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Generation of mouse oocytes defective in cAMP synthesis and degradation: endogenous cyclic AMP is essential for meiotic arrest

Sergio Vaccari[1,3], **Kathleen Horner**[1,3], **Lisa M. Mehlmann**[2], and **Marco Conti**[1,3,*]

[1]Division of Reproductive Biology, Department of Obstetrics and Gynecology Stanford University 94305

[2]Department of Cell Biology, University of Connecticut Health Center, Farmington, CT 06032

Abstract

Although it is established that cAMP accumulation plays a pivotal role in preventing meiotic resumption in mammalian oocytes, the mechanisms controlling cAMP levels in the female gamete have remained elusive. Both production of cAMP via GPCRs/Gs/adenylyl cyclases endogenous to the oocyte as well as diffusion from the somatic compartment through gap junctions have been implicated in maintaining cAMP at levels that preclude maturation. Here we have used a genetic approach to investigate the different biochemical pathways contributing to cAMP accumulation and maturation in mouse oocytes. Because cAMP hydrolysis is greatly decreased and cAMP accumulates above a threshold, oocytes deficient in PDE3A do not resume meiosis *in vitro* or *in vivo*, resulting in complete female infertility. *In vitro*, inactivation of Gs or downregulation of the GPCR GPR3 causes meiotic resumption in the *Pde3a* null oocytes. Crossing of *Pde3a*$^{-/-}$ mice with *Gpr3*$^{-/-}$ mice causes partial recovery of female fertility. Unlike the complete meiotic block of the *Pde3a* null mice, oocyte maturation is restored in the double knockout, although it occurs prematurely as described for the *Gpr3*$^{-/-}$ mouse. The increase in cAMP that follows PDE3A ablation is not detected in double mutant oocytes, confirming that GPR3 functions upstream of PDE3A in the regulation of oocyte cAMP. Metabolic coupling between oocytes and granulosa cells was not affected in follicles from the single or double mutant mice, suggesting that diffusion of cAMP is not prevented. Finally, simultaneous ablation of GPR12, an additional receptor expressed in the oocyte, does not modify the *Gpr3*$^{-/-}$ phenotype. Taken together, these findings demonstrate that *Gpr3* is epistatic to *Pde3a* and that fertility as well as meiotic arrest in the PDE3A-deficient oocyte is dependent on the activity of GPR3. These findings also suggest that cAMP diffusion through gap junctions or the activity of additional receptors is not sufficient by itself to maintain the meiotic arrest in the mouse oocyte.

Keywords

cAMP; GPCR; PDE3A; oocyte; meiotic arrest

*To whom correspondence should be addressed: Marco Conti, Director of Center for Reproductive Sciences, School of Medicine, UCSF, 513 Parnassus Ave, HSW 1656, San Francisco, CA 94143-0556. Tel.: 415-476-9214, Fax: 415-476-3121, Email:contim@obgyn.ucsf.edu.

[3]Present address: Center for Reproductive Sciences, UCSF, 513 Parnassus Ave, HSE 1645, San Francisco, CA 94143-0556

Introduction

Following the initial observations implicating somatic cell signals and cAMP in oocyte maturation (Edwards, 1965; Pincus and Enzmann, 1934), numerous studies have confirmed the importance of somatic-germ cell interactions in the control of meiosis (Conti et al., 2002; Eppig and Telfer, 1993). Pharmacological manipulations that increase cAMP levels in the oocyte prevent spontaneous maturation *in vitro* (Conti et al., 2002; Dekel and Beers, 1978; Eppig et al., 1993; Vivarelli et al., 1983), as well as *in vivo* maturation induced by the endogenous LH surge (Wiersma et al., 1998). Direct measurements of cAMP in oocytes removed from the antral follicle also show a correlation between cAMP levels and reentry into the meiotic cell cycle (Aberdam et al., 1987; Anderson and Albertini, 1976; Dekel and Piontkewitz, 1991; Schultz et al., 1983; Tornell et al., 1990; Vivarelli et al., 1983). Although conflicting observations were initially reported (Dekel et al., 1981; Dekel and Sherizly, 1983; Hillensjo et al., 1978a; Hillensjo et al., 1978b; Tsafriri et al., 1972; Yoshimura et al., 1992a; Yoshimura et al., 1992b), more recent data involving selective manipulation of cAMP levels in the somatic and germ cell compartments have confirmed a link between cAMP concentration in the oocyte and meiotic arrest (Tsafriri et al., 1996). The genetic inactivation of the major phosphodiesterase (PDE) form responsible for cAMP degradation in the oocyte has further consolidated the concept that cAMP plays an inhibitory role in meiotic resumption *in vivo* (Masciarelli et al., 2004). Finally, important strides have been made in identifying the biochemical steps in frog and mouse oocytes that link cAMP and the downstream kinase PKA to the inhibition of MPF, the cdc2/cyclin B complex, which is the master orchestrator of the cell cycle (Duckworth et al., 2002; Han et al., 2005; Newhall et al., 2006).

In spite of the consensus on the inhibitory function of cAMP on meiotic G2/M transition, there is considerable uncertainty on how cAMP levels necessary for the prevention of meiotic maturation are maintained in germinal vesicle (GV) oocytes that are competent to reenter the cell cycle. For many years, a common tenet has been that oocytes are not able to produce cAMP sufficient to maintain the meiotic blockade (Dekel et al., 1984) and that the somatic compartment provides the oocyte with a pool of cAMP through the extensive network of transzona projections and gap junctions connecting the oocyte to cumulus cells (Dekel et al., 1981). In support of this view, several reports have documented the transfer of cAMP generated in the cumulus cells to the oocyte during FSH stimulation (Bornslaeger and Schultz, 1985; Webb et al., 2002). As an extension of this concept, it has been proposed that reentry into the cell cycle is accompanied by the interruption of the communication between the oocyte and the somatic compartment or between somatic cumulus cells and granulosa cells (Larsen et al., 1986; Larsen et al., 1987; Racowsky et al., 1989). It is well established that pharmacological manipulation of gap junction permeability causes meiotic maturation in oocytes still in the follicle, suggesting an important role for these cell/cell contacts (Sela-Abramovich et al., 2006). However, the nature of inhibitors used in those studies could not distinguish between gap junctions among follicle cells and those between follicle cells and oocytes. Alternative possibilities proposed are that somatic cells produce hypoxanthine, which blocks the PDE present in the oocyte (Eppig and Downs, 1987) or that they produce an inhibitor of meiosis transferred to the oocyte through gap junctions (Eppig et al., 1983).

More recent studies have revisited the idea of the oocyte's autonomous production of cAMP and its role in maintaining meiotic arrest. Mouse and rat oocytes express active adenylyl cyclases, including ADCY3 and ADCY9. Analysis of adenylyl cyclase 3 deficient oocytes shows a precocious maturation, suggesting that cAMP accumulation within the oocyte is required to maintain the meiotic arrest (Horner et al., 2003). Moreover, injection of Gs inhibitory antibody into oocytes within the follicle causes meiotic resumption (Mehlmann et al., 2002). Since the antibody cannot diffuse through gap junctions, this observation strongly implies that Gs regulation of adenylyl cyclase and cAMP production is endogenous to the

oocyte. In the same vein, oocytes deficient in phosphodiesterase 3A (*Pde3a*$^{-/-}$) remain in meiotic arrest after isolation from preovulatory follicles and culture up to 48hr or after ovulation, suggesting that they continue to produce cAMP after release from the follicle (Masciarelli et al., 2004). Finally, receptors coupled to Gs and adenylyl cyclase have been described in the oocyte (Hinckley et al., 2005; Ledent et al., 2005; Mehlmann et al., 2004). G-protein coupled receptor 3 knock-out (*Gpr3*$^{-/-}$) females have a phenotype of premature GVBD in the absence of LH stimulation at the time when oocytes become competent for meiotic maturation (Ledent et al., 2005; Mehlmann et al., 2004). Similarly, downregulation of GPR3 or GPR12 by the use of RNAi or morpholino oligonucleotides causes spontaneous maturation in mouse and rat oocytes, respectively (Hinckley et al., 2005; Mehlmann, 2005). From these studies, it has been inferred that GPR3 activation of Gs in the oocyte causes cAMP accumulation above a threshold that precludes oocyte re-entry into the cell cycle. In support of subunit from the cytoplasm to the plasma membrane (Freudzon et al., 2005); however, no evidence is available as to whether cAMP levels are indeed reduced in the *Gpr3*$^{-/-}$ oocytes. Interestingly, the *Gpr3*$^{-/-}$ ovary shows a small population of oocytes that do not prematurely resume meiosis (Ledent et al., 2005; Mehlmann, 2005; Mehlmann et al., 2004), opening the possibility that other mechanisms may compensate for the loss of *Gpr3*. We and Mehlmann et al. have established the potential expression of up to 30 G-protein coupled receptors (GPCRs) in rodent oocytes (Mehlmann et al., 2004) and demonstrated the importance of GPR12 in maintaining rat oocyte meiotic arrest (Hinckley et al., 2005). Thus, additional GPCRs may contribute to the control of oocyte maturation in rodents.

To further define how cAMP levels are regulated in the rodent oocyte, we have taken advantage of genetic models that disrupt cAMP homeostasis within the oocyte. By comparing the phenotypes of disrupted cAMP synthesis and degradation in vivo in the oocyte, we propose that GPR3 and PDE3A are primary determinants of cAMP levels necessary to maintain meiotic arrest in mouse oocytes and that other mechanisms contributing to cAMP levels in the oocyte are not sufficient to maintain meiotic arrest.

Materials and Methods

Animals and Materials

Gpr3$^{+/-}$ mice on a C57BL/6 background were obtained from Dr. Laurinda Jaffe (originally provided by Deltagen, Inc., San Carlos, CA) and the colony was established at Stanford University. The *Pde3a*$^{-/-}$ colony (C57BL/6 x 129Sv), generated as previously described (Masciarelli et al., 2004), was established and maintained through heterozygous breeding. All the effect of the different alleles were assessed using mice of (C57BL/6 x 129Sv) mixed background. All animal procedures were in accordance with accepted standards of humane animal care and were approved by the Institutional Animal Care and Use Committee at Stanford University. All reagents were purchased from Sigma (St. Louis, MO, USA) or as otherwise specified. Mice were genotyped by PCR using specific primers designed to detect wild-type and targeted alleles from extracted tail DNA, as already described (Masciarelli et al., 2004; Mehlmann et al., 2004).

Collection of oocytes, cumulus cells, granulosa cells and follicles

Oocytes and granulosa cells were collected as described previously (Hinckley et al., 2005). Immature 22-23 day-old mice were primed with 5IU of PMSG (Calbiochem, San Diego, CA, USA), and 42-44 hours later, the ovaries were excised and collected in M2 media (Quinn et al., 1982) supplemented with 4 mg/ml of fatty acid-free BSA. When needed, hypoxanthine was added at a final concentration of 3.5 mM. Preantral, antral or preovulatory follicles were isolated with the use of a stereomicroscope (Olympus, Melville, New York) using a 30 G needle and sorted in groups according to size and morphology. Preovulatory follicles were punctured

with a 30 G needle, and cumulus—oocyte complexes (COCs) were aspirated and stripped of cumulus cells by repeated pipetting with a pulled Pasteur pipette. Denuded oocytes were washed free of all cells by transferring to different plates of fresh medium. For cumulus cell collection, COCs were separated from mural granulosa cells and cumulus cells were stripped from oocytes. Oocyte-granulosa cell complexes from preantral follicles were obtained by modifying a protocol used for primordial follicle culture (O'Brien et al., 2003). Preantral follicles were isolated in M2/BSA (4 mg/ml) from 11-12-day-old mice and incubated for 20 min at 37°C in the presence of 0.1% type I collagenase and 0.01 U/ul DNaseI. During a 20 min incubation with enzymes, the preantral follicles were subjected to repetitive pipetting with a pulled Pasteur pipette whose internal diameter was selected to be slightly larger than the follicle. This combined digestion/mechanical treatment causes the release of the theca and outer layers of granulosa cells, yielding oocytes surrounded by several layers of granulosa cells. At the end of the incubation, the resulting oocyte-granulosa cell complexes were transferred to fresh drops of M2/BSA media and cleaned from theca/interstitial cells debris by repeated pipetting with a pulled Pasteur pipette.

Maturation rate measurements

Ovaries from 22-day-old mice were collected 42-44 hours after an intraperitoneal injection of 5 IU PMSG. The ovaries were either processed to obtain denuded oocytes or washed in phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde (PFA) overnight (O/N) at 4° C. After fixation, the ovaries were dehydrated in several steps in increasing concentrations of ethanol, embedded in paraffin, then sectioned and stained with Hematoxylin and Eosin (Dako, Carpinteria, CA) (H&E). The entire ovary was sectioned and each section recorded in digital format using a camcorder (Carl Zeiss AxioCam HRc) connected to a transmitted microscope at 20X magnification. Every section was then analyzed and every oocyte present was classified according to the size of the follicle. In addition, the morphology, maturation stage, and size of each oocyte were recorded (data not shown). Axioprism software (version 3.1.0.1.8) from Leica was used for the analysis of follicle and oocyte size.

Mouse oocyte microinjections

Antibody microinjections were performed as previously described by Mehlmann et al. (Mehlmann and Kline, 1994). Dr. Teresa Jones (National Institutes of Health, Bethesda, MD) provided the antibody against Gs. This affinity purified antibody, produced against the carboxyl terminal 10 amino acids of mammalian Gs (Simonds et al., 1989), specifically recognizes Gs protein in mouse oocytes (Mehlmann et al., 2002), inhibits G_s activity (Gallo et al., 1995), and causes GVBD when injected into frog and mouse oocytes (Gallo et al., 1995; Mehlmann et al., 2002). The medium for the antibody injections was Hepes-buffered MEM (Mehlmann and Kline, 1994). Following microinjection, oocytes were placed in 200 μl drops of MEM-Hepes, incubated on a 37°C tray, and scored for GVBD.

Morpholino oligonucleotide oocyte microinjection was performed as previously described (Hinckley et al., 2005). Oocytes were collected from 22-day-old mice previously primed with PMSG. In all experiments, oocytes from wild type (WT) mice were maintained in meiotic arrest with 3.5 mM hypoxanthine added to the media. All morpholino (MO) oligonucleotides were dissolved in nuclease-free water at a concentration of 1 mM and stored at -80°C in 2 μl aliquots. All aliquots were heated at 60°C for 5 min, briefly vortexed and cooled to room temperature (RT) for 10 min prior to injection. 10–15 pl of the indicated MO was injected into the cytoplasm of the oocytes. Immediately after injection, oocytes were transferred drops of α-MEM (GIBCO, Carlsbad, CA) medium supplemented with 0.3% fatty acid-free (FAF) BSA under a layer of mineral oil and incubated at 37°C in 5% CO_2. Meiotic progression was scored up to 24 hours with an inverted microscope (Olympus) fitted with a Hoffman contrast lens.

Xenopus laevis *oocyte microinjections*

Ovary fragments were surgically removed from PMSG-primed *X. laevis*, and defolliculated oocytes were isolated after treatment with collagenase (2.5 mg/ml) in MBS buffer [10 mM HEPES (pH 7.4), 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM Na_2 HCO_3] for 1–1.5 hours. Dumont Stage VI oocytes were selected for all experiments. All experimental procedures were carried out in OR2 solution (5 mM HEPES (pH 7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4). Oocytes were either injected with mRNA encoding *Gpr3* using pGEMHE-GPR3 construct as previously described (Hinckley et al., 2005) or with increasing volumes of GPR3 MO oligos. All injections into defolliculated *Xenopus* oocytes were performed using a micromanipulator (Narishige USA Inc., Long Island, NY). Sixteen hours after the injections, oocyte maturation was induced by stimulation with 500 nM progesterone, and resumption of meiosis was scored by the appearance of a white spot on the animal pole of the oocyte.

cAMP measurements

Levels of cAMP from denuded oocytes and granulosa cells were assayed as previously described (Horner et al., 2003), with slight modification. Ovaries from 11-12 day-old mice were harvested in M2 media without any phosphodiesterase inhibitors, then preantral follicles isolated and punctured to obtain granulosa cells and denuded oocytes. Only those oocytes and granulosa cells collected within 10 minutes from the ovary isolation were used for the assay. All samples were washed 3 to 5 times in PBS supplemented with 1 mg/ml BSA, collected in 3 μl, 14000 rpm for 10 min at 4°C, and the supernatant was evaporated while centrifuging under a radioimmunoassay (Harper and Brooker, 1975).

Metabolic coupling assay

Follicles were isolated from 11-12 day-old mice and incubated in M2/BSA supplemented with 0.1% type I collagenase and 0.01U/ul DNaseI for 20 min at 37°C. At the end of the incubation, the metabolic coupling assay was performed as described by Schultz (Schultz et al., 1983) with slight modifications. Half of the oocyte-granulosa complexes were used to obtain denuded oocytes, then the two groups were incubated for 1 hr in the presence of [^{3}H] uridine (10μCi/group) at 37°C. At the end of the incubation both groups were washed several times in fresh media without radiolabeled uridine, and granulosa-oocyte complexes were stripped of granulosa cells. After addition of 7 ml scintillation fluid (Econo-Safe, RPI Inc., Mt. Prospect, IL) [^{3}H] uridine incorporation into denuded oocytes was measured by counting for 10 min using a beta counter (Beckman Coulter, Inc., Fullerton, CA).

Statistical analyses

Values were compared by Student's *t* test, and $P < 0.05$ was considered statistically significant.

Results

GPR3 and Gs are required to maintain meiotic arrest in the Pde3a null oocytes —To test whether cAMP produced endogenously is required for the meiotic arrest in *Pde3a*$^{-/-}$ oocytes, receptor expression and Gs function in these oocytes were blocked either by a GPR3 morpholino oligonucleotide or Gs neutralizing antibody treatment, respectively. *Pde3a*$^{-/-}$ oocytes, which do not spontaneously re-enter the meiotic cell cycle, underwent maturation and extruded a polar body when GPR3 expression was downregulated (Fig.1A) or when Gs function was blocked with a specific antibody (Fig.1B). The specificity of Gs antibody for mouse oocyte Gs has been reported (Mehlmann et al., 2002), whereas the properties of morpholino oligos were further investigated by injection into frog oocytes (see Supporting Fig. 1).

Together with the finding that the expression of recombinant PDE3A on a *Pde3a*$^{-/-}$ background induces maturation (Han et al., 2006), these initial *in vitro* findings strongly indicate that GPR3 and Gs functions are required to maintain the meiotic arrest observed in the *Pde3a*$^{-/-}$ oocytes through regulation of cAMP levels.

GPR3 ablation rescues the fertility phenotype of Pde3a$^{-/-}$ mice—To further define the relationship between GPR3 and PDE3A *in vivo*, we have generated mice with combined deletions in the *Gpr3* and *Pde3a* loci. We hypothesized that if prevention of cAMP degradation by inactivating PDE3A were to rescue the precocious meiotic resumption of *Gpr3*$^{-/-}$ mice, it would indicate the presence of alternative sources of cAMP in the oocyte. Thus, the model would test whether diffusion through gap junctions or the expression of other GPCRs, such as GPR12, would be sufficient to maintain meiotic arrest when cAMP synthesis is decreased (GPR3 ablation) and cAMP degradation in the oocyte is absent (*Pde3a*$^{-/-}$ oocytes).

To this aim, double heterozygous *Gpr3*$^{+/-}$ *Pde3a*$^{+/-}$ mice were generated by mating *Pde3a*$^{-/-}$ males to *Gpr3*$^{+/-}$ females. The double heterozygous mice obtained were then mated to produce male and female homozygous null for the two genes. The *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$ mice were generated at the normal Mendelian frequency (5%) and had normal body development, and no major defects were observed over a period of 2 years. Fertility of these mice was recorded over a period of five months using double homozygous females mated to wild type (*Gpr3*$^{+/+}$ *Pde3a*$^{+/+}$) males of proven fertility. As previously published, *Pde3a*$^{-/-}$ females are completely sterile because they ovulate immature oocytes (Masciarelli et al., 2004) (Table 1), whereas *Gpr3*$^{-/-}$ females display reduced fertility (Ledent et al., 2005). Double mutant *Pde3a*$^{-/-}$ *Gpr3*$^{-/-}$ females produced pups, demonstrating a rescue of the complete infertility of *Pde3a*$^{-/-}$ females. Their fertility was reduced compared to wild type controls but was comparable to that of the *Gpr3*$^{-/-}$ females (Ledent et al., 2005). Double heterozygous females mated with wild type males generated a normal number of litters and pups (Table 1), demonstrating that hemizygosity does not affect fertility. Thus, *in vivo Gpr3* ablation compensates for the *Pde3a*$^{-/-}$ infertility, showing that oocyte maturation is restored in the double knockout mice.

Defective meiotic arrest in Gpr3$^{-/-}$ Pde3a$^{-/-}$ mice—The state of maturation of oocytes in the different genetic backgrounds was next investigated using two complementary strategies. In the first approach, antral follicles were punctured to extrude the oocyte-cumulus complexes (Fig. 2 and supporting Fig. 2), and meiotic resumption was assessed immediately after denudation. Of the total number of oocytes recovered, 52-56% of the oocytes removed from antral follicles of *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$, *Gpr3*$^{-/-}$ *Pde3a*$^{+/+}$, and *Gpr3*$^{-/-}$ *Pde3a*$^{+/-}$ were in GVBD, 28-34% showed an intact GV (GVI), and 15-20% were degenerated (Fig. 2). Thus, *Gpr3*$^{-/-}$ *Pde3a*$^{+/+}$ and *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$ oocytes undergo untimely maturation at comparable rates.

In order to define the exact timing of this aberrant oocyte reentry into the cell cycle during follicle growth, a second approach was used to score maturation in the different genetic backgrounds. The meiotic stage of maturation of oocytes within follicles of different diameter was evaluated in ovary sections from PMSG-primed *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$ mice and matched wild type controls. From the analysis of entire ovaries from double KO mice, 66% ± 6.51 (mean ± SEM; N = 3 mice) of the oocytes in antral follicles (diameter > 251 μm) had resumed meiosis (Fig. 3), whereas no oocytes in GVBD could be detected in wild type antral follicles. Again, the number of GVI and GVBD oocytes in double KO was not statistically different from that of *Gpr3*$^{-/-}$ *Pde3a*$^{+/-}$ mice (Fig. 3), although it was slightly lower that the 82% reported by Mehlmann et al. for antral follicles (see Discussion). Increased oocyte degeneration was evident in single and double KO oocytes, particularly in follicles of 141-250 μm diameters. This finding is consistent with data reported by Ledent et al. for immature mice (Ledent et al., 2005), even though different methods were used to score the state of maturation and degeneration. Taken together, these measurements indicate that GPR3 activity in follicle-

enclosed oocytes is necessary for maintaining meiotic arrest, even in the absence of PDE3A activity.

It should be noted that none of the oocytes from preantral follicles (diameter < 140 μm, or follicles with less than three layers of granulosa cells around the oocyte) either in single or double *Gpr3* null mice showed signs of precocious meiotic resumption.

Ablation of GPR12 does not influence Gpr3$^{-/-}$ meiotic phenotype—We have previously reported that mice deficient in *Gpr12* do not show altered meiotic maturation (Hinckley et al., 2005). However, GPR12 function could become significant in oocytes deficient in *Gpr3*. Therefore, the state of meiotic progression in oocytes from mice lacking the expression of *Gpr12* on *Gpr3* null background was studied in 22-day-old immature mice (Fig. 4). In the double knockout mice, 67% ± 6.2, (mean ± SEM) of *Gpr3*$^{-/-}$ *Gpr12*$^{-/-}$ oocytes from antral follicles were GVBD, 15% ± 2.4 were GVI and 18% ± 6.5 were considered degenerated. Thus, the percentage of oocytes that are GVI in *Gpr3*$^{-/-}$ *Gpr12*$^{-/-}$ does not differ from *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$, *Gpr3*$^{-/-}$ *Pde3a*$^{+/+}$, and *Gpr3*$^{-/-}$ *Pde3a*$^{+/-}$, indicating that GPR12 does not contribute significantly to the maintenance of meiotic arrest.

Decreased levels of cAMP in oocytes from Gpr3 $^{-/-}$ Pde3a$^{-/-}$ mice—Previous reports from our laboratory have shown that cAMP accumulation in *Pde3a*$^{-/-}$ oocytes is significantly increased in isolated oocytes (Masciarelli et al., 2004). The analysis of meiotic maturation in the double mutant mice suggests that an increase in cAMP either does not occur in these mice or is not sufficient to maintain the meiotic arrest. To distinguish between these possibilities, oocytes from preantral follicles of mice double KO as well as single KO for each locus as controls were isolated, and the levels of cAMP in denuded oocytes were measured by RIA (Fig. 5). The rationale for the use of preantral follicles rather than preovulatory follicles is that of all the different genotypes have not resumed meiosis at this stage oocytes, allowing direct comparison among the different groups. GPR3 is present and active in oocytes within preantral follicles (Hinckley et al., 2005; Freudzon et al., 2005). In all experiments, oocytes were collected in the absence of any phosphodiesterase inhibitors in less than 10 min to avoid any bias due to PDE inhibition. Because of the detection of a small amount of mRNA for *Gpr3* in granulosa cells (Mehlmann et al., 2004), parallel measurements of cAMP levels in these somatic cells were also performed. In addition, mural granulosa cells were collected from double KO and single mutant mice, and cAMP was measured for additional controls (Supporting Fig. 3). In accordance with previous publications (Masciarelli et al., 2004), cAMP hydrolyzing PDE activity was undetectable in denuded oocytes from double mutant mice (Supporting Fig.4).

In agreement with our preceding reports (Masciarelli et al., 2004), oocytes lacking PDE3A (*Gpr3*$^{+/+}$ *Pde3a*$^{-/-}$) had considerably higher levels of cAMP (0.50 ± 0.062 fmole/oocyte) than wild type controls (Fig. 5). Conversely, the cAMP content in *Gpr3*$^{-/-}$ *Pde3a*$^{+/-}$ oocytes (0.086 ± 0.02 fmole/oocyte) was significantly lower than control *Gpr3*$^{+/+}$ *Pde3a*$^{+/+}$ oocytes (0.22 ± 0.02 fmole/oocyte). Most importantly, the cAMP levels in double knockout oocytes were greatly reduced (0.084 ± 0.04 fmole/oocyte) in comparison to the single *Pde3a* knockout but similar to the *Gpr3*$^{-/-}$ oocytes (Fig.5). The cAMP levels in the cumulus or mural granulosa cells were not affected by the ablation of either *Gpr3* or *Pde3a* genes, singly or together in the double knockout (Supplemental Fig. 3).

GPR3 *and* PDE3A do not influence metabolic coupling between oocyte and granulosa cells—In order to confirm that the differences in levels of cAMP are due to the absence of GPR3 and PDE3A in the oocyte and not to an indirect effect of the coupling between oocytes and the surrounding somatic compartment, we tested the functionality of gap junctions between germinal and somatic compartments in single and double mutant mice. These

measurements are based on the inability of denuded oocytes to uptake uridine (Eppig et al., 1983), whereas oocytes within a complex accumulate uridine from cumulus cells through gap junctions. Preantral follicles instead of preovulatory follicles were again used to avoid possible bias due to precocious meiotic maturation and loss of connection in the *Gpr3*$^{-/-}$ or double mutant oocytes. Groups of preantral follicles from 11- to 12-day-old mice were briefly processed as described in materials and methods to obtain oocyte-granulosa complexes and denuded oocytes. At the end of a one-hour incubation in the presence of [^{3}H] uridine, the oocyte-granulosa complexes were denuded, washed several times, and levels of radioactivity present in the two groups were measured (Fig. 6). To confirm the involvement of gap junctions in the transfer, oocyte-cumulus complexes were incubated in the presence of increasing concentrations of carbenoxolone (CBX), known to block gap junction-mediated communication (de Groot et al., 2003). At the end of the incubation, the effect of the gap junction block was assayed as described above (Supporting Fig. 4). At concentrations up to 500 μM, this inhibitor progressively prevented transfer of radiolabeled uridine from the cumulus cells to the oocytes. When oocyte-granulosa complexes from different genotypes were tested, single or combined ablation of *Gpr3* and *Pde3a* did not influence the gap junction functionality between the oocyte and the somatic compartments. Comparable transfer of uridine was detected in all samples. Although a complete time course of [^{3}H] uridine incorporation was not performed, previous reports with COC have demonstrated linearity of [^{3}H] uridine uptake for at least 2 h (Wassarman and Letourneau, 1976). Barring possible differences in kinetics of uptake among the different genotypes, this experiment indicates that the gap junction permeability is not affected in single and double mutants, at least in preantral follicles.

Discussion

The *in vivo* genetic approach and *in vitro* manipulations of cAMP signaling in the oocyte described in this study demonstrate that the oocyte meiotic arrest that follows PDE3A ablation is dependent on the expression of the receptor GPR3 and the presence of an active Gs protein. This conclusion has several important implications for GPR3 and PDE3A functions in the oocyte and provides an insight into the mechanisms maintaining cAMP levels and meiotic arrest in the female gamete.

In vitro down regulation of GPR3 expression with morpholino oligonucleotides or inhibition of Gs activity by antibody injection causes meiotic resumption in *Pde3a*$^{-/-}$ oocytes, which would otherwise remain arrested in meiotic prophase for up to 48 hours after isolation. These experiments on a *Pde3a*$^{-/-}$ genetic background complement the observations that a Gαs antibody or a dominant negative form of Gαs causes meiotic resumption in denuded oocytes arrested in meiotic prophase with a cAMP PDE inhibitor (Kalinowski et al., 2004; Mehlmann et al., 2002). Taken together, these findings strongly indicate that the oocyte continues to synthesize cAMP when separated from the somatic compartment and this continuous accumulation is required for maintaining an intact GV in the *Pde3a*$^{-/-}$ background. Thus, our findings further support the concept that mouse oocytes are endowed with the machinery that produces cAMP. The meiotic resumption after morpholino treatment also implies that GPR3 is the receptor that maintains Gs and adenylyl cyclase in the active state in isolated *Pde3a*$^{-/-}$ oocytes, conclusions consistent with the increased partition of GFP-tagged Gs and endogenous Gαs to the membrane after ablation of GPR3 (Freudzon et al., 2005). That the GPR3/Gs interaction affects oocyte cAMP concentration is verified by our cAMP measurements (see below), thus further strengthening the argument of an oocyte-autonomous regulation of cAMP levels. Whether the activity of GPR3 in the denuded oocyte is comparable to that in the oocyte still in the follicle cannot be addressed by our experimental paradigm and remains unclear. Indirect measurements of GPR3 activity by monitoring the translocation of Gs from the

membrane to the oocyte cytosol have suggested that signaling from this receptor is not modified by contact with somatic cells (Freudzon et al., 2005).

Similar conclusions on the dominant function of GPR3 in oocyte meiotic arrest can be drawn from the analysis of the *in vivo* phenotypes of double knockout mice. The complete infertility of *Pde3a* null mice is rescued by concomitant ablation of *Gpr3*, suggesting that maturation in a significant group of oocytes is restored *in vivo* under these conditions. Morphological analyses performed show that ablation of *Gpr3* on a *Pde3a* null background produces meiotic resumption as soon as the oocytes become competent for reentry into the cell cycle, whereas *Pde3a* null oocytes remain arrested in prophase even after ovulation. Quantitatively, $Gpr3^{-/-}$ and $Gpr3^{-/-}$ $Pde3a^{-/-}$ double mutant oocytes show comparable rates of premature meiotic resumption, confirming the dominant effect of $Gpr3^{-/-}$ on meiosis. This finding implies that cAMP levels in the double mutant, where both synthesis and degradation are disrupted, are not sufficient to maintain MPF inactive. Consistent with our reasoning, elevated cAMP levels associated with PDE3A ablation are not detected in the double mutant oocytes. Thus, these multiple independent observations strongly indicate that GPR3 activity is required to generate the *Pde3a* null phenotype and for cAMP accumulation in the oocyte also *in vivo*. The low cAMP levels in the $Gpr3^{-/-}$ $Pde3a^{-/-}$ oocytes associated with precocious meiotic reentry strongly suggest that other possible sources of cAMP accumulation in the oocytes, if present, are unable to maintain cAMP at levels sufficient for meiotic arrest. A caveat to the above conclusion is that cAMP was measured in oocytes from preantral and not antral follicle because oocytes from antral follicle have mostly resumed meiosis. Thus, cAMP levels would have been compared in oocytes at different stages of maturation, rendering the interpretation of the data difficult. Nevertheless, this extrapolation from prenatral to antral oocyte is probably legitimate in view of the fact that cAMP levels in preantral and antral oocytes are comparable in both wild type and $Pde3a^{-/-}$ oocytes (Fig.5 and Masciarelli et al., 2004).

Although the number of $Gpr3^{-/-}$ GVI oocytes in our study is identical to that of two previous reports, if we compare the data on premature maturation (GVBD) in preantral and antral $Gpr3^{-/-}$ mice with those of Mehlmann et al, some differences are evident. Mehlmann et al. reported 82% maturation in large antral follicles, whereas we scored 66% ± 6.51 maturation. This difference is likely due to the method used for calculation of the percentage meiotic maturation, as we included degenerating oocytes in the total number, whereas Mehlmann et al. did not include this group in the calculation (Mehlmann et al., 2004). It should be pointed out that an increase in degenerating oocytes in $Gpr3^{-/-}$ mice has been observed also by Ledent et al. (Ledent et al., 2005). The total number of oocytes undergoing parthenogenetic division, lysis, or fragmentation (27-30%) reported by these authors is in the same range as our observation if we average early antral and large antral follicle data (average 30% degeneration). This degeneration may represent oocytes that have prematurely resumed meiosis and are undergoing parthenogenetic division, or the fragmentation or degeneration may be a sign of increased oocyte attrition in the single and double KO. Although apoptosis of granulosa cells was not specifically studied, cells with pycnotic nuclei were often associated with follicles enclosing degenerated oocytes. It is then possible that oocyte viability is decreased in single and double knockout mice and these are signs of the reported premature ovarian failure. Also similar to Ledent et al. is the decrease in litter size observed in our studies. In addition to loss of oocytes with premature maturation, a decrease in meiotic and developmental competence of the $Gpr3^{-/-}$ oocytes may contribute to the reduced litter size. Indeed, it has been demonstrated that cAMP is necessary to develop competence to undergo maturation (Chesnel et al., 1994; Eppig and Downs, 1984) and *Gpr3* mRNA levels are detected throughout the growth phase of the oocyte (Hinckley et al., 2005). Further experiments are required to assess this possibility, and the *Gpr3* null model may be useful to probe the effect of cAMP accumulation during growth.

Data accumulated during the past three decades suggest several mechanisms controlling cAMP levels in the oocyte, the most widely accepted being the diffusion from the somatic compartment through gap junctions. However, the analysis of the double mutant oocytes indicates that cAMP diffusion through gap junctions, although clearly demonstrated by several experimental paradigms, is not sufficient by itself to maintain cAMP levels and meiotic arrest. It is unlikely that the *Gpr3* ablation affects cAMP accumulation in the somatic compartment in view of the findings of no change in cAMP levels in cumulus or granulosa cells, which is consistent with the low abundance of *Gpr3* mRNA in somatic cells of the follicles. It is also unlikely that the single and double knockout affect gap junction functionality because we show here that follicle cell-oocyte communication is comparable in the different genetic backgrounds, at least in preantral follicles and in the assay conditions used. Thus, the low cAMP levels in the double knockout mice are due to defective cAMP synthesis endogenous to the oocyte and are associated with precocious maturation in early antral follicles. The cAMP that may diffuse through junctional communication is not sufficient to compensate for the loss of endogenous cAMP synthesis that follows *Gpr3* ablation even on a background of decreased degradation.

Different connexins are specifically implicated in gap junction intracellular communication in the different cellular compartments of the follicle (Ackert et al., 2001; Teilmann, 2005; Veitch et al., 2004). Gap junction intercellular communication is affected positively or negatively by the phosphorylation status of its subunits according to cell type, nature of the stimulus, and type of connexin involved in the homo- or heteromeric assembly (Faucheux et al., 2004; Granot and Dekel, 1994; Lampe and Lau, 2000; Lampe and Lau, 2004; Lampe et al., 2000; TenBroek et al., 2001; Urschel et al., 2006; Yogo et al., 2006). Thus, the low cAMP levels in the double knockout oocyte may affect the gap junction functionality. However, this possibility was not verified by measuring the uridine uptake. Oocytes with high (*Pde3a*$^{-/-}$) and low (*Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$) cAMP show comparable uridine uptake and presumably gap junction permeability. Whereas it has been proposed that an increase in cAMP causes gap junction closure in rat oocytes, treatment of oocytes with PDE inhibitors maintains or upregulates (or both) cumulus-oocyte gap junctional communication, which is associated with or may actually cause a delay in meiotic resumption (Thomas et al., 2004). Moreover, transfer of information through gap junctions may be essential for both meiotic arrest and meiotic maturation.

The presence of several GPCRs in addition to *Gpr3* has been inferred by gene expression analysis (Wang et al., 2004). These additional receptors may stimulate Gs and therefore adenylyl cyclase in the oocyte. GPR12 in particular has been shown to have a function homologous to GPR3 in rat oocytes. However, other GPCRs coupled to cyclase, such as GPR12, are not sufficient in sustaining cAMP above a threshold required for meiotic arrest in the mouse oocyte deficient in *Gpr3* and *Pde3a*. This conclusion is further supported by direct analysis of the meiotic phenotype in the double mutant *Gpr3*$^{-/-}$ *Gpr12*$^{-/-}$ generated in our laboratory. The ablation of *Gpr12* on a *Gpr3* null background did not modify the meiotic phenotype of the single *Gpr3*$^{-/-}$, indicating that GPR12 activity does not contribute to the meiotic blockade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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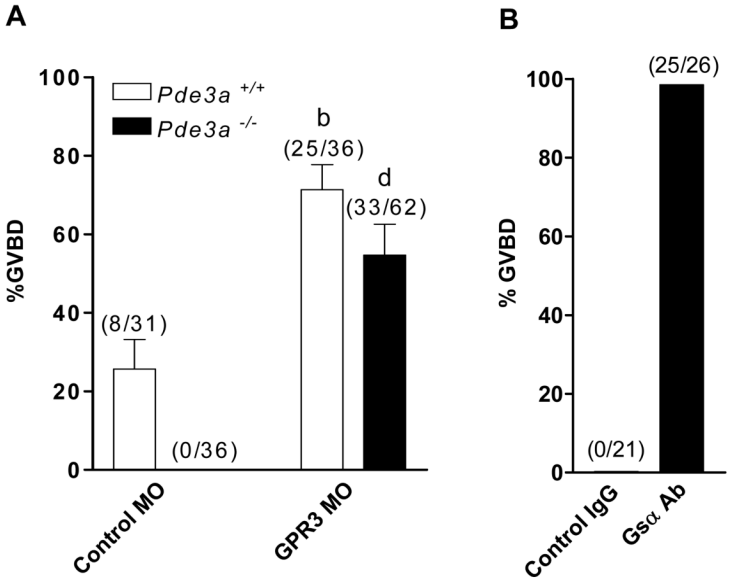

Fig.1. Injection of Gs-neutralizing antibody or GPR3 morpholino oligonucleotides in *Pde3a*$^{-/-}$ null oocytes induces meiotic resumption

A. Groups of wild type (WT) oocytes maintained in meiotic arrest with 3.5 mM hypoxanthine or *Pde3a*$^{-/-}$ oocytes were injected with GPR3 morpholino oligonucleotide or a control scrambled morpholino as described (Hinckley et al., 2005). The effects of the injection on meiotic maturation were followed up to 24 hours. The data reported are the mean ± SE of the analysis from three experiments, and the meiotic maturation stage after 18 hours from the injections is reported as percentage of GVBD. The numbers at the top of the bars indicate the total number of oocytes injected. Superscripts indicate statistical difference vs. controls for each genotype: a, $p<0.05$; b, $p<0.01$; c, $p<0.005$ and d, $p<0.001$. **B**. Denuded oocytes from *Pde3a*$^{-/-}$ mice were injected with antibody against Gs or control rabbit IgG at a final concentration of 1.3 μM. Oocytes were monitored for meiotic resumption every 30 min for 3 hours and at 21-24 hours for PB extrusion. Meiotic maturation was scored after 2 hours from the injections and reported as percentage of GVBD. After 2 hours of culture, 96% of Gs antibody injected oocytes had resumed meiosis. The numbers above the bars indicate the total number of oocytes injected. The data are the average of two independent experiments.

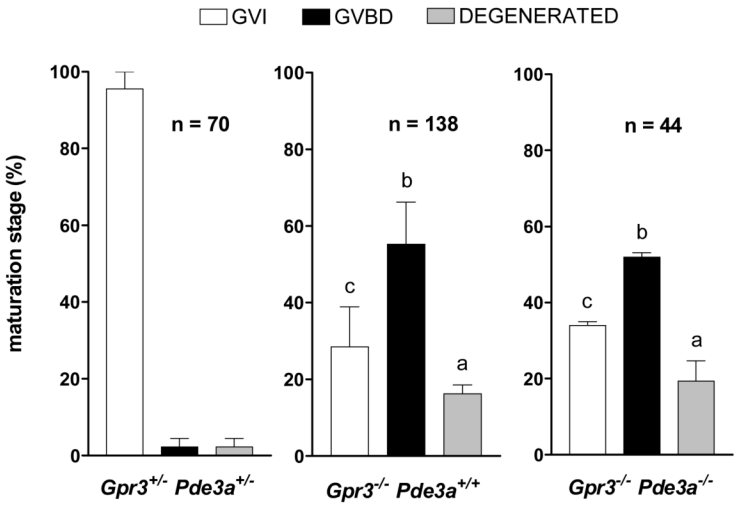

Fig.2. Meiotic resumption in immature ovaries from mice with different genetic backgrounds

22-day-old mice were primed with 5 IU PMSG, ovaries were collected 42-44 hours later, and preovulatory follicles punctured to obtain COCs. Cumulus cells were stripped from oocytes with a pulled Pasteur pipette, and cell cycle progression immediately scored. Only well formed COCs were used for meiotic progression analysis. In all experiments M2 media supplemented with 3 mg/ml BSA was used. The bar graph represents mean ± SE of up to 4 different mice of the indicated genotype. A maximum of 15 min elapsed from the beginning of COC collection from each ovary to the end of the oocyte scoring. The letters above the bars indicate statistical difference within the same oocyte maturational stage, between the indicated genotype and *Gpr3*$^{+/-}$ *Pde3a*$^{+/-}$. a, $p<0.05$; b, $p<0.01$; c, $p<0.005$ and d, $p<0.001$. n = number of oocytes scored for each genotype.

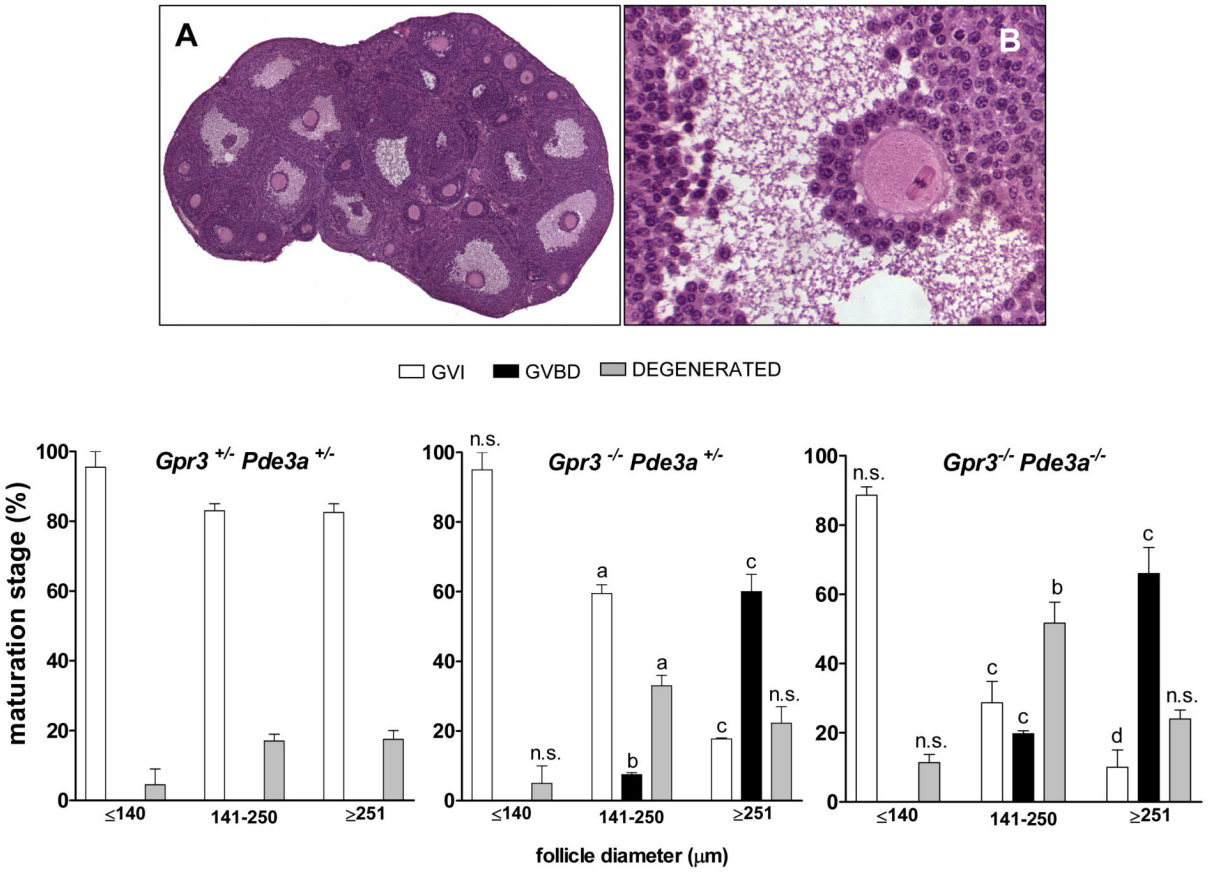

Fig.3. Ovarian morphology and state of oocyte maturation in *Grp3*$^{-/-}$ *Pde3a*$^{-/-}$ mice

22-day-old mice were primed with 5 IU PMSG; after 42-44 hours, ovaries were collected, fixed in 4% PFA and meiotic progression scored in follicles from HE sections. **A**. Median section of whole ovary from double mutant mice. **B**. Metaphase spindle in follicle enclosed oocytes from double null mice. **C**. Scoring of meiotic maturation in double heterozygous, *Gpr3*$^{-/-}$ *Pde3a*$^{+/-}$, and *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$ mice. The state of oocyte maturation was related to the diameter of the follicle by measuring follicle size not including theca cells, as detailed in the methods. The data represent the mean ± SE of the analysis from three to five mice for each genotype. The letters above the bar indicate statistical difference between the oocyte maturation state in any given group of follicles of the indicated genotype and the corresponding maturation state follicles of the same diameter of *Gpr3*$^{+/-}$ *Pde3a*$^{+/-}$. n.s., non significant; a, $p<0.05$; b, $p<0.01$; c, $p<0.005$ and d, $p<0.001$. No significant differences between *Gpr3*$^{-/-}$ and *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$ were observed.

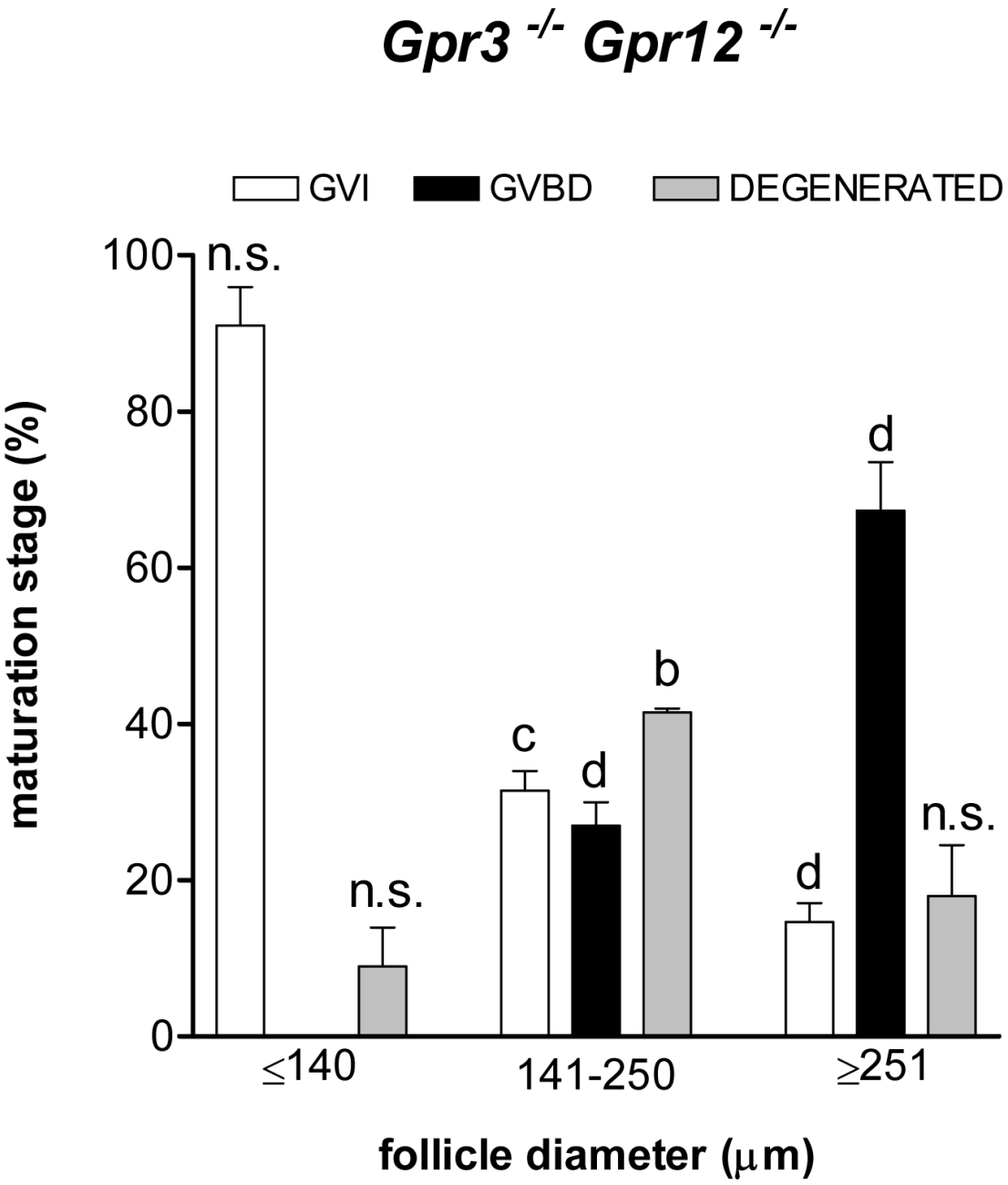

Fig.4. State of oocyte maturation in *Grp3*$^{-/-}$ *Gpr12*$^{-/-}$ mice

The state of oocyte maturation was scored in *Gpr3*$^{-/-}$ *Gpr12*$^{-/-}$ and control mice. Every follicle and oocyte was analyzed using a 20X magnification. Follicle size, not including theca cells, was determined using the AxioVision 3.1.0.1.8 software (Carl Zeiss) and refers to the average of measurements in two dimensions. The graph represents the mean ± SE of the analysis from three mice for each genotype. The letters above the bar indicate statistical difference between the oocyte maturation state in any given group of follicles and the corresponding maturation state follicles of the same diameter of *Gpr3*$^{+/-}$ *Pde3a*$^{+/-}$. n.s., non significant; a, $p<0.05$; b, $p<0.01$; c, $p<0.005$ and d, $p<0.001$. No significant differences among *Grp3*$^{-/-}$ *Gpr12*$^{-/-}$ and *Gpr3*$^{-/-}$ oocytes were observed.

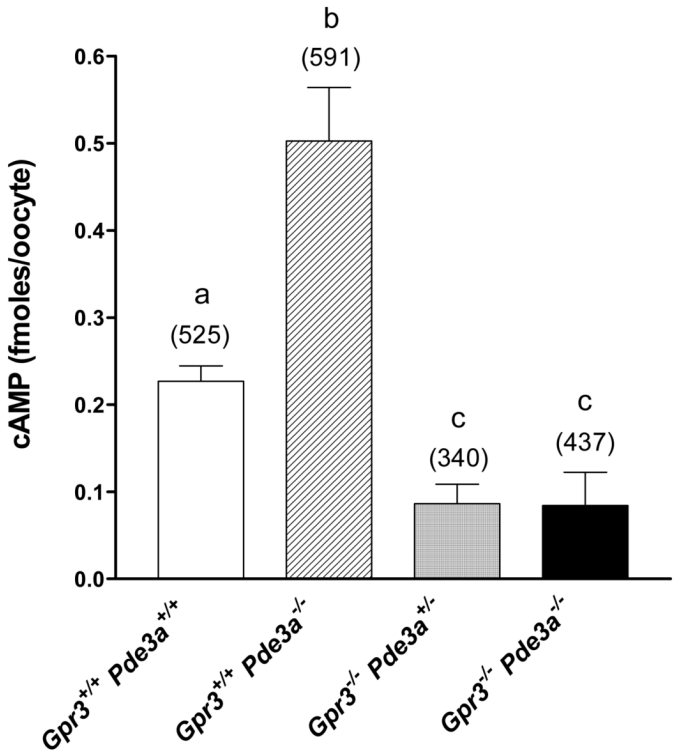

Fig.5. cAMP levels in denuded oocytes from wild type and mice with ablation of *Gpr3* and *Pde3a*

All oocytes analyzed were harvested from preantral follicles of 11- to12-day-old mice. Follicles isolated from ovaries were considered as preantral if smaller than 140 μm and without a visible antrum. Oocytes were collected without PDE inhibitors in M2 media supplemented with 3 mg/ml BSA. The collecting/denuding process was completed in less than 10 minutes/ovary. The graph reports mean ± SE of three to four different experiments. Numbers on the top of the bars indicate the total number of oocytes used. Different superscripts denote statistical difference between $Gpr3^{+/+}$ $Pde3a^{+/+}$ and the indicated genotype. a, $p<0.05$; b, $p<0.01$ and c, $p<0.005$.

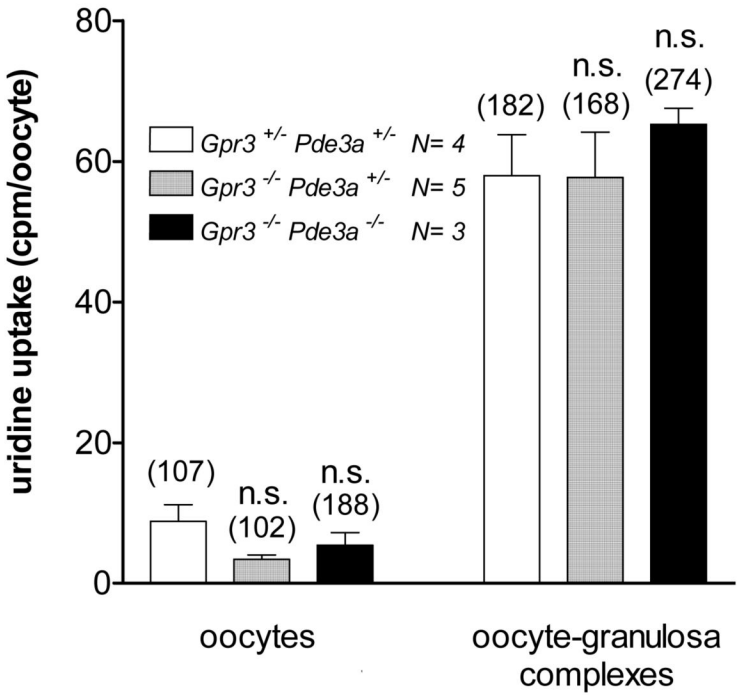

Fig. 6. Gap junction permeability is intact in double mutant mice

Metabolic coupling between oocyte and granulosa cells was measured in preantral follicles. Follicles isolated from 11- to 12-day-old mice were incubated in M2/BSA supplemented with 0.1% type I collagenase and 0.01 U/ul DNaseI for 20 min at 37°C. Half of the oocyte-granulosa cell complexes were used to obtain denuded oocytes, and then the two groups were incubated for 1 hour in the presence of [^{3}H] uridine (10μCi/group). The difference of uptake between the groups reflects the transport of [^{3}H] uridine through gap junctions. The graph shown represents the mean ± SE of three independent experiments using quadruplicates for each group of oocytes. The numbers at the top of the bars indicate the total number of oocytes tested. No significant differences between *Gpr3*$^{+/-}$ *Pde3a*$^{+/-}$ and *Gpr3*$^{-/-}$ *Pde3a*$^{+/-}$ or *Gpr3*$^{-/-}$ *Pde3a*$^{-/-}$ were observed. N refers to the final number of animals used for each genotype.

Table 1

Crossing between females of different genotypes with wild type males

Two-month-old females were mated with four-month-old males of proven fertility. After 3 months of mating, the WT males were replaced with four-month-old WT males. The result represents the mean ± SE of three mating couples studied over a period of five months. A total of 5 litters were obtained from the mating of double KO females and WT males. Different superscripts mean statistical difference, $P<0.05$.

♀ genotype	Litters/month	Pups/litter
Gpr3 $^{+/+}$ *Pde3a* $^{+/+}$	0.8 ± 0.1 ^{a}	9.5 ± 0.7 ^{a}
Gpr3 $^{+/-}$ *Pde3a* $^{+/-}$	0.8 ± 0.1 ^{a}	10.2 ± 1.2 ^{a}
Gpr3 $^{+/-}$ *Pde3a* $^{-/-}$	0 ^{b}	0 ^{b}
Gpr3 $^{-/-}$ *Pde3a* $^{+/-}$	0.4 ± 0.1 ^{c}	4.6 ± 0.5 ^{c}
Gpr3 $^{-/-}$ *Pde3a* $^{-/-}$	0.3 ± 0.1 ^{c}	4.5 ± 0.5 ^{c}