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Oocyte-specific expression of *Gpr3* is required for the maintenance of meiotic arrest in mouse oocytes

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Abstract

The maintenance of meiotic prophase arrest in mouse oocytes within fully grown follicles, prior to the surge of luteinizing hormone (LH) that triggers meiotic resumption, depends on a high level of cAMP within the oocyte. cAMP is produced within the oocyte, at least in large part, by the G_s-linked G-protein-coupled receptor, GPR3. *Gpr3* is localized in the mouse oocyte but is also present throughout the follicle. To investigate whether *Gpr3* in the follicle cells contributes to the maintenance of meiotic arrest, RNA interference (RNAi) was used to reduce the amount of *Gpr3* RNA within follicle-enclosed oocytes. Follicle-enclosed oocytes injected with small interfering double-stranded RNA (siRNA) targeting *Gpr3,* but not control siRNAs, stimulated the resumption of meiosis in the majority of oocytes following a 3-day culture period. Reduction of RNA was specific for *Gpr3* because an unrelated gene was not reduced by microinjection of siRNA. Meiotic resumption was stimulated in isolated oocytes injected with the same siRNA and cultured for 1 to 2 days, but at a much lower rate than in follicle-enclosed oocytes that could be cultured for longer. These results demonstrate that GPR3 specifically in the oocyte, rather than in the follicle cells, is responsible for maintenance of meiotic arrest in mouse oocytes. Furthermore, the method developed here for specifically reducing RNA in follicle-enclosed oocytes, which can be cultured for a sufficient time to reduce the level of endogenous protein, should be generally useful for targeting a wide range of other proteins that may be involved in meiotic arrest, the resumption of meiosis, fertilization, or early embryonic development.

Keywords

Gpr3; RNAi; siRNA; Oocyte; Meiotic arrest

Introduction

Mammalian oocytes arise and enter meiosis during embryogenesis, reaching the dictyate stage of prophase I, at which point meiosis arrests (Eppig et al., 2004). They remain arrested in prophase I until a pre-ovulatory surge of luteinizing hormone (LH) from the pituitary acts on the follicle cells surrounding the oocyte to stimulate meiotic resumption, as well as cytoplasmic changes that prepare the oocyte for fertilization (Mehlmann et al, 1996; Ducibella, 1996, 1998; Eppig et al, 2004). During the extended period of storage prior to follicular growth, and up until the surrounding follicle cells begin to develop an antrum, immature oocytes are held in prophase arrest by inherent factors, such as subthreshold levels of cell cycle regulatory proteins (De Vantery et al, 1996, 1997; Kanatsu-Shinohara et al, 2000). As follicles begin to form an antrum, the oocyte acquires the ability to mature into a fertilizable egg and will mature spontaneously if it is removed from the follicle (Pincus and Enzmann, 1935; Edwards, 1965; Erickson and Sorensen, 1974; Sorensen and Wassarman, 1976; Mehlmann, 2005). Thus, after

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antrum formation, the follicle cells provide a signal to the oocyte that keeps it arrested at prophase I until the pre-ovulatory surge of LH.

It is well established that maintenance of meiotic arrest in meiotically competent oocytes prior to the LH surge depends on high levels of cAMP within the oocyte (Conti et al, 2002; Eppig et al, 2004; Mehlmann, 2005). In support of this idea, maintaining high levels of cAMP within oocytes isolated from their follicles prevents oocytes from resuming meiosis spontaneously (Cho et al, 1974; Schultz et al, 1983; Eppig et al, 1985), and cAMP levels fall upon oocyte isolation from follicles (Törnell et al, 1990). Cyclic AMP activates protein kinase A (PKA), which ultimately affects the activity of protein kinases and phosphatases that favor a net phosphorylation of the key cell cycle regulatory protein, CDK1, rendering it inactive (Duckworth et al., 2002; Lincoln et al., 2002; Eppig et al., 2004; Mehlmann, 2005). A decrease in the amount of cAMP following LH stimulation ultimately leads to a net dephosphorylation of CDK1, thereby activating it and permitting meiotic resumption.

The oocyte produces the cAMP needed for meiotic arrest through the stimulation of a G_s G protein (Mehlmann et al., 2002; Kalinowski et al., 2004). G_s stimulates at least one form of adenylyl cyclase, type 3 (Horner et al., 2003), to produce cAMP. G_s is stimulated, in turn, by an orphan G-protein-coupled receptor in the oocyte, GPR3 (Mehlmann et al., 2004; Freudzon et al., 2005). GPR3 has been characterized in cultured cells as a constitutive activator of adenylyl cyclase (Eggerickx et al., 1995; Uhlenbrock et al., 2002), and in mouse oocytes, it has recently been shown to activate G_s and elevate cAMP (Freudzon et al., in press). If any of the components of this signaling pathway are eliminated in the follicle-enclosed oocyte, it will mature spontaneously (Mehlmann et al., 2002, 2004; Horner et al., 2003; Kalinowski et al., 2004; Ledent et al., 2005). So far, it is unclear what role the follicle cells play in the maintenance of meiotic arrest. Follicle cells could produce a ligand that elevates cAMP production by stimulating GPR3, and/or inhibit the activity of cAMP phosphodiesterases within the oocyte, which would help maintain high cAMP levels (see Mehlmann, 2005).

RNA encoding *Gpr3* is expressed primarily in the oocyte but is also present at lower levels in the surrounding follicle cells (Mehlmann et al., 2004). Mouse oocytes from animals in which the *Gpr3* gene is knocked out by homologous recombination undergo LH-independent meiotic resumption within pre-ovulatory as well as early antral stage follicles (Mehlmann et al., 2004; Ledent et al., 2005). Although the ability to maintain meiotic arrest can be rescued in *Gpr3*$^{-/-}$ oocytes by microinjecting follicle-enclosed oocytes with RNA encoding *Gpr3* (Mehlmann et al., 2004), a potential role for GPR3 in the follicle cells is not completely ruled out. In theory, any receptor overexpressed in the oocyte that has the ability to stimulate cAMP production should rescue the ability of the oocyte to maintain arrest. Because *Gpr3* is expressed in the follicle cells, albeit at low levels, it is possible that the follicle cells could produce cAMP and provide it to the oocyte through gap junctions that are present between the oocyte and the somatic cells (Albertini and Anderson, 1974; Anderson and Albertini, 1976; Piontkewitz and Dekel, 1993).

The goal of the present study was to resolve the issue of whether GPR3 within the oocyte or from the surrounding somatic cells is required for the maintenance of meiotic arrest. The technique of RNA interference (RNAi), which involves the introduction of small, interfering double-stranded RNA (siRNA) into oocytes to trigger sequence-specific degradation of mRNA (Svoboda et al., 2000, 2001; Wianny and Zernicka-Goetz, 2000; Xu et al., 2003), was used to reduce levels of mRNA encoding *Gpr3* in follicle-enclosed oocytes. Specific reduction of *Gpr3* mRNA from mouse oocytes after 72 h of culture resulted in the loss of the ability of follicle-enclosed oocytes to maintain meiotic arrest. In contrast, RNAi was less effective when siRNA was introduced into isolated oocytes, which cannot be cultured beyond ~48 h and, therefore, undergo less turnover of RNA and protein.

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These results demonstrate that GPR3 within the oocyte, rather than in the surrounding follicle cells, is necessary to maintain meiotic arrest. In addition, the method of injecting siRNA into follicle-enclosed oocytes that may be cultured for several days after microinjection should be generally useful in studying a wide range of proteins involved in meiotic arrest, the resumption of meiosis, fertilization, or early embryonic development.

Materials and methods

Follicle and oocyte isolation, microinjection, and culture

The mice used for experiments in which follicles were isolated and cultured were unprimed, 20- to 24-day-old B6SJLF1s (Jackson Laboratories, Bar Harbor, ME). The medium used for follicle culture was MEMα (#12000-022, Invitrogen, Carlsbad, CA) supplemented with 25 mM $NaHCO_3$, 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (all from Sigma), 5% fetal bovine serum (#16000-044, Invitrogen), and 10 ng/ml ovine FSH (provided by A. F. Parlow, National Hormone and Peptide Program, Torrance, CA), and equilibrated with 5% CO_2 and 95% air.

Mice were sacrificed using CO_2 asphyxiation. Pre-antral follicles (140–180 μm in diameter) were isolated in 3 ml of MEMα using watchmaker's forceps and a 30-gauge syringe needle, as previously described (Mehlmann et al., 2002, 2004). Follicles were transferred to 2 ml MEMα and maintained in an atmosphere of 5% CO_2 and 95% air. Follicle-enclosed oocytes were microinjected essentially as previously described (Jaffe and Terasaki, 2004; Kalinowski et al., 2004); the microinjection chamber consisted of two coverslips separated by a 100-μm spacer formed by a single piece of doublestick tape, and mounted on a U-shaped plastic slide over a reservoir of medium. The chamber was observed using a Zeiss Axioskop upright microscope with a 20× lens (0.5 N.A.). Follicle-enclosed oocytes could readily be seen inside these chambers, and all microinjected oocytes contained a visible nucleolus. Four to five follicles were placed in the chamber at one time and were kept in the chamber at ~22°C for ~10–20 min. Following microinjection, follicles were removed from the chamber and placed in 2 ml medium. An equal number of uninjected follicles similar in size and appearance to the injected follicles was put aside into 2 ml of medium, and served as parallel controls. When all microinjections were completed, injected and uninjected follicles were placed on Millicell culture plate inserts (PICMORG50, Millipore Corp., Bedford, MA) in 35-mm Petri dishes, as previously described (Nayudu and Osborn, 1992). Four to five follicles were placed on each Millicell filter, and 1.6 ml of medium was placed in the dish underneath the filter. The culture medium was changed 2 days later by removing 1.2 ml medium and replacing it with 1.2 ml freshly prepared MEMα containing supplements. During the entire incubation period, follicles were cultured in a humidified atmosphere containing 5% CO_2 and 95% air.

After a 3-day culture period, oocytes were removed from follicles, the cumulus cells were removed by repeated pipetting through a small-bore pipet, and the oocytes were scored for the presence or absence of a nucleus (germinal vesicle, GV). In some experiments, isolated oocytes were saved and prepared for RT-PCR by pelleting them briefly at 3000 rpm in 0.6-ml microfuge tubes. Most of the medium was removed, and the oocytes were quick frozen in liquid nitrogen and stored at −80°C until use.

For experiments using isolated oocytes, mice were 6- to 12-week-old B6SJLF1s. The medium used for oocyte collection was MEM with Earle's salts, L-glutamine, nonessential amino acids (Sigma), 120 U/ml penicillin G (potassium salt), 50 μg/ml streptomycin sulfate, 0.24 mM sodium pyruvate, 0.1% polyvinyl alcohol, and 20 mM HEPES, pH 7.2, and was supplemented with 250 μM dibutyryl cyclic adenosine monophosphate (dbcAMP) to prevent spontaneous oocyte maturation (Cho et al., 1974). Mice were sacrificed and their ovaries punctured with a 26-gauge needle in 2 ml medium. Full-grown (70–75 μm in diameter) oocytes were collected

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and washed through several washes of MEM. Oocytes were microinjected with siRNA as described previously (Mehlmann et al., 1996). Following microinjection, oocytes were transferred to MEM in which the HEPES was replaced with 25 mM $NaHCO_3$, and the dbcAMP was replaced by 4 mM hypoxanthine. Oocytes were cultured for 26–45 h in an atmosphere containing 5% CO_2 and 95% air, after which they were scored for the presence or absence of a GV. At the end of the culture period, oocytes were saved and prepared for RT-PCR by pelleting them gently in small microfuge tubes. Most of the culture medium was removed, oocytes were quick frozen in liquid nitrogen and were stored at −80°C until use.

Follicles and isolated oocytes were photographed with a Nikon Coolpix 5000 digital camera coupled to a Zeiss Axiovert 200M microscope, using either a 20×, 0.5 NA or a 40×, 0.75 NA lens.

Production of siRNA

Target sequences for the production of siRNA, consisting of two, 21-bp regions from mouse *Gpr3,* were selected using the siRNA Target Finder at the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). As a control, one of the *Gpr3* targets was mutated in 5 regions (mut*Gpr3*). For *Gpr3,* the target sequences were 5′-AAGCUUUGAUUUACUUCUUUG-3′ and 5′-AAUGCUCUCACUUACUACUCA-3′; for mut*Gpr3,* the target sequence was 5′-AAUCUGUGCUUGACGUCGUUA-3′. Double-stranded RNA was produced from the template DNA using the *Silencer* siRNA Construction Kit (Ambion), according to the manufacturer's instructions. The concentration of each siRNA was determined by comparing the intensity on a 5% agarose gel, stained with SYBRGold nucleic acid stain (Molecular Probes Inc., Eugene, OR), with the intensity of a known amount of siRNA against mouse GAPDH (Ambion); this GAPDH siRNA was also used in some experiments as a control.

For all microinjection experiments, 10 pl of siRNA solution was injected into the oocyte (volume of 200 pl). For *Gpr3,* both siRNAs were mixed together and diluted to a stock concentration of ~1 μM or 0.1 μM, yielding a final concentration in the oocyte of 50 or 5 nM siRNA, respectively. The siRNAs against mut*Gpr3* and GAPDH were diluted to ~2 μM, for a cytoplasmic concentration of ~100 nM. The total number of molecules injected for all siRNAs ranged from 3×10^4 to 6×10^5.

RT-PCR

cDNA was produced using the Superscript II Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions and using oligo-dT as the primer. In most cases, cDNA was produced from isolated oocytes by resuspending frozen, pelleted oocytes in 10 μl nuclease-free water, without purifying the RNA. In some cases, RNA was first purified from isolated oocytes using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Both methods yielded similar results. The samples were paired such that in each experiment, there was an equal number of siRNA-injected and uninjected oocytes. 9–30 oocytes were processed at a time.

Following reverse transcription, 0.5–2 oocyte equivalents were used for PCR to amplify *Gpr3* and the internal standard ribosomal protein L19 (*Rpl19*) gene. *Gpr3* and *Rpl19* were amplified in the same tube using the following primer sequences: *Gpr3:* 5′-TATCCACTCTCCAAGAACCATCTGG-3′ and 5′-GGAATTAAGCCCTGGTGGACCTAAC-3′; *Rpl19:* 5′-GGGAAAAAGAAGGTCTGGTTG-3′ and 5′-TTCAGCTTGTGGATGTGCTC-3′. The cycling parameters were an initial denaturation of 2 min, 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 7 min. PCR products

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were electrophoresed on 1.5% agarose gels, visualized by staining the gel with SYBRGold nucleic acid stain, and photographed using Polaroid film. Photographs were scanned and band intensities quantified using Scion Image (Scion Corporation, Frederick, MD).

Statistical analysis

InStat software (Graph Pad Software Inc., San Diego, CA) was used to perform Chi-square analysis or Student's *t* tests to determine significant differences between different groups. Significance for each experiment is indicated in the figure legends.

Results

Microinjection of follicle-enclosed oocytes with siRNA targeting Gpr3 causes meiotic resumption

To reduce *Gpr3* mRNA, oocytes within pre-antral follicles (140–180 μm in diameter) were microinjected with siRNA targeting mouse *Gpr3.* Follicles were cultured for 3 days, after which time oocytes were isolated and were scored for the presence or absence of a GV, which indicated whether they were still arrested in prophase I (GV intact) or had resumed meiosis (GV breakdown; GVBD). During the 3-day culture period, the follicle diameters increased (Table 1; Fig. 1). Follicles in which the oocyte had been microinjected grew to similar sizes as control, uninjected follicles, demonstrating that the process of microinjection was not damaging to the follicles. In many cases, follicles developed antral spaces and distinct mural granulosa and cumulus cell layers (Fig. 1).

67% of follicle-enclosed oocytes microinjected with 50 nM *Gpr3* siRNA underwent GVBD (Fig. 2). In contrast, oocytes injected with siRNA in which one of the target sequences against *Gpr3* was mutated in several regions (mut*Gpr3*; see Materials and methods), or with siRNA targeting GAPDH, did not undergo GVBD. Likewise, uninjected, follicle-enclosed oocytes isolated on the same day as the injected follicles and cultured in parallel with injected follicles did not resume meiosis (Fig. 2). Resumption of meiosis in *Gpr3* siRNA-injected oocytes was dose-dependent, with only 15% of oocytes injected with 5 nM siRNA undergoing GVBD, compared with 67% of oocytes injected with 50 nM siRNA (Fig. 2). Of the oocytes that resumed meiosis in response to injection of *Gpr3* siRNA, 64% went on to form first polar bodies (see Fig. 2A), demonstrating that they completed the first meiotic division, and indicating that oocyte maturation was physiological rather than a toxic effect of the siRNA. Uninjected oocytes from control follicles, or follicle-enclosed oocytes injected with 100 nM mut*Gpr3* or *GAPDH* siRNA, underwent GVBD much less frequently, with just 2% of uninjected, 5% of mut*Gpr3* siRNA, and 0% of *GAPDH* siRNA-injected oocytes undergoing GVBD. These results demonstrate that reduction of *Gpr3* RNA within the mouse oocyte causes meiotic resumption and indicate that GPR3 specifically in the oocyte, rather than in the follicle cells, is responsible for maintenance of meiotic arrest in mouse oocytes.

Microinjection of Gpr3 siRNA causes specific and effective reduction of Gpr3 RNA in follicle-enclosed mouse oocytes

The ability of the siRNA targeting *Gpr3* to reduce oocyte *Gpr3* mRNA, as well as its specificity, were assessed by performing RT-PCR on oocytes that were saved after scoring for GVBD. For RT-PCR analysis, cDNA was prepared from an equal number of *Gpr3* siRNA-injected, mut*GprS* siRNA-injected, or uninjected oocytes; for each condition, samples were prepared from at least 3 separate injection experiments. PCR reactions were run using primers directed against a combination of *Gpr3* and the ribosomal protein L19 (*Rpl19*) which should not be decreased by *Gpr3* siRNA, and was therefore used to standardize the results.

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Injection of *Gpr3* siRNA reduced the amount of *Gpr3* mRNA to ~10% of that seen in uninjected, control oocytes (Fig. 3). The amount of *Rpl19* mRNA was unaffected. In addition, mut*GprS* siRNA did not decrease the amount of *Gpr3* mRNA (Fig. 3). These results demonstrate that RNAi targeting *Gpr3* is specific.

RNAi causes meiotic resumption and reduces Gpr3 mRNA in isolated oocytes but to a lesser extent than in follicle-enclosed oocytes

RNAi has been used effectively in isolated mouse oocytes to prevent maturation-associated increases in proteins that are normally produced as a result of recruitment of maternal mRNAs (Svoboda et al., 2000; Xu et al., 2003; Anger et al., 2005). However, oocytes can only be cultured for ~2 days following isolation, after which time they will no longer mature in vitro (unpublished results). Therefore, in some cases, it may be impossible to sufficiently reduce the amount of the corresponding proteins in isolated oocytes, particularly if the proteins have low turnover rates. To compare the effectiveness of RNAi in follicle-enclosed vs. isolated oocytes, isolated oocytes were microinjected with 50 nM *Gpr3* siRNA and were cultured in the presence of 4 mM hypoxanthine to maintain meiotic arrest (Mehlmann et al., 2002). Oocytes were cultured for 26–45 h and then were scored for the presence or absence of a GV. GVBD occurred in isolated oocytes, though at a much lower rate than in follicle-enclosed oocytes cultured for 72 h (16% GVBD in isolated oocytes, compared with 67% GVBD in follicle-enclosed oocytes; Fig. 4A).

The lower percentage of isolated oocytes that resumed meiosis probably resulted because the oocytes were not cultured long enough to reduce the amount of pre-existing GPR3 protein. Indeed, RT-PCR analysis showed that RNAi-mediated reduction of *Gpr3* RNA in isolated oocytes that were cultured for 26–45 h was less complete than in follicle-enclosed oocytes that were cultured for 3 days (59 ± 13% (mean ± SEM, $n = 3$) for isolated oocytes, vs. 88 ± 3% ($n = 4$) for follicle-enclosed oocytes). These results show that RNAi is more effective in reducing RNA (and therefore protein) in follicle-enclosed oocytes than in isolated oocytes, due to the limited period for which isolated oocytes can be cultured lacking granulosa cell support.

Discussion

Recent characterization of knockout mice has demonstrated a critical role for the G-protein-coupled receptor, GPR3, in the maintenance of meiotic arrest (Mehlmann et al., 2004; Ledent et al., 2005). However, while *Gpr3* RNA is primarily localized in the oocyte, it is also present in lower quantities in the surrounding follicle cells (Mehlmann et al., 2004), leaving open the question of whether GPR3 in the follicle cells is essential for the maintenance of meiotic arrest, perhaps by producing cAMP that enters the oocyte via gap junctions (Anderson and Albertini, 1976; Bornslaeger and Schultz, 1985; Piontkewitz and Dekel, 1993; Webb et al., 2002; Eppig et al., 2004). Here, *Gpr3* was reduced specifically within the oocyte by microinjecting follicle-enclosed oocytes with siRNA targeting *Gpr3.* Within 3 days of culture following microinjection, oocytes injected with *Gpr3* siRNA, but not with control siRNA, lost their ability to maintain meiotic arrest. The ~70% GVBD observed following siRNA injection into oocytes is very close to the ~80–90% seen with deletion of *Gpr3* RNA from both oocyte and follicle cells in *Gpr3* knockout mice (Mehlmann et al., 2004). Therefore, the phenotype of the knockout mice can be mimicked by specific reduction of *Gpr3* in the oocyte, demonstrating that GPR3 specifically within the oocyte is necessary to maintain meiotic arrest prior to the LH surge.

GVBD was also observed in a small percentage of isolated oocytes injected with siRNA targeting *Gpr3.* Because these oocytes had no surrounding follicle cells but were maintained in meiotic arrest by hypoxanthine, this result further supports the hypothesis that elimination of GPR3 itself is sufficient to trigger the resumption of meiosis. *Gpr3* mRNA was less effectively reduced in isolated oocytes cultured for 1–2 days following injection of the same

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amount of siRNA than in follicle-enclosed oocytes that were cultured for 3 days (compare Figs. 3A and 4B). This is likely due to the longer period afforded by growing oocytes within their follicles that provides more time for the reduced mRNA levels to result in a reduction in GPR3 protein. Native levels of G-protein-coupled receptors are generally difficult to detect using antibodies because the amounts of these proteins in cells are low (Daly and McGrath, 2003); we have been unable to detect specific labeling of GPR3 using commercially available antibodies used by others (Ledent et al., 2005), or with 3 custom synthesized antibodies (L.M. Mehlmann, L.A. Jaffe, S. Tanaka, and Y. Saeki, unpublished results). For this reason, protein levels of GPR3 could not be directly measured in this study. However, the resumption of meiosis that was observed in siRNA-injected oocytes is an indirect indicator of a physiologically significant decrease in GPR3 protein leading to a decrease in cAMP.

In isolated mouse oocytes, RNAi has previously been used effectively to prevent the expression of proteins that accumulate during oocyte maturation (Svoboda et al., 2000, 2001; Wianny and Zernicka-Goetz, 2000; Xu et al., 2003; Anger et al., 2005). However, the technique is limited using isolated oocytes because they can only be cultured for a relatively short period that may not be sufficient to reduce the amount of preexisting proteins to a level needed to observe a phenotype, particularly if the proteins in question are stable and/or are present before and during oocyte maturation. Indeed, in one study utilizing RNAi to prevent the oocyte maturation-associated increase in the IP_3 receptor in isolated oocytes, the increase in receptor protein was prevented, but the level of existing protein did not change during the 14- to 16-h culture period (Xu et al., 2003).

To combat this problem, transgenic mice have been produced in which the synthesis of siRNA is driven by an oocyte-specific promoter, such that specific reduction of mRNA can begin earlier in oogenesis (Stein et al., 2003; Fedoriw et al., 2004; Yu et al., 2004). However, this method results in different degrees of mRNA targeting, yielding variable amounts of protein reduction (Stein et al., 2003; Fedoriw et al., 2004; Yu et al., 2004). Oocyte-specific, conditional knockouts have been shown to effectively eliminate oocyte proteins (He et al., 2003), but this method is expensive and time consuming. In contrast, the method presented here utilizes an effective, alternative approach that has several advantages. The ability to introduce a known amount of siRNA directly into oocytes is an advantage over transgenic RNAi approaches in which the amount of dsRNA is not known. The ability to microinject oocytes within pre-antral follicles and culture the follicles for several days following microinjection allows for a more complete reduction of the targeted RNA and protein. Furthermore, with this method, the oocytes are cultured within follicles that more closely approximate their natural environment, thereby obviating the need for a culture system that maintains high levels of cAMP using artificial means. In addition, the method is relatively easy, requires only a few animals, and results are obtained within a matter of days.

The method used here employed synthetically produced dsRNAs 21 bp in length. In contrast, in other studies, longer dsRNAs were used to effectively reduce mRNA levels in oocytes (Svoboda et al., 2000, 2001; Wianny and Zernicka-Goetz, 2000; Xu et al., 2003; Anger et al., 2005). Such long, dsRNAs are cleaved within the oocyte to yield fragments 21–23 nucleotides in length (Zamore et al., 2000). Both methods appear to be effective in reducing specific mRNAs within mouse oocytes.

The results presented here demonstrate that reduction of GPR3 specifically in the follicle-enclosed mouse oocyte causes meiotic resumption. Combined with our previous study showing that expression of GPR3 specifically into oocytes within follicles of *Gpr3* knockout mice can rescue the ability of the *Gpr3*$^{-/-}$ oocytes to maintain meiotic arrest (Mehlmann et al., 2004), they provide strong support for the hypothesis that the oocyte is primarily responsible for producing the cAMP that leads to meiotic arrest. It is possible that the follicle cells also

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contribute cAMP to the oocyte, but clearly, the amount of cAMP produced by the follicle cells is insufficient to maintain meiotic arrest. In addition, the ability to inject substances into follicle-enclosed oocytes and grow follicles for several days in culture establishes a method that could be generally useful for targeting a wide variety of proteins specifically within the oocyte. Follicles grown in culture can respond to LH, and matured eggs obtained from them are fertilizable (Eppig, 1991; Rose et al, 1999). Thus, this method of microinjection of follicle-enclosed oocytes, followed by in vitro follicle culture, could be used to examine a variety of questions pertaining to meiotic maturation, fertilization, and early development.

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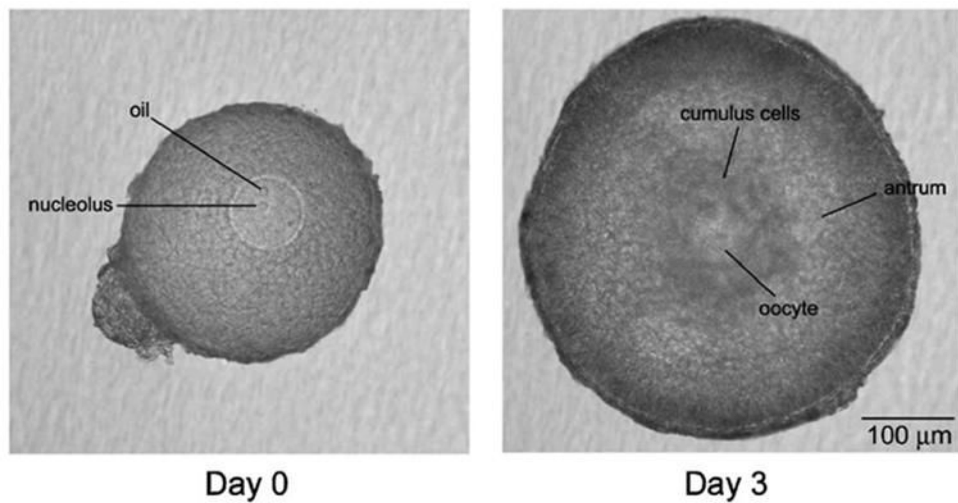

Fig. 1.

Follicles containing *Gpr3* siRNA-injected oocytes grow in culture. Pre-antral follicles, 140–180 µm in diameter, were dissected from mouse ovaries. Shortly after isolation, follicle-enclosed oocytes were injected with *Gpr3* siRNA. Follicles were subsequently cultured for 3 days on Millicell culture plate inserts. Growth of a single follicle is shown here shortly following microinjection (Day 0) and after 3 days of culture (Day 3).

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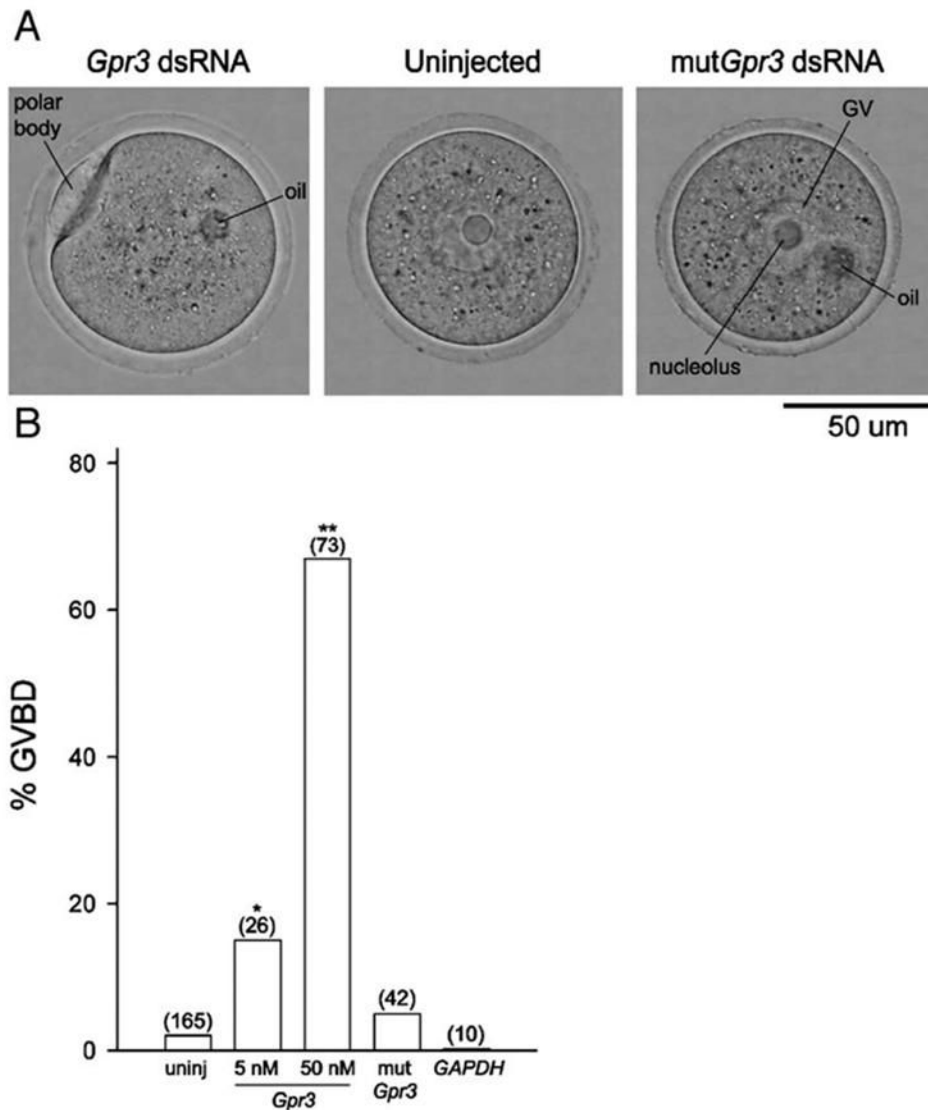

Fig. 2.

Microinjecting follicle-enclosed oocytes with *Gpr3* siRNA stimulates the resumption of meiosis. Follicle-enclosed oocytes were microinjected with siRNA, follicles were cultured for 3 days, and oocytes were removed and immediately photographed. (A) Oocytes injected with *Gpr3* siRNA underwent GVBD and first polar body formation. Uninjected follicle-enclosed oocytes, or follicle-enclosed oocytes injected with a mutated *Gpr3* siRNA sequence (mut*Gpr3*), did not undergo GVBD during the culture period. (B) Percentage of follicle-enclosed oocytes that underwent resumption of meiosis when injected with different siRNAs. All follicles were cultured for 3 days following isolation and microinjection, except the follicles injected with siRNA targeting GAPDH, which were cultured for 4 days. Each experiment was repeated at least 3 separate times. The total number of follicles per condition is indicated above each bar, and significant differences compared to uninjected control oocytes are indicated above each bar (Chi-square analysis, *$P<0.05$; **$P<0.0001$).

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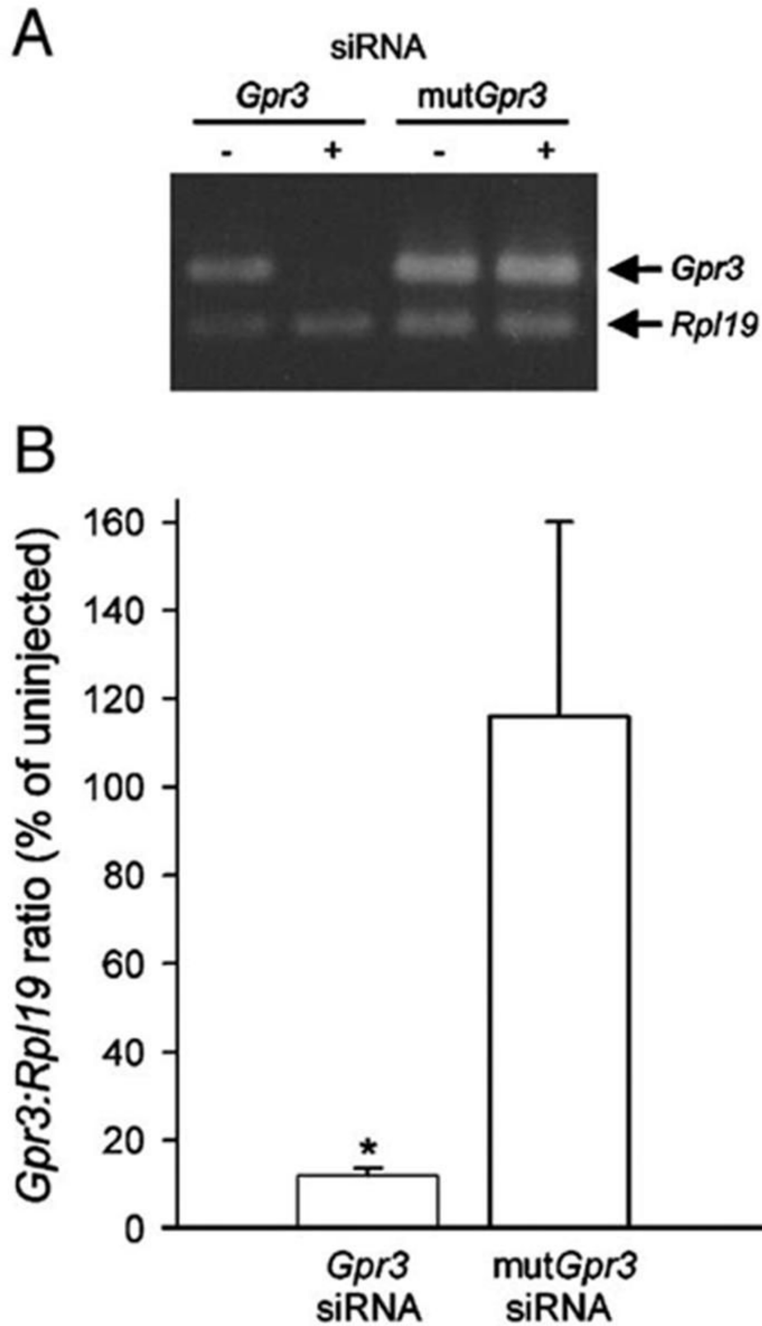

Fig. 3.

(A) *Gpr3* siRNA specifically reduces *Gpr3* mRNA in follicle-enclosed oocytes. RT-PCR amplifying *Gpr3* and *Rpl19* from cDNA prepared from uninjected oocytes or oocytes which had been injected 3 days earlier with *Gpr3* siRNA, or with mut*Gpr3* siRNA. Only the *Gpr3* band was reduced, and only in oocytes that had been injected with *Gpr3* siRNA. (B) Densitometry values quantifying the reduction of *Gpr3* mRNA in oocytes. Data are reported as the *Gpr3:Rpl19* RT-PCR product ratio in siRNA-injected oocytes, as a percentage of that in uninjected oocytes. Mean ± SEM is shown. The statistical significance of the effect of the siRNA injection was determined using a Student's *t* test, $*P < 0.05$. Each experiment was performed 3 times, on 3 different batches of cDNA per group.

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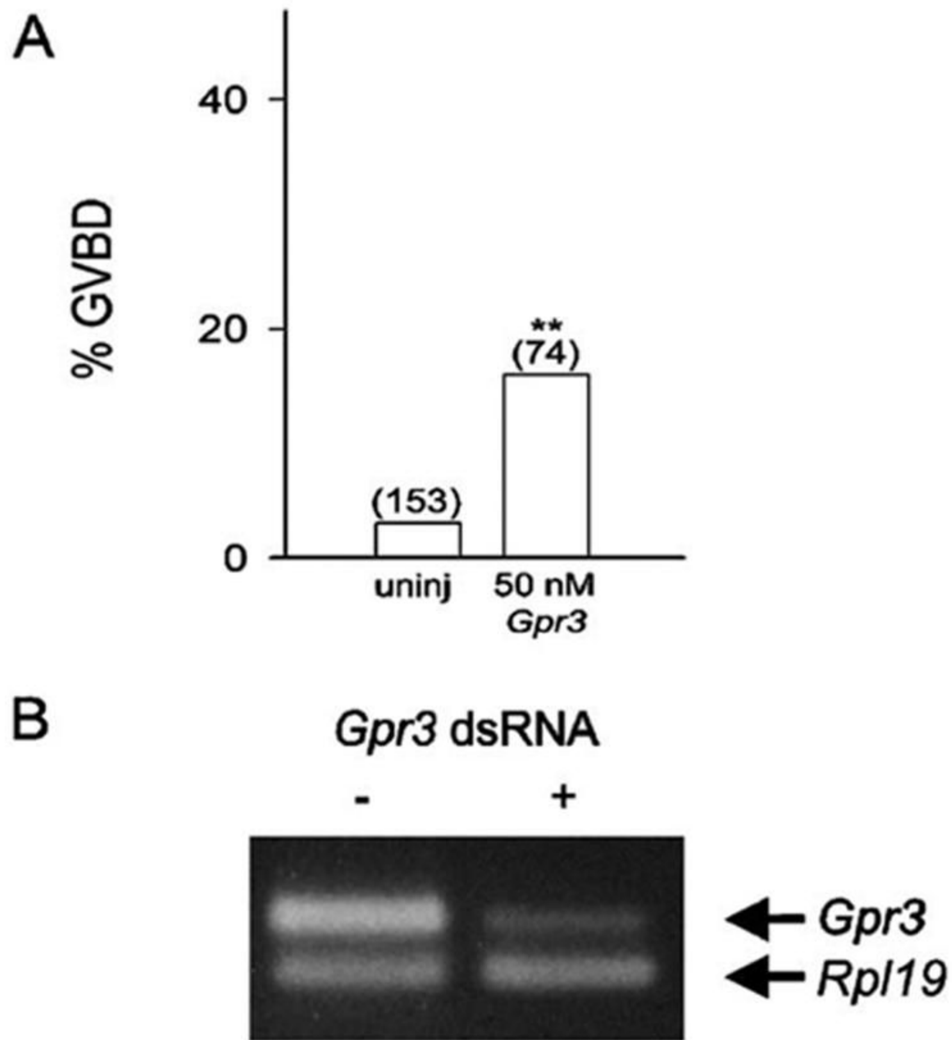

Fig. 4.

RNAi is also partially effective in isolated oocytes. (A) Percentage of isolated oocytes that underwent resumption of meiosis when injected with 50 nM *Gpr3* siRNA. Oocytes were cultured for 1–2 days following microinjection of siRNA. The total number of oocytes from 3 separate experiments is indicated above the bars, and ** indicates significance between the two groups ($P < 0.0001$). (B) Specific reduction of *Gpr3* RNA in oocytes microinjected with siRNA targeting GPR3. RT-PCR amplifying *Gpr3* and *Rpl19* from cDNA prepared from uninjected or *Gpr3* siRNA-injected, isolated oocytes following 45 h of culture. The reduction in *Gpr3* RNA is less than in follicle-enclosed oocytes cultured for 72 h.

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Table 1

Average diameter of follicles grown in culture for at least a 3-day period

Group	Diameter (μm) (number of follicles)
Uninjected	276 ± 37 (165)
Low Gpr3 (5 nM)	236 ± 50 (26)
High Gpr3 (50 nM)	271±35 (73)
mutGpr3 (100 nM)	269 ± 38 (42)
GAPDH (100 nM)	292 ± 40 (10)