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Androgens Promote Maturation and Signaling in Mouse Oocytes Independent of Transcription: A Release of Inhibition Model for Mammalian Oocyte Meiosis

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Normal fertility in females depends upon precise regulation of oocyte meiosis. Oocytes are arrested in prophase I of meiosis until just before ovulation, when meiosis, or maturation, is triggered to resume. Whereas sex steroids appear to promote maturation in fish and amphibians, the factors regulating mammalian oocyte maturation have remained obscure. We show here that, similar to lower vertebrates, steroids may play a role in promoting the release of meiotic inhibition in mammals. Specifically, testosterone induced maturation of mouse oocytes arrested in meiosis, as well as activation of MAPK and cyclin-dependent kinase 1 signaling. These responses appeared to be transcription independent and might involve signaling through classical androgen receptors expressed in the oocytes. Our results are the first to show that sex steroids can modulate meiosis in mammalian oocytes and suggest a model whereby dominant ovarian follicles in mammals may produce sufficient androgen and/or other steroids to overcome constitutive inhibitory signals and allow oocyte maturation and subsequent ovulation to occur. (*Molecular Endocrinology* 18: 97–104, 2004)

FEMALES FROM NEARLY every species of animal are born with their full complement of oocytes; however, these immature oocytes are arrested in prophase I of meiosis. Just before ovulation, gonadotropins stimulate ovarian follicular development, which in turn promotes oocytes to reenter the meiotic cycle, or mature, until they arrest again in metaphase II of meiosis. These mature oocytes are then competent for ovulation and subsequent fertilization (1–3). To date, little is known about the ovarian signals that both inhibit and promote oocyte maturation in mammals.

In contrast, oocyte maturation in the frog *Xenopus laevis* is much better understood, as it has served as one of the best models for studying meiosis for many decades (4). *Xenopus* oocytes remain in meiotic arrest after removal from the ovary, and can be induced *in vitro* to mature by addition of various steroids, including progesterone, corticosteroids, and androgens. Meiotic arrest appears to be maintained in part by constitutive $G\beta\gamma$- and/or $G\alpha_s$-mediated signaling that keeps intracellular cAMP levels high (5–7). Steroids promote maturation through a release of inhibition mechanism whereby these constitutive repressive signals are antagonized, thus allowing meiosis to progress. Maturation is accompanied by several signaling events, including activation of cyclin-dependent kinase 1 (CDK1), or cdc2, and the MAPK cascade (4, 8, 9). Steroids induce all of these processes in a transcription-independent, or nongenomic, fashion. Further, recent evidence suggests that androgens may be the primary physiologic mediators of *Xenopus* oocyte maturation *in vivo*, and are likely signaling in part through classical androgen receptors (ARs) expressed in oocytes (10–12).

Interestingly, regulation of mammalian oocyte maturation is quite similar to that in *X. laevis*. Meiotic arrest of mouse oocytes also appears to be regulated by constitutive signals that elevate intracellular cAMP, and the same signaling pathways are activated when mouse oocytes mature (13–15). In contrast to *Xenopus*, however, mouse oocytes spontaneously mature when removed from the ovary, suggesting that the primary signal maintaining meiotic arrest of mouse oocytes comes from the ovary rather than being endogenous to the oocyte itself, as with frog oocytes. This spontaneous maturation of mouse oocytes upon removal from the ovary has complicated efforts to determine the ovarian factors that can promote maturation in mice and other mammals; thus, the role of steroids in mediating mammalian oocyte maturation has yet to be determined.

Given the similarities in the signaling pathways associated with meiotic arrest and maturation in frogs and mice, we postulated that, as in frog oocytes, ste-

Abbreviations: AR, Androgen receptor; CDK1, cyclin-dependent kinase 1; DHT, dihydrotestosterone; GVBD, germinal vesicle breakdown; IBMX, 3-isobutyl-1-methylxanthine; SDS, sodium dodecyl sulfate.

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roids may be signaling and promoting maturation by releasing inhibitory signals in mouse oocytes as well. We focused on androgens because they appear to be the primary physiologic regulators of oocyte maturation in frogs, and high androgen states in mammals such as polycystic ovarian syndrome are clearly associated with abnormal ovarian growth and development (16). We found that isolated mouse oocytes responded to androgens similarly to frog oocytes. First, testosterone induced maturation of mouse oocytes arrested in prophase I in a dose-dependent fashion. This testosterone-induced maturation appeared to occur in the absence of transcription, and was accompanied by activation of MAPK and CDK1/cdc2 signaling, suggesting that the mechanisms of steroid-induced oocyte maturation are conserved from frogs to mammals. Second, testosterone-mediated signaling was attenuated by the AR antagonist flutamide, indicating that, as with frog oocytes, androgen-triggered signaling may be occurring at least in part via classical ARs. Finally, R1881, a potent activator of AR-mediated transcription, was a poor promoter of oocyte maturation, and actually partially inhibited testosterone-induced activation of MAPK. This suggests that selective AR modulators might be used to specifically promote genomic *vs.* nongenomic androgen-mediated effects in mammals.

RESULTS

Testosterone Triggers Maturation and Activation of MAPK and CDK1/cdc2 Signaling in Mouse Oocytes Arrested in Prophase I of Meiosis

To determine whether androgens could promote mouse oocyte maturation, spontaneous maturation was inhibited *in vitro* by raising intracellular cAMP levels using the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and the ability of testosterone to overcome this inhibition was examined. Oocytes were removed from 4- to 5-wk-old prepubertal mice ovaries to ensure that they had been exposed to minimal steroids before our studies. In the absence of IBMX, nearly 100% of isolated oocytes underwent germinal vesicle breakdown (GVBD) (Fig. 1A, *left*). IBMX concentrations were then titrated to inhibit maturation by approximately 60% as determined by visual examination of GVBD. Interestingly, addition of testosterone to IBMX-treated cells completely reversed IBMX-mediated inhibition of maturation, resulting in nearly 100% of the oocytes reaching GVBD (Fig. 1A, *left*). This result confirmed that androgens were indeed capable of overcoming the inhibitory effects of elevated intracellular cAMP to promote mouse oocyte maturation *in vitro*.

As a more objective measurement of steroid-mediated signaling in mouse oocytes, testosterone-induced activation of the MAPK cascade and CDK1/cdc2 were examined. Removal of mouse oocytes from the ovary resulted in activation of the MAPK cascade, as demonstrated by increased phosphorylation of p42/p44 (Fig. 1B, *top panel*). This activation could be detected after approximately 4 h and peaked at 12 h (data not shown), which is a similar time course to that observed in steroid-induced maturation of *Xenopus* oocytes. IBMX significantly attenuated p42/p44 phosphorylation in mouse oocytes, whereas addition of testosterone in the presence of IBMX led to a dose-dependent increase in p42/p44 phosphorylation that peaked at 500 nM testosterone. The levels of total p42/p44 were relatively similar in each lane, indicating that equal amounts of sample were loaded for each condition (Fig. 1B, *bottom panel*). Similarly, CDK1/cdc2 activity, as measured by the ability of ooctye extracts to phosphorylate histone H1, was elevated in untreated oocytes, inhibited by IBMX, and increased by adding testosterone (Fig. 1C). These results indicate that, in addition to promoting oocyte maturation, testosterone activates some of the same signaling pathways in mouse oocytes as previously described in *X. laevis*. Notably, similar experiments using surrounding follicular cells rather than oocytes revealed no activation of the MAPK or CDK1/cdc2 signaling pathways (data not shown), indicating that these pathways are being specifically activated in the oocytes.

Testosterone-Mediated Events in Mouse Oocytes Occur Independent of Transcription

One of the most fascinating aspects of steroid-induced maturation of *Xenopus* oocytes is that the entire process occurs independent of transcription (4, 12). To test whether testosterone-induced maturation of mouse oocytes was also transcription independent, oocytes were pretreated with the potent transcriptional inhibitor actinomycin D before addition of steroid. Actinomycin D was added at a concentration known to significantly reduce transcription in mouse (17) and *X. laevis* oocytes (12). Both spontaneous and testosterone-mediated oocyte maturation and activation of the MAPK signaling pathway were completely unaffected by actinomycin D (Fig. 1, A, *right*, and B, *right*, respectively), suggesting that, as with frog oocytes, androgen-induced signaling and maturation in mouse oocytes appears to occur independent of transcription.

The Classical AR May Play a Partial Role in Nongenomic Testosterone-Mediated Signaling in Mouse Oocytes

Recent work by several laboratories has shown that many nongenomic steroid-induced events are mediated by classical steroid receptors signaling in the plasma membrane (12, 18–22). To begin to address the possibility that the nongenomic effects of androgens in mouse oocytes might be regulated through classical ARs, mouse oocytes were treated with the classical AR antagonist flutamide before addition of testosterone. Addition of 10 μM flutamide consistently reduced testosterone-

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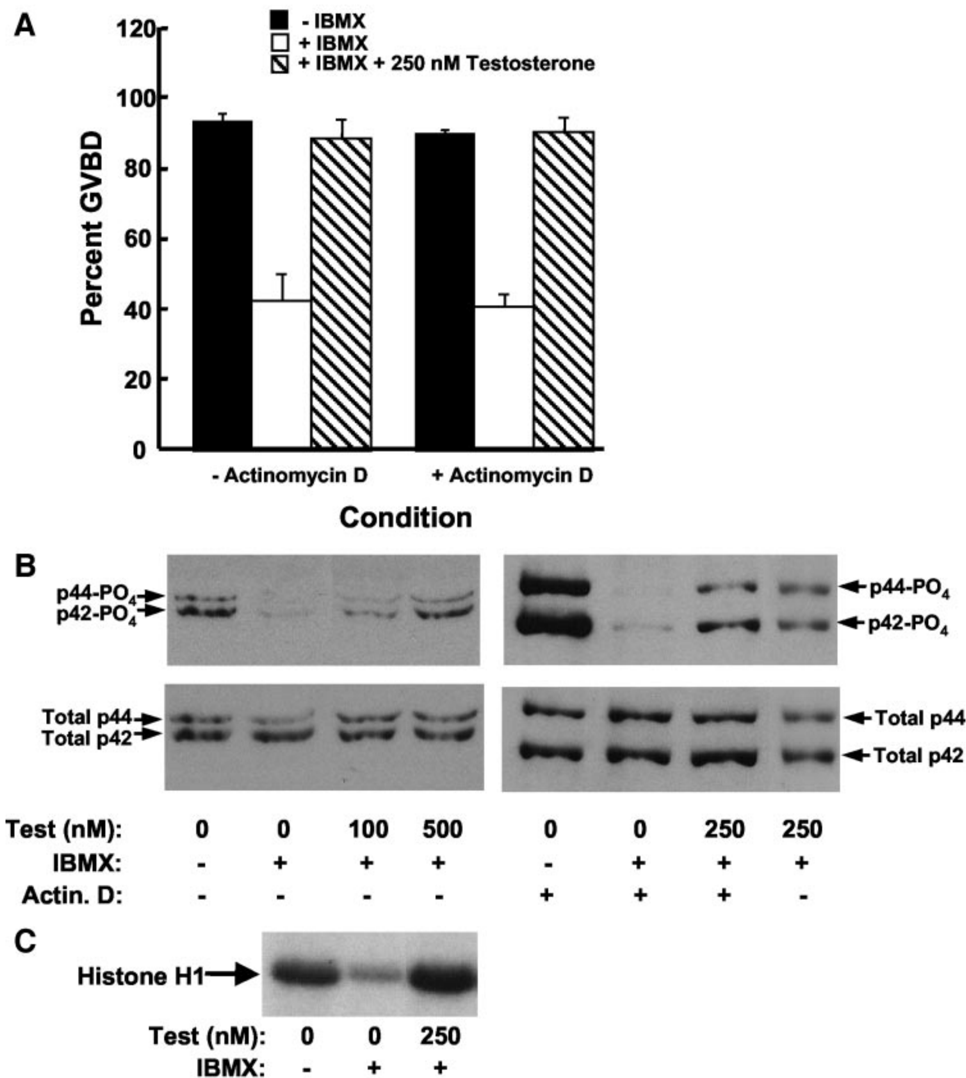

Fig. 1. Testosterone Promotes Mouse Oocyte Maturation and Activation of the MAPK and CDK1/cdc2 Signaling Pathways in a Transcription-Independent Manner

A, Equal numbers (50–60) of mouse oocytes were placed in M16 media with ethanol, IBMX, or IBMX plus 250 nM testosterone. The *bars on the right* represent the same experiment performed in the presence of 10 μg/ml actinomycin D. The percent of oocytes that had undergone GVBD was determined (y-axis). The *bars on the left* (− actinomycin D) represent the average of five experiments ± SEM, whereas the *bars on the right* (+ actinomycin D) represent the average of two experiments ± SEM. B, Equal numbers (approximately 50) of oocytes were treated as described in (A) using testosterone at the indicated concentrations. At the end of 16 h, oocytes were placed in 2× SDS sample buffer and extracts separated by gel electrophoresis. After transfer to PVDF membranes, blots were probed with rabbit anti-phospho-p42/p44 (*top*), stripped, and reprobed with rabbit anti-total p42/p44 antibody (*bottom*). Oocytes treated with actinomycin D are indicated (*right*). C, Twelve oocytes were incubated in M16 media for 7 h with ethanol, IBMX, or IBMX plus 250 nM testosterone. Oocytes were lysed, and the ability of the lysates to phosphorylate histone H1 *in vitro* was determined using radiolabeled ATP. Equal amounts of the final products for each condition were separated by gel electrophoresis and examined by autoradiography. All experiments in this figure were performed at least four times with virtually identical results.

mediated phosphorylation of p42/p44 by 50–70%, as determined by densitometry (Fig. 2A, *upper panel*), whereas total p42/p44 levels were similar (Fig. 2A, *lower panel*). This result confirms that the classical AR may be playing at least a partial role in testosterone-mediated signaling and maturation of mouse oocytes. Similar results were obtained using hydroxyflutamide (data not shown). The lack of complete inhibition by flutamide was most likely due to its lower affinity for and higher dissociation rate from the AR relative to testosterone; thus, a 40-fold excess of flutamide may not have been sufficient to completely block testosterone binding over the course of the MAPK assays. Flutamide concentrations significantly above 10 μM appeared to be toxic to oocytes, and therefore could not be used for these studies (data not shown).

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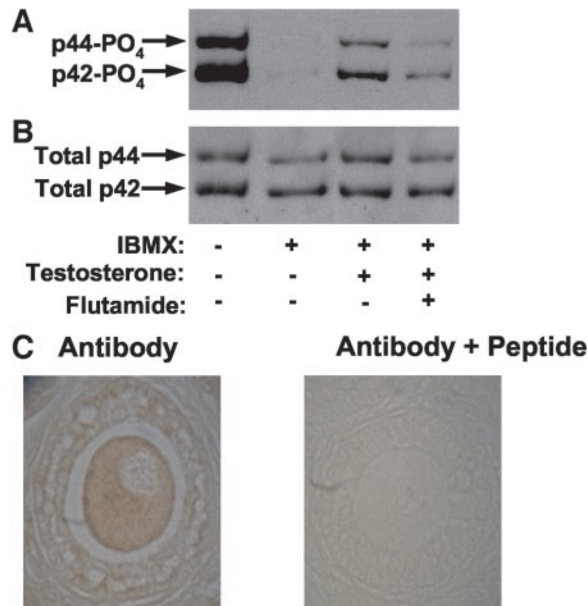

Fig. 2. The AR May Be Playing a Role in Testosterone-Mediated Signaling in Oocytes

A. Flutamide attenuates testosterone-induced activation of the MAPK cascade. Oocytes were treated as described in Fig. 1, only they were pretreated for 30 min with either ethanol or 10 μM flutamide before addition of 250 nM testosterone. Flutamide reduced testosterone-mediated phosphorylation of p42/p44 by 50%. This experiment was repeated multiple times with 50–70% reduction in testosterone-induced p42/p44 phosphorylation. B, Mouse oocytes express the AR. Mouse ovarian sections were treated as described in the supplemental methods section. The anti-AR antibody stained the oocytes *brown*, whereas antibody pretreated with the target peptide did not significantly stain the oocytes (C).

The ability of flutamide to attenuate testosterone-mediated activation of MAPK suggested that the AR was present in mouse oocytes. Expression of the AR was confirmed by immunohistochemistry of mouse ovarian sections using an anti-AR antibody. Oocytes stained positive for the AR, with expression mainly in the cytoplasm and virtually absent in the nucleus (Fig. 2B). Although difficult to confirm, a small amount of AR expression may have been present the plasma membrane as well, which would be consistent with the AR expression pattern in *Xenopus* oocytes. Pretreatment of the antibody preparation with the peptide to which the antibody was directed completely abolished staining of the oocytes, demonstrating the specificity of this result (Fig. 2C).

Isolated Mouse Oocyte Preparations Do Not Significantly Metabolize Testosterone

Although testosterone appeared to be promoting mouse oocyte maturation, testosterone metabolites such as dihydrotestosterone (DHT) or estradiol could still be responsible for the observed effects on maturation and signaling. This prospect is particularly intriguing in that *Xenopus* oocytes have been shown to express the enzyme CYP17, which converts progesterone to androstenedione, both of which are equal promoters of *Xenopus* oocyte maturation *in vitro* (10, 11). Treatment of mouse oocytes with radiolabeled testosterone for 16 h revealed no significant conversion to either DHT or estradiol over 12 h, indicating that little steroid metabolism was occurring in the mouse oocyte preparations (Fig. 3). In addition, both testosterone-mediated maturation and phosphorylation of p42/p44 were minimally affected by the potent CYP19 (aromatase) inhibitor Anastrazole (data not shown), further suggesting that testosterone was not signaling through conversion to estradiol. Anastrazole at these concentrations completely blocked CYP19 activity measured in gonadotropin-stimulated ovaries (data not shown).

DHT and R1881 Are Weak Promoters of Nongenomic Signaling in Mouse Oocytes

Testosterone did not appear to be metabolized in our assay system; however, other steroids might still have been capable of signaling in mouse oocytes. Whereas DHT was significantly more potent at promoting AR-mediated transcription relative to testosterone in somatic cells (data not shown), it was a weaker activator of the MAPK cascade in oocytes when compared with testosterone (Fig. 4). Furthermore, the even more potent promoter of AR-mediated transcription, R1881, induced little to no phosphorylation of p42/p44 in oocytes (Fig. 4). In fact, as seen with *Xenopus* oocytes (12), 3 μM R1881 consistently attenuated testosterone-induced activation of MAPK by approximately 50% (Fig. 4), but not spontaneous maturation (data not shown). Again, complete inhibition would be unexpected with only 10-fold excess of R1881 over the course of a 16-h experiment. These results indicate that, regardless of the species, R1881 appears to act as a selective AR modulator that promotes genomic, but not nongenomic, AR-mediated signaling. Of note,

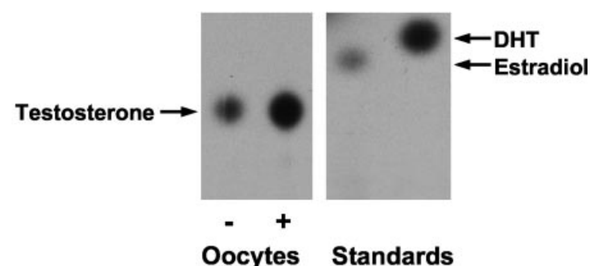

Fig. 3. Testosterone Is Not Significantly Metabolized by Isolated Oocytes *in Vitro*

A 10-nM concentration of radiolabeled testosterone was incubated in M16 medium alone (−) or M16 containing 60 oocytes (+) for 16 h. Steroids were then extracted from the media and oocytes and resolved using thin-layer chromatography. *Arrows* indicate the location of testosterone, as well as the DHT and estradiol standards.

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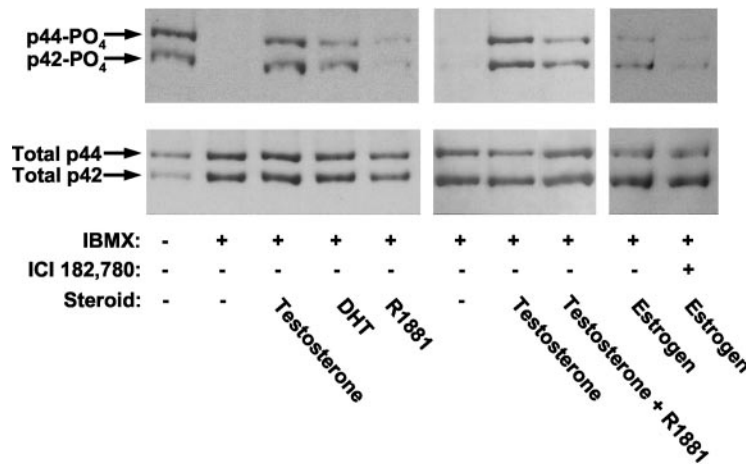

Fig. 4. Different Androgens and Ovarian Steroids Have Variable Abilities to Activate the MAPK Cascade

Equal numbers of oocytes (~50) were treated for 16 h with ethanol, IBMX, or IBMX plus 250 nM of testosterone, DHT, or R1881 (*left panel*). In addition, equal numbers of oocytes pretreated for 1 h with either ethanol or 3 μM R1881 were incubated with IBMX plus 300 nM testosterone (*middle panel*). Finally equal numbers of oocytes pretreated for 30 min with ethanol or ICI, 182, 780 were incubated with IBMX plus 250 nM estradiol (*right panel*). Equal amounts of oocyte extracts were loaded and resolved on polyacrylamide gels, as described. Gels were probed for phosphorylated p42/p44 (*top*), stripped, and probed for total p42/p44 (*bottom*). These experiments were all repeated at least three times with essentially identical results.

incubation of mouse oocytes with radiolabeled DHT or R1881 revealed that similar amounts of both steroids were taken up by the oocytes when compared with radiolabeled testosterone, and that neither was detectably metabolized over the course of 16 h (data not shown).

Estradiol Promotes Nongenomic Signaling in Oocytes, Perhaps through the Classical Estrogen Receptor

In addition to androgens, other ovarian steroids were examined for their abilities to promote phosphorylation of p42/p44. Progesterone was a variable but weaker promoter of p42/p44 phosphorylation relative to testosterone (data not shown), whereas estradiol was a moderate promoter of both oocyte maturation (data not shown) and phosphorylation of p42/p44 (Fig. 4). This estradiol-induced phosphorylation of p42/p44 was attenuated by addition of the antiestrogen ICI 182, 780 (Fig. 4), as was estradiol-induced activation of CDK1/cdc2 (data not shown). These results suggest that, similar to testosterone, estradiol-mediated signaling in mouse oocytes may be mediated at least in part through its classical receptor.

DISCUSSION

In summary, these studies are the first to demonstrate that steroids can promote maturation and signaling in mammalian oocytes *in vitro*. Attempts to demonstrate steroid-mediated maturation of mammalian oocytes have been difficult in past (23–26), perhaps due in part to the background of spontaneous oocyte maturation upon removal from the ovary, as well as methods of oocyte removal that pre-expose oocytes to sex steroids (*e.g*. pretreatment of mice with gonadotropins or removal of oocytes from mice that are postpubertal). Although further work is needed to confirm the physiologic relevance of these steroid-mediated activities, several pieces of evidence support an *in vivo* role for steroid-induced maturation. First, cAMP-mediated meiotic arrest is generally considered to be a physiologically relevant phenomenon (15); thus, the ability of testosterone to overcome this inhibitory signal *in vitro* suggests that similar processes could be occurring *in vivo*. Second, our results correlate with the ovarian phenotype of the recently described AR knockout mice. Female mice lacking the AR have significant reproductive defects that include reduced frequency of pregnancy, smaller litter sizes, and ovaries with small follicles and few mature oocytes (27, 28). Although other steroid binding proteins, such as the recently described membrane progesterone receptor family (29–31), may also be involved in steroid-induced maturation, the phenotype of the AR knockout female mice combined with our *in vitro* data are consistent with androgens and the AR having important physiologic roles in oocyte maturation in mice. Third, the close parallels of these results in mice to earlier studies in *Xenopus*, including the nongenomic nature of signaling, the similarities in steroid-mediated signaling pathways, and the selectivity of various AR ligands, imply a conserved mechanism of oocyte maturation from amphibians to mammals. Fourth, high serum levels of androgens in women, regardless of the source, promote abnormal ovarian growth with development of polycystic ovaries, and the AR antagonist

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flutamide improves infertility in some of these individuals (32, 33). These observations in humans confirm that androgen actions through the AR might be mediating critical signals in the ovary. Given the importance of cross-talk between oocytes and surrounding follicular cells for normal follicular growth (34, 35), it is interesting to speculate that nongenomic androgen signaling in oocytes might be contributing to the polycystic phenotype induced by excess androgens, perhaps by promoting the growth of multiple follicles while preventing the development of dominant ones.

To accommodate our data with other studies of mouse oocyte maturation, we propose a model of *in vivo* mouse oocyte maturation whereby constitutive inhibitory signals within the ovary (I) hold meiosis in prophase I (Fig. 5). Although the precise intracellular mechanisms regulating this meiotic arrest are not know, they may involve G protein-induced elevations in cAMP levels. Before ovulation, gonadotropins promote follicular growth and production of sex steroids (T, testosterone; E, estrogen). In frogs, many follicles are stimulated to grow, resulting in maturation and ovulation of hundreds of oocytes. In contrast, only a few dominant follicles in mice or other mammals might be sufficiently stimulated by gonadotropins, as well as by cross-talk between oocytes and surrounding follicle cells, to produce enough steroid to overcome the inhibitory signals and allow meiosis and subsequent ovulation to progress. In individuals with androgen excess, one might speculate that multiple ovarian follicles would be equally stimulated with androgens, which might result in unregulated growth and the lack of dominant follicle production. This inability to produce dominant follicles might explain the anovulatory state of many individuals with androgen excess, as well as their relative resistance to gonadotropin therapy. Whether the ovaries of patients with androgen excess contain more mature oocytes is not known, as their inability to ovulate would likely lead to oocyte atresia before they could be detected.

Signals that accompany steroid-induced maturation include activation of CDK1/cdc2 and the MAPK cascade. Although elevated intracellular cAMP inhibits maturation, whether a decrease in cAMP is necessary or sufficient to promote maturation remains controversial (36, 37); thus, the effects of steroids on cAMP levels, as well as their significance, have not yet been determined. The ability of both estradiol and testosterone to promote oocyte maturation *in vitro* suggests a redundancy in the steroid-mediated maturation pathway; however, the higher potency of testosterone *in vitro*, combined with the reduced fertility and ovarian phenotypes of mice lacking the AR and of women with excess androgens, suggests that androgen action through the AR plays an important role in normal oocyte and ovarian development. If so, given the nongenomic nature of androgen-induced oocyte maturation in mammals, novel treatments using selective AR modulators such as R1881 that specifically alter nongenomic signaling might prove useful in regulating oocyte maturation and follicular development both *in vitro* and in women with reduced fertility.

MATERIALS AND METHODS

Oocyte Isolation

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ovaries were removed directly from 4- to 5-wk-old mice and placed in M2 medium (Cell and Molecular Technologies, Lavallette, NJ) containing 200 μM IBMX (Calbiochem, La Jolla, CA). Ovaries were punctured and oocytes removed from follicles using 30-gauge needles. Oocytes were denuded by gentle pipetting with pulled glass pasteur pipettes, washed with M2 medium, and placed in M16 medium (Sigma, St. Louis, MO) containing 200 μM IBMX (M16/IBMX) for maturation and signaling assays.

Oocyte Maturation, MAPK, and CDK1/cdc2 Assays

Equal numbers of isolated and washed oocytes (approximately 50–60) were placed in M16/IBMX and incubated at 37

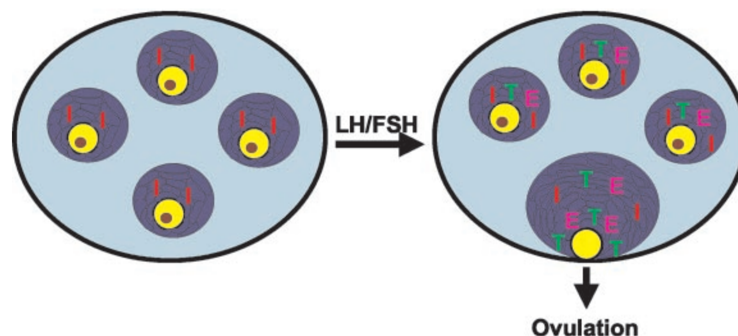

Fig. 5. Model of *in Vivo* Mouse Oocyte Maturation

Unknown inhibitory signals (I) within ovarian follicles maintain meiotic arrest of oocytes (*yellow with brown nucleus*), perhaps by keeping intracellular cAMP levels high. Before ovulation, gonadotropins promote follicular growth and production of sex steroids (T, testosterone; E, estrogen). Large, dominant follicles (*bottom right*) produce sufficient amounts of steroid to overcome and release the inhibitory signals, thus allowing GVBD (loss of defined *brown nucleus*), meiosis, and subsequent ovulation to progress.

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C with either ethanol or steroids (Sigma, St. Louis, MO, and Steraloids, Newport, RI) at the concentrations described in the figure legends. Ethanol concentrations were kept at 0.4% in all samples. An equal number of oocytes were also placed in M16 mediate lacking IBMX as a positive control for maturation and signaling. After 16 h, oocytes were examined under a dissecting microscope and the number of oocytes with GVBD was determined. All oocytes were then placed in 2× sodium dodecyl sulfate (SDS)-sample buffer and phosphorylation of p42/p44 was detected by Western Blot analysis as described (5). After immunoblotting for phosphorylated p42/p44, blots were stripped and reprobed with anti-total p42/p44 as a control for gel loading. The strength of the signals were then determined using the National Institutes of Health Image program (12).

For the actinomycin D experiments, oocytes were treated as above, only they were preincubated for 4–5 h with either ethanol or 10 μg/ml actinomycin D (Sigma) followed by addition of steroid in the continued presence of ethanol or actinomycin D. For other experiments, oocytes were preincubated for 30 min with ethanol, 10 μM flutamide [a gift from M. McPhaul, University of Texas Southwestern Medical Center at Dallas, Dallas, TX (UTSW)], 3 μM R1881 (NEN Life Science Products, Boston, MA), or 10 μM ICI 182, 780 (AstraZeneca, London, UK), before addition of steroids.

To measure CDK1/cdc2 activity, 12 oocytes per condition were treated with steroid as above for 7 h at 37 C. Oocytes were then lysed and extracts examined for their ability to phosphorylate histone H1 (Sigma) *in vitro* as described (38). Reactions were run for 30 min and stopped by addition of 2× SDS-sample buffer. Extracts were then resolved on 12% polyacrylamide gels and inspected by autoradiography.

Immunohistochemistry

Ovaries were removed from mice, fixed in paraffin, sectioned, and mounted on slides by the Molecular Pathology Core Facility at University of Texas Southwestern Medical Center. Slides were incubated overnight with a rabbit anti-carboxyl-terminal AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that had been previously treated with either PBS or excess neutralizing peptide (Santa Cruz Biotechnology). The AR was then detected using the Vectasin ABC kit (Vector Laboratories, Burlingame, CA), and slides were viewed and photographed using a Nikon stereoscope and digital camera.

Testosterone Metabolism

Sixty isolated oocytes were incubated in M16 medium for 16 h at 37 C with [1,2,6,7-^{3}H(N)]testosterone (NEN Life Science Products). Steroids were extracted and examined by thin-layer chromatography as described (11).

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