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Androgen and Follicle-Stimulating Hormone Interactions in Primate Ovarian Follicle Development

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ABSTRACT

We have previously shown that androgens stimulate early stages of follicular development and that granulosal androgen receptor (AR) gene expression is positively correlated with follicular growth. The present study was aimed at elucidating potential interactions between FSH and androgens in follicular development. Study groups included eight normal cycling rhesus monkeys (five follicular and three luteal-phase), eight testosterone (T)-treated, and four FSH-treated animals. Examination of sequential ovary sections revealed selective colocalization of AR and FSH receptor (FSHR) messenger RNAs (mRNAs) in healthy, growing follicles. Moreover, individual follicles demonstrate a highly significant ($P < 0.001$) positive correlation between FSHR and AR mRNA levels in all study groups. Androgen treatment significantly increased granulosa cell FSHR mRNA abundance (by approximately 50–100%, depending on follicle size). FSH treatment increased granulosa AR mRNA levels only in primary follicles. The finding that T augments follicular FSHR expression suggests that androgens promote follicular growth and estrogen biosynthesis indirectly, by amplifying FSH effect, and may partially explain the enhanced responsiveness to gonadotropin stimulation noted in women with polycystic ovary syndrome. (*J Clin Endocrinol Metab* **84:** 2951–2956, 1999)

WE HAVE recently shown that androgens stimulate early stages of follicular growth in the rhesus monkey ovary (1, 2). Primary, secondary, and tertiary (small antral) follicles are significantly increased in number, and granulosa and thecal cell proliferation are significantly increased in T- and dihydrotestosterone-treated animals (1, 2). Furthermore, granulosa cell androgen receptor (AR) gene expression is positively correlated with proliferation and negatively correlated with apoptosis in the monkey ovary (3). Evidence from *in vitro* models is conflicting, with some data suggesting antiproliferative or atretogenic effects (4), whereas other data indicate that androgens promote follicular growth (5, 6). Women with hyperandrogenism have impaired ovulatory function, but this may be caused by excessive numbers of small growing follicles disrupting normal hypothalamic-pituitary-ovary interaction, as opposed to atretogenic effects by androgen. Supporting this view, ovaries from women with polycystic ovary syndrome (PCOS) have increased numbers of small growing follicles (7). Furthermore, granulosa proliferation and steroidogenesis seem robust in PCOS follicles (8, 9), and androgen blockade results in reduction in follicle number and resumption of ovulatory cycles (10).

The mechanism(s) whereby androgens stimulate follicular growth remain unclear. Because infertile women with PCOS frequently hyperrespond to FSH treatment for ovulation induction (11, 12), and granulosa cells from PCOS ovaries are hyperresponsive to FSH treatment *in vitro* (13), we considered the possibility that androgens might promote granulosa FSH receptor (FSHR) expression. Therefore, in the present work, we have investigated the relation between follicular AR and FSHR expression, and we examined the effects of androgens on follicular FSHR messenger RNA (mRNA) levels as well as the effects of FSH on AR mRNA levels.

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Materials and Methods

Animals

Female Rhesus monkeys, 6–13 yr of age (from the NIH Poolesville, MD, colony) were studied under a protocol approved by the NICHD Animal Care and Use Committee. Monkeys were treated with sc pellets (Innovative Research of America, Toledo, OH) containing vehicle (n = 8) or sustained release T (4 mg/kg for 3 days, n = 4; or 0.4 mg/kg for 10 days, n = 4), as previously described (3). Another group (n = 4) received sc injections of recombinant FSH (Metrodin, Serono, Norwell, MA, 35 IU) for 2 days. Ovariectomies were performed under ketamine anesthesia via a ventral laparotomy. Ovaries were removed, snap frozen on dry ice, and stored at −70 C. Serial sections of 10-μm thickness were cut at −15 C, thaw-mounted onto poly-L-lysine-coated slides, and stored at −70 C until used for *in situ* hybridization. Serum for hormone measurements was obtained at the time of ovariectomy. Estradiol (E2), T, and FSH were measured by RIA at Covance Laboratories, Inc. Vienna, VA. In the group of eight random cycling control monkeys, five were in the follicular phase of the menstrual cycle, as determined by progesterone levels less than 3.0 ng/dL (E2 = 70 ± 11 pg/mL). Just these follicular-phase animals were used for quantitative analyses comparing AR, FSHR, and aromatase mRNA levels in size-matched follicles in the different treatment groups.

In situ hybridization

The human AR (3), aromatase, and FSHR cDNAs (14) used as templates for riboprobe synthesis were as previously described. ^{35}S-labeled RNA probes were synthesized to an SA of approximately 2×10^8 dpm/μg, as previously described (15). The sections were fixed; soaked for 10 min in 0.25% acetic anhydride, 0.1 mol/L triethanolamine hydrochloride, and 0.9% NaCl; washed; and dehydrated. ^{35}S-labeled probes (10^7 cpm/mL) were added to hybridization buffer composed of 50% formamide, 0.2 mol/L NaCl, 50 mmol/L Tris HCL (pH 8), 2.5 mmol/L EDTA, 250 μg transfer RNA/mL, 10% dextran sulfate, 10 mmol/L dithiothreitol, and 0.02% each of BSA, Ficoll, and

TABLE 1. Follicle classification

Follicle class	Name	Diameter (μ)	Description
A	Primary	50–100	≤2 Layers of cuboidal GC with no thecal layer
B	Preantral	101–380	3–6 Layers of cuboidal GC; definitive thecal layer emerges
C	Periantral	381–620	>6 Layers of cuboidal GC; definitive thecal layer; antral cavities begin to form
D	Small antral	621–1000	>6 Layers of cuboidal GC with columnar appearing GC at border of basement membrane; definitive thecal layer; all follicles have antral cavities
E	Large antral	>1000	Mature graffian follicles with well-developed granulosa, thecal, and antral elements

The largest diameter measured from basement membrane to basement membrane (not including the thecal layer) was used to categorize each follicle. GC, granulosa cells.

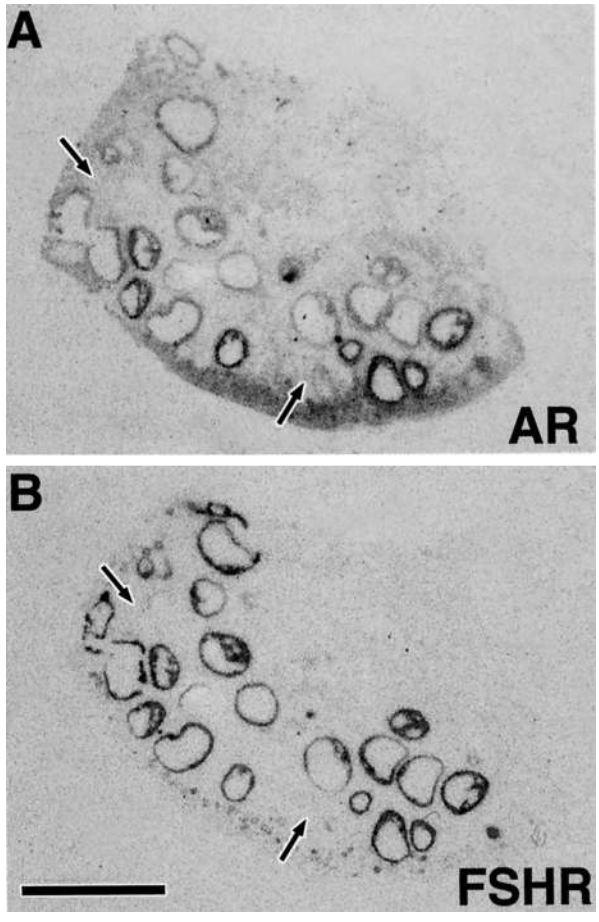

FIG. 1. AR (A) and FSHR (B) mRNAs are colocalized in the monkey ovary. These are representative film autoradiographs taken from sequential ovary sections. The *arrows* point to follicles that are negative for both mRNAs. There is a so-called edge artifact noted along the *lower border* of the AR autoradiograph. Bar = 2.5 mm.

polvinlpryrolidone. Control sections were hybridized with sense probes in the same experiments. Coverslips were placed over the sections, and the slides were incubated in humidified chambers overnight (14 h) at 55 C. Slides were washed several times in 4× SSC (NaCl and sodium citrate, Biofluids, Rockville, MD) to remove coverslips. They were then washed in hybridization buffer, dehydrated, and immersed in 0.3 mmol/L NaCl, 50% formamide, 20 mmol/L Tris HCL, 1 mmol/L EDTA at 60 C for 15 min. Sections were then treated with ribonuclease A (20 μg/mL) for 30 min at room temperature, followed by a 15- min wash in 0.1× SSC at 50 C. Slides were air dried and exposed to Hyperfilm-beta Max (Amersham Pharmacia Biotech, Arlington Heights, IL) for 7 days, dipped in Kodak NTB2 nuclear emulsion, stored with desiccant at 4 C for 14 days, developed, and stained with Mayer's hematoxylin and eosin for microscopic evaluation.

Quantitative analyses

FSHR, aromatase, and AR mRNA levels were quantified in granulosa cells of follicles classified into groups by diameter: A (≤100 μm), B (101–380 μm), C (381–620 μm), D (621–1000 μm), and E (>1 mm), as described in Table 1 and Ref. 1. Hybridization signal was quantified using darkfield illumination on a Laborlux microscope (Leitz, Rockleigh, NJ). Grains overlying an area of 500 μ^2 were captured at 400× magnification via a solid-state monochrome video camera, and the data was analyzed with a Macintosh PowerPC system using NIH Image v 1.57 (NIH, Bethesda, MD). Background or nonspecific signal was obtained by similar measurements on sections hybridized to a control, sense probe. The background counts were subtracted from experimental data before further analysis. Data on mRNA signal in follicles from both right and left ovaries were meaned for each animal. Group means were statistically compared using ANOVA followed by Fisher's least-significant-difference test. A P value < 0.05 was considered significant. Correlation between AR and FSH mRNA levels was analyzed using Spearman's rank correlation.

Results

AR mRNA is concentrated in a subpopulation of follicles in the primate ovary (Fig. 1A). We have previously shown that these AR-expressing follicles are healthy and growing, as determined by high proliferation and low apoptosis indices (3). To investigate potential interactions between FSH and androgen in follicle growth, we compared FSHR and AR mRNA localization in sequential ovary sections (Fig. 1). This comparison shows that AR and FSHR mRNAs are selectively coexpressed in the same subpopulation of follicles. Moreover, the abundance of FSHR mRNA is positively correlated with that of AR mRNA in follicles from random-cycling, androgen-treated, and FSH-treated monkeys ($P < 0.001$, Fig. 2).

Given these observations, we considered that androgens might regulate FSHR gene expression. To test this hypothesis, we compared granulosa FSHR mRNA levels in size-matched follicles from androgen-treated monkeys and follicular-phase control animals (Fig. 3). Monkeys were treated with T for 3 and 10 days. Circulating T levels were very elevated, and E2 levels were suppressed in T-treated monkeys (Table 2). FSHR mRNA levels were significantly increased in large antral follicles after just 3 days of T treatment (Fig. 4A). After 10 days, FSHR mRNA levels were increased, from approximately 50% to 100% in follicles of all sizes in the monkey ovary (Fig. 4A).

We also considered the possibility that FSH stimulates AR gene expression. Thus, we compared granulosa AR mRNA levels in control, follicular-phase monkeys, and FSH-treated

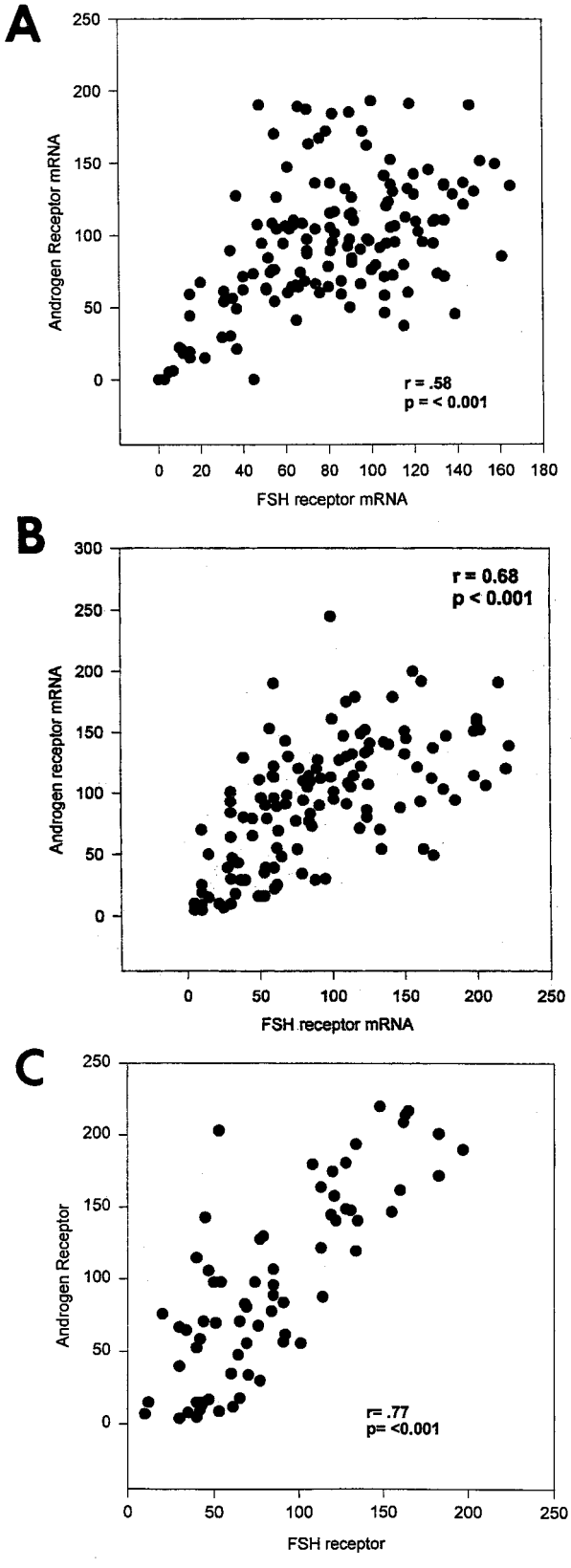

FIG. 2. Correlation between AR and FSHR mRNA levels in individual follicles. A, Data from untreated, random cycling monkeys (n = 8); B, data from T-treated (both 3- and 10-day) monkeys (n = 8); C, data from FSH-treated monkeys (n = 4).

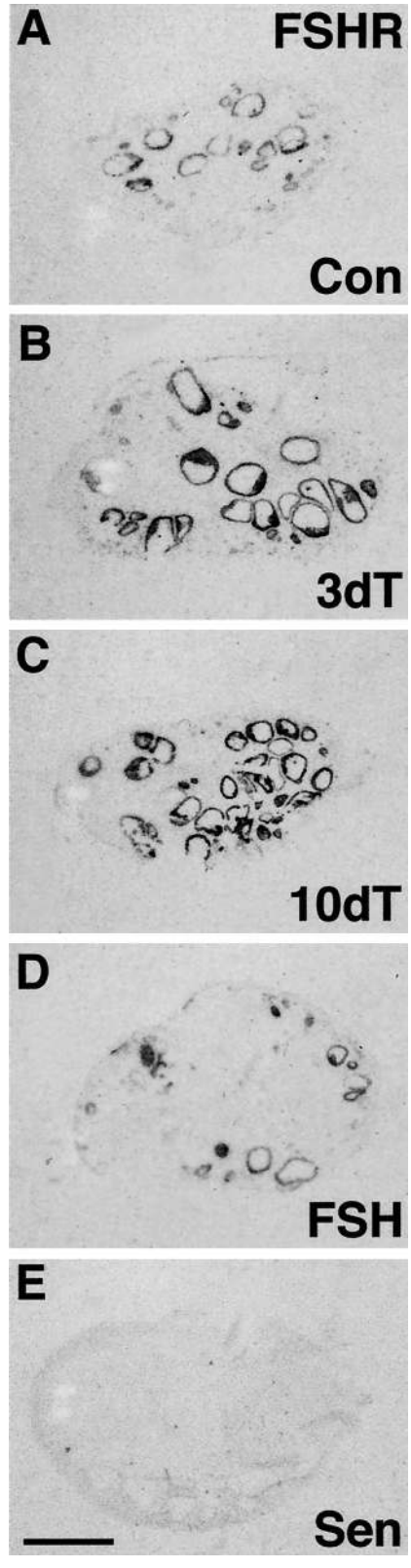

FIG. 3. Increased FSHR gene expression in follicles from T-treated monkeys. Representative film autoradiographs from follicular-phase control (Con) (A), 3-day T-treated (3dT) (B), 10-day T-treated (10dT) (C), and FSH-treated monkeys (D) are shown. E shows an autoradiograph from a section hybridized to a sense (Sen) probe. Note the increased number of follicles in the androgen-treated ovaries. Bar = 2 mm.

TABLE 2. Hormone levels

	Control (8)	T3-day (4)	T10-day (4)	FSH (4)
T (ng/dL)	38.2 ± 5.5	3170 ± 682	1345 ± 233	nd
E2 (pg/mL)	114 ± 30.5	25.3 ± 7.3	18.1 ± 3.1	32 ± 12
P4 (ng/mL)	2.3 ± 1.4	1.0 ± 0.50	1.2 ± 0.30	1.1 ± 0.56
FSH mIU/mL				21.3 ± 4.9

Endogenous monkey FSH is not detected with this RIA. The recombinant human FSH is detected in the treated animals. Data is expressed as means ± SEM. P4, progesterone; nd, not done.

animals. FSH treatment did not alter AR mRNA levels in larger follicles (Fig. 4B) but did result in a dramatic increase in AR mRNA in primary follicles (Figs. 4B and 5). FSH treatment had only modest effects on FSHR mRNA levels (Fig. 3D), which did not achieve statistical significance (data not shown).

Aromatase mRNA is selectively expressed in AR/FSHR-positive follicles (Fig. 6). Aromatase mRNA levels are not significantly altered in T-treated animals (data not shown) but are predictably increased in FSH-treated animals (Fig. 6D). LH receptor mRNA levels were also examined in these treatment groups, and no significant differences were obtained (not shown).

Discussion

This study presents evidence of positive, complementary interactions between FSH and androgen effects in primate follicle development *in vivo*. We have shown that androgen- and FSHR mRNAs are selectively colocalized in growing follicles in the normal cycling primate ovary. Moreover, AR mRNA levels are positively correlated with FSHR mRNA levels in granulosa cells from normal cycling-, androgen-treated, and FSH-treated animals. T increases FSHR mRNA levels in follicles at all stages of development, whereas FSH increases AR mRNA in primary follicles. Our previous work demonstrated that androgens increase follicle cell proliferation and suppress granulosa cell apoptosis (1) and that AR gene expression is positively correlated with granulosa proliferation and negatively correlated with apoptosis (3). Taken together, these observations strengthen the view that androgens (in addition to serving as precursors for ovarian estrogen synthesis) also have a fundamental trophic role in primate ovarian follicular development.

The observation that FSH treatment markedly increases AR gene expression in primary follicles is novel and interesting. The factors regulating follicular AR expression have been unknown. AR mRNA (3) and immunoreactivity (16, 17) range from low to undetectable in primordial and primary follicles of normal-cycling monkeys. Furthermore, androgen treatment stimulates a slight increase in granulosa cell AR mRNA level in larger follicles but is without effect on AR expression in primary follicles (3). Notably, androgen-treatment is associated with a marked decrease in thecal and interstitial AR mRNA levels (3). The present data, showing a robust, FSH-induced induction of AR gene expression in the smallest ovarian follicles, suggests a potential physiological mechanism whereby FSH may promote early follicular development.

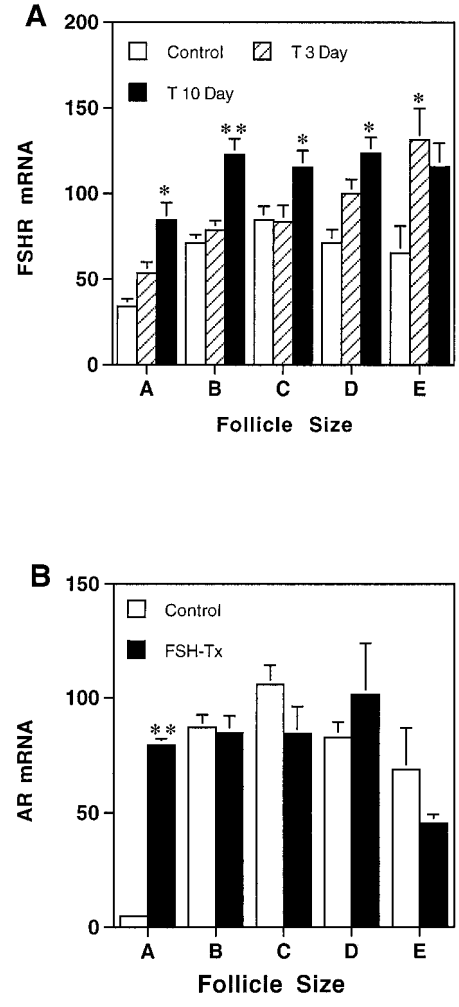

FIG. 4. A, Effect of T treatment on follicular FSHR mRNA levels. Data are means ± SEM for five animals in the control group and four in each of the T-treatment groups. B, Effect of FSH treatment (FSH-Tx) on follicular AR mRNA levels. Data are means ± SEM for five animals in the control group and four in the FSH-treated group. RNA levels were quantified by grain counting, as described in Materials and Methods. *, $P < 0.05$; **, $P < 0.01$, compared with control.

Androgen-induced increases in granulosal FSHR expression are expected to promote FSH action, leading to increased aromatase expression and conversion of androgen to estrogen. Indeed, androgens amplify FSH-induced aromatase expression in cultured rat (18) and primate (19) granulosa cells. The present data suggest that this *in vitro* effect may be caused by androgen augmentation of FSHR expression. Consistent with this indirect mode of action, we found that T is without effect on follicular aromatase gene expression in a situation where FSH is presumably suppressed because of high circulating T levels (see suppressed E2 levels, Table 2). The fact that the aromatase substrate T facilitates (albeit indirectly) aromatase production provides yet another regulatory element to the complex two-cell paradigm of ovarian estrogen biosynthesis.

The androgen-induced augmentation of granulosa FSHR gene expression shown in the present study could explain enhanced follicular growth as well as estrogen biosynthesis

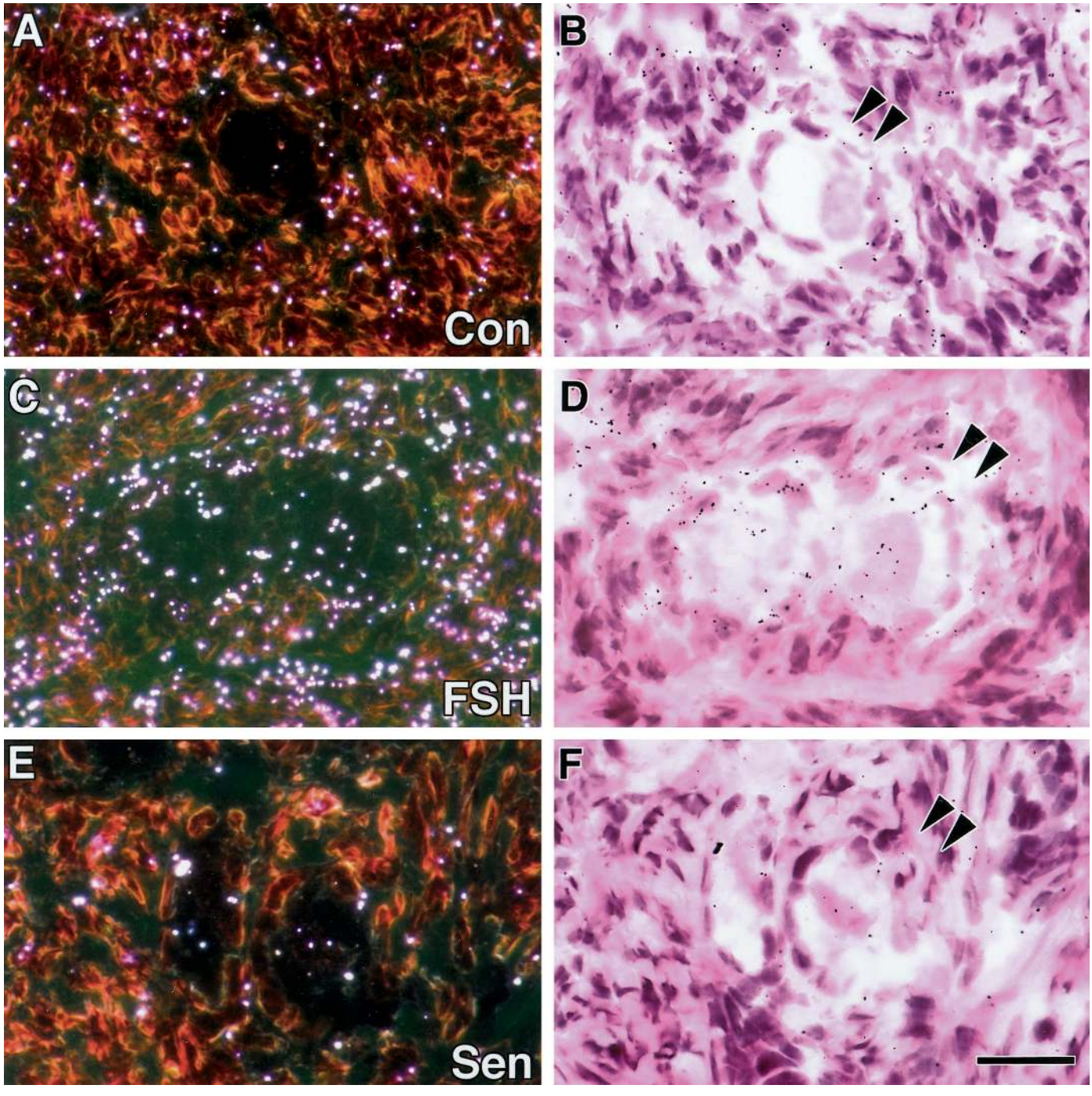

FIG. 5. AR mRNA in primary follicles of control (A and B) and FSH-treated (C and D) monkeys. Signal is concentrated primarily over granulosa cells in primary follicles, two of which are seen in C and D (*double arrowheads*). E and F show nonspecific signal in sense probe hybridized tissue. Bar = 50 μ.

in response to FSH. The mechanism whereby androgen increases granulosa FSHR gene expression is unclear. This could be an indirect effect, caused, for example, by increased local IGF1 production. Supporting this possibility, we have shown that IGF1 stimulates granulosa FSHR gene expression in the mouse (14). Moreover, IGF1 and IGF1 receptor expression are increased in granulosa and thecal cells in virtually all follicles in the androgen-treated monkeys (Ref. 2, Vendola *et al.*, manuscript in preparation).

Hyperandrogenism is the cardinal clinical feature of PCOS, and recent genetic evidence suggests that it is also a primary etiology of the disorder (20, 21). Mason *et al.* (13) have shown that granulosa cells from women with PCOS hyperrespond to FSH *in vitro*, and the present data suggest that this heightened responsiveness could be attributable to enhanced granulosa FSHR expression caused by hyperandrogenism in these women. Women with PCOS are also prone to hyperrespond to FSH stimulation for ovu-

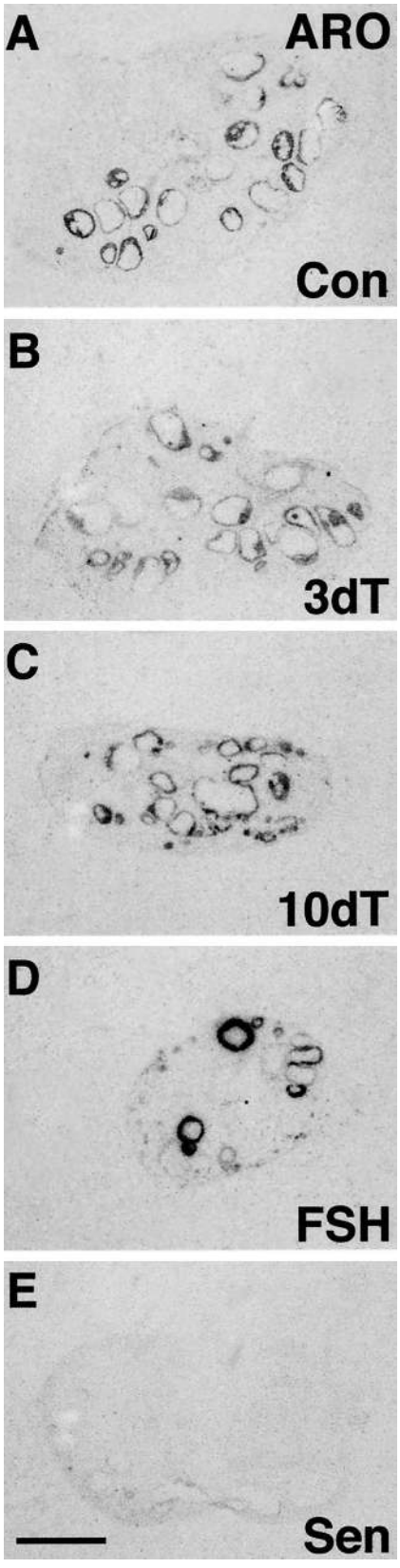

FIG. 6. Effects of androgen and FSH treatment on aromatase gene expression in the primate ovary. Representative film autoradiographs from follicular-phase control (A), 3-day T- (B), 10-day T- (C), and FSH-treated (D) animals.

lation induction *in vivo* (11, 12), and this could be caused by androgen-induced heightened follicular FSHR expression, as well as to increased numbers of FSH-responsive follicles (22). These observations support the view that PCOS ovulatory dysfunction is not attributable to any intrinsic defect in follicular development, but rather to disordered relations between too many or too-sensitive developing follicles and gonadotropin orchestration of ovulation (23).

References

1. **Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA.** 1998 Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest. 101:2622–2629.
2. **Vendola KA, Zhou J, Wang J, Famuiya OA, Bievre M, Bondy CA.** 1999 Androgens stimulate primordial follicle development in the primate ovary. Biol Reprod. 61:353–357.
3. **Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Bondy CA.** 1998 Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J Clin Endocrinol Metab. 83:2479–2485.
4. **Billig H, Furuta I, Hsueh JW.** 1993 Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology. 133:2204–2212.
5. **Murray AA, Gosden RG, Allison V, Spears N.** 1998 Effect of androgens on the development of mouse follicles growing *in vitro*. J Reprod Fertil. 113:27–23.
6. **Hillier SG, Tetsuka M.** 1997 Role of androgens in follicle maturation, and atresia. Baillieres Clin Obstet Gynaecol. 11:249–260.
7. **Hughesdon PE.** 1982 Morphology and morphogenesis of the Stein-Leventhal ovary and of so-called "hyperthecosis". Obstet Gynecol Surv. 37:59–77.
8. **Pache TD, Hop WC, de Jong FH, et al.** 1992. 17β-Oestradiol, androstenedione and inhibin levels in fluid from individual follicles of normal and polycystic ovaries, and in ovaries from androgen treated female to male transsexuals. Clin Endocrinol. 36:565–571.
9. **Takayama K, Fukaya T, Sasano H, et al.** 1996 Immunohistochemical study of steroidogenesis and cell proliferation in polycystic ovarian syndrome. Hum Reprod. 11:1387–1392.
10. **de Leo V, Lanzetta D, D'Antona D, la Maarca A, Morgante G.** 1998 Hormonal effects of flutamide in young women with polycystic ovary syndrome. J Clin Endocrinol Metab. 83:99–102.
11. **Farhi J, Jacobs HS.** 1997 Early prediction of ovarian multifollicular response during ovulation induction in patients with polycystic ovary syndrome. Fertil Steril. 67:459–462.
12. **Wada I, Matson PL, Macnamee MC, Brinsden PR, Lieberman BA.** 1994 High ovarian response in Yoruba African women during ovulation induction for assisted conception. Hum Reprod. 9:1077–1080.
13. **Mason HD, Willis DS, Beard RW, Winston RM, Margara R, Franks S.** 1994 Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. J Clin Endocrinol Metab. 79:1355–1360.
14. **Zhou J, Rajendra Kumar T, Matzuk MM, Bondy CA.** 1997 Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. Mol Endocrinol. 11:1924–1997.
15. **Bondy CA, Zhou J, Lee WH.** 1993 *In situ* hybridization histochemistry. In: de Pablo F, Scanes CG, Weintraub BD, eds. Handbook of endocrine research techniques. San Diego, CA: Academic Press, 266–288.
16. **Hillier SG, Tetsuka M, Fraser HM.** 1997 Location and developmental regulation of androgen receptor in primate ovary. Hum Reprod. 12:107–111.
17. **Hild-Petito S, West NB, Brenner RM, Stouffer RL.** 1991 Localization of androgen receptor in the follicle and corpus luteum of the primate ovary during the menstrual cycle. Biol Reprod. 44:561–568.
18. **Hillier SG, De Zwart FA.** 1981 Evidence that granulosa cell aromatase induction/activation by follicle-stimulating hormone is an androgen receptor-regulated process *in vitro*. Endocrinology. 109:1303–1305.
19. **Harlow CR, Hillier SG, Hodges JK.** 1986 Androgen modulation of follicle-stimulating hormone-induced granulosa cell steroidogenesis in the primate ovary. Endocrinology. 119:1403–1405.
20. **Legro RS, Driscoll D, Strauss III JF, Fox J, Dunaif A.** 1998 Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. Proc Natl Acad Sci USA. 95:14956–14960.
21. **Govind A, Obhrai MS, Clayton RN.** 1999 Polycystic ovaries are inherited as an autosomal dominant trait: analysis of 29 polycystic ovary syndrome and 10 control families. J Clin Endocrinol Metab. 84:38–43.
22. **Van Der Meer M, Hompes PGA, De Boer JAM, Schats R, Schoemaker J.** 1998 Cohort size rather than FSH threshold level determines ovarian sensitivity in polycystic ovary syndrome. J Clin Endocrinol Metab. 83:423–426.
23. **Chavez-Ross A, Franks S, Mason HD, Hardy K, Stark J.** 1997 Modelling the control of ovulation and polycystic ovary syndrome. J Math Biol. 36:95–118.