

Expression of *interferon regulatory factor-1* in the mouse cumulus-oocyte complex is negatively related with oocyte maturation

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Objective: We found previously that *interferon regulatory factor (Irf-1)* is a germinal vesicle (GV)-selective gene that highly expressed in GV as compared to metaphase II oocytes. To our knowledge, the function of *Irf-1* in oocytes has yet to be examined. The present study was conducted to determine the relationship between retinoic acid (RA) and RA-mediated expression of *Irf-1* and the mouse oocyte maturation.

Methods: Immature cumulus-oocyte-complexes (COCs) were collected from 17-day-old female mice and cultured *in vitro* for 16 hours in the presence of varying concentrations of RA (0-10 μ M). Rate of oocyte maturation and activation was measured. Gene expression was measured by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and cytokine secretion in the medium was measured by Bio-Plex analysis. Apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Results: The rates of oocyte maturation to metaphase II and oocyte activation increased significantly with RA treatment (10 nM-1 μ M). With 100 nM RA treatment, lowest level of *Irf-1* mRNA and cumulus cell's apoptosis was found. Among 23 cytokines measured by Bio-Plex system, the substantial changes in secretion of tumor necrosis factor- α , macrophage inflammatory protein-1 β , eotaxin and interleukin-12 (p40) from COCs in response to RA were detected.

Conclusion: We concluded that the maturation of oocytes and *Irf-1* expression are negatively correlated, and RA enhances the developmental competence of mouse immature oocytes *in vitro* by suppressing apoptosis of cumulus cells. Using a mouse model, results of the present study provide insights into improved culture conditions for *in vitro* oocyte maturation and relevant cytokine production and secretion in assisted reproductive technology.

Keywords: Apoptosis; Immature oocyte; *In vitro* maturation; *Interferon regulatory factor-1*; Retinoic acid; Mouse

Introduction

Oocyte maturation is a progression of developmental programs

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that are essential for the production of an oocyte that is competent to undergo fertilization and embryogenesis [1]. There are three different components of oocyte maturation: nuclear, cytoplasmic and epigenetic maturation [2]. Nuclear maturation refers to the resumption of meiosis and progression to metaphase II (MII) [3]. Cytoplasmic maturation includes all of the processes that occur in the cytoplasm, exclusive of meiotic changes in the nucleus, which prepare the oocyte for fertilization and embryo development. Cytoplasmic maturation occurs throughout oocyte growth and is concurrent with nuclear maturation [4]. Epigenetic maturation also occurs during oocyte growth, and refers to sequence-independent modifications of DNA structure that regulate gene expression during oocyte development, oocyte maturation and post-fertilization [5].

Previously, in an effort to better understand the molecular mechanisms involved in the acquisition of oocyte competence, we used an annealing control primer-polymerase chain reaction (PCR) to identify differentially expressed genes (DEGs) in the germinal vesicle (GV) and in MII mouse oocytes [6]. Among the DEGs that were identified, *interferon regulatory factor (Irf)-1* emerged as a GV-selective gene that was highly expressed in GV as compared to MII oocytes. To our knowledge, the function of *Irf-1* as it relates to the meiotic cell cycle and maturation in oocytes has yet to be examined.

Extracellular growth factors that modify *Irf-1* expression include common mediators of a variety of biologic processes, including cell cycle progression, DNA damage and apoptosis, cell differentiation, cell-to-cell contact inhibition, and cell senescence. The involvement of *Irf-1* in cell cycle regulation and apoptosis has been studied extensively in cells and tissues, and its function as a tumor suppressor can be explained at least in part by its function at the mitotic checkpoint [7].

One of the well-known inducers of *Irf-1* is retinoic acid (RA), one of the active forms of vitamin A. Vitamin A plays an essential role in vertebrate physiology, in such processes as cellular growth, development, differentiation, including oocyte maturation [8-11]. The active forms of vitamin A, namely all-trans-RA (atRA) and 9-cis-RA (9cRA), bind to their cognate ligand-activated transcription factors, RA receptor (*Rar*) and retinoid X receptor (*Rxr*), each of which exists as one of three isoforms (α , β , and γ). Retinoid signals are reached at the gene level by RAR-RXR heterodimers, which activate or repress specific target genes through binding to specific response elements near promoter regions [12]. The production and secretion of several cytokines, including interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-2, IL-3, IL-4, and transforming growth factor- β (TGF- β), have been shown to be modulated by RA and RA metabolites in neutrophils, natural killer cells, and T- and B-lymphocytes [13].

It has also been reported that vitamin A supplementation has a positive effect on bovine fertility using artificial reproductive methods, and it has been suggested that RA might promote cytoplasmic maturation of bovine oocytes through the modulation of gene expression in cumulus-granulosa cells [9]. Addition of 9cRA into the IVM medium of bovine cumulus-oocyte-complexes (COCs) increased rates of embryo development and hatching [14].

Since RA and the induction of *Irf-1* by RA can establish or change the pattern of gene expression in various cells, we interested in whether these events also influenced the maturation and subsequent capacity of the mouse oocytes to progress through embryonic development. In the current study, we investigated the effects of RA treatment on immature COCs on the oocyte maturation, the expression of many cytokines and genes including *Irf-1* and the cumulus cells' apoptosis.

Methods

1. Animals

ICR mice were housed in the breeding facility at the CHA Stem Cell Institute of CHA University (Seoul, Korea) in a temperature-controlled environment under a 12 hours light/12 hours dark cycle. Animals were fed *ad libitum*. All animal procedures were reviewed and approved by the University of Science Institutional Animal Care and Use Committee, and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

2. Collection of mouse oocytes, IVM, and oocyte activation

Immature GV stage oocytes were obtained from 17-day-old ICR female mice 44 hours after the injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Intervet, Boxmeer, Holland). COCs were collected in M2 medium (Sigma, Louis, MO, USA) containing 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma) to maintain the oocytes at the GV stage. COCs were recovered from the ovaries by puncturing the surface of the ovary, and then cultured at 37°C in a 5% CO₂ incubator in M16 medium (Sigma) containing 3 mg/mL bovine serum albumin (BSA; Sigma) and increasing concentrations (0 nM, 10 nM, 100 nM, 1 μ M, 10 μ M) of RA, as indicated. The vehicle solution was 0.01% ethanol. After 16 hours in culture, oocytes were denuded and examined using a stereomicroscope to determine maturation stage. Emission of the first polar body was used as an indicator of progression to MII. Oocytes without GVs or polar bodies were scored as MI. Isolated oocytes, remnant cumulus cells and culture medium were snap-frozen and stored at -70°C.

To determine cytoplasmic maturation status, oocytes were parthenogenetically activated by incubation in activating medium (Ca²⁺-free CZB supplemented with 10 mM SrCl₂ [Sigma] containing 5 μ g/mL cytochalasin B [Sigma]) at 37°C in a humidified atmosphere of 5% CO₂. 6 hours after the onset of activation, oocytes were examined by microscopy.

3. RNA isolation and reverse transcription (RT)-PCR

Messenger RNA (mRNA) was isolated from the same number of oocytes and cumulus cells (derived from the same COCs) using a Dynabeads mRNA Direct Kit (Dyna, Oslo, Norway), according to the manufacturer's instructions. Cells were resuspended in lysis/binding buffer (containing lithium dodecylsulphate/lithium chloride) for 5 minutes at room temperature, and then the solution was incubated with Dynabeads oligo (dT)₂₅ at room temperature for 5 minutes to separate mRNA from total RNA. Bound mRNA was immobilized against the wall of a microfuge tube using a magnet (DynaL MPC-S; DYNAL A.S, Oslo, Norway), and the supernatant was removed. Dynabead/mRNA complexes were washed twice with lysis buffer and then three

times with washing buffer. The complexes were resuspended in 10 μ L of Tris-HCl (10 mM Tris-HCl, pH 7.5) at 65°C for 2 minutes, and then an aliquot was used for reverse transcription.

PCR was carried out in a reaction mixture consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTPs, 25 pmol of each primer, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI, USA) in a final volume of 20 μ L. Amplified products (20 μ L) were separated by 1.2% agarose gel electrophoresis and assessed using an Image Analyzer (Viber Lourmat). The sequences of the gene-specific primers (*β -Actin* was used as an internal control), as well as the PCR conditions, are listed in Table 1.

4. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was also accomplished as described previously [15]. Quantitative real-time RT-PCR analysis was performed using an iCycler system (Bio-Rad, Hercules, CA, USA) and the results were evaluated using iCycler iQ real-time detection system software. The template was amplified by 40 cycles of denaturation at 95°C for 40 second, annealing at 60°C for 40 second, and extension at 72°C for 40 second. At the completion of the PCR, we monitored fluorescence continuously while slowly heating the samples from 60°C to 95°C at 0.5°C intervals, producing melting curves to identify any nonspecific products.

Table 1. Sequences of oligonucleotide primers, annealing temperatures (AT) and expected reverse transcription-polymerase chain reaction product sizes

Genes	GeneBank accession No.	Oligonucleotide sequences	AT (°C)	Size (bp)
<i>IRF-1</i>	NM_008390	F 5'-CCCTGGCTAGAGATGCAGATTA-3' R 5'-TAGAGCTGCTGAGTCCATCAGA-3'	60	439
<i>RARα</i>	NM_009024	F 5'-GCCTGAGCAAGACACAATGA-3' R 5'-CCTGGGATCTCCATCTTCAA-3'	60	378
<i>RARβ</i>	NM_011243	F 5'-GGACCTTGAGGAACCAACAA-3' R 5'-ATGAGAGGTGGCATTGATCC-3'	60	210
<i>RARγ</i>	NM_011243	F 5'-AAGCCCACCAGGAGACTTTT-3' R 5'-GATCCGCAGCATTAGGATGT-3'	60	242
<i>RXRα</i>	NM_011305	F 5'-GCTACCAAATGACCCTGT-3' R 5'-CGTCTTGCCATCTGCATGT-3'	60	320
<i>RXRβ</i>	NM_011306	F 5'-CTGCAAGGTTTCTTCAAGC-3' R 5'-GGGGCTGAAGGAGGGAGG-3'	60	280
<i>RXRγ</i>	NM_009107	F 5'-AATGCTCTTGGCTCTCCGTA-3' R 5'-AATGCTCTTGGCTCTCCGTA-3'	60	301
<i>Fshr</i>	NM_013523	F 5'-AGAGGCTCCCTGVAAAACAT-3' R 5'-TCCTTCATGGGACTGAGCTT-3'	60	165
<i>Gdf9</i>	NM_008110	F 5'-GGTTCTATCTGATAGGCGAGG-3' R 5'-GGGGCTGAAGGAGGGAGG-3'	60	472
<i>β-Actin</i>	NM_007393	F 5'-GGGTGTGATGGTGGGAATGGG-3' R 5'-GCTGTGGTGGTGAAGCTGTAG-3'	60	314
<i>Gapdh</i>	BC093598	F 5'-ACCACAGTCCATGCCATCAC-3' R 5'-TCCACCACCCTGTTGCTGTA-3'	60	512

tify any nonspecific products. Gene amplification was quantified by determining the cycle threshold (C_T), based on the level of fluorescence within the geometric region of the semilog amplification plot. The mRNA level of each target gene was calculated using the comparative C_T method, and the experiments were repeated at least three times using different sets of oocytes and cumulus cells.

5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

All COCs, including the control and treatment groups, were washed in PVP (1 mg/mL polyvinyl-pyrrolidone in phosphate buffered saline) and then fixed in a solution of 3.7% paraformaldehyde (w/v) and 0.2% Triton X-100 for 40 minutes at room temperature. The fixed COCs were washed twice in PVP and then incubated in 0.5% Triton X-100 for 1 hour at room temperature. The samples were then incubated for 1 hour at 37°C in TUNEL reaction mixture (*In situ* Cell Death Detection Kit, Roche, Mannheim, Germany). After the TUNEL reaction was completed, nuclei were stained with Hoechst 33342 (2 μ g/mL) for 30 minutes, and the COCs were mounted on slides using Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). Slides were observed by a laser-scanning confocal microscopy (LSM 510, Carl Zeiss, Dublin, CA, USA). Simulated 3D images and sections were constructed using the software Volocity (PerkinElmer, Waltham, MA, USA).

6. Bio-Plex analysis

Changes in the amounts of cytokines in culture media during IVM were measured using the Bio-Plex 2200 system (Bio-Rad). The Bio-Plex filter plate was pre-wet with buffer prior to the addition of samples. Antibody-coupled beads for 23 different cytokines (IL-1 α , IL1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 [p40], IL-12 [p70], IL-13, IL-17, G-CSF, GM-CSF, INF- γ , KC, macrophage inflammatory protein [MIP]-1 α , RANRES, TNF- α , eotaxin, MCP-1, and MIP-1 β) were premixed (06-0143; Bio-Rad), and assays were carried out according to the manufacturer's protocol. After loading of the bead solutions and samples, the plate was incubated for 30 minutes at room temperature with shaking. After washing three times, detection antibodies were added and the plate was incubated for 30 minutes, followed by the addition of streptavidin-PE for 10 minutes. The plate was agitated to resuspend the beads, and the amount of cytokine was measured using the Bio-Plex 2200 system. Each reaction was carried out in duplicate and the experiment was repeated three times.

7. Statistical analysis

All statistical analyses were performed on a CHA University main-frame computer using the Statistical Analysis System (SAS; SAS Korea Inc., Seoul, Korea), version Enterprise 4.0. At least three independent

experiments were conducted for each treatment condition. The rate of maturation, oocyte activation, the rate of apoptosis and cytokine levels were evaluated using a mixed model analysis of variance procedure (PROC MIXED; SAS) followed by Fisher's least significant difference. Data are expressed as mean \pm SE, and a *p*-value of < 0.05 was considered significant.

Results

1. Characterization of *Irf-1* and RAR expression

Expression of *Irf-1* mRNA was analyzed by quantitative real-time PCR

(Figure 1A). *Irf-1* was highly expressed in GV oocytes as compared to MII oocytes. In each case of melting curve, there was a single peak that indicated the absence of nonspecific amplicons (inset, Figure 1A). *Irf-1* mRNA was ubiquitously expressed in ovarian follicular cells (Figure 1B).

Rara and *Rxra* mRNAs were expressed in both oocytes and cumulus cells. *Rary* and *Rxr β* were highly expressed in cumulus cells as compared to oocytes, while the expression of *Rxr γ* was not detected in oocytes or cumulus cells (Figure 1C). Growth differentiation factor 9 (*Gdf9*) and FSH receptor (*Fshr*) expression was measured as an indicator for clear cell separation between oocytes and granulosa cells.

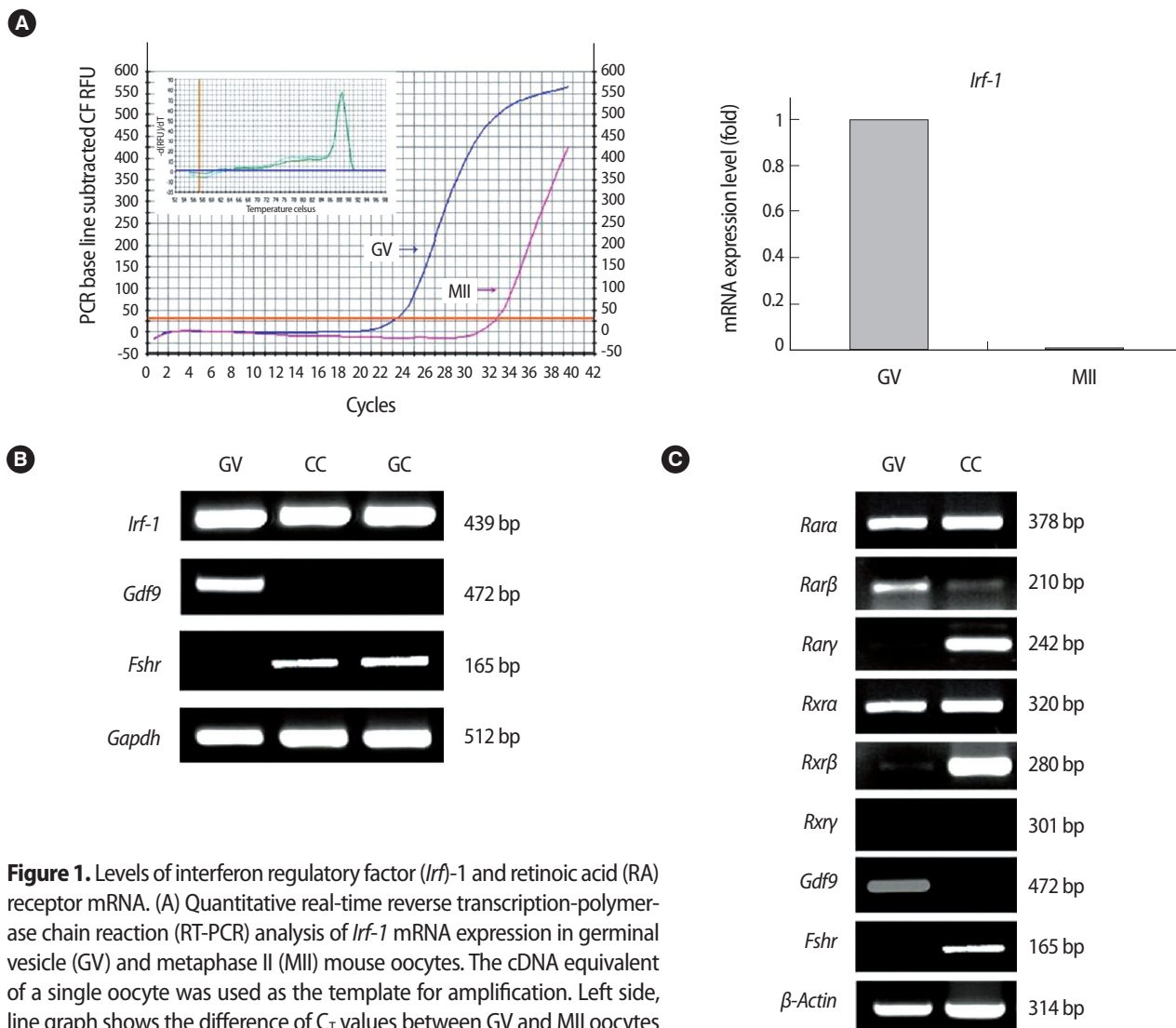


Figure 1. Levels of interferon regulatory factor (*Irf*)-1 and retinoic acid (RA) receptor mRNA. (A) Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *Irf-1* mRNA expression in germinal vesicle (GV) and metaphase II (MII) mouse oocytes. The cDNA equivalent of a single oocyte was used as the template for amplification. Left side, line graph shows the difference of C_T values between GV and MII oocytes and right side, bar graph shows expression levels calculated using its C_T value. (B) Analysis of *Irf-1* mRNA level in mouse follicular cells using RT-PCR. (C) Levels of *Rara*, *Rar β* , *Rary*, *Rxra*, *Rxr β* , *Rxr γ* , *Gdf9*, *Fshr*, and β -Actin mRNA transcripts. *Gdf9* mRNA was used as a specific marker for oocytes, and *Fshr* was used as a marker for cumulus and granulosa cells. GV, GV oocytes; CC, cumulus cells; GC, granulosa cells, *Gdf9*, growth differentiation factor; *Fshr*, follicle stimulating hormone receptor; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Rar*, retinoic acid receptor; *Rxr*, retinoid X receptor.

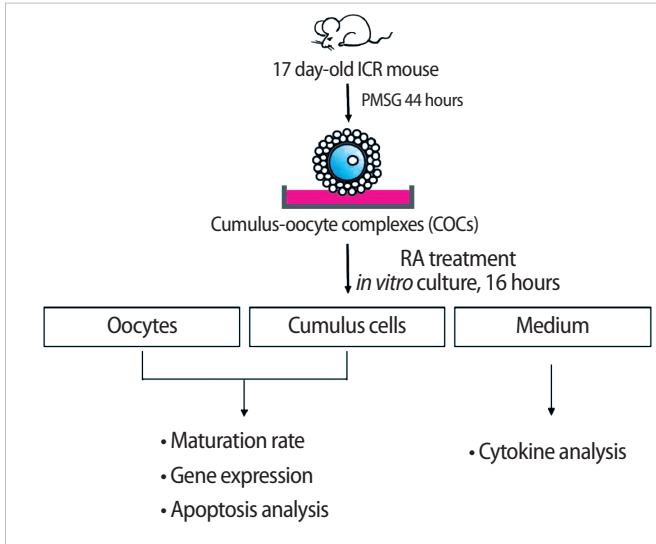


Figure 2. Schematic diagram of the experimental strategy for evaluating the effects of retinoic acid (RA) on the oocyte maturation in mouse. Cumulus-oocyte-complexes (COCs) from preovulatory follicles were collected from the ovaries of 17 day-old mice 44 hours after PMSG injection. COCs were incubated with various doses (0-10 μ M) of RA. At the end of culture period for 16 hours, the oocytes were denuded and examined using a stereomicroscope to determine the progression of meiotic maturation. PMSG, pregnant mare serum gonadotropin; Oocyte, cumulus cells, and conditioned medium were sampled and snap frozen for further analysis.

2. The effect of RA on nuclear and cytoplasmic maturation *in vitro*

To examine the effects of RA on the maturation of mouse oocytes, Immature COCs from 17-day-old mice were incubated with 0, 10 nM, 100 nM, 1 μ M and 10 μ M RA as summarized in Figure 2. As a control for the effect of the vehicle solution, COCs were treated with 0.1% ethanol alone. The maturation rates of the control and vehicle groups were 79.6% and 75.2%, respectively. The rate of maturation to MII was highest in the presence of 100 nM RA (91.9%), and maturation rates of the 10 nM and 1 μ M groups were comparable as 86.9% and 85.4% (Figure 3A).

The oocyte activation rate was also highest in the presence of 100 nM RA (56%), as compared to control (46.89%) and vehicle (34.91%) groups (Figure 3B). Post hoc analysis showed that the vehicle group was significantly different from the three conditioned group including 10 nM, 100 nM, and 1 μ M ($p < 0.03$). The 10 μ M group differed significantly from all the other groups with the exception of the vehicle group ($p < 0.06$).

3. The effect of RA on *Irf-1* mRNA and cytokine expression

To determine the effect of RA on *Irf-1* mRNA expression, we performed quantitative real-time PCR analysis using cDNA from separated oocytes and cumulus cells. In oocytes, *Irf-1* mRNA expression was markedly decreased by 100 nM (Figure 4A), as compared to that of

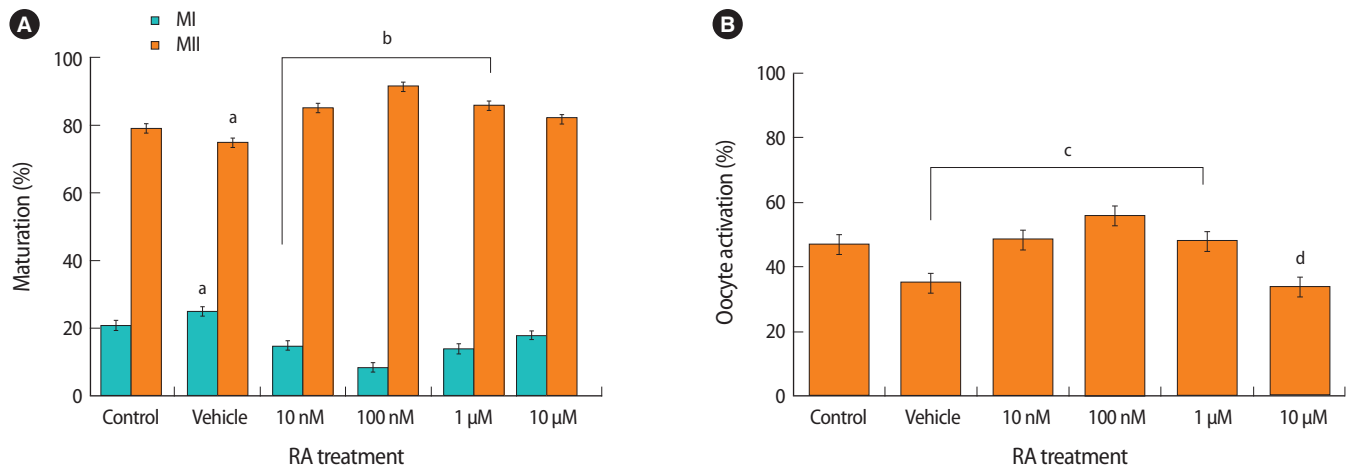


Figure 3. The effect of retinoic acid (RA) treatment on the oocyte maturation *in vitro*. (A) The effect of RA on nuclear maturation. Mouse cumulus-oocyte-complexes (COCs) obtained from 17-day-old mice were incubated with increasing doses of RA. The oocytes were then denuded, and examined using a stereomicroscope to score the progression of meiotic maturation. Emission of the first polar body was used as an indicator of progression to metaphase II (MII). Oocytes without germinal vesicle (GV) or polar bodies were scored as metaphase I (MI). Data represents the means \pm SE of at least three experiments. Letter a, indicates that group means were significantly different from the control group. Letter b, indicates that the conditions, 10 nM, 100 nM, and 1 μ M are significantly different from the control group for both MI and MII ($p < 0.001$). (B) The effect of RA on cytoplasmic maturation. Activation of IVM mouse oocytes after treatment with RA was measured. Six hours after the onset of activation, oocytes were observed under a microscope. Oocytes with pronuclei, or two cells with each one nucleus, were considered activated. Data represents the mean \pm SE of at least three experiments. Letter c, indicates that group means were significantly different from the vehicle group ($p < 0.05$). Letter d, indicates that the 10 μ M group is significantly different from the all other groups except for the vehicle group ($p < 0.06$).

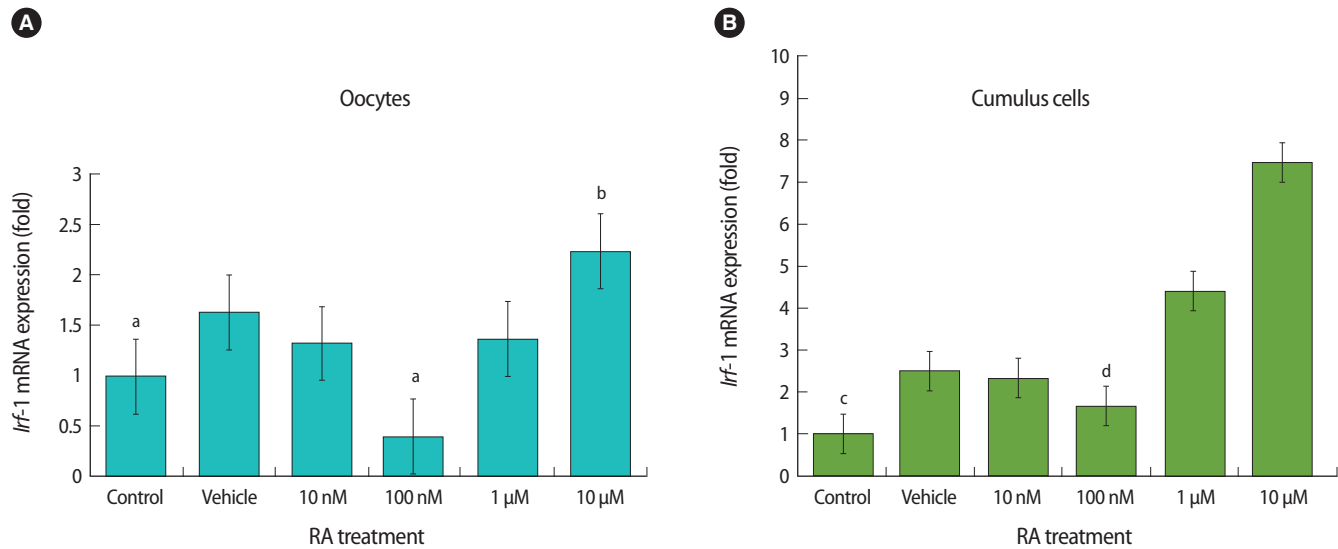


Figure 4. The effect of retinoic acid (RA) on interferon regulatory factor (*lrf*)-1 mRNA expression in oocytes (A) and cumulus cells (B). Data represents the means \pm SE of at least three experiments. (A) Letter a, indicates that 100 nM is significantly different from all other groups except for the control group. Letter b, indicates that the 10 μ M group is significantly different from the control and 100 nM groups ($p < 0.05$). (B) Letter c, indicates that control group is significantly different from all other groups except for the 100 nM group ($p < 0.05$); while letter d, indicates 100 nM group is significantly different from all other groups except for the control group.

control. There were significant differences in mRNA expression levels between 100 nM and all the other groups. The mRNA expression level of the 10 μ M group was about twice as much as the control group. In cumulus cells, the pattern of expression of *lrf-1* mRNA was similar to that of oocytes, but the fold changes were much larger than in oocytes (Figure 4B). As shown in Figure 4B, the control group expressed the lowest level of mRNA among six groups ($p < 0.002$). Contrast analyses showed there were significant differences in mRNA expression level between control group and all the other groups with exception of 100 nM group ($p < 0.02$).

We examined the expression levels of 23 cytokines in culture medium using Bio-Plex system and detected substantial changes in the expression of 4 cytokines, such as TNF- α , MIP-1b, eotaxin and IL-12 (p40) in the presence of RA. The secretion profiles of these four cytokines are summarized in Figure 5.

In the case of TNF- α , the 100 nM group showed the lowest level among six groups with the highest level in 10 μ M group. The level of TNF- α of 100 nM was significantly less than those of 10 nM and 1 μ M ($p < 0.05$). There was significant group difference in the MIP-1b level ($p < 0.004$). The 10 μ M group exhibited the highest level of MIP-1b with a significant difference compared to all the other groups. The level of eotaxin and IL-12 in the control group was significantly higher than those in any other conditioned groups ($p < 0.001$). Results clearly showed that all the conditions decrease the level of eotaxin and IL-12 in comparison to control group.

4. The effect of RA on apoptosis in CCs

To determine the effect of RA on apoptosis of COCs, we performed the TUNEL assay. A representative result, showing red-stained apoptotic cells, is shown in Figure 6A. The rate of COC apoptosis was lowest in the presence of 100 nM RA (20.86%), and this was significantly lower than that of the control (43%) or vehicle (43.02%) groups. The rates of apoptosis increased significantly in the presence of 1 μ M and 10 μ M RA (57.33% and 76.17%, respectively, Figure 6B).

Discussion

In the current study, we demonstrated that 1) RA treatment of immature COCs *in vitro* has a beneficial effect on increasing oocyte maturation rates; 2) RA may reduce cumulus cell apoptosis through the reduction of *lrf-1* expression and destructive cytokine secretion from oocytes, in particular from cumulus cells; and 3) these changes may be favorable for producing high quality oocytes.

Retinoid, in particular RA, can induce differentiation in a number of cells, and plays a role in the regulation of the cell cycle [16]. However, while RA can induce or silence the expression of hundreds of genes, little is known about the molecular mechanism of action of RA within intact tissues [17,18]. In the current study, treatment of immature COCs with RA during IVF, especially at 100 nM concentration, improved both nuclear and cytoplasmic oocyte maturation with lowest apoptosis in cumulus cells.

Ovarian follicular cells are regulated through a variety of mecha-

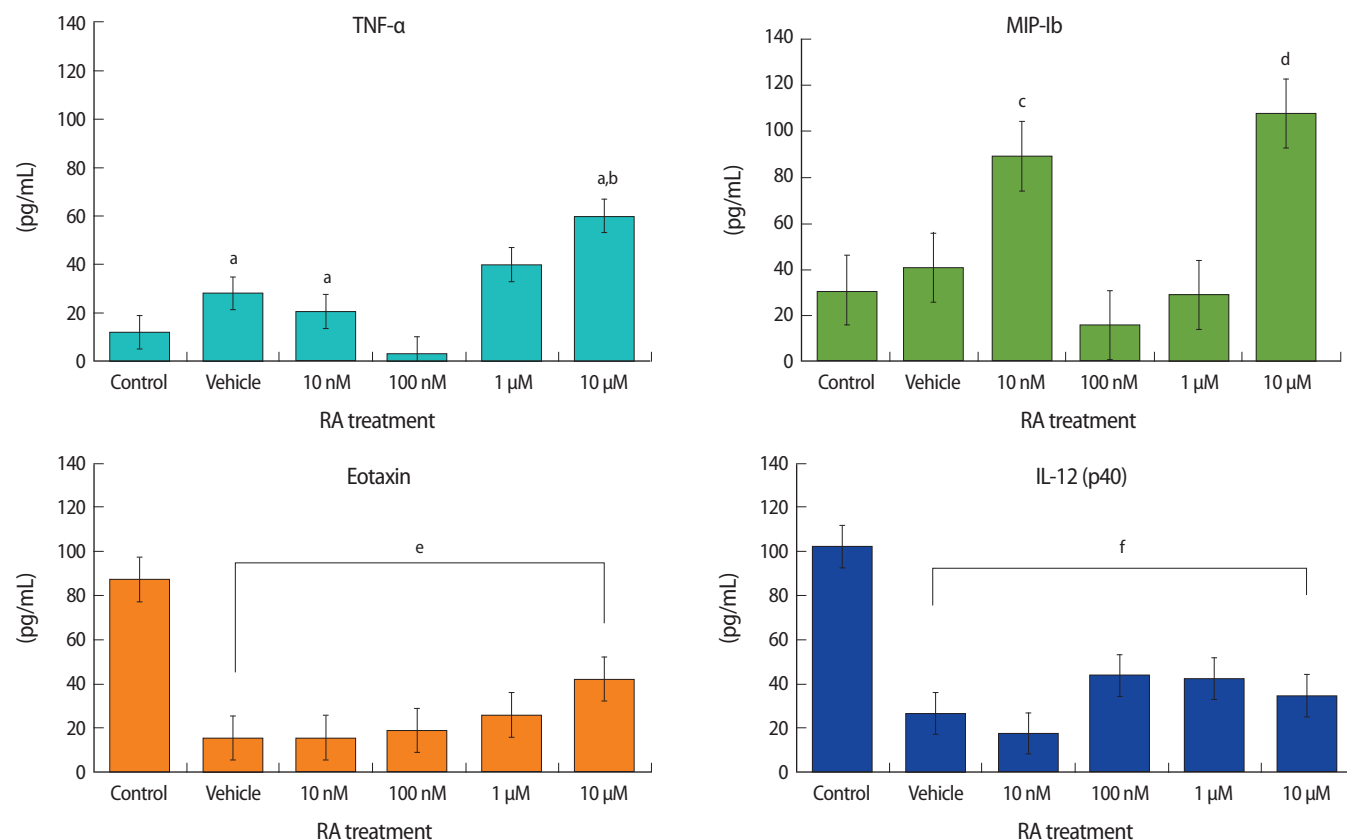


Figure 5. The secretion of tumor necrosis factor (TNF)- α , macrophage inflammatory protein (MIP)-1b, eotaxin and interleukin-12 (p40) after *in vitro* culture of cumulus-oocyte-complexes (COCs) with increasing doses of retinoic acid (RA). After a 16 hours incubation period, the culture medium was collected and assayed using the Bio-Plex assay system. Data represents the mean \pm SE of at least three experiments. For TNF- α , letter a, indicates that the 100 nM is significantly different from all these groups ($p < 0.05$). Letter b, indicates that the 10 μ M group is significantly different from all groups except 1 μ M groups ($p < 0.0004$). For MIP-1b, letter c, indicates that the 10 nM is significantly different from all other groups except for 10 μ M groups ($p < 0.05$). Letter d, indicates that the 10 μ M group is significantly different from the all other groups except for the 10 nM group ($p < 0.001$). For eotaxin and IL-12 (p40), the control group is significantly different from all other groups (e and f, $p < 0.001$).

nisms that involve the action and interactions of endocrine, paracrine and autocrine factors. To date, gene expression studies of *Rar* and *Rxr* family have not focused on ovarian follicular cells, including oocytes. In the current study, we investigated the role of RA during spontaneous IVM, and characterized the expression patterns of RARs and RXRs in ovarian follicular cells. Retinoids function through the RAR and RXR families of transcription factors, each of which is comprised of multiple isoforms (α , β , γ). RAR and RXR form heterodimers (RAR-RXR) that function in transmitting RA signals [19]. Previous studies have shown that *Rxra* and *Rxr β* are ubiquitously expressed in the mouse embryo during development, whereas *Rxry* exhibits a restricted pattern of expression during embryogenesis [20]. We found that *Rxra* and *Rxr β* , not *Rxry*, are present in ovarian follicles. The expression of RAR and RXR family transcription factors in ovarian follicles suggests that certain receptor isoforms might function in RA signaling during oocyte maturation, fertilization and embryo development. RXR γ , however, does not appear to be involved in these processes.

IRF-1 is a regulator of the cell cycle and apoptosis, and inactivation of IRF-1 accelerates the process of cellular transformation. IRF-1 functions as a transcription factor to induce genes involved in strengthening immune responses and promoting cancer prevention [21]. BRCA1-induced IRF-1 nuclear localization and its transcriptional activity activate a multi-component network containing OAS-2 and RNase L [22]. TNF-related apoptosis inducing ligand (TRAIL) and other IRF-1 responsive genes may function in concert to induce apoptosis in cancer cells. The tumor suppressor functions of RNase L can also amplify the apoptotic signals generated by TRAIL [23]. At a concentration of 10-100 nM, RA significantly reduced the rate of apoptosis of cumulus cells. Cumulus cells undergo apoptosis during IVM, and it has been proposed that there is a negative correlation between the degree of cumulus cell apoptosis during IVM and the developmental competence of COCs [24].

A complex array of regulatory factors, including hormones, cytokines and growth factors, influence oocyte viability and development

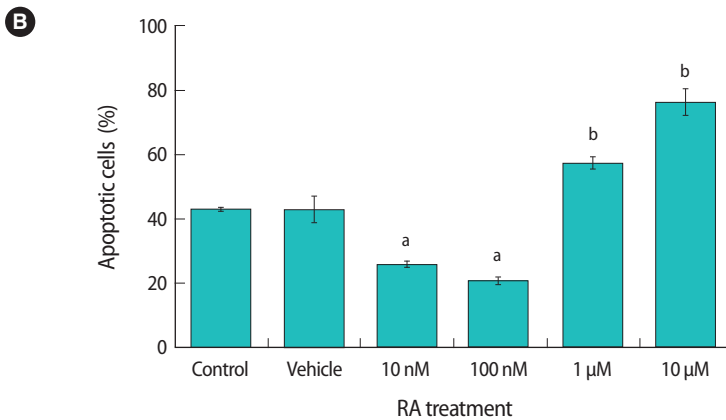
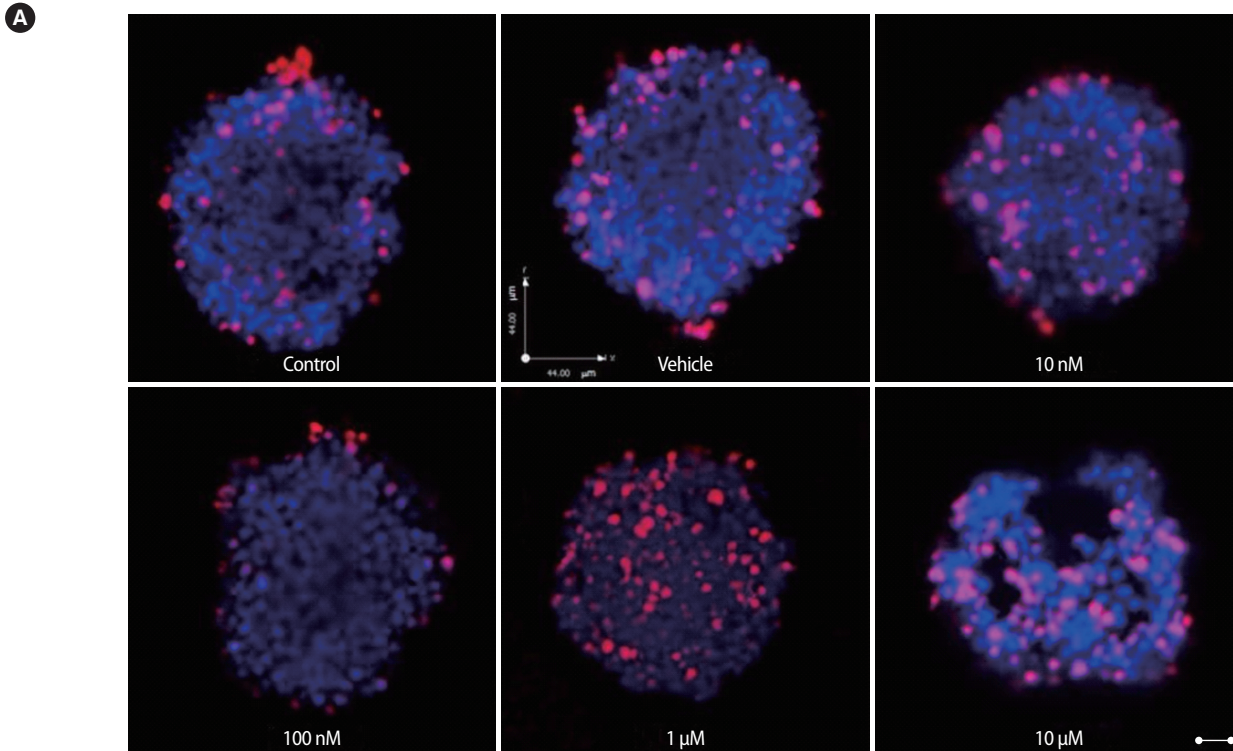


Figure 6. The effect of retinoic acid (RA) treatment on apoptosis of cumulus cells. (A) Representative pictures of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showing cumulus-oocyte-complexes (COCs) after IVM in the presence of increasing doses of RA. The red color indicates DNA fragmentation. COCs were counter stained with Hoechst 33342 (blue). (B) The apoptotic rate was calculated as the number of cells with positive TUNEL staining (red) divided by the number of cells with positive Hoechst staining (blue). Letters a and b, indicate that the group mean was significantly decreased and increased, respectively, than that of the control cells ($p < 0.05$). Scale bar indicates 25 μ m.

potential, either directly or indirectly [25,26]. Cytokines, in particular, play an important role in the regulation of ovarian function, gonadal steroid secretion, corpus luteum function, embryonic development and implantation. To our knowledge, this is the first report of the simultaneous measurement of multiple cytokines from a single COC culture after treatment with RA during oocyte maturation. Using the Bio-Plex system, which is a bead-based ELISA, the levels of 23 cytokines were assessed. A substantial level and regular pattern of secretion was detected for 4 of the 23 cytokines examined, namely TNF- α , MIP-1b, eotaxin and IL-12 (p40).

TNF- α is a 14 kDa pleiotropic cytokine produced by several cell types, particularly the macrophage [27]. During oocyte maturation, TNF- α compromises oocyte developmental potential and increases blasto-

mere apoptosis, resulting in reduced embryo survival [28]. MIP-1b is an 8 kDa member of the CC chemokine family, and is synthesized by a variety of cells, including monocytes and activated T- and B-cells [29]. TNF- α and MIP-1b levels were decreased by treatment with 100 nM RA, and this decrease correlated with decreased *lrf-1* expression. These results suggest a close functional relationship between the regulation of *lrf-1* expression and the secretion of TNF- α and MIP-1b.

Eotaxin is an 8.3 kDa protein that also belongs to the CC family of chemokines. It acts through CC chemokine receptor 3 (CCR3) on eosinophils [30]. The accumulation of eosinophils within human tissue has been correlated with increased eotaxin expression in a number of inflammatory conditions, including atopic asthma, nasal polyps and ulcerative colitis [30]. IL-12 is a pleiotropic type 1 cytokine that

mediates several biological activities in human T-cells and natural killer (NK) cells, including the induction of INF γ production and the enhancement of cell mediated cytotoxicity [31]. The presence of IL-12 in follicular fluid is associated with a negative outcome during IVF, which could reflect the immaturity of follicles as well as the negative effect of IL-12 on folliculogenesis, oocyte maturation and implantation [32]. Eotaxin and IL-12 (p40) were decreased by all doses of RA examined, as compared to control cells, suggesting that RA has some positive effect of the induction of oocyte maturation *in vitro* by inhibiting these cytokines.

In the present study, while the dose of 100 nM RA treatment showed consistent results, dose of 10 nM or 1 μ M showed incompatible fluctuating results fatefully. Therefore even though the maturation and activation rate was increased from 10 nM to 1 μ M RA treatment (Figure 3), we advocate 100 nM treatment for maintain steadiness of the culture system.

In conclusion, RA enhanced the developmental competence of mouse oocytes through the suppression of apoptosis in the mouse COCs, oocytes as well as cumulus cells, during IVM. We also presented evidence that RA promotes the cytoplasmic maturation and viability of mouse oocytes through the modulation of *Irf-1* gene expression in cumulus cells and oocytes. The production and secretion of several cytokines was also modulated by RA during oocyte maturation, and the secretion pattern of TNF- α and MIP-1b strongly suggests a functional relationship between these cytokines and RA through *Irf-1*.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

References

- van den Hurk R, Zhao J. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology* 2005;63:1717-51.
- Eppig JJ, Viveiros MM, Matin-Bivens C, De La Fuente. Regulation of mammalian oocyte maturation. In: Leung PC, Adashi EY, editors. *The ovary*. 2nd ed. California: Elsevier Academic Press; 2004. p.113-29.
- Schmitt A, Nebreda AR. Signalling pathways in oocyte meiotic maturation. *J Cell Sci* 2002;115:2457-9.
- Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 1998; 49:83-102.
- Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;293:1089-93.
- Yoon SJ, Chung HM, Cha KY, Kim NH, Lee KA. Identification of differential gene expression in germinal vesicle vs. metaphase II mouse oocytes by using annealing control primers. *Fertil Steril* 2005;83 Suppl 1:1293-6.
- Romeo G, Fiorucci G, Chiantore MV, Percario ZA, Vannucchi S, Afbabis E. IRF-1 as a negative regulator of cell proliferation. *J Interferon Cytokine Res* 2002;22:39-47.
- Bowles J, Koopman P. Retinoic acid, meiosis and germ cell fate in mammals. *Development* 2007;134:3401-11.
- Ikeda S, Kitagawa M, Imai H, Yamada M. The roles of vitamin A for cytoplasmic maturation of bovine oocytes. *J Reprod Dev* 2005; 51:23-35.
- Mohan M, Thirumalapura NR, Malayer J. Bovine cumulus-granulosa cells contain biologically active retinoid receptors that can respond to retinoic acid. *Reprod Biol Endocrinol* 2003;1:104.
- Wellik DM, Norback DH, DeLuca HF. Retinol is specifically required during midgestation for neonatal survival. *Am J Physiol* 1997; 272:E25-9.
- Gronemeyer H, Gustafsson JA, Laudet V. Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov* 2004;3:950-64.
- Devaux Y, Grosjean S, Seguin C, David C, Dousset B, Zannad F, et al. Retinoic acid and host-pathogen interactions: effects on inducible nitric oxide synthase in vivo. *Am J Physiol Endocrinol Metab* 2000;279:E1045-53.
- Hidalgo CO, Díez C, Duque P, Facal N, Gómez E. Pregnancies and improved early embryonic development with bovine oocytes matured in vitro with 9-cis-retinoic acid. *Reproduction* 2003;125: 409-16.
- Yoon SJ, Koo DB, Park JS, Choi KH, Han YM, Lee KA. Role of cytosolic malate dehydrogenase in oocyte maturation and embryo development. *Fertil Steril* 2006;86:1129-36.
- Old RW, Smith DP, Mason CS, Marklew S, Jones EA. Effects of retinoic acid on *Xenopus* embryos. *Biochem Soc Symp* 1996;62:157-74.
- Gudas LJ. Retinoids and vertebrate development. *J Biol Chem* 1994;269:15399-402.
- Means AL, Gudas LJ. The roles of retinoids in vertebrate development. *Annu Rev Biochem* 1995;64:201-33.
- Mark M, Ghyselinck NB, Chambon P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol* 2006;46:451-80.
- Dollé P, Fraulob V, Kastner P, Chambon P. Developmental expression of murine retinoid X receptor (RXR) genes. *Mech Dev* 1994; 45:91-104.

21. Luo XM, Ross AC. Retinoic acid exerts dual regulatory actions on the expression and nuclear localization of interferon regulatory factor-1. *Exp Biol Med (Maywood)* 2006;231:619-31.
22. Mullan PB, Hosey AM, Buckley NE, Quinn JE, Kennedy RD, Johnston PG, et al. The 2,5 oligoadenylate synthetase/RNaseL pathway is a novel effector of BRCA1- and interferon-gamma-mediated apoptosis. *Oncogene* 2005;24:5492-501.
23. Malathi K, Paranjape JM, Ganapathi R, Silverman RH. HPC1/RNASEL mediates apoptosis of prostate cancer cells treated with 2',5'-oligoadenylates, topoisomerase I inhibitors, and tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* 2004;64:9144-51.
24. Ikeda S, Imai H, Yamada M. Apoptosis in cumulus cells during in vitro maturation of bovine cumulus-enclosed oocytes. *Reproduction* 2003;125:369-76.
25. Mendoza C, Cremades N, Ruiz-Requena E, Martinez F, Ortega E, Bernabeu S, et al. Relationship between fertilization results after intracytoplasmic sperm injection, and intrafollicular steroid, pituitary hormone and cytokine concentrations. *Hum Reprod* 1999;14:628-35.
26. Mendoza C, Ruiz-Requena E, Ortega E, Cremades N, Martinez F, Bernabeu R, et al. Follicular fluid markers of oocyte developmental potential. *Hum Reprod* 2002;17:1017-22.
27. Tabibzadeh S. Human endometrium: an active site of cytokine production and action. *Endocr Rev* 1991;12:272-90.
28. Soto P, Natzke RP, Hansen PJ. Actions of tumor necrosis factor-alpha on oocyte maturation and embryonic development in cattle. *Am J Reprod Immunol* 2003;50:380-8.
29. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* 2000;18:593-620.
30. Zepeda M, Wilson JM. Neonatal cotton rats do not exhibit destructive immune responses to adenoviral vectors. *Gene Ther* 1996;3:973-9.
31. Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ. IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol Rev* 2004;202:96-105.
32. Gazvani MR, Bates M, Vince G, Christmas S, Lewis-Jones DI, Kingsland C. Follicular fluid concentrations of interleukin-12 and interleukin-8 in IVF cycles. *Fertil Steril* 2000;74:953-8.