# Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and *microRNA-125b* expression

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Edited by John J. Eppig, Jackson Laboratory, Bar Harbor, ME, and approved January 15, 2014 (received for review October 8, 2013)

**Although androgen excess is considered detrimental to women's health and fertility, global and ovarian granulosa cell-specific androgen-receptor (AR) knockout mouse models have been used to show that androgen actions through ARs are actually necessary for normal ovarian function and female fertility. Here we describe two AR-mediated pathways in granulosa cells that regulate ovarian follicular development and therefore female fertility. First, we show that androgens attenuate follicular atresia through nuclear and extranuclear signaling pathways by enhancing expression of the microRNA (miR) *miR-125b*, which in turn suppresses proapoptotic protein expression. Second, we demonstrate that, independent of transcription, androgens enhance follicle-stimulating hormone (FSH) receptor expression, which then augments FSH-mediated follicle growth and development. Interestingly, we find that the scaffold molecule paxillin regulates both processes, making it a critical regulator of AR actions in the ovary. Finally, we report that low doses of exogenous androgens enhance gonadotropin-induced ovulation in mice, further demonstrating the critical role that androgens play in follicular development and fertility. These data may explain reported positive effects of androgens on ovulation rates in women with diminished ovarian reserve. Furthermore, this study demonstrates mechanisms that might contribute to the unregulated follicle growth seen in diseases of excess androgens such as polycystic ovary syndrome.**

Other than the obligatory role of androgens as estrogen precursors in steroidogenesis (1), little is known about the direct involvement of androgens in the female ovary. For many decades, excess androgens in women have been considered detrimental to women's health, as diseases such as polycystic ovary syndrome (PCOS) are associated with reduced fertility. In the past, these negative effects of androgens on female fertility were thought to occur primarily at the level of the hypothalamus and pituitary (2, 3), although important data across different species (4–7) suggested that androgens could also directly promote follicle growth (8, 9). Attitudes about androgen actions in female fertility changed with the development of global androgen-receptor knockout (ARKO) mice (10–12). The female ARKO mice had considerable reproductive defects, with decreased fertility, defective follicular development, reduced ovulation, and premature ovarian failure. In other words, the phenotype of these ARKO mice suggested that androgen signaling might actually be important for normal female reproductive health. Intriguingly, through the generation of granulosa cell (GC)-specific ARKO mice, we (13) and then others (14) demonstrated that essentially all the observed reproductive phenotypes in the complete AR-null mice are caused by androgen actions in GCs. These results highlighted that, with regard to fertility, androgen signaling in the ovary is at least as important as androgen signaling in the pituitary or hypothalamus. By using this GC-specific ARKO mouse model, we further demonstrated that androgens regulate follicular development and female fertility by attenuating follicular atresia while simultaneously promoting preantral follicle growth and development into antral follicles. However, these in vivo studies did not elucidate specific mechanisms used by androgens and ARs to mediate these processes.

Here we performed an in-depth analysis of androgen-induced signaling pathways that regulate ovarian folliculogenesis. Androgens signal via extranuclear (nongenomic) and nuclear (genomic) pathways. In fact, previous work by others and ourselves in androgen-sensitive prostate cancer cells showed that these two processes are tightly linked, with maximal AR-mediated nuclear transcription requiring extranuclear AR signaling (15). We reported that, through membrane-localized ARs, androgens trigger matrix metalloproteinase (MMP)-mediated transactivation of the epidermal growth factor receptor (EGFR), which in turn leads to cytoplasmic Akt and MAPK3/1 signaling. MAPK3/1 signaling is then required for normal nuclear AR transcriptional effects (16, 17). Interestingly, we have shown (16) that paxillin, a scaffold protein traditionally thought to regulate cytoskeletal remodeling and focal adhesion function, is an essential mediator of extranuclear and nuclear AR signaling. Paxillin first regulates Src-induced MAPK3/1 activation at the membrane. Upon activation, MAPK3/1 promotes serine phosphorylation of paxillin, resulting in the nuclear localization of phosphoserine (PS)-paxillin. Once in the nucleus, PS-paxillin then complexes with the AR to retain it in the nucleus, allowing AR-mediated transcription to occur. In fact, in the absence of paxillin, AR is no longer retained in the nucleus of prostate cancer cells or primary ovarian GCs,

## Significance

**Androgens are primarily considered detrimental to women's health. However, androgen-receptor KO mouse models have been used to establish that androgens are actually necessary for normal ovarian function and female fertility. Despite these observations, how androgens regulate female fertility is not known. Here we show that androgens promote follicular development via two mechanisms: (*i*) prevention of follicular atresia by inducing the expression of an antiapoptotic microRNA (miR), *miR-125b*; and (*ii*) promotion of follicle growth by increasing follicle-stimulating hormone receptor levels in a transcription-independent fashion. These data considerably change our understanding of androgen effects in female reproduction, and help explain the ovarian physiology seen in patients with too little or too much androgen.**

Author contributions: A.S. and S.R.H. designed research; A.S., H.P., A.L., A.B., and E.H. performed research; H.-J.L., D.B., and N.G. contributed new reagents/analytic tools; A.S. and S.R.H. analyzed data; and A.S. and S.R.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318978111/-/DCSupplemental.

even in the presence of androgen (16). Thus, paxillin serves as a critical "liaison" between extranuclear kinase signaling and intranuclear transcription (17).

Here we describe two paxillin-dependent AR-induced regulatory mechanisms in GCs that use extranuclear and nuclear AR signaling. These pathways likely account for the majority of the observed androgen actions in follicular development. First, we show that ARs enhance the expression of the antiapoptotic microRNA (miR) *miR-125b*, which likely contributes to androgen-induced follicular survival. Second, we demonstrate that androgens increase follicle-stimulating hormone (FSH) receptor (FSHR) protein, but not mRNA, expression, thus sensitizing follicles to FSH actions and potentially contributing to androgen-mediated follicle growth. These positive effects of androgens on follicle development may play a critical role in gonadotropin-induced ovulation, and may serve as potential targets to enhance fertility in women with decreased follicle growth and ovulation as a result of diminished ovarian reserve.

## Results

**Nuclear and Extranuclear Actions of ARs Regulate Expression of *miR-125b* in GCs.** In this study we report that androgens regulate follicular atresia by inducing the expression of *miR-125b*, an miR known to suppress proapoptotic protein expression (18). Results show that *miR-125b* is expressed in mouse GCs (Fig. 1*A*), human KGN GC tumor cells (Fig. 1*B*), and primary human GCs isolated during oocyte retrieval from women undergoing in vitro fertilization (Fig. S1*D*). Importantly, the androgens dihydrotestosterone (DHT) and testosterone, in contrast to other steroids (estradiol and R5020), induce expression of *miR-125b* (Fig. 1*A*), but not *miR-125a*, a related miR of the same miR family (Fig. S2*B*), in primary GCs.Notably, basal expression of *miR-125b* is high in KGN cells. Thus, DHT-induced induction of *miR-125b* expression is subdued but still significant. This may be because tumor cells such as KGN have suppressed apoptosis, perhaps maintained in part through increased expression of antiapoptotic miRs such as *miR-125b*.

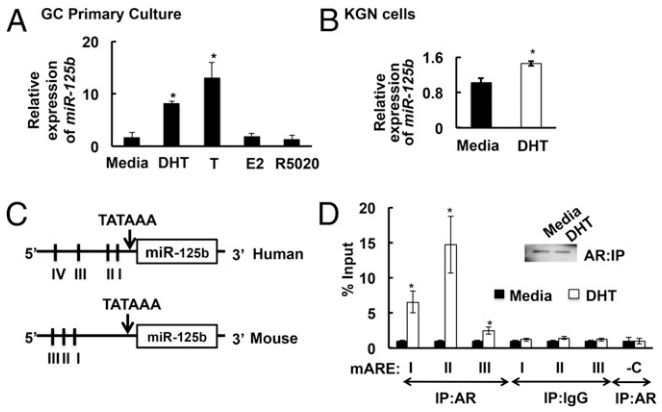


**Fig. 1.** (*A* and *B*) Androgens [DHT and testosterone (T)] but not estradiol (E2) or the progestin R5020 induce *miR-125b* expression in GCs after 18 h. Relative expression of *miR-125b* in (*A*) primary mouse GCs and (*B*) the human granulosa-like tumor cell line, KGN. Data are displayed as means ± SEM ($n = 3$) and normalized to GAPDH levels (*$P \leq 0.05$ vs. media). (*C* and *D*) DHT stimulates AR association with the mouse *miR-125b-2* promoter: (*C*) Schematics showing that the human and mouse 5′ UTRs of the *miR-125b-2* promoter contain four (I, −316/−266; II, −616/−567; III, −816/−767; IV, −2,628/−2,579) and three (I, −2,400/−2,350; II, −2,533/−2,483; III, −2,733/−2,783) AREs, respectively. (*D*) ChIP assay in primary mouse GCs showing that ARs bind to the AREs in the *miR-125b-2* promoter after 45 min with DHT (25 nM). Immunoprecipitation of AR was equal between media- and DHT-treated cells (*Inset*). IgG represents nonspecific antibody and "-C" represents nonspecific primers within the promoter. Values represent percentage input (means ± SEM, $n = 3$; *$P \leq 0.05$ vs. media).

Mature *miR-125b* originates from two precursors: *miR-125b-1* and *miR-125b-2*. In mice and humans, the 5′ UTRs of the *miR-125b-2*, but not *miR-125b-1*, promoters contain three and four androgen responsive elements (AREs; Fig. 1*B*), respectively, suggesting that the AR might interact directly with these AREs to induce *miR-125b* expression. In fact, ChIP assays in primary mouse GCs reveal that ARs associate with all three AREs in the 5′ UTR region of the mouse *miR-125b-2* promoter in a DHT-dependent fashion (Fig. 1*C*); thus, androgens likely up-regulate *miR-125b* expression through AR-mediated transcription of *miR-125b-2*.

As mentioned, our previous work in prostate cancer cells showed that AR-mediated transcription in the nucleus requires extranuclear AR signaling through transactivation of the EGFR and subsequent MAPK3/1 signaling in the cytoplasm (16, 17, 19). Similarly, in primary mouse GCs, DHT promotes rapid extranuclear MAPK3/1 signaling (Fig. S1*E*). Addition of an AR antagonist (flutamide), an MMP inhibitor (Galardin), or an EGFR inhibitor (AG1478) inhibits DHT-induced MAPK3/1 activation (Fig. S1*E*), confirming that, as seen in prostate cancer cells, androgen-induced MAPK3/1 signaling occurs via transactivation of the EGFR through MMP-mediated release of membrane bound EGFR ligands in GCs. Furthermore, as in prostate cancer cells, androgen-triggered extranuclear MAPK3/1 signaling is critical for nuclear AR-mediated transcription in GCs, as the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 completely abrogates DHT-induced up-regulation of *miR-125b* (Fig. 2*A*). Finally, consistent with our observation that paxillin is required for AR nuclear localization in GCs (16), siRNA-mediated knockdown of paxillin in primary mouse GCs inhibits DHT-induced MAPK3/1 signaling, whereas reexpression of paxillin restores MAPK3/1 signaling (Fig. 2*B*). Paxillin knockdown also blocks DHT-induced *miR-125b* expression, and EGF alone is not sufficient to promote *miR-125b* expression (Fig. 2*A*), confirming the need for paxillin-mediated nuclear and extranuclear AR crosstalk to regulate *miR-125b* expression in GCs.

**Androgen-Induced *miR-125b* Suppresses Proapoptotic Protein Expression in GCs, Resulting in Follicular Survival.** We used TargetScan algorithm 6.2 (Whitehead Institute for Biomedical Research) to find that Bcl-2 homologous antagonist/killer (*BAK1*), Bcl-2 modifying factor (*BMF*), Bcl-2-associated X protein (*BAX*), and the tumor suppressor protein p53 (*TP53*), are potential targets of *miR-125b*. As these are antiapoptotic proteins, we tested whether androgens protect GCs from apoptosis. In fact, DHT significantly reduces apoptosis in primary mouse GCs, as determined by flow cytometry for Annexin V expression (Fig. 3*A*). Moreover, down-regulation of *miR-125b* in primary GCs using an *miR-125b-2* inhibitor (Fig. S2*A*) increases expression of proapoptotic proteins (Fig. 3*B*) as well as the number of apoptotic cells (Fig. 3*C*). Notably, with reduction of *miR-125b* expression, DHT no longer protects cells from apoptosis (Fig. 3*C*), confirming that *miR-125b* at least in part mediates DHT-induced suppression of apoptosis.

To determine the in vivo effects of *miR-125b* on ovarian apoptosis, we knocked down *miR-125b* expression in vivo (Fig. 3*D*) by injecting locked nucleic acid (LNA)-containing oligonucleotides targeting *miR-125b-2* into one ovarian bursa whereas the other corresponding ovary received vehicle. Ovaries were collected 72 h postsurgery and analyzed for apoptosis. Levels of proapoptotic proteins BAK, BAX, BMF, and p53 in GCs (Fig. 3*E*), as well as follicular atresia (measured by TUNEL assay; Fig. 3*F*), are significantly elevated in *miR-125b*–knockdown ovaries compared with corresponding vehicle-treated ovaries. Morphological analysis ($n = 3$) reveals that 54.7% of follicles are atretic in LNA-oligonucleotide–injected vs. 33% in control ovaries ($P \leq 0.05$).

Finally, in ovaries of GC-specific ARKO mice, our reported high rate of follicular atresia (13) corresponds with lower expression of *miR-125b* (Fig. 4*A*) as well as higher expression of proapoptotic proteins and caspase 3 activity compared with control littermates (Fig. 4 *B* and *C*). Thus, in vitro and in vivo

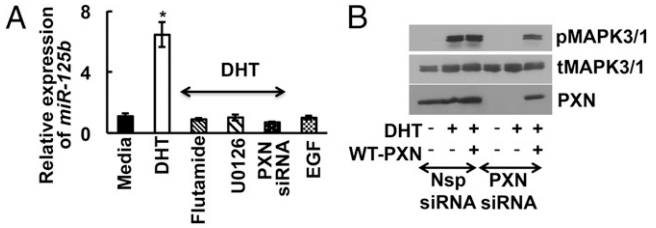


**Fig. 2.** Extra- and intranuclear androgen signaling regulate *miR-125b* expression in GCs. (*A*) Relative expression of *miR-125b* in primary mouse GCs treated with vehicle (0.1% DMSO), flutamide (AR inhibitor; 100 nM), or U0126 (MEK inhibitor; 10 μM), and in siRNA-mediated paxillin (PXN) knockdown GCs stimulated with/without DHT (25 nM) for 30 min. EGF is a negative control. Data are displayed as means ± SEM ($n = 3$) and normalized to GAPDH levels (*$P \leq 0.05$ vs. media). (*B*) Representative immunoblots of total MAPK3/1, phosphorylated MAPK3/1, and paxillin in nonspecific (Nsp) or paxillin-specific siRNAs treated mouse primary GCs. For rescue experiments, following siRNA treatment GCs were transfected with WT paxillin. Results are representative of three separate experiments.

results indicate that, in GCs, paxillin-mediated nuclear and extranuclear androgen signaling induces *miR-125b*, which then suppresses proapoptotic protein expression, leading to decreased follicular atresia and increased follicular survival.

**Androgens Increase FSHR Protein Levels in a Transcription-Independent (Nongenomic) Fashion.** Our GC-specific ARKO mouse model revealed that, whereas androgens suppress follicular atresia, they might also promote follicle growth and development. Previous work in animals (4–8, 20, 21) and humans (22, 23) suggested that androgens might augment FSH function in the ovary. To test whether androgens promote follicle growth by enhancing FSH actions, we examined androgen-induced FSHR mRNA and protein levels in primary mouse GCs. Neither DHT (Fig. 5*A*) nor testosterone (Fig S2*D*) alters *FSHR* mRNA levels, but both significantly increase FSHR protein expression (Fig. 5*B* and Fig. S2*D*), suggesting that androgens promote FSHR protein expression independent of transcription. Pulse-chase studies with cells exposed to the translation inhibitor cycloheximide (CHX) with or without DHT reveal no difference in FSHR protein degradation (Fig. 5*C*; quantified in Fig. S3*A*), indicating that DHT promotes FSHR protein expression by enhancing translation through extranuclear AR signaling. Accordingly, inhibition of the nongenomic AR-induced signaling by using flutamide, the MEK inhibitor U0126, or siRNA-mediated paxillin knockdown abrogates DHT-induced FSHR expression, whereas reexpression of paxillin in paxillin-knockdown GCs partially rescues DHT-induced FSHR expression (Fig. 5*D*; quantified in Fig. S3*B*).

**Androgen Treatment Enhances Follicular Sensitivity Toward FSH Actions.** As androgens increase FSHR expression in primary mouse GCs, we postulated that this might enhance FSH-mediated follicle growth. Preantral follicles were collected and treated with FSH (10 ng/mL) plus increasing amounts of DHT. Although FSH alone promotes follicular growth, addition of DHT in a dose-dependent manner significantly increases follicular growth (Fig. 6*A*), suggesting that androgens indeed enhance FSH-mediated follicular growth.

To determine whether androgens similarly enhance FSH-mediated transition from preantral to antral follicles in a whole ovary, intact mouse ovaries were cultured (Fig. 6*B*) with different concentrations of FSH in the presence or absence of DHT (25 nM). After culture and treatment, ovaries were fixed, sectioned, and stained for morphological analysis. DHT alone has no effect on antral follicle formation relative to untreated ovaries, whereas FSH alone increases follicle progression to the antral stage in a dose-dependent manner (Fig. 6*B*). Importantly, addition of DHT significantly enhances FSH-induced antral follicle formation (Fig. 6*B*). Thus, although androgens are neither sufficient to promote follicle progression nor necessary for FSH-induced follicle progression, they increase the sensitivity of preantral follicles toward FSH, thus optimizing transition from preantral to antral follicles.

**Preovulatory Androgen Priming Enhances Oocyte Ovulation.** As androgens enhance gonadotropin-induced follicle growth and development, we determined whether androgens augment gonadotropin-mediated ovulation. We injected mice for 3 d with DHT (0, 0.25, or 25 mg) before superovulation with pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin. To avoid overwhelming DHT effects with excess stimulation, 2 IU of PMSG and 2 IU of hCG were administered. Ovulated oocytes were then isolated from the oviducts of the animals and counted. DHT pretreatment has no effect on the number of ovulated oocytes in prepubertal (4 wk old) animals (Table 1), consistent with our previous data showing that GC-specific ARKO ovaries of 4-wk-old animals are normal. In contrast, pretreatment with 0.25 mg of DHT significantly increases the number of ovulated oocytes in 8- to 12-wk-old (13.6 ± 0.8 vs. 19 ± 0.6) and 24- to 28-wk-old (12.2 ± 0.5 vs. 16.6 ± 1.2) animals (Table 1), consistent with our model that androgens enhance gonadotropin-mediated follicle progression. Intriguingly, animals pretreated with higher amounts of DHT (25 mg) trend toward fewer superovulated oocytes compared with controls (Table 1).

## Discussion

Although global (11, 12) and GC-specific (13) ARKO mouse models, as well as in vitro studies (4–6, 8, 9, 20, 21, 24, 25), established the importance of androgens and ARs in follicular development, the present study provides insight into the molecular mechanisms of AR actions in the ovary. Previous work (26, 27) established that the balance between follicular atresia and follicle growth is essential for normal ovarian function, and we find that androgens play a key role in this regulation. We show that the physiological effects of androgens in GCs

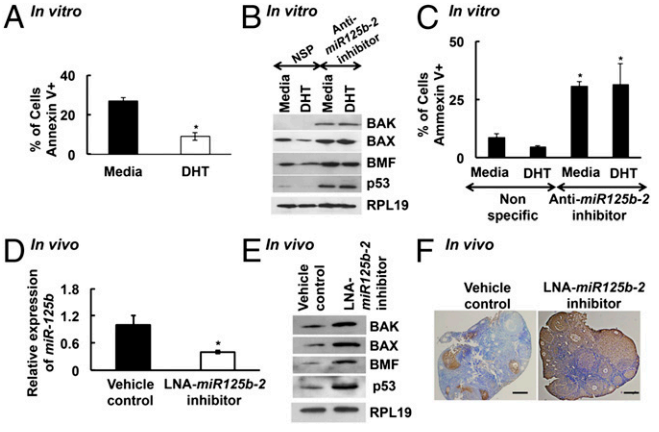


**Fig. 3.** (*A*) DHT protects GCs from apoptosis. Percentage of apoptotic cells measured by flow cytometry for Annexin V in primary mouse GCs treated with media or 25 nM DHT for 24 h. Data are means ± SEM ($n = 5$; *$P \leq 0.05$ vs. media). (*B* and *C*) *miR-125b* suppresses proapoptotic proteins in GCs in vitro. Proapoptotic protein levels (*B*) and percentage of apoptotic cells (*C*) in primary mouse GCs transfected with mouse *miR-125b-2* mirVana miR inhibitor or nonspecific control and stimulated with media or 25 nM DHT for 24 h. For *C*, data are means ± SEM ($n = 3$; *$P \leq 0.05$ vs. nonspecific). (*D–F*) In vivo knockdown of *miR-125b-2* increases follicular atresia. Relative expression of *miR-125b* (*D*) and proapoptotic protein levels and RPL19 control (*E*) in GCs and representative TUNEL-stained ovarian sections (*F*) from animals ($n = 3$) injected with LNA-containing oligonucleotides targeting miR-125b-2 into one ovarian bursa and vehicle control injected in the other corresponding ovary. For *D*, data are means ± SEM ($n = 3$) and normalized to GAPDH (*$P \leq 0.05$ vs. control).

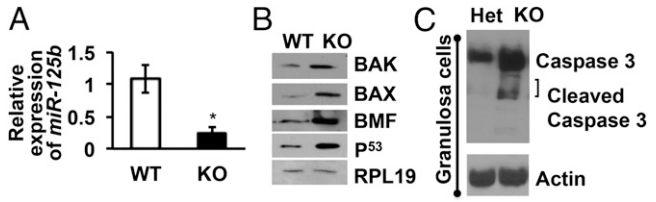


**Fig. 4.** Expression of *miR-125b* and proapoptotic proteins are significantly low and high, respectively, in primary GCs isolated from GC-specific ARKO mice. (*A*) Relative expression of *miR-125b* as measured by quantitative real-time PCR. Data are displayed as means ± SEM ($n = 3$ mice per genotype) and normalized to GAPDH levels (*$P \leq 0.05$ vs. WT). (*B*) Representative Western blots (from $n = 3$ mice per genotype) for proapoptotic proteins and RPL19 in GCs isolated from WT and GC-specific ARKO (KO) ovaries. (*C*) Representative Western blots (from $n = 3$ mice per genotype) detecting for caspase 3 activity and actin control in GCs isolated from ovaries of GC-specific ARKO (KO) mice and heterozygous (Het) littermates.

involve paxillin-mediated synergistic action between nuclear and extranuclear signaling of ARs that induces expression of the antiapoptotic *miR-125b* and up-regulates expression of the proproliferative FSHR.

First, we use primary GCs, GC-specific AR-null mice and in vivo ovary knockdown experiments to show that androgens promote *miR-125b* expression, which then suppresses expression of proapoptotic protein markers and subsequent apoptosis and follicle atresia. Notably studies report (18, 28) that *miR-125b* is similarly up-regulated by androgens in prostate cancer, and may play a crucial role in cancer cell proliferation and metastasis (29). With respect to miRs in female reproductive tissue, most work has focused on miR profiling rather than mechanism, and *miR-125b* is reported to be one of the most abundant miRs in the ovary among different species (30, 31). In fact, little is known about the expression, regulation, and function of miRs in the ovary (32). Exceptions include *miR-17-5p* and *let-7b*, which regulate corpus luteum development and function (33); *miR-21*, an LH-induced miR (34) that may play an antiapoptotic role during ovulation (35); *miR-224*, a TGF-β1–induced miR that targets Smad4 (36); and *miR-378*, which regulates estradiol synthesis by targeting aromatase (37). Our study not only demonstrates *miR-125b* as an important regulator of follicular atresia, but also provides detailed insight as to the hormonal regulation of *miR-125b* by androgens in the ovary. Importantly, few transcriptional targets of androgens have been identified in the ovary (10–12, 38–40); our results add to this short list by establishing *miR-125b* as a direct target of AR in GCs.

Second, we perform an in-depth analysis of the androgen signaling pathways that regulate *miR-125b* expression in GCs, demonstrating the importance of extranuclear androgen signaling in ovarian follicles. We show that, in GCs, androgens use a conserved nongenomic pathway (16, 17) to rapidly activate kinase signaling via transactivation of the EGF receptor. Furthermore, we show that extranuclear AR signaling is required for AR-mediated transcriptional effects on *miR-125b*, confirming and adding to earlier data (16) demonstrating that extranuclear androgen signaling is essential for AR nuclear localization and subsequent genomic androgen signaling (16).

Third, we show that paxillin is a critical regulator of extranuclear, and therefore nuclear, AR signaling in primary mouse GCs. These results mirrors previous work in prostate cancer cells (17) showing that paxillin is required for AR-induced Erk activation, after which Erk-dependent serine phosphorylation of paxillin leads to nuclear localization of PS-paxillin. Once in the nucleus, PS-paxillin then regulates AR nuclear localization and AR-mediated transcription (16). This demonstration of a conserved function of paxillin in vastly different cell types validates the importance of paxillin as a general liaison between extranuclear and nuclear AR signaling.

Fourth, we examine the molecular mechanisms behind observations (6, 41, 42) that androgens can up-regulate FSHR expression. We use mouse primary GCs, whole ovary culture, and primary follicle culture experiments to show that androgens promote FSHR protein translation in an exclusively nongenomic fashion, by using the same paxillin-dependent extranuclear pathway described earlier (Fig. 6*C*). By increasing FSHR expression, we show that androgens then enhance sensitivity to FSH-induced follicle growth and progression to the antral stage. Detailed mechanisms explaining how AR and paxillin selectively regulate FSHR protein are still not known; however, previous studies (43, 44) suggest that paxillin might up-regulate protein expression by complexing with polyadenylate-binding protein 1 (PABP1) to assist in translation. Notably, some studies (24, 45) report that androgens may induce *FSHR* mRNA expression. However, these studies were performed in prepubertal animals (45) or in human luteinized GCs isolated during oocyte retrieval for in vitro fertilization (24). Furthermore, concentrations of androgens used to stimulate these cells were quite high. Thus, androgenic effects on follicle development may be concentration- and age-dependent, consistent with reports of age-dependent differential effects of hormones and growth factors (46, 47). For our studies, we did not use prepubertal animals because prepubertal GC-specific ARKO animals still had normal follicular development (13). Furthermore, to avoid unwanted effects caused by pretreatment with pharmacologic gonadotropin concentrations, we used freshly isolated primary GCs from unprimed mice. Although androgen-induced increases in FSHR levels may be one mechanism by which androgens facilitate follicular growth, it is likely that androgens also modulate post-FSHR signaling, such as FSH-induced induction of cAMP (25, 48, 49).

In summary, our studies show that androgens regulate two critical stages of follicular development: follicular atresia and preantral follicle growth. In GCs, androgens through extranuclear AR signaling and nuclear AR actions synergistically induce the

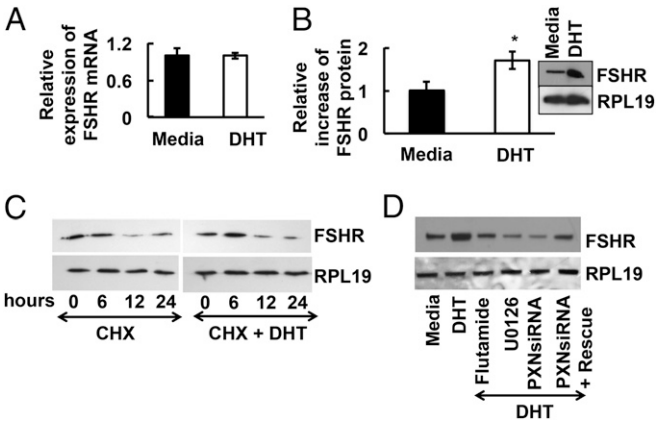


**Fig. 5.** Androgens increase FSHR protein, but not mRNA, levels in a transcription-independent fashion. (*A*) Relative expression of *FSHR* mRNA levels by quantitative PCR in primary mouse GCs stimulated with or without DHT for 24 h. Results are normalized to *GAPDH*. (*B*) Quantitative densitometric analysis of immunoblots from three separate experiments (means ± SEM; *$P \leq 0.05$) showing relative increase in FSHR protein levels in mouse primary GCs upon DHT stimulation. Representative Western blot for FSHR and RPL19 expression (*Inset*). (*C*) Androgens do not suppress FSHR protein degradation. Time course of FSHR protein degradation in presence of the translational inhibitor cycloheximide (CHX) (1 μM) in primary mouse GCs treated with or without DHT. (*D*) Extranuclear androgen signaling regulates DHT-induced increase in FSHR protein levels. Representative Western blots of FSHR and RPL19 protein levels ($n = 3$ with similar results) in primary mouse GCs treated with flutamide (100 nM), U0126 (10 μM) or in siRNA-mediated paxillin (PXN) knockdown primary mouse GCs following DHT for 30 min. For rescue experiments, paxillin-knockdown GCs were transfected with WT paxillin (PXN). Quantitative analysis for *C* and *D* is shown in Fig. S3.

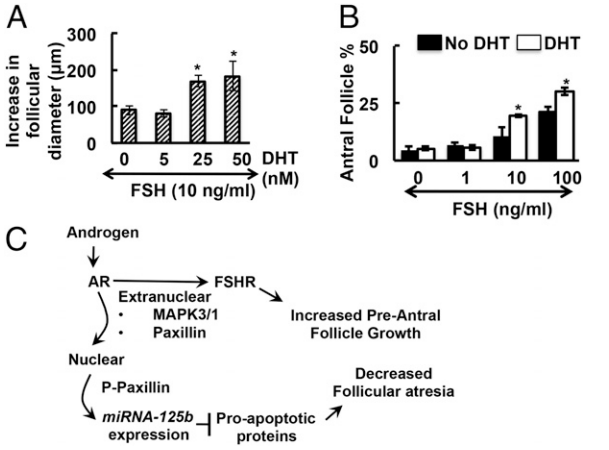


**Fig. 6.** (*A*) Androgens enhance FSH-mediated in vitro follicle growth in a dose-dependent manner. Increase in diameter of follicles cultured 4 d with 10 ng/mL FSH and with or without different concentrations of DHT. The difference in follicular diameter from day 0 and day 4 of culture is presented as a measure of follicle growth (*$P \leq 0.05$ vs. no DHT, n = 15 follicles per treatment). (*B*) Androgens enhance FSH-mediated preantral to antral follicle transition in a whole ovary culture. Percentage of antral follicles in ovaries cultured with different concentrations of FSH in presence or absence of DHT (25 nM) for 5 d. Data are presented as percent of antral follicles relative to total follicles counted (*$P \leq 0.05$ vs. no DHT, $n = 3$ ovaries per treatment). (*C*) Proposed model for androgen actions in follicular development. Based on work in prostate cancer cells (16, 17), androgens signal via classical ARs and extranuclear paxillin to promote MAPK3/1 signaling. MAPK3/1 then promotes increased FSHR protein expression that enhances the sensitivity of preantral follicles to FSH-mediated growth. In addition, extranuclear AR-mediated activation of MAPK3/1 leads to phosphorylation of paxillin on serines (P, paxillin), allowing paxillin to enter the nucleus and mediate AR-induced transcription of *miR-125b*. This miR suppresses apoptosis, thus attenuating preantral follicle atresia. The resultant androgen-mediated increased follicle growth plus decreased follicular atresia promotes follicle development and subsequent ovulation.

expression of *miR-125b*. The latter targets proapoptotic proteins, thus suppressing follicular atresia (Fig. 6*C*). In addition, androgens increase FSHR protein levels in a transcription-independent (nongenomic) fashion that increases the sensitivity of the follicles toward FSH, promoting preantral follicle growth and progression to antral follicles. Importantly, both these AR actions are regulated by paxillin (Fig. 6*C*). As we show that preovulatory androgens can promote follicle development and increase ovulation rates, whereas androgen excess is well established as one of the major causes of PCOS, we propose that there exists a critical balance between the essentiality of androgens in normal follicular development and the detrimental effects of androgens in the setting of androgen excess. In fact, the concept that androgens can promote follicle development is supported by the reported positive effects of androgen treatment in animals (7) as well as in patients with diminished ovarian reserve (22, 23, 50), in whom androgen priming is now being used in clinical settings to enhance ovulation (51, 52).

**Table 1. Preovulatory androgen treatment enhances ovulation**

| | DHT (mg) | | |
|---|---|---|---|
| Time point | 0 | 0.25 | 25 |
| Prepubertal | 14 ± 0.6 | 13.5 ± 0.6 | 14.1 ± 0.8 |
| 8–12 wk | 13.6 ± 0.8 | 19.0 ± 0.6* | 11.2 ± 1.0 |
| 24–28 wk | 12.2 ± 0.5 | 16.6 ± 1.2* | 11.0 ± 1.0 |

Mice were injected i.p. for 3 d with DHT as indicated before superovulation (i.p. injection of 2 U PMSG followed by 2 U hCG 48 h later). At 18 h after the hCG injection, ovulated oocytes were counted. *$P \leq 0.05$ vs. no DHT ($n = 10$ mice per condition).

## Materials and Methods

**Animals and Cell Culture.** Mouse studies were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the University Committee on Animal Resources at the University of Rochester. Unless otherwise mentioned, mouse experiments were performed in 8- to 12-wk-old C57BL/6J mice (Jackson Laboratories). Collection and culture of mouse GCs were performed as described previously (13, 16). KGN cells (RIKEN BioResource Center) were cultured in DMEM:F-12 medium containing 10% (vol/vol) FBS and 1% penicillin and streptomycin. Cells were serum starved for 4 h, followed by 25 nM of DHT stimulation for 18 h unless mentioned otherwise. GCs were also stimulated with 100 nM testosterone (Steraloids), 50 nM estradiol (Sigma), or 10 nM R5020 (Steraloids) for 18 h. In studies examining MAPK3/1 activation, GCs were stimulated with 25 nM DHT for 30 min. The concentration of DHT was based on dose–response experiments (Fig. S1 *A–C*). For experiments involving GC-specific ARKO animals, RNA and protein samples were obtained from GCs of GC-specific ARKO animals generated previously by crossing AR-flox and MisRII-Cre mice (13).

***miR-125b* Isolation and Detection.** Total RNA was isolated by using mirVANA miRNA Isolation Kit (Ambion/Life Technologies). Quantitative RT-PCR was performed using TaqMan MicroRNA reverse transcription kit and mouse/human *miR-125b* TaqMan MicroRNA assays (Applied Biosystems). *GAPDH* was used as an endogenous control and relative expression of *miR-125b* was calculated by using the Δ/Δ Ct method.

**ChIP Assay.** GCs were stimulated with DHT for 45 min, and ChIP was performed as described (16). Quantitative PCR was performed using EXPRESS SYBR GreenER qPCR SuperMixes (Invitrogen) with primers designed for ARE I, ARE II, ARE III, and control regions (Table S1).

**Paxillin Knockdown and Rescue Experiments.** siRNA-mediated paxillin knockdown and rescue experiments were performed as described previously (16) using nontargeting siRNA pool or mouse paxillin siRNA ON-TARGET plus SMARTpool according to the manufacturer's instructions (Themo Fisher Scientific).

***miR-125b* Inhibition.** In vitro, primary mouse GCs were transfected with mouse *miR-125b-2* mirVana miR inhibitor (mmu-*miR-125b-2*–3p) or with nonspecific control (Ambion). After 48 h, GCs were serum-starved for 4 h and then stimulated with DHT for 18 h. Specificity of knockdown was determined by measuring *miR-125b* and *miR-125a* expression levels (Fig. S2 *A* and *B*). In vivo, ovarian bursal injections were performed as described previously (33, 35). miRCURY LNA *miR-125b-2* inhibitor (Exiqon) or vehicle control were injected into ovarian bursa. The LNA *miR-125b-2* inhibitor (0.5 nM) was mixed with FuGENE 6 (Promega) before bursal injection. Saline solution plus FuGENE 6 was used as vehicle control. Each animal ($n = 6$) served as its own control, with one ovary receiving the blocking oligonucleotide and the other vehicle control. Animals were euthanized 72 h after injection and ovaries processed for TUNEL assay as well as GC isolation to detect *miR-125b* and proapoptotic protein expression.

**Western Blot Analysis.** Western blots were performed as described previously (13, 16, 53). Primary antibodies used were as follows: anti-rabbit BAX, anti-rabbit BMF (G81), anti-rabbit BAK (D2D3), anti-rabbit p53 and anti-rabbit Caspase-3 (Cell Signaling Technology), and anti-rabbit FSH receptor anti-RPL19 (Abcam).

**TUNEL Assay.** TUNEL assay was performed by using an ApopTag Plus Peroxidase In Situ Apoptosis detection kit (EMD Millipore) as described previously (13).

**In Vitro Follicle Culture.** In vitro follicle culture was performed as described previously (13).

**In Vitro Organ Culture.** Mouse whole ovaries ($n = 3$ ovaries per treatment) were cultured as described previously (54) with 0, 1, 10, and 100 ng/mL of recombinant FSH (National Hormone and Peptide Program, Harbor–University of California, Los Angeles, Medical Center) in the presence or absence of DHT. Ovaries were cultured for 5 d at 37 °C under 5% $CO_2$ in air with media replaced every 48 h. Ovaries were then processed for sectioning (5-μm sections taken at 30-μm intervals) and stained with H&E for morphological analysis by using previously published criteria (13).

**Oocyte Collection and Counting.** Hormonal stimulation for superovulation as well as oocyte collection and counting were performed as described previously (13).

**Flow Cytometry.** Flow cytometry was performed in the University of Rochester Medical Center flow cytometry core facility by using Alexa Fluor 488 Annexin V/dead cell apoptosis kit (Invitrogen).

**Statistical Analysis.** Data are displayed as means ± SEM of replicate samples. Statistical analysis was performed using Prism version 6 (GraphPad). ANOVA was used to detect differences between treatments. $P \leq 0.05$ was considered significant.

**ACKNOWLEDGMENTS.** This study was supported by a Foundation for Reproductive Medicine Grant (to A.S. and S.R.H.).

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