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ORIGINAL ARTICLE ***Reproductive biology***

Direct actions of androgens on the survival, growth and secretion of steroids and anti-Müllerian hormone by individual macaque follicles during three-dimensional culture

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STUDY QUESTION: What are the direct effects of androgens on primate follicular development and function at specific stages of folliculogenesis?

SUMMARY ANSWER: Androgen addition altered primate follicle survival, growth, steroid and anti-Müllerian hormone (AMH) production, and oocyte quality *in vitro*, in a dose- and stage-dependent manner.

WHAT IS KNOWN ALREADY: Androgens have local actions in the ovary, particularly in the developing follicles. It is hypothesized that androgen promotes early follicular growth, but becomes detrimental to the antral follicles in primates.

STUDY DESIGN, SIZE, DURATION: *In vitro* follicle maturation was performed using rhesus macaques. Secondary (125–225 μm) follicles were mechanically isolated from 14 pairs of ovaries, encapsulated into alginate (0.25% w/v), and cultured for 40 days.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Individual follicles were cultured in a 5% O_2 environment, in alpha minimum essential medium supplemented with recombinant human FSH. Follicles were randomly assigned to experiments of steroid ablation by trilostane (TRL), testosterone (T) replacement and dihydrotestosterone (DHT) replacement. Follicle survival and growth were assessed. Follicles with diameters ≥500 μm at Week 5 were categorized as fast-grow follicles. Pregnenolone (P5), progesterone (P4), estradiol (E2) and AMH concentrations in media were measured. Meiotic maturation and fertilization of oocytes from recombinant human chorionic gonadotrophin-treated follicles were assessed at the end of culture.

MAIN RESULTS AND THE ROLE OF CHANCE: Compared with controls, TRL exposure reduced ($P < 0.05$) follicle survival, antrum formation rate and follicle diameters at Week 5. While P5 concentrations increased ($P < 0.05$) following TRL treatment, P4 levels decreased ($P < 0.05$) in fast-grow follicles at Week 5. Few healthy oocytes were retrieved from antral follicles developed in the presence of TRL. T replacement with TRL increased ($P < 0.05$) follicle survival and antrum formation at Week 5, compared with TRL alone, to levels comparable to controls. However, high-dose T with TRL decreased ($P < 0.05$) diameters of fast-grow follicles. Although P4 concentrations produced by fast-grow follicles were not altered by T in the presence of TRL, there was a dose-dependent increase ($P < 0.05$) in E2 levels at Week 5. High-dose T with TRL decreased ($P < 0.05$) AMH production by fast-grow follicles at Week 3. More healthy oocytes were retrieved from antral follicles developed in TRL+T compared with TRL alone. DHT had the similar effects to those of high-dose T, except that DHT replacement decreased ($P < 0.05$) E2 concentrations produced by fast-grow follicles at Week 5 regardless of TRL treatment.

LIMITATION, REASONS FOR CAUTION: This study reports T and DHT actions on *in vitro*-developed individual primate (macaque) follicles, which are limited to the interval from the secondary to small antral stage.

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WIDER IMPLICATION OF THE FINDINGS: The above findings provide novel information on the role(s) of androgens in primate follicular development and oocyte maturation. We hypothesize that androgens promote pre-antral follicle development, but inhibit antral follicle growth and function in primates. While androgens can act positively, excess levels of androgens may have negative impacts on primate folliculogenesis.

STUDY FUNDING/COMPETING INTEREST(S): NIH U54 RR024347/RL1HD058294/PL1EB008542 (Oncofertility Consortium), NIH U54 HD071836 (SCCPIR), NIH ORWH/NICHD 2K12HD043488 (BIRCWH), NIH FIC TW/HD-00668, ONPRC 8P51OD011092. There are no conflicts of interest.

Key words: androgens / testosterone / dihydrotestosterone / follicle culture / follicle development

Introduction

In addition to androgen's role as a precursor for estradiol (E2) biosynthesis in females, considerable evidence suggests that androgen has local actions in the ovary, particularly in developing follicles (Drummond, 2006). Reports of androgen receptor (AR) expression in developing follicles are consistent with a paracrine/autocrine role for androgen in female reproductive physiology (Lebbe and Woodruff, 2013). ARs have been detected in growing follicles from all stages in various species, including mouse (Sen and Hammes, 2010), pig (Cárdenas and Pope, 1997), cow (Hampton *et al.*, 2004), nonhuman primates (Duffy *et al.*, 1999) and humans (Suzuki *et al.*, 1994); this suggests that androgen has effects throughout the follicular development. In women, AR protein expression was observed in granulosa cells, theca cells and stromal cells of ovarian follicles during almost all stages of the menstrual cycle. However, there appear to be differences in immunohistochemical staining intensity between different-sized follicles (Horie *et al.*, 1992), suggesting that AR expression levels fluctuate in cells as a function of the developmental stage of the follicle (Drummond, 2006).

Studies in mice discovered that gene mutations inactivating ARs result in premature ovarian failure, indicating that normal folliculogenesis requires AR-mediated androgen actions (Sen and Hammes, 2010). This concept is supported by the evidence that androgen promotes early follicular growth. Androgen treatment increased the diameter of mouse pre-antral follicles *in vitro* (Wang *et al.*, 2001). It is also reported that exogenous testosterone promoted early development of bovine (Yang and Fortune, 2006) and macaque (Vendola *et al.*, 1999) follicles. These data provide the basis for clinical efforts to determine if pretreatment with transdermal testosterone improves ovarian response to gonadotrophins in 'poor-responder' *in vitro* fertilization (IVF) patients (Wiser *et al.*, 2010).

Androgen becomes detrimental to antral follicles, exhibiting anti-maturation and anti-growth effects at this stage. It is proposed that androgen can be, depending on the follicular stages, a promoter or inhibitor of follicular growth (Hillier and Tetsuka, 1997). In addition, androgen actions may depend on its intrafollicular levels, with lower levels required for a normal folliculogenesis and increased levels causing follicular dysfunction. Notably, studies in women with polycystic ovary syndrome suggest that the associated hyperandrogenemia adversely affects follicular development and oocyte maturation (Lebbe and Woodruff, 2013).

Therefore, further studies are needed to define the direct effects of androgen on follicles and their enclosed oocytes at specific stages of folliculogenesis, especially in primates. We recently developed a three-dimensional culture system to support the growth of primate (macaque) follicles from the pre-antral (primary and secondary) to small antral (>1 mm in diameter) stage, with achievement of follicular function and oocyte maturation (Xu *et al.*, 2011, 2013a). The present study was designed to assess androgen actions on development and function (survival, growth, hormone and local factor production, and oocyte maturation) of individually cultured primate (macaque) follicles via ablation and replacement of testosterone (T) and dihydrotestosterone (DHT).

Materials and Methods

Animal use and ovary collection

The general care and housing of rhesus macaques (*Macaca mulatta*) was provided by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC), Oregon Health & Science University. Animals were pair-caged in a temperature-controlled (22°C), light-regulated (12L:12D) room. Diet consisted of Purina monkey chow (Ralston-Purina, Richmond, IN, USA) provided twice a day, supplemented with fresh fruit or vegetables once a day and water *ad libitum*. Animals were treated according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Protocols were approved by the ONPRC Institutional Animal Care and Use Committee (Xu *et al.*, 2011, 2013a).

Fourteen adult, female rhesus macaques exhibiting regular menstrual cycles, with the first day of menstruation considered Day 1 of the cycle, provided ovarian tissue. Ten of the 14 animals were assigned specifically to the study. Ovariectomies were conducted on anesthetized monkeys by laparoscopy at early follicular phase (Day 1–4 of the cycle) as previously described (Duffy and Stouffer, 2002). Ovaries were also collected from four additional animals at necropsy due to reasons unrelated to reproductive health. Ovaries were immediately transferred into HEPES-buffered holding media (Cooper Surgical, Inc., Trumbull, CT, USA) supplemented with 0.2% (v/v) human serum protein supplement (SPS, Cooper Surgical, Inc.) and 10 µg/ml gentamicin (Sigma-Aldrich, St Louis, MO, USA), and kept at 37°C for follicle isolation (Xu *et al.*, 2011, 2013a).

Follicle isolation, encapsulation and culture

The process of follicle isolation, encapsulation and culture was previously reported (Xu *et al.*, 2011, 2013a). Briefly, the ovarian cortex was cut into $1 \times 1 \times 1$ mm cubes. Follicles were mechanically isolated using 31-gauge needles. Secondary follicles (diameter 125–225 µm) met criteria for encapsulation if they exhibited an intact basement membrane, 2–3 layers of granulosa cells, and a healthy, centrally located oocyte. Follicles from individual monkeys were divided among the treatment groups. Each treatment group consisted of 12–36 follicles from every monkey.

Follicles were individually transferred into 5 µl 0.25% (w/v) sterile sodium alginate (FMC BioPolymers, Philadelphia, PA, USA)-PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, Invitrogen, Carlsbad, CA, USA). The droplets were cross-linked in 50 mM $CaCl_2$, 140 mM NaCl, 10 mM HEPES solution (pH 7.2) for 1 min. Each encapsulated follicle was placed in an individual well of 48-well plates containing 300 µl alpha

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minimum essential medium (Invitrogen) supplemented with 0.3% (v/v) SPS, 0.5 mg/ml bovine fetuin, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma-Aldrich) (Xu et al., 2011, 2013a). Recombinant human FSH (NV Organon, Oss, Netherlands) was added at 3 ng/ml during the first 20 days, and then decreased to 0.3 ng/ml for the remainder of the culture interval (Sánchez et al., 2010; Xu et al., 2013b).

Follicles were cultured at 37°C in a 5% O_2 environment (balance 6% CO_2/89% N_2) for 40 days. Follicles that reached the antral stage were treated with 100 ng/ml recombinant human chorionic gonadotrophin (hCG, Merck Serono, Geneva, Switzerland) for 34 h before removing the enclosed oocyte for evaluation of meiotic maturation. Media (150 μl) was collected and replaced every other day, and stored at −20°C for analysis of steroid hormone and anti-Müllerian hormone (AMH) concentrations (Xu et al., 2011, 2013a).

Experiment 1: steroid ablation

To evaluate the role of steroids in primate folliculogenesis, trilostane (TRL; Vetoryl, Dechra Veterinary Products, Overland Park, KS, USA), a steroid synthesis inhibitor, was used to block the action of 3 beta-hydroxysteroid dehydrogenase (3βHSD), an enzyme converting pregnenolone (P5) to progesterone (P4). TRL was added to the culture media at a dose of 250 ng/ml, which effectively blocks P4 production by primate follicular cells *in vitro* (Duffy et al., 1996). Secondary follicles from six animals were randomly assigned to three groups with ∼170 follicles/group: (i) CTRL group: control media plus TRL vehicle (100% ethanol) for 40 days; (ii) TRL group: TRL addition for 40 days; and (iii) TRL2 group: TRL addition from the end of Week 2 to Day 40 of culture.

Experiment 2: T replacement

To examine androgen effects during follicular development, T was added at a low (10 ng/ml) or high (50 ng/ml) dose in the culture media with TRL. Secondary follicles from four animals were randomly assigned to four groups with ∼85 follicles/group: (i) CTRL group; (ii) TRL group; (iii) TRL+TL group: TRL plus low-dose T addition for 40 days; and (iv) TRL+TH group: TRL plus high-dose T addition for 40 days.

Experiment 3: DHT replacement

DHT (50 ng/ml) was added to the culture media to confirm the androgen actions, independent of possible conversion to estrogen. Secondary follicles from four animals were randomly assigned to four groups with ∼70 follicles/group: (i) CTRL group; (ii) TRL group; (iii) DHT group: DHT addition for 40 days; and (iv) TRL+DHT group: TRL plus DHT addition for 40 days.

Follicle survival and growth

Follicle survival and growth were assessed weekly using an Olympus CK-40 inverted microscope and an Olympus DP11 digital camera (Olympus Imaging America, Inc., Center Valley, PA, USA) as described previously (Xu et al., 2011, 2013a). Follicle sizes were determined by measuring the distance from the outer layer of cells at the widest diameter and then the diameter perpendicular to the first measurement. The mean of the two values was considered the follicle's overall diameter. The measurements were performed using Image J 1.44 software (National Institutes of Health, Bethesda, MD, USA). Follicles were considered atretic if the oocyte was dark or not surrounded by a layer of granulosa cells, the granulosa cells appeared dark or fragmented, or the follicle diameter decreased.

Macaque follicles developed *in vitro* can be divided into three distinct cohorts based on their growth rates by Week 5 (Xu et al., 2010, 2011): (i) fast-grow follicles: follicles with diameters ≥500 μm; (ii) slow-grow follicles: follicles with diameters of 250–499 μm; and (iii) no-grow follicles: follicles with diameters <250 μm.

Ovarian steroid and AMH assays

One media sample collected weekly from each follicle culture was analyzed for pregnenolone (P5), progesterone (P4) and estradiol (E2) concentrations by the Endocrine Technology Support Core at ONPRC. P5 was measured by RIA as previously described (Duffy et al., 1994). P4 and E2 were assayed using an Immulite 2000, a chemiluminescence-based automatic clinical platform (Siemens Healthcare Diagnostics, Deerfield, IL, USA), validated for macaques (Xu et al., 2009).

Another media sample collected weekly was analyzed for AMH concentrations by ELISA using a DSL-10-14400 kit (Diagnostic Systems Laboratories, Inc.) based on the manufacturers' instructions (Fréour et al., 2007), and validated for macaques (Xu et al., 2010).

Oocyte retrieval, maturation and fertilization

Oocyte retrieval and evaluation were performed on a 37°C warming plate as previously described (Xu et al., 2011, 2013a). Briefly, cumulus-oocyte complexes were released in Tyrode's albumin lactate pyruvate (TALP)-HEPES-BSA (0.3% w/v) medium, and then treated with 2 mg/ml hyaluronidase (Sigma-Aldrich) in TALP-HEPES-BSA for 30 s to dissociate cumulus cells and obtain denuded oocytes. Retrieved oocytes were transferred to TALP medium and photographed. Oocyte diameters (excluding the zona pellucida) and meiotic status were assessed using the same camera and software as described above.

Metaphase II (MII) oocytes were maintained in TALP medium at 37°C in a 20% O_2/5% CO_2/75% N_2 environment for conventional IVF as previously described (Wolf et al., 1989) within 3 h of oocyte retrieval. Semen collection was performed by the Assisted Reproductive Technologies (ART) Support Core at ONPRC as previously reported (Lanzendorf et al., 1990). The resulting zygotes were transferred to 500 μl hamster embryo culture medium-9 with 5% v/v fetal bovine serum and cultured at 37°C in 5% O_2/6% CO_2/89% N_2 as previously described (Weston et al., 1996). Embryos were photographed daily to document development. Reagents and protocols for embryo culture were provided by the ART Support Core.

Statistical analysis

Statistical significance was determined with R2.15.3 (Revolution Analytics, Mountain View, CA, USA) software using binomial logistic regression to analyze survival and antrum formation data between groups, and marginal log-linear regressions to analyze steroid hormone and AMH data. Differences were considered significant at $P < 0.05$ and values were presented as mean ± SEM. Follicle survival and antrum formation represent six (Experiment 1) or four (Experiment 2 and 3) individual animals in each treatment group. Follicle growth, steroid and AMH production, and oocyte maturation were analyzed for each individual follicle.

Results

Experiment 1: steroid ablation

The percentages of follicle survival at Week 5 were low ($P < 0.05$) in the presence of TRL, either throughout the culture period (TRL) or beginning at Week 2 (TRL2), compared with the control group (Fig. 1A). The percentage of surviving follicles achieving antrum formation decreased ($P < 0.05$) in the TRL, but not TRL2, group relative to controls (Fig. 1B).

Similar percentages of no- and slow-grow follicles were noted in the control and TRL2 groups at Week 5 (Table I). In contrast, the percentage of no-grow follicles increased ($P < 0.05$) in the TRL group relative to controls. Fast-grow follicles were reduced ($P < 0.05$) in the TRL2 group

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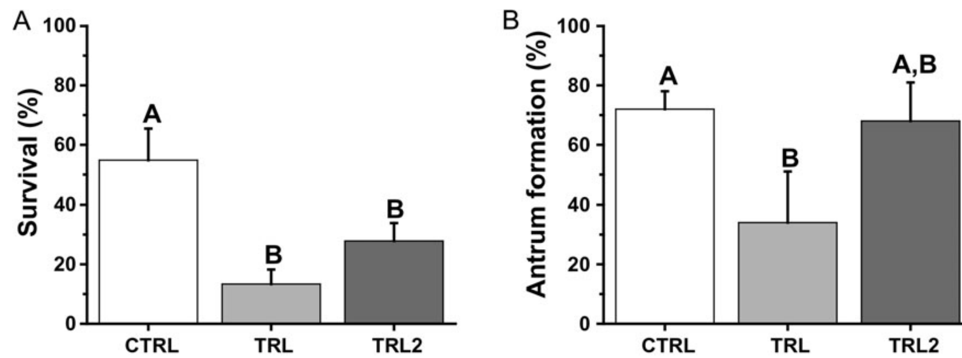

Figure 1 The effects of trilostane on macaque follicle survival (percentage of those cultured; **A**) and antrum formation (percentage of those survived; **B**) *in vitro* at Week 5. CTRL, control; TRL, trilostane exposure throughout 40 days of culture; TRL2, trilostane added from the end of Week 2 through Day 40 of culture. Significant differences among culture conditions are indicated by different letters ($P < 0.05$). Data are presented as the mean ± SEM with six animals per treatment group.

Table I Steroid ablation with trilostane: growth characteristics of surviving follicles at Week 5.

Follicle culture	Total	No-grow (%)	Slow-grow (%)	Fast-grow (%)
CTRL	98	11 (11%)[a]	60 (61%)[a]	27 (28%)[a]
TRL	26	7 (27%)[b]	19 (73%)[b]	0 (0%)[b]
TRL2	49	7 (14%)[a]	33 (67%)[a,b]	9 (19%)[c]

Data are presented as number and percent total follicles per growth category. CTRL, control (vehicle); TRL, trilostane (250 ng/ml) exposure through 40 days of culture; TRL2, trilostane (250 ng/ml) added from the end of Week 2 through Day 40 of culture. Different letters (a, b and c) indicate significant differences within the column ($P < 0.05$).

compared with controls and were absent in the TRL group. Moreover, though starting at equivalent sizes at the beginning of culture, slow-grow follicles were smaller ($P < 0.05$) in the TRL group compared with controls (235 ± 17 versus 330 ± 11 μm), but were comparable between the control and TRL2 group (283 ± 17 μm).

P4 concentrations in the culture media at Week 5 were appreciable in the control group for slow-grow follicles, and markedly reduced ($P < 0.05$) by TRL exposure, either throughout the culture period (TRL) or beginning at Week 2 (TRL2) (Fig. 2A). While fast-grow follicles were absent from the TRL group, a similar treatment effect was observed when comparing P4 levels for fast-grow follicles between the control and TRL2 groups (6.3 ± 1.0 versus 0.2 ± 0.1 ng/ml, $P < 0.05$). Notably, TRL exposure also reduced E2 levels compared with controls, especially in slow-grow follicles (data not shown; see Experiment 2 and 3). Conversely, P5 concentrations were low in slow-grow follicles of the control group at Week 5 (Fig. 2B), but markedly increased ($P < 0.05$) in the TRL and TRL2 groups. Likewise, P5 levels in fast-grow follicles were 29-fold higher ($P < 0.05$) in the TRL2 group relative to controls (1.17 ± 0.42 versus 0.04 ± 0.04 ng/ml).

In the control group, antral follicles typically provided healthy oocytes (12 of 13 follicles; Table II), though they rarely reinitiated meiotic maturation in response to hCG to achieve the metaphase II (MII) stage (1 of 12). The diameter of the MII oocyte was greater than that of any germinal vesicle (GV)-stage oocytes. Due to few antral follicles, only one oocyte was retrieved from the TRL group which was degenerating. Four of seven oocytes retrieved from the TRL2 group were healthy, but none achieved the MII stage.

Experiment 2: T replacement

The high-dose T with TRL increased ($P < 0.05$) the follicle survival at Week 5 compared with the TRL group, with the percent survival in TRL+TL intermediate between TRL alone and TRL+TH (Fig. 3A). Although, unlike in Experiment 1, some follicles in the TRL group achieved antrum formation, the percentage of antral follicles was lower ($P < 0.05$) with TRL alone (65 ± 22%) than that of the TRL+TH group (83 ± 8%). Moreover, the percentage of antral follicles in the TRL+TL group (92 ± 8%) was greater ($P < 0.05$) than that of controls (70 ± 14%).

As in Experiment 1, the percentage of no-grow follicles was greatest in the TRL group at Week 5 (Table III, T replacement), though some fast-grow follicles were evident. Remarkably, no-grow follicles were absent from both the TRL+TL and TRL+TH groups, and the percentages of fast-grow follicles were comparable to the controls. For slow-grow follicles, their diameters in the TRL+TL and TRL+TH groups were greater ($P < 0.05$) than those of TRL alone (350 ± 15 and 361 ± 14 versus 260 ± 12 μm), but comparable to controls (318 ± 14 μm). In contrast, the high-dose T with TRL decreased ($P < 0.05$) diameters of fast-grow follicles compared with other groups (572 ± 42 versus 773 ± 56 μm in controls).

Similar to Experiment 1, TRL alone decreased ($P < 0.05$) P4 concentrations produced by slow- (data not shown) and fast-grow follicles at Week 5 (Fig. 4A). Unexpectedly, P4 levels were comparable between the control and TRL+TL groups, especially for fast-grow follicles. In contrast, P4 concentrations in the TRL+TH groups were reminiscent of TRL alone, which were not restored to control levels. When measuring E2 as a marker of follicular endocrine function, TRL alone reduced ($P < 0.05$) E2 concentrations compared with controls in slow-grow follicles at Week 5 (63 ± 13 versus 247 ± 47 pg/ml). Exposure to low- or high-dose T markedly increased ($P < 0.05$) E2 levels in the presence of TRL (2734 ± 338 or 18 723 ± 1758 pg/ml). Results from fast-grow follicles displayed a similar trend except TRL alone did not decrease the 14-fold higher E2 levels relative to slow-grow follicles (Fig. 4B). Nevertheless,

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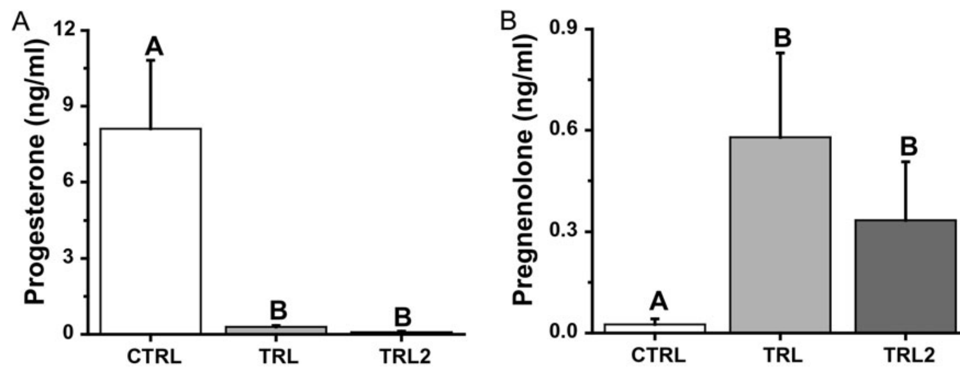

Figure 2 The effects of trilostane on progesterone (**A**) and pregnenolone (**B**) levels produced by macaque slow-grow follicles *in vitro* at Week 5. CTRL, control; TRL, trilostane exposure throughout 40 days of culture; TRL2, trilostane added from the end of Week 2 through Day 40 of culture. Significant differences among culture conditions are indicated by different letters ($P < 0.05$). Data are presented as the mean ± SEM with six animals per treatment group.

Table II Steroid ablation with trilostane: characteristics of oocytes retrieved from antral follicles at Week 5 (34 h after addition of recombinant human chorionic gonadotrophin).

Follicle culture	Number (*n*) of					Diameter (μm)*	
	Follicles harvested	Oocytes retrieved	Degenerate oocytes	Healthy oocytes		GV oocytes	MII oocytes
				GV	MII		
CTRL	13	13	1	11	1	111 ± 2	117
TRL	2	1	1	0	0	–	–
TRL2	10	7	3	4	0	92 ± 18	–

CTRL, control (vehicle); TRL, trilostane (250 ng/ml) exposure through 40 days of culture; TRL2, trilostane (250 ng/ml) added from the end of Week 2 through Day 40 of culture. GV germinal vesicle MII metaphase II.
*Values are the mean ± SEM with each oocyte diameter as an individual data point.

there was a dose-dependent increase ($P < 0.05$) in E2 levels compared with TRL alone, following the T addition.

When measuring AMH as a marker of follicular paracrine function, no differences were noted in AMH concentrations among groups for slow-grow follicles (data not shown). However, while TRL alone did not alter AMH levels for fast-grow follicles, the addition of high-dose T reduced ($P < 0.05$) AMH levels compared with controls at Week 3 (peak AMH level; Fig. 5A).

As in Experiment 1, few antral follicles and hence oocytes were obtained after hCG exposure following TRL treatment (Table IV; T replacement). However, T exposure during TRL treatment restored the numbers of antral follicles that consistently provided healthy oocytes, albeit at the GV stage. Both the control and T-replacement groups generated follicles that yielded MI and/or MII oocytes after hCG exposure. Notably, MII oocyte diameters were >130 μm. Following IVF, fertilization was confirmed by the presence of two polar bodies and two pronuclei (data not shown). However, zygotes arrested without cell division.

Experiment 3: DHT replacement

Addition of DHT with TRL returned the percent survival of follicles to control levels at Week 5 (Fig. 3B), which was similar to that of T exposure (Fig. 3A). Notably, DHT alone increased follicle survival to over 80%, which was higher ($P < 0.05$) relative to the control group. Moreover, the percentage of antral follicles in the DHT alone group was greater ($P < 0.05$) than that of controls (93 ± 3% versus 82 ± 4%).

Unlike in Experiments 1 and 2, TRL alone did not alter the number/percent of no-grow follicles, likely due to the greater numbers of no-grow follicles in the control group, and treatment with DHT in the presence of TRL did not reduce no-grow follicles (Table III; DHT replacement). However, the addition of DHT alone decreased ($P < 0.05$) the number/percent of no-grow follicles at Week 5 compared with the other groups. TRL again reduced ($P < 0.05$) the diameter of slow-grow follicles compared with controls (315 ± 13 versus 357 ± 16 μm), but this difference disappeared with combined TRL+DHT (373 ± 14 μm). Notably, DHT alone had no effect on diameters of slow-grow follicles (data not shown), but decreased ($P < 0.05$) the sizes of fast-grow follicles compared with controls (607 ± 29 versus 726 ± 50 μm).

As in Experiment 2, TRL alone decreased ($P < 0.05$) P4 concentrations produced by slow-grow follicles at Week 5, and the DHT replacement restored P4 levels to those of controls (data not shown). Likewise, neither DHT (Fig. 4C) nor low-dose T addition (Fig. 4A) altered P4 levels generated by fast-grow follicles when combined with

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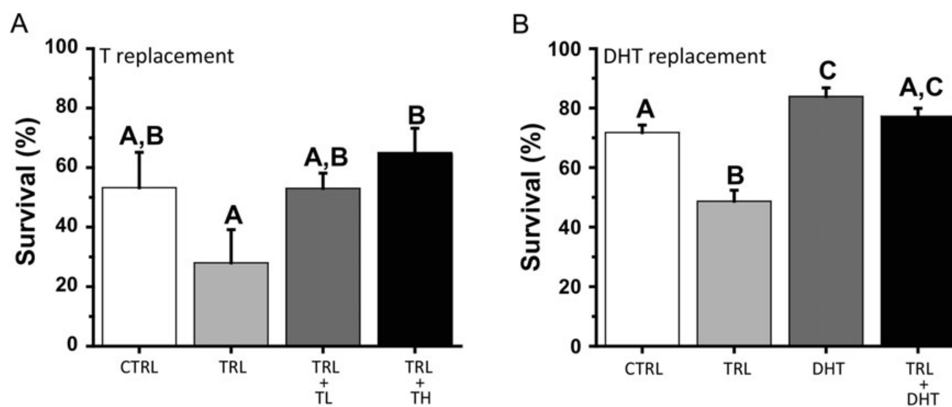

Figure 3 The effects of testosterone (**A**) and dihydrotestosterone (**B**) replacement on macaque follicle survival (percentage of those cultured) *in vitro* at Week 5. CTRL, control; TRL, trilostane exposure throughout 40 days of culture; TRL+TL, trilostane plus low-dose testosterone exposure for 40 days; TRL+TH, trilostane plus high-dose testosterone exposure for 40 days; DHT, dihydrotestosterone alone for 40 days; TRL+DHT, trilostane plus dihydrotestosterone exposure for 40 days. Significant differences among culture conditions are indicated by different letters ($P < 0.05$). Data are presented as the mean ± SEM with four animals per treatment group.

Table III Androgen replacement: growth characteristics of surviving follicles at Week 5.

Follicle culture	Total	No-grow	Slow-grow	Fast-grow
T replacement				
CTRL	47	3 (6%)^{a,b}	31 (66%)^{a,b}	13 (28%)^{a,b}
TRL	23	5 (21%)^{b}	13 (58%)^{b}	5 (21%)^{a}
TRL+TL	47	0 (0%)^{a}	29 (62%)^{a,b}	18 (38%)^{b}
TRL+TH	53	0 (0%)^{a}	37 (70%)^{a}	16 (30%)^{a,b}
DHT replacement				
CTRL	51	9 (18%)^{a}	22 (43%)^{a}	20 (39%)^{a}
TRL	30	5 (17%)^{a,b}	16 (53%)^{b}	9 (30%)^{b,c}
DHT	58	4 (7%)^{c}	35 (60%)^{c}	19 (33%)^{b}
TRL+DHT	51	11 (22%)^{d}	26 (51%)^{b,d}	14 (27%)^{c}

Data are presented as number and percent total follicles per growth category.
CTRL, control (vehicle); TRL, trilostane (250 ng/ml) exposure through 40 days of culture; TRL+TL, trilostane (250 ng/ml) plus low-dose testosterone (10 ng/ml) exposure for 40 days; TRL+TH, trilostane (250 ng/ml) plus high- dose testosterone (50 ng/ml) exposure for 40 days; DHT, dihydrotestosterone (50 ng/ml) alone for 40 days; TRL+DHT, trilostane (250 ng/ml) plus dihydrotestosterone (50 ng/ml) exposure for 40 days. Different letters (a, b, c and d) indicate significant differences within the column for T or DHT replacement experiment, respectively ($P < 0.05$).

TRL. However, the addition of DHT alone increased ($P < 0.05$) P4 levels produced by slow- (data not shown) and fast-grow (Fig. 4C) follicles. Unlike T addition (Fig. 4B), the combination of TRL+DHT reduced ($P < 0.05$) E2 concentrations produced by slow- (data not shown) and fast-grow (Fig. 4D) follicles compared with controls. Moreover, DHT alone replicated this effect.

Notably, DHT alone markedly reduced ($P < 0.05$) AMH levels produced by slow- (data not shown) and fast-grow (Fig. 5B) follicles at Week 3 compared with controls. AMH levels in the TRL+DHT group were also lower ($P < 0.05$) than those of the control and TRL alone groups.

As noted in Experiments 1 and 2, few healthy oocytes were derived from antral follicles in the TRL group compared with controls (Table IV; DHT replacement). In contrast, the TRL+DHT group yielded follicles with typically (10 of 13) healthy follicles, including 1 MII oocyte. Notably, the diameter of healthy GV oocytes from the TRL+DHT group was greater ($P < 0.05$) than those of controls. Exposure to DHT alone yielded oocytes with similar characteristics to those of the control and TRL+DHT groups, except no MII oocyte was observed. Figure 6 illustrates a secondary follicle (Fig. 6A) developing to the antral stage at Week 3 (Fig. 6B). Oocytes were harvested from antral follicles at Week 5 (Fig. 6C). After IVF, three MII oocytes (Fig. 6D; two from the control group and one from the TRL+DHT group), fertilization was confirmed by the presence of two polar bodies and two pronuclei (data not shown). One of the fertilized oocytes from the control group cleaved and the embryo developed to the morula stage at Day 5 post-IVF (Fig. 6E).

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Figure 4 The effects of testosterone (**A** and **B**) and dihydrotestosterone (**C** and **D**) replacement on progesterone (A and C) and estradiol (B and D) levels produced by macaque fast-grow follicles *in vitro* at Week 5. CTRL, control; TRL, trilostane exposure throughout 40 days of culture; TRL+TL, trilostane plus low-dose testosterone exposure for 40 days; TRL+TH, trilostane plus high-dose testosterone exposure for 40 days; DHT, dihydrotestosterone alone for 40 days; TRL+DHT, trilostane plus dihydrotestosterone exposure for 40 days. Significant differences among culture conditions are indicated by different letters ($P < 0.05$). Data are presented as the mean ± SEM with four animals per treatment group.

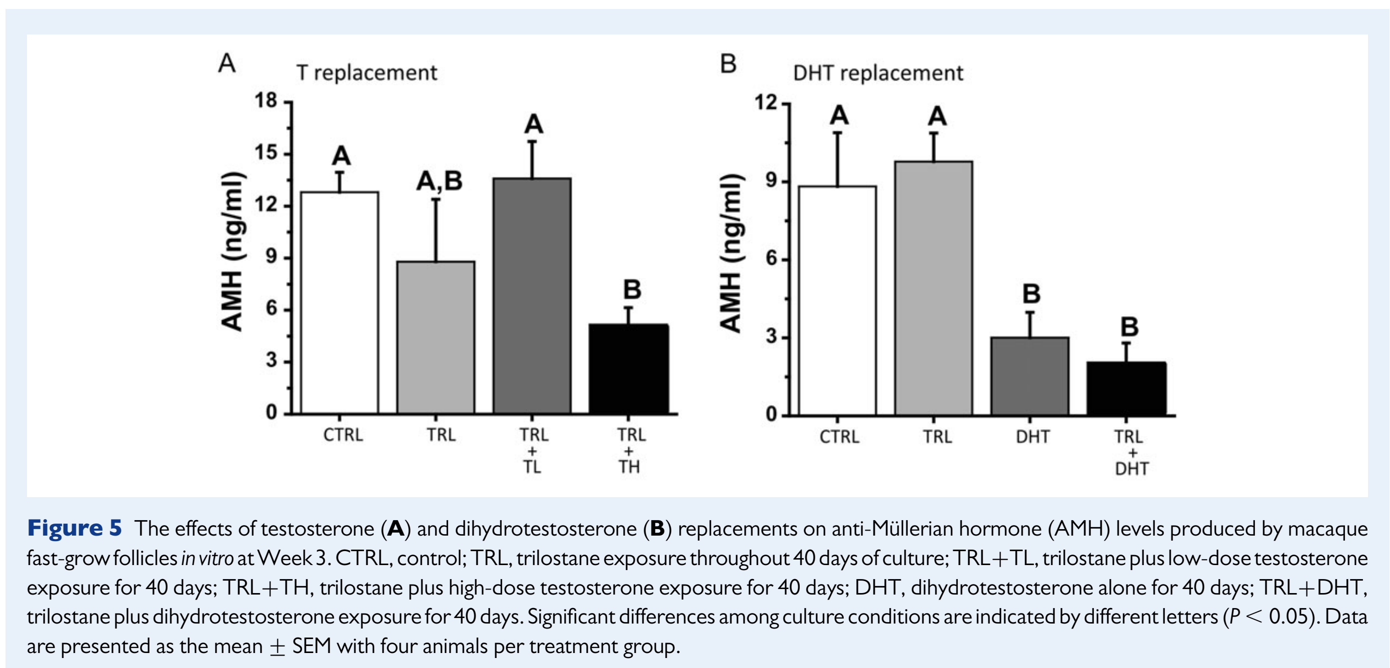

Figure 5 The effects of testosterone (**A**) and dihydrotestosterone (**B**) replacements on anti-Müllerian hormone (AMH) levels produced by macaque fast-grow follicles *in vitro* at Week 3. CTRL, control; TRL, trilostane exposure throughout 40 days of culture; TRL+TL, trilostane plus low-dose testosterone exposure for 40 days; TRL+TH, trilostane plus high-dose testosterone exposure for 40 days; DHT, dihydrotestosterone alone for 40 days; TRL+DHT, trilostane plus dihydrotestosterone exposure for 40 days. Significant differences among culture conditions are indicated by different letters ($P < 0.05$). Data are presented as the mean ± SEM with four animals per treatment group.

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Table IV Androgen replacement: characteristics of oocytes retrieved from antral follicles at Week 5 (34 h after addition of recombinant human chorionic gonadotrophin).

Follicle culture	Number (n) of						Diameter (μm)*		
	Follicles harvested	Oocytes retrieved	Degenerate oocytes	Healthy oocytes			GV oocytes	MI oocytes	MII oocytes
				GV	MI	MII			
T replacement									
CTRL	15	15	2	11	1	1	119 ± 5^{a}	143	131
TRL	4	4	2	2	0	0	117 ± 2^{a}	–	–
TRL+TL	16	16	1	14	1	0	119 ± 14^{a}	131	–
TRL+TH	13	13	0	12	0	1	102 ± 14^{a}	–	147
DHT replacement									
CTRL	21	21	6	13	0	2	110 ± 2^{a}	–	120, 127
TRL	8	8	6	2	0	0	112, 114	–	–
DHT	10	10	4	6	0	0	$116 \pm 4^{a,b}$	–	–
TRL+DHT	13	13	3	9	0	1	118 ± 2^{b}	–	117

CTRL, control (vehicle); TRL, trilostane (250 ng/ml) exposure through 40 days of culture; TRL+TL, trilostane (250 ng/ml) plus low-dose testosterone (10 ng/ml) exposure for 40 days; TRL+TH, trilostane (250 ng/ml) plus high-dose testosterone (50 ng/ml) exposure for 40 days; DHT, dihydrotestosterone (50 ng/ml) alone for 40 days; TRL+DHT, trilostane (250 ng/ml) plus dihydrotestosterone (50 ng/ml) exposure for 40 days. Different letters (a and b) indicate significant differences within the column for T or DHT replacement experiment, respectively ($P < 0.05$).
*Values are the mean ± SEM with each oocyte diameter as an individual data point.

Discussion

In vitro follicle maturation during three-dimensional culture permits one to monitor individual follicles and their response to *in vitro* manipulations (e.g. O_2 milieu) or *in vivo* conditions (e.g. reproductive age) (Xu *et al.*, 2010, 2011). We previously reported that pre-antral follicle survival and growth were critically dependent on the presence and dose of FSH (Xu *et al.*, 2010). The current investigation is our first attempt to modulate the paracrine environment of the primate follicle and evaluate the effects. Parameters of macaque follicle culture vary among animals depending on their ages (Xu *et al.*, 2010), menstrual cycle stages (Xu *et al.*, 2009) and sources. The variation may be augmented when necropsy animals with unknown cycle stages are used or sample sizes are relatively small due to the limited animal resources. Therefore, a control group was included in every experiment of the current study. Follicles obtained from every animal were assigned to both the control and treatment groups. Data were blocked for statistical analysis of differences between the control and treatment groups within the same experiment. The data strongly suggest that steroid (progestin, androgen and estrogen) depletion markedly suppresses the survival, growth and maturation of primate follicles *in vitro*. Moreover, the replacement of one steroid family, androgens either as T or its active nonaromatizable metabolite DHT, restored pre-antral follicle survival and growth. However, their actions on follicular function could be inhibitory, in a dose- or stage-dependent manner, to reduce estrogen and AMH production by small antral follicles.

A steroid-depleted milieu was created for individually cultured macaque follicles by administering TRL, a 3βHSD enzyme inhibitor, to block directly the biosynthesis of P4, 17-hydroxyprogesterone, androstenedione and T (Schane *et al.*, 1979). Subsequently, the formation of estrogens (e.g. E2) and DHT from androgen substrates will also be compromised. The reduced levels of P4 in culture media from slow and fast-grow follicles indicated that TRL was effective in blocking the conversion of P5 (whose levels were elevated) into P4 by 3βHSD. TRL's effect on the steroidogenic cascade was also reflected via the reduction of subsequent steroid production by slow-grow follicles, including androstenedione (data not shown) and E2. The results are consistent with *in vivo* studies in monkeys in which TRL blocked ovulation via inhibiting progestin production/action (Hibbert *et al.*, 1996). TRL effectively inhibited P4 production and action in macaque granulosa cells during culture (Duffy *et al.*, 1996).

TRL exposure from the beginning of the culture, to sustain a steroid-depleted milieu, markedly suppressed the survival and growth of macaque pre-antral follicles. TRL also negatively impacted oocyte quality and meiotic maturation in antral follicles developed *in vitro*. The data are consistent with the concept that ovarian steroids play a critical, local role in follicle growth and oocyte maturation (Drummond, 2006). These effects of steroid depletion could be due to a loss of local actions of progestins, androgens, or estrogens, acting alone or in concert. In addition to AR, as summarized in the Introduction, there is evidence for the presence of P4 receptor (Peluso, 2006) and estrogen receptor (Drummond and Fuller, 2010) in primate follicles. In this initial study, we focused on the possible role(s) of androgens by replacing T and its active metabolite DHT. The addition of T or DHT to the steroid-depleted milieu markedly improved the survival and antrum formation of primate pre-antral follicles *in vitro*. Follicle growth was also recovered to the pattern observed in controls. Moreover, the treatment of T or DHT in presence of TRL promoted oocyte viability and maturity. Perhaps androgen action in the follicle promotes oocyte-somatic cell interactions via oocyte-secreted factors (Drummond, 2006).

E2 levels produced by cultured follicles markedly increased upon exposure to low- or high-dose T, but not DHT, in presence of TRL.

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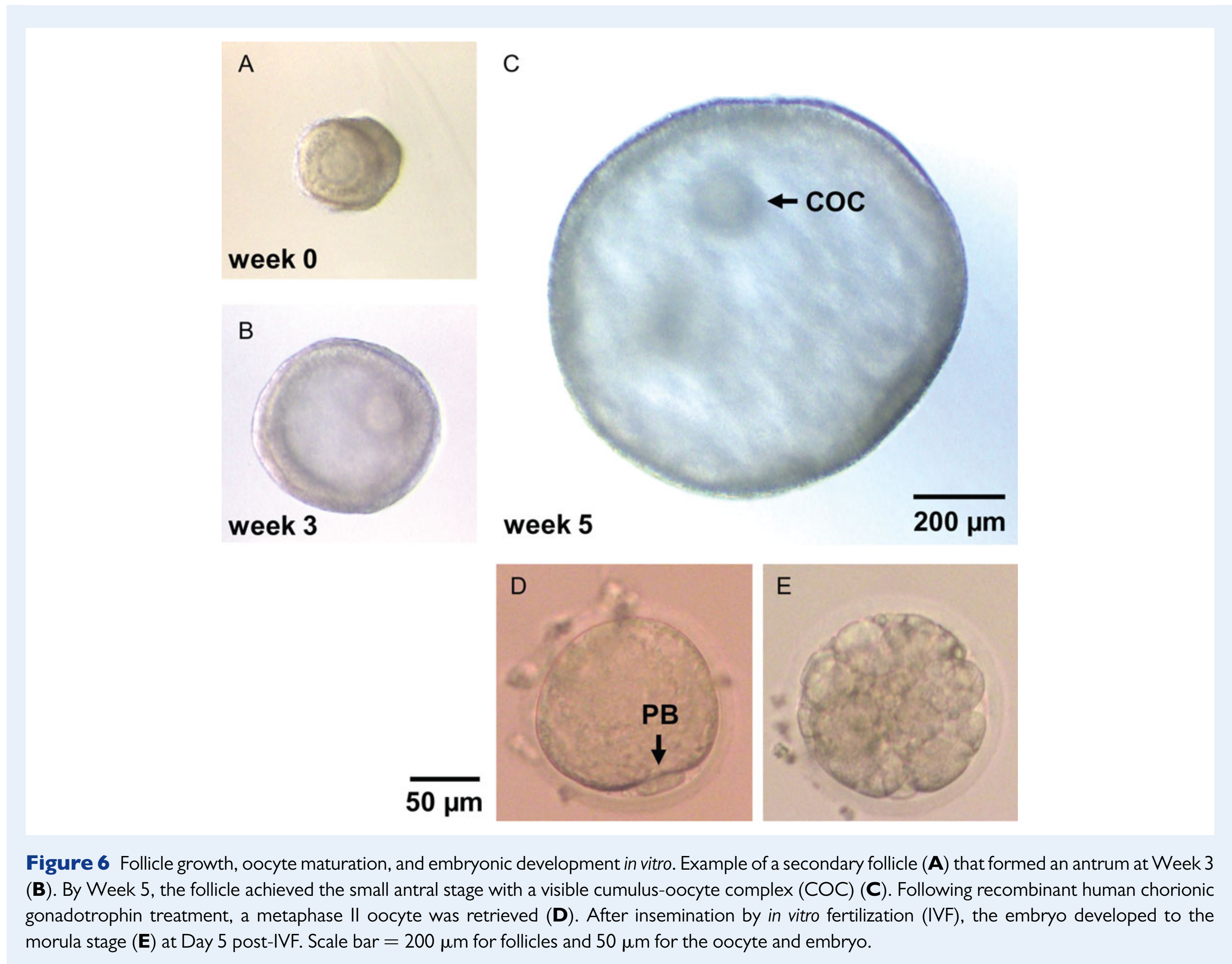

Figure 6 Follicle growth, oocyte maturation, and embryonic development *in vitro*. Example of a secondary follicle (**A**) that formed an antrum at Week 3 (**B**). By Week 5, the follicle achieved the small antral stage with a visible cumulus-oocyte complex (COC) (**C**). Following recombinant human chorionic gonadotrophin treatment, a metaphase II oocyte was retrieved (**D**). After insemination by *in vitro* fertilization (IVF), the embryo developed to the morula stage (**E**) at Day 5 post-IVF. Scale bar = 200 μm for follicles and 50 μm for the oocyte and embryo.

Therefore, the elevated E2 levels during T treatment are presumably due to aromatization of T by granulosa cells in the antral follicles (Simpson, 2002). The recovery of follicle survival, growth and antrum formation by T replacement could be caused in part from the conversion of T to E2 and estrogen receptor signaling (Rosenfeld *et al.*, 2001). However, the direct androgenic effects on *in vitro*-developed follicles and their enclosed oocytes were further clarified in the current study by DHT, a nonaromatizable androgen, replacement. The results strongly support direct androgen actions to promote early follicular development and oocyte maturation in primate follicles.

However, some androgens effects on cultured primate follicles were inhibitory. Notably, androgen addition (high-dose T and DHT) reduced the size obtained by antral follicles, especially fast-grow follicles. Also, unlike T which was aromatized to E2, DHT reduced E2 production by *in vitro*-developed antral follicles regardless of TRL addition. This androgen action may specifically alter aromatization as P4 and androstenedione (data not shown) levels were not changed. The elevated P4 in the DHT alone group may reflect accumulated substrates due to insufficient E2 production. The modest increase in P4 levels upon addition of low-dose T in the presence of TRL is an enigma. The possible mechanisms for the active conversion of androgens to P4, or androgen receptor signaling that promotes 3βHSD independent production of P4 are unclear. Notably, exposure to high-dose T or DHT in a steroid-depleted milieu, or DHT alone, also significantly decreased AMH levels produced by antral follicles at Week 3. AMH generated by granulosa cells of growing follicles is considered a local regulator of follicular development (Visser and Themmen, 2005). We previously reported that macaque secondary follicles produce AMH *in vitro* as a function of their growth potential; levels rise during the first 3 weeks of culture to peak at antrum formation (Xu *et al.*, 2011, 2013a). In the current study, androgen decreased the peak levels of AMH, which further supports the negative impact of androgen on antral follicle growth.

These findings support the concept that androgen actions in the primate follicle may be dose- and stage-dependent. The high, but not low, androgen levels in developing antral follicles could serve as a brake on antral follicle growth and E2 production, which is consistent with the hyperandrogenemia associated limitation of antral follicle growth and E2 production in women with polycystic ovary syndrome (Agarwal *et al.*, 1996). The current evidence that androgens promote early follicular development, in terms of survival and numbers of growing follicles (with healthy oocytes), but inhibit select activities after reaching the antral stage (E2 and AMH production) agree with previous

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studies in which the stage-dependent effects of androgens could be either positive or negative (Harlow et al., 1988; Hickey et al., 2004). Notably, the positive effects of androgens on primate pre-antral follicles *in vitro* are consistent with the observations of Vendola et al. (1998) that acute T or DHT administration increased the number of growing pre-antral and small (≤1 mm in diameter) antral follicles, but not those >1 mm, in normal cycling rhesus monkeys. The current results are also similar to those of Lenie and Smitz (2009) who reported that treatment of mouse pre-antral follicles with antiandrogens during culture reduced follicle growth, altered steroidogenic function, and arrested oocyte meiotic maturation. The observation of dose- and stage-dependent androgen effects on primate follicular development may provide an explanation for controversies in androgen supplementation in reproductive medicine. The disparate outcomes of treatment in human studies (Wiser et al., 2010; Yeung et al., 2014) may due to discrepancies in timing and/or therapeutic range of androgen supplementation.

The cellular pathways and molecular processes, which are regulated by androgens and mediate their effects in primate follicles, await investigation. There are reports of androgen regulation of select gene products, including the FSH receptor (Weil et al., 1999), insulin-like growth factor I (IGF-1) and IGF-1 receptor (Vendola et al., 1999) in primate follicles, as well as other species (Hickey et al., 2004). Given the importance of FSH action in promoting macaque follicle survival, growth and function *in vitro* (Xu et al., 2011), it is possible that T and DHT modulate FSH receptor signaling in a stage-dependent manner. Local paracrine factors (e.g. AMH or IGFs) could also mediate the inhibitory or stimulatory effects of androgen on primate folliculogenesis. A comparison of the actions of androgens with other steroids, notably P4 and E2, that reportedly modulate follicular development is also needed. This study reinforces the value of the three-dimensional culture of primate follicles to evaluate the actions and mechanisms whereby hormonal and local factors control follicular development either by manipulating the *in vitro* milieu at specific stages of follicle/oocyte maturation, or by studying follicles from control or treated monkey (e.g. hyperandrogenemia; Xu et al., in review).

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Authors' roles

J.K.R. provided contributions to (i) experimental design, (ii) follicle collection, culture and oocyte retrieval, (iii) follicle growth recording and diameter measurement, (iv) data analysis and interpretation on follicle development, oocyte maturation, steroid and AMH production, (v) manuscript drafting and critical revising and (vi) final approval of the version to be submitted for publication. P.A.N. provided contributions to (i) data interpretation on follicle development, oocyte maturation, steroid and AMH production, (ii) critical manuscript revising for important intellectual content and (iii) final approval of the version to be submitted for publication. M.B.Z. provided contributions to (i) conception and design of the experiments, (ii) follicle collection, (iii) data interpretation on follicle development, oocyte maturation, steroid and AMH production, (iv) critical manuscript revising for important intellectual content and (v) final approval of the version to be submitted for publication. R.L.S. provided contributions to (i) conception and design of the experiments, (ii) data interpretation on follicle development, oocyte maturation, steroid and AMH production, (iii) critical manuscript revising for important intellectual content and (iv) final approval of the version to be submitted for publication. J.X. provided contributions to (i) experimental design, (ii) follicle collection, culture and oocyte retrieval, (iii) follicle growth recording and diameter measurement, (iv) data analysis and interpretation on follicle development, oocyte maturation, steroid and AMH production (v) manuscript drafting and critical revising and (vi) final approval of the version to be submitted for publication.

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Conflict of interest

None declared.

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