The Histone Deacetylase Inhibitor Trichostatin A Induces Retrogressive Chromatin Decondensation in the Germinal Vesicle of Porcine Cumulus-enclosed Oocytes

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Abstract: *In porcine growing oocytes, chromatin remains diffuse. As the oocytes approach their final size, 120 μm, the chromatin becomes partly condensed and forms a perinucleolar sheath. These changes occur simultaneously with a decrease in transcriptional activity. In many other cell types, it has been shown that the state of acetylation of nucleosome core histones is essential in chromatin remodeling and transcription so that partial chromatin condensation in oocytes may involve the recruitment of histone deacetylases. In order to test this hypothesis, porcine oocyte-cumulus cell complexes were treated with a specific inhibitor of histone deacetylases, trichostatin A (TSA). The perinucleolar sheath loosened or disappeared after 24 hr culture with 100 nM TSA, but after further culture in TSA-free medium, about 40% developed the perinucleolar sheath again. In the presence of 4 mM hypoxanthine, the decondensation induced by TSA progressed rather slowly, but continuously, for 72 hr. When oocytes were denuded before culture, spontaneous maturation occurred in the presence of TSA. Thus, the inhibitor-induced decondensation is not attributed to the inhibition of the maturation-promoting factor. These results suggest that deacetylation of histones may be involved in chromatin remodeling in oocytes near the end of the growth phase.*

Key words: *Porcine oocyte, Germinal vesicle, Chromatin, Histone deacetylation, Trichostatin A*

Introduction

In mammalian oocytes progression of meiosis remains arrested for a long time at the prophase of the first meiotic division [1]. During this period, chromatin in the germinal vesicle (GV) is not static: first, growing oocytes display very strong transcriptional activity [2–7]; second, chromatin configuration is greatly altered in the latter half of the growth period [2, 6–9]; and third, recent studies have proven the existence of growth-dependent regulation of gene imprinting [10]. In the second of these activities, chromatin configuration is categorized broadly into two kinds by the presence or absence of a condensed mass of chromatin around the nucleolus. In brief, growing oocytes contain stringy chromatins [8, 9], while fully grown oocytes develop a continuous perinucleolar chromatin sheath [8, 9].

Because changes in chromatin configuration are a continuous process, there are also intermediate stages between the above two types. Growing porcine oocytes

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smaller than 110 μm in diameter possess diffuse and filamentous chromatin [8, 9]. As the oocytes grow to 115–120 μm, the chromatin thickens, yet remains still stringy [8]. Only after they attain full size, >120 μm, do we see the condensed chromatin sheath around the nucleolus [8, 9]. This stage has been categorized as GVI by Motlik and Fulka [11], and the appearance of this morphology has been correlated with oocyte competence to progress to metaphase II after meiotic resumption *in vitro* [8].

It has been shown that changes in chromatin configuration in oocytes correlate with a decline in transcriptional activity [2, 7, 12]. This is consistent with the basic processes that are already well known in other cell types and in which acetylation of nucleosome core histones is essential in both chromatin remodeling and transcription. The balance between the activities of histone acetyltransferases and histone deacetylases determines the acetylation status of chromatin [13] and histone deacetylation may therefore be directly involved in chromatin behavior in mammalian oocytes at the end of the growth phase. Previous studies have shown that histone acetyltransferases and histone deacetylases are present and play a role in oocytes and preimplantation embryos in mice and cattle [14–16], though it is not known whether these enzymes are involved in the regulation of chromatin behavior in growing oocytes. To test this hypothesis, deacetylation was artificially abrogated by treating oocytes with a specific inhibitor of histone deacetylation, trichostatin A (TSA).

The experimental design was to treat fully grown porcine oocytes with TSA for up to 72 hr to determine whether or not the inhibitor induced retrogressive decondensation of the chromatin. To achieve this, spontaneous meiotic maturation was prevented by culturing oocytes with mural granulosa cells [17]. In some experiments, 4 mM hypoxanthine was supplemented in the medium to ensure the maintenance of meiotic arrest [18]. Our results clearly suggest that TSA is effective in diffusing the perinucleolar condensed chromatin sheath and that TSA-induced decondensation is reversible by the removal of the TSA.

Materials and Methods

Collection and culture of oocytes

Porcine ovaries were obtained from prepubertal gilts at a local abattoir. From the ovaries, antral follicles 4–6 mm in diameter, each of which contained the fully grown oocyte (>120 μm), were dissected in phosphate-buffered saline containing 0.1% polyvinylalcohol according to the techniques described by Moor and Trounson [19]. The follicles were opened in HEPES-buffered TCM 199 (Earl's salt, Nissui Pharmaceutical, Tokyo, Japan) and oocyte-cumulus cell complexes associated with a group of mural granulosa cells, hereinafter referred to as complexes, were isolated from the follicles with fine forceps [17]. Only oocytes with a healthy appearance were used for culture. The basic culture medium was TCM199 (Nissui) supplemented with 0.46 mM sodium pyruvate (Wako, Osaka, Japan), 80 mg/l kanamycin sulfate, and 3 mg/ml bovine serum albumin (Sigma, St Louis, MO, USA). Cultures were performed in an incubator maintained at 38.5°C under an atmosphere of 5% CO_2 and 95% air. Approximately 10 complexes were cultured in each culture dish (Falcon 1008; Becton Dickinson Labware, Bedford, MA, USA) containing 3 ml of medium. Under these conditions, complexes did not adhere to the surface of the culture dish.

Trichostatin A treatment

TSA (Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 1 mM and stored at –30°C until use. Just prior to use, the stock solution was diluted with DMSO to give a final concentration of 0 (control), 10, 50 or 100 nM TSA. The final concentration of DMSO was 0.1% for all groups.

Examination of chromatin configuration

At the end of the culture period, oocytes were denuded of cumulus cells by drawing them in and out of small-bore pipettes. The oocytes were then mounted onto slides and fixed with acetic alcohol. The following day, the chromatin was stained with acetic orcein and examined under an inverted microscope. The chromatin configuration at the GV stage was classified according to the definition of Motlik and Fulka [11]. Fully grown oocytes possess the condensed chromatin sheath around the nucleolus, and such configuration was categorized as GVI [11]. Stage GVI is followed by Stage GVII, in which chromatin clumps in the periphery of the nuclear membrane and the perinucleolar chromatin sheath are seen [11]. In the present study, Stage GVI and Stage GVII were combined and designated GVI–II. Two additional chromatin configurations, GVIII and GVIV, also were distinguished before the completion of the germinal vesicle breakdown (GVB) [11]. In the present study, we added two more classes, fibril-GVI–II (F-GVI–II) and "stringy"

chromatin. The stringy chromatin is commonly seen in the growing oocytes, and Stage F-GVI–II, which is characterized by a loosened chromatin sheath, may represent an intermediate status between Stage GVI and the stringy configuration.

Statistical analysis

Data were pooled from at least three independent experimental replicates in which all groups were included in each experiment. The groups were compared by means of Fisher's exact probability test. When $P<0.05$, the difference was considered to be significant.

Experiment 1

Complexes were cultured for 24 hr in medium supplemented with 0, 10, 50 or 100 nM TSA. Six experiments were conducted.

Experiment 2

All complexes were cultured with 100 nM TSA for the first 24 hr, and then were transferred to fresh medium either supplemented with 100 nM TSA or without TSA. At 0, 24 or 48 hr after the beginning of culture, oocytes were denuded and fixed for examination. Four experiments were conducted.

Experiment 3

Hypoxanthine, a natural potent inhibitor of meiotic resumption, was added to the medium to prevent spontaneous meiotic resumption during culture [18]. Although the original formulation for TCM 199 includes 0.0025 mM hypoxanthine, we further supplemented 0, 0.04, 0.1, 0.8 or 4 mM hypoxanthine. Complexes were cultured for 24 hr in the medium supplemented with 100 nM TSA and with various concentrations of hypoxanthine. Four experiments were conducted.

Experiment 4

Complexes were cultured for up to 72 hr with 4 mM hypoxanthine and 100 nM TSA. The oocytes recovered at 0, 24, 48 or 72 hr of culture were fixed for examination as described above. Three experiments were conducted.

Experiment 5

At the time of isolation from the follicles, the oocytes were denuded of cumulus cells by pipetting, and were cultured for 24 hr as described above with 100 nM TSA or without TSA. Oocytes were classified according to their stage of meiotic maturation: GV, diakinesis or prometaphase I, metaphase I, anaphase I or telophase I, or metaphase II.

Results

In preliminary experiments, growing oocytes and fully grown oocytes were fixed just after isolation from the follicles. As shown in Fig. 1A, the diffuse status of chromatin was obvious in growing oocytes about 105 μm in diameter. The chromatin in nearly full-sized oocytes, about 115 μm, was a bit thicker, yet was still stringy (Fig. 1B). In contrast, fully grown oocytes, >120 μm, had a condensed chromatin sheath around the nucleolus (Fig. 1C). About 90% of oocytes were at Stage GVI–II. As shown in Fig. 1D, after 24 hr in medium without TSA, the chromatin rim appeared the same as it had been before culture, suggesting that granulosa cells surrounding the oocytes prevented spontaneous meiotic resumption. On the other hand, after 24 hr culture in medium supplemented with 100 nM TSA, oocytes had a loosened chromatin sheath (Fig. 1E), and were designated F-GVI–II. Some oocytes had more decondensed chromatin, so that the chromatin sheath completely disappeared (Fig. 1F), and were designated as having stringy chromatin. These configurations resembled those observed in growing oocytes.

Table 1 shows the effect of different concentrations of TSA on the oocyte chromatin configuration after 24 hr culture of the complexes. In control medium, 76.5% of oocytes still showed the GVI–II configuration. As the concentration of TSA increased, the numbers of oocytes with the perinucleolar sheath intact decreased, but more oocytes had the loosened chromatin. At a concentration of 100 nM TSA, 40.7% of oocytes had stringy chromatin, whereas the rates of stringy chromatin in oocytes treated with the medium with 0 and 10 nM TSA were only 8.6% and 10.0%, respectively. The percentages of oocytes that had undergone GVB and that had degenerated were 2–7% and 2–4%, respectively, regardless of the dose of TSA.

Our next experiments were designed to determine whether culturing TSA-treated complexes in TSA-free medium could reverse the chromatin decondensation in the oocytes (Fig. 2). During the first 24 hr in medium with 100 nM TSA, the frequency of GVI–II decreased from 88.4% to 8.3, but when the complexes were cultured in TSA-free medium, this frequency increased again to 42.6%. On the other hand, when complexes were then transferred to fresh medium with 100 nM TSA, there were no GVI–II oocytes after 24 hr. It is

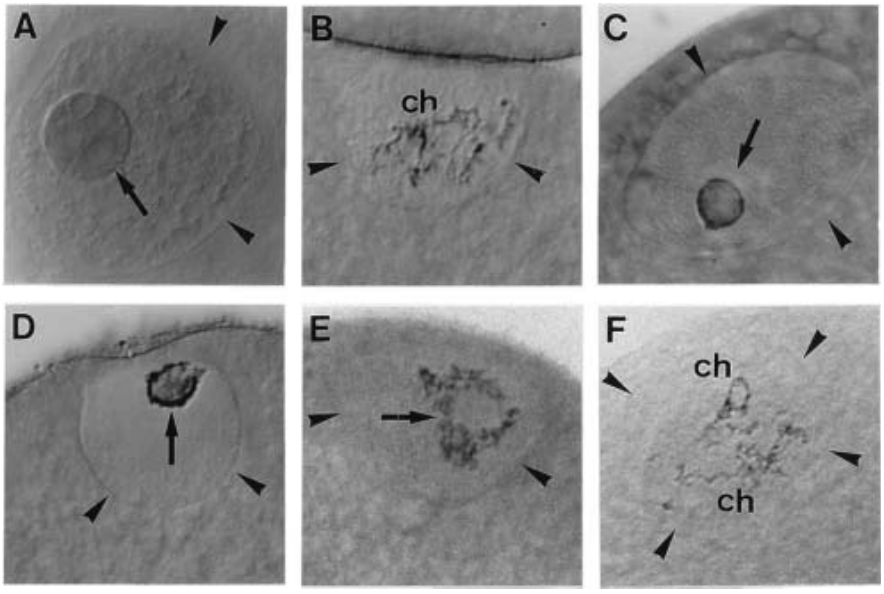

Fig. 1. The chromatin configuration of porcine germinal vesicle stage oocytes before and after treatment with TSA. The nuclear membrane is indicated with arrow heads. (A) The germinal vesicle in a growing oocyte isolated from an early antral follicle about 1 mm in diameter. An orcein-stained nucleolus is visible (arrow), and the decondensed chromatin is diffused over the entire region of the germinal vesicle. (B) The germinal vesicle of a growing oocyte obtained from a mid-antral follicle about 3 mm in diameter. The chromatin strings (ch) are distributed. (C) The perinucleolar condensed chromatin sheath (arrow) in a fully grown oocyte, GVI–II, isolated from a fully grown follicle larger than 5 mm in diameter. (D) The perinucleolar chromatin sheath (arrow) remained present for 24 hr in control medium. (E) Partially diffused chromatin (arrow) designated F-GVI–II in the oocytes cultured for 24 hr in the presence of 100 nM TSA. (F) The stringy chromatin (ch) after 24 hr of TSA treatment.

Table 1. Chromatin configurations of the porcine oocytes evaluated after 24 hr of culture in medium supplemented with different concentrations of TSA

TSA (nM)	No. of Oocytes[a]	No. of oocytes (%)					
		At the germinal vesicle stage				Undergoing GVB	Degene-rating
		GVI–II	F-GVI–II	Stringy	GVIII-IV		
0	81	62 (76.5)^{b}	4 (4.9)^{d}	7 (8.6)^{c}	4 (4.9)	2 (2.5)	2 (2.5)
10	80	46 (57.5)^{c}	16 (20.0)^{c}	8 (10.0)^{c}	2 (2.5)	5 (6.3)	3 (3.8)
50	79	20 (25.3)^{d}	29 (36.7)^{b}	21 (26.6)^{b}	1 (1.3)	5 (6.3)	3 (3.8)
100	81	12 (14.8)^{d}	28 (34.6)^{bc}	33 (40.7)^{b}	3 (3.7)	3 (3.7)	2 (2.5)

The designations of GVI–II, F-GVI–II, Stringy and GVIII–IV are described in the text. GVB, germinal vesicle breakdown. [a]: Data from 6 experiments, [b–d]: Values with different superscripts in the same column are significantly different (P<0.05).

important to note that the stringy chromatin after 48 hr of TSA treatment was observed to be extremely fine threads. We thus conclude that the reappearance of the perinucleolar sheath was not due to the simple elongation of the culture period. By the end of 48 hr of culture altogether, 14.6% of oocytes had reached metaphase II, suggesting that in some complexes granulosa cells had lost the ability to maintain meiotic arrest. Alternatively, the connection between oocytes and granulosa cells could have been lost as the culture proceeded, possibly due to the increasing fragility of the granulosa cell mass, as judged by the ease with which

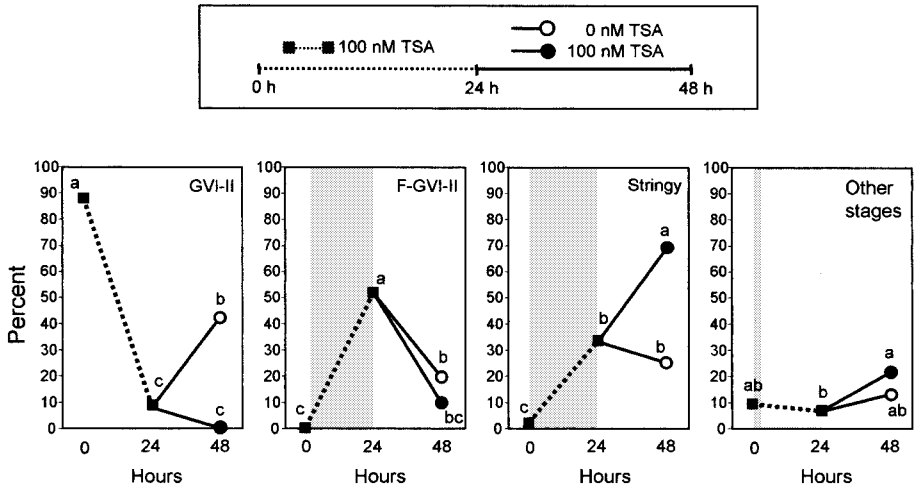

Fig. 2. Oocyte-cumulus/granulosa cell complexes were cultured for 24 hr with TSA and then transferred into medium supplemented either with 100 nM TSA or without TSA for another 24 hr culture. The designations of GVI–II, F-GVI–II and Stringy are described in the text and in Fig. 1 above. Ten to 12 oocytes were included in each group in each experiment. Different letters in the same graph indicate significant differences (P<0.05).

Table 2. Effects of hypoxanthine on the chromatin configuration of porcine oocytes cultured in the TSA-supplemented medium[a]

Hypoxanthine (mM)	No. of Oocytes[b]	No. of oocytes (%)				
		At the germinal vesicle stage				Undergoing GVB
		GVI–II	F-GVI–II	Stringy	GVIII-IV	
0	48	3 (6.2)[d]	13 (27.1)	26 (54.2)[c]	2 (4.2)	4 (8.3)
0.04	47	3 (6.4)[d]	14 (29.8)	26 (55.3)[c]	1 (2.1)	3 (6.4)
0.1	47	9 (19.1)[d]	16 (34.1)	15 (31.9)[d]	4 (8.5)	3 (6.4)
0.8	50	7 (14.0)[d]	19 (38.0)	21 (42.0)[cd]	2 (4.0)	1 (2.0)
4	49	20 (40.8)[c]	14 (28.6)	13 (26.6)[d]	1 (2.0)	1 (2.0)

The designations GVI–II, F-GVI–II, Stringy and GVIII–IV are described in the text. GVB: germinal vesicle breakdown. [a]: Complexes were cultured for 24 hr, [b]: Data from 4 experiments, [c–d]: Values with different superscripts in the same column are significantly different (P<0.05).

they could be torn off by pipetting.

The experiments described above suggest that TSA may also influence the health and/or function of the granulosa cells. In order to provide conditions that assure the maintenance of meiotic arrest, hypoxanthine was added to the culture medium. First, effects of various concentrations of hypoxanthine were examined (Table 2). When no hypoxanthine was added at all, only 6.2% of oocytes exhibited the GVI–II configuration after 24 hr of 100 nM TSA treatment. Unexpectedly, however, 40.8% of oocytes treated with 4 mM hypoxanthine exhibited the GVI–II configuration even in the presence of TSA. Accordingly, a lower percentage of oocytes possessed the stringy chromatin than in medium without the addition of hypoxanthine.

To further assess the combined effects of 100 nM TSA and 4 mM hypoxanthine, complexes were cultured for 72 hr. As shown in Fig. 3, the majority of oocytes eventually had possessed the stringy chromatin.

Finally, the effect of TSA on spontaneous meiotic maturation was examined. As shown in Table 3, spontaneous maturation to metaphase II was evident regardless of the presence or absence of 100 nM TSA in culture medium. Thus, TSA does not affect the chromatin configuration by modulating/interfering $p34^{cdc2}$/cyclin B kinase activity in the oocytes.

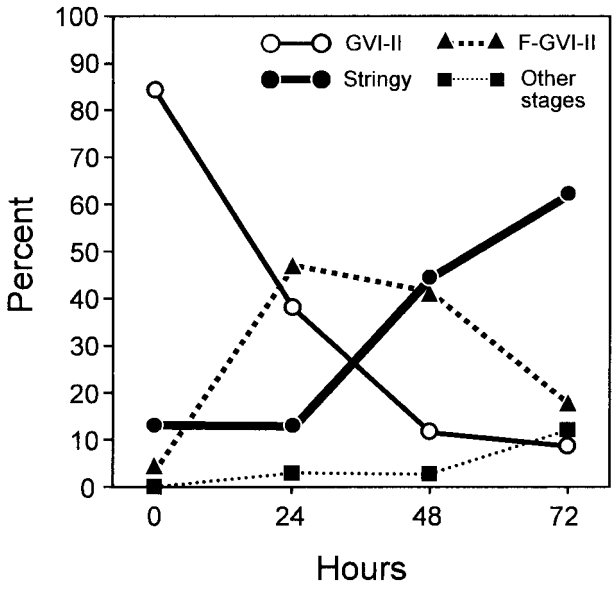

Fig. 3. Oocyte-cumulus/granulosa cell complexes were cultured in medium supplemented with 100 nM TSA and 4 mM hypoxanthine for up to 72 hr. The number of oocytes included at each time point in each experiment is: 0 hr = 10, 10, 11; 24 hr = 11, 11, 10; 48 hr = 12, 11, 11; 72 hr = 12, 11, 11.

Discussion

Little is known about the regulation of chromatin behavior in growing oocytes in mammals. As suggested by studies with mouse maturing oocytes [16], acetylation of histones probably exerts a regulatory effect. In the present study, the perinucleolar condensed chromatin mass in the fully grown porcine oocytes was dissolved after TSA treatment. The perinucleolar chromatin mass appears in oocytes at nearly the end of the growth phase, so that a mechanism that thickens a some of the oocyte chromatin may be activated at this stage. In many cell types, the reversible acetylation of core histones in nucleosomes plays an important role in the folding of the chromatin [20, 21]. When histones become deacetylated, the chromatin fiber is modified to be packed [20, 21]. Since TSA is a specific inhibitor of the histone deacetylases, this reagent may have induced a hyper-acetylated status in the oocyte chromatin. Overall modification of the chromatin would have been reflected in the disappearance of the condensed mass in the oocytes treated with TSA. Considering that hyper-acetylation of core histones is associated with active transcription, it seems reasonable to speculate that the transition from hyper-acetylation to hypo-acetylation of the core histones occurs in the porcine oocytes when they complete the growth phase.

There are a number of conditions that may be responsible for inducing the disappearance of the perinucleolar sheath. In maturing oocytes, for example, the sheath disappears as part of the maturation process. The chromatin at Stage GVIII somewhat resembles the stringy chromatin observed in the present study, but we observed a return to Stage GVI–II after the removal of TSA. In sharp contrast, extended TSA treatment for 48 or 72 hr resulted in the formation of the fine thread-like chromatin in the majority of oocytes. A proportion of these had extremely fine chromatin that does not fit any configurations in the oocytes undergoing GVB. Therefore, the TSA-induced decondensation was not a sign of the onset of meiotic maturation.

It has been well documented that both the nucleolar structure and the chromatin configuration change in conjunction with oocyte growth and the decline of transcriptional activity [3, 5, 22–24]. Interestingly, inhibition of rRNA synthesis by actinomycin D can modify the morphology of the nucleolus [25]. The nucleolus of growing oocytes in wholemount preparations can be stained clearly with aceto-orcein, though in fully grown oocytes the nucleolus is much denser and does not stain. When the perinucleolar sheath is formed, the nucleolus is usually seen as a transparent sphere located in the nucleoplasm lightly stained with orcein (compared to the chromatin). In the

Table 3. Meiotic progression by denuded porcine oocytes in culture medium supplemented with TSA or without TSA[a]

TSA (nM)	No. of Oocytes[b]	No. of oocytes (%) at the stage of				
		GV	Diakinesis-Pro-MI	MI	AI-TI	MII
0	37	2 (5.4)	3 (8.1)	10 (27.0)	8 (21.6)	14 (37.9)
100	35	2 (5.7)	1 (2.9)	8 (22.9)	6 (17.1)	18 (51.4)

GV: germinal vesicle; MI: metaphase I; AI-TI: anaphase I - telophase I; MII: metaphase II. [a]: Oocytes were cultured for 24 hr, [b]: Data from 3 experiments.

present experiments, the chromatin became diffused after treatment with TSA, though the nucleolus was still unstainable by orcein. In those oocytes, therefore, TSA probably affected only some of the extensive alterations that had occurred. A significantly longer time could be necessary to induce retrogressive modification in the nucleolus.

In mice, the formation of the perinucleolar sheath is coincidental with the acquisition and expression of meiotic competence [26]. In the pig, however, the competence to undergo GVB is developed when oocytes reach about 105 μm in diameter [8, 9]. The chromatin of those oocytes is still diffuse, and there is therefore no major difference in chromatin before and after the acquisition of competence [8, 9]. Rather, the ability to progress to metaphase II seems to be correlated with the appearance of the perinucleolar chromatin sheath [8]. This change in morphology might include a topological organization of the chromatin. For example, the decondensed chromatin could become physically tangled during maturation, preventing chromatin from separating at the transition from metaphase I to anaphase I. If this is the case, the phenomenon is similar to that observed in maturing oocytes whose anaphase I entrance was prevented by an inhibitor of topoisomerase II [27], but recent studies suggest that the chromatin in growing oocytes may not be tangled even if it is forced to condense to become chromosomes. Bao *et al.* [28, 29] transferred germinal vesicles of oocytes of various sizes into enucleated fully grown oocytes, and found that progression to metaphase II was not affected by the size of donor oocytes. Thus, it appears that chromatin in growing oocytes is not sufficiently intertwined to prevent meiotic division, or if it is, that it can untangle easily in the cytoplasm of fully grown oocytes. In either case, it is important to resolve the mechanism(s) whereby the cytoplasm of fully grown oocytes can transform the diffuse chromatin into highly condensed chromosomes.

In the present experiments, the granulosa cells were used primarily to prevent precocious meiotic resumption. TSA treatment, however, could have affected also these cells and diminished the ability to sustain the meiotic arrest. To overcome this potential problem, 4 mM hypoxanthine was utilized. This was successful, and almost 90% of oocytes had remained in the GV stage for as long as 72 hr in the presence of TSA. In the meantime, however, hypoxanthine obviously retarded the decondensation of the GVI–II chromatin for the first 24 hr, although it eventually allowed the formation of the stringy configuration. It is not known whether this stabilizing effect was mediated by the granulosa cells or hypoxanthine could directly modify the oocyte chromatin under the influence of TSA.

Recent studies on mice indicate that companion granulosa cells play an important role in modifying certain aspects of chromatin remodeling as well as the onset of global transcriptional repression during the final stages of oocyte growth [12]. The culture condition in our experiments was not optimal for granulosa cells, and some complexes lost the functional integrity that they had had prior to culture. Supplementation of hypoxanthine does not seem to be sufficient. Improvement of culture conditions is necessary to determine the role of granulosa cells in the regulation of oocyte development.

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