Zygote **17** (November), pp. 307–314. © Cambridge University Press 2009
doi:10.1017/S0967199409005437 First Published Online 22 May 2009

Histone deacetylase inhibition improves meiotic competence but not developmental competence in growing pig oocytes

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Date submitted: 7.10.08. Date accepted: 24.02.09

Summary

In fully grown pig oocytes, meiotic maturation *in vitro* is retarded by inhibition of histone deacetylases by trichostatin A (TSA). In growing oocytes with partial meiotic competence, culture with TSA has no significant effect on the meiotic maturation. Growing oocytes treated with TSA mature mainly to metaphase I. The ratio of oocytes that mature to metaphase II is very limited. After transient exposure to TSA, the maturation of growing oocytes with partial meiotic competence takes a different course. When these oocytes are first cultured in a TSA-free medium, then cultured for another 24 h with 100 nM TSA and finally again in a TSA-free medium for 24 h, the ratio of oocytes that mature to metaphase II significantly increases reaching 59%. When oocytes were cultured for the same length of time without transient exposure to TSA, only 19% matured to metaphase II. Those oocytes that matured to metaphase II after transient exposure to TSA were successfully activated using calcium ionophore. However, the subsequent cleavage was very limited. We can conclude that transient exposure of growing pig oocytes with partial meiotic competence to TSA increases oocyte meiotic competence, but it does not enhance developmental competence after parthenogenetic activation.

Keywords: Activation, Meiotic competence, Oocyte, Pig, Trichostatin A

Introduction

Meiotic maturation of mammalian oocytes starts during the fetal development of the female, but it is arrested at late diplotene. The ability to resume meiosis and to continue in oocyte maturation beyond this stage is acquired gradually during oocyte growth. In fully grown oocytes, meiosis can progress through the stages of germinal vesicle breakdown, metaphase I, anaphase I and telophase I to metaphase II, when oocyte maturation is again arrested. The capability of passing through all these stages of oocyte maturation is called full meiotic competence. During growth, the oocyte enters a stage when meiotic competence develops only partially (Wassarman, 1988). These oocytes acquire the ability to undergo germinal vesicle breakdown and to progress to metaphase I. However, these oocytes are unable to exit from metaphase I, reach metaphase II and to complete their maturation (Szybek, 1972; Sorensen & Wassarman, 1976; Motlik & Fulka, 1986; Motlik, 1989).

Current methods for *in vitro* embryo production or cloning using the transfer of nuclei of somatic cells depend on sufficient numbers of fully grown oocytes with full meiotic competence. However, the pool of such oocytes in the ovary is limited (Ock *et al.*, 2007). The pool of growing oocytes with partial meiotic competence is abundant, but it cannot be used for these purposes. Not only is there limited meiotic competence, but the developmental competence of these oocytes is compromised as well (Lee *et al.*, 2007; Wu & Tian, 2007).

Numerous precisely timed and coordinated events must occur in the nucleus and cytoplasm for the full meiotic and developmental competence of the oocyte. Histone acetylation, which plays an important role in oocyte maturation, belongs among these events occurring in the oocyte nucleus (Kim *et al.*, 2003; Wang

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et al., 2006a,b). The importance of histone acetylation was also demonstrated during the maturation of pig oocytes (Endo *et al.*, 2005; Bui *et al.*, 2007). The level of histone acetylation results from the action of two types of enzymes – histone acetyltransferases and histone deacetylases (Bertos *et al.*, 2001; Marmorstein & Roth, 2001).

During the growth phase of the oocyte, the histones are very heavily acetylated and the level of histone acetylation culminates when the oocyte completes its growth (Kageyama *et al.*, 2007b). After resumption of meiosis, the histones are intensely deacetylated both in fully grown and growing oocytes (Spinaci *et al.*, 2004; Bui *et al.*, 2007; Ola *et al.*, 2007). In oocytes with full meiotic competence, the histones are acetylated again when the oocytes progress through metaphase I (Tang *et al.*, 2007). This pattern of acetylation is not followed by maturing growing oocytes and this fact could be the reason for their low meiotic and developmental competence (Ola *et al.*, 2007).

Based on these data we have postulated the hypothesis that artificial intervention, which has an effect on histone acetylation, can increase meiotic and developmental competence in growing oocytes. When cultured *in vitro*, maturation in the majority of oocytes with partial meiotic competence is arrested at metaphase I. These oocytes only seldom enter anaphase I. The maturation of fully grown oocytes with full meiotic competence is accompanied by histone acetylation when these oocytes enter the stages of anaphase I or telophase I (Tang *et al.*, 2007). We can conjecture that histones may be acetylated in growing oocytes with maturation blocked at metaphase I, when these oocytes are transiently exposed to the inhibitor of histone deacetylases. This treatment can mimic the increase in histone acetylation observed at anaphase I or telophase I during maturation in fully grown oocytes with full meiotic competence. This situation could facilitate the complete maturation of growing oocytes to metaphase II.

The objective of the present study was to monitor the effect of permanent or transient inhibition of histone deacetylases in growing pig oocytes with partial meiotic competence, in which maturation was spontaneously arrested at metaphase I. We used trichostatin A (TSA) for the inhibition of histone deacetylases.

Materials and methods

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the estrous cycle and transported to the laboratory within 1 h in a saline solution (0.9% natrium chloride) at 39°C. Fully grown oocytes were collected from the follicles by aspirating follicles that were 2–5 mm in diameter with a 20-gauge needle. Only oocytes with compact cumuli were chosen for further study.

Growing oocytes of different size categories were obtained from thin strips (10–15 mm long, 1–2 mm wide) dissected from the surface of the ovaries using a scalpel. The strips of ovarian tissue were placed in Petri dishes containing a culture medium. The oocytes were released from their follicles by opening the follicular wall using the tip of a 25-gauge needle. The internal diameter of the oocytes (without zona pellucida) was measured with an ocular micrometer mounted on a microscope. Only those oocytes surrounded by several layers of cumulus cells were chosen for further experiments. Before culture, the oocytes were washed three times in a culture medium.

Modified M199 medium was used as the culture medium. Medium M199 (GibcoBRL, Life Technologies) was supplemented with sodium bicarbonate (0.039 ml of a 7.0% solution per millilitre of the medium), calcium lactate (0.6 mg/ml), gentamycin (0.025 mg/ml), HEPES (1.5 mg/ml), 13.5 IU eCG, 6.6 IU hCG/ml (P.G. 600 Intervet) and 10% fetal calf serum (GibcoBRL).

The oocytes were cultured for 48 h in 3.5 cm diameter Petri dishes (Nunc) containing 3.0 ml of the culture medium at 39°C in a mixture of 5.0% CO_2 in the air.

Evaluation of oocytes

At the end of the culture, the oocytes or embryos were mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h and stained with 1.0% orcein. The oocytes were examined under a phase contrast microscope. Activation was considered to have occurred if the oocytes were in the pronuclear stage. Oocytes remaining at metaphase II or arrested at anaphase II or at telophase II were not considered as activated. In cleaved parthenogenetic embryos, the number of nuclei was counted at the end of the culture.

Oocyte activation and culture of embryos

Oocytes were activated using the method described by Jilek *et al.* (2001). Briefly, oocytes matured *in vitro* were denuded from their cumulus cells and were subjected to a 5 min treatment with 25 μM calcium ionophore A23185. The calcium ionophore was diluted in modified M199 medium without fetal calf serum and without bovine serum albumin. After the treatment, the oocytes were washed in M199 medium supplemented with bovine serum albumin and cultured for 2 h in the NCSU23 culture medium (Petters & Wells, 1993) supplemented with 2 mM 6-dimethylaminopurine (DMAP). The oocytes were then carefully washed and were cultured in DMAP-free

https://doi.org/10.1017/S0967199409005437 Published online by Cambridge University Press

NCSU23 medium in 4-well Petri dishes (Nunc), each well containing 1.0 ml of the culture medium. The eggs were cultured at 39°C in a mixture of 5.0% CO_2 in the air for 7 days.

Arrangement of experiments

Experiment 1 was performed to confirm meiotic competence in growing oocytes or fully grown oocytes. Oocytes with an internal diameter of 110 μm were cultured for 24, 48 or 72 h. At the end of the culture, the maturation of oocytes was assessed as described above. Fully grown oocytes (internal diameter 120 μm) were cultured for 24 or 48 h and their maturation was assessed at the end of the culture.

In Experiment 2, the effect of the histone deacetylase inhibitor TSA on the maturation of growing oocytes with partial meiotic competence (internal diameter of 110 μm) and on the maturation of fully grown oocytes with full meiotic competence was monitored. The growing oocytes were cultured in the medium supplemented with 0, 100 or 1000 nM of TSA for 24 or 48 h. Similarly, fully grown oocytes were cultured with 0, 100 or 1000 nM of TSA for 24 or 48 h.

Experiment 3 was performed to evaluate the effects of transient exposure to TSA in growing oocytes with partial meiotic competence. To observe the effect of TSA at the beginning of the 72 h culture, the growing oocytes were cultured with TSA (100 or 1000 nM) for 24 h. Then, the TSA was carefully washed out and the oocytes were cultured for another 48 h with the TSA-free medium. To evaluate the effects of TSA at the middle of the 72 h culture time, the growing oocytes with partial meiotic competence were cultured for 24 h in the TSA-free medium. Then these oocytes were exposed to TSA (100 or 1000 nM) for another 24 h. Following this, TSA was washed out and the oocytes were cultured in the TSA-free medium for 24 h. To test the effects of TSA at the end of the 72 h culture, the oocytes were cultured in the TSA-free medium for 48 h and then exposed to the TSA-supplemented medium (100 or 1000 nM) for 24 h. In control experiments, the growing oocytes with partial meiotic competence were cultured for 72 h in the TSA-free medium or in the TSA-supplemented medium (100 or 1000 nM).

In Experiment 4, we tested parthenogenetic activation of oocytes transiently treated with TSA at the middle of the 72 h culture. Growing oocytes with partial meiotic competence were cultured for 24 h in the TSA-free medium. Then, these oocytes were exposed to 100 nM TSA for another 24 h. Subsequently TSA was washed out and the oocytes were cultured in the TSA-free medium for 24 h. Following this, the oocytes were activated using calcium ionophore as described above and cultured for another 22 h in the TSA-free medium. In the control experiment, we activated fully grown oocytes after their 48 h culture *in vitro* and then cultured these oocytes for 22 h in the TSA-free medium. To address spontaneous activation of growing oocytes after transient treatment with TSA, we performed another control experiment. During this experiment, the growing oocytes with partial meiotic competence were cultured for 24 h in the TSA-free medium. These oocytes were then exposed to 100 nM TSA for another 24 h, after which TSA was washed out and the oocytes were cultured in the TSA-free medium for 24 h. These oocytes were then cultured for another 24 h in the culture medium without previous parthenogenetic activation.

Experiment 5 was performed to evaluate the parthenogenetic development of embryos produced from growing oocytes with partial meiotic competence. The oocytes were cultured for 24 h in the TSA-free medium and then exposed to 100 nM TSA for another 24 h. After this, TSA was washed out and the oocytes were cultured in the TSA-free medium for 24 h. Subsequently, they were activated using calcium ionophore as described above and cultured for another 7 days in the NCSU23 medium. In the control experiment, we activated fully grown oocytes after their 48 h culture *in vitro* and then cultured these oocytes for another 7 days in the NCSU23 medium.

Statistical analysis

All experiments were repeated four times. The results were subjected to analysis of variance using Statistica 6.0, followed by the Tukey test. p-values <0.05 were designated as significant.

Results

In Experiment 1 we confirmed the maturation of growing and fully grown pig oocytes under our culture conditions. These data were used for the preparation of the experiment design. We observed that growing oocytes with an internal diameter of 110 μm have partial meiotic competence. The majority of these oocytes are unable to mature beyond metaphase I. This category of oocytes was chosen for further experiments. As revealed in further experiments, the maturation of oocytes with partial meiotic competence is not only delayed, but actually blocked at metaphase I (see Table 1). About 63% of growing oocytes reached metaphase I after 24 h culture. The same percentage of growing oocytes remained at metaphase I after 48 h of culture with approximately 17% of the oocytes proceeding to metaphase II. This situation was unchanged even after 72 h culture. Under our culture conditions, 99% of fully grown oocytes reached metaphase I after 24 h culture.

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Table 1 Maturation of growing pig oocytes with partial meiotic competence.

Time of culture (h)	Stage of maturation (%)		
	GV	MI	MII
0	100 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}
24	36.7 ± 2.7^{b}	63.3 ± 4.1^{b}	0 ± 0.0^{a}
48	$20.8 \pm 3.3^{b,c}$	62.5 ± 3.5^{b}	16.7 ± 1.1^{b}
72	14.3 ± 1.6^{c}	66.7 ± 1.7^{b}	19.0 ± 2.2^{b}

Growing pig oocytes with an internal diameter of 110 µm were cultured for 24, 48 or 72 h *in vitro*. Nuclear maturation was assessed at the end of the culture. The results are presented as mean ± SEM. Each experimental group contained 120 oocytes.
[a–c]Statistically significant differences in the percentage of the respective stage of oocyte maturation (i.e., differences within columns) are indicated by different superscripts.
GV, germinal vesicle; MI, metaphase I; MII, metaphase II.

After 48 h culture, 92% of fully grown oocytes matured to metaphase II (the remaining oocytes were mainly at the stages of anaphase I or telophase I).

In Experiment 2, we tested the effects of the histone deacetylase inhibitor on the maturation of fully grown pig oocytes or growing pig oocytes with partial meiotic competence. TSA delayed maturation in fully grown oocytes. After 24 h culture with 100 nM TSA, 22% of the oocytes remained at the germinal vesicle stage. The remaining oocytes were observed at metaphase I. When cultured with 1000 nM of TSA, about 40% of fully grown oocytes were at the germinal vesicle stage. After 48 h culture with TSA, no fully grown oocytes remained at the germinal vesicle stage. The percentage of oocytes matured to metaphase I increased and the percentage of oocytes matured to metaphase II decreased (Table 2).

We did not observe a significant effect of TSA on the maturation of growing oocytes with partial meiotic competence. Neither the concentration of TSA (100 or 1000 nM) nor the culture time (24 or 48 h) had any effect.

In Experiment 3 we tested maturation in growing oocytes exposed transiently to the histone deacetylase inhibitor TSA. The results are shown in Table 3.

We examined maturation in growing oocytes with an internal diameter of 110 µm that were first cultured with TSA (100 or 1000 nM) for 24 h and then cultured in the TSA-free medium for another 48 h. In contrast with oocytes cultured for the same total time (i.e., 72 h) in the TSA-free or TSA-supplemented medium, all the oocytes transiently treated with TSA underwent germinal vesicle breakdown. However, the percentage of oocytes that matured to metaphase II did not change.

A similar situation was observed in growing oocytes cultured first for 48 h in the TSA-free medium and then exposed to TSA (100 or 1000 nM) for 24 h. All these oocytes underwent germinal vesicle breakdown. The percentage of oocytes that matured to metaphase II did not change.

A different situation was observed in growing oocytes with partial meiotic competence cultured first for 24 h with the TSA-free medium, then exposed to TSA (100 or 1000 nM) for another 24 h and after washing out of TSA further cultured for 24 h in the TSA-free medium. Oocytes exposed transiently to 100 nM of TSA in the middle of 72 h culture increased their maturation rate (59% of oocytes were at metaphase II). The remaining oocytes reached metaphase I. Those oocytes exposed transiently to 1000 nM of TSA in the middle of the culture did not increase their maturation rate to metaphase II (9% of oocytes at metaphase II). However, a significant percentage of these oocytes degenerated (27%). For further experiments, we chose

Table 2 Effects of the histone deacetylase inhibitor (TSA) on maturation in growing or fully grown oocytes.

Category of oocytes	Concentration of TSA (nM)	Maturation stage (%) (24 h culture)			Maturation stage (%) (48 h culture)		
		GV	LD–MI	MII	GV	LD–MI	MII
Growing	0	35.4 ± 3.1^{a}	63.2 ± 5.2^{a}	0 ± 0^{a}	19.1 ± 3.5^{a}	65.3 ± 0.9^{a}	15.6 ± 2.2^{a}
	100	37.1 ± 4.9^{a}	62.2 ± 1.1^{a}	0 ± 0^{a}	22.2 ± 2.1^{a}	59.5 ± 3.7^{a}	18.3 ± 2.9^{a}
	1000	45.6 ± 4.2^{a}	53.8 ± 2.5^{a}	0 ± 0^{a}	16.9 ± 4.3^{a}	55.8 ± 4.1^{a}	17.3 ± 4.5^{a}
Fully grown	0	2.9 ± 2.2^{a}	97.1 ± 4.2^{a}	0 ± 0^{a}	0 ± 0^{a}	5.7 ± 2.4^{a}	94.3 ± 1.9^{a}
	100	22.2 ± 3.2^{b}	77.8 ± 2.9^{b}	0 ± 0^{a}	0 ± 0^{a}	19.5 ± 4.7^{a}	80.5 ± 3.6^{a}
	1000	41.7 ± 1.8^{c}	58.3 ± 0.9^{c}	0 ± 0^{a}	0 ± 0^{a}	52.1 ± 1.1^{b}	47.8 ± 2.3^{b}

The oocytes were cultured respectively for 0, 24 or 48 h, with a respective concentration of TSA (0, 100 or 1000 nM). The results are presented as mean ± SEM. Each experimental group contained 120 oocytes.
[a,b]Statistically significant differences in the percentage of the respective stage of oocyte maturation in growing or fully grown oocytes (i.e., differences within columns within each size category of oocytes) are indicated by different superscripts.
GV, germinal vesicle; LD, late diakinesis; MI, metaphase I; MII, metaphase II.

https://doi.org/10.1017/S0967199409005437 Published online by Cambridge University Press

Table 3 Effect of transient exposure of growing pig oocytes to trichostatin A (TSA).

Type of culture	Oocyte maturation (%) (mean ± SEM)			
	GV	MI	MII	Degenerated
72 h TSA 0 nM	12.5 ± 1.3^a	71.9 ± 3.4^a	16.6 ± 4.1^a	0 ± 0^a
72 h TSA 100 nM	15.6 ± 3.6^a	72.1 ± 3.9^a	12.3 ± 4.9^a	0 ± 0^a
72 h TSA 1000 nM	12.9 ± 2.9^a	70.3 ± 3.7^a	8.5 ± 4.3^a	8.3 ± 2.5^a
24 h TSA 100 nM + 48 h TSA 0 nM	0 ± 0^b	86.7 ± 2.1^b	13.3 ± 4.1^a	0 ± 0^a
24 h TSA 1000 nM + 48 h TSA 0 nM	0 ± 0^b	73.1 ± 3.8^a	4.8 ± 4.5^a	22.1 ± 5.3^b
24 h TSA 0 nM + 24 h TSA 100 nM + 24 h TSA 0 nM	0 ± 0^b	41.3 ± 3.6^c	58.7 ± 3.8^b	0 ± 0^a
24 h TSA 0 nM + 24 h TSA 1000 nM + 24 h TSA 0 nM	0 ± 0^b	63.5 ± 4.2^a	8.7 ± 5.2^a	27.8 ± 5.6^b
48 h TSA 0 nM + 24 h TSA 100 nM	0 ± 0^b	88.0 ± 2.7^b	12.0 ± 3.1^a	0 ± 0^a
48 h TSA 0 nM + 24 h TSA 1000 nM	0 ± 0^b	68.4 ± 3.9^a	8.5 ± 4.9^a	23.1 ± 5.5^b

The total culture time in all oocytes was 72 h. During this time, experimental oocytes were treated for 24 h with 100 nM TSA (TSA 100) or 1000 nM TSA (TSA 1000) at the beginning, in the middle and at the end of the total 72 h culture. For the remainder of the culture time, the oocytes were cultured in a medium without TSA (TSA-free). In control experiments, the oocytes were cultured in the TSA-supplemented medium (100 or 1000 nM) for 72 h or in the TSA-free medium for 72 h. The total number of oocytes for each treatment was 120.

[a–c]Statistically significant differences in the percentage of the respective stages of oocyte maturation (i.e., differences within columns) are indicated by different superscripts.

GV, germinal vesicle; MI, metaphase I; MII, metaphase II.

only treatment during which the oocytes were first cultured in the TSA-free medium for 24 h, then treated with 100 nM of TSA for another 24 h and finally cultured for 24 h in the TSA-free medium.

In Experiment 4, the oocytes treated transiently with 100 nM TSA were parthenogenetically activated. They were cultured for 24 h in the TSA-free medium, then exposed to 100 nM for another 24 h and finally cultured for another 24 h in the TSA-free medium. The oocytes were then activated using calcium ionophore A23187 in accordance with Materials and methods. Twenty-four hours later, approximately 52% of the oocytes ($n = 120$) were activated. This figure roughly corresponds to the percentage of growing oocytes that matured to metaphase II after transient TSA treatment. 87% of fully grown oocytes ($n = 120$) matured for 48 h and treated with calcium ionophore were activated. The activation rate was significantly lower in growing oocytes activated after maturation induced by transient treatment with TSA than in matured fully grown oocytes subjected to the same ionophore treatment.

The control experiment on growing oocytes excluded the possibility that the observed activation was a result of spontaneous activation resulting from the aging of those oocytes that matured to metaphase II after transient 24 h TSA treatment. When we cultured these oocytes ($n = 120$) for another 24 h without calcium ionophore treatment, there was no activation.

Parthenogenetic development was tested during experiment 5. The oocytes were cultured for 24 h in the TSA-free medium, then exposed to 100 nM for another 24 h and finally cultured for another 24 h in the TSA-free medium. They were then activated using calcium ionophore A23187 and cultured for 7 days in the NCSU23 medium. Cleavage was observed in 22% of the oocytes ($n = 120$). Cleavage was blocked mainly at the 4-cell stage (17% of all oocytes). The remaining cleaved oocytes reached the 8-cell stage.

In the control experiment with fully grown oocytes cultured in the TSA-free medium for 48 h and then activated using calcium ionophore, cleavage was observed in 52% of the oocytes. The morula stage was reached by 17% of the oocytes and 20% reached the blastocyst stage. The cleavage rate and the development to the morula stage and blastocyst stage were significantly higher in fully grown oocytes activated using calcium ionophore than in growing oocytes activated after maturation induced by transient treatment with TSA.

Discussion

In the present study, the histone deacetylase inhibitor TSA delayed maturation in fully grown pig oocytes. In growing pig oocytes with partial meiotic competence, transient exposure to TSA resulted in enhanced meiotic competence. However, the developmental competence of these oocytes remained very low.

The delay in meiotic maturation in fully grown oocytes observed in our study is in accordance with the results of Wang *et al.* (2006b), who also described the delayed maturation of pig oocytes after TSA treatment. Bui *et al.* (2007) observed complete inhibition of germinal vesicle breakdown in pig oocytes treated

https://doi.org/10.1017/S0967199409005437 Published online by Cambridge University Press

with 100 nM TSA. In somatic cells, it was observed that TSA regulates molecules that are involved in the transition from the G_2 to the M phase of the cell cycle (Noh & Lee, 2003). However, this regulation acts on the transcription of genes regulating the cell cycle. As there is no transcriptional activity during oocyte maturation (Bachvarova, 1985; Kageyama *et al.*, 2007a), the above-mentioned effects of TSA on oocyte maturation would not be associated with changed gene expression. Wang *et al.* (2006b) assumed that TSA influences chromosome condensation and this can affect kinetochore–microtubule interaction, induce checkpoint activation and postpone the onset of metaphase.

On the other hand, Kim *et al.* (2003) did not observe meiotic blockage in TSA-treated mouse oocytes. This finding can reflect interspecies differences. However, Endo *et al.* (2005) did not observe any effect of TSA on the maturation of pig oocytes. This situation is in striking contrast with the effects of TSA on the maturation of pig oocytes observed in the present study and also studies by Wang *et al.* (2006b) and Bui *et al.* (2007). This finding can be at least partially explained by different culture conditions. Bui *et al.* (2007) cultured pig oocytes with cumulus cells and a piece of attached parietal granulosa tissue. It is known that parietal granulosa can inhibit the maturation of pig oocytes *in vitro* and this inhibitory effect can be reversed by protein synthesis inhibitors (Motlik *et al.*, 1991). However, Bui *et al.* (2007) did not observe the inhibition of germinal vesicle breakdown in oocytes with parietal granulosa in the TSA-free medium. It cannot be excluded that parietal granulosa enhances the effect of TSA. However, Wang *et al.* (2006b) demonstrated that TSA acts directly on the pig oocyte and its effects are not mediated by somatic cells of the ovarian follicle. This finding was demonstrated for cumulus cells, but it is not clear to what extent it is relevant for the complex formed by oocyte, cumulus cells and parietal granulosa.

All these data indicate that the effect of TSA and the subsequent histone hyperacetylation on cell cycle progression greatly depends on the cell type and other factors. This finding is also demonstrated in our study, in which growing oocytes with partial meiotic competence react differently to TSA treatment when compared with fully grown oocytes with full meiotic competence. The maturation of growing oocytes was not influenced by TSA. This difference may be due to the different initial level of histone acetylation in growing and fully grown oocytes. Several authors have demonstrated that histone acetylation increases during oocyte growth and is at its maximum in fully grown oocytes (Kageyama *et al.*, 2007b; Bui *et al.*, 2007; Meglicki *et al.*, 2008). Growing oocytes have high expression of genes regulating histone acetylation. However, the activity of histone deacetylases may be lower than in fully grown oocytes (Kageyama *et al.*, 2007b). Under such conditions, the inhibition of histone deacetylases could have no significant effect on growing oocytes. With regard to transcriptional activity of growing oocytes (Bachvarova, 1985) we cannot exclude the possibility that TSA acts on gene expression. The effect of TSA on the expression of genes regulating the cell cycle was clearly demonstrated in somatic cells (Noh & Lee, 2003).

We observed that transient exposure of growing oocytes to the histone deacetylase inhibitor TSA increased the ratio of oocytes that mature to metaphase II. Growing oocytes are able to resume meiosis and this resumption is accompanied by vast histone deacetylation (Kim *et al.*, 2003; Akiyama *et al.*, 2004; Spinaci *et al.*, 2004; Endo *et al.*, 2005; Wang *et al.*, 2006a; Bui *et al.*, 2007; Kageyama *et al.*, 2007b; Tang *et al.*, 2007). We can speculate that this histone deacetylation also occurs in maturing growing oocytes. The reversal of histone acetylation is typical for maturing, fully grown pig oocytes with full meiotic competence during anaphase I and telophase II with subsequent histone deacetylation at metaphase II (Endo *et al.*, 2005; Bui *et al.*, 2007). Similarly, transient histone acetylation at anaphase I and telophase I was observed in mouse (Akiyama *et al.*, 2004) and sheep oocytes (Tang *et al.*, 2007). The oocytes of these species also undergo histone deacetylation at metaphase II (Akiyama *et al.*, 2004; Tang *et al.*, 2007).

We can conjecture that transient treatment of growing pig oocytes with TSA can allow certain histone acetylation due to the inhibition of histone deacetylases. This treatment can counteract initial spontaneous histone deacetylation. After washing out TSA, histone deacetylation can occur again. This sequence of events may mimic the time sequence of histone acetylation observed by others (Akiyama *et al.*, 2004; Endo *et al.*, 2005; Bui *et al.*, 2007) and this situation could improve the meiotic competence of growing oocytes. This finding is in accordance with a study by Ola *et al.* (2007), which suggested that abrupt histone deacetylation of oocytes that did not complete all the processes typical for oocyte growth might explain their low meiotic and developmental competence.

We successfully activated growing pig oocytes that matured to metaphase II after transient treatment with TSA. The subsequent parthenogenetic development was, not surprisingly, very limited. Akiyama *et al.* (2006) demonstrated that manipulation of histone acetylation in maturing oocytes induces aneuploidy and has a detrimental effect on subsequent embryonic development. On the other hand, TSA is able to enhance the viability of oocytes matured to metaphase II that were not fertilized and underwent a spontaneous detrimental process designated as aging (Jeseta *et al.*,

2008). Also, Rybouchkin *et al.* (2006) described the positive effects of histone deacetylase inhibition on the development of embryos that had been created using somatic cell nuclear transfer. However, this positive effect was observed only when histone deacetylases were inhibited after oocyte activation, i.e., at the final stage of meiosis and at the onset of the first mitotic cycle of embryo cleavage. The inhibition of histone deacetylases has different effects on meiotic oocyte maturation and the mitotic cell cycle of embryo cleavage because histone deacetylases are localized differently in the oocyte and in early stages of the embryo (Kim *et al.*, 2003). Histone acetylation strongly influences the gene expression in mitotic cells (Clarke *et al.*, 1993; O´Neill and Turner, 1995; Grunstein, 1997). However, this effect of elevated histone acetylation was not observed in oocytes (Kim *et al.*, 2003). This finding is in accordance with observations of histone deacetylation during oocyte meiotic maturation and histone acetylation in fertilized or parthenogenetically activated oocytes (Spinaci *et al.*, 2004; Bui *et al.*, 2007).

On the basis of our data, we can conclude that transient inhibition of histone deacetylases has a positive effect on the meiotic maturation in growing oocytes with partial meiotic competence. The developmental competence of the oocytes remained low after this treatment.

Acknowledgement

We thank Mrs Lois Russell for her editorial assistance with this manuscript. This project was supported by grants MZE 0002701404, NAZV QG50052 and MSM 6046070901.

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