A role for retinoids in human oocyte fertilization: regulation of connexin 43 by retinoic acid in cumulus granulosa cells

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ABSTRACT: Retinoids are essential for ovarian steroid production and oocyte maturation in mammals. Oocyte competency is known to positively correlate with efficient gap junction intercellular communication (GJIC) among granulosa cells in the cumulus-oocyte complex. Connexin 43 (Cx43) is the main subunit of gap junction channels in human cumulus granulosa cells (CGC) and is regulated by all-trans retinoic acid (ATRA) in other hormone responsive cell types. The objectives of this study were to quantify retinoid levels in human CGC obtained during IVF oocyte retrievals, to investigate the potential relationship between CGC ATRA levels and successful oocyte fertilization, and to determine the effects of ATRA on Cx43 protein expression in CGC. Results showed that CGC cultures actively metabolize retinol to produce ATRA. Grouped according to fertilization rate tertiles, mean ATRA levels were 2-fold higher in pooled CGC from women in the highest versus the lowest tertile (P₀.05). ATRA induced a rapid dephosphorylation of Cx43 in CGC and granulosa cell line (KGN) cultures resulting in a 2-fold increase in the expression of the functional non-phosphorylated (P₀) species (P₀.02). Similar enhancement of P₀ by ATRA was shown in CGC and KGN cultures co-treated with LH or hCG which, by themselves, enhanced the protein levels of Cx43 without altering its phosphorylation profile. Correspondingly, the combination of ATRA + hCG treatment of KGN caused a significant increase in GJIC compared with single agent treatments (P₀.025) and a doubling of GJIC from that seen in untreated cells (P₀.01). These findings indicate that CGC are a primary site of retinoid uptake and ATRA biosynthesis. Regulation of Cx43 by ATRA may serve an important role in folliculogenesis, development of oocyte competency, and successful fertilization by increasing GJIC in CGC.

Key words: all-trans retinoic acid / connexin 43 / cumulus granulosa cells / oocyte competency

Introduction

Emerging evidence indicates important effects of vitamin A compounds (retinoids) on oocyte maturation and function (Zheng et al., 1999; Livera et al., 2000; Brown et al., 2003; Ikeda et al., 2005). To this end, in vivo administration of retinoids in cattle, sheep and pigs have been shown to enhance oocyte fertilization competence (Eberhardt et al., 1999; Whaley et al., 2000; Ikeda et al., 2005) while in vitro studies identified all-trans retinoic acid (ATRA) as the active vitamin A metabolite in this activity (Alminana et al., 2008; Nasiri et al., 2011; Tahaei et al., 2011). Given the demonstrated importance of ATRA in oocyte competency in animals, we have been interested in providing evidence of its role and mechanism of action in human oocyte development. Our previous work demonstrated that high follicular fluid (FF) ATRA concentrations at the time of oocyte retrieval were associated with oocytes giving rise to the highest quality (grade I) embryos in our in vitro fertilization (IVF) program (Pauli et al., 2013). In that investigation, follicular fluid ATRA concentrations also were shown to correlate with patient plasma levels. As the relative contribution of plasma to follicular fluid ATRA is unknown, we evaluated active intra-follicular biosynthesis of ATRA. In this regard, as far as we know, active synthesis of ATRA by ovarian follicle cells has not been reported.
The mechanism(s) by which retinoids may affect oocyte development has received only scant attention. It was suggested that ATRA may promote cytoplasmic maturation of bovine oocytes via its modulatory effects on the gene expression of gonadotrophin receptors, midkine (neurite outgrowth-promoting factor 2), cyclooxygenase-2, and/or nitric oxide synthase in cumulus granulosa cells (CGC) (Ikeda et al., 2005). We have previously shown that ATRA can rapidly up-regulate gap junction intercellular communication (GJIC) in human endometrial stromal cells through a post-translational mechanism of action that involves rapid dephosphorylation of gap junction alpha-1 protein (GJα1) also known as connexin 43 (Cx43) (Wu et al., 2013). In ovarian granulosa cells, Cx43 is the most widely expressed Cx, and plays an important role in regulating the micronutrient environment of the oocyte by allowing transfer of ions, metabolites, and small molecules up to 1 kDa (Goldberg et al., 2004; Wang et al., 2009). Multiple animal studies have demonstrated the regulation of ovarian Cx43 by luteinizing hormone (LH); exposure of pre-ovulatory follicles to LH deactivates gap junctions through induced phosphorylation of Cx43 (Sela-Abramovich et al., 2005; Norris et al., 2008). This activity prevents the intercellular transport of cAMP, a meiosis inhibitor, leading to resumption of meiosis (Dekel, 2005). The importance of this activity was highlighted by showing that Cx43 knockout mice have impaired folliculogenesis as the follicles are unable to proceed beyond the pro-antral follicular stage (Ackert et al., 2001). Mechanisms that reestablish GJIC in the corpus luteum, an action that is necessary for progesterone production, are still unresolved although cAMP and oxytocin are thought to cooperate with LH/hCG signaling in this process. However, the rapid increase of GJIC during the early postovulatory luteal phase (Grazul-Bliska et al., 1996b, c) suggests the involvement of other, as yet unknown, regulatory co-factors that can quickly modulate Cx43 function. In light of the importance of retinoids in oogenesis and the recognized action of ATRA on Cx43, we were interested in the possibility that ATRA functions in the post-meiotic follicle through its ability to enhance GJIC in human cumulus granulosa cells. As such, our objectives for this study were to (i) quantify retinoid levels in human CGC; (ii) investigate the potential relationship between CGC ATRA levels and successful oocyte fertilization; and (iii) determine the effects of ATRA on Cx43 in CGC and in the immortalized KGN granulosa cell line. The findings reported herein provide evidence of a novel role for ATRA in regulating CGC-oocyte interactions during human ovarian folliculogenesis.

Materials and Methods

Experimental participants and collection of CGC

CGC were obtained from patients undergoing fresh IVF at the Emory Reproductive Center. Written informed consent was obtained from all participants prior to the procedure. Retinoid levels in CGC were measured in samples collected from participants of a previous study conducted between August 2008 and July 2009 (Pauli et al., 2013). Subsequent CGC used for the in vitro studies of ATRA effects on Cx43 were obtained from new patients enrolled between November 2011 and March 2013. Each participant’s cycle parameters were collated from clinical records as previously reported (Pauli et al., 2013). All patients underwent gonadotrophin stimulation administered by subcutaneous injection. Serial E2 levels and 2-dimensional transvaginal ultrasound follicle measurements were performed until at least two follicles reached 18 mm or more in diameter. Final oocyte maturation was induced by administering hCG (10 000 IU intramuscular) followed by transvaginal follicle aspiration and oocyte retrieval 35 h later.

Primary human CGC were collected from women undergoing IVF by gently removing them from the cumulus-oocyte complex following egg retrieval. CGC from all cumulus-oocyte complexes retrieved from each patient were pooled in order to obtain enough material for retinoid analysis. Human endometrial and mammary tissues were used for comparison of retinoid levels with CGC. Endometrial biopsies were obtained from patients (n = 42) undergoing surgery for infertility or pelvic pain as part of a previously published study (Pierzchalski et al., 2014). Normal mammary tissue was obtained from the Marlene and Stewart Greenebaum Cancer Center tissue bank (n = 9).

Cell cultures and chemicals

The human granulosa cell line KGN, originally provided to us by Drs Nishi and Yanase (Graduate School of Medical Sciences, Kyusyu University, Fukuoka, Japan), was derived from a patient with invasive ovarian granulosa cell carcinoma (Nishi et al., 2001). This cell line has been used as a model for human granulosa cell steroidogenesis, cell growth and apoptosis (Nishi et al., 2001; Woods et al., 2008). Primary CGC and KGN cells were cultured in 6-well plates in Dulbecco’s Modified Eagle Medium/F12 media (Cellgro, Manassas, VA, USA) containing 1% insulin-transferin-selenium, 1% penicillin/streptomycin, 10% fetal bovine serum (complete medium) for 24 h prior to treatment. For evaluating Cx43 expression, cells were treated for up to 72 h with all-trans retinoic acid (ATRA), LH, hCG (all from Sigma Chemical Co., St. Louis, MO, USA) or in combinations as indicated. A 50 mM stock of ATRA was made in dimethyl sulphoxide (DMSO), aliquoted, and stored frozen at −20 °C for up to 2 months for future use. For experiments, ATRA stock was further diluted in complete medium to the concentrations indicated. Final solvent concentrations (<0.02% DMSO) were contained in the vehicle controls. To assess the ability of CGC to synthesize ATRA, cells were washed with phosphate-buffered saline (PBS) and then cultured in serum-free medium in the presence of 2 μM retinol (ROL, Sigma Chemical Co., St. Louis, MO, USA) or solvent control for 16 h. ATRA concentration was then quantified in the culture supernatant and cell pellets (below).

Western blot analysis

Western blot analysis was performed as previously described (Wu et al., 2013). Briefly, whole cell extracts of CGC and KGN were derived by dissolving cell pellets in Cell Extraction Buffer (Invitrogen, Grand Island, NY, USA) and protein concentrations determined using a bicinchoninic acid (BCA) protein assay kit (Sigma Chemical Co.). Protein (20 μg) from cells treated with medium (control), ATRA, LH, hCG, and combinations were loaded on 10% SDS–PAGE gel, transferred to nitrocellulose membrane and blocked with 4% bovine albumin in PBS. Rabbit polyclonal anti-Cx43 antibody (1:500, Zymed, Grand Island, NY, USA) and sequential secondary antibody linked with horseradish peroxidase was used to detect total Cx43. The non-phosphorylated form of Cx43 was detected using mouse monoclonal anti-Cx43 antibody (1:1,000, Invitrogen, Cat. No. 13-8300). Enhanced Chemiluminescence System (Amersham Biosciences Piscataway, NJ, USA) was utilized to visualize immunoreactive bands. Even loading was ensured by washing, stripping and reprobing blots with anti-β-actin antibody.

Scrape loading/dye transfer

Relative GJIC in control and treated cultures was determined by the Scrape Loading/Dye Transfer (SL/DT) technique previously described (Tanmahasamut and Sidell, 2005; Wu et al., 2013) by using the gap junction-permeable fluorescent dye Lucifer Yellow (LY) (Molecular Probes, Life Technologies, Grand Island, NY, USA). KGN cells were cultured as described above and were washed with PBS before performing the assay. The SL was done by making a razor blade cut across the cell monolayer and then 1 mg/ml LY
was added to the culture at the area of the defect. Dye was rinsed after 10 min. Cells were washed three times with PBS and fixed with 4% paraformaldehyde. Fluorescence emission with an inverted microscope equipped with a camera detected the lateral diffusion of LY into cells with GJC. Cells that received LY from an adjacent scrape-loaded cell were considered communicating and the numbers of communicating (fluorescent) cells in the untreated and treated cultures were counted. For each condition, five randomly selected fields were counted. GJC was reported as the number of LY positive cells.

**Determination of retinoid levels**

Biopsy specimens, cultured cell pellets or culture supernatant were prepared for retinoid analysis under yellow lights. In the case of CGC, cells from all cumulus-oocyte complexes retrieved from each patient were pooled in order to obtain enough material for analysis. Retinoids were extracted with a two-step liquid-liquid extraction that has been described in detail (Kane et al., 2008a, Kane and Napoli, 2010). Total protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Retinoid acid was quantified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) with atmospheric pressure chemical ionization in positive-ion mode on a 5500 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer operated in MRM mode (AB Sciex, Foster City, California) (Kane et al., 2008b). Retinol and total retinyl ester (RE) were quantified by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) on an Aquity H-Class UPLC (Waters, Milford, MA, USA) (Kane et al., 2008a).

**Statistical analysis**

Statistical analysis was performed using GraphPad Software (San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM). Differences between groups as indicated were analyzed by t-test (unpaired, two-tailed), where P < 0.05 was considered statistically significant. Each experiment was replicated a minimum of three times.

**Results**

**Retinoid levels in primary human endometrial and mammary cells used as sources of hormone sensitive tissue known to actively synthesize ATRA from ROL (Pierzchalski et al., 2014).** 13-cis RA was not detected (ND) in 66 of the samples and had an average concentration of 94.5 ± 38.4 pmol/g protein in the remaining 11 samples. Neither was 9-cis RA detected in any of the patient samples. Limits of detection for both 13-cis and 9-cis RA in tissue were ~12 pmol/g protein (Kane et al., 2008b). As expected, ROL and RE were more abundant than ATRA, falling in the nmol/g protein range. ATRA levels ranged from 127 to 7552 pmol/g protein while ROL levels ranged from 23.2 to 3915.2 pmol/g protein. RE levels ranged from 49.0 to 337.3 nmol/g protein. CGC ATRA concentrations from the same women correlated weakly with ROL levels (r² = 0.41) but did not correlate with RE levels (r² = 0.00). There was also no correlation between granulosa ATRA and plasma ATRA levels (r² = 0.02).

**Oocyte fertilization rate**

Previous studies showed the positive effects of vitamin A supplementation on bovine fertility in artificial insemination while in vitro oocyte studies provide evidence of a role for retinoids in oocyte cytoplasmic maturation and fertilization competence (Ikeda et al., 2005; Alminana et al., 2008; Nasiri et al., 2011; Tahaei et al., 2011). In our previous work, we determined that follicular fluid from follicles yielding high quality (grade I) had higher mean levels of ATRA but not ROL than fluid from follicles that yielded nongrade I embryos (Pauli et al., 2013). In the present study, we sought to assess the potential relationship between retinoid levels in CGC and the fertilization competence of that subject’s oocytes. However, a direct evaluation of this relationship in individual follicles was not possible due to insufficient cumulus material necessary for retinoid measurements in single-follicle CGC. Thus, to address this question, retinoid levels in CGC pooled from all retrieved oocytes from each patient were measured and results stratified by ter tiles according to the percent of successfully fertilized oocytes (i.e. the number of oocytes that were successfully fertilized compared to the total retrieved from each patient; #2PN/total retrieved × 100). Figure 1 shows that the mean ATRA level was significantly (>2-fold) higher in the CGC pool from patients in the highest fertilization tertile compared with those in the lowest tertile. In contrast, there were no significant differences in ROL concentrations across the different fertilization tertiles. The clinical characteristics of known confounding factors between the highest and lowest fertilization groups were not significantly different, and both groups had similar stimulation parameters and response to gonadotrophins (Table II). Furthermore, there were no differences in CGC ATRA levels in patients with varying infertility diagnosis (unexplained, tubal or male factor infertility, polycystic ovarian syndrome, endometriosis, diminished ovarian reserve) although this stratification lacked adequate power with the sample size studied.

**Table I Mean retinoid levels in cumulus granulosa cells, endometrial and mammary tissue. Data are mean (SEM).**

<table>
<thead>
<tr>
<th>Granulosa cells</th>
<th>Endometrial tissue</th>
<th>Mammary tissue</th>
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<tbody>
<tr>
<td>N = 77</td>
<td>N = 42</td>
<td>N = 9</td>
</tr>
<tr>
<td>All-trans retinoic acid (pmol/g protein)</td>
<td>1140 (176)</td>
<td>330 (43)</td>
</tr>
<tr>
<td>Retinol (nmol/g protein)</td>
<td>603 (108)</td>
<td>55 (12)</td>
</tr>
<tr>
<td>Retinyl esters (nmol/g protein)</td>
<td>216 (81)</td>
<td>56 (10)</td>
</tr>
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</table>

**Synthesis of ATRA by CGC**

ATRA production was quantitated in CGC cultures by the addition of 2 μM ROL to the culture medium under serum-free conditions. After an additional 16 h (overnight) culturing, the medium and cells were harvested and analyzed for ATRA (Fig. 2). While little ATRA was detected in the absence of added ROL, treatment of the cultures with ROL induced a >3-fold increase in ATRA concentration in both the cell pellets and...
These findings indicate that CGC both synthesize and actively secrete ATRA.

**Effects of ATRA on Cx43**

We examined the effects of ATRA on Cx43 mRNA levels and protein expression in primary CGC. Dose–response experiments showed only a marginal (<50%) increase in mRNA levels at concentrations up to 10 μM ATRA and incubation times up to 3 days (data not shown). Figure 3 indicates that CGC exhibited three distinct immunoreactive Cx43 protein bands (41, 42 and 45 kDa; designated P0, P1 and P2, respectively). As shown by us and others in various human tissues, the lower band (P0) corresponds to the non-phosphorylated and most biologically active form of Cx43 (Tanmahasamut and Sidell, 2005; Solan and Lampe, 2007; Wu et al., 2013). Treatment of these cells for 2 days with 10 μM ATRA induced a change in the relative levels of the Cx43 species; P0 is enhanced, whereas P2 shows a dramatic decrease compared with control cultures (Fig. 3A, inset). Using the human granulosa cell line KGN as a model of primary CGC (Nishi et al., 2001; Woods et al., 2008), we observed similar results. The shift in band intensities induced by ATRA to the biologically active non-phosphorylated species was quantified by expressing the relative intensity of the three Cx43 species as the ratio (P1 + P2)/P0 (Fig. 3A). Using a non-phosphorylated Cx43 specific antibody, we confirmed that this P0 isoform of Cx43 was increased 2-fold by ATRA treatment (Fig. 3B).

**Combined effects of ATRA and LH/hCG on Cx43 expression**

Animal studies have demonstrated modulation of Cx43 in granulosa cells by LH or hCG; exposure of the ovarian follicle to LH leads to an increase in the phosphorylation of Cx43 on serines 255, 262, and 279/282, resulting in an increase of Cx43 with mobility corresponding to the P2 position and inhibitory effects on GJIC (Norris et al., 2008). In our human CGC, LH was found to time-dependently enhance the overall protein levels of Cx43 without significant changes in the relative distributions of the three phosphorylation species (Fig. 4A). Similar results were obtained when CGC were stimulated with hCG, consistent with the fact that both gonadotrophins utilize the same transmembrane receptor (the luteinizing hormone/choriogonadotropin receptor, LhCGR) and activate identical signaling pathways (Menon et al., 2004). The CGC often showed intense P2 Cx43 bands even under control culture conditions, such that increased expression by LH and hCG was not always apparent (Fig. 4B). This may be due to the fact that all the patients were treated with hCG 35 h before retrieval as part of their IVF protocol, so that...
Cx43 expression in CGC may have been near maximally stimulated in vivo. Addition of ATRA treatment, both in the absence and presence of LH or hCG, induced a marked shift in the relative levels of the phosphorylated Cx43 species, as evidenced by a dramatic enhancement of P0 with a corresponding decrease in P2 (Fig. 4B). Similar results were obtained with KGN cells although up-regulation of Cx43 by hCG alone was more modest than that seen with primary CGC (Fig. 4C). Whether this difference is due to a relative paucity of LhCGR on KGN cells is unknown.

Modulation of GJIC

Previous studies in endometrial stromal cells showed that ATRA increases GJIC through dephosphorylation of Cx43 as reflected by a similar decrease in the intensity of the P2 species and a reciprocal increase in P0 (Tanmahasamut and Sidell, 2005; Wu et al., 2013). To determine the extent to which Cx43 expression and phosphorylation status is related to GJIC in CGC, we performed SL/DT assays in KGN cells using the gap junction-permeable fluorescent dye LY. As assessed by this technique, serum-starved cells were communication-competent and transferred LY to numerous cells distant to the wound edge (Fig. 5).

Figure 3 Effect of all-trans retinoic acid (ATRA) on Cx43 phosphorylation. Total cellular protein was isolated from cumulus granulosa cells (CGC) and human granulosa cell line (KGN) cells and assayed for Cx43 expression by western blotting. (A) Representative experiments of primary CGC from two patients and KGN cells treated with 10 μM ATRA (RA) or vehicle control (C) as indicated for 48 h show changes in the band intensity and distribution of non-phosphorylated (P0) and phosphorylated (P1 and P2) species of Cx43 (inset). Figure in the lower panel represents the relative intensity of the three Cx43 phosphorylation species expressed as the ratio (P1 + P2)/P0 in order to quantify the sensitivity of Cx43 dephosphorylation to ATRA treatment. Columns represent the mean (± SEM) from seven CGC patient samples (black bars) and three independent experiments with KGN cells (gray bars). Significant difference when compared with untreated controls, +p < 0.02, **p < 0.0001. (B) Representative western blot analysis using a non-phosphorylated Cx43 (Non-P Cx43)-specific antibody showing the effects of ATRA on Non-P Cx43 in CGC from three patients. Culture conditions were as in part (A). Human endometrial stromal cells (HESC) were previously determined to show up-regulation of non-phosphorylated Cx43 by ATRA (Tanmahasamut and Sidell, 2005; Wu et al., 2013) and were used as positive control cells for this effect. Quantification of six patient samples showed a mean (± SEM) fold increase of 2.31 ± 0.39 induced by ATRA (p < 0.02).

Figure 4 Western blot analysis showing the combined effects of all-trans retinoic acid (ATRA) and LH or hCG on Cx43 phosphorylation. (A) Cumulus granulosa cells (CGC) were cultured for up to 72 h with vehicle control or LH (2.2 IU/ml) added at different time points before the end of the culture period as indicated (representative experiment of four patient samples). Similar enhancements of Cx43 protein levels without alteration of relative intensities of the three phosphorylation species were seen in cultures treated with LH for > 24 h (not shown). (B) Representative experiment of five patient samples where CGC were treated with 10 μM ATRA (RA), 80 IU/ml hCG, 2.2 IU/ml LH, or combinations as indicated for 48 h. Western blot analysis was performed for both total Cx43 and non-phosphorylated Cx43 (Non-P Cx43) using a Non-P Cx43 specific antibody. (C) Representative analysis of five independent experiments showing total Cx43 in KGN cells treated for 48 h with 80 IU/ml hCG, 10 μM ATRA (RA), or hCG + ATRA as indicated.
of ATRA + hCG caused a further significant increase from single agent treatments, yielding a doubling of the number of LY positive cells compared with controls.

**Discussion**

Although concentrations of retinol and expression of retinoid-metabolizing enzymes in granulosa cells from animals have previously been assessed (Zheng et al., 1999; Ikeda et al., 2005; Kipp et al., 2011), a determination of retinoids or its signaling or metabolic pathway in human granulosa cells has not, to our knowledge been reported. Our results showed high levels of retinoids in CGC and active production of ATRA from its retinol substrate. As a comparison, we also quantified retinoid levels in human endometrium and mammary glands, tissues previously shown to actively synthesize ATRA from ROL (Pierzchalski et al., 2013, 2014). In the endometrium, the action of ATRA is essential for proper decidualization (Zheng and Ong, 1998) while in the mammary gland, ATRA signaling plays an essential role in ductal morphogenesis (Wang et al., 2005). Our results showing higher concentrations of retinoids in CGC compared with these other ROL metabolizing tissues (Table I) support the contention that CGC also are a primary target of retinoid uptake and an important source of ATRA biosynthesis. Our previous work demonstrated that high follicular fluid ATRA concentrations at the IVF retrieval were associated with oocytes giving rise to higher quality (grade I) embryos (Pauli et al., 2013). We now show that those patients yielding the highest percentage of successfully fertilized oocytes had significantly higher mean levels of ATRA in their CGC. Our analysis did not show significant differences in retinol levels between the groups supporting the contention that increased retinoid biosynthesis, rather than uptake of the retinol precursor, may be important for influencing oocyte fertilization competency. The finding that ATRA is actively produced and secreted from CGC suggests that the concentrations previously determined in follicular fluid at the time of oocyte retrieval may, at least partially, reflect retinoid biosynthesis by CGC, which, as shown here, is positively associated with the most successful fertilization rates. This conclusion proposes that high follicular fluid ATRA levels associated with grade I embryos is a consequence of efficient retinoid metabolism within that follicle’s CGC, which in turn is important for production of competent oocytes.

Ovarian folliculogenesis and the production of fertilizable oocytes depend on GJIC within both the developing and the mature follicle (Khan-Dawood et al., 1998; Johnson et al., 2002; Borowczyk et al., 2007). Many connexins are expressed within the oocyte-granulosa cell complex with Cx43 being the primary connexin in CGC (Wang et al., 2009) as well as in mural granulosa cells (Kalma et al., 2004). Work over the last 30 years has suggested a well-orchestrated sequence of events mediated by GJIC between the CGC and oocyte that results in successful oocyte maturation and ovulation (Kidder and Mhawi, 2002; Senbon et al., 2003; Kalma et al., 2004; Kidder and Vanderhyden, 2010). During follicle growth, gap junction coupling between CGC and oocytes play an essential role in transferring nutrients to the oocyte necessary for its development and to maintain the differentiated state of granulosa cells by preventing them from premature luteinization. In the pre-ovulatory follicle, the LH surge then triggers deactivation of gap junctions through Cx43 phosphorylation, resulting in resumption of meiosis, extrusion of the first polar body and creation of a mature oocyte. In contrast to this negative regulation of Cx43 in the pre-ovulatory ovarian follicle, LH has been reported to have a stimulatory effect on Cx43 production in the postovulatory follicle upon the development of mural granulosa cells into granulosa lutein cells where an increase in non-phosphorylated Cx43 is noted 1–5 days after the midcycle LH surge (Khan-Dawood et al., 1998). In the developed corpus luteum, gap junctions mediated by Cx43 are critical for efficient progesterone production by luteal cells (Borowczyk et al., 2007). Animal studies have indicated that cumulus cells, prior to ovulation express little if any LhCGR (Diaz et al., 2007). However, studies of material from human subjects undergoing IVF have shown a similar regulation of LhCGR expression in CGC as occurs in luteinised granulosa cells (Hauzui et al., 2009; Jeppesen et al., 2012; Maman et al., 2012; Papamentzelopoulou et al., 2012). It has also been demonstrated that, while in vivo, CGC ovulate and have no steroidogenic function, post-IVF cumulus cells in culture spontaneously luteinize and

![Figure 5](image-url)
produce large amounts of progesterone (Hughes et al., 1990). These cells also respond to estrogenic agents in a manner previously seen with mural granulosa cells (Hughes et al., 1990). Thus, it appears that regardless of their origin, stimulation of CGC by an LH or an hCG surge during ovulation induction leaves them with the same capability as mural granulosa cells for steroidogenesis. Based on the timing of our in vitro experiments with relation to the hCG administration before egg retrieval (cultures evaluated 3–5 days post ‘pharmacologic’ LH surge), we conjecture that the primary CGC evaluated in our studies correspond to an early luteal phase phenotype (1–5 days post LH surge). Our results showed that ATRA induced a dephosphorylation of Cx43 in both primary CGC and KGN cells, which was associated with an increase in GJIC as assessed by SL/DT experiments. Similar effects on Cx43 phosphorylation by ATRA have been observed in human endometrial stromal cells and were shown to be a post-translational effect mediated through increased interaction of Cx43 with its primary phosphatase, PP2A (Wu et al., 2013). The rapidity of this mechanism of action suggests that ATRA may serve an important regulatory role in hormone-responsive tissue to increase quickly GJIC in cooperation with the hypothalamic-pituitary-ovarian axis. Our data showing a further dramatic increase in the non-phosphorylated Cx43 species when ATRA treatment was combined with LH/hCG support this hypothesis. This mechanism may help mediate the rapid increase in GJIC noted in posto-vulatory granulosa cells in the face of comparatively slower changes in gonadotrophins and steroid hormone levels (Grazul-Bilska et al., 1996a, Khan-Dawood et al., 1998). In this way, regulation of Cx43 by ATRA may play an important role in the luteinization of antral follicles and oocyte competence. This hypothesis predicts that reduced follicle ATRA production may contribute to reduced fecundity in certain reproductive disorders, such as endometriosis, via adverse effects on luteinization and oocyte competence. Support for this contention has now been provided by our recent finding that follicular fluid ATRA than control subjects (Pauli et al., 2013).

Taken together, these results present a mechanism by which retinoid metabolism in CGC may play a dynamic role in oogenesis and successful fertilization. Together with an increased understanding of the role of retinoids in ovarian follicle processes, this information may lead to a new appreciation of the importance of micronutrient repletion in normal reproductive physiology and potential cause(s) of impaired oocyte development in some women.

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Authors’ roles


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Conflict of interest

None declared.

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