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Mouse versus Rat: Profound Differences in Meiotic Regulation at the Level of the Isolated Oocyte

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Abstract:

Cumulus cell-enclosed oocytes (CEO), denuded oocytes (DO) or dissected follicles were obtained 44–48 h after priming immature mice (20–23-days-old) with 5 IU or immature rats (25–27-days-old) with 12.5 IU of equine chorionic gonadotropin, and exposed to a variety of culture conditions. Mouse oocytes were more effectively maintained in meiotic arrest by hypoxanthine, dbcAMP, IBMX, milrinone, and 8-Br-cGMP. The guanylate cyclase activator, atrial natriuretic peptide, suppressed maturation in CEO from both species, but mycophenolic acid reversed IBMX-maintained meiotic arrest in mouse CEO with little activity in rat CEO. IBMX-arrested mouse, but not rat, CEO were induced to undergo germinal vesicle breakdown (GVB) by follicle stimulating hormone (FSH) and amphiregulin, while human chorionic gonadotropin (hCG) was ineffective in both species. Nevertheless, FSH and amphiregulin stimulated cumulus expansion in both species. FSH and hCG were both effective inducers of GVB in cultured mouse and rat follicles while amphiregulin was stimulatory only in mouse follicles. Changing the culture medium or altering macromolecular supplementation had no effect on FSH-induced maturation in rat CEO. The AMP-activated protein kinase (AMPK) activator, AICAR, was a potent stimulator of maturation in mouse CEO and DO, but only marginally stimulatory in rat CEO and ineffective in rat DO. The AMPK inhibitor, compound C, blocked meiotic induction more effectively in

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hCG-treated mouse follicles and heat-treated mouse CEO. Both agents produced contrasting results on polar body formation in cultured CEO in the two species. Active AMPK was detected in germinal vesicles of immature mouse, but not rat, oocytes prior to hCG-induced maturation in vivo; it colocalized with chromatin after GVB in rat and mouse oocytes, but did not appear at the spindle poles in rat oocytes as it did in mouse oocytes. Finally, cultured mouse and rat CEO displayed disparate maturation responses to energy substrate manipulation. These data highlight significant differences in meiotic regulation between the two species, and demonstrate a greater potential in mice for control at the level of the cumulus cell-enclosed oocyte.

INTRODUCTION

Nearly a decade after the mouse genome had been sequenced (Mouse Genome Sequencing Consortium, 2002), the topic of sequencing the rat genome became somewhat controversial. Some argued the two species are so similar the genomes would likely prove to be redundant, thereby rendering such pursuit a waste. When an initial rat genome sequence analysis was published two years later, however, significant differences were reported between the two species (Rat Sequencing Project Consortium, 2004). Rats have apparently evolved more quickly than mice (Mullins and Mullins, 2004), and it has been suggested that the rat genome more closely resembles the human genome than that of mice (Zhao et al, 2004). It is logical to assume then that a variety of phenotypic differences would be manifest as a result.

For many years, mice and rats have been used as model organisms to study the mechanisms regulating meiotic maturation in mammals. In fact, much of what is understood concerning meiotic control has been gleaned from studies involving these two species. Results from studies in one species have often been used to corroborate findings in the corresponding species due to the tacit assumption that the physiology in one is similar, if not equivalent, to that in the other. Yet, experiments that directly compare the meiotic behavior of mouse and rat oocytes under a set of identical experimental conditions have rarely been conducted. It was the purpose of the present study to do just that: parallel groups of isolated

mouse and rat oocytes were simultaneously tested and compared for their meiotic response to a variety of specific culture conditions. The results document profound differences in numerous parameters of meiotic regulation in these two species, demonstrating effective regulation at the level of the oocyte-cumulus cell complex in mice, but suggesting a more important regulatory site at the cumulus-mural granulosa interface in rats.

RESULTS

Experimental System

For most of the experiments carried out in this study, the focus was on isolated cumulus cell-enclosed oocytes (CEO) or denuded oocytes (DO). Though the contribution of the mural granulosa follicular component has been eliminated under these conditions, this approach addresses the potential direct action of numerous agents on the oocyte. Nevertheless, the behavior of rat and mouse oocytes has been compared in cultured preovulatory follicles in some experiments.

Maintenance of Meiotic Arrest

Initial experiments were carried out to compare the efficacy of meiotic inhibitors on the spontaneous maturation of mouse and rat oocytes in vitro. CEO were cultured 17–18 h in increasing concentrations of dibutyryl cyclic adenosine monophosphate (dbcAMP), hypoxanthine, 3-isobutylmethylxanthine (IBMX) or milrinone, and then assessed for germinal vesicle breakdown (GVB). As shown in Fig. 1, each of these inhibitors suppressed maturation more effectively in mouse than in rat oocytes over the range of concentrations tested.
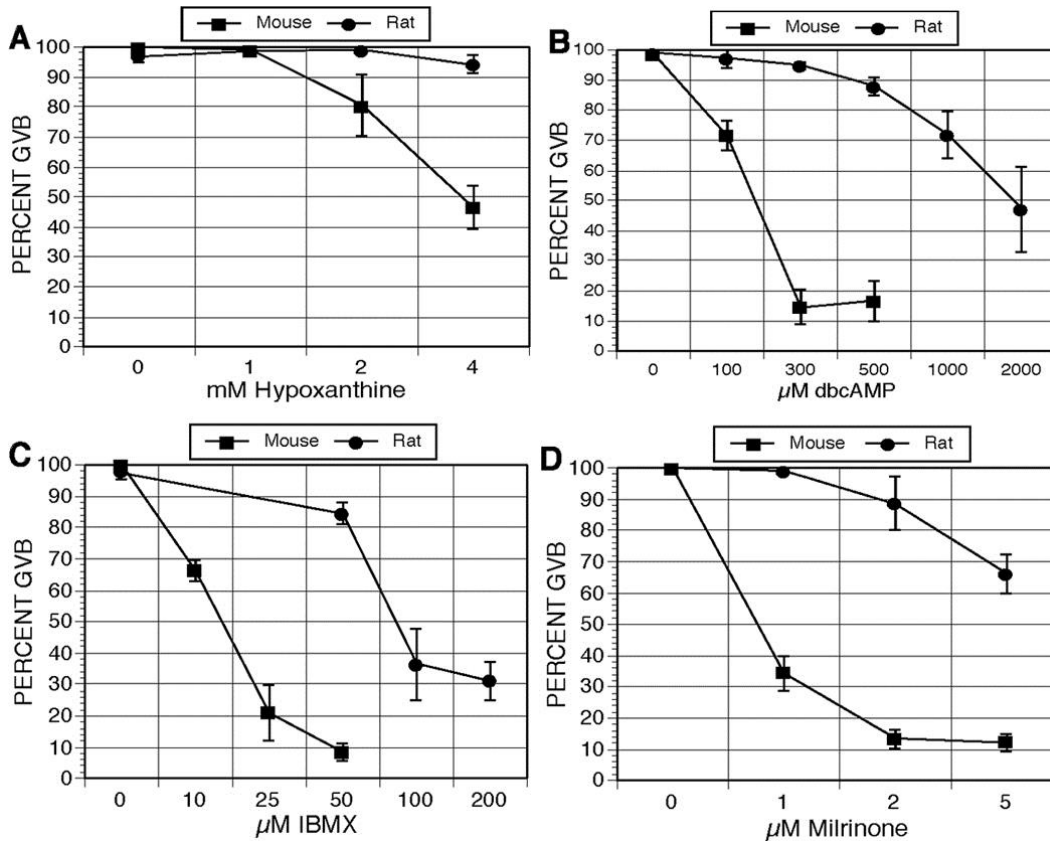
Fig. 1 Dose response effect of meiotic inhibitors on mouse and rat oocyte maturation. Mouse and rat CEO were cultured 17–18 h in increasing concentrations of one of four meiotic inhibitors, and then assessed for germinal vesicle breakdown (GVB).

To determine if more comparable inhibitory potency could be observed during shorter-term culture, both CEO and DO were cultured 3 h in each of these inhibitors and then assessed for GVB. As with overnight cultures, the inhibitors were much less potent in rat oocytes, whether denuded or cumulus cell-enclosed, and removal of the cumulus cells lessened the inhibitory effect in both species (Fig. 2). All four inhibitors significantly suppressed GVB in both groups of mouse oocytes, while only hypoxanthine and IBMX were inhibitory in rat CEO and none were inhibitory in rat DO. Because IBMX was the least-discrepant inhibitor between the two species of oocytes, it was used for subsequent experiments; specifically, 50 µm IBMX was used to maintain meiotic arrest in mouse oocytes, while 100 µM was used in rat oocytes.
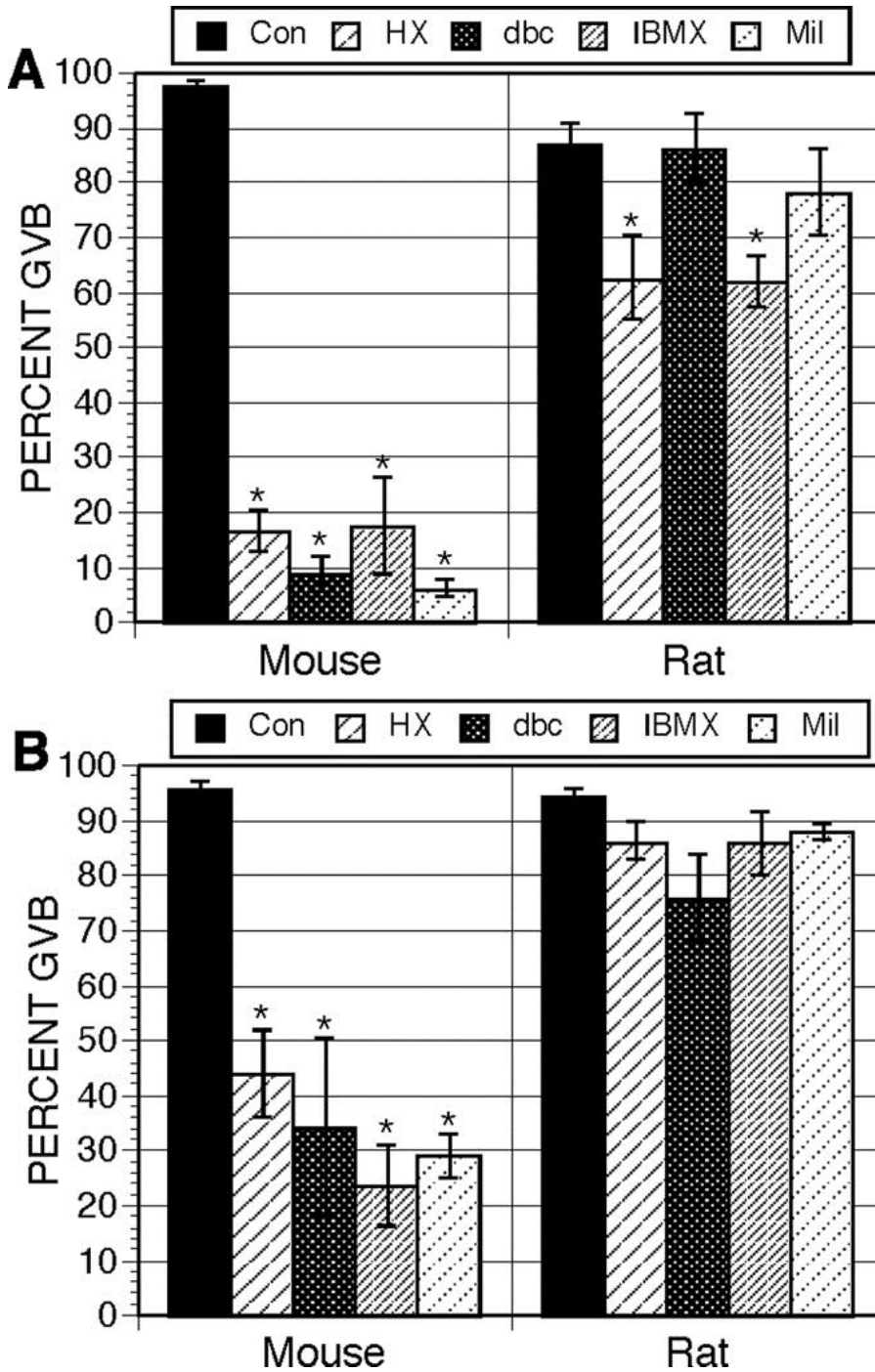
Fig. 2 Effect of short-term exposure to meiotic inhibitors on GVB. Mouse and rat CEO (Panel A) or DO (Panel B) were cultured 3 h in control medium or in medium supplemented with 4 mM hypoxanthine (HX), 250 µM dbcAMP, 25 µm 3-isobutylmethylxanthine (IBMX) or 1 µM milrinone (Mil). An asterisk denotes a significant difference from controls, by Student's *t*-test.

Spontaneous maturation of mouse and rat CEO during overnight culture was tested by treating populations with 8-Bromo-cyclic guanosine monophosphate (8-Br-cGMP). Because preliminary experiments revealed a limited capacity for inhibition by this agent alone, an initial experiment was carried out with mouse oocytes to establish a possible synergistic relationship between dbcAMP and 8-Br-cGMP. Mouse CEO were cultured 17–18 h in control medium or medium containing 100 μM dbcAMP alone, increasing concentrations of 8-Br-cGMP alone, or a combination of the two inhibitors. The suboptimal concentration of dbcAMP modestly reduced the maturation percentage to 80% from 100% GVB in the control group (Fig. 3A). The two lowest concentrations of 8-Br-cGMP (500 and 1000 μM) had negligible inhibitory activity; however, a concentration of 2000 μM reduced the maturation percentage by 67%. When cultured in combination with 100 μM dbcAMP, the two lower concentrations of 8-Br-cGMP demonstrated significant synergism, producing synergism quotients (SQ) of 3.0 and 2.4 for 500 and 1000 μM, respectively. This indicates that the degree of inhibition with the two agents together was 2.4–3.0 times the additive effect. Inhibition by 2000 μM 8-Br-cGMP was additive with dbcAMP (of 0.9).
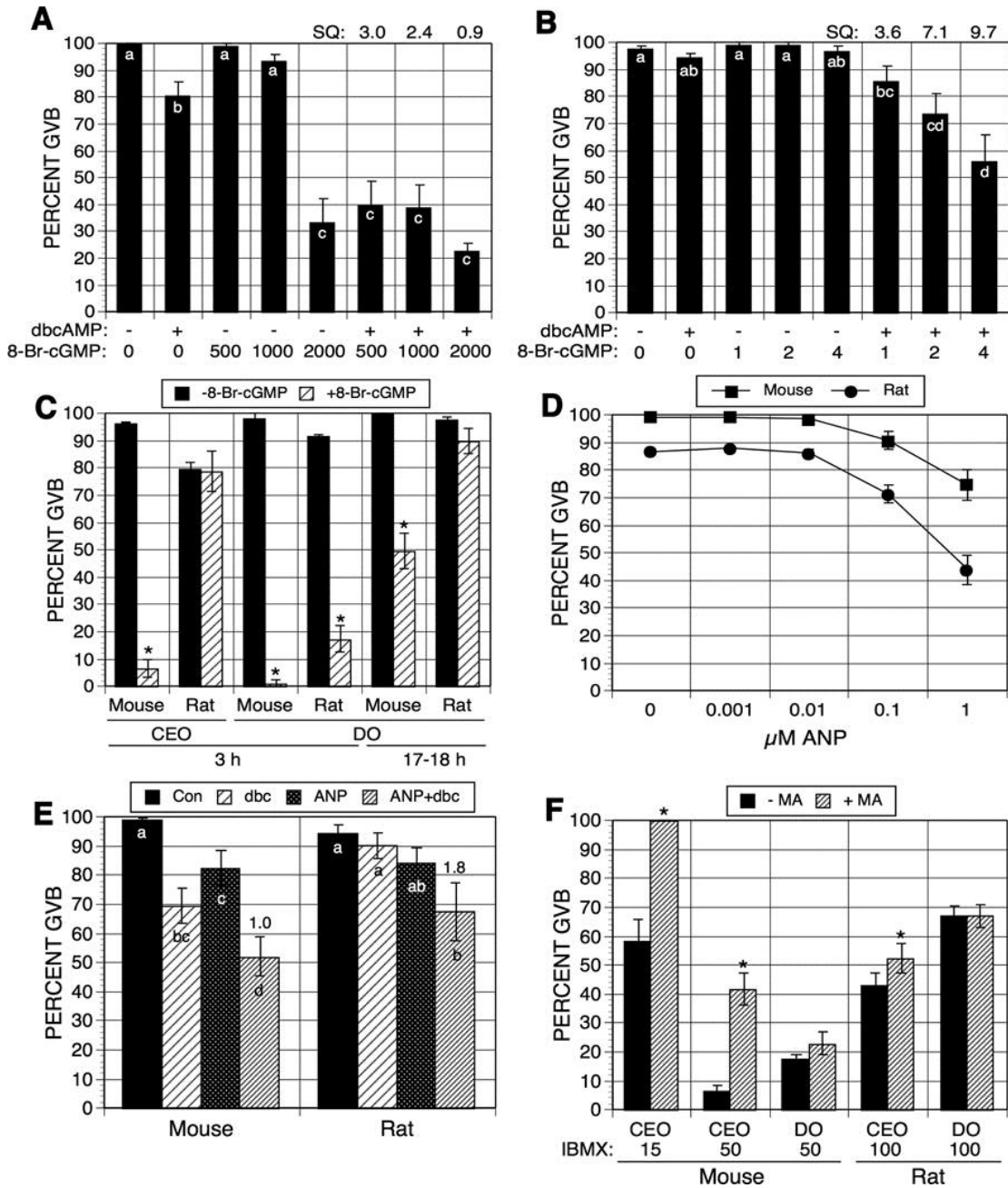
Fig. 3 Possible involvement of cGMP and guanyl compounds in maintaining meiotic arrest. Mouse (Panel A) or rat (Panel B) CEO were cultured 17–18 h in medium containing dbcAMP alone, 8-Br-cGMP alone, or the two in combination before assessment of GVB and determination of the synergism quotient. Groups with no common letter are significantly different. C, mouse and rat CEO or DO were cultured 3 h in the absence or presence of 2 mM 8-Br-cGMP before assessment of GVB. An asterisk denotes a significant difference from controls, by Student's *t*-test. D, Mouse and rat CEO were cultured 3 h in medium containing increasing concentrations of atrial

natriuretic peptide (ANP) before assessment of GVB. E, Mouse and rat CEO were cultured 17–18 h in control medium or medium containing dbcAMP alone, ANP alone, or dbcAMP + ANP before assessment of GVB. The number at the top of the bar represents the synergism quotient (SQ). Within each species, groups with no common letter are significantly different. F, mouse and rat CEO or DO were cultured 17–18 h in IBMX-supplemented medium in the absence or presence of mycophenolic acid (MA) before assessment of GVB. An asterisk denotes a significant difference, by Student's *t*-test.

When this relationship was examined in rat oocytes, a similar interaction was observed, but it required higher concentrations of both cyclic nucleotides. GVB was suppressed by only 3.4% in rat CEO exposed overnight to 500 µM dbcAMP and none of the 8-Br-cGMP concentrations (1, 2 and 4 mM) alone had any significant inhibitory effect; however, all three combinations of dbcAMP plus 8-Br-cGMP produced a synergistic inhibitory response in dose-dependent fashion (Fig. 3B), though the maturation percentages were still well above those achieved in mouse CEO with lower concentrations of inhibitors. The synergistic quotients were larger in the rat cultures (3.6–9.7), but this was due to the fact that so little inhibition occurred in either inhibitor alone.

To assess the short-term action of cGMP alone, mouse and rat CEO and DO were cultured 3 h in control medium or medium containing 2 mM 8-Br-cGMP, then scored for GVB. As shown in Fig. 3C, 8-Br-cGMP almost completely blocked meiotic resumption in mouse CEO after 3 h, but had no effect in rat; however, maturation was effectively suppressed in both mouse and rat DO. When cultured 17–18 h in 2 mM 8-Br-cGMP, only mouse DO exhibited significant suppression of GVB (Fig. 3C).

Recent evidence implicates the participation of atrial natriuretic peptide (ANP), a stimulator of guanylate cyclase, in the maintenance of meiotic arrest that acts at the level of the cumulus cell (Zhang et al, 2010). A dose-response experiment was therefore carried out in which mouse and rat CEO were cultured 3 h in medium containing increasing concentrations of a rat ANP peptide. Both mouse and rat CEO exhibited dose-dependent suppression of maturation in response to ANP, but in this experiment rat oocytes demonstrated greater sensitivity (43.9% GVB at 1 µM versus 74.8% GVB in mouse CEO; Fig. 3E).

To determine if ANP would synergize with cAMP, overnight cultures of CEO were carried out with dbcAMP and ANP, either alone or in combination. As shown in Fig. 3E, the effect of the two agents was additive in mouse CEO (synergism quotient of 1.0) and synergistic in rat CEO (synergism quotient of 1.8).

To compare how perturbation of guanyl nucleotide biosynthesis affects meiotic arrest, IBMX-arrested mouse and rat CEO and DO were treated overnight with the inosine monophosphate (IMP) dehydrogenase inhibitor, mycophenolic acid (MA), which prevents the conversion of IMP to xanthosine monophosphate, an intermediate in the pathway to guanyl nucleotides. Consistent with previous studies (Downs et al, 1986), MA stimulated GVB in mouse CEO but not DO (Fig. 3F). The extent of stimulation (35–42%) was similar whether a low or high dose of IBMX was used to maintain arrest. MA had a small, but significant, effect in rat CEO (an increase in GVB of 10%), with no effect in DO.

## Hormonal Induction of Maturation

In the first set of induction experiments, IBMX-arrested CEO were cultured 17–18 h in medium containing follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG) or the EGF-like peptide, amphiregulin. Luteinizing hormone/hCG has previously been reported to stimulate GVB in arrested rat CEO (Dekel and Beers, 1978), while FSH and EGF-like peptides have well documented stimulatory activity in mouse CEO (Downs et al, 1987; Downs and Chen, 2008). In mouse oocytes, both FSH and amphiregulin were potent inducers of GVB, while hCG was without effect; however, none of the hormones had any stimulatory effect on rat oocyte maturation (Fig. 4A–C). Several different forms of FSH, including ovine, rat, and human, had similar potency on mouse oocyte maturation, but even rat-derived FSH was unable to stimulate GVB in rat CEO (Fig. 4A).
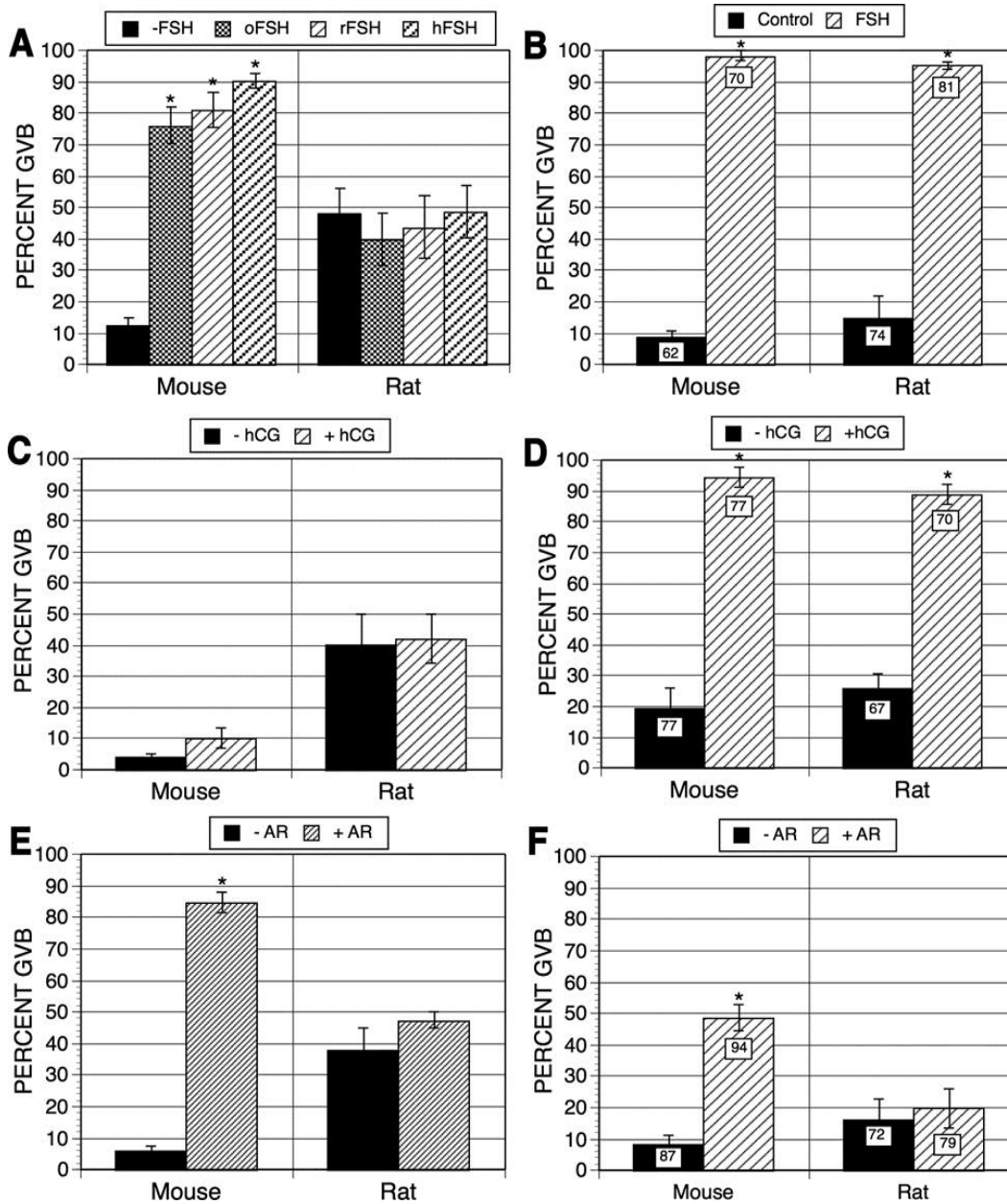
Fig. 4 Hormonal responsiveness of CEO (A, C, E) and FEO (B, D, F). Mouse and rat CEO were cultured 17–18 h in IBMX-supplemented medium (mouse, 50 µM; rats, 100 µM) containing 0.1 µg/ml follicle-stimulating hormone (ovine, rat or human FSH; Panel A), 5 IU/ml human chorionic gonadotropin (hCG; Panel C) or 0.01 µg/ml amphiregulin (AR; Panel E) before assessment of GVB. Mouse or rat FEO were cultured 3.5 h in control medium or medium containing highly purified FSH (Panel B), 5 IU/ml hCG (Panel D) or 1 µg/ml AR (Panel F) before assessment of GVB. The total number of follicles analyzed is provided for each group. For each experiment, an asterisk denotes a significant difference from the no hormone control, by Student's *t*-test.

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In the next induction experiments, intact preovulatory follicles were dissected and cultured 3.5 h in the presence or absence of 0.1 µg/ml highly purified ovine FSH, 5 IU/ml hCG, or 1 µg/ml amphiregulin. hCG and highly purified FSH were potent inducers of meiotic resumption in both mouse and rat follicle-enclosed oocytes (FEO) (Figs. 4D, E). Preliminary experiments with amphiregulin at a concentration shown to be effective in isolated mouse CEO (0.01 µg/ml) failed to trigger meiotic resumption in mouse FEO, prompting the testing of higher concentrations. While 0.1 µg/ml had a nominal stimulatory effect on mouse FEO (data not shown), 1 µg/ml stimulated about 40% of mouse FEO to resume maturation, while this same concentration failed to consistently drive meiotic resumption in rat FEO (Fig. 4F).

Because of the significant difference in meiotic outcome between the two groups of oocytes, it was important to further compare the extent of meiotic competence between mouse and rat oocytes in our equine chorionic gonadotropin (eCG)-primed model system. Although the dose-response experiments above demonstrated the ability of rat oocytes to resume maturation spontaneously in culture, they did not assess their ability to complete meiotic maturation. Thus, CEO and DO from both species were cultured 17–18 h prior to assessing their progression to metaphase II (MII). The results of this experiment show that both groups of rat oocytes complete meiotic maturation at levels comparable to mouse oocytes, and, like the results with mice, the denuded group had a significantly higher percentage of MII-stage oocytes than the cumulus cell-enclosed group (Fig. 5A).
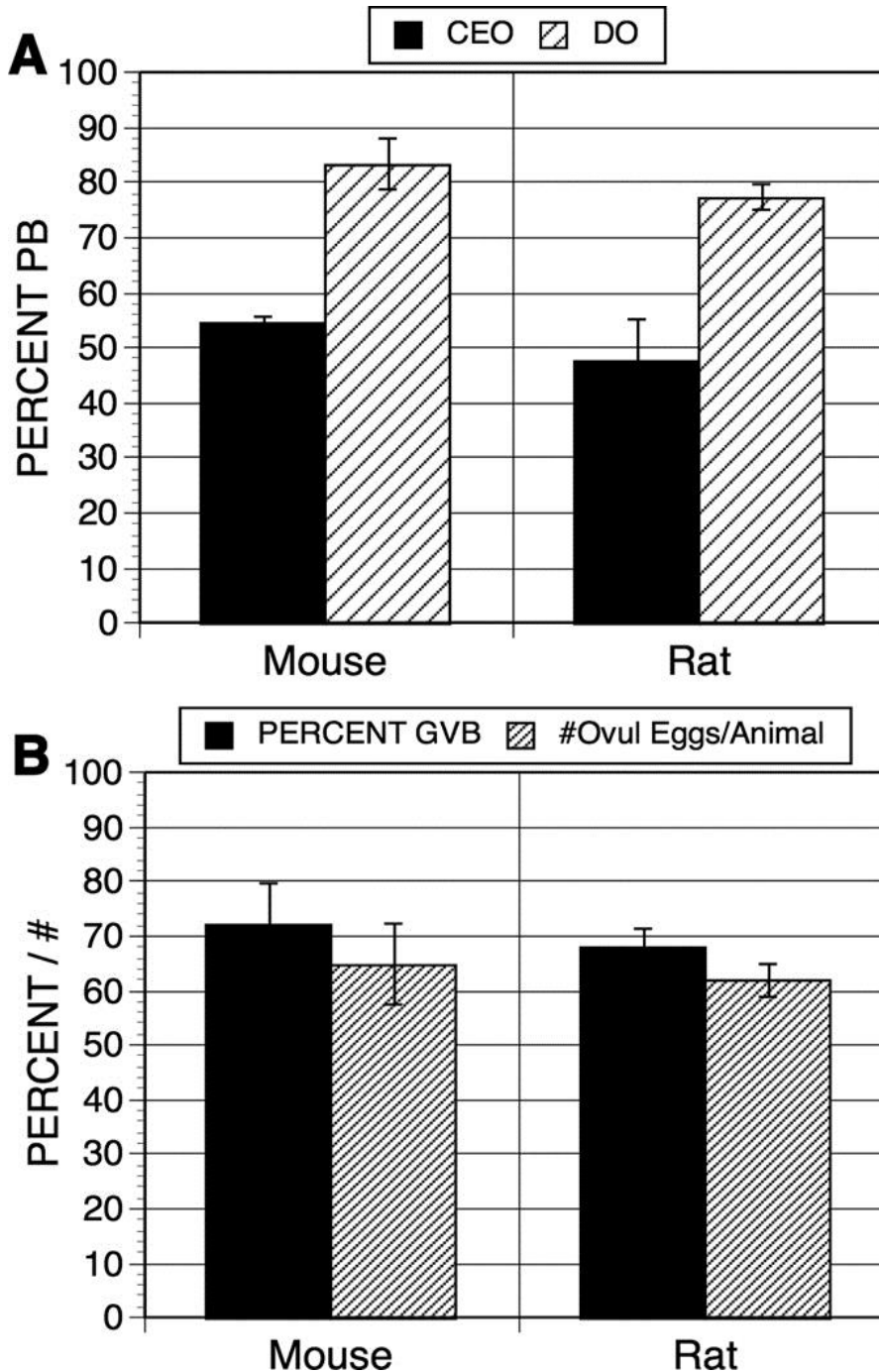
Fig. 5 Analysis of polar body formation and hCG responsiveness. A, Mouse and rat CEO and DO were cultured 16 h in control medium and assessed for polar body (PB) formation. B, Primed mice and rats were administered 5 IU and 12.5 IU hCG, respectively, and were either killed 2.5 h later to determine the extent of GVB in isolated CEO or were killed 16 h later and assessed for ovulation. Number of animals used: mice, 8; rats, 14.

To test sensitivity to hCG stimulation in vivo, eCG-primed animals received an injection of hCG, and then oocytes were isolated 3 h later and scored for GVB. Maturation rates in the two groups were virtually identical (68–72% GVB), as were ovulation rates in superovulated animals (62–65; Fig. 5B). These results show that the in situ responsiveness of the rat to hCG is comparable to that of the mouse, and that culture conditions support intrafollicular meiotic induction by gonadotropin in both species.

To determine the sensitivity of isolated CEO to hormonal stimulation by an alternative endpoint, mouse and rat CEO were cultured in serum-supplemented medium and tested for cumulus expansion. In mouse CEO, FSH, dbcAMP and amphiregulin were all potent stimulators of expansion, and the cumulus expansion index for FSH- and dbcAMP-treated CEO was increased by inclusion of IBMX (Fig. 6A). Rat CEO also responded to FSH and amphiregulin, but not dbcAMP, with significant expansion; however the expansion index was increased in both the FSH and dbcAMP groups by IBMX (Fig. 6B). IBMX alone had no effect on CEO from either species. These results demonstrate that, despite the inability of rat CEO to respond meiotically to FSH and amphiregulin, the cumulus cells are nevertheless responsive to these agents as measured by cumulus expansion.
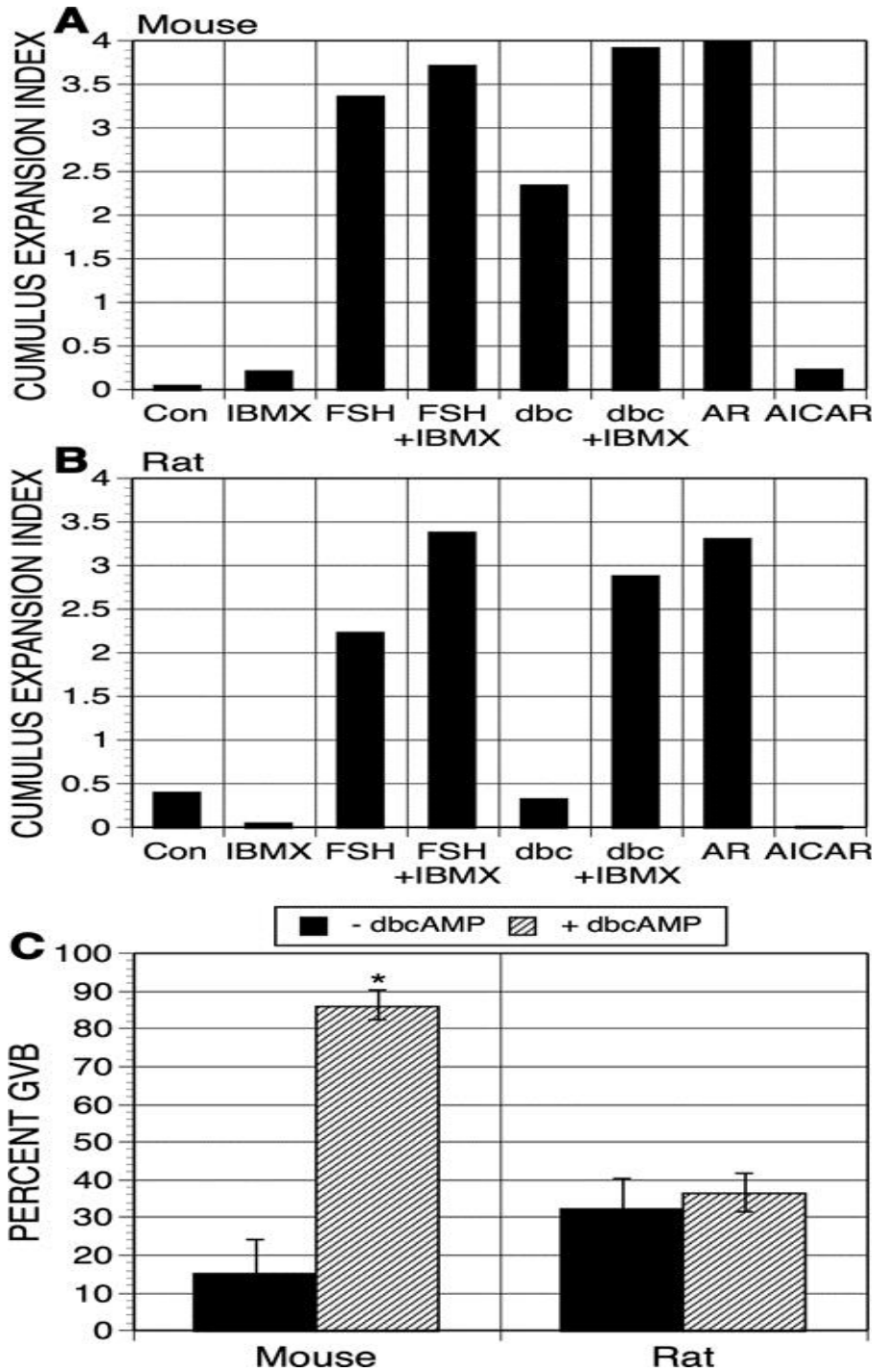
Fig. 6 Cumulus expansion and dbcAMP pulsing effects on meiotic maturation. A, Mouse CEO were cultured 16 h in medium containing 50 µM IBMX, 0.1 µg/ml FSH, FSH + IBMX, 2 mM dbcAMP, dbcAMP + IBMX, 0.01 µg/ml AR, or 1 mM AICAR and assessed for cumulus expansion, from which a cumulus expansion index was calculated. B, Rat CEO were cultured under identical conditions, except that the IBMX concentration was

100 µM. C, Mouse and rat CEO were cultured 3 h in medium containing IBMX (mouse, 50 µM; rat, 100 µM) plus 2 mM dbcAMP, then washed free of dbcAMP and returned to medium containing IBMX alone for 14–15 h before assessment of GVB.

To achieve meiotic induction in mouse CEO, hormonal stimulation can be bypassed by pulsing with high concentrations of cAMP analogs (Downs et al, 1988). To test whether or not rat oocytes would respond in a similar fashion, IBMX-arrested CEO from both species were exposed to 2 mM dbcAMP for 3 h and then returned to medium containing IBMX alone for an additional 14–15 h of culture. A parallel group of control oocytes was cultured 17–18 h in IBMX without pulsing. In mouse CEO, dbcAMP pulsing stimulated an increase in maturation from 16% to 86% GVB, whereas the effect was negligible in rat CEO (Fig. 6C). Thus, despite the fact that rat CEO can respond to IBMX plus dbcAMP exposure with significant cumulus expansion, dbcAMP pulsing in the presence of IBMX is unable to trigger meiotic resumption.

Effects of L-15 Medium and Serum on Meiotic Arrest and Induction

It is well established that medium composition has a dramatic effect on the meiotic behavior of mouse oocytes in vitro (Downs and Mastropolo, 1994,1997; Downs and Verhoeven, 2003). Many of the previous studies that have examined meiotic regulatory mechanisms in rat oocytes have used Leibovitz's L15 medium, a phosphate-buffered formulation that differs from minimal essential medium (MEM) in that it does not require a CO_2 gas phase and has a significantly different energy substrate composition than MEM—i.e., an absence of glucose, the inclusion of 900 mg/L galactose, and an exceptionally high concentration of pyruvate (5mM compared to 0.23 mM in MEM). In addition, for macromolecule supplementation, bovine serum albumin (BSA) is often replaced by serum. Thus, it was possible that the differences observed between rat and mouse oocytes in meiotic regulation in the above experiments were due to the use of a comparatively suboptimal culture system for rat oocytes. To test this idea, the effects of L15 and serum on (1) meiotic arrest maintained by hypoxanthine or IBMX and (2) meiotic resumption induced by FSH were compared in mouse and rat CEO. CEO were cultured 17–18 h in either MEM or L15 medium supplemented with BSA or 5% fetal bovine

serum (FBS). Meiotic arrest was maintained with either 4 mM hypoxanthine or IBMX at 50 µM (mouse) or 100 µM (rat).

Hypoxanthine-supplemented medium

Results with hypoxanthine as a meiotic inhibitor are shown in Suppl. Fig. S1A,B. In MEM, good inhibition of maturation in mouse CEOs was achieved with either BSA or fetal bovine serum (FBS) as supplement (34–35% GVB; Suppl. Fig. S1A. In addition, FSH was an effective stimulator of maturation, though to a lesser extent in FBS-supplemented medium (83% GVB compared to 99% GVB in BSA-supplemented medium). Hypoxanthine was a much less potent inhibitor of maturation in rat CEO, with better inhibition observed in FBS (75% GVB) compared to BSA (95% GVB). The addition of FSH tended to be inhibitory in BSA-containing medium, while there was a tendency for stimulation when FBS was present (a 10% increase in GVB; $P > 0.05$).

In L15 medium, hypoxanthine was a slightly less potent suppressor of maturation in mouse CEO, and meiotic induction by FSH was significantly reduced; in fact, no significant stimulation occurred in FBS-containing medium (Suppl. Fig. S1B). In rat CEO, hypoxanthine was slightly more potent as an inhibitor of maturation in L15 than in MEM (8–14% reduction in GVB), and the same pattern of maturation in response to FSH as that occurring in MEM was observed. It should be noted, however, that the overall level of maturation in response to FSH was reduced in FBS-supplemented medium (67–71% GVB compared to 85–90%).

IBMX-Supplemented Medium

Inhibition of maturation was more effective in both species when IBMX was used to maintain meiotic arrest. The pattern in mouse oocytes mimicked that seen in hypoxanthine-supplemented medium, with more effective meiotic induction occurring in MEM, and in medium containing BSA compared to FBS (Suppl. Fig. S1C,D). Similar levels of meiotic arrest were maintained in rat oocytes in MEM and L15 (27–43% GVB), and little change occurred in response to FSH stimulation regardless of the macromolecule supplementation, the most significant being a 19% increase in L15/FBS. These results show that neither

changing the culture medium to L15 nor altering the supplementation from BSA to FBS improved the responsiveness of rat CEO to FSH.

AMPK and Meiotic Maturation

We have previously demonstrated stimulation of meiotic induction and completion of meiotic maturation by AMP-activated kinase (AMPK) in mouse oocytes (Downs et al, 2002; Chen et al, 2006; Downs et al, 2010). It was therefore important to test if this kinase has a similar function in rat oocytes. IBMX-arrested CEO and DO from mice and rats were cultured 17–18 h in the presence or absence of the AMPK activator, 5-aminoamidazole-4-carboximide (AICAR). Consistent with our previous reports, AICAR was a potent stimulator of GVB in both CEO and DO from mice, with increases in maturation frequency of 76–83%. This agent had no effect in rat DO, however, and only a modest stimulatory action on rat CEO, increasing the GVB percentage from 28% to 54% (Fig. 7A), which coincided with the maturation frequency in DO cultured in IBMX alone. Similar results were obtained when an alternative inhibitor (dbcAMP) was utilized: while 300 µM was used to maintain arrest in mouse oocytes and 2 mM was used in rat oocytes, AICAR at a concentration of 1 mM increased the maturation frequency in mouse CEO from 18% to 98% and in rat CEO from 37% to 45%.
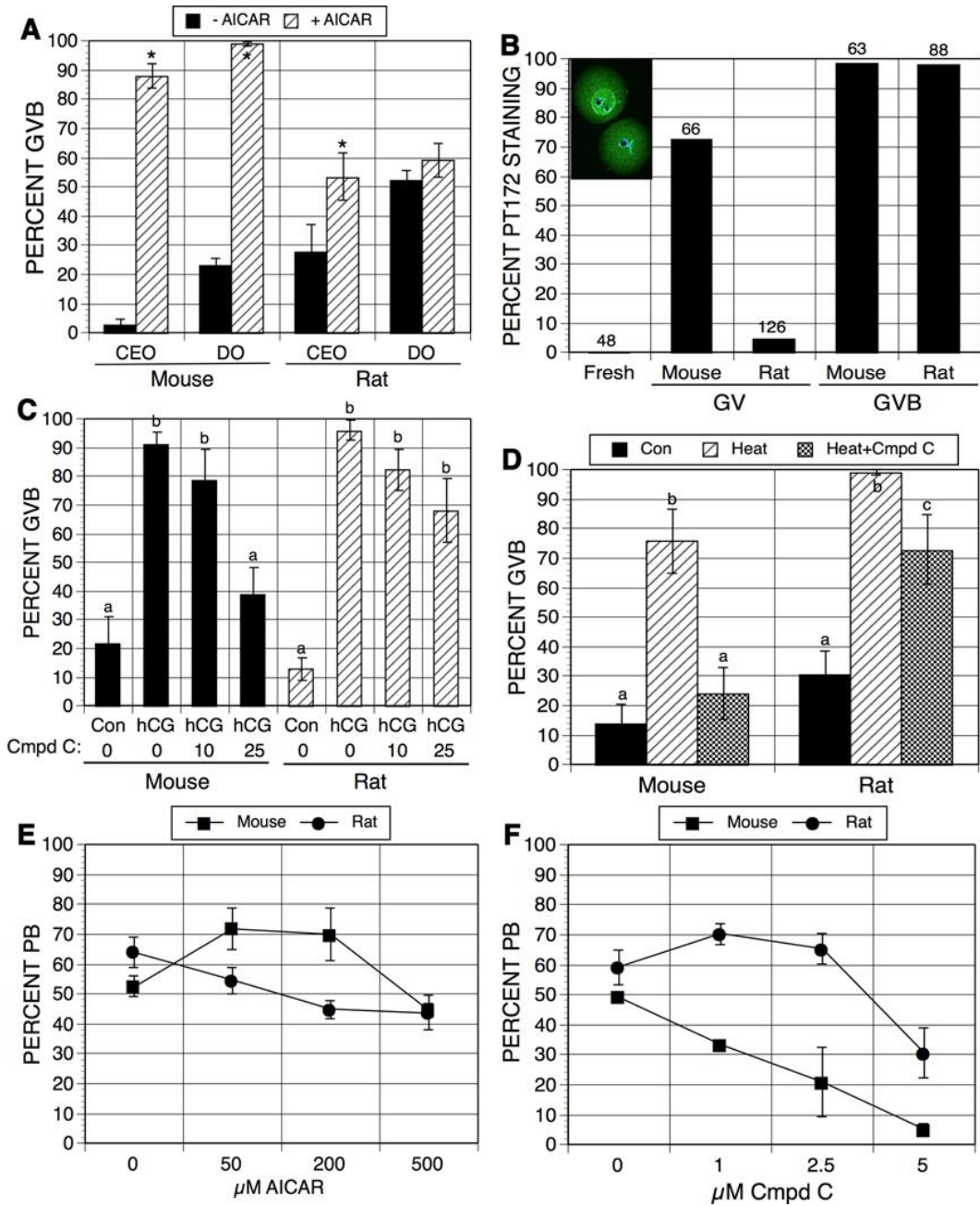
Fig. 7 Effects of AICAR and modulators of AMPK on oocyte maturation. A, Mouse and rat CEO and DO were cultured 17–18 h in medium containing IBMX (mouse, 50 μM; rat, 100 μM) plus or minus AICAR (CEO, 1 mM, DO, 0.5 mM) before assessment of GVB. An asterisk denotes a significant difference from the –AICAR group, by Student's *t*-test. B, Primed mice and rats were administered hCG and 2.5 h later CEO were isolated from the ovaries, fixed and stained for active AMPK. The percent of oocytes exhibiting active AMPK was determined in both germinal vesicle-stage and GVB-stage oocytes. The number of oocytes examined in each group is displayed above

each bar. Inset, immunofluorescence staining of phospho-AMPK, showing positive (top) and negative (bottom) oocytes. Blue, chromatin; green, phospho-AMPK. C, Mouse and rat follicles were cultured 3.5 h in medium containing 5 IU/ml hCG plus or minus compound C at 10 or 25 µM. Oocytes were then assessed for GVB. For each species, groups without a common letter are significantly different. D, Mouse and rat CEO were cultured 17–18 h in medium containing IBMX, with some being pulsed during the first hour at 42°C in the presence or absence of 2.5 µM compound C. Oocytes were then assessed for GVB. For each species, groups without a common letter are significantly different. E, F, Mouse and rat CEO were cultured 16 h in medium containing increasing concentrations of AICAR (Panel E) or compound C (Panel F) before assessment of polar body (PB) formation.

Primed mice and rats were next injected with hCG, then oocytes were retrieved from the ovaries 2.5 h later and processed for double immunofluorescence using an anti-PT172 antibody that recognizes active AMPK. The percentage of positively staining germinal vesicle-stage and GVB-stage oocytes from each species was then determined. As shown in Fig. 7B, mouse oocytes from primed mice not injected with hCG showed no active staining, but 73% of germinal versicle-stage oocytes from hCG-injected mice demonstrated active AMPK in the nucleus, while 99% of GVB-stage oocytes demonstrated active AMPK associated with the condensed chromatin. On the other hand, very few oocytes (<5%) from hCG-injected rats demonstrated active staining in the germinal vesicle prior to GVB, whereas most (98%) of GVB-stage oocytes had active AMPK colocalized with condensed chromatin. In the small percentage of germinal vesicle-stage rat oocytes encountered showing condensing chromatin, and thus actively engaged in GVB, no AMPK staining was observed whereas active AMPK was always observed in similarly staged mouse oocytes (Fig. 9). These results are consistent with the AICAR maturation data in that they implicate AMPK as a regulator of hormone-induced GVB in mice but not in rats.
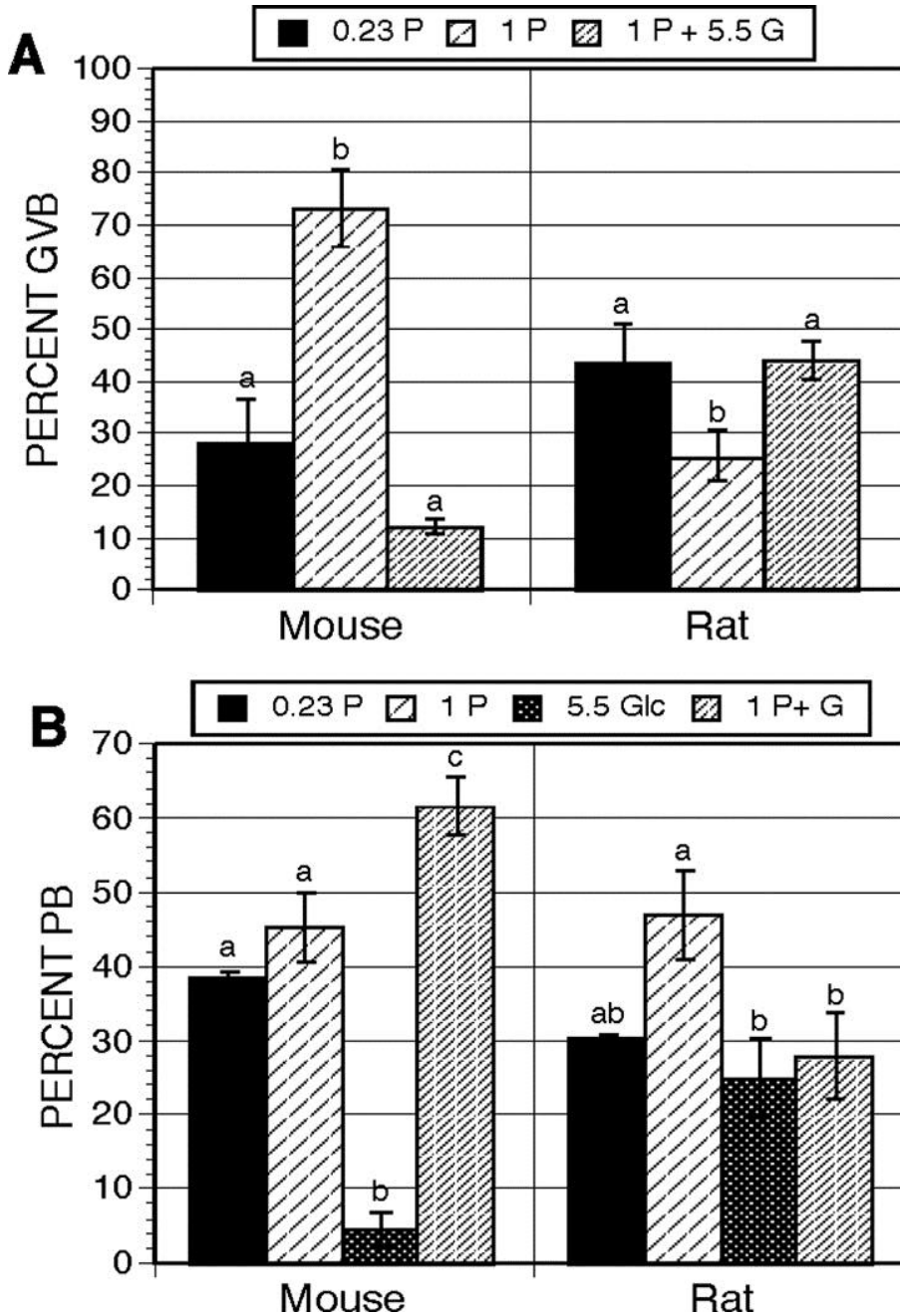
Fig. 9 Effects of energy substrates on oocyte maturation. A, Mouse and rat CEO were cultured 17–18 h in medium containing IBMX (50 µM, mouse; 100 µM rat) and supplemented with 0.23 or 1 mM pyruvate alone or 1 mM pyruvate plus 5.5 mM glucose before assessment of GVB. Groups without a common letter are significantly different. B, CEO were cultured 16 h in inhibitor-free medium containing 0.23 or 1 mM pyruvate alone, 5.5 mM glucose alone or 1 mM pyruvate plus 5.5 mM glucose before assessment of polar body (PB) formation. Groups without a common letter are significantly different.

To examine this further, cultured follicles were employed to test if the AMPK inhibitor, compound C, could block hCG-induced follicular oocyte maturation. As shown in Fig. 7C, hCG-induced maturation in mouse follicles was dose-dependently suppressed by compound C, with 25 µM reducing GVB from 91% to 39%, which was not significantly different from controls (22%). In rat follicles, compound C also suppressed hCG-induced maturation, but the effect was not as pronounced as it was in mice (a reduction from 96% to 68% GVB) and still differed significantly from controls (13% GVB).

AMPK is a stress response enzyme that is activated under a variety of conditions. We have shown that several different stresses trigger resumption of meiosis in meiotically arrested mouse oocytes in an AMPK-dependent manner (LaRosa and Downs, 2006, 2007). We therefore tested one of these stresses—heat—on meiotic arrest maintained by IBMX in mouse and rat CEO. Meiotically arrested CEO were initially heat stressed at 42°C for 60 min, and then placed in 37°C for the remainder of the culture period (16–17 h); controls were maintained at 37°C for the full culture period (17–18 h). An additional group was heat-treated in the presence of 2.5 µM compound C. Maturation was significantly stimulated in heat-treated mouse CEO groups (an increase in GVB from 14% to 76%), and this response was prevented by compound C (24% GVB; Fig. 7D). Interestingly, heat treatment also induced meiotic resumption in rat CEO (an increase from 30% GVB to 99% GVB), and compound C had a partial inhibitory effect on the maturation response (73% GVB).

The association of active AMPK with condensed chromatin in both mouse and rat oocytes raised the possibility that AMPK participates in oocyte maturation post-GVB. Previous data indicate that AMPK is involved throughout the entire maturation period in mouse oocytes from GVB to MII (Downs et al, 2010). Two types of experiments were performed to test this idea. First, mouse and rat CEO were exposed to increasing concentrations of AICAR or compound C, and oocytes were examined 16–17 h later for polar body formation. Interestingly, opposite response patterns were observed for both agents. In mouse oocytes, 50 and 200 µm AICAR increased the number of oocytes reaching MII (by 17–19%), while 500 µM was without significant effect (Fig. 7E). In rat oocytes, all concentrations of AICAR were inhibitory (average decreases of 9–20%). Compound C

dose-dependently suppressed polar body formation in mouse CEO, but the lower two doses in rat CEO either had no effect or produced a modest stimulation in polar body number (Fig. 7F). These results support a positive effect of AMPK on spontaneous oocyte maturation in mouse, but not rat, oocytes.

In the second set of experiments, mouse and rat oocytes were retrieved at varying periods after hCG injection of primed animals, and processed for immunofluorescence using the anti-PT172 antibody for active AMPK. As previously described (Downs et al, 2010), active AMPK in mouse oocytes localized to condensed chromatin after GVB, to spindle poles at metaphase I (MI) and –II (MII), and to the midbody during anaphase (Fig. 8). Some faint staining associated with metaphase chromosomes was also discernible. In rat oocytes, active AMPK also colocalized with condensed chromatin and appeared at the midbody during anaphase, but showed a different pattern at metaphase. Discrete punctate staining was apparent among the chromosomes at both MI and MII, but staining was absent at the spindle poles.
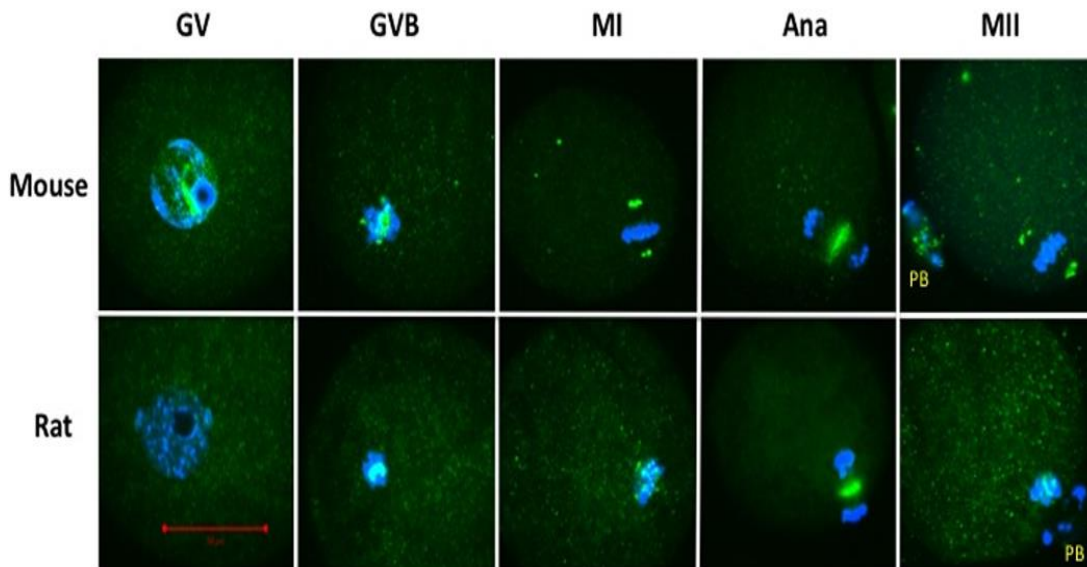
Fig. 8 Comparison of phospho-AMPK localization in oocytes throughout maturation. PMSG-primed mice and rats were administered hCG and oocytes were isolated at varying times post-injection, fixed and stained for active AMPK (anti-PT172 antibody). The different stages shown are germinal vesicle (GV), germinal vesicle breakdown (GVB), metaphase I (MI), anaphase (Ana) and metaphase II (MII) with polar body (PB). Blue, chromatin; green, phospho-AMPK. The bar represents 50 μM.

Effects of Energy Substrates on Oocyte Maturation

Varying the concentration of energy substrates in the culture medium has previously been shown to dramatically influence the meiotic status of mouse oocytes in culture (Downs and Mastropolo, 1994; Downs and Hudson, 2000). The effect of varying glucose and pyruvate supplementation on the inhibitory potency of IBMX was therefore tested during overnight incubation. Mouse and rat CEO were cultured 17–18 h in medium containing IBMX plus one of three energy substrate supplementations: 0.23 mM pyruvate (no glucose), 1 mM pyruvate (no glucose) or 1 mM pyruvate plus 5.5 mM glucose. In 0.23 mM pyruvate alone, mouse CEO underwent GVB at a rate of 28%, and raising the concentration to 1 mM increased the maturation frequency to 73%; however, the addition of glucose to 1 mM pyruvate reduced the meiotic response to only 12% GVB (Fig. 9A). Results with rat oocytes showed the opposite pattern: 0.23 mM pyruvate alone supported a maturation percentage of 44%, but increasing this concentration to 1 mM significantly lowered the response to 26% GVB; furthermore, the addition of glucose to 1 mM pyruvate did not reduce maturation but, rather, restored the maturation percentage to 44% (Fig. 9A).

The effects of energy substrates on PB formation during spontaneous maturation were assessed next. In mouse oocytes, polar body formation in the presence of 0.23 and 1 mM pyruvate was comparable (40–52%), while this number was negligible when maturation was carried out in 5.5 mM glucose alone (5%; Fig. 9B). The addition of glucose to medium containing 1 mM pyruvate increased the polar body percentage by 16% to 67%. Rat oocytes reached MII at a rate of 36–47% in 0.23 and 1 mM pyruvate, while this number was 27% in the presence of glucose alone (Fig. 9B). The addition of glucose to 1 mM pyruvate reduced the MII percentage by 18% to 29%. These results show that, like their response to AMPK-modifying agents, mouse and rat oocytes displayed opposing maturation responses to energy substrate manipulation during spontaneous maturation.

DISCUSSION

In this study, the meiotic behavior of mouse and rat oocytes has been compared under a variety of culture conditions. When isolated from preovulatory follicles and cultured in vitro, meiotic arrest was maintained much less effectively in rat oocytes by established meiotic inhibitors that act by modulating cAMP levels. Cyclic GMP and an activator of cGMP production suppressed maturation in oocytes of both species, but with less potency in rat oocytes. When maintained in meiotic arrest with IBMX, rat oocytes were unresponsive to hormones and other agents that were potent inducers of meiotic resumption in mouse oocytes, this in spite of comparable meiotic competence when oocytes from these two species were retained within the preovulatory follicle. Of particular interest was the finding that stimulation of AMPK triggered GVB in arrested mouse oocytes, but had only a nominal effect in arrested rat oocytes. Moreover, opposite effects on polar body formation during spontaneous maturation were manifest in rat oocytes compared to mouse oocytes upon treatment with stimulators and inhibitors of AMPK. Immunofluorescent localization of active AMPK showed activation of this kinase in the germinal vesicle prior to GVB in mouse oocytes, but not in rat oocytes, stimulated to resume maturation in vivo with hCG. Immediately following GVB, active kinase was localized to condensed chromatin in oocytes from both species, and thereafter colocalized with chromatin in rat oocytes but with components of the meiotic apparatus in mouse oocytes. Further, manipulation of pyruvate and glucose supplementation produced opposing effects on meiotic resumption. These characteristics of maturation indicate that significant differences exist between the rat and mouse in meiotic regulation.

Maintenance of Meiotic Arrest

We have utilized cAMP-elevating agents for many years to maintain isolated mouse oocytes in the germinal vesicle stage in vitro. Indeed, a significant decrease in oocyte cAMP levels is an important event preceding GVB in both mouse and rat oocytes (Schultz et al, 1983; Vivarelli et al, 1983; Dekel et al, 1984; Racowsky, 1984). cAMP analogs, purines, and phosphodiesterase inhibitors have previously been shown to block the spontaneous maturation of rat oocytes (Magnusson and Hillensjo, 1977; Dekel and Beers, 1978; Brannstrom

et al, 1987; Tornell et al, 1990a; Tsafriri et al, 1996) as well as mouse oocytes (Cho et al, 1976; Downs et al, 1985; Downs and Hunzicker-Dunn, 1995). In the present study, these agents suppressed the spontaneous maturation of oocytes from both species, but rat oocytes were much less effectively maintained in meiotic arrest than mouse oocytes in all experiments, whether cultured for 3 h or overnight. These results therefore highlight a profound difference between the two species in oocyte sensitivity to meiotic inhibitors that act via elevated cAMP.

cGMP also has an important regulatory function in oocyte maturation. More than three decades ago, it was reported that ovarian cGMP levels declined in response to luteinizing hormone (LH) stimulation in rats (Ratner, 1976; Ratner and Sanborn, 1980) and hamsters (Makris and Ryan, 1978; Hubbard, 1980). It was later shown in rat oocytes that a decrease in oocyte cGMP accompanies GVB (Tornell et al, 1990b), and this has recently been demonstrated in mouse oocytes as well (Norris et al, 2009; Vaccari et al, 2009). Exposure of oocytes to cGMP suppresses spontaneous oocyte maturation (Downs et al, 1988; Tornell et al, 1984, 1990b, 1991), and blocking guanylate cyclase in rat preovulatory follicles induces GVB (Sela-Abramovich et al, 2008). Tornell et al (1991) proposed that granulosa cell cGMP could reach the oocyte through gap junctions, suppress cAMP degradation, and thereby help maintain meiotic arrest. The results of two recent studies (Norris et al, 2009; Vaccari et al, 2009) strongly support this idea and show that cGMP acts by blocking the activity of PDE3A, the primary oocyte cAMP phosphodiesterase (Richard et al, 2001; Shitsukawa et al, 2001). It follows that the decrease in ovarian cGMP at the time of the preovulatory gonadotropin surge accounts for the resulting increase in cAMP phosphodiesterase activity that drives meiotic resumption (Masciarelli et al, 2004, Han et al, 2006).

Since cGMP can suppress cAMP degradation, it was reasoned that agents elevating cGMP within the oocyte should synergize with cAMP to maintain meiotic arrest, much like the interaction between cAMP and the phosphodiesterase inhibitors, IBMX and hypoxanthine (Downs et al, 1985; Eppig et al, 1985; Tornell et al, 1990a). When cultured overnight in the presence of increasing concentrations of 8-Br-cGMP, mouse CEO were dose-dependently maintained at the

germinal vesicle stage, but no inhibitory effect was observed in rat CEO, consistent with the findings of Tornell et al (1984). Nevertheless, 8-Br-cGMP synergized with dbcAMP to block spontaneous maturation in CEO from both species, though the absolute level of inhibition was greater in mouse oocytes, even when lower concentrations of cyclic nucleotide were used. The synergism quotients obtained were higher with rat oocytes, but this was due to the relative insensitivity of the CEO to either cyclic nucleotide alone. Denuded rat oocytes were maintained in meiotic arrest by 8-Br-cGMP alone, as were mouse oocytes, but the arrest was transient. The greater sensitivity of DO to 8-Br-cGMP identifies the oocyte as the site of action of cGMP, and further supports a role for this cyclic nucleotide in meiotic regulation. Further, while the effective suppression of GVB in rat DO by 8-Br-cGMP during short-term culture confirms that the cyclic nucleotide is accessible to the oocyte, we cannot discount the possibility that some of the differences observed in this study between the two species are due to differential permeability to the inhibitory agents.

Atrial natriuretic peptide (ANP) stimulates cGMP production via activation of natriuretic peptide receptors (NPRs) that constitute the membrane-bound form of guanylate cyclase, and this interaction serves as an important regulator of intraoocyte cGMP levels (Zhang et al, 2007). Previous studies have demonstrated ANP suppression of meiotic resumption in rat (Tornell et al, 1990b) and porcine (Zhang et al, 2005) oocytes. A recent study carried out in mice (Zhang et al, 2010) provided convincing evidence that ANP-stimulated production of cGMP in the somatic compartment diffuses to the oocyte, where it acts to block cAMP degradation and thereby maintain meiotic arrest; most compelling was the loss of meiotic arrest in situ upon genetic ablation of either receptor or ligand.

A potential synergystic interaction between dbcAMP and ANP was therefore tested in mouse and rat CEO. ANP alone dose-dependently maintained meiotic arrest in both oocyte groups during a 3-h culture period, though the extent of inhibition was limited. When dbcAMP was combined with ANP during overnight culture, the effects on mouse oocytes were additive, while those on rat oocytes were synergistic, yielding a synergism quotient of 1.8; nevertheless, the level of inhibition was greater in mouse oocytes. These data are

consistent with the idea that cAMP and cGMP act cooperatively to maintain meiotic arrest in oocytes from both species.

As a further test of the importance of guanyl compounds in meiotic regulation, IBMX-arrested CEO and DO were treated with the inosine mohophosphate (IMP) dehydrogenase inhibitor, mycophenolic acid. As reported in earlier studies (Downs et al, 1985), this inhibitor induced maturation in CEO but not DO, and was stimulatory in both species, although the effect in rats was barely significant. This indicates that guanyl compound production occurs in the cumulus cells and suggests rat CEO have a limited ability to generate guanyl compounds de novo when compared to their mouse counterparts.

Meiotic Induction and Hormone Responsiveness

The isolated mouse oocyte has been a popular model system for investigating hormone-induced meiotic induction. It was therefore important to test the ability of different hormones to stimulate meiotic resumption in meiotically arrested mouse and rat CEO. When IBMX-arrested oocytes were treated overnight with hormones, FSH and amphiregulin produced significant induction of GVB in mouse oocytes, but had no effect in rat oocytes, while hCG was ineffective in both groups. This corroborates previous reports in the mouse (Downs et al, 1988; Downs and Chen, 2008), but conflicts with an earlier study in rats (Dekel and Beers, 1978) in which both FSH and LH triggered GVB in IBMX-arrested CEO. When follicle-enclosed oocytes (FEO) were cultured, both mouse and rat oocytes responded to hCG and highly purified FSH with significant meiotic resumption. The collective data from CEO and FEO demonstrate effective responsiveness of rat tissues to gonadotropin, but suggest that the primary site of action for meiotic induction is the mural granulosa compartment.

Higher concentrations of amphiregulin were required to stimulate mouse FEO (1 μg/ml compared to 0.01 μg/ml in CEO) and the extent of GVB was less than that seen in CEO. Although a previous report (Dekel and Sherizly, 1983) showed effective induction of GVB in rat FEO by epidermal growth factor, the EGF-like peptide, amphiregulin had no effect in the present study. This is surprising in light of the finding that LH-induced maturation of rat FEO required prolonged activation of the EGF receptor (Reizel et al, 2010). It may be that

amphiregulin penetrates follicles poorly (c.f., Norris et al, 2010), and particularly so in rat follicles. Indeed, Romero and Smitz (2009) have reported a failure of the EGF-like peptide, epiregulin, to stimulate oocyte maturation in mouse follicles grown in vitro.

Experiments comparing relative meiotic competence and hormonal responsiveness revealed no differences in the ability of mouse and rat oocytes to resume maturation in vitro and to progress meiotically to MII; in addition, hCG administration to primed animals led to identical levels of meiotic resumption and ovulation. These are important observations because they confirm that rats were fully responsive to a superovulation protocol and demonstrate meiotic competence comparable to that observed in mice.

When cumulus expansion in vitro was analyzed, rat complexes were capable of significant expansion in response to FSH and dbcAMP, particularly when IBMX was also present, and amphiregulin alone was also very effective, although in all instances the level of expansion was less than that observed in mouse complexes. Nevertheless, despite the fact that both mouse and rat isolated complexes are responsive to hormonal stimulation as determined by cumulus expansion, only mouse complexes were able to generate ample downstream signal to drive meiotic resumption. Consistent with this were the results of the dbcAMP pulsing experiment, in which direct, short-term exposure to dbcAMP, presumably mimicking the effect of, and bypassing the need for, gonadotropin, stimulated GVB in IBMX-arrested mouse, but not rat, CEO. It is important to note that the concentrations of dbcAMP (2 mM) and IBMX (100 µM) used in the rat pulsing experiment were identical to those used for cumulus expansion. Interestingly, pulsing of cultured rat follicles with dbcAMP or IBMX is able to drive meiotic resumption (Tsafriri et al, 1972; Dekel et al, 1988), again suggesting a different site of action in the follicle.

A difference between many of the previous studies that have been conducted in mice and rats is the choice of culture medium. While most of the studies with mice have been carried out with conventional media such as MEM or MEMalpha supplemented with BSA and buffered with bicarbonate, those with rats have often utilized Liebovitz's L15 medium, usually supplemented with FBS and buffered with phosphate. MEM contains 5.5 mM glucose and we supplement it

with 0.23 mM pyruvate, while L15 medium omits glucose, has very high pyuruvate (5 mM), and also contains galactose as an energy source. In a previous study (Downs and Mastropolo, 1997), we reported that L15 medium supported poor induction of mouse oocyte maturation in vitro, with effective induction achieved by replacing galactose with glucose and reducing pyuruvate to 0.23 mM. To determine if the disparity in meiotic behavior between rat and mouse oocytes observed herein is due to different media requirements, the effect of MEM versus L15 medium and BSA versus serum were compared in both hypoxanthine- and IBMX-arrested CEO in the presence or absence of FSH. Although some minor differences were observed between the two media and macromolecule supplementations in the extent of inhibition and meiotic induction, the overall data indicate that the differences in meiotic behavior between mouse and rat oocytes are not attributable to the choice of culture media and supplements therein. Although mouse oocytes prefer MEM, rat oocytes exhibit no obvious preference.

Role of AMPK in Maturation

Experiments with AICAR implicated AMPK activation as a potent trigger of GVB in mouse, but not rat, oocytes. Modest induction of rat CEO was observed, but not at levels greater than the maturation observed in DO not exposed to AICAR; moreover, AICAR had no effect on rat DO but completely overcame meiotic arrest in mouse DO. This was supported by immunofluorescent labeling of active AMPK that showed germinal vesicle staining in a high percentage of mouse oocytes, but not rat oocytes, 2.5 h after hCG injection. Heat stress, previously shown to induce AMPK-dependent maturation in mouse oocytes (LaRosa and Downs, 2007), was equally potent in stimulating GVB in mouse and rat oocytes; this stimulation was completely blocked by compound C in mouse oocytes, but only partially blocked in rat oocytes. The possibility exists that maturation triggered by heat in rat oocytes can be mediated by AMPK. Similar results were obtained with follicle culture, in which treatment with hCG led to comparable levels of meiotic induction in mouse and rat FEO, but compound C was more effective in blocking mouse oocyte GVB. These results, which suggest that at least a portion of the maturation in hCG-stimulated rat FEO may be AMPK-mediated, are curious in light of the finding that no activation was detected in rat oocyte GVs in situ following injection of

primed rats with hCG. Alternatively, it is possible that some of the effects of compound C are unrelated to its action on AMPK.

Recent work indicated that activating AMPK in vitro augmented polar body formation in spontaneously maturing mouse oocytes, while suppressing AMPK activity had the opposite effect (Downs et al, 2010). When this relationship was compared in mouse and rat oocytes, our earlier work on mouse oocytes was confirmed, yet contrary results were observed in rat oocytes—specifically, AICAR was inhibitory to polar body formation in rat oocytes at concentrations that were stimulatory in mouse oocytes, while compound C was stimulatory or had no effect on rat oocytes at concentrations that were inhibitory to mouse oocytes. Although active AMPK was not detected in rat oocytes by immunostaining prior to GVB, active AMPK was detected upon GVB and was associated with condensed chromatin, but it was not localized to meiotic spindle poles as in mouse oocytes. It is tempting to speculate that the localization pattern of active AMPK is related to its potential influence on the successful completion of meiotic maturation to MII.

Energy Substrate Supplementation and Meiotic Maturation

It is well established that both the completion of spontaneous maturation and the meiosis-arresting capability of meiotic inhibitors in mouse oocytes is profoundly influenced by the relative levels of pyruvate and glucose in the culture medium (Downs and Mastropolo, 1994; Downs and Hudson, 2000). Herein, mouse and rat CEO displayed profound differences in their meiotic response to energy substrate manipulation. In IBMX-arrested mouse CEO, raising the pyruvate concentration from 0.23 mM to 1 mM promoted maturation of mouse oocytes by 45%, whereas glucose had an opposite, inhibitory influence when added to medium containing 1 mM pyruvate (61% decrease in maturation). These results are consistent with previous reports (Fagbohun and Downs, 1992; Downs and Mastropolo, 1994). On the other hand, identical manipulation produced the opposite responses in rat CEO (decrease of 18% and increase of 19%, respectively). When the completion of maturation was assessed in spontaneously maturing CEO, results in the two species were again contradictory. The addition of 5.5 mM glucose to 1 mM pyruvate

improved the percentage of mouse CEO progressing to MII by 22%, but reduced the percentage in rat CEO by 19%. Our results indicate significant differences in metabolism between the two species, and some of these observations are no doubt due to the fact that rat oocytes cannot use pyruvate as a sole energy source (Zeilmaker and Verhamme, 1974) whereas mouse oocytes can (Biggers et al, 1967). It will be particularly interesting to determine the extent of these metabolic differences and the degree to which they influence meiotic regulation.

Conclusions

A table has been prepared (Table 1) that summarizes the major findings of this study. Effective meiotic regulation in rats was lost upon removal of CEO from the follicle, while control in mouse oocytes was largely retained. This was manifest in rats as a reduced ability of meiotic inhibitors to maintain meiotic arrest and an inability of stimulatory ligands to induce meiotic resumption in arrested oocytes, even though significant cumulus expansion was observed. These results raise the possibility that the mural granulosa compartment exerts a more important meiotic regulatory influence in rat follicles, whereas in mice significant regulatory potential still resides in the cumulus cells. It has been proposed that the loss of gap junctional communication between the somatic compartment and the oocyte is a primary mechanism for gonadotropin-stimulated meiotic resumption (Dekel and Beers, 1978), and there is ample evidence to support this idea (Sela-Abramovich et al, 2005, 2006). Larsen et al (1987) proposed that gap junction disruption at the cumulus/mural granulosa interface may be pivotal in driving GVB following the ovulatory gonadotropin stimulus. Additional support comes from work in mice (Norris et al, 2008). Indeed, it has been suggested that the mural granulosa, and not cumulus oophorus, provides the inhibitory cyclic nucleotide responsible for meiotic arrest in rats (Sela-Abramovich et al, 2008). While such a mechanism may contribute to the meiotic resumption in both mice and rats, our results support the idea that it plays a more prominent role in rats, but confirmation of this idea must await further experimentation. In addition, results of this study indicate fundamental differences in metabolism between the two species as shown by the different responses to AMPK-modulating agents and energy substrate manipulation. It is anticipated that

further inquiry into these different aspects of metabolic behavior will shed light on existing similarities and differences between the two species and lead to a better understanding of meiotic regulation.

TREATMENT	MICE	RATS
Inhibition of GVB by cAMP-elevating agents	Strong	Weak
Inhibition of GVB by cGMP-elevating agents	Modest	Modest
Hormone induction of GVB in isolated CEO	Yes	No
hCG induction of GVB in follicle-enclosed oocytes	Yes	Yes
Meiotic induction by cAMP pulsing in CEO	Yes	No
cAMP stimulation of cumulus expansion	Yes	Yes
Superovulation response	GVB good; Ovulation good	GVB good; Ovulation good
AMPK induction of GVB in isolated oocytes	Yes	Weak or nonexistent
Cmpd C effect on hCG-induced GVB in follicles	Strong inhibition	Moderate inhibition
Effect of AMPK modulators on PB formation	AICAR promotes; Cmpd C suppresses	AICAR suppresses; Cmpd C promotes
Localization of active AMPK (PT172 Ab) during maturation	GV; w/chrom at GVB; strong at spindle poles, weak at chrom at MI & MII; midbody at Ana	No GV stain before GVB; w/chrom at GVB, MI & MII; midbody at Ana
Effects of energy substrates on IBMX-treated oocytes	Pyr promotes GVB; Glc suppresses GVB	Pyr suppresses GVB; Glc promotes GVB
Support of PB formation by energy substrates	Pyr alone good; Glc alone poor; Pyr + Glc optimal	Pyr alone good; Glc alone good; Pyr + Glc suboptimal

TABLE 1 Summary of Results in Mice and Rats

MATERIALS AND METHODS

Chemicals

Hypoxanthine, dbcAMP, 3-isobutylmethylxanthine (IBMX), milrinone, 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP), rat atrial natriuretic peptide (ANP) fragment 3–28, mycophenolic acid (MA), human chorionic gonadotropin (hCG), glucose and pyruvate were purchased from Sigma-Aldrich Co. (St. Louis, MO). Compound C was obtained from Calbiochem (EMD Chemicals, Gibbstown, NJ) and 5-aminoimidazole-4-carboximide (AICAR) from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Amphiregulin was supplied by R&D Systems (Minneapolis, MN), while rat, human, ovine and highly purified ovine follicle-stimulating hormone (FSH), as well as equine chorionic gonadotropin (eCG), were supplied by Dr. A.F. Parlow at the National Hormone and Peptide Program (Torrance, CA).

Animals and Culture System

C57BL/6J X SJL F_1 mice and Sprague Dawley rats were used for all experiments. To stimulate follicle development, 20–23-day-old mice were primed with 5 IU eCG and 25–27-day-old rats were primed with 12.5 IU eCG. To induce meiotic induction and/or ovulation in vivo 2 days after priming, mice were injected with 5 IU hCG, while rats received 12.5 IU hCG. For isolation of preovulatory oocytes, animals were killed 44–48 h after priming and ovaries were removed and placed in culture medium. Cumulus cell-enclosed oocytes (CEO) were isolated by puncturing the antral follicles with sterile needles; denuded oocytes (DO) were obtained by repeated pipetting of CEO through fine bore pipets.

Oocytes were isolated at room temperature in inhibitor-free medium. This reduced the total isolation time, since there was no need to wash oocytes free of inhibitor prio to allocation to the different experimental groups. We have found that oocytes do not commit to resume maturation during isolation at room temperature, and this is demonstrated in Supplemental Figure 2, where identical frequencies of maturation were observed in mouse and rat CEO and DO after 17–18 h of culture in IBMX-supplemented medium whether isolated in the presence or absence of IBMX.

Most experiments utilized Eagle's minimum essential medium (MEM; Sigma-Aldrich Co., St. Louis, MO) that was supplemented with 0.23 mM pyruvate, penicillin, streptomycin sulfate and 3 mg/ml crystallized lyophilized bovine serum albumin (BSA; MP Biomedicals, Solon, OH). For follicle culture and cumulus expansion experiments, medium was supplemented with 5% fetal bovine serum (FBS). MEM is a bicarbonate-buffered medium, whereupon CEO and DO cultures were gassed with a humidified mixture of 5% O_2, 5% CO_2 and balanced nitrogen, while follicle cultures were gassed with a humidified mixture of 95% O_2 and 5% CO_2. Some experiments involved the use of Leibovitz's L15 medium (GIBCO, Invitrogen, Carlsbad, CA), a phosphate-buffered medium that does not require a special gas phase to maintain pH, and, consequently, these cultures were maintained in air.

CEO and DO were cultured in 1 ml medium in capped plastic Falcon culture tubes in a 37°C water bath. Follicle cultures were carried out as previously described (Downs, 2000) with minor modifications. Briefly, follicles were dissected from ovaries of primed animals and placed in stoppered 10-ml glass Erlenmeyer flasks in 1 ml medium and gassed with 95% O_2 and 5% CO_2. The flasks were placed in a 37°C incubator with continuous gentle agitation.

Cumulus Expansion

Oocyte-cumulus cell complexes were cultured in Falcon petri dishes containing 2.5 ml medium. These dishes were cultured in a modulator incubator (Billups-Rothenburg, Del Mar, CA) that was gassed, sealed, and placed in a 37°C incubator. At the end of culture, the complexes were scored for expansion using a previously described scale (Fagbohun and Downs, 1990) ranging from 0 (no expansion) to 4 (complete expansion), and an average expansion index was calculated.

Immunofluorescence

Oocytes were fixed and processed for immunofluorescence as previously described (Downs et al, 2010), using rabbit anti-PT172 antibody (1:100; #2535, Cell Signaling Technology, Danvers, MA) followed by FITC-labeled sheep anti-rabbit antibody (1:1000; Sigma-

Aldrich, St. Louis, MO). The primary antibody recognizes phospho-threonine 172 of the catalytic subunit, and thus binds only to active kinase. Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) contained DAPI to stain DNA.

Statistics

All oocyte maturation experiments were conducted at least three times with at least 25 oocytes per group per experiment, and means ± SEM were calculated for presentation of data. For statistical analysis, all maturation frequencies were subjected to arcsin transformation and groups of three or more were analyzed by ANOVA followed by Duncan's multiple range test, while paired comparisons were made by Student's *t*-test. P values less than 0.05 were considered significant.

Supplementary Material

Supp Figure S1

Effects of medium and macromolecule supplementation on meiotic arrest and meiotic induction. Mouse and rat CEO were cultured in MEM or Leibovitz's L15 medium containing either 4 mM hypoxanthine or IBMX (mice, 50 µM; rats, 100 µM). In addition, medium was supplemented with either 3 mg/ml bovine serum albumin (BSA) or 5% fetal bovine serum (FBS) and contained no FSH or 0.1 µg/ml FSH. Oocytes were assessed 17–18 h later for GVB.
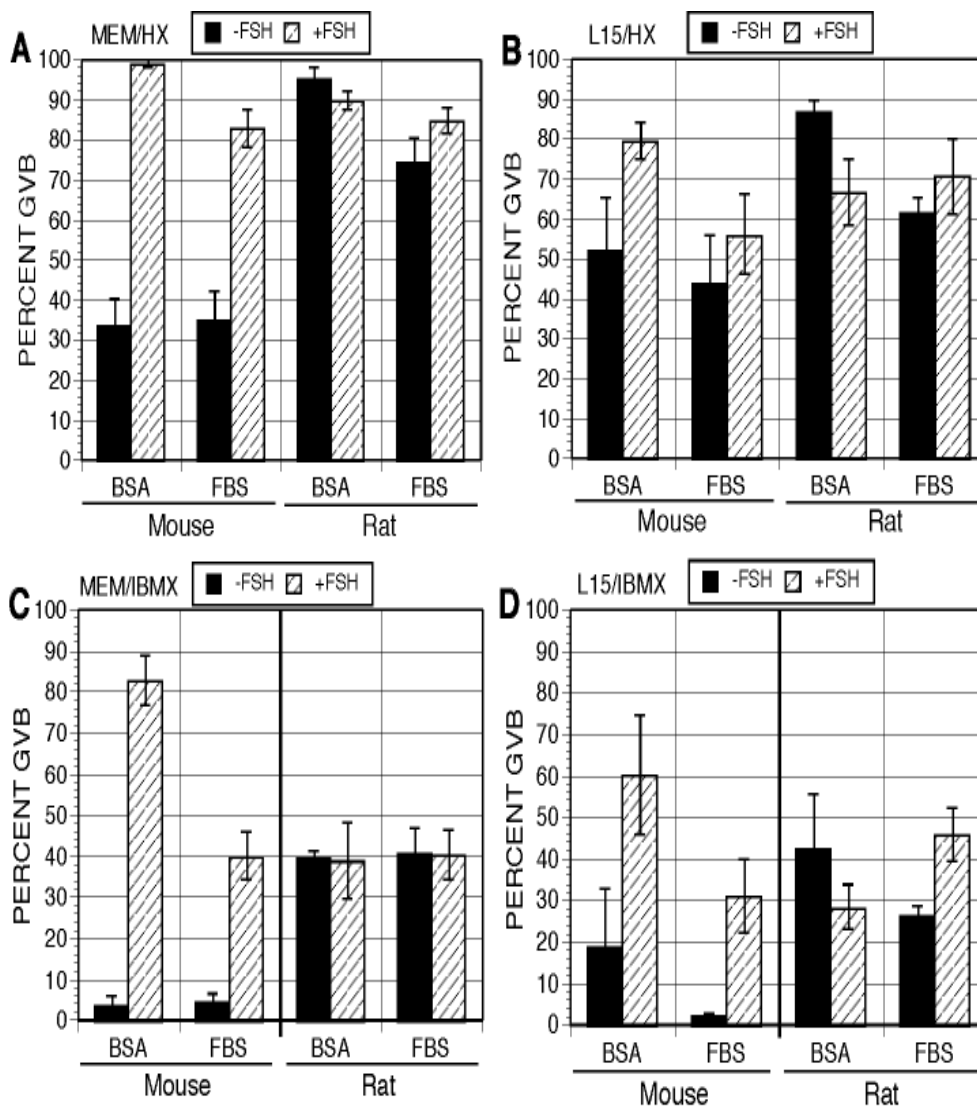
Supp Figure S2

Effect of isolation of oocytes in control or IBMX-supplemented medium on meiotic arrest maintained by IBMX. Mouse and rat CEO and DO were isolated in control, inhibitor-free medium or medium supplemented with 50 µM (mouse) or 100 µM (rat) IBMX before transfer to IBMX-containing medium (M/I and I/I groups, respectfully). Another set of oocytes was isolated and cultured in control, inhibitor-free medium (M/M). GVB was assessed after 17–18 h of culture.
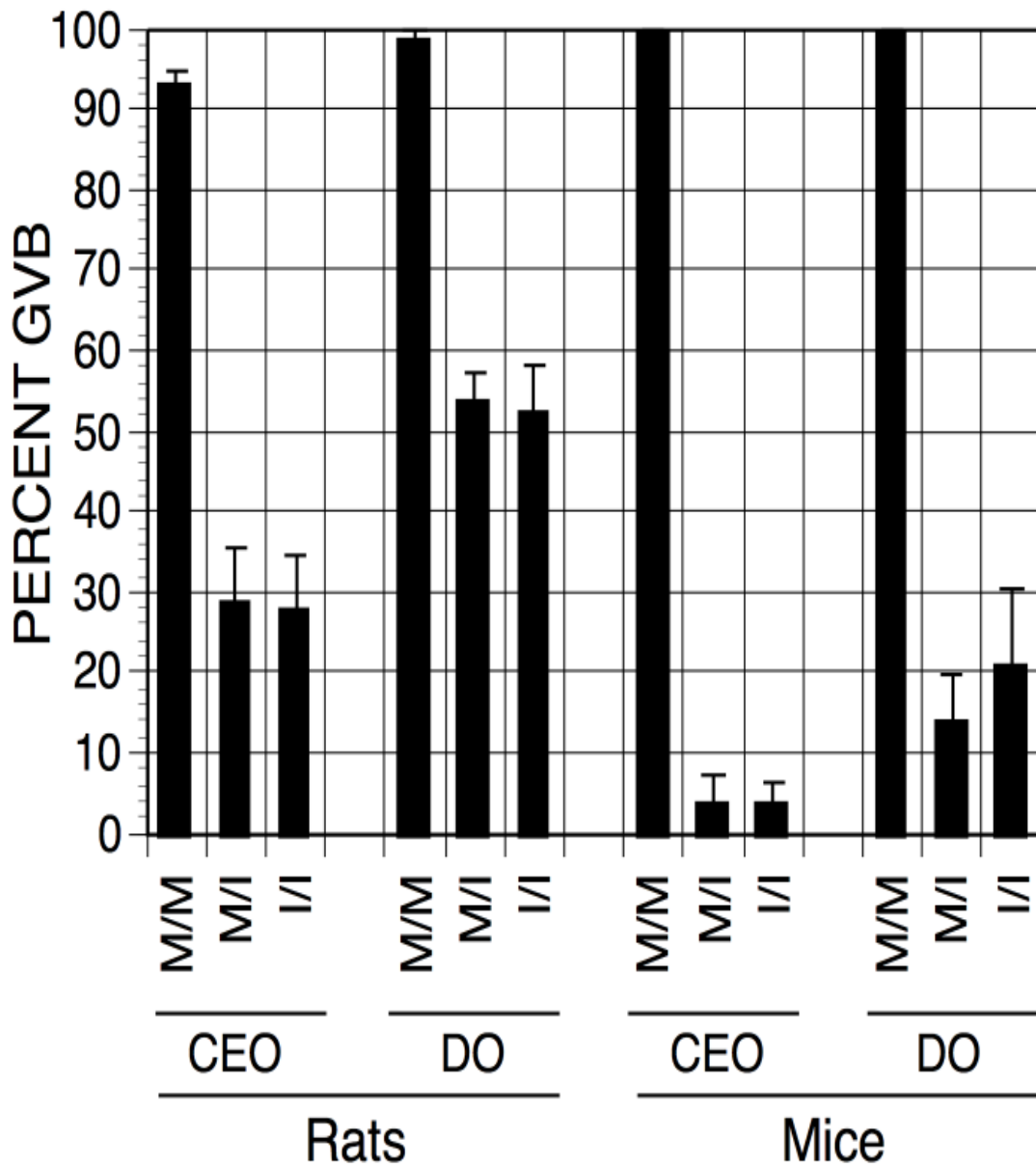
37

Acknowledgements

Abbreviations used

8-Br-cGMP : 8-bromo-cyclic guanosine monophosphate

AICAR : 5-aminoamidazole-4-carboximide

AMPK : AMP-activated protein kinase

BSA : bovine serum albumin

CEO : cumulus-enclosed oocytes

dbcAMP : dibutyryl cyclic adenosine monophosphate

DO : denuded oocytes

FBS : fetal bovine serum

FSH : follicle-stimulating hormone

GVB : germinal vesicle breakdown

hCG : human chorionic gonadotropin

IBMX : isobutylmethylxanthine

LH : luteinizing hormone

MEM : minimal essential medium

MI/II : metaphase I/II

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