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Research

Bovine cumulus-granulosa cells contain biologically active retinoid receptors that can respond to retinoic acid
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Abstract

Retinoids, a class of compounds that include retinol and its metabolite, retinoic acid, are absolutely essential for ovarian steroid production, oocyte maturation, and early embryogenesis. Previous studies have detected high concentrations of retinol in bovine large follicles. Further, administration of retinol in vivo and supplementation of retinoic acid during in vitro maturation results in enhanced embryonic development. In the present study, we hypothesized that retinoids administered either in vivo previously or in vitro can exert receptor-mediated effects in cumulus-granulosa cells. Total RNA extracted from in vitro cultured cumulus-granulosa cells was subjected to reverse transcription polymerase chain reaction (RT-PCR) and mRNA expression for retinol binding protein (RBP), retinoic acid receptor alpha (RARalpha), retinoic acid receptor beta (RARbeta), retinoic acid receptor gamma (RARgamma), retinoid X receptor alpha (RXRalpha), retinoid X receptor beta (RXRbeta), retinaldehyde dehydrogenase-2 (RALDH-2), and peroxisome proliferator activated receptor gamma (PPARgamma). Transcripts were detected for RBP, RARalpha, RARgamma, RXRalpha, RXRbeta, RALDH-2, and PPARgamma. Expression of RARbeta was not detected in cumulus-granulosa cells. Using western blotting, immunoreactive RARalpha, and RXRbeta protein was also detected in bovine cumulus-granulosa cells. The biological activity of these endogenous retinoid receptors was tested using a transient reporter assay using the pAAV-MCS-betaRARE-Luc vector. Addition of 0.5 and 1 micro molar all-trans retinoic acid significantly (P < 0.05) increased the activity of the pAAV-MCS-betaRARE-Luc reporter compared to cells transfected with the control reporter lacking a retinoic acid response element. Addition of 5 or 10 micro molar all-trans retinol stimulated a mild increase in reporter activity, however, the increase was not statistically significant. Based on these results we conclude that cumulus cells contain endogenously active retinoid receptors and may also be competent to synthesize retinoic acid using the precursor, retinol. These results also indirectly provide evidence that retinoids administered either in vivo previously or in vitro may have exerted a receptor-mediated effect on cumulus-granulosa cells.

Background

Retinoids, which include vitamin A and its active metabolite, retinoic acid (RA) are unstable hydrophobic compounds essential for cell growth and differentiation [1] and more importantly, for embryonic and placental development [2]. Various retinoid binding proteins such
as the 21 kDa plasma retinol binding protein (RBP), cellular retinol binding protein (CRBP-I & II) and cellular retinoic acid binding proteins (CRABP-I & II) both of ~16 kDa molecular weight, exist in the cell. RBP is extracellular and functions in the intercellular transport of retinol. On the other hand, CRBP-I & II functions in the intracellular transport of retinol and its metabolism to retinoic acid. CRABP-I & II not only regulates retinoic acid availability to retinoic acid receptors but also modulates its metabolism [3]. Biologically active retinoids mediate their effects on target cells through binding to two sets of nuclear receptors, namely, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), that are members of steroid/thyroid hormone nuclear receptor superfamily. Both RARs and RXRs have three subtypes, α, β, γ. Ligand-bound RARs and RXRs function as transcription factors by binding to cis-acting DNA sequences called retinoic acid response elements (RAREs). RAREs comprise directly repeated hexameric half-sites with consensus sequences (5'-PuG(G/T)TC-3') and are located within the transcriptional regulatory regions of target genes and facilitate transcriptional regulation of these genes [4]. The first step in the synthesis of retinoic acid is the oxidation of retinol to retinaldehyde by alcohol dehydrogenases [5]. Both medium and short chain retinol dehydrogenases can perform this function. The next step involves the oxidation of retinaldehyde to retinoic acid by aldehyde dehydrogenases [5]. Several aldehyde dehydrogenases (ALDH) including three NAD-dependent enzymes specific for retinaldehyde called ALDH-1, -2 and -3, have been isolated and characterized [5].

We had earlier shown that both immature oocytes and the early preattachment bovine embryo, from the 2-cell to the hatched blastocysts, express mRNA for RBP, RARα & γ, RXRα & β, and RALDH-2 [6,7]. In addition, we also detected the immunoreactive protein for RARα, γ2 and RXRβ in both inner cell mass and trophectoderm cells of intact and hatched blastocysts. Recently, Duque et al. [8] showed that addition of 5 nM 9-cis retinoic acid (9-cis RA) during prematuration of cumulus-oocyte complexes (COCs) in the presence of roscovitine improved cytoplasmic maturation and had a positive effect on blastocyst development and freeze-thaw survival rates. COCs treated with 9-cis RA had higher total cell numbers than untreated controls. In addition, the same authors also provided evidence to show that 9-cis RA induced trophectoderm differentiation, altered inner cell mass to trophectoderm cell ratio and also increased pregnancy rates following transfer of 9-cis RA treated day 7 blastocysts [9]. Based on these and our earlier studies we hypothesize that the cumulus-granulosa cells may be the predominant targets for retinoic acid added during in vitro prematuration. The objective of the present study is to investigate the presence of the retinoid signaling pathway in cumulus-granulosa cells, and retinoic acid responsiveness in cumulus-granulosa cells.

**Methods**

**Cell culture**

Experiments were carried out using cumulus cells harvested from follicles utilized for our routine in vitro fertilization studies. Briefly, ovaries were collected from cows at a local abattoir and transported to the laboratory in 0.9% normal saline supplemented with penicillin-G (100 IU/ml) and streptomycin sulfate (0.2 mg/ml) (Sigma Chemical Co, MO) at 26–30°C within 5 h. Cumulus oocyte complexes (COCs) were aspirated from follicles ranging in diameter from 2 to 8 mm using an 18-gauge needle into modified PBS solution (Invitrogen Life Technologies, CA). Following 24 hr of in vitro maturation, the expanded cumulus cells were removed from the oocyte. The matured oocyte with at least 2–3 layers of cumulus cells were then subjected to in vitro fertilization. Cumulus cells thus obtained were cultured in Hyclone's high glucose Dulbecco's modified Eagle's Medium (Hyclone Laboratories Inc, Logan, UT) containing 1x antibiotic-antimyocytic (Sigma Chemical Company), and supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories Inc, Logan, UT). Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO₂ and fed every 48 h.

**RNA extraction and reverse transcription polymerase reaction**

RNA extraction and reverse transcription were performed according to the methods described in Mohan et al. [6] with the following modifications. In the present study total RNA was extracted from cumulus-granulosa cells and only one round of PCR was performed owing to the large amount of RNA available from cumulus-granulosa cells. Primer sequences and the protocols used for RT-PCR are described in Mohan et al. [6,7].

**Western blotting**

Presence of the immunoreactive protein for RARα and RXRβ in cumulus-granulosa cells were examined by western blotting of nuclear extracts. Briefly, cumulus-granulosa cell cultures were treated with 1 µM RA for 24 hr. This approach was taken since a previous study had shown that RARs in the absence of RA remained cytoplasmic and in the presence of the ligand localized to the nucleus [10] further concentrating the receptor in the nucleus. Nuclear extracts were obtained using the NE-Perl Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL), as described in the manufacturer's protocol. Protein concentrations were determined using the DC protein assay kit (BioRad Laboratories, Hercules, CA). Approximately, 25 µg of nuclear protein, was separated by 12% sodium dodecyl sulfate-polyacrylamide gel
electrophoresis and subsequently transferred to Nitrocellulose membranes (BioRad) using the Transblot-SD Semi Dry Transfer Cell (BioRad). The membrane was blocked overnight at 4°C with Tris-buffered saline (Tris-HCL 20 mM, NaCl 500 mM pH 7.5) (TBS) containing 7.5% non-fat milk powder. The primary polyclonal antibodies against RARα and RXRβ (ABR-Affinity Bioreagents, Golden, CO) were used at a 1:2000 dilution in TBS containing 0.1% Tween 20 (TBST) and incubated with the membrane for 1 hr. Unbound primary antibodies were removed by washing thrice with TBST with each wash lasting about 15 minutes. Bound primary antibodies were detected using horseradish peroxidase labeled goat anti-Rabbit IgG secondary antibody. The secondary antibody was used at a 1:5000 dilution and incubation lasted about 1 hr. Membranes were washed thrice with TBST to remove unbound secondary antibody. Signals were developed using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) at two different concentrations (0.5 and 1 µM), 5 or 10 µM all-trans retinol, or alcohol vehicle. One micromolar and five micromolar concentrations were selected for all-trans RA and all-trans retinol, respectively, because these concentrations have been previously shown to enhance in vitro bovine embryo development [8,12]. The specified concentration of each ligand was added to triplicate wells and the entire assay was repeated twice. Twelve hours post-treatment, the culture medium was removed and the cells were washed with Ca2+-, Mg2+- containing Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen Corporation, CA). Luciferase assays were performed using the LucLite Luciferase Reporter gene assay kit according to the manufacturer’s instructions (Perkin Elmer Life Sciences, Boston, MA). The cell lysates were transferred to a 96-well Microtiter luminescence microplate (Microlite 1+, Thermo Biosystems, Vantaa, Finland). Luminescence in each well was recorded by counting the plate on a Top-Count NXT Microplate scintillation and luminescence counter (Packard Instrument Company, IL) for 30 seconds. For each treatment, luminescence recordings were obtained from the mean of triplicate wells. Luminescence measurements were normalized to the protein content and are expressed as luminescence units per microgram protein. The protein content in the cell lysate was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Statistical analyses
Luminescence measurements for all five ligand concentrations including alcohol from cells transfected with either pAAVMCS-pGL3-βRARE-Luc or the control vector were analyzed as a 2 × 5 factorial experiment with subsamples in a completely randomized design using Proc Mixed. Mean luminescence measurements for each ligand concentration for both pAAVMCS-pGL3-βRARE-Luc and the control vector transfected cells were compared using the Tukey’s procedure. A probability value of P < 0.05 was considered significant.

Reporter plasmid constructs
The plasmid pGL3 promoter vector (Promega Corporation, Madison, WI) contains the SV40 promoter driving expression of the firefly luciferase reporter gene. To confer retinoic acid responsiveness, a 37 bp consensus retinoic acid response element (RARE) from the human retinoic acid receptor β2 [11] carrying KpnI linkers on the 5’ prime end of both strands was cloned into the multiple cloning region located upstream of the SV40 promoter to produce the pGL3 promoter βRARE plasmid. The expression cassette from the pGL3 promoter RARE plasmid was isolated by cutting with the restriction enzyme ClaI. A second vector, pAAVMCS (4.7 kb) (Stratagene Corporation, La Jolla, CA) containing inverted terminal repeats (ITRs) was digested with NotI to remove the expression cassette (1.8 kb). The remaining backbone of the pAAVMCS vector containing the ITRs (2.9 kb) was ligated to the expression cassette of the pGL3 promoter vector to generate the final reporter construct, hereafter called pAAVMCS-pGL3-βRARE-Luc vector (Fig. 1). pAAVMCS-pGL3-βRARE-Luc vector was digested with Kpn1 to remove the RARE for control studies.

Transient transfection assays
Cumulus cells were cultured in 48-well plates (Costar, Corning Inc., Corning, NY) at a density of 35,000 cells per well in 500 µl of DMEM (Hyclone Laboratories Inc., Logan, UT) containing 5% charcoal dextran-extracted FCS, 1× antibiotic-antimycotic and 2 mM glutamine (Sigma Chemical Company). At 85–90% confluence, the cells were transfected with pAAVMCS-pGL3-βRARE-Luc or control reporter vector. For each well, approximately 1 µl of the transfection reagent (Lipofectamine 2000 Reagent, Invitrogen Corp, CA) was diluted in 37 µl of serum-and antibiotic-free DMEM. DNA was then diluted at a concentration of 500 ng in 37 µl of serum-and antibiotic-free DMEM in a separate tube. The DNA was then mixed with the liposomes and the transfection mixture was incubated at room temperature for about 20 minutes for the DNA-liposome complexes to form. Before transfection, the culture media in each well was replaced with 200 µl of serum-and antibiotic-free DMEM and 74 µl of the DNA-liposome complex was added to achieve a final volume of 274 µl. The transfection media was removed 6–8 h later and replenished with 500 µl of antibiotic-antimycotic free fresh media and the cells were allowed to recover for 24 h. Twenty four hours post-transfection the cells received one of the five treatments: all-trans retinoic acid (Sigma Chemical Co) at two different concentrations (0.5 and 1 µM), 5 or 10 µM all-trans retinol, or alcohol vehicle. One micromolar and five micromolar concentrations were selected for all-trans RA and all-trans retinol, respectively, because these concentrations have been previously shown to enhance in vitro bovine embryo development [8,12]. The specified concentration of each ligand was added to triplicate wells and the entire assay was repeated twice. Twelve hours post-treatment, the culture medium was removed and the cells were washed with Ca2+-, Mg2+- containing Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen Corporation, CA). Luciferase assays were performed using the LucLite Luciferase Reporter gene assay kit according to the manufacturer’s instructions (Perkin Elmer Life Sciences, Boston, MA). The cell lysates were transferred to a 96-well Microtiter luminescence microplate (Microlite 1+, Thermo Biosystems, Vantaa, Finland). Luminescence in each well was recorded by counting the plate on a Top-Count NXT Microplate scintillation and luminescence counter (Packard Instrument Company, IL) for 30 seconds. For each treatment, luminescence recordings were obtained from the mean of triplicate wells. Luminescence measurements were normalized to the protein content and are expressed as luminescence units per microgram protein. The protein content in the cell lysate was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).
The plasmid pAAV-MCS-βRARE-Luc was generated by inserting a 37 bp consensus retinoic acid response element (RARE) from the human retinoic acid receptor β2 carrying Kpn1 linkers on the 5' prime end of both strands into the pGL3 promoter vector (Promega Corporation, Madison, WI). The expression cassette with the RARE sequence was then ligated to the back bone of the pAAVMCS vector carrying the ITR sequences. This construct was characterized by restriction mapping and by dideoxy chain-termination sequencing to verify that the β2 RARE was intact. The SV40 promoter drives expression of the firefly luciferase reporter.
mouse and human RBP, RAR exhibited a very high (>90%) homology to published sequence analysis. The isolated bovine cDNA sequences (Fig. 2). The identity of the PCR products was verified by of the predicted size were detected for each target cDNA previously described (Mohan et al., 2001; 2002). Products Primer sequences and PCR conditions used to amplify western blotting Reverse transcription polymerase chain reaction and Results

**Expression of transcripts for RBP (311 bp, Lane 2), RARα (392 bp, Lane 3), RARγ (795 bp, Lane 4), RXRα (415 bp, Lane 6), RXRβ (Lane 7, 207 bp), RALDH-2 (531 bp, Lane 8) and PPARγ (332 bp, Lane 9) using RT-PCR in cumulus cells. Lanes 1 and 5 contain DNA marker. Negative control lane (not shown here) contained the product of RT-PCR under identical conditions in the absence of RNA template. Products of RT-PCR were resolved in 2% TAE-agarose gel and visualized by ethidium bromide staining.

Out of the three known isoforms for both RARs and RXRs, the expression of two were observed in cumulus granulosa cells. A 392 bp and 795 bp product was detected for RARα and RARγ (Fig. 2), respectively. In addition to the 795 bp product corresponding to RARγ, a second product migrating between 400 and 500 bp was also detected. We have not sequenced the lower molecular weight product and therefore, it is not clear if this product represents a different isoform of RARγ or if it is the result of non-specific amplification. A 311 bp product was detected for retinol binding protein. The intensity of the band was, however, very weak. Similarly, a 415 bp and 207 bp product was detected for RXRα, and RXRβ, respectively. On the other hand, expression of RARβ and RXRγ was not detected (data not shown). A 432 bp product representing the heterodimerization partner for RXRs, namely, PPARγ was also detected in cumulus-granulosa cells. Cumulus-granulosa cells also expressed RALDH-2, the enzyme responsible for the conversion of retinaldehyde to retinoic acid that was detected as a 531 bp fragment.

The primary polyclonal antibodies developed by Affinity Bioreagents identifies an approximately 45 kDa and 55 kDa against RARα, and RXRβ respectively, in COS cells. According to the manufacturer's observations we were also able to detect an approximately 45 kDa and 55 kDa band for RARα, and RXRβ respectively, in bovine cumulus-granulosa cells (Fig 3). For RXRβ, in addition to the specific product we also observed a few weak non-specific products. Control blots without secondary antibody treatment did not exhibit any signal (data not shown). Western blots were repeated twice.

**Transient reporter assays**

To understand the transcriptional properties of the endogenous retinoid receptors, a retinoid responsive reporter construct, pAAVMCS-pGL3-βRARE-Luc vector was introduced using liposomes into cumulus granulosa cells. A similar vector lacking the βRARE was also transfected into cells to serve as a control. The assay was performed twice and a similar trend in luminescence activity as presented here was observed on both occasions. The data on fold increase provided here is from a single assay and is presented in Figure 4. There was a vector by ligand concentration interaction and therefore, for each vector, mean luminescence measurements at each of the ligand concentrations were compared using the Tukey's procedure. In Figure 4, both 0.5 and 1 µM all-trans RA significantly increased (P < 0.05) pAAVMCS-pGL3-βRARE-Luc activity by 3.87- and 2.46- fold, respectively, compared to the cells transfected with the control vector. Supplementation of 5 µM retinol caused a mild increase in pAAVMCS-pGL3-βRARE-Luc activity (1.63-fold) (P > 0.05). Increasing the concentration of retinol from 5 µM to 10 µM accordingly increased pAAVMCS-pGL3-βRARE-Luc activity (2.14-fold) (P > 0.05) compared to the cells transfected with the control vector. However, the increase in reporter activity observed following retinol treatment at both concentrations was not statistically significant. Addition of alcohol vehicle did not stimulate an increase in pAAVMCS-pGL3-βRARE-Luc activity.
Discussion
Cumulus-granulosa cells play a very important role during maturation so that the oocyte acquires competence for further development [13]. Since mammalian oocytes develop in the follicular environment tightly surrounded by cumulus cells, these cells directly exert their effects on the developing oocyte. Cumulus cells support oocyte maturation before ovulation, facilitate oocyte transport into the oviduct during ovulation and thereafter orchestrates the complex mechanisms that control the interaction of spermatozoa with the oocyte [14]. At least under in vitro conditions these observations are exemplified by the fact that cumulus removal before IVF reduced cleavage rate in the cow [13]. Administration of retinol to cows in conjunction with superovulation protocols increased the number of transferable blastocysts on day 7 [15] and more recently improved the yield of cumulus oocyte complexes from heifers following transvaginal ultrasound aspiration [16].

Earlier, Scheigwert et al. [17] and more recently, Brown et al. [18] reported detecting retinol in the follicular fluid of bovine dominant follicles. Both these authors observed vitamin A concentrations to be highest in large nonatretic follicles and lowest in small atretic follicles and correlated their findings with follicular fluid estradiol concentrations. In the later study, both the mRNA and immunoreactive RBP were detected in granulosa, theca cells, and the blood vessels lining the follicle [18]. In addition, RBP concentrations were also found to be elevated in follicles containing high retinol concentrations [18]. These findings indicate that retinol is available in high concentrations in the follicular fluid of large ovulatory follicles and the follicular cells were equipped to take up retinol from the follicular fluid. In the present study, we also detected mRNA expression for RBP in cumulus cells, which is in agreement with the findings of Brown et al. [18], and suggests the capacity for intercellular binding and transport of retinol. The presence of RBP does not signify the possibility for retinoic acid synthesis or provide information about the fate of retinol following uptake by cumulus-granulosa cells from the follicular fluid. No effort was made in the present study to investigate the expression of alcohol dehydrogenases responsible for the oxidation of retinol to retinaldehyde. We earlier reported detecting a product very close to the expected size for alcohol dehydrogenase IV using nested PCR in bovine preattachment embryos [7]. However, BLAST searches revealed less than 45% homology to alcohol dehydrogenase IV. Using RTPCR, we detected RALDH-2 in cumulus granulosa cells indicating the possibility of retinoic acid synthesis at least from retinaldehyde. Data from our transient reporter assays show that addition of retinol at 5 and 10 µM concentrations to cells transfected with the pAAVMCS-pGL3-βRARE-Luc vector did not cause a significant increase in reporter activity compared to controls (Fig. 3). Ethanol was used as a solvent for retinoids used in this study and it is possible that ethanol decreased retinoic acid synthesis in competition with all-trans retinol to inhibit alcohol dehydrogenase [19]. This reduction in reporter activity was not observed with all-trans retinoic acid since it acts at a step downstream of the inhibitory point. Based on these results it is not clear if cumulus cells can oxidize retinol to retinaldehyde or in other words if alcohol dehydrogenase activity is existent in cumulus cells. Therefore, in the future it may be a good idea to test the effect of retinol dissolved in another solvent such as dimethyl sulfoxide on activation of the reporter. The detection of RALDH-2, nevertheless, indirectly points towards the likely oxidization
of retinol to retinoic acid by cumulus-granulosa cells since retinaldehyde, the substrate for RALDH-2 is generated from retinol. Retinoic acid thus generated may then activate the reporter.

Transcripts for RARα, RARγ, RXRα and RXRβ were also expressed in cumulus cells (Fig. 2). The presence of both RAR and RXRs would mean that both all-trans and 9-cis RA have the potential to exert receptor mediated effects on transcriptional regulation in cumulus-granulosa cells. Interestingly, presence of the mRNA does not specify the presence of the mature protein. Using western blotting we identified the immunoreactive protein for both RARα, and RXRβ in cumulus-granulosa cells employing polyclonal antibodies. Furthermore, presence of the immunoreactive protein does not guarantee biological activity. Therefore, we tested the biological activity of both RARs and RXRs using a transient reporter assay. Addition of both 0.5 and 1 μM RA significantly increased reporter activity in cells transfected with the pAAVMCS vector carrying the RARE sequence compared to the cells transfected with the control vector. The main reason for performing a retinoid sensitive reporter assay is to only show that the retinoid receptors expressed by the cumulus-oocyte complexes are biologically active or in other words capable of binding retinoic acid. Therefore, caution should be exercised while interpreting the data from the reporter assay since it neither represents a dose-response nor a time course study. These results basically show that cumulus-granulosa cells contain endogenous retinoid receptors capable of binding all-trans retinoic acid and these receptors may transduce the retinoid signal further downstream. Based on our transient reporter assay results it appears that addition of 5 nM 9-cis and 1 μM all-trans retinoic acid to the in vitro maturation medium [8] had a direct positive effect on the cumulus oocyte complex, thereby enhancing embryo development. We also detected transcripts for PPARγ in cumulus cells. The expression of PPARγ further suggests the possibility for heterodimerization between RXRs and PPARs.

The mechanisms by which addition of retinoic acid brought about positive effects on embryonic development in earlier studies is unclear and needs further investigation. Exposure of immature porcine granulosa cells to 1 μM retinoic acid for 15 hrs inhibited mRNA expression of LH receptor via downregulation of c-fos mRNA [20]. Consequently, RA-treated immature granulosa cells failed to

Figure 4
Transactivation of retinoid-responsive reporter gene pAAVMCS-pGL3-βRARE-Luc (blue bars) versus pAAVMCS-pGL3-Luc (red bars) by endogenous retinoid receptors in bovine cumulus-granulosa cells. The Y axis represents luciferase activity in luminescence units (LU) per microgram total protein following treatment with all-trans retinoic acid (RA) (0.5 or 1 μM), all-trans retinol (ROH) (5 or 10 μM) and alcohol vehicle control. Each sample was accordingly normalized and means ± SEs were evaluated for LU/μg of total protein. Within each treatment (ligand concentration) means with different superscripts differ significantly (P < 0.05). The assay was carried out in triplicate wells for each treatment and performed twice.
differentiate into mature cells. Similarly, RA also inhibited transcription of FSH receptor mRNA in a dose-dependent manner [21]. Follicle stimulating hormone is partly responsible for the differentiation process initially through the induction of FSH receptors and later of LH receptors [22]. It is likely that retinoic acid could have exerted a very similar negative effect on bovine cumulus-granulosa cells during in vitro maturation [8]. However, the significance of retinoic acid induced downregulation of both LH and FSH receptor mRNA and its consequent effects on in vitro oocyte maturation needs future investigation. Further, retinoic acid receptors being ligand activated transcription factors are involved in regulating the transcription of several genes. One such target gene is midkine, initially identified in a teratocarcinoma cell line as a retinoic acid inducible gene [23]. Midkine belongs to the family of heparin-binding growth/differentiation factors. Midkine has been detected and reported to be present at a concentration of 125 ng/mL in the bovine follicular fluid [24]. Further, in situ hybridization studies have shown that midkine mRNA is restricted to the granulosa cells of healthy rat follicles [25] and RA has been shown to induce a 2-fold increase in midkine mRNA at a concentration of 0.3 μM [26]. Similarly, treatment of bovine cumulus-granulosa cells with 5 nM 9-cis retinoic acid increased the expression of midkine mRNA [27]. More recently, addition of midkine during in vitro oocyte maturation influenced cytoplasmic maturation of oocytes and increased blastocyst yields compared to untreated controls [28,29].

**Conclusions**

We detected mRNA expression for RBP, nuclear retinoic acid and retinoid X receptors, PPARγ and RALDH-2 in bovine cumulus granulosa cells. The activation of the reporter construct following the addition of all-trans retinoic acid would indicate that these endogenous retinoid receptors are competent to bind the ligand and may be capable of transducing the biologically active retinoid signal further downstream. Presence of biologically active retinoic acid and retinoid X receptors also suggests that retinol and its metabolite retinoic acid may exert transcriptional regulation during in vivo/in vivo oocyte maturation in the bovine. Retinoic acid is believed to be a potent regulator of cell differentiation, cell proliferation and apoptosis by regulating the expression of specific genes. Identification of functional retinoic acid responsive genes and their downstream products will throw more light into the mechanisms by which retinoic acid addition during maturation enhanced blastocyst development rates and increased blastocyst cell numbers [8]. Therefore, elucidation of the molecular pathways involved in retinoid-mediated regulation of gene expression in COCs remains a high priority in the future.

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