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Role of cyclic AMP in the maturation of *Ciona intestinalis* oocytes

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Summary

Immature oocytes are arrested at prophase I of the meiotic process and maturation onset is indicated by oocyte nuclear disassembly (germinal vesicle breakdown or GVBD). Signaling pathways that elevate intracellular cyclic AMP (cAMP) may either prevent or induce oocyte maturation depending on the species. In some marine invertebrates and, in particular, in ascidian oocytes, cAMP triggers GVBD rather than blocking it. In this paper, we tested different cAMP elevators in fully grown oocytes at the germinal vesicle stage (GV) of the ascidian *Ciona intestinalis*. We demonstrated that through the activation of adenylate cyclase or the inhibition and phosphodiesterases the oocyte remained at the GV stage. This effect was reversible as the GV-arrested oocytes, rinsed and incubated in sea water, are able to undergo spontaneous maturation and extrusion of follicle cells. In addition, oocytes acquire the ability to be fertilized and start early development. However, morphology of follicle cells, embryos and larvae from *in vitro* matured oocytes showed different morphology from those derived from *in vivo* mature oocytes. The role and the transduction mechanism of cAMP in the regulation of oocyte maturation were discussed. Finally, we indicated a variation of biological mechanisms present in the ascidian species; moreover, we sustain evidence proving that tunicates share some biological mechanisms with vertebrates. This information provided new hints on the importance of ascidians in the evolution of chordates.

Keywords: Ascidians, cAMP, *Ciona intestinalis*, GVBD, Oocyte maturation

Introduction

Oocyte maturation is the last phase of oogenesis, where the oocyte grows and acquires the competence to be ovulated and fertilized. Different mechanisms drive the oocyte into maturation including nuclear and cytoplasmic changes.

Nuclear maturation is underlain by the meiotic process and is defined as the period of progression from the first to the second meiotic arrest. In most of the species at the onset of meiosis, the oocyte undergoes a first meiotic arrest in the first prophase (PI) identified by the presence of a large nucleus known as germinal vesicle (GV). Meiosis is then resumed in response to a stimulus that is species-specific and the first sign of meiosis progression is the breakdown of the germinal vesicle (GVBD); after that, meiosis progresses up to metaphase I (MI) or II (MII) depending on the species. Here the meiosis arrests again up to the definitive removal of the block induced by the fertilizing spermatozoon (Tsafriri, 1979; Monroy, 1985; Voronina & Wessel, 2003; Richard, 2007). Resumption of meiosis after the first arrest in PI is induced by an external stimulus via hormones, neurotransmitters, pH increase and different molecules in echinoderms (Yamashita, 1988; Mita, 2000), mollusks (Dubè, 1992; Guerrier *et al*., 1993; Deguchi & Osanai, 1994; Deguchi & Morisawa, 2003), coelenterates (Takeda *et al*., 2006), amphibians (Schorderet-Slatkine, 1972; Sánchez-Toranzo *et al*., 2007) and mammals (Moor *et al*., 1981; Mattioli & Barboni, 2000; for review see Tripathi *et al*., 2010).

Cytoplasmic maturation changes are related to many metabolic processes that involve cell cycle control proteins, mRNAs, plasma membrane permeability and calcium release (Ducibella *et al*., 1988; Whitaker

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& Patel, 1990; Homa, 1995; Masui, 1996; Whitaker, 1996; Dale & Elder 1997; Wessel *et al.*, 2001; Tosti, 2006). The control of oocyte maturation involves the participation of numerous metabolic pathways; in particular, meiosis arrest and resumption rely on two different mechanisms: a positive stimulation and the removal of an inhibitory signal.

Oocyte maturation may be driven by either physiological inducers, collectively known as MIS (maturation inducing substances) (Kanatani, 1983; Lambert, 2008), or inhibitory substances. Among these, a key role is played by intracellular concentrations of cyclic AMP (cAMP), whose action depends on interplay between the cAMP-synthesizing enzymes (adenylate cyclases) and the cAMP-degrading enzymes (phosphodiesterases) (Mehlmann, 2005; Sun *et al.*, 2009). In many animal species, oocytes undergo spontaneous maturation when they are deprived of their accessory cells (Edwards, 1965; Foote & Thibault, 1969; Eppig, 1991; Sánchez-Toranzo *et al.*, 2007), or when they are removed from their ovarian environment (Lambert, 2008), suggesting an interplay among the oocyte, its accessory cells and the extracellular environment. In mammals, accessory cells may be responsible for the meiotic block by transferring small molecules to the oocyte through gap junctions connecting the two compartments (Eppig *et al.*, 1983). In fact, it is known that loss of gap junctional communication triggers GVBD in hamster, bovine and mouse (Racowsky & Satterlie, 1985; Dekel *et al.*, 1988; Thomas *et al.*, 2004). In particular, cAMP produced in the follicle cells diffuses through the gap junctions, playing a fundamental role in maintaining meiotic arrest in PI (Downs *et al.*, 1989) and may even prevent premature spontaneous maturation (see Richard, 2007 for a review). An alternative mechanism for meiotic arrest involving endogenous production of cAMP has been proposed by Vaccari *et al.* (2008).

Initiation of meiosis may therefore result from a negative signal, such as the removal of inhibitory factors (cAMP) whose degradation has been shown to stimulate AMP-activated kinase (AMPK), a well documented inducer of GVBD in mice (Chen & Downs, 2008). This is also supported by the spontaneous maturation that in marine animals occurs when oocytes are removed from the ovary and put in sea water (Cuomo *et al.*, 2006; Stricker & Smythe, 2006; Lambert, 2008).

Alternatively, GVBD may be induced by a positive signal, such as growth factors provided by accessory cells and transmitted to the oocyte through gap junctions (O'Donnel *et al.*, 2004; Park *et al.*, 2004).

In the ascidian *Ciona intestinalis*, the ovary contains immature oocytes of various size and stages of oogenesis, but only full vitellogenic oocytes were shown to be meiotic competent (Silvestre *et al.*, 2009). Although the natural signal responsible for triggering maturation in ascidians remains unknown, we showed that GVBD is modulated by an interplay between pH, trypsin-like molecules and calcium, whereas we excluded a role of the follicle cells in transmitting the signal for oocyte maturation (Silvestre *et al.*, 2009).

In this study, we show that treatment that elevates cAMP levels in immature oocytes of *C. intestinalis* inhibits the onset of GVBD, in a manner that is more similar to vertebrates than to other ascidian species.

Materials and methods

Chemicals

All the materials were purchased from Sigma-Aldrich.

Animals and oocytes

The *C. intestinalis* ascidians were collected from the Bay of Naples and kept in tanks with running sea water until use. The single bag-like ovary was removed with forceps and fine scissors, cut, and transferred directly to Petri dishes containing a solution of artificial sea water (ASW: 400 mM NaCl; 50 mM $MgCl_2$; 10 mM KCl; 10 mM $CaCl_2$; 10 mM HEPES) at pH 5.0. Among all the stages present in the ovary (Silvestre *et al.*, 2009), we selected the fully grown oocytes showing a clear GV and characterized by a diameter of the nude oocyte >120 μm, a brown cytoplasm, and a layer of cube-shaped follicle cells up to 35 μm ca. width. The size of nude oocytes and follicle layers was evaluated with a millimetre grid.

Spontaneous maturation and cAMP level maintenance

In vitro GVBD was performed according to Cuomo *et al.* (2006) by incubating GV oocytes in ASW at pH 8.2 as control.

In order to maintain high cAMP levels, the immature oocytes at the GV stage were incubated with the following substances:

- the activator of adenylate cyclase, forskolin at different final concentrations from 50 to 500 μM in ASW at pH 8.2;
- three inhibitors of phosphodiesterases: theophylline at 2 mM final concentration, caffeine at 25 mM final concentration and isobutylmethylxanthine (IBMX) at 1 mM final concentration, all in ASW at pH 8.2;
- the membrane permeable cAMP analogue, 8-bromo cAMP at 4 and 8 mM final concentration in ASW at pH 8.2.

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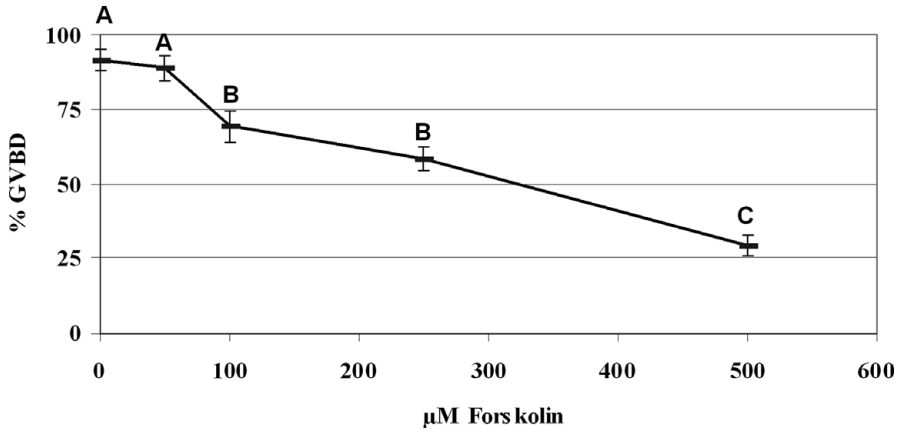

Figure 1 Forskolin-induced GVBD percentage (mean ± SE) in ASW pH 8.2 after 3 h. A vs. B vs. C ($p < 0.01$).

All the incubations were performed for 3 h, after that oocytes were scored for GVBD occurrence at the inverted microscope.

Spontaneous maturation and fertilization test

We tested potential toxicity of the substances used to maintain high cAMP levels. GV oocytes after the incubation time were transferred in ASW pH 8.2 in order to allow the spontaneous maturation. *In vitro* GVBD oocytes were followed at the inverted microscope in order to assess the disappearance of the GV, the extrusion of follicle cells and the chorion elevation. After 3 h of incubation in ASW, GVBD oocytes were then fertilized by adding 10^6 spermatozoa/ml. After the assessment of post-fertilization oocyte contraction the zygotes were put in an incubator at 18 °C and followed for embryo development up to hatched larvae (18–24 h). Vitality and morphology of the hatched larvae were observed at the inverted microscope.

As a control we submitted to spontaneous maturation and fertilization the untreated GV oocytes.

Statistical analysis

Differences between GVBD percentages were analyzed with ANOVA (SAS, 1988). In the case of values expressed as percentages, we proceeded to analyze data after arcsine transformation.

Pairwise comparisons of means were analyzed by the least significant difference (LSD) test.

Results

The results were expressed as mean ± standard error (SE).

Adenylate cyclase activator

GV stage oocytes in ASW treated with forskolin gave rise to GVBD % in a dose-dependent manner as follows: 88.8 ± 4.2 at 50 µM ($n = 5$), 69.3 ± 5.0 at 100 µM ($n = 5$), 58.3 ± 4.1 at 250 µM ($n = 5$) with a maximum inhibition value of 29.4 ± 3.6 at 500 µM ($n = 8$), compared with 91.3 ± 3.6 in control oocytes ($n = 8$; Fig. 1). The data obtained was significant ($p < 0.01$) starting from a concentration of 100 µM.

Phosphodiesterase inhibitors

GV stage oocytes incubated in ASW with phosphodiesterase inhibitors showed significant differences in maturation occurrence.

Theophylline gave rise to 25.0 ± 2.0% GVBD vs. 85.9 ± 3.4% in the control ($p < 0.01$; $n = 9$; Fig. 2). Similar results were obtained also by using caffeine that reduced GVBD to 26.0 ± 4.0% vs. 95.0 ± 5.0% of the control ($p < 0.01$; $n = 8$; Fig. 2).

The last inhibitor tested, IBMX, also decreased the maturation percentage leading to 52.4 ± 4.7% GVBD vs. 93.4 ± 2.7% of the control ($p < 0.01$; $n = 11$; Fig. 2).

The cAMP analogue

The cAMP analogue, 8-bromo cAMP, was used at two different concentrations, but in both cases we did not

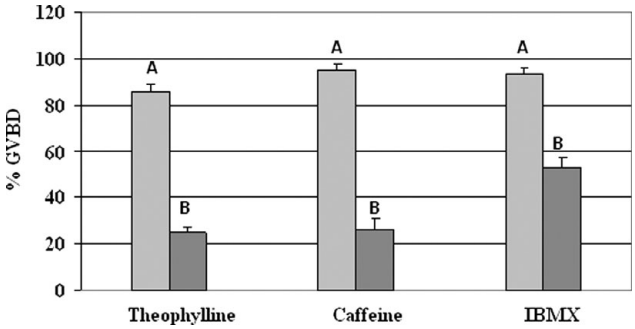

Figure 2 Occurrence (mean ± SE) of GVBD percentage in GV stage after 3 h of incubation in: ASW pH 8.2 (control, light shading), theophylline 2 mM, caffeine 25 mM and IBMX 1 mM (dark shading). A vs. B ($p < 0.01$).

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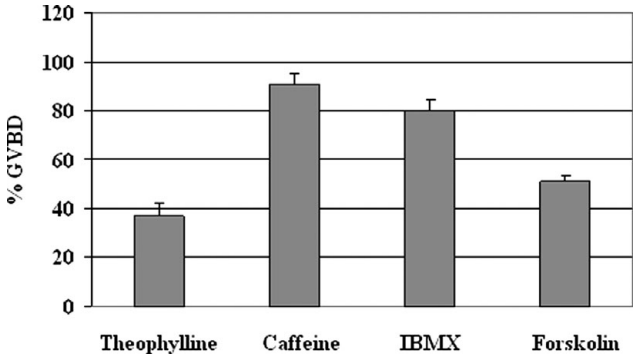

Figure 3 Occurrence (mean ± SE) of GVBD percentage in oocytes removed from the treatment bath and incubated in sea water pH 8.2. The concentrations of substances in the treatment bath were: theophylline 2 mM, caffeine 25 mM, IBMX 1 mM and forskolin 500 μM. The reversible effect was evaluated after 3 h of incubation.

observe any significant reduction in GVBD percentage ($n = 7$; data not shown).

Spontaneous maturation and fertilization test

Oocytes treated with cAMP-elevating substances remained blocked at the GV stage up to the removal from the treatment bath and the incubation in ASW. Here, they retained the ability to undergo spontaneous maturation with higher GVBD percentage in IBMX and caffeine treated oocytes (Fig. 3).

Figure 4 reports time lapse sequence of spontaneous maturation in control and treated oocytes after incubation in ASW. Both the two groups extruded follicle cells within 7 h, but the shape and length of the follicle cells were different from those of ovulated MI oocytes. By fertilizing control and treated oocytes after spontaneous maturation, we observed 2-cell stage and hatched larvae 50 min and 24 h after fertilization, respectively (Table 1). However, hatched larvae were

Table 1 Percentage of 2-cell stage and hatched larvae obtained from GV oocytes matured *in vitro* and then fertilized (control) or from GV oocytes blocked by the substances, rinsed and incubated in sea water for 3 h and then fertilized.

	% 2-cell stage	% hatched larvae (% coiled tails)
Control	60	30 (70)
Theophylline (2 mM)	62	0
Caffeine (25 mM)	44	10 (100)
IBMX (1 mM)	43	14 (100)
Forskolin (500 μM)	22	0

Percentage of 2-cell and hatched larvae was calculated over the total of oocytes fertilized, while percentage in parentheses was calculated over the total of hatched larvae.

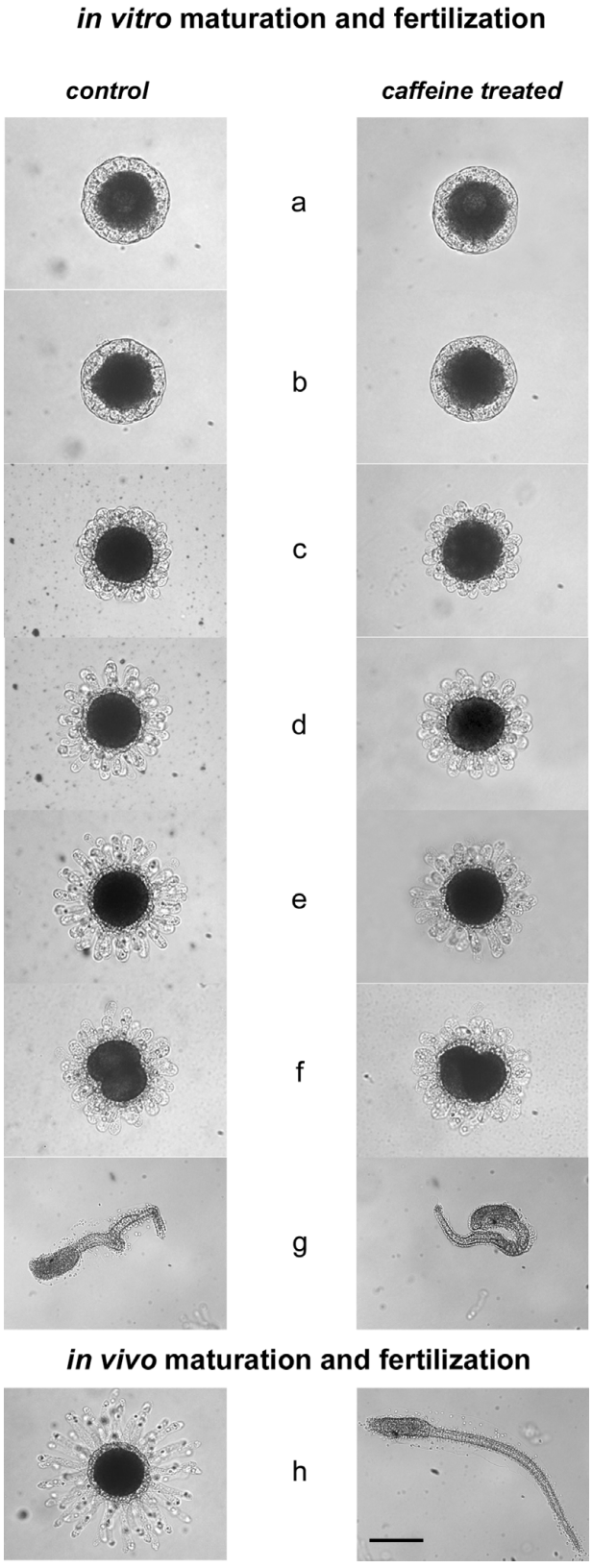

Figure 4 Representative experiment of time lapse sequence of spontaneous maturation in control and treated (caffeine 25 mM) oocytes after incubation in ASW: (a) GV oocytes at the beginning of incubation time; (b) GVBD oocytes after 1–2 h of incubation showing the disappearance of GV;

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obtained only from oocytes treated with caffeine or IBMX showing 100% abnormal coiled tails with respect of 70% of the control (Table 1 and Fig. 4).

Discussion

In this paper we have shown that high cytoplasmic levels of cAMP in *C. intestinalis* immature oocytes maintain the meiotic arrest at the PI stage.

Many studies have provided evidence of the involvement of cyclic nucleotides in the maintenance of meiotic arrest (Sun *et al*., 2009 for a review) and in particular, it is known that the intracellular second messenger cAMP plays a significant role in the regulation of mammalian oocyte maturation (Gilchrist & Thompson, 2007). High levels of cAMP, some analogues, cAMP-dependent protein kinase (PKA) and related substances such as GPR3, act by preventing spontaneous maturation and/or blocking GVBD *in vitro* or, on the contrary, may release oocyte from meiotic arrest (Mehlmann *et al*., 2004; Richard, 2007 for a review; Vaccari *et al*., 2008).

In fact, contrasting results exist in the literature regarding the inhibitory role of cAMP elevation (Eppig, 1989; Conti *et al*., 1998; Nogueira *et al*., 2003; Voronina & Wessel, 2003; Mehlmann, 2005) or its role in induction of GVBD (Freeman & Ridgway, 1988; Yamashita, 1988; Yi *et al*., 2002; Stricker & Smythe, 2006; Takeda *et al*., 2006; Lambert, 2008).

In ascidian oocytes, spontaneous maturation occurs in natural sea water, resulting in very fast morphological changes such as extrusion of test cells, elevation of the chorion, and extrusion of follicle cells.

In previous papers (Cuomo *et al*., 2006; Silvestre *et al*., 2009), we showed that the largest stage of the *Ciona* GV oocytes was the only immature stage that is competent for meiosis progression and was able to undergo spontaneous maturation.

Here, we show that treatment of these immature oocytes with cAMP-elevating substances maintained the meiotic block at the GV stage in a reversible manner. In fact, after removal of the inhibiting substances and incubation in sea water, the GV-arrested oocytes were able to undergo *in vitro* GVBD and extrusion of the follicle cells. However, the resulting size and morphology of the follicle cells was different from those *in vivo* matured. We demonstrated that the substances used, even at high concentrations, did not interfere with the acquired meiotic competence of the oocyte: in fact, all the oocytes matured after removal of the substances showing a reversible effect; furthermore, they were successfully fertilized showing high percentage of first cleavage. Hatched larvae were obtained in good percentage especially from caffeine or IBMX-incubated oocytes, but in a lower percentage than the control and showing an abnormal morphology. All together, these results suggest that although spontaneous oocyte maturation occurs in sea water, a different follicle cell formation and abnormal larval development may be due to a long-term effect of the lack of the physiological stimulus and related process of meiosis resumption.

Cyclic AMP has been defined as a paradox (Tsafriri & Dekel, 2010) since its action is differs with species. In the ascidian *Boltenia villosa* (Lambert, 2008) and in the echinoderm *Amphipholis kochii* (Yamashita, 1988), it has been observed that different substances elevating cAMP levels, i.e. forskolin, theophylline, caffeine and IBMX, stimulate GVBD even at low pH values normally used in the ascidians to fully block spontaneous maturation. Here, we demonstrated that, in the *C. intestinalis*, as in *Halocynthia roretzi* (Sakairi & Shirai, 1991), the same compounds exerted an opposite effect as they inhibited GVBD.

These data provide support that some physiological mechanisms of *Ciona* are different from some other invertebrates, but similar to those of some vertebrates such as amphibians and mammals (Kren *et al*., 2004; Sánchez-Toranzo *et al*., 2007; Ozawa *et al*., 2008; Chen *et al*., 2009).

Although *Ciona* shows many similarity with mammals, in the latter as in other species, the maintenance of elevated cAMP levels rely on a complex communication between oocyte and follicle cells. In *Ciona*, we previously demonstrated that defolliculated oocytes could directly respond to external stimuli and that functional gap junctions between oocyte and follicle cells were absent, indicating that follicle cells do not play a role in oocyte spontaneous maturation (Silvestre *et al*., 2009). Although a similar mechanism has been recently proved in marine worms suggesting that GVBD may be induced by a deactivation of AMPK (Stricker *et al*., 2010), in *Ciona* an unresolved question remains: how the signal coming from cAMP is transduced to the oocyte. A possible mechanism could involve the test cells that in *Ciona* exhibit tachykinin receptors, whose activation induces expression of several proteases. These enzymes were found to be responsible for

Figure 4 (continued) (c) oocytes after 2–3 h showing early follicle cells extrusion; (d) oocytes after 3–5 h with longer follicle cells and beginning of chorion elevation; (e) oocytes after 5–7 h reaching the maximum extrusion of follicle cells *in vitro*; (f) 2-cell stage 50 min after fertilization of GVBD oocytes (row d); (g) abnormal hatched larvae 24 h after fertilization; (h) mature oocytes collected from the oviduct (on the left) and normal hatched larva (on the right) showed for comparison. Scale bar is 120 μm for the oocytes and 2-cell stage, and 240 μm for the larvae.

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multiple biological events in the growth and maturation of oocytes and follicle cells in various animal species, including *Ciona* (Aoyama *et al*., 2008; Silvestre *et al*., 2009). The lack of action of cAMP analogue 8-bromo cAMP may be attributed to its limited inhibitory capability as shown by Downs & Chen (2006). Moreover, similar results were obtained by using another cAMP analogue in the *H. roretzi* oocytes (Sakairi & Shirai, 1991).

Although we show that cAMP plays a role in the process inducing GVBD, at present, the mechanism that influences cAMP increase and hence its role in maintaining maturation arrest remains to be clarified.

In this paper we indicate a variability of biological mechanisms existing among the ascidian species, and on the other hand we support increasing evidence that tunicates share many common biological mechanisms with vertebrates (Delsuc *et al*., 2006). This close relationship with vertebrates makes ascidians a suitable model for developmental and genomic studies.

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