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Tissue-Specific Expression of Inhibin/Activin Subunit and Follistatin mRNAs in Mid- to Late-Gestational Age Human Fetal Testis and Epididymis

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Inhibin/activin subunit (α, βA, and βB) immunoreactive protein localization patterns and cell type specific inhibin α-subunit mRNA expression have been examined in early- to midgestational age human fetal testes. The scarcity of available third trimester human fetal tissue has, however, prevented a complete examination throughout the gestational period and the cell specific expression of follistatin and βA- and βB-subunit mRNAs are currently unknown at any gestational age. In the present study, this gap is filled and report mRNA expression patterns of inhibin/activin subunits in mid- and late-gestational age (21–33 wk) human fetal testes and testicular duct system. We also report the first examination of follistatin mRNA signals in the human fetal gonad is also reported. Inhibin/activin α-subunit mRNA signal is present in both tubular and interstitial cells, and βB-subunit mRNA is expressed in seminiferous tubules, in mid- and late-gestational age human fetal testes. Inhibin/activin βA-subunit mRNA was detected in the interstitial cells of remarkably well preserved mid (21 and 22 wk) and late (29 wk) gestational age testis, and is the only activin-system factor mRNA also expressed in tissue of the duct system of the testis (smooth muscle cells of the epididymis). Follistatin mRNA signal was equal to background levels in testicular and duct tissues at all ages examined. These cell specific expression patterns suggest prominent and possibly differential roles for the inhibins and activins, unopposed by gonadal follistatin, in the human fetal male reproductive system.

Key Words: Inhibin; activin; follistatin; testis; human; fetus.

Introduction

Numerous studies have examined the functional roles and cell type specific expression patterns of inhibin/activin subunits (α, βA, and βB), their binding protein (follistatin), and type II activin receptors (ActRII and ActRIIB) in adult animals. Inhibin (α/β) and activin (β/β) are glycoprotein dimers originally isolated from bovine or porcine ovarian follicular fluid and ovine rete testis fluid. Inhibin specifically downregulates and activin upregulates follicle-stimulating hormone (FSH) production and secretion *(1)*. Although it has been shown that inhibin and activin are produced by the fetal gonads of rats, chick, ovine, bovine, human, and subhuman primates *(2–9)*, the tissue specific expression and physiological roles of these factors in the fetus is virtually unexplored. It has been found that in the fetal rat testis, mRNAs encoding activin-system factors are expressed in cell type specific patterns similar to that observed in immature and adult rats *(10–12)*. In vivo manipulations with inhibin or FSH have provided evidence that the FSH-inhibin feedback system and intragonadal communications are functionally active in the ovine fetus *(2,3)*.

In the human, Erämaa et al. *(13)* reported high levels of α- and βB-subunit mRNA expression, with Northern blot analysis, in the 13–25 wk gestational age testis (Term ~40 wk). Low levels of βA-subunit mRNA were detected with reverse-transcription polymerase chain reaction (RT-PCR). At the beginning of the second trimester (13 wk gestational age) a strong hybridization signal was observed for the α-subunit in seminiferous tubules and a weak signal was found in interstitial cells *(13)*. Rabinovici et al. *(4)* reported positive cytoplasmic staining for the inhibin-α subunit in interstitial and intratubular cells, and immunopositive staining for the βA- and βB-subunits in clusters of Leydig cells in midgestational age (16–23 wk) human fetal testis. Cultured testicular cells from midgestational age human fetal testis secreted detectable levels of radioimmunoassayable inhibin-α subunit following gonadotropic (FSH or hCG) treatment *(4)*.

The tissue specific mRNA expression patterns of inhibin/activin βA- and βB-subunits, and follistatin, are

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Table 1
Distribution of mRNAs Encoding Activin-System Factors in Human Fetal Testis and Testicular Duct System as Determined by *In Situ* Hybridization Analysis

	α	βA	βB	Follistatin
Seminiferous tubules	21–33	–	21–33	–
Interstitial tissue	21–33	21–29	–	–
Epididymis	–	22–29	–	–

Numbers indicate gestational age mRNA signal is detected. Not detected (–).

currently unknown at any gestational age in the human fetal gonad. Moreover, because late-gestational age human fetal tissue is scarce and difficult to obtain, studies examining the activin-system in third trimester human fetal testis have not been done. Utilizing cDNA clones specific for human activin-system factors available at the time this study was initiated (inhibin/activin α-, βA-, βB-subunits, and follistatin), cell specific expression patterns of inhibin/activin-subunit and follistatin mRNAs in mid- and late-gestational age human fetal testes and testicular duct system are reported.

Results

The localization of mRNAs encoding inhibin/activin subunits (α, βA, and βB) and follistatin was determined in the developing human fetal testis and duct system from 21 to 33 wk gestational age. The mRNA signals were considered positive if grain density was at least four times the control grain density over a 100,000 μm^2 area *(14)*. A summary of the temporal and spatial distributions of the mRNA signals is shown in Table 1.

Inhibin-α Subunit

Moderate to abundant mRNA signal for the inhibin-α subunit was observed in the seminiferous tubules of each of the mid- and late-gestational age (21, 22, 29, 30, and 33 wk) human fetal testis examined. Weak to moderate mRNA expression was also seen over individual cells in the interstitial tissue of the testis at each of these ages (Figs. 1–3). Inhibin-α subunit mRNA signal was not found in tissues of the testicular duct system, including the epididymis, at any age (Fig. 1).

Inhibin/Activin-βA Subunit

Weak to moderate mRNA signal for the inhibin/activin-βA subunit was detected in the interstitial tissue of remarkably well preserved mid- (21 and 22 wk) and late-gestational age (29 wk; both samples) human fetal testis (Figs. 1–3). The cell specific βA-subunit mRNA signal was not detected in the interstitial tissue of the 30 and 33 wk fetal gonads or in the seminiferous tubules at any gestational age. The mRNA signal encoding the βA-subunit was abundantly expressed in the smooth muscle cells of the epididymis of the 22 and both 29 wk gestational age testes (Figs. 1 and 4).

Inhibin/Activin-βB Subunit

Abundant levels of mRNA encoding the βB-subunit were expressed exclusively in the seminiferous tubules of 21, 22, 29, 30, and 33 wk human fetal testes (Figs. 1–3). βB-subunit mRNA expression was not observed in interstitial tissue of the testis or in tissues of the testicular duct system, including the epididymis, (Figs. 1 and 4) at any of these ages.

Follistatin

A positive follistatin mRNA signal was not detected in mid- or late-gestational age human fetal testis or in any tissues of the duct system of the testis (Figs. 1–3). These results are in agreement with previous studies showing high levels of expression of follistatin mRNA in fetal rat ovary, and undetectable levels of expression in the fetal rat testis *(12,15)*.

Discussion

This study was undertaken to simultaneously determine the spatiotemporal patterns of expression of mRNAs encoding activin-system factors in the human fetal testis and testicular duct system. The localization patterns are similar to findings in the fetal rat *(11,12,15)*. In both species, α-subunit mRNA expression is found in tubular and interstitial cells, βB-subunit mRNA expression is present only in tubular cells, and βA-subunit mRNA is expressed in testicular interstitial tissue and in the epididymis, and follistatin mRNA signal is undetectable in any testicular tissue. Localization patterns of activin-system mRNAs (α- and βA-subunits) have also been examined in the ovine fetal gonad. In agreement with the aforementioned results, inhibin-α subunit mRNA was localized in the seminiferous tubules, and βA-subunit mRNA was below *in situ* hybridization assay detection limits at all mid- and late-gestational ages examined *(16)*. Similarly, the pattern of inhibin-α subunit mRNA expression in seminiferous tubules and interstitial cells of human fetal testes generally coincides with the localization of immunoreactive inhibin α-subunit protein in the interstitial and intratubular cells of fetal and adult humans, nonhuman primates, sheep, and rats *(4,10,16–18)*. Results are, therefore, supportive of a gen-

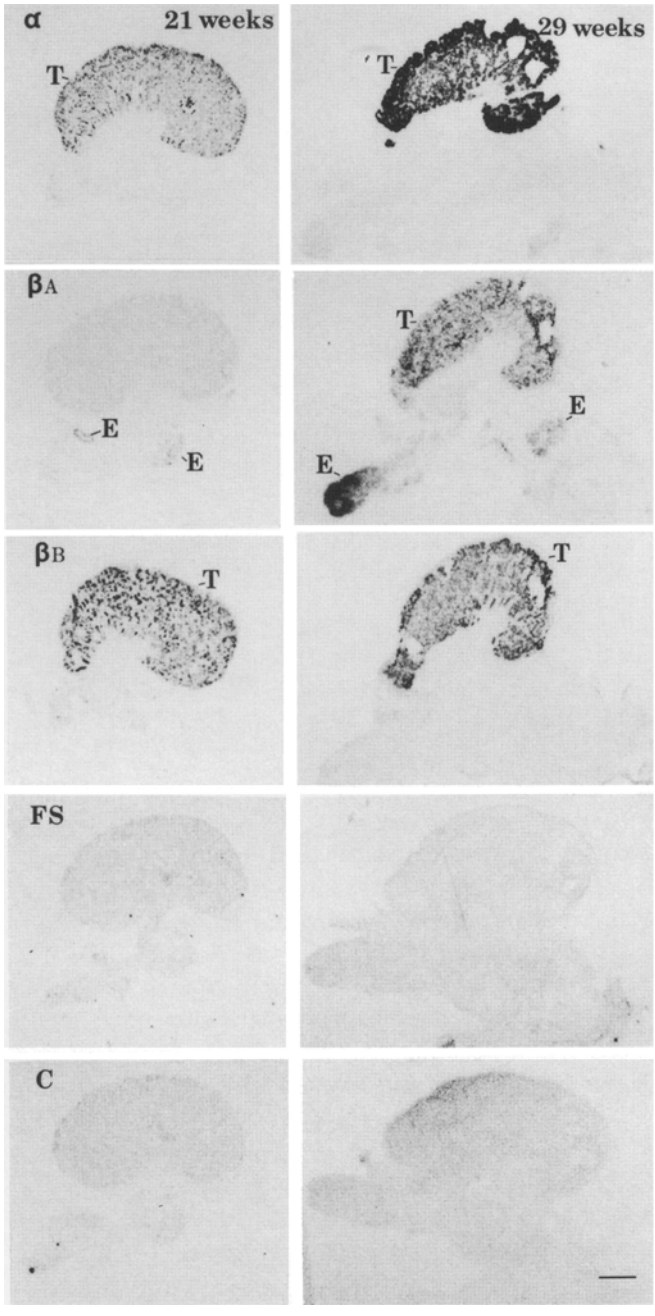

Fig. 1. *In situ* hybridization localization of activin-system mRNA signals in a representative midgestational age (21 wk; left column) and a late gestational age (29 wk; right column) human fetal testis and epididymis using ^{35}S-labeled antisense probes specific for follistatin (FS) or inhibin/activin-α, -βA, or -βB subunit mRNAs. The βA figure in the left column is of a 22 wk testis and duct system. Low magnification of autoradiographic images on X-ray film show α- and βB-subunit mRNA expression in the testis (T). The βA-subunit mRNA signal can be seen in the 29 wk testis and in the epididymis (E) of both gestational ages. The follistatin mRNA signal is not significantly different from background hybridization levels. Background levels are shown in representative sections (C) using a ^{35}S-labeled sense βA-subunit RNA probe. Bar, 1 mm.

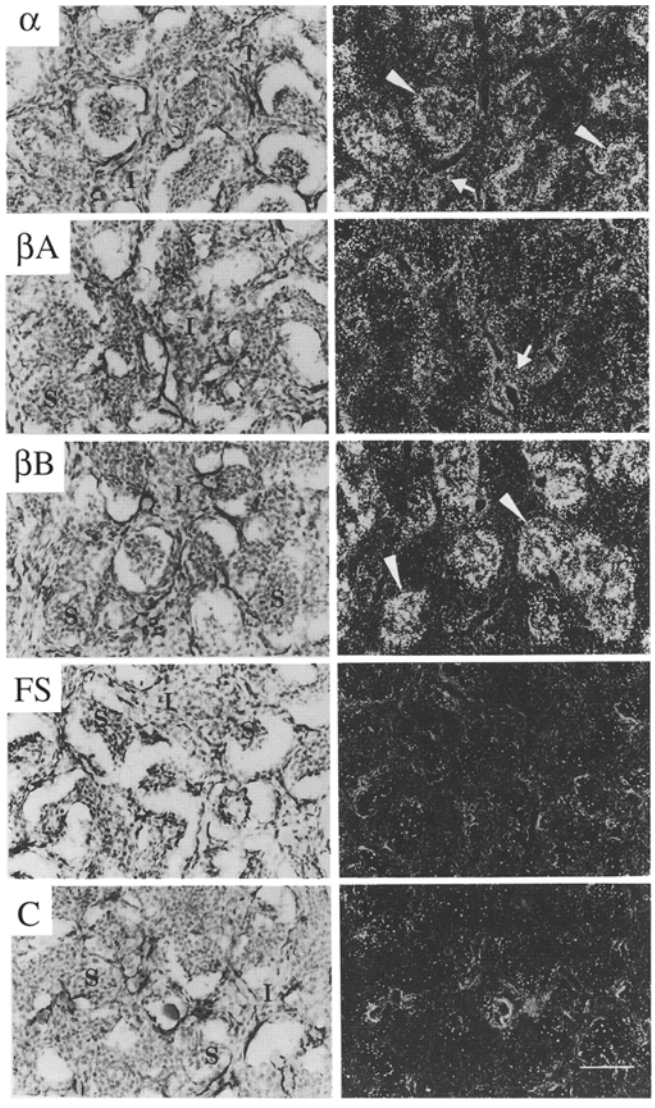

Fig. 2. High magnification *in situ* hybridization localization of mRNAs encoding follistatin (FS) or inhibin/activin-α, -βA, or -βB subunits in a mid gestational age (21 wk) human fetal testis using specific ^{35}S-labeled antisense probes. Photomicrographs of emulsion-dipped sections show the morphology (bright-field, left column) and the mRNA signals (white grains in dark-field, right column). Moderate to abundant α- and βB-subunit mRNA signals can be seen over the seminiferous tubules (S; arrowheads). Weak α- and βA-subunit mRNA signals are present in interstitial tissue (I; arrows). The bottom row shows the density of grains using a labeled sense strand mRNA probe for the inhibin/activin βA-subunit (C) and is representative of the grain density for each of the sense strand mRNA probes. Bar, 100 μm.

eral similarity in the tissue specific expression patterns of activin-system factors in human fetal gonads and other mammalian species. Consequently, in vivo and in vitro manipulations with animals and animal tissues are likely to provide crucial information toward understanding the functional significance of the activin-system in the human fetal reproductive axis.

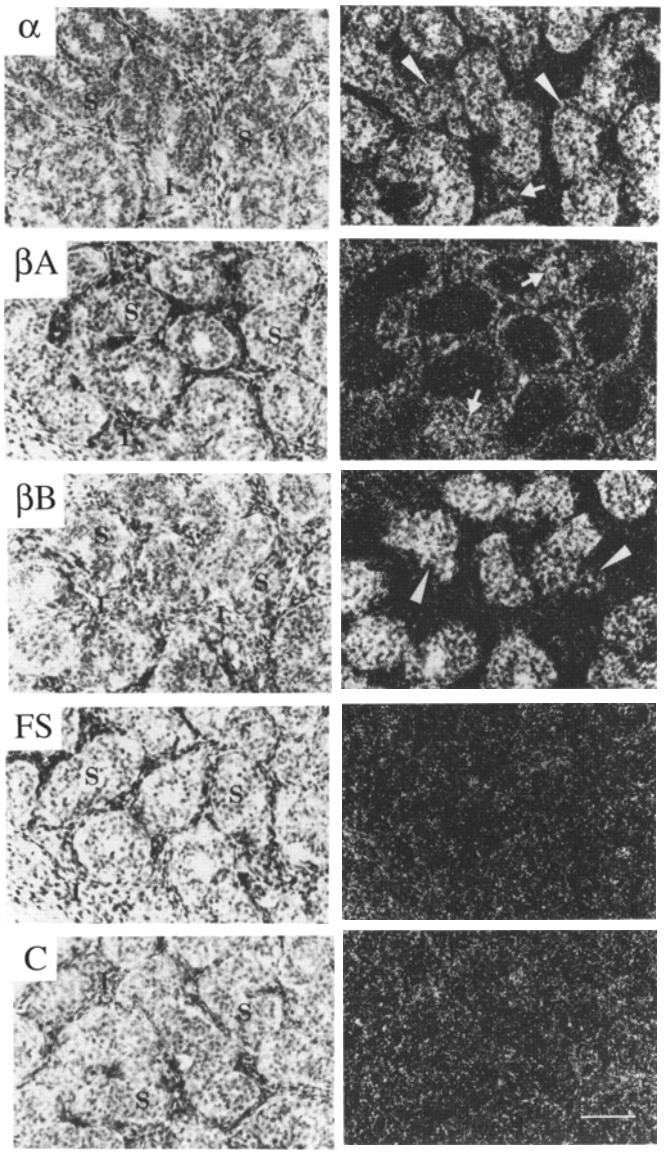

Fig. 3. High magnification *in situ* hybridization localization of activin-system factors in a late gestational age (29 wk) human fetal testis using ^{35}S-labeled antisense probes specific for mRNAs encoding follistatin (FS) or inhibin/activin-α, -βA, or -βB subunits. Photomicrographs of emulsion-dipped sections show the morphology (bright-field, left column) and the mRNA signals (white grains in dark-field, right column). Abundant α- and βB-subunit mRNA signals can be seen over the seminiferous tubules (S; arrowheads). Moderate α- and βA-subunit mRNA signals are present in interstitial tissue (I; arrows). The bottom row shows the density of grains using a labeled sense strand mRNA probe for the inhibin α-subunit (C) and is representative of the grain density for each of the sense strand mRNA probes. Bar, 100 μm.

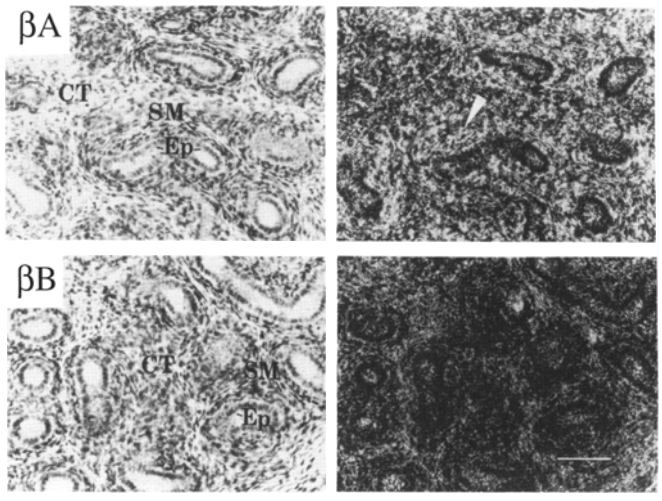

Fig. 4. High magnification *in situ* hybridization localization of activin-system factors in a late gestational age (29 wk) human fetal epididymis using ^{35}S-labeled antisense probes specific for inhibin/activin-βA or -βB subunit mRNAs. Photomicrographs of emulsion-dipped sections show the morphology (bright-field, left column) and the mRNA signals (white grains in dark-field, right column). Abundant βA-subunit mRNA signal can be seen over smooth muscle tissue (SM; arrowhead). Grain density over connective tissue (CT) or epithelial tissue (Ep) is not significantly different from background levels. The βB-subunit mRNA signal is not significantly different from, and is therefore representative of, background hybridization levels. Bar, 100 μm.

Physiological studies show that, as in the adult primate and juvenile rat *(19,20)*, the FSH-inhibin feedback loop is functionally active in the mammalian fetus. Pulsatile administration of FSH to chronically catheterized late gestational age ovine fetuses stimulates production of biologically active inhibin, and decreases testosterone synthesis, in the ovine fetal gonad *(3)*. Moreover, bolus administration of inhibin-rich charcoal-treated porcine follicular fluid to ovine fetuses selectively suppresses plasma FSH whereas LH levels are not affected *(2)*. Treatment of testicular cells from midgestational age human fetal gonads with FSH or hCG significantly increases radioimmunoreactive inhibin α-subunit levels in the culture media. FSH treatment also induces a significant increase in the secretion of immunoreactive inhibin α-subunit levels from late-gestational age rhesus monkey testicular cells *(4)*.

The tissue specific expression of inhibin/activin βA- and βB-subunit mRNAs in the human fetal testis was previously unknown at any gestational age. The data, demonstrating relatively abundant βB-subunit mRNA signal in the seminiferous tubules and low to moderate levels of βA-subunit mRNA expression in interstitial tissue of mid- to late-gestational age human fetal testis, is compatible with an earlier report showing high levels of βB-subunit mRNA expression, using Northern blot analysis, and low levels of βA-subunit mRNA expression, only detectable with RT-PCR, in human fetal testis 13–25 wk old *(13)*.

Although the βA-subunit mRNA signal was found only in the testis of 21, 22, and 29 wk old fetuses and in the epididymis of 22 and 29 wk old fetuses, it is likely that βA-subunit mRNA expression is present, but is below the detection limit of our *in situ* hybridization assay, at the other gestational ages. The relative abundance of mRNA signals detected by *in situ* hybridization is dependent upon

tissue preservation and integrity. In contrast to studies using animals, acquisition, and preservation of human autopsy tissue is variable and difficult to manage.

In men, circulating inhibin-A (α/βA) is undetectable whereas inhibin-B (α/βB) is measurable by enzyme-linked immunosorbent assay *(21)*. Inhibin-B is not detectable in the sera of orchidectomized men *(22)*. An inverse relationship between inhibin-B and FSH in the circulation of men is also found and demonstrates that inhibin-B is the predominant form of bioactive inhibin in men *(21,22)*. Physiological studies, described above, show that the FSH-inhibin feedback loop is functionally active in the mammalian fetus, but the predominant form of bioactive inhibin has not been determined *(2–4,19,20)*. Consistent with localizations in adult men *(17)*, the relatively abundant expression of inhibin/activin βB-subunit mRNA in the seminiferous tubules of the human fetal gonad, and low expression of βA-subunit mRNA in interstitial tissue, is supportive of inhibin-B as the predominant form of bioactive inhibin in the male fetus.

Activin-A is a member of the transforming growth factor β (TGFβ) superfamily, and numerous studies implicate activin-A in the regulation of growth and differentiation of various testicular cells. Activin-A stimulates spermatogonial and Sertoli cell proliferation in cultured testicular cells isolated from immature or adult rats *(23–25)*. In fetal rat testis organ culture, recombinant bovine activin-A inhibited thymidine incorporation in testes and male mesonephroi on day 14 pc in a dose-dependent manner *(15)*. Type II activin receptor knock-out mice have reduced seminiferous tubule volume, consistent with an overall decrease in Sertoli cell number, and are delayed in reaching fertility *(26)*. It is proposed that an intragonadal role of activin-A may be to function as a regulator of growth and development in the human fetal testis. Likewise, results suggest that activin-A modulates DNA synthesis in rat vascular smooth muscle cells in an autocrine manner *(27,28)*. These studies and the expression of mRNA encoding the inhibin/activin βA-subunit, and not the βB- or α-subunit, in human fetal epididymal smooth muscle is evidence for a potential role of activin-A in smooth muscle cells of the epididymus.

Follistatin binds to the inhibin/activin β-subunit and consequently modulates the biological actions of activin *(29–31)*. In the rat, follistatin mRNA is specifically detected in the fetal and immature ovary, but not in the testis using Northern blot analysis or *in situ* hybridization *(12,15,32)*. Studies in adult rats, on the other hand, have found high levels of follistatin mRNA expression in the ovary and relatively low levels in the testis using S1-nuclease analysis *(33)*. Correspondingly, in the adult human, follistatin mRNA and protein was found to be abundantly expressed in the ovary *(34)*, and human follistatin was originally cloned from a testicular cDNA library prepared from the testis of a 50-yr-old man *(35)*. It is reported here the first analysis of follistatin mRNA localization in human fetal gonads and, consistent with studies in the rat, follistatin mRNA is not detected, in the human fetal testis.

In summary, the tissue-specific expression of activin-system factors in human fetal testis generally coincides with localization patterns in the gonads of other mammalian species. Studies show that the FSH-gonadal inhibin feedback loop is functionally active in the fetus and it is suggested that inhibin-B may be a primary endocrine regulator of this system. Activin-A may serve an intragonadal role, unopposed by follistatin, as a regulator of growth and development in the human fetal testis and epididymis.

Materials and Methods

Tissue and Tissue Preparation

Human fetal testes were obtained (over the course of approx 5 yr), at the time of autopsy from spontaneously aborted 21, 22, 29 (×2, from different fetuses), 30, and 33 wk gestational age fetuses. Approval for use of human fetal autopsy tissue was granted from the Committee on Investigations Involving Human Subjects, UCSD, in accordance with the requirements of the code of Federal Regulations on the Protection of Human Subjects. Gestational age was determined by fetal foot length. Autopsy was performed 3–10 h postmortem. Tissue was placed in ice-cold 10% neutral buffered formalin for 1–4 wk. Sucrose (10%) was added 24 h before freezing the tissue in a 50/50 mixture of aquamount (Lerner Laboratories, Pittsburgh, PA) and OCT (Miles, Elkhart, IN) embedding medium. Frozen sections were cut on a cryostat at 25-μm thick, mounted on Fisher brand superfrost slides, and stored desiccated for 48 h at room temperature.

In Situ *Hybridization*

Antisense ^{35}S- or ^{33}P-labeled RNA probes specific for the human inhibin/activin α-, βA-, βB-subunits, and follistatin, were generated by transcription from the SP6, T7, or T3 promoters of linearized pGEM4 or pBluescript SK$^+$ subclones, each to a specific activity of 10^8–10^9 cpm/μg using an excess of ^{35}S-UTP (~1200 Ci/mol·L). Human RNA probe complementary to the α-subunit comprised 500 nucleotides, the βA-subunit 350 nucleotides, the βB-subunit 750 nucleotides, and follistatin 259 nucleotides. Sense ^{35}S-labeled RNA probes specific for each of the subunits, and follistatin, were used for control hybridizations. The mRNAs were detected in adjacent fixed-frozen sections using well-established *in situ* hybridization techniques *(11)*. Slide mounted sections were counterstained with H&E. Emulsion-dipped slide mounted sections and autoradiographic images on β-max film were photographed with Ilford (Paramus, NJ) XP2 35-mm film.

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