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Abstract
Purpose This study was aimed to test the hypothesis that gap junction mediated communications (GJC) are required to allow the progressive chromatin configuration remodeling (from GV1 to GV3) process to occur in fully grown oocytes in order to gain the final step of developmental competence acquisition, and that a premature disruption of GJC can alter this process.
Methods Bovine cumulus-oocytes complexes collected from medium antral follicles were cultured for 2, 4, 6 and 8 h in the presence of 10^{-4} IU/ml of r-hFSH and with 2 mM of the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) to prevent meiotic resumption. GJC functionality and chromatin configuration were monitored during the culture period. After meiotic arrest, the developmental capability of oocytes was assessed after IVM and IVF.
Results IBMX was effective in significantly sustaining GJC up to 6 h and maintaining meiotic arrest, when compared to control group. Moreover, the percentage of oocytes with less condensed chromatin (GV1) decreased within 4 h of culture, while the proportion of GV2 oocytes gradually increased up to 6 h. Interestingly, a decline in the proportion of GV2 oocytes and an increase in the proportion of GV3 oocytes were observed after 6 h of culture, when the major drop of GJC occurred. On the contrary, when GJC were uncoupled by adding 3 mM of 1-heptanol or through cumulus cells removal, chromatin condensation occurred rapidly throughout the culture period, more promptly in denuded oocytes. Moreover, the maintenance of GJC during meiotic arrest was accompanied by a significant increase of developmental competence compared to the control, as indicated by a higher percentage of hatched blastocysts and blastocyst cell number.
Conclusions Altogether, our data indicate that both paracrine and junctional mechanisms are involved in modulating large-scale chromatin structure during the final phase of oocyte differentiation.

Keywords Meiotic arrest · Phosphodiesterase (PDE) · 3-isobutyl-methyl-xanthine · Cumulus cells · Gap junctions · Chromatin · Oocyte maturation · Embryo development

Capsule Oocyte-cumulus cells coupling plays a key role in large-scale chromatin configuration changes during the final phase of oocyte differentiation.

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Introduction

The mammalian oocyte nucleus or germinal vesicle (GV) is modified at the level of chromatin patterning during oogenesis and alterations in the location and extent of heterochromatization have been related to the oocyte metabolic properties and competencies [1–3], (reviewed in [4–7]). During meiotic arrest at Prophase I, the oocyte's chromosomes lose their individuality and form a loose chromatin mass, which in turn undergoes profound and dynamic rearrangements within the GV concentrating in a small area of the nucleoplasm before the meiotic resumption. These large- scale chromatin configuration changes have been studied in several mammals and

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progressive condensation of the chromatin has been related to the achievement of meiotic and developmental potential [7]. In cow, uncondensed chromatin (GV0 configuration) is found in growing oocytes from early antral follicle of small diameter (0.5–2 mm), while three patterns of chromatin configuration (from GV1 to GV3) have been characterized in fully-grown oocytes isolated from 2–6 mm middle antral follicles [3]. The gradual chromatin configuration remodeling process starts with the appearance of few foci of condensation in GV1 oocytes and proceeds with the formation of distinct clumps of condensed chromatin in GV2 oocytes; the GV3 is the stage where the maximum level of condensation is reached with chromatin organized into a single clump.

This remodeling process has been related to the progressive increase of embryonic developmental competence [3, 8]. However, the mechanisms and factors controlling large-scale chromatin configuration changes have remained elusive.

In the follicle, the oocyte is arrested at phophase I (GV stage) of meiotic division. In assisted reproductive technologies (ART), the physical removal of the cumulus-oocyte complex (COC) from middle size antral follicles initiate oocyte's meiotic resumption before the journey of the oocyte development has been completed and causes asynchrony between cytoplasmic and nuclear maturation, thus decreasing oocyte developmental competence (Reviewed in [9–11]). Spontaneous resumption of meiosis is mainly due to a decrease in the concentration of intra-oocyte cyclic adenosine monophosphate (cAMP), which has a major role in controlling mammalian oocyte maturation [12–14].

Several attempts have been made in order to mimic a physiological system for oocyte in vitro culture that could take into account the time for completing the developmental competence acquisition. These culture systems (namely pre-maturation systems) that precede the in vitro maturation [10], are based on the control of spontaneous meiosis resumption through the addition of either cAMP analogues or adenylate cyclase activator or PDE inhibitors (specific or general) or through a combination of these treatments [15–22].

Recent findings indicated that in cow the highest level of chromatin condensation in oocytes isolated from mid-antral follicles (2–6 mm) corresponded to the highest incidence of gap junction mediated communications interruption [3]. Likewise, the achievement of meiotic and developmental capability of growing oocytes isolated from early antral follicles depends on GJ-mediated communications functionality [19]. We recently demostrated the role of cAMP and of gap-junction functionality on large-scale chromatin remodeling and developmental capability acquisition in oocytes with a GV0 chromatin configuration isolated from early antral follicles (0.5–2 mm), when oocytes are still growing, transcriptionally active and meiotically non competent [19]. In particular, the preservation of functional coupling during a follicle-stimulating hormone (FSH)-based culture system before in vitro maturation has been shown to promote oocyte growth and chromatin transition from uncondensed (GV0) to higher stages of condensation and to enhance the ability of oocytes collected from early antral follicles to undergo meiosis and early embryonic development [19]. In particular, when GJ functionality was experimentally interrupted, chromatin rapidly condensed, and RNA synthesis suddenly ceased [19].

Starting from the above observations, this study was conducted to test the hypothesis that functional coupling is required to allow the chromatin remodeling process to occur also in oocytes at more advanced stage of differentiation, i.e. in fully grown oocytes isolated from 2–6 mm antral follicles in order to gain the final step of developmental competence acquisition and that a premature disruption of GJC can alter this process.

Material and methods

Oocyte collection and culture

All the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned. Bovine ovaries were recovered at the abattoir (INALCA S.p.A., Ospedaletto Lodigiano, LO, IT 2270 M CE, Italy) from pubertal females (4–8 years old) subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. Ovaries were transported to the laboratory within 2 h in sterile saline (NaCl, 9 g l^{-1}) maintained at 26 °C. All subsequent procedures, unless differently specified, were performed at 35–38 °C. As previously described [23], COCs were retrieved from mid-sized antral follicles (2–6 mm) with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane QLD, Australia) in M199 supplemented with 20 mM of HEPES, 1790 units/L of Heparin and 0.4 % of BSA (M199-H). For in vitro meiotic arrest (IVA) experiments, COCs were aspirated in M199-H supplemented with the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) at the final concentration of 0.5 mM [12, 14]. After examination under a stereomicroscope, only COCs medium-brown in color, with five or more complete layers of cumulus cells enclosing an oocyte with finely granulated homogenous ooplasm were used. Groups of COCs were cultured in M-199 added with 0.68 mM L-glutamine, 25 mM $NaHCO_3$, 0.4 % BSA fatty acid free, 0.2 mM sodium pyruvate (M199-C) and depending on the experimental design, supplemented either with 10^{-4} IU/ml of r-hFSH [19] (Gonal-F, Serono, Rome, Italy) and with 2 mM of IBMX (In vitro Arrest group, IVA) or with 10^{-1} IU/ml r-hFSH (control group, CTRL), in humidified air under 5 % CO_2 at 38.5 °C. In vitro maturation was performed in M199-C supplemented either with 10^{-1} IU/ml of r-hFSH.

Control COCs and IVA COCs were matured for 24 h and 22 h, respectively.

In vitro fertilization and embryo culture

After in vitro maturation, oocytes were fertilized as previously described [24]. Briefly, the contents of a straw of cryopreserved bull spermatozoa (CIZ, S. Miniato Pisa, Italy) was thawed and cells separated on a 45–90 % Percoll gradient. Sperms were counted and diluted to a final concentration of 0.5×10^6 spermatozoa/ml in fertilization medium that was a modified Tyrode's solution (TALP) supplemented with 0.6 % (w/v) BSA fatty acid free, 10 μg/ml heparin, 20 μM penicillamine, 1 μM epinephrine, and 100 μM hypotaurine. COCs and sperms were incubated for 18 h at 38.5 ° C under 5 % CO_2 in humidified air. After in vitro fertilization, presumptive zygotes were washed and cumulus cells were removed by vortexing for 2 min in 500 μl of a modified synthetic oviduct fluid (SOF) supplemented with 0.3 % (w/v) BSA fraction V, fatty acid free, MEM essential and non-essential aminoacids, 0.72 mM of sodium pyruvate, and buffered with 10 mM of HEPES and 5 mM of $NaHCO_3$. Presumptive zygotes were rinsed and transferred in embryo culture medium, which was SOF buffered with 25 mM of $NaHCO_3$, supplemented with MEM essential and non-essential amino acids, 0.72 mM of sodium pyruvate, 2.74 mM of myo-inositol, 0.34 mM of sodium citrate and with 5 % of calf serum (Gibco, Invitrogen, San Giuliano Milanese, Milan, Italy). Incubation was performed at 38.5 ° C under 5 % CO_2, 5 % O_2 and 90 % N_2 in humidified air. After 48 h post insemination, the number of cleaved embryos was rapidly recorded. At the end of culture period, blastocyst rate was assessed and embryos were fixed in 60 % methanol in PBS. Cell nuclei were counted under fluorescence microscope after staining with 0.5 mg/ml of propidium iodide [23].

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 [14]. Briefly, a 3 % solution of LY in 5 mM lithium chloride was pressure injected into the oocytes and the spread of the dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope (Nikon Diaphot; Nikon Corp., Tokyo, Japan). A microinjection apparatus (Narishige Co. Ltd.) was used to guide the holding and injecting micropipettes into a 50 μl drop of M199-H 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. COCs were classified as open, partially open, or closed as previously described [14].

Assessment of chromatin configuration and meiotic progression

Changes of large-scale chromatin configuration were assessed at the time of collection and after different times of culture by Hoechst 33342 staining and fluorescence microscopy analysis. COCs were freed of cumulus cells by vortexing [23]. Denuded oocytes were incubated for 10 min in the dark in M199-H containing 1 μg/ml Hoechst 33342 and transferred into a 5 μl drop of the same medium. Chromatin configuration was evaluated under an inverted fluorescence microscope (Olympus IX50, magnification 40X, Milan, Italy) and was classified according to the degree of chromatin mass condensation within the GV as previously described [3, 19]. 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].

Statistical analysis

All the experiments were repeated three to five times. Observations from all the experiments were pooled. Statistical analyses were performed using Prism GraphPad (GraphPad Software, San Diego, CA, USA). Differences in GJ functionality were analyzed by Fisher's exact test. In the other experiments statistical significances were tested using one-way ANOVA followed by Fisher's protected LSD post hoc test or by Student t-Test, when means of two groups were compared. Values of $P < 0.05$ were considered significant.

Results

The presence of IBMX maintains meiotic arrest, promote the functional coupling between oocyte and surrounding cumulus cells and accompanies a progressive chromatin remodeling

A total of 998 bovine COCs collected from medium antral follicles were cultured for 2, 4, 6 and 8 h in the presence of low concentration of r-hFSH (10^{-4} IU/ml) and in the absence (control) or in the presence of 2 mM of IBMX to maintain meiotic arrest in vitro (IVA). During the culture period GJC functionality was monitored by means of LY microinjection and chromatin configuration was assessed by Hoechst staining. As shown in Table 1, when compared to control group where IBMX was absent, the presence of the PDE inhibitor was effective in significantly sustaining oocyte-cumulus cells coupling up to 6 h of culture. Moreover, as indicated in Table 2, IBMX maintained COCs meiotically arrested at the GV stage up to 8 h of culture, similar to the time of collection (0 h).

Table 1 Effect of the administration of IBMX on gap junction functionality

Treatment	Time	n*	Open (%)	Partial (%)	Closed (%)	Open+Partial (%)
CTRL	0 h	44	23 (52.27)[a]	13 (29.55)[a]	8 (18.18)[e]	36 (81.82)[a]
CTRL	4 h	43	9 (20.93)[b]	9 (20.93)[a]	25 (58.14)[bc]	18 (41.86)[bc]
IVA	4 h	41	18 (43.90)[a]	12 (29.27)[a]	11 (26.83)[de]	30 (73.17)[a]
CTRL	6 h	31	3 (9.68)[c]	5 (16.13)[a]	23 (74.19)[ab]	8 (25.81)[c]
IVA	6 h	31	10 (32.26)[b]	9 (29.03)[a]	12 (38.71)[cd]	19 (61.29)[ab]
CTRL	8 h	37	2 (5.41)[c]	1 (2.7)[b]	34 (91.89)[a]	3 (8.11)[d]
IVA	8 h	30	3 (10.0)[c]	5 (16.67)[a]	22 (73.33)[ab]	8 (26.67)[c]

*Total number of oocytes analyzed in each treatment. Data were analyzed by Fisher exact test

[a,b,c] Values with different superscripts within columns are significantly different ($P<0.05$)

During IVA, the percentage of oocytes with less condensed chromatin (GV1) decreased within 4 h of culture, while the proportion of GV2 oocytes gradually increased up to 6 h (Table 3a). Interestingly, a decline in the proportion of GV2 oocytes and an increase in the proportion of GV3 oocytes were observed between 6 and 8 h of culture, when the major drop of GJC occurred (Table 1).

Disruption of gap junctional coupling induces a premature chromatin condensation

Compared to COCs during IVA (Table 3a), when GJC were uncoupled by adding to the culture medium 3 mM of 1-heptanol (N=317 total oocyte, Table 3b) or through cumulus cells removal (N=306 total oocytes, Table 3c), chromatin condensation occurred rapidly throughout the culture period, more promptly in denuded oocytes.

Table 2 Effect of the administration of IBMX on the maintenance of meiotic arrest in intact cumulus-oocyte complexes

Treatment	Time	n*	GV (%)	Intermediate (%)	Degenerate (%)
CTRL	0 h	107	98.4±1.6[a]	0.8±0.8[a]	0.8±0.8
CTRL	2 h	78	89.6±2.8[a]	9.3±3.4[a]	1.2±1.2
IVA	2 h	82	97.5±1.5[a]	1.1±1.1[a]	1.3±1.3
CTRL	4 h	78	64.7±4.0[b]	33.0±5.2[b]	2.3±1.5
IVA	4 h	92	95.9±1.9[a]	3.1±2.0[a]	1.0±1.0
CTRL	6 h	75	33.2±5.2[c]	62.9±5.5[c]	3.9±1.6
IVA	6 h	82	92.7±4.3[a]	6.1±3.2[a]	1.2±1.2
CTRL	8 h	74	24.8±5.2[c]	71.2±2.6[c]	4.1±1.4
IVA	8 h	73	89.9±1.8[a]	7.2±2.6[a]	2.9±1.8

*Total number of oocytes analyzed in each treatment

Data are expressed as mean ± s.e.m

Data were analyzed by one-way ANOVA Fisher's Protected LSD test

[a,b,c] Values with different superscripts within columns are significantly different ($P<0.05$)

The progressive chromatin remodeling is related to an increase in embryonic developmental potential

After 6 h of IVA in the presence of IBMX, COCs were matured for 22 h and fertilized in vitro. Blastocyst rate and embryo cell number were assessed after 7 days of culture. As shown in Table 4, the maintenance of oocyte-cumulus cells coupling during meiotic arrest was accompanied by a significant increase of developmental competence compared with control, as indicated by a higher percentage of hatched blastocysts and mean blastocyst cell number.

Discussion

The molecular mechanisms regulating changes in large-scale chromatin configuration during mammalian oocyte growth and differentiation remain poorly understood, nonetheless it is clear that multiple factors can contribute to this process.

In the present study we confirmed and expanded our previous findings in growing oocytes isolated from early antral follicles (0.5–2 mm), in which we demonstrated that the maintenance of a functional bidirectional coupling between the oocytes and cumulus cells is crucial for an orderly chromatin transition. In fact, data presented here indicate that the maintenance of a proper oocyte-cumulus cells coupling affects chromatin configuration changes also in oocytes at more advanced stages of folliculogenesis (i.e. fully grown oocytes isolated from 2–6 antral follicles), which in turn increase embryonic developmental competence. This is of particular importantce since these two populations of oocytes (growing and fully grown) are metabolically and functionally different. In fact, while growing oocytes are transcriptionally active and meiotically non competent, fully grown oocytes are transcriptionally silent and have acquired both meiotic and developmental competence.

Our data indicate that the administration of the nonspecific PDE inhibitor IBMX is able to reversibly maintain oocytes in meiotic arrest and to prolong, in combination with low-FSH

Table 3 Chromatin remodeling during in vitro meiotic arrest (IVA) of intact cumulus-oocyte complexes (A) and after blocking GJC with Heptanol (B) or denuded oocytes (DO) after cumulus cells removal (C)

Treatment	Time	n*	% GV1	% GV2	% GV3	% Interm	% Deg
A							
	0 h	75	14.8±3.4^{a}	34.5±2.2^{a}	50.7±3.5	0±0^{a}	0±0^{a}
IVA COC	2 h	87	14.0±3.1^{a}	41.9±1.7^{abc}	44.1±1.8	0±0^{a}	0±0^{a}
IVA COC	4 h	81	5.4±2.6^{b}	47.5±4.6^{bc}	46.2±6.6	1±1^{a}	0±0^{a}
IVA COC	6 h	80	2.2±1.3^{b}	51.4±4.7^{c}	39.9±4.6	5.3±0.8^{b}	1.2±1.2^{ab}
IVA COC	8 h	76	1.2±1.3^{b}	38.0±3.8^{ab}	52.5±6.4	4.7±2.7^{b}	3.6±2.4^{b}
B							
	0 h	75	14.8±3.4^{a}	34.5±2.2^{a}	50.7±3.5^{a}	0±0^{a}	0±0^{a}
IVA COC Hep	2 h	92	10.6±2.7^{a}	44.8±7.6^{ab}	39.9±7.3^{ab}	4.7±2.3^{ab}	0±0
IVA COC Hep	4 h	90	4.0±1.8^{b}	54.4±10.9^{ab}	33.1±7.4^{ab}	6.9±4.3^{ab}	1.6±1.6
IVA COC Hep	6 h	62	0±0^{b}	53.5±4.0^{ab}	41.6±4.2^{ab}	1.6±1.6^{ab}	3.3±1.7
IVA COC Hep	8 h	73	0±0^{b}	59.3±5.9^{b}	22.9±3.2^{b}	12.9±5.4^{b}	5±3.5
C							
	0 h	75	14.8±3.4^{a}	34.5±2.2	50.7±3.5^{ab}	0±0^{a}	0±0
IVA DO	2 h	78	5.8±1.7^{b}	30.9±6.0	61.9±6.2^{ab}	1.3±1.3	0±0
IVA DO	4 h	84	0±0^{c}	43.3±10.1	49.4±10.0^{a}	5.1±3.2	2.2±2.2
IVA DO	6 h	70	0±0^{c}	31.2±7.9	66±5.7^{ab}	2.8±2.8	0±0
IVA DO	8 h	74	0±0^{c}	24±5.3	71.2±7.4^{b}	2.3±1.3	2.6±1.5

*Total number of oocytes analyzed in each treatment

Data are expressed as mean ± s.e.m

Data were analyzed by one-way ANOVA Fisher's Protected LSD test

[a,b,c] Values with different superscripts within columns are significantly different ($P<0.05$)

administration, the functional oocyte-cumulus cells coupling that in turn guarantees gradual chromatin remodeling (from GV1 to GV3) during the final phase of fully grown oocyte differentiation. The direct involvement of GJC is further supported by the fact that the disruption of gap junctional coupling induced a rapid premature chromatin condensation. Interestingly, the removal of cumulus cells altered the process of chromatin remodeling with different dynamics compared to 1-heptanol induced breakdown of communication. Since paracrine factors secreted by oocytes and somatic cells regulate many important aspects of early ovarian follicle development and oocyte growth [10, 25–28], we hypothesize that both paracrine and junctional mechanisms are involved in modulating large-scale chromatin configuration changes.

Table 4 Effect of the administration of IBMX during IVA on embryonic developmental competence after fertilization

Treatment	n*	Blastocyst (%)	Hatched on total Blastocyst (%)	Mean cell number per embryo
CTRL	292	32.4±4.7^{a}	41.3±9.1^{b}	82.2±4^{b}
IVA	349	29.7±3.5^{a}	57.1±6.7^{a}	91.3±4.3^{a}

*Total number of oocytes analyzed in each treatment

Values are expressed as mean ± s.e.m. Data were analyzed by Student t-test

[a,b] Values with different superscripts within columns are significantly different ($P<0.05$)

As already mentioned, the present work is in agreement with previous studies that pointed out the central role of GJC in the coordination of both large-scale chromatin configuration and transcriptional activity during oocyte growth in mouse [29, 30] and cow [3, 19]. Earlier experiments in the bovine model, where low doses of FSH sustained GJC functionality in cumulus oocytes complexes from early antral follicles, provided further insight into the mechanisms by which GJC may modulate gradual changes in large-scale chromatin configuration and transcription in growing oocytes [19]. In that system, treatment with the uncoupler 1-heptanol induced abrupt chromatin condensation and decreased in transcription. However, the addition of the oocyte-specific PDE3 inhibitor cilostamide [31–33] to the culture medium nullified this effect indicating that the functional status of GJC may affect both transcriptional activity and remodeling of large-scale chromatin configuration in growing oocytes, potentially through cAMP-dependent mechanism(s) [19].

Besides the well-characterized mechanisms of action by which cAMP is known to regulate meiotic resumption (Reviewed in [34, 35]) the results of the present work further indicate that cAMP could be also involved in the control of the activity of factors that modulate large-scale chromatin remodeling during the final differentiation of fully grown oocytes, right before the resumption of meiosis. Intra-oocyte cAMP level is maintained by endogenous adenylate cyclases and constitutively active G-protein-coupled receptors [36]. cAMP is generated also by cumulus cells and then transported into the oocyte through

gap junctions [37, 38]. The manipulation of intracellular cAMP concentration has been demonstrated to influence functional coupling in bovine COCs [14, 39]. In our culture system, the maintenance of open GJ-mediated communications in cumulus oocyte complexes isolated from mid-antral follicles was achieved with the combined administration of IBMX and of r-hFSH at a concentration lower than what is usually adopted during IVM. This is consistent with previous findings in cow, where low concentrations of r-hFSH have been proved to be effective in promoting intercellular GJ-mediated coupling during IVM of COCs isolated from mid-antral follicles [14, 17]. On the contrary, higher doses of r-hFSH triggered a drop in patent bidirectional communications [14, 19].

In our study, prolonging the coupling between oocytes and cumulus cells during oocyte pre-maturation increased embryonic developmental potential after standard in vitro maturation and fertilization. This is in agreement with the general concept that coupling between oocytes and granulosa cells must be maintained to promote the final growth and differentiation of the oocyte, and with previous studies in which treatments that maintained the intra-oocyte level of cAMP have the capability to prevent the loss of cumulus-oocyte GJ mediated communications and to increase oocyte developmental competence [14–16, 18, 22, 40–43]. Moreover, in our as well as in previous systems [19, 44], the maintenance of a proper cAMP concentration seems to be the main requirement to promote regular chromatin transition thus endorsing oocyte differentiation.

No doubt that the experimental manipulation of large-scale chromatin configuration in vivo and in vitro will provide a tool to determine the key cellular pathways and oocyte-derived factors involved in genome-wide chromatin modifications. Yet, assessment of large-scale chromatin configurations has also key implications in ARTs both in human and domestic mammals. It has been shown that different patterns of chromatin configuration are indicative of different metabolic properties; thus, they could represent a morphological marker to select a population of oocytes with different cultural needs. Several studies support the notion that in vitro treatments aimed to improve the developmental capability of immature oocytes can have a different effect with pre-maturation culture depending on the metabolic status of the oocyte at the time of its removal from the follicular environment [20, 42, 45]. This has been confirmed also by morphological studies in cow, which demonstrated that pharmacological pre-maturation system can negatively affect oocyte belonging to medium antral follicles when compared with those isolated from earlier stages [46].

Growing oocytes isolated from early antral follicles are an homogeneous population characterized by filamentous dispersed chromatin (GV0, [3]) while fully grown oocytes isolated from 2–6 mm follicles show different pattern of chromatin organization, GV1, GV2 and GV3, which represented 24, 31, and 45 % respectively. Moreover, 98 % of GV0 growing oocytes have fully open communications while 64 % of GV3 isolated from 2–6 mm follicles showed functionally closed communications with cumulus cells, significantly higher than in group GV1 and GV2 [3]. Therefore an important aspect that must be considered is the actual growth and differentiation stage of the immature oocytes subjected to the PDE inhibitor-based treatment since oocytes isolated from 2–6 mm antral follicles represents a population of oocytes with different metabolic properties that may require different cultural conditions [7].

In particular, in human ART this must be taken into account when considering the origin of the oocyte after repetitive FSH treatment [11]. In this case, success in attempting to improve the oocyte developmental capability in vitro may be strongly dependent on the large-scale chromatin configuration and junctional coupling status once the oocytes have been recovered from the follicle.

It is of extreme importance noticing that efforts to manipulate in vitro large-scale chromatin configuration must be performed prudently. In fact, even though it is true that the chromatin configuration of an oocyte is symptomatic of its developmental capability at the time of its isolation from the follicle, pharmacological treatments forcing chromatin abruptly into a high-condensed state may not necessarily be beneficial to the oocyte competence [47]. Therefore, design of pre-maturative strategies must take into account that chromatin condensation and spatial reorganization should occur gradually and orderly, recapitulating the process that naturally occurs in vivo.

As in earlier work [19], we restate that the maintenance of a proper functional coupling between oocyte and cumulus seems to be crucial in sustaining an orderly chromatin condensation in vitro. Thus, if coupling is prematurely interrupted - i.e., when oocytes have not yet acquired full competence—sudden chromatin condensation can be triggered, thus preventing proper and gradual differentiation of large-scale chromatin configuration and function [11]. In view of all given considerations, knowledge of the molecular mechanism(s) leading the oocyte to remodel its chromatin configuration under physiological conditions will be beneficial for ARTs.

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