	CCAGGCTGAGTCATATCATCAATAC

Co., Beijing, China) at room temperature for 1 h. The HRP activity was detected using an enhanced chemiluminescence detection kit (Zhongshan Biotechnology Co.).

Immunofluorescence staining and immunohistochemistry

Cells cultured on Lab-Tek Chamber Slides (Nunc, Naperville, IL, USA) were fixed with pre-chilled methanol at -20°C for 3 min and subsequently permeabilized with 0.2% Triton X-100 in $1\times$ PBS for 15 min. After blocking with 10% normal goat serum in PBS for 30 min at room temperature, the cells were incubated with primary antibodies for 1 h at 37°C in a humid chamber. Then, the cells were washed three times with PBS and incubated with the appropriate fluorescein isothiocyanate-conjugated secondary antibodies (Zhongshan Biotechnology Co.) for 30 min at 37°C . Negative control cells were incubated with pre-immune rabbit serum instead of the primary antibodies. After co-staining with DAPI, the cells were mounted with a mounting solution (Vector Laboratories, Inc., Burlingame, CA, USA) for observation under a fluorescence microscope (IX-71; Olympus).

Ovaries from 5-week-old mice were used for immunohistochemistry studies according to previously described procedures (Wang *et al.* 2005). Briefly, after 24 h of fixation in Bouin's solution, the ovaries were embedded in paraffin and cut into $5\text{-}\mu\text{m}$ -thick sections. The sections were incubated for 15 min with $1\times$ PBS containing 3% H_2O_2 to inhibit endogenous peroxidase activity, soaked in citrate buffer and then microwaved at 100°C for 10 min to retrieve antigens. After blocking with 5% normal rabbit sera in PBS for 1 h at room temperature, the sections were incubated with primary antibodies overnight at 4°C . After rinsing with PBS, the sections were incubated with biotinylated secondary antibodies at room temperature for 30 min. The streptavidin-peroxidase activity was visualized using the diaminobenzidine method. Negative control sections were incubated with pre-immune sera instead of the primary antibodies. The sections

were counter-stained with hematoxylin and mounted with a mounting solution.

Enzyme-linked immunosorbent assay (ELISA)

The ovaries were lysed by grinding in $1\times$ PBS and centrifuged at 800 g for 5 min, and the supernatants were collected for ELISA. The granulosa cells were cultured in six-well plates at a density of 5×10^5 cells and then transfected with VACV70 in serum-free F12/DMEM. At 24 h after transfection, the concentration of cytokines in the culture medium was measured using ELISA kits according to the manufacturer's instructions. Kits for IL6 (BMS603/2), TNFA (BMS607/3) and IFNA (BMS6027) were purchased from eBioscience (San Diego, CA, USA), the kit for IFNB (42400) was purchased from R&D Systems and kits for estradiol (582251) and testosterone (582701) were purchased from Cayman Chemical Company. The concentration detection limits of the kits for IL6, TNFA, IFNA, IFNB, estradiol and testosterone were 6.5, 3.7, 7.5, 1.0, 3.7 and 4.2 pg/mL respectively.

Local injection of VACV70 into the ovarian bursa

For the local injection of VACV70 into the ovarian bursa, four-week-old female mice were anesthetized using pentobarbital sodium (50 mg/kg), and the ovaries were surgically exposed. One ovary was injected with a mixture of $0.2\text{ }\mu\text{g}$ of VACV70 and $1\text{ }\mu\text{L}$ of Lipofectamine RNAiMAX in a total volume of $10\text{ }\mu\text{L}$ with $1\times$ PBS using a 31-gauge insulin syringe through the ovarian fat into the ovarian bursa according to previously described procedures (Zhang *et al.* 2013). The contralateral ovary was injected with an equal volume of PBS and $1\text{ }\mu\text{L}$ of Lipofectamine RNAiMAX to serve as the control.

MTT assay

Cell viability was assessed using a 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (ATCC)

according to the manufacturer's instructions. Briefly, granulosa cells were cultured in 96-well microplates at a density of 2×10^4 cells/well. After treatment with the same conditions as those for the innate immune response induction, the cells were incubated with 10 μ L of MTT solution for 2 h. After the removal of the culture medium, 100 μ L of detergent reagent was added in each well to lyse the cells. The absorbance at 570 nm was recorded by a microplate reader (BioTek).

Statistical analysis

The data are presented as the mean $\pm$ S.E.M. Student's *t*-test was used to determine the significance between treatments. A one-way ANOVA test with Bonferroni's correction was used for multiple comparisons. The data were analyzed using the statistical software SPSS, version 11.0. Values of $P < 0.05$ were considered to be statistically significant.

Results

Expression of cytosolic DNA sensors in mouse ovarian granulosa cells

The purity of granulosa cells was more than 95%, which was confirmed by examining the expression of FSHR, a marker of granulosa cells (Camp *et al.* 1991) (Fig. 1A). The real-time quantitative reverse-transcription PCR (qRT-PCR) results showed that granulosa cells express *Fshr* (Fig. 1A, left panel), whereas *Fshr* mRNA was not detected in peritoneal macrophages from the same mice. Indirect immunofluorescence staining confirmed that FSHR protein was located in the granulosa cells (Fig. 1A, middle panel), which was not detected in macrophages (Fig. 1A, right panel). The expression of cytosolic viral DNA sensors, including p204, DAI, cGAS and Pol III, and their common adaptor STING was examined in mouse ovarian granulosa cells. Peritoneal macrophages from the same mice were used as positive controls. The real-time quantitative reverse-transcription PCR results showed that *p204*, *cGas* and *Sting* were expressed at comparable levels in granulosa cells and macrophages (Fig. 1B). In contrast, the *DAI* and *Pol III* mRNA levels were relatively low in granulosa cells compared with the macrophages. The protein levels of the DNA sensors were confirmed using Western blot analysis (Fig. 1C). Indirect immunofluorescence staining (Fig. 1D, upper panels) showed that p204 was distributed in both the cytoplasm and nucleus of the granulosa cells, whereas cGAS was mainly located in the cytoplasm of the granulosa cells (Fig. 1D, lower panels). Immunohistochemistry confirmed that the p204 and cGAS proteins were predominantly located in the granulosa cells (black arrows) (Fig. 1E), whereas the stromal (white arrows) and theca (black arrowheads) cells only faintly expressed these two proteins.

Figure 1 Expression of cytosolic DNA sensors. (A) Expression of follicle-stimulating hormone receptor (FSHR). Total RNA was extracted from mouse ovarian granulosa cells (Gc) and peritoneal macrophages (Mφ) and analyzed for relative mRNA levels of *Fshr* by normalizing to β -actin (left panel). Immunofluorescence staining using specific antibodies against mouse FSHR was performed in Gc (middle panel) and Mφ (right panel). (B) The mRNA levels of the cytosolic DNA sensors. Total RNA was extracted from Gc and Mφ. The relative mRNA levels of *p204*, *DAI*, *cGas*, *Pol III* and their common signaling adaptor *Sting* were determined using real-time quantitative RT-PCR by normalizing to β -actin. (C) The protein levels of the cytosolic DNA sensors and STING were determined by Western blot analysis using specific antibodies. β -Actin was used as loading control. (D) The distribution of p204 and cGAS proteins in granulosa cells. Immunofluorescence staining was performed using specific antibodies. The cells were co-stained with 4',6-diamidino-2-phenylindol. (E) The localization of p204 and cGAS in the mouse ovary. Immunohistochemistry was performed on sections of paraffin-embedded ovaries using specific antibodies. The black arrows, white arrows and black arrowheads indicate granulosa cells, stromal cells and theca cells, respectively. The insets in the upper right corners of the images represent negative controls for immunostaining. The images are representative of at least three experiments. The data represent the mean $\pm$ S.E.M. of three experiments. Bar = 20 μ m.

VACV70 induces innate immune responses in granulosa cells

Cytosolic viral DNA sensors can recognize VACV70 and thereby initiate the innate immune response (Unterholzner *et al.* 2010). To assess the cytosolic DNA sensor functions in granulosa cells, we examined the innate immune response after transfection with 2 µg/mL VACV70. We found that VACV70 transfection significantly upregulated the expression of p204 and cGAS at both the mRNA (Fig. 2A) and protein (Fig. 2B) levels. The mRNA levels of *p204* and *cGas* peaked at

18 h after VACV70 transfection. By contrast, Pol III, DAI and STING expressions were not changed after VACV70 transfection (data not shown). The expression of *Ifna*, *Ifnb*, *Il6* and *Tnfa* was dramatically induced by VACV70 in a time-dependent manner (Fig. 2C). Peak mRNA levels appeared at 6 h after VACV70 transfection. VACV70 also induced the expression of these cytokines in a dose-dependent manner (Fig. 2D). The plateaus of mRNA levels were observed after the cells were transfected with 2 and 5 µg/mL VACV70. Therefore, we performed *in vitro* experiments with 2 µg/mL VACV70 in this study.

Figure 2 VACV70-induced innate immune responses. (A) *P204* and *cGas* upregulation. Granulosa cells were transfected with VACV70 for specific durations. The relative mRNA levels of *p204* and *cGas* were determined by real-time quantitative RT-PCR. (B) The granulosa cells were lysed 24 h after VACV70 transfection. The *p204* and *cGAS* protein levels were determined by Western blot analysis. β -Actin was used as loading control. (C) Time-dependent cytokine expression. Total RNA was extracted from granulosa cells at the indicated time points after VACV70 transfection. The relative mRNA levels of *Ifna*, *Ifnb*, *Il6* and *Tnfa* were determined using real-time quantitative RT-PCR by normalizing to β -actin. (D) VACV70 dose-dependent cytokine expression. Granulosa cells were transfected with specific doses of VACV70 for 6 h. The relative *Ifna*, *Ifnb*, *Il6* and *Tnfa* mRNA levels were determined by real-time quantitative RT-PCR. (E) Cytokine secretion. Granulosa cells were transfected with VACV70 for the indicated durations. The IFNA, IFNB, IL6 and TNFA levels in the culture medium were measured using an ELISA. The Western blot images represent at least three independent experiments. The data are presented as the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$.

Enzyme-linked immunosorbent assay (ELISA) results showed that the protein levels of these cytokines in the culture medium were significantly increased (Fig. 2E). The peak levels were detected 18h after VACV70 transfection.

Various antiviral proteins can be upregulated in response to viral infections (Diamond & Farzan 2013). Major antiviral protein genes, including *Oas1*, *Mx1* and *Isg15*, were dramatically induced by VACV70 in a time-dependent manner (Fig. 3A). The highest mRNA levels were detected 16h after VACV70 transfection. The upregulation of these antiviral proteins was confirmed at the protein level by Western blot analysis 24h after VACV70 transfection (Fig. 3B).

NF- κ B and IRF3 activation

Given that cytosolic DNA sensor signaling can induce both IL6/TNFA expression via NF- κ B activation and type I IFN expression via IRF3 activation (Unterholzner et al. 2010), we examined NF- κ B and IRF3 activation in granulosa cells after VACV70 transfection. The Western blot results showed that VACV70 induced NF- κ B and IRF3 phosphorylation in a time-dependent manner (Fig. 4A). The expression of phospho (p)-p65 (Ser536) and p-IRF3 (Ser396) was detected 1h after VACV70 transfection, peaked at 2h and declined at 3h. In the control cells, adding transfection reagent alone to the medium for 2h did not induce the phosphorylation of

Figure 3 Expression of antiviral proteins. (A) RNA levels. Total RNA was extracted from granulosa cells at specific time points after transfection with VACV70. The relative mRNA levels of *Oas1*, *Mx1* and *Isg15* were examined using real-time quantitative RT-PCR. (B) Protein levels. Granulosa cells were transfected with VACV70. After 24h, the cell lysates were subjected to Western blot analysis to probe antiviral proteins using specific antibodies. Cells treated with Lipofectamine RNAiMAX alone served as Ctrl. β -Actin was used as the loading control. The images are representative of at least three independent experiments. The data are presented as the mean $\pm$ S.E.M. of three independent experiments. ** $P < 0.01$.

Figure 4 Activation of interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF- κ B). (A) Phosphorylation of NF- κ Bp65 (p65) and IRF3. Granulosa cells were transfected with VACV70 for the specified durations. Cells that were transfected with Lipofectamine RNAiMAX alone for 2h served as Ctrl. The cell lysates were analyzed via Western blot using specific antibodies against phospho-p65 (p-p65), p65, phospho-IRF3 and IRF3. β -Actin was used as the loading control. (B) Translocation of IRF3 and p65 from the cytoplasm to the nucleus. Granulosa cells were treated as in (A). The cellular distributions of IRF3 and p65 were determined by immunofluorescence staining using specific antibodies. The dynamics of IRF3 and p65 nuclear translocation were quantitatively analyzed by counting 300 cells at the indicated time points after VACV70 transfection (right panel). The images are representative of three separate experiments. The data represent the mean $\pm$ S.E.M. of three experiments. Bar = 20 μ m.

NF- κ B and IRF3. Phosphorylated NF- κ B and IRF3 must translocate into nuclei to induce cytokine expression. Immunofluorescence staining showed that NF- κ B and IRF3 efficiently translocated into the cell nuclei in a time-dependent manner (Fig. 4B). Peak levels of nuclear translocation were observed 2h after transfection, corresponding with the timing of peak phosphorylation efficiencies. In the control cells, the transfection reagent alone did not induce the nuclear translocation of NF- κ B and IRF3.

To clarify the involvement of NF- κ B and IRF3 activation in the VACV70-induced innate immune response in ovarian granulosa cells, the signaling pathways that are responsible for VACV70-induced gene expression were examined. VACV70-induced phosphorylation of NF- κ B and IRF3 in granulosa cells was efficiently blocked by pre-incubating the cells with the appropriate inhibitors (10 μ M BAY11-7082 for NF- κ B activation and 1 μ g/mL BX795 for IRF3 activation) (Fig. 5A). A 2-h pretreatment with the inhibitors differentially decreased the VACV70-induced TNFA, IL6 and IFNA/B secretion into the culture medium (Fig. 5B). BAY11-7082 significantly decreased VACV70-induced TNFA and IL6 levels;

Figure 5 Involvement of NF-κB and IRF3 activation in VACV70-induced gene expression. (A) Inhibition of NF-κB and IRF3 activation by appropriate inhibitors. Granulosa cells were transfected with 2 µg/mL VACV70 alone or after 2 h of pre-incubation with inhibitors (10 µM BAY11-7082 (BAY) for NF-κB, 1 µg/mL BX795 for IRF3). At 2 h after transfection, the cell lysates were subjected to Western blot analysis using specific antibodies to probe the indicated proteins. (B) Secretion of cytokines. Granulosa cells were cultured in 6-well plates at a density of 5×10^5 cells/well and treated as in (A). At 18 h after transfection, the cytokine concentrations in the culture medium were measured by ELISA. ND, not detectable. (C) Expression of antiviral proteins. Granulosa cells were treated as in (A) for 16 h. The relative mRNA levels of *Oas1*, *Mx1* and *Isg15* were determined using real-time quantitative RT-PCR. (D) Cell viability. Granulosa cells were treated as in (A) for 18 h. After treatment with MTT assay agents, the cells were lysed, and the absorbance at 570 nm was recorded. The images are representative of at least three experiments. The data represent the mean $\pm$ S.E.M. of three experiments. ** $P < 0.01$.

however, it did not affect the IFNα/B secretion. By contrast, BX795 markedly inhibited the secretion of IFNα/B and did not affect TNFA and IL6 expression. Moreover, the VACV70-induced upregulation of *Oas1*, *Mx1* and *Isg15* was significantly inhibited by BX795 but not by BAY11-7082 (Fig. 5C). In the control cells, the inhibitors did not affect cytokine production or antiviral protein expression in basal conditions. The MTT assay results showed that the cell viability was not significantly affected by VACV70 or the chemical inhibitors (Fig. 5D). These results imply that VACV70 induces TNFA and IL6 expression through NF-κB activation and induces the expression of IFNα/B and antiviral proteins by IRF3 activation.

Involvement of p204 and cGAS signaling

P204 and cGAS initiate innate immune responses through the adaptor STING (Ishikawa *et al.* 2009). To further verify the involvement of p204 and cGAS signaling in the VACV70-induced antiviral response, we examined the effect of VACV70 after the individual knockdown of *p204*, *cGas* and *Sting* in granulosa cells using specific small-interfering RNAs (siRNAs). Each siRNA inhibited >70% of the target mRNA 24 h after transfection (Fig. 6A). Accordingly, the protein levels of p204, cGAS and STING were significantly reduced by siRNA (Fig. 6B). As a control, a siRNA targeting a scrambled sequence (siCtrl) had no effect on p204, cGAS

and STING expression. At 24 h after siRNA transfection, the cells were then transfected with VACV70. The pretreatment of cells with siRNAs targeting *p204* (*sip204*), *cGas* (*sicGas*) or *Sting* (*siSting*) significantly inhibited p-IRF3 (Ser396) and p-p65 (Ser536) levels at 2 h after VACV70 transfection (Fig. 6C). The secretion of cytokines was significantly inhibited when the cells were pretreated with *sip204*, *sicGas* or *siSting* compared with siCtrl at 18 h after VACV70 transfection (Fig. 6D). Moreover, the antiviral protein levels were also dramatically reduced by *sip204*, *sicGas* or *siSting* (Fig. 6E). Notably, the knockdown of p204 and cGAS had comparable inhibitory effects on the VACV70-induced innate immune response. However, the knockdown of STING resulted in a more severe inhibition compared with p204 and cGAS silencing. Cell viability was insignificantly affected by siRNA transfection (Fig. 6F).

VACV70 inhibits P450 aromatase expression in ovarian granulosa cells

Many studies have demonstrated that inflammatory factors, especially TNFA, can inhibit estradiol synthesis

in granulosa cells (Morales *et al.* 2006, Yan *et al.* 2013, 2014, 2015). To further confirm the impact of VACV70-induced TNFA production on the expression of P450 aromatase (P450arom), which is the key enzyme for estradiol synthesis in granulosa cells, we examined P450arom expression in different conditions. VACV70 markedly inhibited the mRNA level of *P450arom* in a time-dependent (Fig. 7A) and dose-dependent (Fig. 7B) manner. The lowest mRNA level in granulosa cells was detected at 16 h after VACV70 transfection. Both 2 and 5 µg/mL VACV70 significantly suppressed *P450arom* expression. Notably, the VACV70-mediated inhibition of *P450arom* expression was significantly reversed by the presence of AnTNFA, the TNFA-neutralizing antibody that can neutralize the cytotoxic activity of mouse TNFA (Fig. 7C). An MTT assay showed that VACV70 and AnTNFA did not affect granulosa cell survival at 24 h after treatment (Fig. 7D). Accordingly, the protein level of P450arom was also markedly inhibited by VACV70 transfection, and this inhibition was significantly reversed by adding AnTNFA to the medium (Fig. 7E). Furthermore, the estradiol secretion in media was similar to the expression of P450arom. VACV70 transfection

Figure 6 Roles of DNA sensors in VACV70-induced innate immune responses. (A and B) Knockdown of p204, cGAS and STING using specific siRNAs. Granulosa cells were transfected with individual siRNAs that targeted a scrambled sequence (siCtrl), *p204* (*sip204*), *cGas* (*sicGas*) or *Sting* (*siSting*). After 24 h, the *p204*, *cGAS* and *STING* expression levels were determined using real-time quantitative RT-PCR (A) and Western blot analysis (B). (C) NF-κB and IRF3 activation. Granulosa cells were transfected with the indicated siRNAs. Twenty-four hours later, the cells were transfected with VACV70. At 2 h after transfection, the cell lysates were subjected to Western blot analysis using specific antibodies to probe the indicated proteins. (D) Cytokine secretion. Granulosa cells were treated as in (C). At 18 h after VACV70 transfection, the cytokine levels in the culture media were measured by an ELISA. (E) Antiviral protein expression. Granulosa cells were treated as in (C). At 24 h after VACV70 transfection, antiviral protein levels were determined by Western blot analysis. (F) Cell viability. Granulosa cells were treated as in (C). At 24 h after VACV70 transfection, the cells were lysed after treatment with MTT assay agents. The absorbance at 570 nm was recorded. The images are representative of at least three independent experiments. The data represent the mean ± S.E.M. of three independent experiments. **P* < 0.05, ***P* < 0.01.

Figure 7 Inhibition of *P450arom* expression. (A and B) VACV70 inhibited *P450arom* expression. Granulosa cells were seeded in 6-well plates at a density of 2×10^5 cells/well. After 24 h, the cells were transfected with VACV70. The effect of VACV70 on *P450arom* expression in a time-dependent (A) and dose-dependent (B) manner was examined using real-time quantitative RT-PCR by normalizing to β -actin. (C) Granulosa cells were seeded as in A and B. After 24 h, the cells were transfected with 2 μ g/mL VACV70 alone or VACV70 with 30 ng/mL AnTNFA. At 16 h after treatment, the total RNA was extracted, and the relative mRNA levels of *P450arom* were determined using real-time quantitative RT-PCR. Cells that were transfected with Lipofectamine RNAiMAX alone served as Ctrl. (D) Cell viability. Granulosa cells were treated as in (C). The cell viability was assessed using an MTT assay at 24 h after treatment. (E) Protein levels of *P450arom*. Granulosa cells were treated as in (C). At 24 h after treatment, the cell lysates were subjected to Western blot analysis using specific antibodies to *P450arom*. β -Actin was used as a loading control. (F) Estradiol secretion in media. Granulosa cells were treated as in (C). The culture media were subjected to ELISA to determine estradiol level at 24 h after treatment. The images are representative of at least three experiments. The data represent the mean $\pm$ S.E.M. of three experiments. ** $P < 0.01$.

significantly inhibited estradiol secretion, and this inhibition was reduced by AnTNFA (Fig. 7F).

VACV70 induces the ovarian innate immune response and suppresses steroidogenesis in vivo

To confirm the effect of VACV70 *in vivo*, a mixture of VACV70 and Lipofectamine RNAiMAX was locally injected into the ovarian bursa of one ovary. Noticeably increased p-p65 (Ser536) and p-IRF3 (Ser396) levels were detected at 3 h after VACV70 injection (Fig. 8A). In the contralateral ovary, the injection of Lipofectamine RNAiMAX alone did not induce p65 and IRF3 phosphorylation. We further examined the expression of cytokines and antiviral proteins *in vivo*. At 24 h after VACV70 injection, the levels of IFNA, IFNB, IL6 and TNFA in the ovary lysates were significantly increased (Fig. 8B). The expression of antiviral proteins was also dramatically upregulated by VACV70 injection (Fig. 8C). Furthermore, the mRNA levels of steroidogenic enzymes, including *P450arom*, cytochrome P450 side-chain cleavage enzyme (*P450scc*), 3 β -hydroxysteroid dehydrogenase (*Hsd3b*) and steroidogenic acute regulatory protein (*Star*), were evidently reduced at 24 h after VACV70 injection (Fig. 8D). The Western blot results showed that all these steroidogenic enzymes were significantly inhibited by VACV70 injection at the protein levels (Fig. 8E). Correspondingly, the ovarian estradiol and testosterone levels were evidently decreased at 24 h after VACV70 injection (Fig. 8F and G).

Discussion

Various viruses, including RNA and DNA viruses, can infect the ovary and affect ovarian functions (Zhao *et al.* 2011, Wang *et al.* 2016). Understanding the mechanisms underlying innate immune responses in the ovary can aid in the development of therapeutic and preventive approaches against viral infections. We recently demonstrated that viral RNA sensors initiate innate immune responses in the mouse ovary, which impair ovarian functions (Yan *et al.* 2013, 2014, 2015, Wang *et al.* 2016). The present study examined the cytosolic DNA sensor-initiated innate immune responses in mouse granulosa cells and their effects on the endocrine function.

In comparison with peritoneal macrophages, we demonstrated that granulosa cells constitutively express p204, cGAS and the signaling adaptor STING. By contrast, DAI and Pol III were not evidently detected in granulosa cells. P204 is located in both the cytoplasm and nuclei of granulosa cells. This cellular distribution corresponds to the previous observation in other cells and is conveniently modulated by protein acetylation (Li *et al.* 2012). VACV70 can trigger the innate immune response after transfection into cells (Unterholzner *et al.* 2010).

Figure 8 The VACV70-induced ovarian innate immune responses *in vivo*. (A) NF-κB and IRF3 activation. A mixture of VACV70 and Lipofectamine RNAiMAX was injected into the ovarian bursa of one ovary, and the contralateral ovary was injected with Lipofectamine RNAiMAX alone as Ctrl. At 3 h after injection, the ovary lysates were analyzed via Western blot using specific antibodies to probe the indicated proteins. (B) Cytokine levels. At 24 h after injection, the levels of cytokines in the ovarian lysates were measured by ELISA. (C) Expression of antiviral proteins. At 24 h after injection, the ovarian lysates were subjected to Western blot analysis to probe the antiviral proteins using specific antibodies. (D and E) Inhibition of steroidogenic enzymes. At 24 h after injection, the mRNA (D) and protein (E) levels of steroidogenic enzymes were examined by real-time quantitative RT-PCR and Western blot respectively. (F and G) Inhibition of estradiol and testosterone synthesis. At 0 and 24 h after VACV70 injection, the levels of estradiol (F) and testosterone (G) in the ovarian lysates were measured by ELISA. The images are representative of at least three experiments. The ovarian lysates of three mice were used in each experiment. The data represent the mean $\pm$ S.E.M. of three experiments. * $P < 0.05$, ** $P < 0.01$.

P204 and cGAS were further upregulated by VACV70 in granulosa cells. The peak mRNA levels of *p204* and *cGas* appeared later than the cytokines after VACV70 transfection. P204 and cGAS were constitutively expressed in granulosa cells under basal condition. These two DNA sensors can recognize transfected VACV70 and immediately induce the expression of cytokines. IFNβ produced by granulosa cells can upregulate DNA sensors expression. The upregulation of DNA sensors may be a positive feedback to amplify innate immune responses. We provide evidence that both p204 and cGAS signaling pathways are involved in innate immune responses in granulosa cells. These results expand our recent observations that dsRNA sensors, including TLR3, MDA5 and RIG-I, initiate the antiviral response in the mouse ovary (Yan *et al.* 2013, 2014, 2015).

The doses of VACV70 used in previous studies are different from 0.5 to 5 μg/mL (Unterholzner *et al.* 2010). The present study found that 2 μg/mL VACV70 can strongly trigger cytosolic DNA sensor signaling in mouse ovarian granulosa cells. This dose has no relevance to natural virus titers because virus titers in natural infections can be different and natural viruses may replicate in hosts. Moreover, viral particles may activate multiple PRRs because viral components may activate other PRRs beyond DNA sensors. Therefore, as a synthetic DNA analog, VACV70 does not equate to infection of natural viruses.

Type I IFN production is a primary response of hosts to viral infection (Randall & Goodbourn 2008). IFNα and IFNβ play crucial roles in the antiviral activities by the upregulation of antiviral proteins and the promotion

of adaptive immune responses against invading viruses (Katze *et al.* 2002). The current study demonstrated that major antiviral proteins, including OAS1, ISG15 and MX1, were dramatically upregulated by VACV70, which would favor the cellular defense against invading viruses. Given that all of these antiviral proteins are encoded by IFN-inducible genes (Sadler & Williams 2008), it is reasonable to speculate that the VACV70-mediated upregulation of the antiviral proteins could be attributed to the production of IFNA/B. Accordingly, the presence of BX795, an inhibitor of IRF3 activation, significantly decreased the VACV70-induced IFNA/B and antiviral protein production. The peak mRNA levels of the antiviral proteins appeared later than *Ifna/b* in granulosa cells after VACV70 transfection, suggesting that IFNA/B may induce the antiviral protein expression in an autocrine manner.

The results of the present study indicate that VACV70 also induced TNFA and IL6 production via NF- κ B activation in granulosa cells. In contrast, our other recent studies demonstrated that DNA sensor signaling does not induce TNFA and IL6 production in Leydig and adipose cells (Zhu *et al.* 2014, Yu *et al.* 2015). These results imply that various viruses may trigger different signaling pathways in different types of cells.

Steroidogenesis is one of the major functions of the ovary. Estradiol is synthesized by granulosa cells through the aromatization of androgens secreted by theca cells (Berisha *et al.* 2002). Multiple steroidogenic enzymes are required for the synthesis of estradiol and androgen, including P450arom, P450SCC, HSD3B and STAR. The present study demonstrated that not only the expression of these steroidogenic enzymes but also the secretion of estradiol and testosterone were inhibited by VACV70.

IFNA/B plays critical roles in the defense against viruses, whereas elevated levels of pro-inflammatory cytokines may perturb tissue functions. High levels of pro-inflammatory cytokines may result in pathological conditions, such as ovarian cancer (Maccio & Madeddu 2012). Moreover, TNFA inhibits estradiol synthesis and induces granulosa cell apoptosis in rat ovaries (Kaipia *et al.* 1996, Morales *et al.* 2006, Yamamoto *et al.* 2015). TNFA also inhibits androgen synthesis in Leydig cells (Bornstein *et al.* 2004, Hong *et al.* 2004). Our previous study demonstrated that TNFA inhibits P450arom expression in granulosa cells in a dose-dependent manner. We speculated that the VACV70-mediated inhibition of estradiol synthesis could be attributed to TNFA production. In agreement with this speculation, we showed that TNFA-neutralizing antibodies significantly reversed the VACV70-mediated inhibition of estradiol synthesis in granulosa cells.

The theca cells express androgen synthetic enzymes and produce androgen (Berisha *et al.* 2002). Our data showed that the theca cells did not express cytosolic

Figure 9 Summary picture. VACV70 transfection can trigger DNA sensor-STING signaling and induce the expression of inflammatory cytokines (TNFA/IL6) and type I interferons (IFNA/B) through NF- κ B and IRF3 activation respectively. Type I interferons initiate antiviral responses by inducing antiviral proteins and facilitating antiviral adaptive immunity. The inflammatory cytokines, especially TNFA, can inhibit estrogen synthesis in granulosa cells and androgen synthesis in theca cells.

DNA sensors. Therefore, we speculated that theca cells do not directly respond to VACV70. However, VACV70 significantly inhibited androgen synthesis and relevant steroidogenic enzymes expression. These effects could be mediated by inflammatory cytokines. In particular, increased TNFA level perturbs steroidogenesis in the theca cells (Bornstein *et al.* 2004). In agreement with this, we found that the theca cells abundantly express TNFA receptor 1 (TNFR1) (Yan *et al.* 2015). Therefore, we hypothesized that TNFA produced by ovarian granulosa cells could inhibit steroidogenesis in the theca cells.

In conclusion, we demonstrated that VACV70 initiates innate immune responses in mouse granulosa cells via cytosolic DNA sensor signaling (Fig. 9), which would contribute to the defense against DNA viral infection in the ovary. Moreover, the DNA sensor-initiated innate immune responses inhibit the steroidogenesis of the ovary. These data provide novel clues into the mechanism underlying the innate immune responses in the ovary to vaccinia viral infection.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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