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Production of Activins by the Human Endosalpinx

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Uterine tubes from 11 premenopausal and 6 postmenopausal women were collected and examined for the presence of inhibin, activin, and follistatin in the endosalpinx. Immunocytochemistry of tissue from both the isthmic and ampullary regions demonstrated clear staining for the β_A- and β_B-subunits that increased in intensity from the isthmus to the ampulla. Staining for follistatin showed a similar pattern, but no staining for the α-subunit was observed. Although staining for the β_A-subunit was seen in almost every epithelial cell, staining for the β_B-subunit was more variable. Western blotting showed a band with an apparent molecular mass of 28 kDa (corresponding to the activin dimer) and a band of approximately 60 kDa (corresponding to the pro-protein of activin). *In situ* hybridization confirmed the presence of mRNA for the β_A- and β_B-subunits in the endosalpinx. These results indicate that the endosalpinx is able to synthesize activin, not inhibin, suggesting that in premenopausal women they may have an important role in the biology of the developing embryo. The role in postmenopausal women is less certain, but could lead to the stimulation of FSH secretion by the pituitary gland or other autocrine/paracrine function within the uterine tube. (*J Clin Endocrinol Metab* 87: 5283–5289, 2002)

INHIBINS AND ACTIVINS are dimeric glycoproteins that are defined by their actions on pituitary gonadotroph cells as either inhibiting (inhibins) or stimulating (activins) the production of FSH. They act through serine/threonine kinase transmembrane receptors, resulting in the phosphorylation of intracellular mediators, S-MAD (proteins related to the *Drosophila* gene mothers against deca-penta-plegic) (1). Follistatin (2) is recognized to be an important regulator of cellular differentiation and secretion through it is potent ability to bind activins. The production of follistatin is, in turn, regulated by activin in the pituitary, gonads, and elsewhere (3). The inhibins and activins are members of the TGFβ superfamily and are known to have a variety of actions concerning hormonogenesis, cellular homeostasis, and differentiation in addition to their classical action on FSH. They also participate in fundamental development processes, including differentiation of embryonic mesoderm, neuronal protection, hemopoiesis, and reproductive function. Deletion or mutation of genes involved in the regulation of synthesis of inhibins and activins results in phenotypic abnormalities, including embryonic lethality, tumor formation, and infertility (4).

Inhibins and activins have been shown in a number of species to participate in the biology of both sperm (5) and oocyte (6) and in early embryonic development (7). Activin A has been shown to enhance postcleavage development of the bovine oocyte (8, 9) and to improve *in vitro* development of embryos in mouse (10) and bovine (8, 11). Inhibin is expressed in fetal testis throughout gestation in both ovine and bovine, with concentrations increasing toward term (12, 13). In the mouse, both β_A- and β_B-subunits are expressed in both oocytes and early preimplantation embryos and the oviduct (14), whereas the α-subunit was not expressed in any of these, suggesting that mouse embryos produce activins, but not inhibins, and activins appearing in the oviduct and in embryos are produced by the oviduct, oocytes, and embryos themselves (14). A recent study showed that neither activin β_A nor β_B mRNA was localized in any human or mouse oocyte, but it is highly expressed in the cumulus cells (15). In the human, the α-, β_A-, and β_B-subunits are expressed in the testis (16), and inhibins, activins, and follistatin have been isolated from follicular fluid and identified in thecal and granulosa-lutein cells of the corpus luteum (17).

The uterine tube is the site of fertilization and early embryogenesis, and it seems likely that the inner epithelial lining of the uterine tube (the endosalpinx) is involved in the biology of gametes and early embryo, providing secretions into the tubal lumen that form the microenvironment of the gametes at the time of fertilization (18, 19). Although inhibins and activins have mostly been studied in the testis and ovary, early studies suggest that both the endosalpinx and the epididymal epithelium express inhibin/activin subunits (20, 21). We therefore decided to study the expression and localization of inhibin/activin subunits and follistatin in the human endosalpinx. Given the decline in circulating concentrations of inhibins A and B after the menopause, we also studied local expression of the α- and β-subunits (and follistatin) in the uterine tubes from postmenopausal women.

Materials and Methods

Patient recruitment and surgery

Uterine tubes were obtained from women undergoing routine total abdominal hysterectomy at the Jessop Hospital for Women and the Northern General Hospital (Sheffield, UK). Before the operation, informed consent was obtained to perform unilateral or bilateral salpingectomy; these procedures were subject to control from the local ethics committee. Details of the surgical procedure and tissue handling arrangements were previously described (22), with all tissue samples arriving in the laboratory within 20 min of their removal from the patient. Uterine tubes from two groups of women were obtained. The first were women who had a regular menstrual cycle (between 25–35 d) and were of proven fertility, with no evidence of tubal disease and with

Abbreviations: SSC, Saline sodium citrate; TBS, Tris-buffered saline.

patent (unsterilized) tubes. Women who were taking drugs to control their menstrual cycle were specifically excluded from the study. The second group consisted of amenorrheic postmenopausal women, aged 55–65 yr, who had not received hormone replacement therapy for at least 2 yr before the time of surgery.

Sampling and processing

For uterine tubes of premenopausal women, the isthmic and ampullary regions were identified, and a small section cut from each region. These were then further cut into two equal pieces, with one piece being fixed in 10% buffered formalin for immunocytochemistry and *in situ* hybridization (see below) and the second part being frozen in liquid nitrogen for Western blot analysis (see below). For the tubes of postmenopausal women, sections were cut from the isthmus and ampulla and fixed in 10% buffered formalin for immunocytochemistry only.

Immunocytochemistry

The tissue samples prepared for immunocytochemistry were processed and embedded in wax according to methods described previously (23). Histological sections of 1 and 5 μm thickness were then cut from the wax blocks and processed as described below.

An indirect avidin-biotin elite horseradish peroxidase technique was used to localize inhibin α, β_A-, and β_B-subunits and follistatin following the protocol described. Briefly, sections were dewaxed, dehydrated in alcohol, and treated with 2% (vol/vol) hydrogen peroxide for 20 min in methanol to block endogenous peroxidase (24). Sections were pretreated in an 850-watt domestic microwave oven in 0.01 M citrate buffers twice for 5 min each time. The sections were incubated for 30 min with normal horse serum and then incubated with the primary antibodies overnight at 4 C. The following day, the section were washed with 20 mM PBS and then incubated with the secondary antibody for 30 min. After a further wash step, the sections were incubated with the avidin-biotin peroxidase complex ELITE system (Vector Laboratories, Inc., Burlingame, CA) with 3,3′-diaminobenzidine (Vector Laboratories, Inc.) as the substrate. Sections were washed in tap water, counterstained with Gill's hematoxylin, then dehydrated in a series of graded ethanols, cleared in xylene, and mounted in piccolyte.

The sections were observed on a Labor Lux microscope (Leitz, Wetzlar, Germany) at a magnification of ×400. A positive reaction was characterized by the presence of brown staining. Each section was examined by two observers who agreed on the intensity of the staining according to the following semiquantitative scale: −, negative; ±, equivocally positive; +, weakly positive; 2+, positive; and 3+++, strongly positive. Representative sections were photographed using a Wild MPS 48/52 Photoautomat (Leica Corp., Heebrugg, Switzerland) at ×250 and ×400 magnification.

Western immunoblot analysis

The frozen tissue (~2 g) was ground to a fine powder in liquid nitrogen using a mortar and pestle and cooled in liquid nitrogen, and the macerated tissue was then lysed in 500 μl triple detergent lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40] containing the enzyme inhibitors aprotinin (1 μg/ml), sodium orthovanadate (100 mM), and phenylmethylsulfonylfluoride (100 μg/ml). Samples were aspirated with a syringe fitted with a 21-gauge needle, left on ice for 30 min, and centrifuged (17,900 × *g* for 10 min). Supernatants were collected and stored at −80 C. Sample protein content was measured using a DC protein assay kit (Bio-Rad Laboratories, Inc., Richmond, CA) according to the manufacturer's directions. Samples were denatured at 95 C for 5 min in sodium dodecyl sulfate loading buffer containing 100 mM dithiothreitol. Equal amounts of protein (20 μg) were resolved by SDS-PAGE using a 4% stacking and 15% resolving gel. Protein was electroblotted using a semidry transfer method onto polyvinylidene fluoride microporous membrane (NEN Life Science Products, Boston, MA) in Tris/glycine buffer containing 10% methanol. Membranes were blocked overnight at 4C in Tris-buffered saline (TBS) containing 5% nonfat milk, and 0.05% Tween 20 and then incubated with mouse antihuman inhibin β_B antibody (C5; 1:200 dilution) or mouse antihuman inhibin β_A antibody (E4; 1:400 dilution), overnight at 4 C. Membranes were washed in TBS with 0.1% Tween 20 (and then incubated with biotinylated antimouse immunoglobulin and avidin-biotin peroxidase complex reagents (Vector Laboratories, Inc.) according to the manufacturer's instructions. Detection signal was developed using an enhanced chemiluminescence detection system (NEN Life Science Products) according to the manufacturer's directions. Biotin broad range protein molecular weight markers were used to determine the molecular weights, and Kaleidoscope polypeptide markers were used to monitor electrophoresis and Western transfer of proteins in the gel (Bio-Rad Laboratories, Inc.).

Antibodies

Monoclonal antibodies to detect the α-subunit and the β_A- and β_B-subunits of inhibin were obtained from Oxford Bio-innovation (Oxford, UK), and the monoclonal to follistatin obtained from R&D Systems, Inc. (Oxford, UK).

In situ hybridization

This was performed using a fluorescent-labeled cDNA oligonucleotide probe directed against mRNA encoding the activin β_A- and β_B-subunits in sections of human uterine tubes. The complete cDNA sequences of the β_A-subunit (accession no. GI = 204936) and β_B-subunit (accession no. GI = 186422) were obtained from GenBank (NIH, Bethesda, MD). Human oligonucleotide probes complementary to the β_A- and β_B-subunits, comprising 30 nucleotides each, were obtained from (Cruachem Ltd., Glasgow, UK).

Sense probes specific for each of the subunits were used for control hybridizations, and in every experiment a negative control sample was included for each set of conditions tested. The *in situ* hybridization procedure was briefly as follows: 5 μm formaldehyde-fixed sections were deparaffinized, rehydrated, and washed in 4× saline sodium citrate (SSC) solution and incubated at 37 C with pepsin (12.5 μg/ml in 0.2 M HCl) for 20 min. The sections were then washed in diethylpyrocarbonate (Sigma, Poole, UK) water and prehybridized for 2 h at 37 C with the hybridization buffer without probe (10% polyvinylpyrrolidone, 20× SSC, 10% Ficoll, and 0.22 ml salmon sperm DNA). Hybridization was performed at 37 C overnight with a final probe concentration of 200–500 ng/ml. Sense probe and hybridization buffer without probe were used as negative controls. Sections were then washed in 4× and 2× SSC at 37 C for 5 min, and then incubated in normal sheep serum (1:5 dilution in TBS with 3% BSA and 0.1% Triton) added at room temperature for 20 min, followed by antifluorescein alkaline phosphatase-conjugated antibody (Roche Molecular Biochemicals, Mannheim, Germany) for 60 min at room temperature (1:200 dilution in TBS with 3% BSA and 0.1% Triton). Sections were visualized with BCIB-nitro blue tetrazolium mixture (Life Technologies, Inc., Uxbridge, UK). Sections were counterstained with methyl green, dehydrated, and mounted with piccolyte.

The sections were observed on a Labor Lux microscope (Leitz) at a magnification of ×400. A positive reaction was characterized by the presence of nuclear purple staining. Representative sections were photographed using a Wild MPS 48/52 Photoautomat (Leica Corp.).

Results

Uterine tubes were obtained from 11 premenopausal women (median age, 37 yr; range, 31–45 yr) and 5 postmenopausal women (median age, 59.5 yr; range, 55–65). Immunohistochemistry using a range of dilutions of antibody specific for the α-subunit failed to demonstrate any positivity in any of the sections of the tube (5 or 1 μm) in either premenopausal (Fig. 1, A and B) or postmenopausal (Fig. 2, A and B) women. However, positive staining was observed in all tissue samples for the inhibin β_A- and β_B-subunits as well as follistatin (Tables 1 and 2).

In both premenopausal (Fig. 1, C and D) and postmenopausal (Fig. 2, C and D) groups, labeling of the 5-μm sections with antibodies directed against the β_A-subunit resulted in the detection of immunoreactivity in both the ampulla (Fig. 2C) and

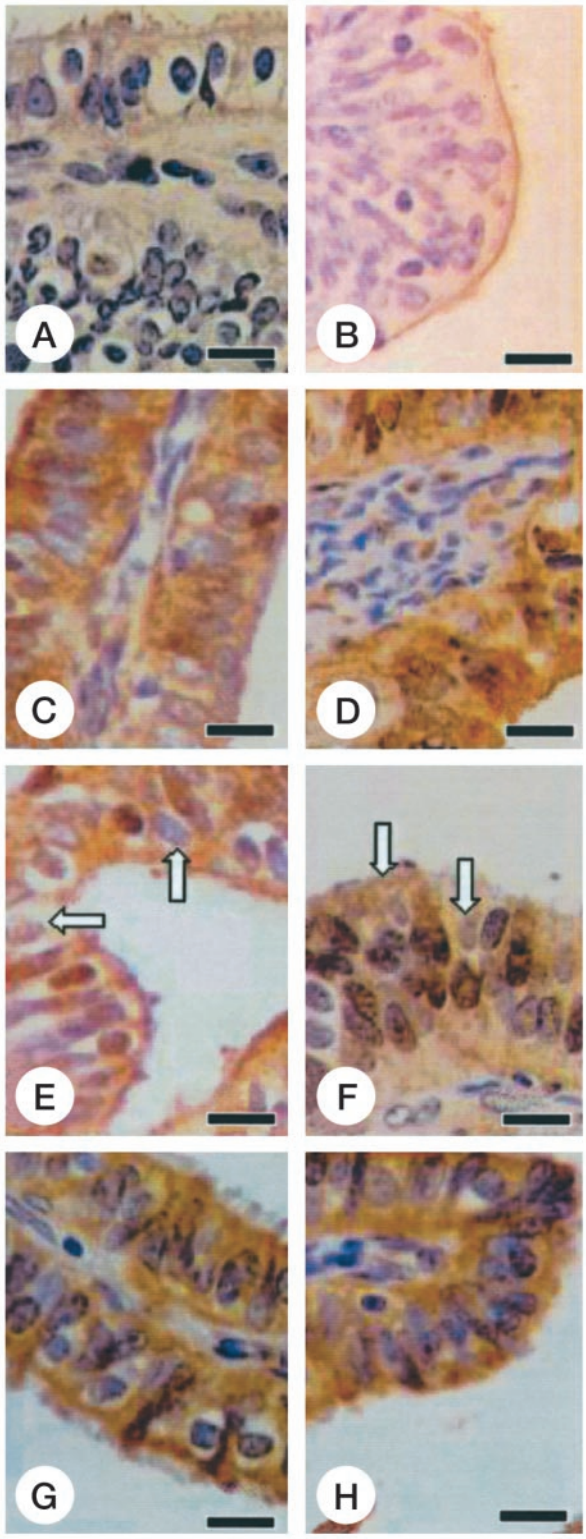

FIG. 1. Immunohistochemical localization of the α-subunit (A and B), the β_A-subunit (C and D), the β_B-subunit (E and F), and follistatin (G and H) in the ampullary (*left series*) and isthmic (*right series*) regions of uterine tube sections from premenopausal women. No immunoreactivity was observed for the α-subunit (A and B). However, staining was observed for β_A-subunit (C and D), β_B-subunit (E and F), and follistatin (E and F). All showed a similar pattern of staining, although with the β_B-subunit not all cells were stained (*arrow*). *Scale bar*, 20 μm.

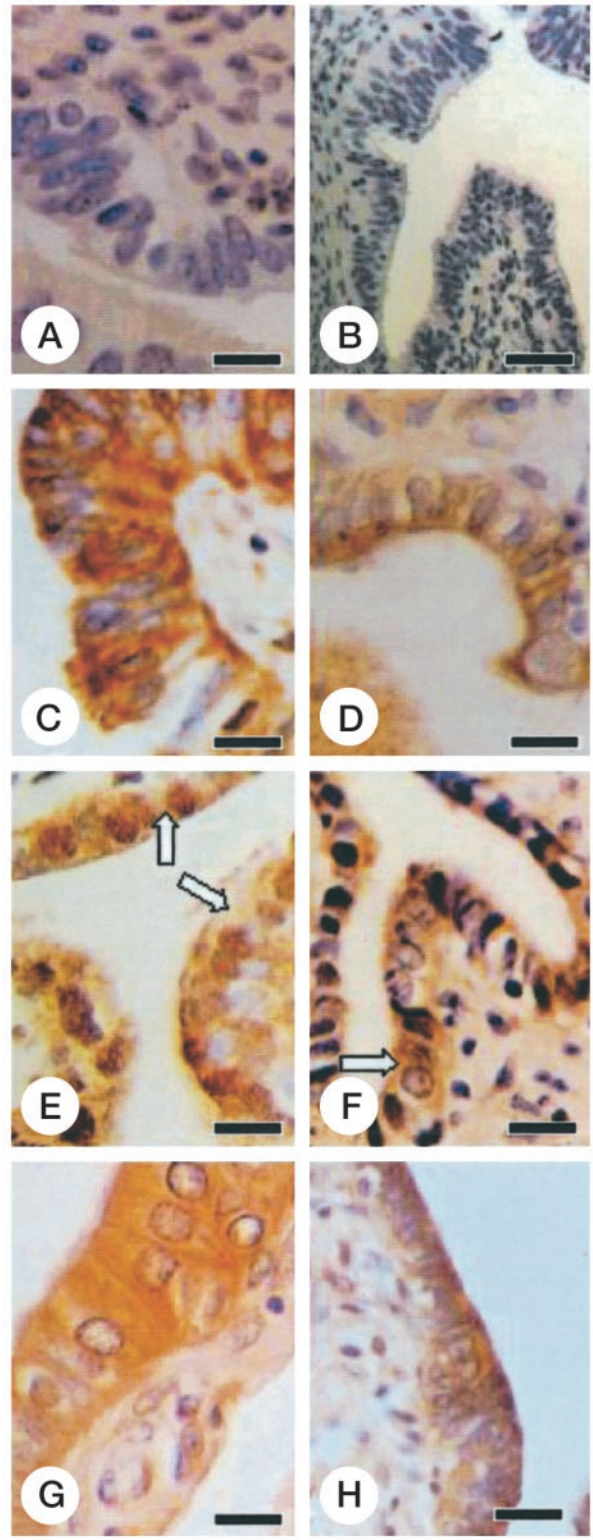

FIG. 2. Immunohistochemical localization of the α-subunit (A and B), the β_A-subunit (C and D), the β_B-subunit (E and F), and follistatin (G and H) in the ampullary (*left series*) and isthmic (*right series*) regions of uterine tube sections from postmenopausal women. No immunoreactivity was observed for the α-subunit. However, staining was observed for the β_A-subunit (C and D), β_B-subunit (E and F), and follistatin (E and F). All showed a similar pattern of staining, although with the β_B-subunit, not all cells were stained (*arrow*). *Scale bar*, 20 μm.

TABLE 1. Summary of the intensity of immunohistochemical expression of inhibin βA, βB, and follistatin in 22 premenopausal human uterine tubes

	Patient number										
	01	02	03	04	05	06	07	08	09	10	11
βA-Subunit											
Ampulla	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	++
Isthmus	+	+	+	+	+	+	+	+	+	+	+
βB-Subunit											
Ampulla	++	++	++	++	++	++	++	++	++	++	++
Isthmus	+	+	+	+	+	+	+	+	+	+	+
Follistatin											
Ampulla	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	++
Isthmus	+	+	+	+	+	+	+	+	+	+	+

For each patient, both tubes were examined.

TABLE 2. Summary of the intensity of immunohistochemical expression of inhibition βA, βB, and follistatin in 12 postmenopausal human uterine tubes

	Patient number					
	01	02	03	04	05	06
βA-Subunit						
Ampulla	++	++	++	++	++	++
Isthmus	+	+	+	+	+	+
βB-Subunit						
Ampulla	++	++	++	++	++	++
Isthmus	+	+	+	+	+	+
Follistatin						
Ampulla	++	++	+	++	++	++
Isthmus	+	+	+	+	+	+

For each patient, both tubes were examined.

isthmus (Fig. 2D). The antibody clearly labeled the cytoplasm and nucleus of the epithelial cells, with the intensity of staining decreasing toward the isthmic region (Tables 1 and 2). Staining intensity for the β_B-subunit in premenopausal (Fig. 1, E and F) and postmenopausal (Fig. 2, E and F) women showed a similar localization to the β_A-subunit (see also Tables 1 and 2). Of particular interest was the pattern of expression observed, where some of the columnar epithelial cells showed nuclear localization of β_B-subunit, whereas adjacent cells of similar morphology sometimes displayed no localization (see *arrow* in Fig. 2). To further characterize the nuclear localization of the β_A- and β_B-subunits, 1-μm sections were cut and immunostained with the respective antibody (data not shown). This confirmed that not all of the epithelial cells were stained for these subunits, and in the majority of cases this staining was nuclear.

Immunoreactivity for follistatin was detected in the cytoplasm of epithelial cells from both premenopausal (Fig. 1, G and H) and postmenopausal (Fig. 2, G and H) women. Immunoreactivity appeared most strongly positive in the ampullary region of the tube and decreased toward the isthmus in both premenopausal (Table 1) and postmenopausal (Table 2) women.

Immunoblot analysis

One form of protein, with an apparent molecular mass of 28 kDa, was present in the rat ovary (positive control) and the human uterine tube (Fig. 3, *arrow A*) when examined under reducing conditions. A further band of approximately 60 kDa

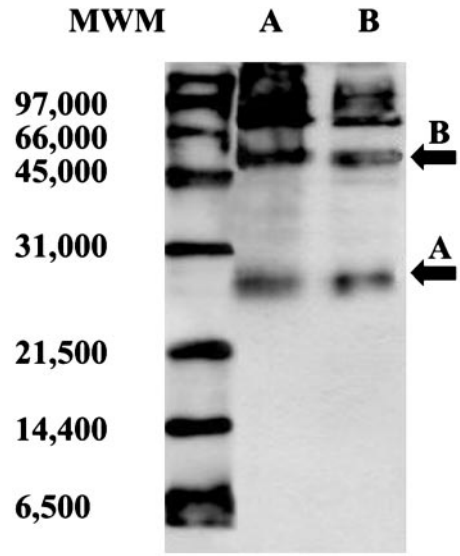

FIG. 3. Western blot of proteins from the rat ovary (lane A) and human uterine tube (lane B) separated by SDS-PAGE under reducing conditions. Both tissues show two major forms of immunoreactive protein at 28 and 60 kDa; they correspond to the activin dimer and the pro-protein, respectively. The high molecular mass protein of about 70 kDa may be another form of the pro-protein of β_A-subunits.

(Fig. 3, *arrow B*) and other of 70 kDa or greater were also detected in both samples at varying density. Under nonreducing conditions no bands were detected (data not shown).

In situ hybridization

Figures 4 and 5 show the results of *in situ* hybridization of premenopausal uterine tube tissue and related controls for the β_A-subunit (Fig. 4) and β_B-subunit (Fig. 5). In both cases, positive staining, shown by a purple color (indicating the presence of mRNA), was associated with the tubal epithelial nuclei (Figs. 4E and 5E). Two-week-old rat testis was used as a positive control tissue for the β_A-subunit (Fig. 4A), and human epididymal tissue was used as a positive control for the β_B-subunit (Fig. 5A). For both subunits, staining was absent in the negative controls (Figs. 4, B and D, and 5, B and D). A poly(deoxythymidine)oligonucleotide probe was used to monitor the sample preparation, and in both cases was positive (Figs. 4C and 5C).

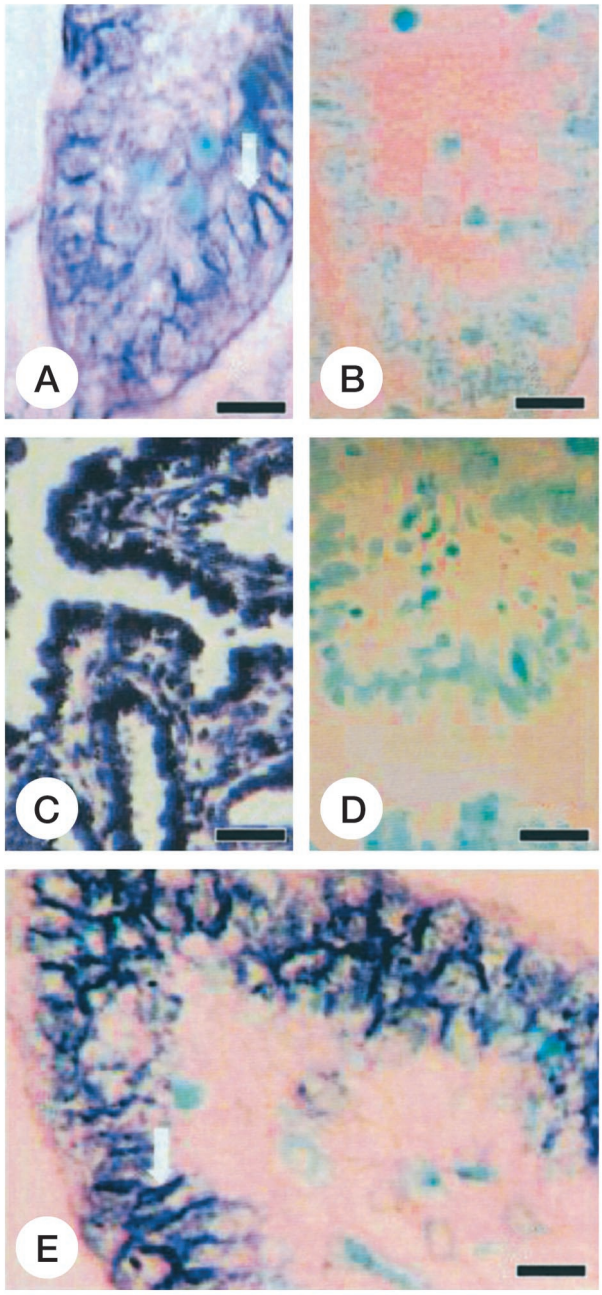

FIG. 4. Localization of activin β_A mRNA in tissue sections of uterine tubes from premenopausal women showing mRNA localization in the nucleus of epithelial cells. A, Rat testis incubated with antisense probe for β_A-subunit (positive control); B, rat testis incubated with sense probe (negative control); C, human uterine tube incubated with poly(deoxythymidine)oligonucleotide probe; D, human uterine tube incubated with hybridization buffer; E, human uterine tube incubated with antisense probe for β_A-subunit. *Scale bar*, 17 μm for A, B, and D; 4 μm for C; and 12.5 μm for E. Positive staining is shown in *purple*.

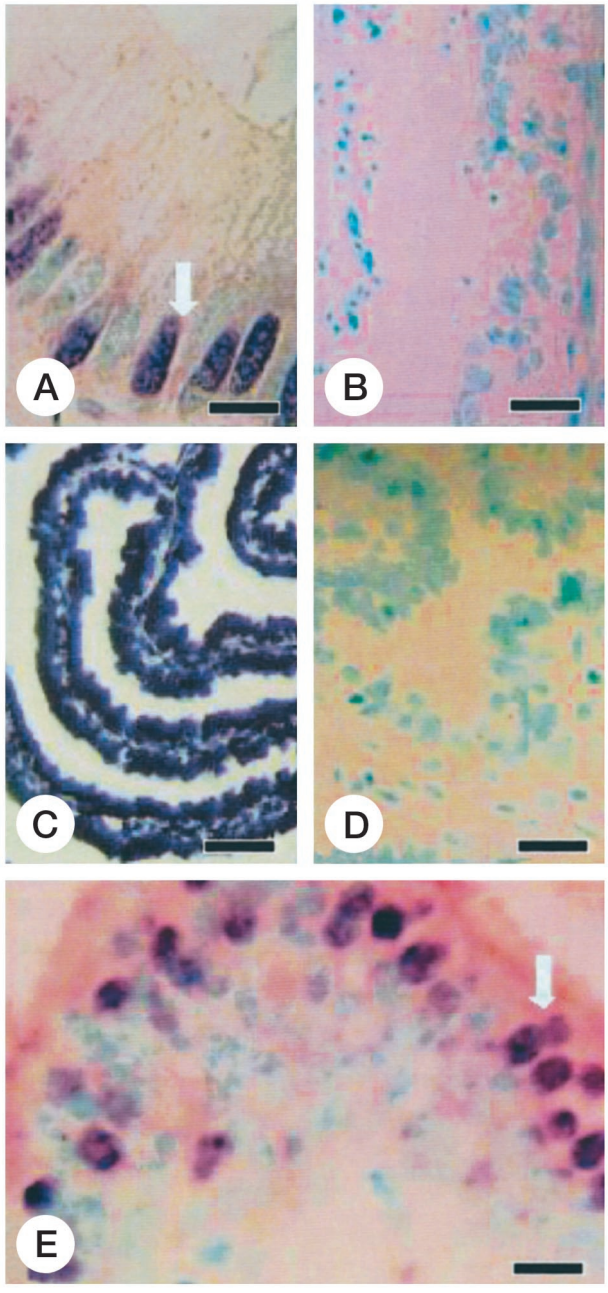

FIG. 5. Localization of activin β_B mRNA in tissue sections of uterine tubes from premenopausal women showing mRNA localization in the nucleus of epithelial cells. Human epididymis incubated with antisense probe for β_A-subunit (A; positive control), human epididymis incubated with sense probe (B; negative control), human uterine tube incubated with poly(deoxythymidine)oligonucleotide probe (C), human uterine tube incubated with hybridization buffer (D), and human uterine tube incubated with antisense probe for β_B-subunit (E). *Scale bar*, 17 μm for A, B, and D; 4 μm for C; and 12.5 μm for E. Positive staining is shown in *purple*.

Discussion

Inhibin and activin are classically involved in the regulation of FSH secretion (1). However, more recently they and other members of the TGFβ superfamily have been implicated in paracrine and autocrine regulation of cellular function in the gonad. To date, several studies have demonstrated synthesis of inhibins and activins in the fetal gonads of rats, chickens, cows, sheep, subhuman primates, and humans (25, 26). This study is the first to identify the presence of inhibin/activin β-subunits in the epithelium of the human uterine tube (the endosalpinx). We were not able to detect the pres-

ence of α-subunit of inhibin, suggesting that the endosalpinx may synthesize activins, not inhibins.

The data presented here demonstrate localization of the activin subunits and follistatin to the epithelium of both isthmus and ampulla. The intracellular localization of the β_B-subunit was particularly unusual, with adjacent epithelial cells appearing to show extreme variation in the level of expression, with intense nuclear staining in one cell and none in the next. This demonstrates a functional pleiomorphism within morphologically similar epithelial cells. In contrast, the majority of immunoreactions for β_A-subunit were seen in cytoplasm, although nuclear staining was also detected. Again, only a proportion of epithelial cells appeared to exhibit positive nuclear staining. Follistatin immunoreaction was restricted to the epithelial cytoplasm.

The intensity of staining for both subunits (and follistatin) was greatest in the ampullary region of the tube. However, in all tissues studied, the β_A- and β_B-subunits were coexpressed with follistatin, the binding protein of activin (2), suggesting that the activin dimer has a local paracrine or autocrine role. Fertilization and the earliest stages of embryo development occur in the tubal ampulla, and it is possible that activin derived from the endosalpinx may participate in this process. A number of studies report a role for activin in early embryo development. The mRNAs for the activin β_A-subunits, follistatins, and activin type I and type II receptors are expressed in bovine oocyte and embryos from immature oocyte to hatched blastocysts (27). Sidis *et al.* (15) and recently Ying *et al.* (18) demonstrated that early mouse and human embryos, from four-cell to morula stage, were unable to synthesize activin, as no mRNA for these proteins was detected; activins that bind to the activin receptor I and activin receptor II on the preimplantation embryo may originate from surrounding tissue, such as the uterine tube; and oocytes are capable of receiving an activin signal emitted by surrounding cumulus cells, but not of transmitting one. Other studies show that follistatin mRNA is expressed early in the gastrula stage of *Xenopus* embryos (28) and in peri- and postimplantation mouse blastocysts (15). Also in humans, the expression of β_A-subunit, activin receptor I and follistatin was hardly detectable in preimplantation embryos before the morula stage, but noticeably increased at the blastocyst stage, indicating that the expression of β_A-subunit activin receptor I and follistatin was dependent on embryonic developmental stage (18). Mouse embryos have been shown to produce activins, but not inhibins (27).

Immunohistochemistry results alone do not provide evidence that the β_A- and β_B-subunits are synthesized by the endosalpinx. To investigate this we performed *in situ* hybridization using the same tissue samples. The results of these experiments provide evidence that activin subunits are synthesized by the endosalpinx. Immunoblotting with antibodies directed against the β_A-subunit under reducing conditions indicates the presence of a 28-kDa protein. This would be equivalent to mature activin. The appearance of the 28-kDa band under reducing conditions might be due to the release of mature activin from follistatin-binding protein. This phenomenon has previously been reported by Rabinovici *et al.* (29) and Klein *et al.* (30), Given the specificity of the monoclonal antibodies to the β-subunits, we doubt that the 28-kDa band represents another protein and propose that the three-dimensional configuration of the activin molecule around the disulfide bonds may impart unusual strength to the adhesion between subunits.

Follistatin has a high affinity for activin under physiological conditions, but activin can be released from binding either through proteolytic action or after a change in follistatin configuration. Either or both of these events may be achieved under reducing conditions (31). Under nonreducing conditions, no free activin A was detected, possibly due to failure of the β-subunit antibody to recognize activin/follistatin complexes (32). A recent study by Klein *et al.* (30) has demonstrated that free inhibin can be detected under reducing conditions, and that the detected 32-kDa inhibin did not reduce further to its α- and β-subunits. Immunoblotting with β_A-subunit antibodies also shows a high molecular mass band of about 70 kDa. This may be a form of pro-β_A resulting from variable proteolytic processing of the β-subunit pro-sequence (pro-β_A) at the surface of the endosalpinx. β-Precursors are known to dimerize with other β-precursors or mature β_A-subunit forms to produce a number of high molecular mass activin molecules (33).

The detection of positive staining for activin subunits and follistatin in the uterine tubes from postmenopausal women is intriguing. Activin secreted from the endosalpinx of postmenopausal women may exert a paracrine/autocrine effect within the uterine tube itself. This suggestion is in agreement with the findings of Menon *et al.* (34), who showed that postmenopausal women have measurable concentrations of activin A, but not inhibin, in the circulation. During perimenopause, decreasing levels of estradiol and inhibin reduce negative feedback inhibition on the hypothalamo-pituitary axis, producing a concomitant rise in FSH secretion into the bloodstream (35–37). At menopause, ovarian inhibin production ceases, and circulatory inhibin becomes undetectable. Small amounts of estradiol, originating from nonovarian sources, remain detectable in the circulation. Taken together, our findings in postmenopausal women show that activin, but not inhibin, is expressed by the endosalpinx, and that significant levels of activin A, but not follistatin, in the postmenopausal circulation may be involved in paracrine/autocrine functions within the uterine tube itself.

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