Gap Junction-Mediated Communications Regulate Chromatin Remodeling During Bovine Oocyte Growth and Differentiation Through cAMP-Dependent Mechanism(s)

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ABSTRACT

Oocyte development is characterized by impressive changes in chromatin structure and function in the germinal vesicle (GV) that are crucial in conferring to the oocyte meiotic and developmental competence. During oogenesis, oocyte and follicular cells communicate by paracrine and junctional mechanisms. In cow, cumulus-enclosed oocytes (CEOs) isolated from early antral follicles have uncondensed chromatin (GV0), functionally open gap junction (GJ)-mediated communications, and limited meiotic competence. The aim of the present study was to analyze the role of GJ communications on the chromatin remodeling process during the specific phase of folliculogenesis that coincides with the transcriptional silencing and the sequential acquisition of meiotic and developmental capability. CEOs were cultured in a follicle-stimulating hormone-based culture system that sustained GJ coupling and promoted oocyte growth and transition from GV0 to higher stages of condensation. When GJ functionality was experimentally interrupted, chromatin rapidly condensed, and RNA synthesis suddenly ceased. These effects were prevented by the addition of cilostamide, a phosphodiesterase 3 inhibitor, indicating that the action of GJ-mediated communication on chromatin structure and function is mediated by cAMP. Prolonging GJ coupling during oocyte culture before in vitro maturation enhanced the ability of early antral oocytes to undergo meiosis and early embryonic development. Altogether, the evidence suggests that GJ-mediated communication between germline and somatic compartments plays a fundamental role in the regulation of chromatin remodeling and transcription, which in turn are related to competence acquisition.

INTRODUCTION

The mammalian oocyte nucleus or germinal vesicle (GV) displays a distinctive chromatin configuration, which is subjected to dynamic modifications during oocyte growth and differentiation (for review, see [1–3]). In both mouse [4] and cow [5], these changes have been related to the achievement of oocyte developmental competences, which are gradually acquired during the long-lasting period of oogenesis [1]. Therefore, the chromatin configuration represents a morphological marker of oocyte differentiation and competence [1–3, 5]. Particularly in cow, analyzing the chromatin morphology of oocytes collected from early antral follicles (diameter, 0.5–2 mm) and middle antral follicles (diameter, 2–6 mm), four discrete stages of GV, from GV0 to GV3, in which chromatin becomes progressively condensed, have been identified [5, 6]. Among these, the GV0 stage, which is characterized by a diffuse filamentous pattern of chromatin in the whole nuclear area, represents the great majority of oocytes in early antral follicles but is absent in middle antral follicles [5, 6]. Notably, GV0 oocytes are transcriptionally active and unable to progress through the metaphase II of the meiotic division. The transition to the GV1 stage represents the establishment of a repressed transcriptional state and the achievement of a full meiotic competence but a limited capacity to complete preimplantation development after in vitro fertilization (IVF) [5, 6]. GV2 and GV3 show the highest developmental capability [5].

To date, the mechanisms that regulate chromatin remodeling and transcriptional silencing in the oocyte are still poorly understood. It has been suggested that companion granulosa cells contribute in modulating these events [7, 8]. Clearly, gap junction (GJ)-mediated communications between the oocyte and the surrounding cumulus cells could have a pivotal role in these processes [9, 10]. Evidence supports this hypothesis. In the mouse model, for example, the presence of oocyte-associated granulosa cells is necessary for the progressive repression of transcription during the in vitro culture of oocytes (IVCO) isolated from preantral follicles [7]. On the contrary, in the absence of a patent GJ-mediated communication with somatic granulosa cells, transcriptional activity remains unabated in denuded oocytes (DOs) [7]. Furthermore, chromatin condensation fails to occur in the knockout mouse, where GJ-mediated communications between oocyte and cumulus cells are interrupted due to targeted deletion of the connexin 37 gene, Gja4 [11]. Finally, in cow, the pattern of uncondensed chromatin in GV0 oocytes associates with a fully open state of GJ-mediated communications, whereas the percentage of oocytes with functionally open communications significantly decreases from GV1 to GV3 oocytes [5].

Starting from these observations, the present study aimed to better clarify the role of GJ-mediated communications in the process of large-scale chromatin remodeling during the specific phase of bovine folliculogenesis that coincides with the transcriptional silencing and the sequential acquisition of meiotic and developmental capability. To this purpose, GJ functionality was modulated during the culture of cumulus-oocyte complexes isolated from early antral follicles. We then assessed the effect on chromatin configuration, transcriptional activity, and meiotic and developmental competences. In
particular, because GJ-mediated intercellular coupling is involved in the metabolic cooperation between compartments [9, 12], mainly through the regulation of cAMP [13–16], we examined the hypothesis that GJ-mediated communications regulate chromatin remodeling and transcription through a cAMP-mediated mechanism. This hypothesis was tested by the use of the oocyte-specific phosphodiesterase 3 (PDE3) inhibitor cilostamide, which is able to keep the level of cAMP high within the oocytes without altering the activity of the surrounding somatic cells [17–19].

MATERIALS AND METHODS

Cumulus-Enclosed Oocyte Collection and In Vitro Culture

All chemicals and reagents were purchased from Sigma Chemical Company except for those specifically mentioned.

Bovine ovaries were recovered at a local abattoir (IT 2270M CE; Inalca JBS S.p.A.) from pubertal females subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. The ovaries were transported to the laboratory within 2 h in sterile saline maintained at 26°C. All the subsequent procedures, unless otherwise specified, were performed between 35°C and 38°C. Small pieces of ovarian cortex were removed and examined under a dissecting microscope. Cumulus-enclosed oocytes (CEOs) were isolated from early antral follicles (diameter, 0.5–2 mm) by rupturing the follicle wall with a scalpel and washed in TCMM supplemented with 20 mM Heps, 1790 IU/L of hCG, and 0.4% bovine serum albumin (BSA) fraction V (M199D) and examined under a stereomicroscope [5]. Only CEOs medullum-brown in color, with five or more compact layers of cumulus cells and a homogenous and finely granulated ooplasm, were used.

The IVCO was performed by culturing the CEOs in 500 μl of TCMM-199, supplemented with 0.68 mM l-glutamine, 25 mM NaHCO3, 0.2% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteine, and 50 μg/ml of kanamycin for 24 h in four-well dishes (Nunc, VWR International) at 38.5°C under 5% CO2 in humidified air. According to the experimental design, IVCO medium was further supplemented with 1 x 10^-4, 1 x 10^-3, or 1 x 10^-4 IU/ml of recombinant human follicle-stimulating hormone (r-hFSH; Gonal F; Serono) and cilostamide, an oocyte-specific inhibitor of PDE3 [19].

Analysis of Functional Status of GJ-Mediated Communication Between Oocytes and Surrounding Cumulus Cells

Intercellular communications between oocytes and cumulus cells were assessed by Lucifer yellow (LY) microinjection as previously described [5, 21]. Briefly, a 3% solution of LY in 5 mM lithium chloride was pressure injected in the oocytes, and the spread of the dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope (Nikon Diaphot; Nikon Corp.). A microinjection apparatus (Narishige Co. Ltd.) was used to guide the holding and injecting micropipettes into a 50-μl drop of M199D supplemented with 5% fetal calf serum (Gibco, Invitrogen s.r.l.) and covered with mineral oil. Analysis of GJ functionality was performed within 10 min after injection by observation of LY spreading from oocytes to cumulus cells. CEOs were classified as open, partially open, or closed as previously described [21].

Assessment of Oocyte Diameter, Chromatin Configuration, and Meiotic Progression

Oocyte diameter was determined after removal of cumulus cells by gentle pipetting in living oocytes as previously described [5]. Bright-field images of DOs were taken by a digital camera (SCCD Color Video Camera; JVC), and the oocyte diameter was measured excluding the zona pellucida using ImageJ 1.44 software (National Institutes of Health) [22].

Chromatin configuration was evaluated in DOs by fluorescence microscopy after fixation in 500 μl of 60% methanol in Dulbecco PBS (DPBS) for 30 min at 4°C and staining with 1 μl/ml of Hoechst 33342. Oocytes at the GV stage were classified according to the degree of chromatin condensation as follows: GV0, with diffuse filamentous pattern of chromatin in the whole nuclear area; GV1, with a few foci of chromatin condensation; GV2, with chromatin further condensed into distinct clumps or strands; and GV3, with chromatin condensed in a single clump [5, 6]. Oocytes from the GV breakdown to the metaphase I stage were classified as intermediate; oocytes at the anaphase I, telophase I, and metaphase II stages as mature; and oocytes that could not be identified as being at any of the previous stages as degenerate [23]. Noticeably, preliminary experiments confirmed that chromatin configuration within the GV was not affected by the method used to fix the samples.

Analysis of Transcriptional Activity

Transcriptional activity was assayed by observing 5-bromouridine 5′-triphosphate (BUTP) incorporation into nascent RNAs using an immunofluorescent method as previously described [24, 25]. Briefly, 100 mM BrUTP in 2 mM PIPES buffer and 140 mM KCl was introduced into the ooplasm (10–15 pl) of a CEO by use of a microinjector (Filmjet: Eppendorf). After 1 h of incubation, CEOs were denuded, rinsed in DPBS, and fixed in 4% paraformaldehyde in DPBS for 30 min at 37°C. Oocytes were then permeabilized by treatment in 0.5% Triton X-100 for 20 min and successively incubated in 2% BSA in DPBS. DOs were incubated overnight at 4°C with a mouse monoclonal antibody raised against 5-bromo 2′-deoxyuridine but also recognizing 5-bromouridine (BrU; Caltag Laboratories) at a final dilution: 1:300 in 2% BSA in DPBS. Samples were rinsed in DPBS and incubated for 1 h at room temperature with the secondary antibody, an Alexa Fluor 488-conjugated donkey anti-mouse (Invitrogen) at a final dilution: 1:400 in DPBS. DNA was stained with 4,6-diamidino-2-phenylindole (Tris-like) in PBS (1 μg/ml). As control, some BrUTP-injected DOs were incubated directly with the secondary antibody, without pretreatment using primary anti-5-bromo 2′-deoxyuridine antibody. All samples were mounted with an antifade medium (Vectashield; Vector Laboratories) and observed by a conventional epifluorescence microscope (Eclipse E 600; Nikon Corp.). Images were taken by a digital camera (DS-5M; Nikon Corp.) while maintaining identical settings for all samples.

In Vitro Maturation, Fertilization, and Embryo Culture

The CEOs were cultured for in vitro maturation (IVM) in 500 μl of medium in four-well dishes at 38.5°C under 5% CO2 in humidified air for 24 h. IVM medium was TCMM-199 supplemented with 0.68 mM l-glutamine, 25 mM NaHCO3, 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteine, 50 μg/ml of kanamycin, and 1 x 10^-1 IU/ml of r-hFSH. After IVM, oocytes were fertilized as previously described [21]. Briefly, the contents of a straw of cryopreserved bull spermatozoa (Centro per l’incremento zootechnico, CIZ, San Miniato, Italy) was thawed and sperm separated on a 45%–90% Percoll gradient. Sperm were counted and diluted to a final concentration of 0.75 x 10^6 spermatoza/ml in fertilization medium that was a modified Tyrode solution (Tyrode albumin lactate pyruvate) supplemented with 0.6% (w/v) fatty acid-free BSA, 10 μg/ml of heparin, 20 μM penicillamine, 1 μM epinephrine, and 100 μM hypotaurine. CEOs were incubated in 300 μl of IVF medium for 18 h in four-well dishes at 38.5°C under 5% CO2 in humidified air.

After IVF, CEOs were vortexed to remove cumulus cells, for 2 min in 500 μl of a modified synthetic oviduct fluid (SOF) supplemented with 0.3% (w/v) BSA fraction V, essential and nonessential amino acids, and 0.72 mM sodium pyruvate and buturated with 10 mM Heps and 5 mM NaHCO3. Presumptive zygotes were rinsed two times in SOF-Hepes buffer and then transferred in 500 μl of embryo culture medium. Embryo culture medium was SOF buffered with 25 mM NaHCO3, supplemented with essential and nonessential amino acids, 0.72 mM sodium pyruvate, 2.74 mM myo-inositol, 0.34 mM sodium citrate, and 5% bovine serum. Incubation was performed at 38.5°C under 5% CO2, 5% O2, and 90% N2 in humidified air, as previously described [23]. Cleavage and blastocyst rates were assessed after 36 and 186 h postfertilization, respectively, and were calculated as the number of cleaved and blastocyst embryos out of the total number of CEOs at the beginning of IVM.

Statistical Analysis

All experiments were repeated three to five times. A minimum of 15 oocytes for each experimental group or treatment was evaluated in each experiment. Differences in GJ functionality at IVCO, chromatin configuration, and transcriptional activity were analyzed by the Fisher exact test. Differences in mean oocyte diameter and maturation, cleavage rates, and blastocyst rates were analyzed by one-way ANOVA followed Newman-Keuls multiple-comparison test (Prism 5; GraphPad Software, Inc.). Regardless of the statistical test, P < 0.05 was considered to be significant.

RESULTS

We first examined the effect of different concentrations of r-hFSH on the persistence of the intercellular coupling between...
oocyte and cumulus cells after 24 h of IVCO of CEOs isolated from early antral follicles. Based on previous studies in mouse [26, 27], CEOs were cultured in control medium, with no hormonal treatment, or in the presence of decreasing concentrations of r-hFSH (1 × 10^{-3}, 1 × 10^{-4}, and 1 × 10^{-5} IU/ml) starting with the concentration that is commonly adopted for IVM (1 × 10^{-1} IU/ml). The functional status of GJ-mediated communications was monitored at the time of isolation (0 h) and after 24 h of IVCO. As shown in Figure 1, the percentage of CEOs with open GJ-mediated communications was significantly higher in the group treated with 1 × 10^{-4} IU/ml of r-hFSH compared with the control and the groups treated with 1 × 10^{-1} and 1 × 10^{-3} IU/ml of r-hFSH (P < 0.05). Moreover, the coupling status of CEOs treated with 1 × 10^{-4} IU/ml of r-hFSH was similar to that observed at the time of isolation from the follicle. As shown in Figure 2, this treatment also promoted the highest significant increase in oocyte diameter after 24 h of culture when compared with other experimental treatments. In fact, after isolation from the follicles, the oocyte diameter (mean ± SEM) was 107.7 ± 0.5 μm, but it reached 111.7 ± 0.7 μm after 24 h of IVCO with 1 × 10^{-4} IU/ml of r-hFSH.

In the subsequent experiment, we evaluated the effect of decreasing concentrations of r-hFSH on the process of large-scale chromatin remodeling by assessing the chromatin configuration at the time of oocyte collection and after 24 h of IVCO in the absence of hormonal stimulation (control) or in the presence of 1 × 10^{-3}, 1 × 10^{-4}, and 1 × 10^{-5} IU/ml of r-hFSH. As shown in Table 1, at the time of collection, 85%, 12.5%, and 2.5% of the oocytes were classified as GV0, GV1, and GV2 respectively, whereas the GV3 stage was absent. Importantly, after 24 h of IVCO, the percentage of oocytes that progressed from the GV0 to the GV1 stage was significantly higher in the group treated with 1 × 10^{-3} IU/ml of r-hFSH when compared to the control and the groups treated with 1 × 10^{-3} and 1 × 10^{-1} IU/ml of r-hFSH, indicating that the maintenance of a patent bidirectional communication is accompanied by the gradual remodeling of the chromatin. On the other hand, the percentages of oocytes that resumed meiosis in the different experimental groups, considered as the sum of oocytes that reached the intermediate and mature stages, was significantly lower in the group treated with 1 × 10^{-4} IU/ml of r-hFSH compared to the groups treated with 1 × 10^{-3} and 1 × 10^{-1} IU/ml of r-hFSH (27.7% vs. 43.6% and 48.3%, respectively; P < 0.05, t-test) and was not significantly different from the percentage of oocytes that resumed meiosis in the control group (23.2%). This indicates that treatment with 1 × 10^{-4} IU/ml of r-hFSH maintained the oocytes in meiotic arrest while promoting chromatin transition from GV0 to GV1, whereas the drop in communications due to high r-hFSH doses was accompanied by premature meiotic resumption without a proper chromatin remodeling.

Having established that the administration of 1 × 10^{-4} IU/ml of r-hFSH during IVCO was effective in maintaining the functional coupling between the oocytes and the surrounding cumulus cells as well as in promoting oocyte growth and gradual chromatin transition, we used this culture system in all following experiments. To examine the relationship between the functional state of GJ-mediated communications and the process of chromatin remodeling, the intercellular coupling between oocytes and cumulus cells was disrupted by adding 1-heptanol [20] during 4 h of IVCO in the presence of 1 × 10^{-4} IU/ml of r-hFSH, and the effect on chromatin configuration was assessed. In these experiments, because GJ-mediated communications contribute to the regulation of cAMP concentration in the oocytes, the involvement of a cAMP-dependent mechanism was also examined. To this aim, CEOs were treated with 10 μM cilostamide, a PDE3 inhibitor able to keep the level of cAMP in the oocytes high without interfering with the functionality of the surrounding somatic cells [17–19]. As shown in Figure 3, treatment of CEOs with 1-heptanol induced an abrupt and premature chromatin condensation after 4 h of culture. The percentage accounted for by the GV-condensed group, being the sum of the GV1, GV2, and GV3 stages, increased significantly, with the majority of oocytes (64.5%) within the GV-condensed group being represented by the GV2 and GV3 stages. However, the presence of 10 μM
cilostamide during uncoupling treatment prevented premature chromatin condensation.

Because companion granulosa cells could play an active role in modulating oocyte transcription through the GJ [7, 25], transcriptional activity was analyzed in oocytes immediately after isolation and after 4 h of culture according to the experimental design described above. As shown in Figure 4, transcriptional activity was classified as absent, moderate, or high on the basis of the intensity of the immunofluorescence signal of incorporated BrUTP. As shown in Figure 5, the presence of 1-heptanol induced a significant decrease in the percentage of oocytes with high and moderate transcriptional activity, with a concomitant significant increase of the percentage of oocytes in which no activity was detected. On the other hand, the presence of cilostamide prevented a drop in transcription, maintaining elevated percentages of transcriptionally active oocytes, which were indeed similar to the group treated with 1 × 10⁻⁴ IU/ml of r-hFSH.

Our data suggest that maintaining a functional coupling between oocytes and surrounding cumulus cells, during the culture of oocytes isolated from early antral follicles, is crucial to allow a proper chromatin remodeling and transcriptional silencing of the oocyte genome, which in turn would account for an increase in the oocyte competencies. To test this hypothesis, CEOs isolated from early antral follicles were matured, fertilized, and cultured in vitro according to the standard protocols adopted for in vitro production (IVP) of bovine embryos or were cultured for 24 h with 1 × 10⁻³, 1 × 10⁻², and 1 × 10⁻¹ IU/ml of r-hFSH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>n*</th>
<th>GV0 (%)</th>
<th>GV1 (%)</th>
<th>GV2 (%)</th>
<th>GV3 (%)</th>
<th>Intermediate (%)</th>
<th>Mature (%)</th>
<th>Total maturing oocytes (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0 h</td>
<td>120</td>
<td>106 (85.0)ᵃ</td>
<td>15 (12.5)ᵃ</td>
<td>3 (2.5)</td>
<td>0 (0.0)⁠</td>
<td>0 (0.0)ᵃ</td>
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<tr>
<td>FSH 10⁻¹ IU/ml</td>
<td>24 h</td>
<td>112</td>
<td>68 (60.7)ᵇ</td>
<td>10 (8.9)ᵇ</td>
<td>4 (3.6)</td>
<td>4 (3.6)</td>
<td>10 (8.9)ᵇ</td>
<td>16 (14.3)ᵇ</td>
<td>26 (23.2)ᵇ</td>
</tr>
<tr>
<td>FSH 10⁻³ IU/ml</td>
<td>24 h</td>
<td>110</td>
<td>54 (49.1)ᶜ</td>
<td>6 (5.4)ᵃ</td>
<td>2 (1.8)</td>
<td>0 (0.0)</td>
<td>44 (37.9)ᶜ</td>
<td>20 (18.2)ᶜ</td>
<td>48 (43.6)ᶜ</td>
</tr>
<tr>
<td>FSH 10⁻⁴ IU/ml</td>
<td>24 h</td>
<td>159</td>
<td>75 (47.2)ᶜ</td>
<td>35 (22.1)ᵇ</td>
<td>3 (1.9)</td>
<td>2 (1.2)</td>
<td>11 (6.9)ᵇ</td>
<td>33 (20.7)ᶜ</td>
<td>44 (27.7)ᵇ</td>
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ᵃ Total number of oocytes analyzed in each treatment. Data were analyzed by Fisher exact test. b,c Values with different superscripts within columns are significantly different (P < 0.05).

discussion

Previous studies have suggested that companion granulosa cells contribute with a signal, still undisclosed, involved with modulating transcription and large-scale chromatin remodeling in oocytes [7, 8]. The present study expands this observation by demonstrating that proper maintenance of GJ functionality during in vitro culture of early antral oocytes guarantees gradual chromatin remodeling, contributes to regulation of transcription during oocyte growth, and promotes sequential achievement of meiotic and developmental competence.

Furthermore, we demonstrate that the functional status of GJ-mediated communications oversees transcriptional activity regulation and large-scale chromatin remodeling. To our knowledge, this has not been previously reported in cow. The specific role of FSH and intraoocyte cAMP level on the GJ and the impact on the chromatin remains to be investigated, however.

In our culture system, maintenance of open GJ-mediated communications in cumulus oocyte complexes isolated from early antral follicles was achieved with the administration of r-hFSH at a concentration lower than what is usually adopted during IVM. Instead, a high dose of r-hFSH causes a drop in patent bidirectional communications and premature meiotic rates were significantly increased by addition of cilostamide during IVCO (Fig. 6C).

Finally, to verify if the increased developmental competence observed in the cilostamide-treated group was possibly due to a prolongation of intercellular coupling between oocytes and cumulus cells during IVCO before IVM, we assessed the status of GJ-mediated communication in CEOs cultured in the presence of cilostamide for 24 h. As expected [17, 18, 28, 29], cilostamide induced a significantly higher percentage of oocytes with open plus partially open GJ-mediated communications when compared to other experimental groups (Fig. 7).

Our data suggest that maintaining a functional coupling between oocytes and surrounding cumulus cells, during the culture of oocytes isolated from early antral follicles, is crucial to allow a proper chromatin remodeling and transcriptional silencing of the oocyte genome, which in turn would account for an increase in the oocyte competencies. To test this hypothesis, CEOs isolated from early antral follicles were matured, fertilized, and cultured in vitro according to the standard protocols adopted for in vitro production (IVP) of bovine embryos or were cultured for 24 h with 1 × 10⁻³ IU/ml of r-hFSH in the presence or absence of 10 µM cilostamide and subsequently treated according to standard IVP protocols (Fig. 6A). As shown in Figure 6B, IVCO with 1 × 10⁻⁴ IU/ml of r-hFSH before IVM significantly increased the percentage of mature oocytes. Meiotic competence was further improved by the presence of cilostamide. Similarly, cleavage and blastocyst formation rates were significantly increased by addition of cilostamide during IVCO (Fig. 6C).

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**DISCUSSION**

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resumption without a proper chromatin remodeling. This is consistent with previous findings in cow, where low concentrations of FSH have been proved to be effective in promoting intercellular GJ-mediated coupling during IVM of CEOs collected from middle antral follicles [30]. Moreover, a low dose of FSH was beneficial to oocyte morphology and health status during bovine preantral follicle growth in vitro, whereas a high dose of FSH induced a premature retraction of transzonal projections and disruption of connexin-mediated somatic-germ cell interactions [31]. These effects can be partially explained by experimental evidence suggesting that FSH plays a role in the control of connexin 43 (Cx43; Gja1) gene expression and cell-to-cell coupling in a rat granulosa cell line (GFSHR-17) [32]. Increased GJ communication was also associated with a general decrease of Cx43 phosphorylation [32]. On the contrary, increased Cx43 phosphorylation is reported to be associated with GJ closure during meiotic resumption [33, 34]. Thus, we can speculate that administration of $1 \times 10^{-4}$ IU/ml of r-hFSH in our system regulates Cx43 phosphorylation in a way that promotes GJ coupling whereas a higher dosage does not.

The GJ functionality plays an important role in chromatin structure and function, as indicated by the fact that proper maintenance of GJ functionality over 24 h of culture guarantees the gradual chromatin remodeling (from GV0 to GV1) and by the evidence that treatment with the uncoupler 1-heptanol suddenly induces condensation of chromatin and decreased transcriptional activity. This is in agreement with the general idea that chromatin remodeling accompanies the sequential achievement of oocyte developmental capacity and with the
hypothesis in which increase of chromatin condensation may represent a consequence of interrupted communication between oocytes and follicular cells, as previously suggested [5]. Thus, if coupling is prematurely interrupted—that is, when oocytes have not yet acquired full competence, such as when they are still committed to accumulating transcripts and other molecules—unexpected chromatin condensation can be triggered, preventing proper and gradual functional differentiation of large-scale chromatin structure, which provides an important epigenetic mechanism for the developmental control of global gene expression during oocyte differentiation. Although the present data as well as previous findings support this hypothesis, further studies are required to confirm this assumption.

In mouse, the absence of cumulus cell investment during the culture of preantral oocytes caused the majority of denuded GV-stage oocytes to remain transcriptionally active [7]. At least three explanations are possible for this discrepancy. The first two relate to the physiological status of the animal model and to the growth phase of the follicle from which an oocyte is isolated. In the present study, early antral oocytes were isolated from pubertal, normal-cycling cows, whereas the study in mouse [7] was performed on preantral oocytes collected from prepubertal, primed mice. Thus, the timing of when the functional coupling between oocytes and cumulus cells is interrupted could determine the effect on chromatin structure and function. Moreover, oocytes from preantral follicles probably are incapable of condensation, because they are far from being fully grown. The third possible explanation relates to paracrine factors secreted by oocytes and somatic cells that regulate many important aspects of early ovarian follicle development and oocyte growth [9, 35–37]. Transcriptional activity in mouse was evaluated in DOs and in the absence of cumulus cells in the culture system. Thus, the possible effect of factors involved in the cross-talk between oocytes and cumulus cells in modulating their functions reciprocally could not be ruled out in this system [38, 39]. On the contrary, in our system, factors secreted by cumulus cells could have had a profound impact on chromatin structure and function.

The effect of 1-heptanol on large-scale chromatin structure and function was nullified by addition of cilostamide to culture medium. This indicates that the functional status of GJ-mediated communications may affect both transcriptional activity and large-scale chromatin remodeling, potentially through cAMP-dependent mechanism(s), because cilostamide acts as a specific inhibitor of the oocyte-specific PDE3, an enzyme-degrading cAMP [40–42]. It is known that cAMP is involved in the transmission of FSH signal transduction during follicle growth [13]. Several mechanisms control intraoocyte cAMP level. It is synthesized in the oocyte by constitutively
active G protein-coupled receptor type 3 (GPR3) [43], and it is supplied to the oocyte by neighboring cumulus cells through the GJ [44, 45]. Intraoocyte cAMP content is also sustained by somatic cells that supply cGMP to the oocyte [46], which inhibits the oocyte-specific PDE3 activity. High levels of oocyte cAMP are known to maintain the oocyte in meiotic arrest [19] by activating protein kinase A, which in turn suppresses the activity of maturation-promoting factor (for review, see [13]). In vivo, ovulatory gonadotropin surge induces the closure of the GJ [33, 47] and causes a decrease in follicular and oocyte cGMP levels [48, 49], which relieves the inhibition of PDE3 [18, 41] that induces a reduction in oocyte cAMP and meiotic resumption. Besides these well-characterized mechanisms of action by cAMP, the present results may suggest that cAMP could be involved in the control of the activity of factors that modulate transcription and large-scale chromatin remodeling during the final phase of oocyte growth and before the resumption of meiosis.

In the present study, prolonging the coupling between oocytes and cumulus cells during oocyte culture before IVM enhanced the ability of early antral oocytes to undergo meiosis after IVM and to develop into a blastocyst before IVF. This is in agreement with the general concept that coupling between oocytes and granulosa cells must be maintained to promote oocyte growth and differentiation and with the results of previous studies in which treatments that maintain the intraoocyte level of cAMP have the capability to prevent the loss of cumulus-oocyte GJ-mediated communications and to increase oocyte developmental competence [17, 21, 28, 29, 50–54]. Moreover, in the present and previous systems [55], maintenance of a proper cAMP concentration seems to be the main requirement to promote regular chromatin transition and gradual transcriptional silencing, thus encouraging final oocyte differentiation.

In conclusion, differentiation of chromatin structure and function in the GV is an important epigenetic mechanism for the control of gene expression during oogenesis supporting mammalian oocytes in achieving successful embryonic development. This process is controlled by intercellular coupling between oocytes and somatic cells that cooperate to the maintenance of an appropriate intraoocyte cAMP level.

In brief, understanding chronological events through oocyte differentiation can have a major impact on research into ovarian physiology as well as on clinical practice. Advancement in knowledge, then, may help us with identifying, and also improving, the vulnerable segment of all biological processes that are recapitulated in vitro through assisted reproductive technology. The implications of the present and future findings are particularly relevant to human IVM practice. As described above, in the present system, a high concentration of r-hFSH disturbs chromatin and interrupts communications, which clearly compromises oocyte quality. In such a context, as also recently highlighted [56], more caution is highly advisable when using a high dose of gonadotropins in human assisted reproductive technology.

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