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Dynamics of Inhibin Subunit and Follistatin mRNA during Development of Normal and Polycystic Ovary Syndrome Follicles

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To further explore the developmental dynamics and possible roles of inhibin, activin, and follistatin in the development of human antral follicles as well as the relationship between mRNA and protein levels of these hormones within follicles, quantitative competitive RT-PCR assays were established to determine mRNA levels for the inhibin/activin subunits and both follistatin splice variants. Granulosa cell RNA was obtained by transvaginally aspirating follicles (6–23 mm) from carefully characterized normal women at different times of the follicular phase. α- and β_A-subunit mRNA levels increased significantly with follicle diameter ($r = 0.56$; $P < 0.01$ and $r = 0.45$; $P < 0.05$, respectively) and follicle maturity ($r = 0.65$; $P < 0.001$ and $r = 0.58$; $P < 0.01$, respectively), but β_B mRNA levels, which were at least 10-fold lower than levels of the other subunits, showed no relationship to size or maturity. Both follistatin 315 and 288 transcripts were detected in granulosa cells, but neither follistatin transcript varied significantly across the range of follicle sizes analyzed. In addition, granulosa cells contained three follistatin 315 mRNA transcripts for each follistatin 288 transcript, and the follistatin 315/288 ratio did not vary with follicle size. α-Subunit mRNA levels were positively associated with dimeric inhibin A protein in human follicular fluid from the same follicle aspirates ($r = 0.71$; $P < 0.001$). Similarly, β_A-subunit mRNA was associated with inhibin A ($r = 0.59$; $P < 0.01$), and β_B mRNA was associated with inhibin B ($r = 0.67$; $P < 0.005$) in these samples. Thus, the increase in inhibin subunit transcription and protein synthesis with follicle size suggests that inhibin biosynthesis might be important for continued development of the dominant follicle.

To explore this hypothesis further, we compared mRNA levels for each of these transcripts in follicles obtained from six polycystic ovary syndrome patients (eight follicles) and compared the results to those from a group ($n = 5$) of normal follicles matched for mean diameter. Comparisons were also performed for a subset of polycystic ovary syndrome follicles ($n = 5$) matched for diameter and size range with the normal group. α-Subunit mRNA levels were 16-fold lower in both polycystic ovary syndrome follicle groups relative to size-matched normal follicles ($P < 0.02$), whereas β_A-subunit mRNA was significantly lower only when all polycystic ovary syndrome follicles were compared. β_B-Subunit and follistatin mRNA levels and the follistatin 315/288 ratio were not statistically different for any group. These results suggest that insufficient production of inhibin α and possibly β_A-subunits, but not follistatin, is associated with follicular arrest in polycystic ovary syndrome follicles. (*J Clin Endocrinol Metab* 86: 4206–4215, 2001)

OVARIAN FOLLICLE development entails both gonadotropin-dependent and gonadotropin-independent processes. Thus, although FSH is required for the development of antral follicles, local ovarian factors appear to be critical for regulating preantral follicle development and for modulating the effects of gonadotropins (reviewed in Ref. 1). For example, it is increasingly evident that selection of the dominant follicle in monoovulatory species involves a careful orchestration between endocrine-derived trophic support and the regulation of that support by steroidal and nonsteroidal factors produced by the follicle itself (2, 3). In fact, the elaboration of inhibins, the follicle-derived members of the TGFβ superfamily that act to suppress FSH secretion from the pituitary (4), may be a critical event in selecting the dominant follicle (2). Thus, understanding the dynamics of inhibin biosynthesis across follicle development and the processes that regulate the biosynthesis of this important endocrine modulator might shed light on pathophysiological situations in which follicle development has been disrupted.

It has been known for some time that inhibin biosynthesis as well as the production of activins, inhibin-related members of the TGFβ superfamily that stimulate FSH biosynthesis and release from pituitary gonadotrophs (4), are regulated during mammalian follicle development. In one of the earliest studies in rodents, inhibin and activin subunit mRNA levels increased up to the evening of proestrus, but fell dramatically after the GnRH surge, only to rise again just after the secondary FSH surge (5). These studies were extended to primates and humans using *in situ* hybridization on surgically derived specimens, which collectively suggest that the β_B-subunit (and presumably activin B) is produced in preantral and/or early antral follicles. As small antral follicles increase in size, biosynthesis of inhibin α- and β_A-subunits increase, suggesting an orderly shift to the production of more dimeric inhibin B, followed by inhibin A and activin A as the preovulatory stage is achieved (6–10). Similarly, follistatin (FS) expression appears to increase with antral follicle

Abbreviations: FS, Follistatin; hFF, human follicular fluid; PCOS, polycystic ovary syndrome.

diameter (6). Thus, this highly coordinated pattern of changing inhibin/activin subunit and FS mRNA expression suggests that preantral follicles might represent a primarily activin B-dominant microenvironment that changes to an inhibin B-, then an inhibin A-dominant environment as α-subunit production becomes more prominent in larger antral follicles.

Support for this concept was obtained by examining concentrations of inhibins and activin in human follicular fluid (hFF) obtained by transvaginally aspirating follicles from normal women across antral follicle development (11). In this study inhibin A increased with follicle size and maturity, whereas activin A was relatively constant, and inhibin B had no relationship with follicle development. Other studies using hFF from surgically derived ovaries observed similar relationships (12). However, as factors regulating the transport of inhibin out of the follicle as well as its metabolism within the follicle are not yet known, it is not clear whether these changing levels of inhibin and activin protein concentrations in hFF are due to regulated biosynthesis or to alterations in clearance, an understanding of which might delineate the physiological and pathophysiological role(s) of inhibins and activins in follicular development.

Although inhibin's endocrine action in regulating FSH biosynthesis and release from the pituitary has been known for some time (4), potential paracrine/autocrine actions of inhibin and activin are not as well defined. Inhibin has been shown to enhance the development of preovulatory follicles when applied to the rat ovary (13) and to increase LH-stimulated thecal androgen production (14), an effect predicted to enhance granulosa cell E2 biosynthesis. Activin induces granulosa cell proliferation, FSH receptor number, and hormone biosynthesis in immature granulosa cells, but inhibits luteinization in more mature granulosa cells, as detected by reduced progesterone biosynthesis (15, 16). This may explain why activin injection into rat ovaries resulted in the appearance of follicle atresia (13). The *in vivo* situation is even more complex, as activin action is regulated by the structurally unrelated protein, FS (17), which is expressed as two alternatively spliced mRNAs encoding for proteins of 288 and 315 amino acids (18). Both forms of FS bind activin with high affinity and nearly irreversible kinetics (19, 20), thereby inhibiting activin's biological activity and making FS a critical factor in determining the overall bioactivity of intrafollicular activin. However, FS288 can bind cell surface proteoglycans (21), a characteristic that affords a differential tissue distribution of the different FS protein forms (22, 23) as well as additional actions involved with regulating paracrine or endocrine-derived activin bioactivity (24) and activin internalization (25). Thus, differences in biosynthesis of the different FS forms could lead to alterations in overall FS activity. Taken together, these studies suggest important local actions for inhibin, activin, and FS in modulating follicle development.

Polycystic ovary syndrome (PCOS) is a common reproductive disorder characterized by irregular menstrual cycles and hyperandrogenism, and associated with insulin resistance and obesity (reviewed in Ref. 26). In addition, ovaries from women with PCOS exhibit a peripheral array of developmentally arrested small, antral follicles (27). Despite intensive effort, it is still unclear why these follicles remain arrested in their development, as PCOS patients typically have normal levels of FSH (26), their follicles respond robustly to exogenous FSH (28), and *in vitro* studies have demonstrated clear, even exaggerated, responsiveness of PCOS granulosa cells to FSH (29). These observations have led to the suggestion that follicular arrest results from either the presence of a growth inhibitor, which would be reduced with treatments such as wedge resection or follicle cauterization, or to the lack of a developmental stimulator. In either case, the action of a local autocrine/paracrine modulator of follicular development has been postulated (30).

To more directly address the question of inhibin, activin, and FS biosynthesis during antral follicle development and examine the hypothesis that coordinate regulation of their production at the mRNA level is critical for normal follicle development, we developed quantitative RT-PCR assays, which were then applied to analysis of mRNA levels in granulosa cells aspirated from normal women across antral follicle development. To investigate the hypothesis that defects in inhibin, activin, or FS biosynthesis might be involved in the follicle developmental arrest associated with PCOS, we also examined mRNA levels in granulosa cells aspirated from PCOS patients and compared these results to those for size-matched normal follicles. Our results indicate that inhibin α- and β_A-subunit biosynthesis increases with size and maturity in normal follicles, consistent with the previously identified increase in inhibin A with antral follicle development (11). Moreover, production of α- and β_A-subunit mRNAs is significantly reduced in PCOS follicles compared with that in size-matched normal follicles, suggesting that a defect in inhibin biosynthesis is associated with PCOS follicular arrest.

Subjects and Methods

Patients

Evaluation and inclusion criteria for normal volunteers was previously described, and the samples studied in this report represent a subsample of those women (11) from whom sufficient numbers of granulosa cells were recovered for mRNA analysis. A total of 21 follicles, representing a mean diameter of 13.1 mm (6–23 mm range), were obtained by transvaginal aspiration of spontaneously maturing follicles from 18 normal volunteers (Table 1). These subjects had a history of regular 26- to 32-d menstrual cycles, were taking no medications for 3 months before this study, received no medication during the study, had normal TSH and PRL levels, and had normal physical examinations. Daily blood samples were drawn across a complete menstrual cycle, continuing up to the day of aspiration in the following cycle. Frequent pelvic ultrasonography was used to document the development of a dominant follicle in the first cycle and to monitor follicle growth during the aspiration cycle. The largest follicle on either ovary was aspirated transvaginally, and for the purposes of this study is referred to as the dominant follicle.

Four follicles were aspirated transvaginally from four women with PCOS. To be included in this study, PCOS patients were otherwise healthy, between the ages of 18–40 yr, had fewer than nine menses per yr, had been taking no medication for 3 months, and had biochemical and/or clinical evidence of hyperandrogenism. Each patient had at least two pelvic ultrasounds and 10 d of daily blood sampling to confirm the absence of follicle maturation and menstrual cyclicity. Four additional follicles were obtained from two PCOS patients whose ovaries had been surgically removed for nonovarian disorders.

To compare normal and PCOS follicles, a subset of normal follicles (n = 5) was selected whose mean diameter was not statistically different from that of the PCOS follicles (n = 8). For a more rigorous comparison,

TABLE 1. Summary of patient and sample characteristics

	All normal subjects (n = 18)	All PCOS patients (n = 6)	Size/range-matched normal subjects (n = 5)	Size/range-matched PCOS patients (n = 5)
Age (yr)[a]	31.2 (1.7)	32 (2.9)	27.6 (2.4)	28.8 (2.9)
BMI[a]	24.0 (1.1)	32 (3.8)	24.7 (1.9)	29.8 (4.5)
Ferriman-Gallwey score[a]	5.7 (0.54)	9.0 (0.5)	5.4 (0.8)	9.0 (0.7)
Number of follicles	21	8	5	5
Follicle diameter range (mm)	6.0–23.0	0.5–8.5	6.0–8.5	5.5–8.5

Samples from all normal subjects were obtained by transvaginal aspiration of the largest follicle in the cycle after a complete normal cycle. Samples from PCOS patients were obtained by transvaginal follicle aspiration (n = 4) after documentation of absence of follicle growth or from surgically removed ovaries from PCOS cases (n = 2). Size/range-matched subjects represent the subset of women from whom follicles were obtained for the size/range-matched follicle groups in Table 3.

[a] Mean (SD).

we also selected a subset of PCOS follicles (n = 5) whose range and mean diameter were nearly identical to those of the normal size-matched group, eliminating the smallest PCOS follicles. Characteristics for normal volunteers and PCOS subjects, in total as well as for the subgroups, are summarized in Table 1.

All subjects gave informed consent, and the entire protocol was approved by the human studies institutional review board at the Massachusetts General and Brigham and Women's Hospitals.

Sample acquisition

The methods for follicle aspiration have been previously described in detail (11). Follicular aspirates were transferred to the laboratory on ice, and the volume of the aspirate was determined. Granulosa cells were then allowed to settle in a plastic petri dish, after which they were manually collected, rinsed in three changes of Dulbecco's PBS (Life Technologies, Inc.-BRL, Grand Island, NY), transferred to a 1.5-ml microcentrifuge tube, and centrifuged at 3000 rpm for 5 min. The supernatant was aspirated, 1 ml TRIzol and 5 μg *Escherichia coli* tRNA (both from Life Technologies, Inc.-BRL) were added, and the tube was stored at −80 C until RNA extraction. The follicular fluid aspirate remaining in the dish after granulosa cell removal was centrifuged, and the supernatant stored at −20 C until assayed as previously described (11). The average time interval between sample collection and completion of sample preparation was 1–1.5 h.

Development of competitive RT-PCR assays

The inhibin/activin subunits α, β_A, and β_B, as well as FS288 and FS315 transcripts were analyzed by competitive, quantitative RT-PCR. Competitors for each target were created by deleting a small portion (~10%) in the middle of each target using PCR to introduce novel restriction sites, followed by excision and ligation of the shortened fragments, so that except for this small deletion, the competitor and target are identical and were similarly amplified by the same primer pair. The religated competitors were then amplified by PCR, cloned into pCRII-Topo (Invitrogen, Carlsbad, CA), amplified, and sequenced. For the quantitative PCR, each target was amplified in a series of four reactions that included increasing amounts of competitor ranging from a dose that showed no competition to a concentration that eliminated amplification of the target, the optimal range being determined experimentally for each target. At the point where the signal from competitor and target were equal, the concentrations of cDNA were equal, thereby allowing calculation of the precise mRNA concentration for each target in the original sample. Because the RNA was extracted in the presence of carrier RNA to prevent loss of the small amounts of granulosa cell RNA, recoveries could not be directly determined, so results for each target were normalized to the β-actin concentration and are reported as ratios. All target mRNA concentrations were in the femtomolar range, with the exception of β_B-subunit mRNA, which was in the attamolar range, indicating that β_B mRNA levels are substantially lower than those of other targets.

RNA extraction/RT-PCR

Granulosa cell RNA was extracted using TRIzol (Life Technologies, Inc.-BRL) according to the manufacturer's protocol. RNA was resolved in 10–15 μl diethylpyrocarbonate H_2O. Five microliters of RNA were treated for 15 min with deoxyribonuclease I (Life Technologies, Inc.-BRL) in a 10-μl reaction and then reverse transcribed using 100 U Superscript II (Life Technologies, Inc.-BRL) in a 21.5-μl reaction for 50 min at 42 C. Ribonuclease H (1.5 U; Life Technologies, Inc.-BRL) was added, and the sample was incubated at 37 C for 20 min, after which the enzyme was inactivated. This RT reaction was diluted 1:10 with water before PCR.

PCR was performed in a volume of 25 μl containing 2 μl diluted RT product, 0.2 mM deoxy-NTPs, 1.74 mM $MgCl_2$, 0.5 μM of each primer, 0.5 U *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA), and 0.25 μCi [α-^{32}P]deoxy-CTP (NEN Life Science Products, Boston MA). All primer pairs used for this analysis have been previously described, and their specificity demonstrated (31–34). Targets were amplified within their respective experimentally determined exponential ranges using a temperature profile of 1 min each at 94, 60, and 72 C. PCR products were resolved on 5% polyacrylamide gels, after which the radioactive bands corresponding to both target and competitor were individually cut and counted in a β-scintillation counter. After subtraction of background radioactivity and adjustment for differences in product size, the ratio of target/competitor was calculated and plotted against the competitor concentration. The competitor amount equivalent to a ratio of 1 was taken as the initial target concentration after compensating for RT dilution. The RNA concentration was then normalized to β-actin for each granulosa cell mRNA sample to control for differences in granulosa cell numbers and for variable RNA recoveries between samples.

To evaluate the precision of the quantitative RT-PCR assays, RNA was extracted from a large pool of granulosa-lutein cells using methods described above, and the cDNA from this pool was analyzed with each PCR assay, providing a measure of interassay coefficient of variation. In addition, this pool was analyzed four times for each target PCR to calculate the intraassay coefficient of variation.

Hormone analysis

Hormone concentrations for the normal follicles represent the subset of previously reported results for which sufficient granulosa cell mRNA was recovered for analysis in this study (11). Hormone concentrations for PCOS follicles were not analyzed, because only two PCOS follicles contained sufficient granulosa cell mRNA and follicular fluid. Follicular fluid inhibin A and B and activin A were measured using commercially available immunoassay kits, whereas free FS was measured using a two-site immunoassay, all as previously reported in detail (11, 35). Pro-αC, a measure of inhibin α-subunit protein containing the pro region of the precursor, was quantified using an immunoassay kit from Serotec (Oxford, UK) as previously described (32). To characterize menstrual cycles, serum samples were analyzed for E2, LH, and progesterone as previously described (11).

Data analysis

For normal subjects, follicle maturity (day of the menstrual cycle) was estimated by determining the number of days before predicted ovulation that each follicle was aspirated using the daily serum hormone levels in the preceding study cycle as a guide, as previously described

in detail (11). Granulosa cell mRNA content, after normalization to β-actin, was then compared with follicle diameter, follicle maturity, or hormone concentration in hFF from the same follicle (11), and correlations were determined using least squares regression analysis. Significant relationships are indicated by inclusion of the regression line along with the corresponding r and *P* values on the appropriate figures. As PCOS patients were anovulatory, results from these follicles were only analyzed by follicle size. Normal and PCOS follicles were analyzed separately for these analyses.

Inhibin A, free FS, and activin A protein results were normally distributed and analyzed directly. Inhibin B, pro-αC, and all mRNA results deviated significantly from a normal distribution and were log transformed before analysis. Results are therefore presented in log scale in appropriate figures and as medians and ranges in tables.

To compare PCOS follicles with normal follicles, the five smallest normal follicles were selected because they closely match both in range and mean follicle diameter with a subset of the five largest PCOS follicles. These size-matched normal follicles were also compared with the whole group of PCOS follicles (n = 8) for which sufficient mRNA was obtained for analysis. *t* tests were used to compare means. $P < 0.05$ was used to indicate statistical significance.

Results

Granulosa cell mRNA

Granulosa cells were obtained from 21 normal and 8 PCOS follicles (Table 1), and steady state mRNA levels for inhibin α-subunit, inhibin/activin β_A- and β_B-subunits, both FS alternative splice variants, and β-actin were assessed by quantitative competitive RT-PCR. Intra- and interassay variations for each target were determined using a pool of granulosa cell mRNA derived from *in vitro* fertilization patients (Table 2). As the assay variability is less than the observed differences between follicles, these results support the utility of the RT-PCR procedures. α-Subunit mRNA levels in granulosa cells from normal women were positively correlated with both follicle diameter (Fig. 1A; r = 0.56; $P < 0.01$) and maturity (Fig. 1B; r = 0.65; $P < 0.001$). Similarly, inhibin/activin β_A-subunit mRNA levels in granulosa cells from normal women were positively associated with both follicle size (Fig. 1C; r = 0.45; $P = 0.05$) and maturity (Fig. 1D; r = 0.58; $P < 0.01$), and there was a strong correlation between α- and β_A-subunit mRNA levels (r = 0.91; $P < 0.001$). These mRNA results are consistent with our previously observed positive association of hFF and serum dimeric inhibin A protein concentration with follicle size and maturity (11, 36). In contrast to α- and β_A-subunits, β_B-subunit mRNA levels varied widely and were not associated with either follicle size or maturity (Fig. 1, E and F, respectively), in agreement with our previously reported lack of association of inhibin B protein with follicle size and maturity (11). However, β_B mRNA levels were more than 10-fold lower than β_A levels. No associations were noted between mRNA levels and follicle size or maturity in granulosa cells from PCOS patients.

TABLE 2. Characterization of intra- and interassay coefficients of variation (CV) for semi-quantitative RT-PCR

	Intraassay CV (%)	Interassay CV (%)
α-Subunit	16.7	21.5
β_A-Subunit	11.8	25.5
β_B-Subunit	14.0	19.1
FS315	2.8	11.0
FS288	11.1	24.6
β-Actin	9.5	13.1

A pool of cDNA that was reverse transcribed from granulosa cell mRNA was assayed four times in a single experiment. The interassay variation was calculated from same pool run in four consecutive experiments.

The FS315 and FS288 mRNA alternatively spliced transcripts were investigated individually so we could determine whether their ratio varied as a function of follicle maturation. Steady state FS315 mRNA levels in granulosa cells from normal or PCOS subjects did not vary consistently when analyzed in relation to either follicle diameter (Fig. 2A) or maturity (Fig. 2B). Similar results were obtained for the less abundant FS288 mRNA transcript (Fig. 2, C and D). Interestingly, the expression of these two transcripts were highly correlated with each other for both normal and PCOS follicles (Fig. 2E; r = 0.85; $P < 0.001$ and r = 0.99; $P < 0.001$ respectively). In addition, the FS315/FS288 ratio was the same for normal and PCOS follicles (mean, 2.98 ± 1.2 and 2.90 ± 1.2, respectively) and did not vary with size (Fig. 2F) or maturity (not shown).

Comparison of mRNA and hormone concentrations in normal follicles

Hormone and mRNA levels from individual follicle aspirates were compared only for samples obtained from normal subjects, as there was insufficient follicular fluid for hormonal analysis in most PCOS follicles from which mRNA was also acquired. Granulosa cell α-subunit steady state mRNA levels were positively associated with dimeric inhibin A concentrations in hFF from the same follicles (Fig. 3A; r = 0.71; $P < 0.001$). However, no association was observed between α-subunit mRNA levels and inhibin B (Fig. 3B). Moreover, there was no association between α-subunit mRNA and pro-αC protein, a measure of α-subunit monomer or dimer containing the pro region of the α-inhibin precursor molecule (Fig. 3C).

As expected, β_A-subunit mRNA levels were positively associated with dimeric inhibin A (Fig. 4A; r = 0.59; $P < 0.01$). Interestingly, β_B mRNA levels were positively associated with dimeric inhibin B concentrations in hFF (Fig. 4B; r = 0.67; $P < 0.005$) despite neither parameter being associated with follicle growth or maturation, indicating that β_B mRNA and inhibin B protein biosynthesis are regulated independently of follicle development. Neither FS288 nor FS315 levels were associated with free FS hormone levels in hFF (Fig. 5, A and B), and activin A was not associated with β_A-subunit mRNA.

Comparison of normal and PCOS follicle mean mRNA levels

A subset of five normal follicles was selected to match the mean follicle diameter for all PCOS follicles. As shown in Table 3, α-subunit mRNA levels were 10-fold ($P < 0.03$) lower in PCOS follicles relative to normal follicles, while β_A-subunit was 5-fold lower in PCOS follicles ($P < 0.02$). Although β_B-subunit mRNA levels were 2.5-fold lower in PCOS follicles, this difference did not reach statistical significance. As the PCOS follicle group contains three very small (0.5–2 mm) follicles that were not directly matched in our normal follicle subset, we also examined a subset of PCOS follicles (n = 5) that overlapped the normal follicle

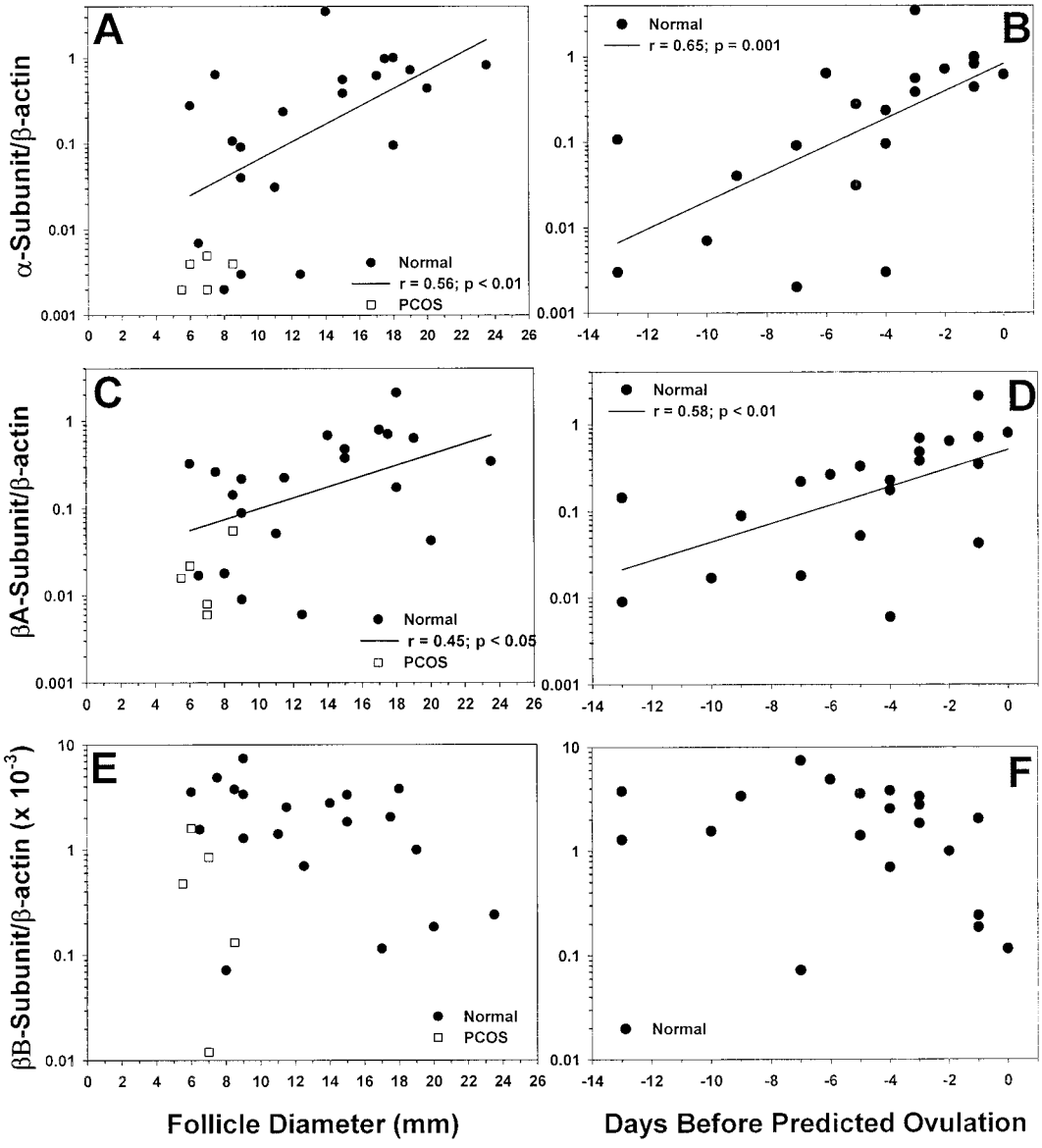

FIG. 1. Inhibin/activin subunit mRNA levels in granulosa cells from aspirated follicles. Quantitative RT-PCR assays were used on RNA extracted from granulosa cells aspirated from normal (●) or PCOS (□) subjects to determine the concentrations of inhibin α-, β_A-, and β_B-subunits relative to that of β-actin. Significant relationships of mRNA level with follicle size or maturity [days before predicted ovulation (11)] are indicated. Note the change of scale for β_B-subunit, because its concentration was at least 10-fold lower than those of the other subunits. No significant relationships were observed for PCOS follicles.

group in both mean follicle diameter and range of follicle sizes. As shown in Table 3, even in this restricted subset of PCOS follicles, α-subunit mRNA levels were 16-fold lower than those in normal follicles ($P < 0.02$), whereas the β_A mRNA levels were no longer significantly different ($P = 0.059$). Thus, at least the reduced α-subunit mRNA level in PCOS follicles appears to be a robust characteristic of these developmentally arrested follicles. Neither the FS mRNA variants nor their ratio were different between normal and PCOS follicles, suggesting that defects in FS biosynthesis are not associated with PCOS.

Discussion

To determine whether biosynthesis of inhibin, activin, and FS is modulated during antral follicle development in normal women, we examined mRNA levels for these hormones in granulosa cells transvaginally aspirated from 6- to 23-mm spontaneously growing normal human follicles. Competitive RT-PCR assays were established for each mRNA target to allow quantitative assessment of each target in the minute quantities of recovered RNA. Consistent with suggestions from earlier *in situ* hybridization and immunocytochemical studies on surgical specimens (6, 9) as well as with measurements of dimeric inhibin A in hFF from follicle aspirates (11), we observed that both α- and β_A-subunit mRNA levels increase as antral follicles grow and mature. In contrast, β_B-subunit RNA levels had no relationship to follicle maturity or size, which also agrees with our previous assessment of inhibin B protein in hFF (11). Thus, relative to inhibin B and possibly activin B, which cannot be quantitated at present,

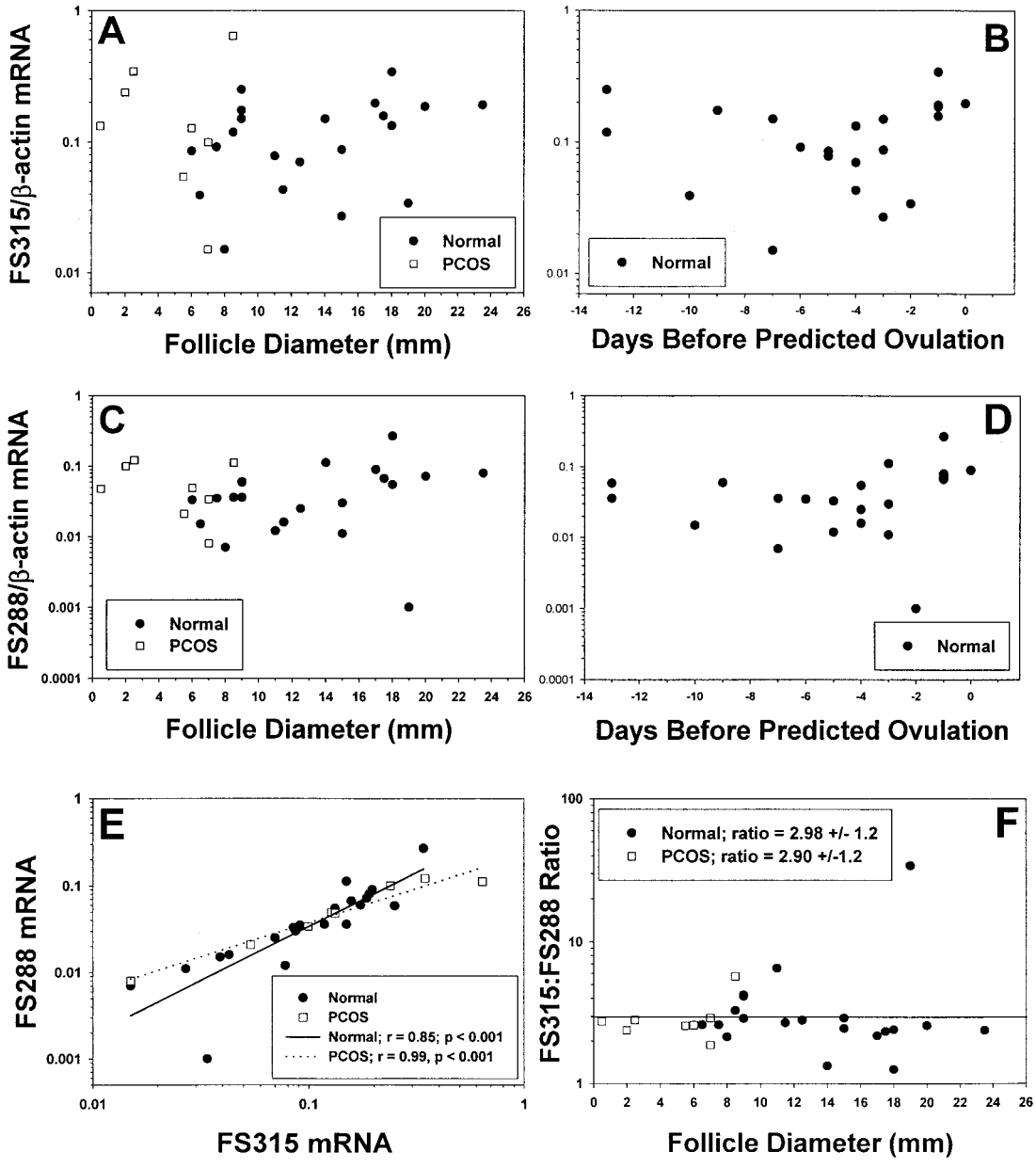

FIG. 2. Analysis of FS315 and FS288 mRNA levels in granulosa cells from aspirated follicles. Quantitative RT-PCR assays were developed for each alternative splice variant, and concentrations were expressed as a ratio with the β-activin concentration. Results are displayed as a function of follicle size (A and C) or maturity (B and D). E, FS315 and FS288 variants are highly correlated for both normal and PCOS follicles. F, The FS315/FS288 ratio does not change across follicle development, and the ratio for both normal and PCOS follicles is about three FS315 transcripts for each FS288 transcript. Significant relationships are indicated by *lines*.

biosynthesis of α- and β_A-subunit mRNA and production of dimeric inhibin A protein is greater in larger follicles, suggesting the hypothesis that increasing inhibin A concentrations are important for maintenance of dominant follicle development.

To further examine this hypothesis, mRNA levels in PCOS follicles, a model for follicular developmental arrest, were compared with those in a group of size-matched normal follicles. Consistent with the hypothesis, α- and β_A-subunit mRNA levels were 10- and 5-fold lower, respectively, in the whole PCOS follicle group ($P < 0.03$ and $P < 0.02$, respectively), and a trend toward reduced β_B-subunit mRNA levels was observed as well. To eliminate the possibility that very small follicles could bias the PCOS follicle group mean analysis, we also examined a subset of five PCOS follicles whose size and diameter range were nearly identical to those in the normal size-matched group. Even with this limited subset of follicles, α-subunit mRNA levels were significantly (16-fold) lower than those in normal follicles ($P < 0.02$), and the β_A mRNA levels were nearly so (5-fold; $P = 0.059$). These results suggest that α-subunit biosynthesis and perhaps β_A- and β_B-subunit production are defective in PCOS follicles and, thus, that PCOS follicular arrest might be associated with inhibin insufficiency.

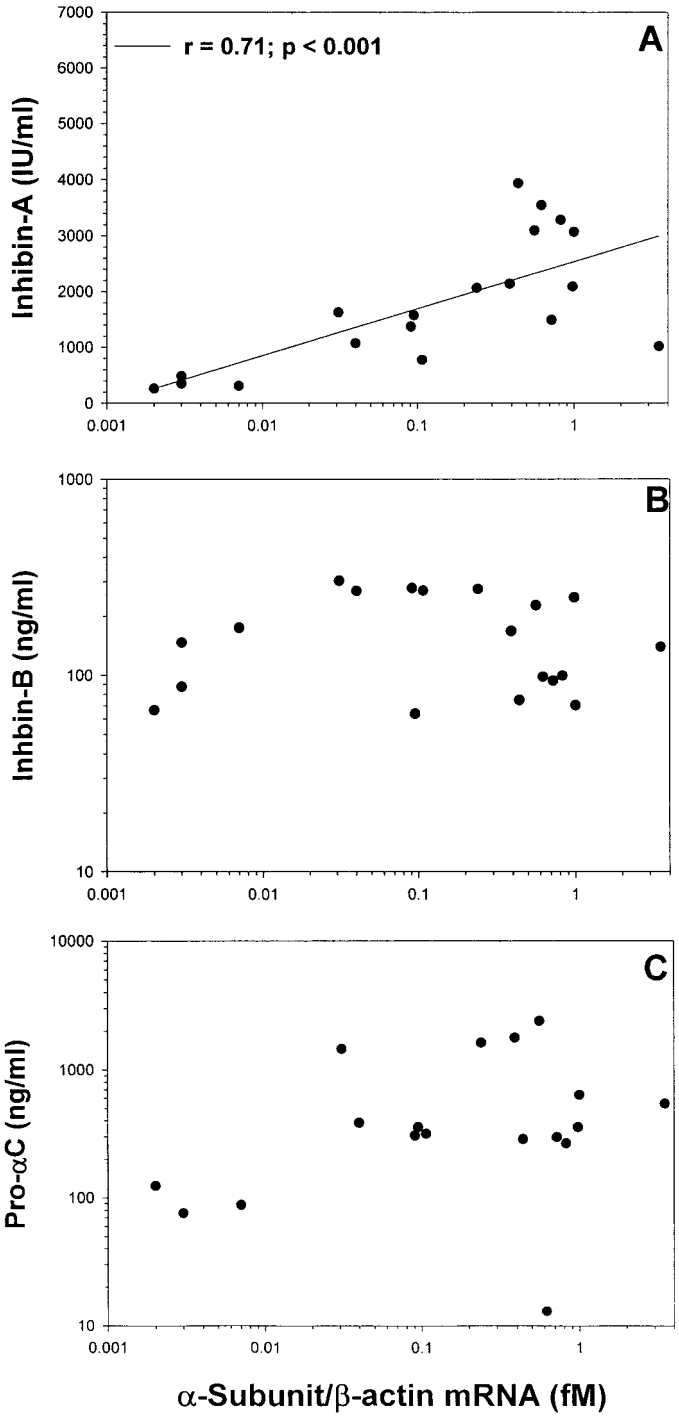

FIG. 3. Relationship of α-subunit mRNA concentration to α-subunit-containing proteins in hFF of the same normal follicle. α-Subunit mRNA levels were correlated with dimeric inhibin A levels in the hFF aspirated from the same follicle (A), but not with inhibin B (B) or pro-αC (C). Significant relationships are indicated by *lines*.

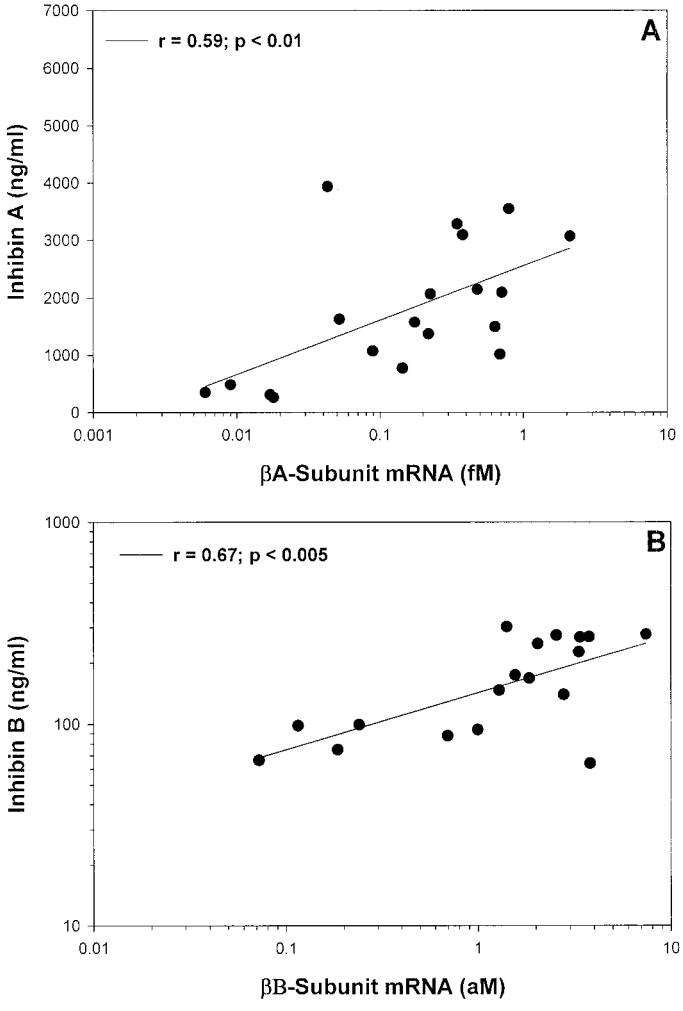

FIG. 4. Relationship of inhibin β-subunits to their respective dimeric hormone in hFF. β_A-Subunit mRNA levels were positively associated with dimeric inhibin A protein concentrations (A), and β_B-subunit mRNA levels were correlated with dimeric inhibin B concentrations (B) in the same follicles. Significant relationships are indicated by *lines*.

It is unclear at this point, however, whether the deficient inhibin subunit biosynthesis in PCOS follicles is secondary to a deficiency of some as yet unidentified factor or if the inhibin deficiency is a primary cause of follicular growth arrest. Inhibin has been previously proposed to function as an autocrine or paracrine regulator of follicular development either by direct action on granulosa cells or by enhancing LH-induced androgen production (14, 37). In addition, treatment of female rats with inhibin A injected under the ovarian bursa resulted in enhanced antral follicle development (13). It is also becoming clear from animal studies that production of inhibin sufficient to reduce circulating FSH is a critical event in selection of the dominant follicle (1, 2). Moreover, in a previous study of hFF inhibin concentrations in women undergoing ovarian hyperstimulation for *in vitro* fertilization, we found that inhibin A concentrations increased with follicle size in non-PCOS patients, but did not vary significantly in women with PCOS (38). Taken together, these results suggest that insufficient production of dimeric inhibin is one potential explanation for follicular arrest typically observed in PCOS patients, indicating that further study into the role of inhibin in follicular development is warranted.

Our examination of both mRNA levels and the corresponding protein hormone concentrations in hFF from the same follicles demonstrates for the first time that inhibin

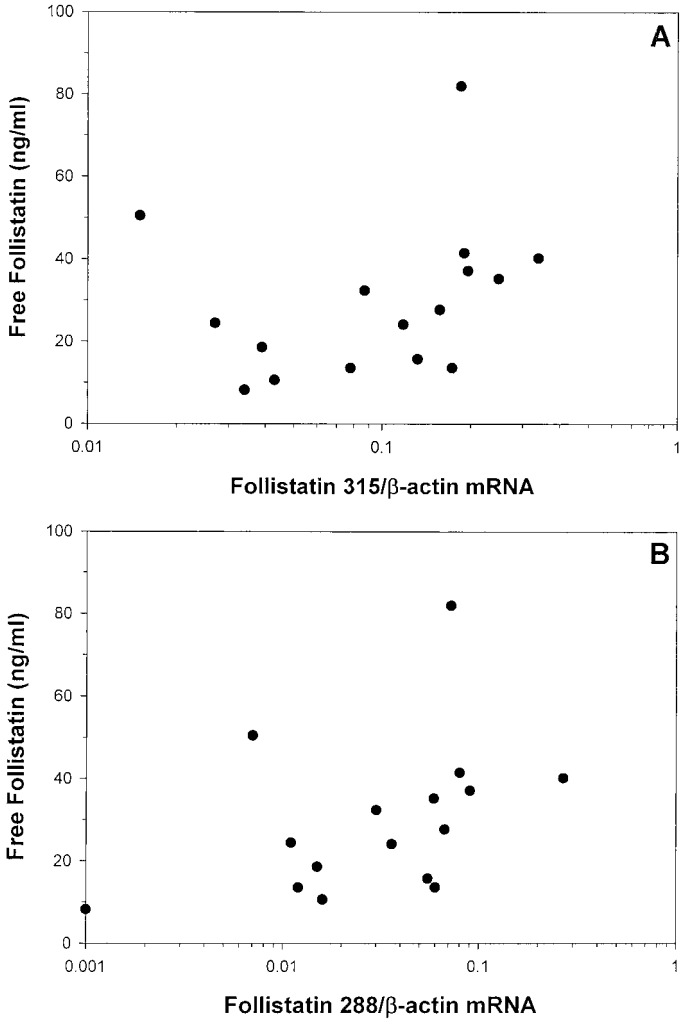

FIG. 5. Relationship of FS mRNA levels to free FS protein concentrations in hFF. Neither FS315 mRNA (A) nor FS288 mRNA (B) were associated with free FS levels in hFF from the same follicles. No significant relationships were noted.

subunit mRNA biosynthesis in granulosa cells is tightly linked to protein hormone production in normally developing follicles *in vivo*. Interestingly, no relationships between granulosa cell mRNA and hFF hormone concentrations were observed when follicles aspirated from gonadotropin-hyperstimulated women were examined (32). These results indicate that administration of exogenous gonadotropins to women stimulates inhibin biosynthesis along with follicular development, but the usual relationship between mRNA and hormone levels is lost. This may be due to hormone biosynthesis in excess of its diffusion rate from the follicle, resulting in an accumulation of inhibin in hFF, or to the production of excess mRNAs that are not directly translated into protein. However, the physiological implication of this loss of association is unclear, as we were unable to identify any relationship between hFF dimeric inhibin concentration and *in vitro* fertilization outcome (32). Nevertheless, the present study indicates that inhibin biosynthesis is strongly regulated at both the mRNA and protein levels during normal antral follicle development.

Although biosynthesis of inhibin α- and β_A-subunits and inhibin A protein appear to be gonadotropin regulated, recent evidence suggests that inhibin B protein and β_B message are not (39–42). In the present study β_B-subunit biosynthesis did not vary with follicle size or maturity, and in our previous study inhibin B protein concentrations in hFF were similarly not associated with follicle development (11). Previous studies have also been unable to detect any association between hFF inhibin B concentrations and follicle size (12, 43). Taken together, these results suggest that the normal increase in serum inhibin B observed during the early follicular phase of the human menstrual cycle (44, 45) may be due to an increased number of developing small antral follicles actively secreting inhibin B, rather than to a direct effect of FSH on inhibin B secretion. However, in the present study, β_B-subunit biosynthesis and inhibin B hormone concentrations were tightly correlated. These results suggest that inhibin B biosynthesis is tightly regulated at the mRNA and protein levels, but that this regulation does not vary with follicle size or maturity. Recent data suggest that, rather than gonadotropins, intrafollicular growth factors such as IGFs and/or other members of the TGFβ superfamily might regulate β_B mRNA transcription and/or inhibin B protein levels in antral follicles (39–42). Interestingly, mean β_B message levels were less than 10% of the β_A levels, consistent with a lower rate of β_B message biosynthesis at this stage of development. In addition, although not significant in this study, β_B mRNA levels were lower in PCOS follicles, suggesting that further analysis of β_B-subunit biosynthesis regulation might yield important clues to the developmental arrest in PCOS follicles.

By designing RT-PCR assays that could differentially quantitate FS315 and FS288 transcripts, we could examine, for the first time, the quantitative relationship between these two transcripts and whether this relationship changes during follicular development. There was no consistent variation in expression for either transcript as follicles matured or grew in size. Based on all normal follicles, the mean FS315:FS288 ratio was 2.98. These observations indicate that the alternative splicing event that produces FS288 is not regulated during follicular development and produces approximately three full-length FS315 transcripts for every one FS288 transcript. It has been previously demonstrated that the major FS protein species in follicular fluid is the 300/303 intermediate size that must be derived by proteolytic processing of the FS315 protein, and that full-length FS315 and FS288 isoforms are relatively rare (23, 46). Moreover, it is currently unknown to what degree the FS288 transcript is actually translated in granulosa cells under normal physiological conditions. Thus, our results do not support the concept that the alternative splicing event itself is important to FS's role in the ovary.

Based on candidate gene analysis of affected sibling pairs, it was previously suggested that a mutation in or near the FS gene was statistically associated with PCOS (47), although a more recent analysis of additional PCOS families casts doubt on this association (48). Consistent with this latter finding, our results fail to support the presence of a defect in FS biosynthesis in PCOS follicles relative to that in size-matched normal follicles, nor was there any difference in the FS315/FS288 transcript ratio. In fact, we recently reported that free

TABLE 3. Comparison of inhibin/activin subunit and follistatin mRNA levels in granulosa cells from size- and range-matched PCOS follicles compared to size-matched normal follicles

	Size/range-matched normal follicles (n = 5)	Size/range-matched PCOS follicles (n = 5)	All PCOS follicles (n = 8)
Mean follicle diameter (mm)[a]	7.3 (0.46)	7.0 (0.57)	5.0 (1.1)
Follicle diameter range (mm)	6.0–8.5	5.5–8.5	0.5–8.5
α-Subunit[b]	4.8 (0.2–6.41)[c,d]	0.3 (0.2–0.5)[c]	0.5 (0.2–2.0)[d]
β_A-Subunit[b]	8.3 (1.7–39.3)[c]	1.6 (0.6–5.6)	1.5 (0.5–5.6)[c]
β_B-Subunit[b]	0.149 (0.07–0.49)	0.03 (0.01–0.2)	0.06 (0.01–0.4)
Follistatin-315[b]	5.6 (1.5–11.8)	9.2 (1.5–63.9)	12.8 (1.5–64.0)
Follistatin-288[b]	2.1 (0.7–3.6)	3.2 (0.8–11.2)	4.5 (0.8–12.2)
Follistatin 315:288 ratio[a]	2.98 (1.2)	2.90 (1.2)	2.80 (1.2)

Also shown are results for all PCOS follicles analyzed. Quantitative, competitive RT-PCR assays were used to determine mRNA levels in granulosa cells obtained from follicle aspirates. mRNA results are expressed as ratios ($\times 10^{-2}$) with β-actin.

[a] Mean (SEM).

[b] Geometric mean (min–max).

[c] $P < 0.02$.

[d] $P < 0.03$.

FS levels in hFF from developing human follicles were not associated with follicle growth or maturity, but were in large excess over activin in nearly all antral follicles examined (11). Taken together, these studies strongly indicate that free FS levels in hFF are not a critical parameter for antral follicle development, nor are they important for PCOS follicular developmental arrest.

By aspirating follicles at precise times from women with well characterized normal menstrual cycles and development of quantitative RT-PCR assays, we were able to determine the developmental pattern of inhibin and activin biosynthesis from a predominantly β_B microenvironment to an α/β_A microenvironment as normal antral follicles mature. In addition, we have clearly demonstrated that FS mRNA levels do not vary with follicle development, that the FS315/FS288 ratio does not change, and that approximately three FS315 transcripts are synthesized for every FS288 mRNA. Moreover, by aspirating developmentally arrested follicles from women with PCOS and comparing them to size-matched normal follicles, we have determined that these PCOS follicles contain vastly reduced α- and β_A-subunit mRNA levels, suggesting that defective inhibin biosynthesis might be related to the aberrant follicle development seen in women with PCOS. Further analysis of relative inhibin protein concentrations between PCOS and size-matched normal follicles should help elucidate the prevalence and importance of this distinction.

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