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Expression, Action, and Regulation of Transforming Growth Factor Alpha and Epidermal Growth Factor Receptor During Embryonic and Perinatal Rat Testis Development

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ABSTRACT: The objective of the current study was to extend previous observations and examine the expression pattern and effects of transforming growth factor alpha (TGFα) and epidermal growth factor receptor (EGFR) on embryonic testis morphogenesis and growth. The expression of TGFα was determined after morphological sex determination (seminiferous cord formation at embryonic day 13 [ED13]) through perinatal testis development (postnatal day 5 [PD5]) with a quantitative reverse transcription-polymerase chain reaction procedure. Expression of messenger RNA (mRNA) for TGFα appeared to be more dynamic during testis development when compared with the expression of mRNA for EGFR. Message for TGFα was reduced at ED16 and PD4, and was elevated at PD0 during testis development. In contrast, EGFR mRNA levels were negligible at ED15 and were elevated constitutively from ED16 through PD5. Immunohistochemistry was conducted at ED14, ED16, ED19, PD0, PD3, and PD5 to localize cellular expression of both TGFα and EGFR. At ED16, positive staining for EGFR was localized to the cords, and by ED19, was mainly in the cords with slight expression in the interstitium. From PD0 to PD5, positive staining for EGFR was detected in the germ, Sertoli, and interstitial cells. Immunohistochemistry for TGFα detected localization at ED14 and ED16 to the Sertoli cells and to specific cells in the interstitium. From ED19 through PD5, TGFα was detected in the Sertoli, germ, and interstitial cells, and in endothelial cells within the interstitium. To determine the effects of TGFα on embryonic testis growth and seminiferous cord formation, ED13 testis organ cultures were treated with sense and antisense TGFα oligonucleotides. Antisense TGFα inhibited testis growth by 25%–30% in ED13 testis organ cultures when compared with sense oligonucleotide control pairs. To examine the effects of TGFα on perinatal testis growth, PD0 testis cultures were treated with different doses of TGFα. TGFα increased thymidine incorporation into DNA in PD0 testis cultures. Therefore, TGFα appears to have actions on both embryonic and perinatal testis growth. The regulation of TGFα and EGFR mRNA levels were examined using PD0 testis cultures treated with hormones that stimulate testis growth. Follicle-stimulating hormone (FSH) stimulated ($P < .05$) and testosterone tended to stimulate ($P < .07$) mRNA expression of EGFR. Epidermal growth factor stimulation of PD0 testis cultures did not affect levels of mRNA expression for EGFR, but did suppress expression of mRNA for TGFα. These results taken together demonstrate that TGFα can act to regulate early embryonic and perinatal testis growth. Furthermore, TGFα and EGFR expression can be regulated through growth stimulatory hormones such as FSH and testosterone.

Key words: FSH, gametogenesis, mesenchymal-epithelial, Sertoli cell.

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Embryonic testis development is dependent on critical cellular differentiation and growth that culminates in morphological sex determination (seminiferous cord formation; Magre and Jost, 1980, 1991) and somatic and germ cell proliferation (Orth et al, 1988). Both cellular differentiation and growth are dependent on the expression and action of paracrine growth factors during testis development (Colenbrander et al, 1979; Orth et al, 1988; Cupp et al, 1999b, 2000; Levine et al, 2000a,b). Morphological differentiation of the testis involves the development of the seminiferous cords containing the germ and Sertoli cells, surrounded by the peritubular myoid cells and interstitium. Seminiferous cord formation occurs at approximately embryonic day (ED) 13.5 in the rat (plug date = ED0; Magre and Jost 1980, 1991) and is the first morphological indication of sex determination. Factors such as neurotropins (Levine et al, 2000a; Cupp et al, 2000), retinoids (Marinos et al, 1995; Cupp et al, 1999a), and hepatocyte growth factor (Ricci et al, 1999) have been implicated to affect seminiferous cord formation.

After the appearance of seminiferous cords all cell populations undergo rapid proliferation (ED14–ED18; Levine et al, 2000b), whereas the greatest levels of Sertoli cell proliferation occur from ED18 to postnatal day (PD) 0 (Levine et al, 2000b) during testis development. Several growth factors have been demonstrated to be important in embryonic and perinatal testis growth after ED17, in-

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cluding bFGF (Koike and Noumura, 1994), NGF, NT3 (Cupp et al, 2000), transforming growth factor beta (TGFβ; Olaso et al, 1998; Cupp et al, 1999b), retinoids (Cupp et al, 1999b), MIS (Munsterberg and Lovell-Badge, 1991), thyroid hormones (Van Haaster et al, 1992; Palmero et al, 1995; Buzzard et al, 2000), and follicle-stimulating hormone (FSH; Orth, 1982). In contrast, TGFβ (Olaso et al, 1998; Cupp et al, 1999b), activins (Boitani et al, 1995; Matzuk et al, 1995) and TGFα (Levine et al, 2000b) have been implicated in the regulation of embryonic testis growth just after seminiferous cord formation (ED14–ED15).

TGFα is a member of the epidermal growth factor (EGF) family, of which there are multiple related ligands including EGF, heparin-binding EGF, crypto, amphiregulin, betacellulin, and heregulin (Marquardt et al, 1984; Massague and Pandiella, 1993). The receptors for the EGF family include the epidermal growth factor receptor (EGFR), HER2, HER3, and HER4 (Holmes et al, 1992; Carraway et al, 1994). TGFα binds specifically to EGFR. The EGFR family of receptors elicit a response by activating a tyrosine kinase in the cytoplasmic domain after ligand binding (Carpenter and Cohen, 1990; Boonstra et al, 1995). The EGF growth factor family has been implicated in inducing cellular proliferation, differentiation, and migration in many different cell types. In addition, the EGF family can stimulate morphogenesis and growth in developing systems such as the kidney, lung, and mammary gland (Taub et al, 1990; Snedeker et al, 1991; Ono et al, 1992; Rogers et al, 1992; Warburton et al, 1992; Seth et al, 1993; Barros et al, 1995; Grandis et al, 1998). In the postnatal testis, TGFα and EGFR are expressed and have been demonstrated to stimulate proliferation, differentiation, and migration of Sertoli, Leydig, and peritubular cells (Tsutsumi et al, 1986; Skinner et al, 1989; Mullaney and Skinner, 1992). The objective of the current study was to extend previous observations on the general growth effects of TGFα during testis development (Levine et al, 2000b) and to further define the expression pattern, action, and potential regulation of TGFα and EGFR expression during embryonic and perinatal testis development.

Materials and Methods

Organ Cultures

Timed pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, Mass). (Plug date was considered to be ED0.) ED13 gonads were dissected out with the mesonephros. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford, Mass) floating on the surface of 0.4 mL of CMRL 1066 media (Gibco BRL, Gaithersburg, Md) supplemented with penicillin-streptomycin, insulin (10 μg/mL), and transferrin (10 μg/mL). Antisense or sense oligonucleotides at 5 μM were added directly to the culture medium every 10–12 hours. The antisense oligonucleotide procedure for uptake, translational inhibition, and mRNA stability effects was used (Chaudhary et al, 2000). The antisense oligonucleotide dose and procedure has been optimized for testis cells as previously described (Chaudhary et al, 2000). The oligonucleotide sequence for each ligand or receptor was as follows: 1) TGFα antisense, 5′-CGAGGGGACCATTTTACGG-3′; 2) TGFα sense, 5′-CCGTAAAATGGTCCCCTCG-3′ (Grandis et al, 1998). The medium was changed every day. ED13 gonad plus mesonephros were typically kept for 3 days, by which point cords were developed in the paired controls (Cupp et al, 1999b). Areas of testis treated with TGFα sense or antisense were measured by use of the National Institutes of Health Image analysis program. Ratios of each testis pair were averaged to determine the average percentage reduction in testis size with the TGFα antisense treatment. Similar measurements have been previously reported for treatment with TGFβ (Cupp et al, 1999b), TGFα antibodies, and EGFR blocking agents (Levine et al, 2000b) on ED13 and ED14 testis organ cultures.

Genomic DNA Isolation and Polymerase Chain Reaction for SRY

To determine the sex of ED13 embryos, polymerase chain reaction (PCR) for SRY was conducted on each embryo. Embryonic tails were collected to isolate genomic DNA by standard procedures. Briefly, the tissue was homogenized through a 25-gauge needle in digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM ethylenediaminetetraacetic acid [EDTA], and 0.5% sodium dodecyl sulfate), and treated with proteinase K (0.15 mg/mL) for at least 4 hours at 60°C. The samples were then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1), and once with chloroform:isoamyl alcohol. The DNA was then precipitated by adding $\frac{1}{10}$ volume of 7.5 M ammonium acetate and 3 volumes cold ethanol and collected by centrifugation at 4°C for 30 minutes after an hour incubation at −20°C. Pellets were dried and resuspended in 10 μL of distilled H_2O. PCR was performed using 1 μL of genomic DNA with primers to SRY. The sequences of the SRY primers were 5′-CGGGATCCATGTCAAGCGCCCCATGAATGCATTTATG-3′ and 5′-GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT-3′. PCR was performed using an annealing temperature of 55°C for 30 cycles to yield a product of 240 base pairs (bp; Giesek et al, 1994).

Testicular Cell Culture and Growth Assay

To generate a testicular culture from PD0 testis, the tunica was removed and the testis digested with 0.125% trypsin, 0.1% EDTA, and 0.02 mg/mL DNase in Hanks balanced salt solution (HBSS), for 15 minutes at 37°C. The trypsin was inactivated with 10% calf serum. The samples were triturated with a pipette tip and washed twice in 1 mL HBSS. The pellet was resuspended and either used in growth assays immediately or placed in 10-mm plates in F12 media supplemented with 10% bovine calf serum until confluent (approximately 2 days). For growth assays, cells were plated at a 25% confluence in 24-well plates and allowed to settle overnight in Dulbecco modified essential medium

(DMEM) without thymidine. Medium was replaced the next day and cells were treated for 24 hours with different hormones or growth factors. Medium was removed after the 24-hour treatment period and medium containing tritiated thymidine (10 μCi/mL) was placed on cells for 5–6 hours. After 5–6 hours the medium was discarded and cells were either frozen or processed using the tritiated thymidine assay. Briefly, a solution of 0.5 M NaH_2P0_4 (pH 7.3; 500 μL) was added to each well, and cells were sonicated. Half of the sonicated cells were placed on DE-81 filters on a manifold and a vacuum was applied. After 3 washes with the NaH_2P0_4 buffer the filters were dried, placed in counting vials with 5 mL of scintillation fluid, and counted. The remaining sonicate was used for DNA assays to normalize the number of cells per well (Cupp et al, 1999b).

RNA Isolation and Quantitative Reverse Transcription-PCR

Total RNA was obtained using Tri Reagent (Sigma Chemical Company, St Louis, Mo). Briefly, tissue or cells were lysed in Tri Reagent (1 mL/50–100 mg tissue or 1 mL/100 mm of a culture plate). After adding 0.2 mL chloroform/mL Tri Reagent, the mixture was centrifuged at 12 000 × *g* for 15 minutes at 4°C, the colorless upper aqueous phase was transferred to a fresh tube, and 0.5 mL isopropanol/mL Tri Reagent was added to pellet the RNA. Reverse transcription (RT) was performed using Maloney murine leukemia virus-reverse transcriptase under standard conditions (Itoh et al, 1998a).

Quantitative RT-PCR (QRT-PCR) procedures were performed as previously described (Itoh et al, 1998a; Cupp et al, 1999b). Briefly, total RNA (1 μg) was reverse transcribed using the specific 3′ primers. Plasmid DNAs containing subclones of interest were used to generate standard curves from 1 ng/μL to 10 pg/μL, each containing 10 ng/μL Bluescript carrier DNA. Identical 10-μL aliquots of each sample were used for PCR amplification. At least 0.25 μCi of ^{32}P-labeled dCTP was included in each sample during amplification. Specific PCR products were quantitated on 4%–5% polyacrylamide gels. The gels were exposed to a phosphor screen for 8–24 hours, followed by quantification of specific bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif) and analyzed with ImageQuant. Equivalent, steady state mRNA levels for each gene were determined by comparing each sample with the appropriate standard curve. All gene expression data was normalized for 1B15 (cyclophilin) mRNA. The optimal cycle number for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity. The sensitivity of each quantitative PCR assay is below 1 fg with intra-assay variabilities of 6%–15%. Primers used for the QRT-PCR were as follows: cyclophilin, 5 prime 5′-ACA CGC CAT AAT GGC ACT GG-3′, 3 prime 5′-ATT TGC CAT GGA CAA GAT GCC-3′; TGFα, 5 prime 5′-TTGCTG TCC TCA TTA TCA CC-3′, 3 prime 5′-CAG AGT GGC AGC AGG CAG TC-3′; EGFR, 5 prime 5′-CTG CTG GGG AAG AGG AGA GGA GAA C-3′, 3 prime 5′-GAG TGG TGG GCA GGT GTC TT-3′ (Itoh et al, 1998a). The size of the PCR products generated are as follows: TGFα, 138 bp; EGFR, 208 bp; and cyclophilin, 105. All have been confirmed with direct sequence analysis (Itoh et al, 1998b).

Embedding, Histology, and Immunohistochemistry

Tissues were fixed in Histochoice (Amresco, Solon, Ohio) and embedded in paraffin according to standard procedures (Akmal et al, 1996, 1997; Cupp et al, 1999b; Levine et al, 2000b). The tissue sections (3–5 μm) were deparaffinized, rehydrated, and microwaved. For the TGFα immunohistochemistry the sections were blocked in 10% donkey serum for 30 minutes at room temperature. The TGFα antibody (S574 raised against rat TGFα made in sheep) was obtained from East Acres Biological (Southbridge, Mass). This TGFα antibody has been shown to be a neutralizing polyclonal antibody that cross-reacts with mouse, rat, human, and bovine TGFα, but not with EGF, amphiregulin, TGFβ, or fibroblast growth factor (FGF) on Western blots (East Acres Biological; Levine et al, 2000b). The secondary antibody was a biotinylated anti-sheep immunoglobulin G made in donkey (Sigma) diluted 1:300. The EGFR immunohistochemistry sections were blocked in 10% goat serum and then incubated with a primary anti-EGFR polyclonal antibody made in rabbit (EGFR peptide antibody, Santa Cruz Biotechnology, Santa Cruz, Calif). The biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif) was diluted 1:300. Both biotinylated secondary antibodies (TGFα and EGFR) were detected by using the histo stain-SP kit (Zymed Laboratories, South San Francisco, Calif) and immunohistochemical images were digitized with a slide scanner. As a negative control, serial sections were put through the same procedure without any primary antibody. Additional negative control sections were incubated with 50×–100× excess of synthetic immunizing peptide anti-EGFR (Santa Cruz Biotechnology), or recombinant TGFα (R&D systems, Minneapolis, Minn). All sections utilized for negative controls (without specific primary antibody or with excess synthetic blocking peptide) had no positive staining. This suggested that the positive staining detected with the TGFα and EGFR antibodies at a 1:1000 dilution appeared to be specific. Four different experiments were conducted for TGFα and EGFR antibodies. In each experiment, 3 serial sections of 4–5 testes for each developmental age were analyzed. One serial section was utilized for the non-immune control for each time period.

Statistical Analysis

Data were analyzed with the JMP 3.1 statistical analysis program (SAS Institute, Cary, NC). All values are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using the Dunnett test for comparison with controls and the Tukey-Kramer honestly significant difference tests for multiple comparisons. Statistical difference was confirmed at $P < .05$.

Results

Pattern of Expression of mRNA for TGFα and EGFR During Testis Development

The expression pattern for TGFα and EGFR mRNA during testis development was determined by using a QRT-PCR assay with the use of RNA extracted from testis at ED15 though PD5 of testis development. The QRT-PCR

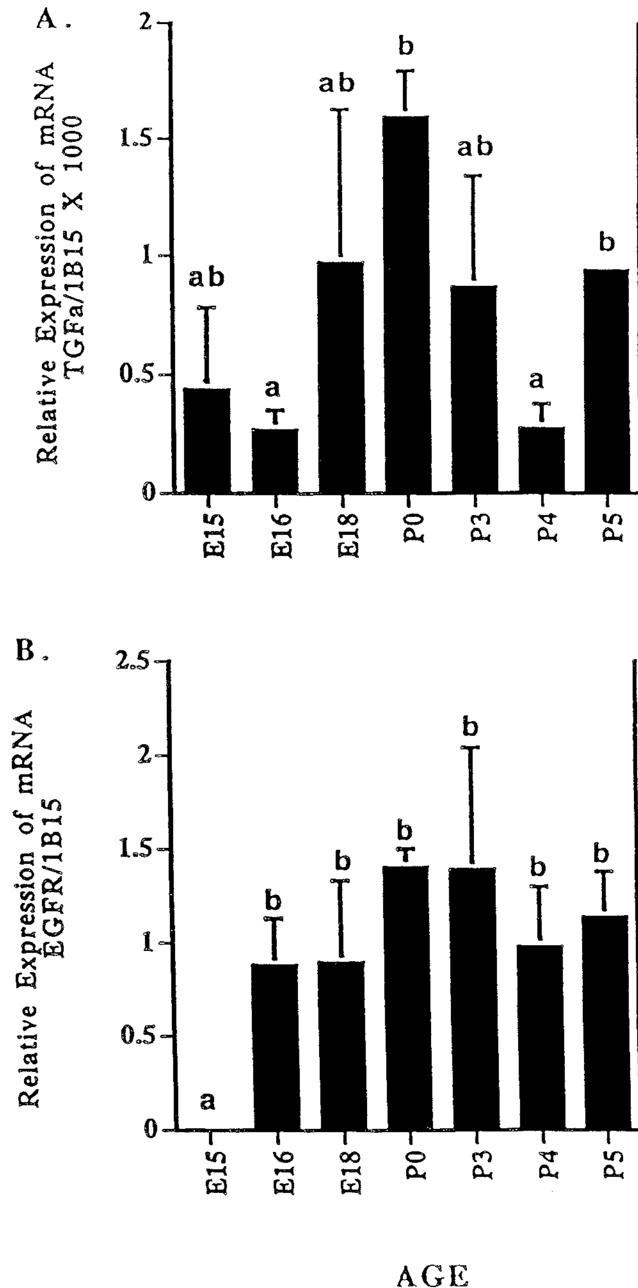


Figure 1. Relative expression of mRNA for **(A)** TGFα and **(B)** EGFR from ED15 through PD5 of testis development. Expression of both TGFα and EGFR mRNA are normalized for amount of cyclophilin (1B15). The mean ± SEM are presented for each time period evaluated. Three to 6 pooled tissue samples were assayed in replicate for each developmental time period. Data from 3 different experiments are combined. Different letters represent a statistical difference at $P < .05$ between amount of mRNA for each developmental time evaluated.

assay utilizes cyclophilin to normalize the data. Cyclophilin has been demonstrated to be constitutively expressed until PD19 of testis development (Wine et al, 1997). A minimum of 3 different experiments with different sample preparations done in replicate (n = 6) were utilized. The concentration of mRNA for TGFα was dynamic, with the lowest concentrations detected at ED16 and PD4, and the highest concentrations at PD0 (Figure 1A). The level of TGFα expression increased at PD5, which had negligible error associated with the data point obtained (Figure 1A). In contrast, the pattern of expression of mRNA for EGFR did not change significantly from ED16 though PD5 (Figure 1B). Concentrations of EGFR were barely detectable with the RT-PCR assay at ED15. In addition, concentrations of both TGFα and EGFR at ED14 in testis development were similar to the concentration depicted for each at ED15 (data not shown). Therefore, it appears that the levels of mRNA for TGFα fluctuate from ED14 to PD5, while concentrations of mRNA for EGFR remain relatively constant after ED16.

Protein Expression and Cellular Localization of TGFα and EGFR During Embryonic and Perinatal Testis Development

To determine the cellular localization of TGFα and EGFR, immunohistochemistry was conducted on testes collected at ED14, ED16, ED19, PD0, PD3, and PD5 of testis development (Figures 2A through L and 3A through F). Localization of EGFR was detected within the wolffian duct and in specific cells (eg, endothelial) in ED14 mesonephros (Figure 2A). In the testis, however, positive EGFR staining was observed in the interstitium and cords as well as endothelial and blood cells (Figure 2B). Greater dilutions of primary EGFR antibody and higher stringency blocking did not provide a more discrete pattern of staining of EGFR in the ED14 testis (data not shown). In addition, excess EGFR peptide blocked the EGFR signal in the ED14 (data not shown), ED16 (Figure 2D), and PD3 (Figure 3A) testis, suggesting that the EGFR staining pattern and localization was specific. By ED16, positive staining for EGFR was detected primarily in the cords and selected cells (eg, endothelial and blood cells; Figure 2E), and by ED19, EGFR was localized to the cords with slight staining in the interstitium (Figure 2K). At PD0, PD3, and PD5, EGFR was detected in the cords, interstitium, and surrounding germ cells with possible positive staining in germ cell cytoplasm (Figures 2K, 3B, and 3E).

Positive staining for TGFα protein was detected faintly in Sertoli cells and more distinctly in selective interstitial cells at ED14 and ED16 (Figure 2C and F). Excess recombinant TGFα blocked the positive staining of Sertoli and interstitial cells at ED14 and ED16 (data not shown), and of Sertoli, endothelial, interstitial, and germ cells at ED19 (Figure 2G), PD0 (Figure 2J), and P5 (Figure 3D). Therefore, the positive staining was determined to be specific for this series of experiments. At ED19, TGFα was detected in Sertoli, interstitial, endothelial, and blood cells (Figure 2I), whereas at PD0, TGFα was present mainly in the cytoplasm of germ cells and in the interstitium,

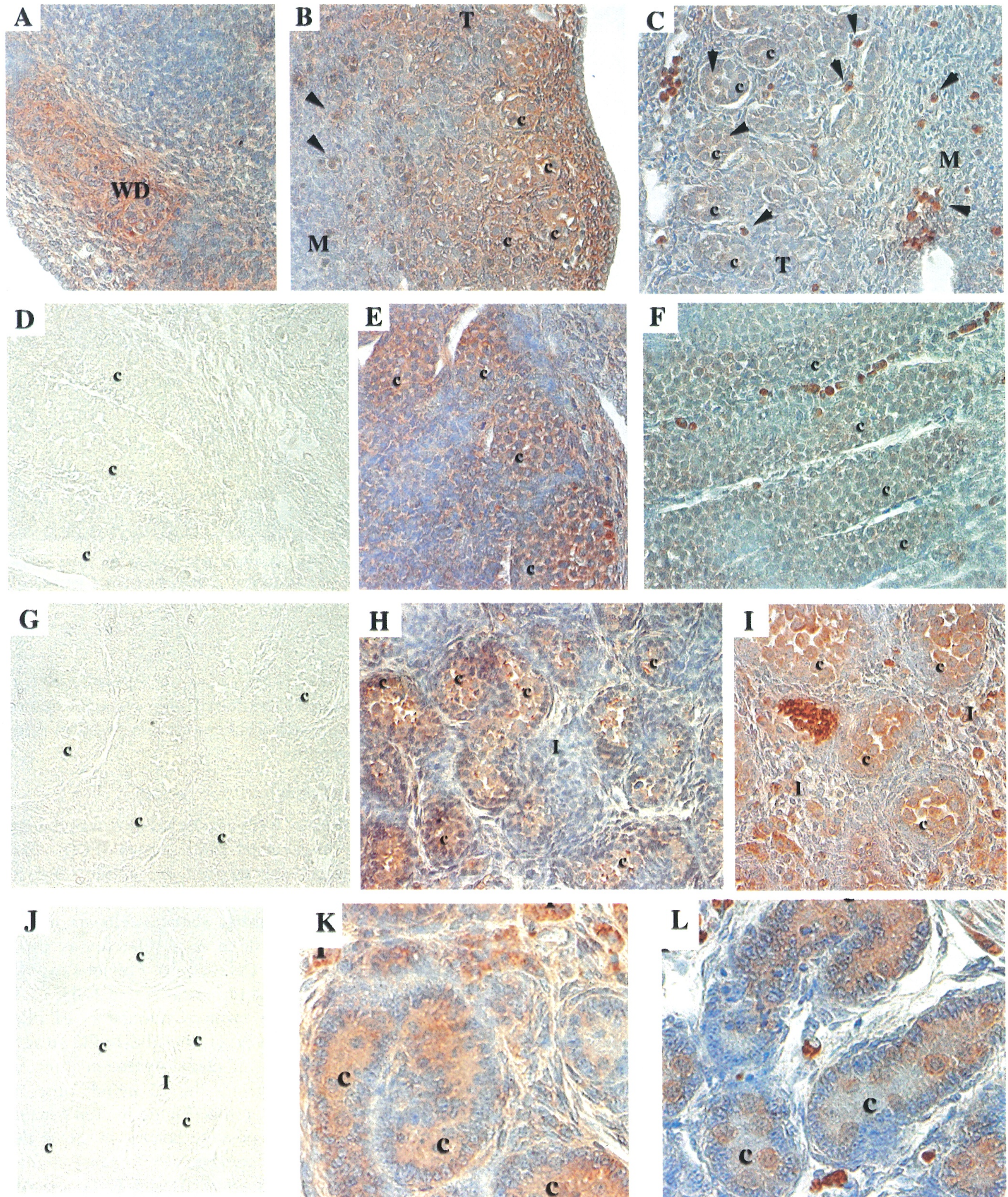


Figure 2. Immunohistochemistry for **(A)** EGFR in ED14 mesonephros, **(B)** EGFR in ED14 testis, **(C)** TGFα in ED14 testis, **(D)** EGFR antipeptide control in ED16 testis, **(E)** EGFR in ED16 testis, **(F)** TGFα in ED16 testis, **(G)** TGFα recombinant blocking peptide control in ED19 testis, **(H)** EGFR in ED19 testis, **(I)** TGFα in ED19 testis, **(J)** TGFα recombinant blocking peptide control in PD0 testis, **(K)** EGFR in PD0 testis, and **(L)** TGFα in PD0 testis. All sections except peptide blocking controls were slightly counterstained with hemotoxylin. Positive staining is reddish brown color. C indicates cords; WD, wolffian duct; M, mesonephros; T, testis; I, interstitium. Arrows point to positively stained cells. These pictures are representative of at least 4 different experiments conducted for all time points. Magnification 400× except for ED14 and ED16 time points, which are 800×.

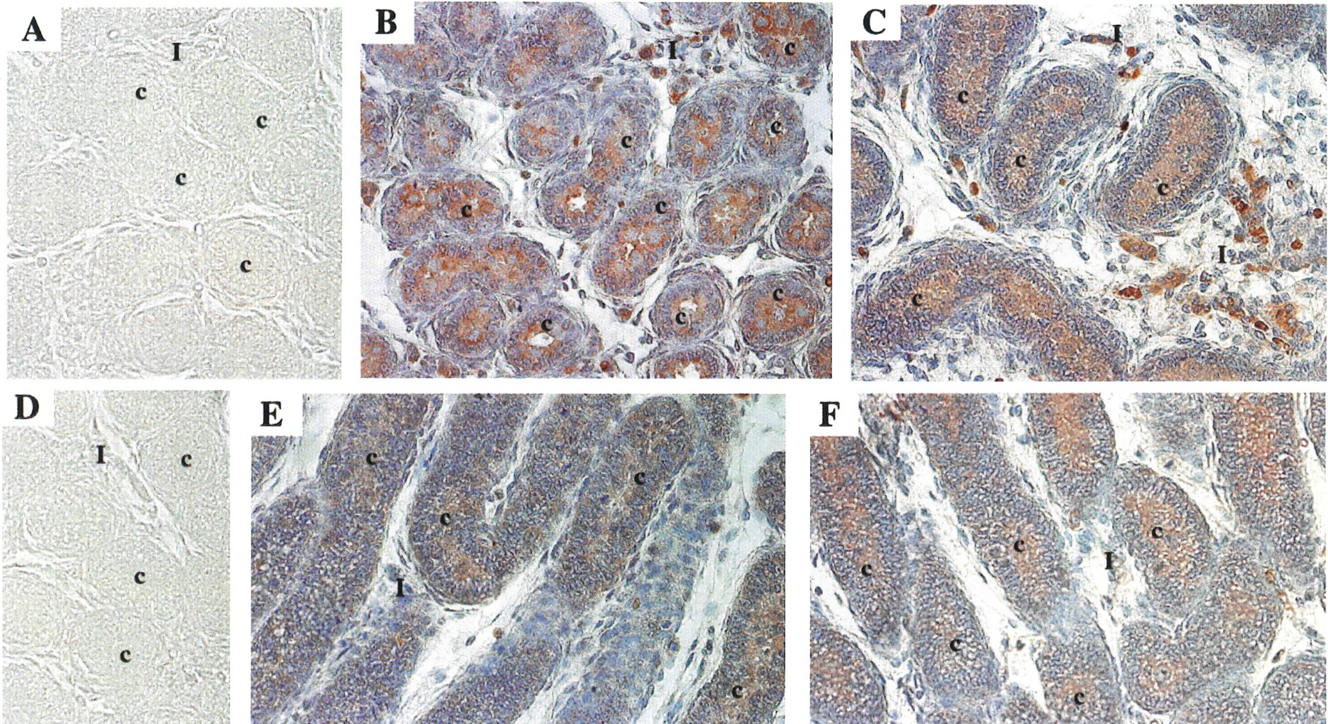


Figure 3. Immunohistochemistry for **(A)** EGFR blocking antipeptide in PD3 testis, **(B)** EGFR in PD3 testis, **(C)** TGFα in PD3 testis, **(D)** TGFα recombinant blocking peptide control in PD5 testis, **(E)** EGFR in PD5 testis, **(F)** TGFα in PD5 testis. Magnification 400×. C indicates cords; I, interstitium. Positive staining is reddish brown color. All sections except excess blocking peptide or antipeptide controls are counterstained with hematoxylin. These pictures are representative of at least 4 different experiments conducted for each time point evaluated.

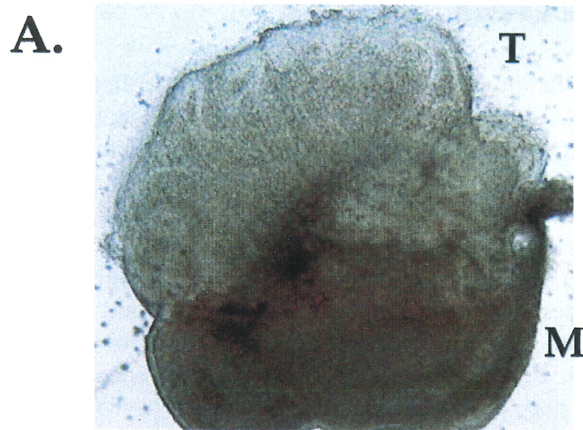
with a decrease staining in Sertoli cells (Figure 2L). At PD3 and PD5 of testis development, TGFα expression was concentrated in Sertoli cells, present in selected interstitial cells, and in germ cells (Figure 3C and F). Therefore, both TGFα and EGFR appear to be localized in different cell populations during testis development. Initially, both proteins are detected within the Sertoli and germ cells, and later, they are detected in most cell types in the testis.

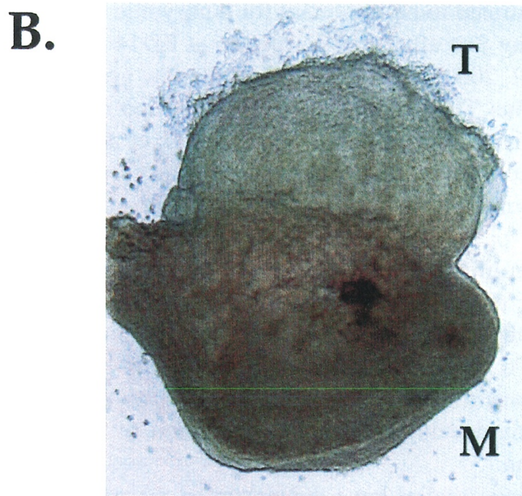
Effects of Antisense Oligonucleotides to TGFα on Seminiferous Cord Formation and Embryonic Testis Growth

ED13 testis organ cultures were used to determine the effect of sense and antisense TGFα oligonucleotides on morphology and growth of embryonic organ cultures. These experiments extended previous results in which antibodies to TGFα and EGFR tyrosine kinase inhibitors reduced the growth of embryonic testes in organ cultures (Levine et al, 2000b). The antisense procedures were optimized as outlined in "Materials and Methods." Antisense TGFα oligonucleotides did not affect formation of seminiferous cords, but they did suppress growth of organ cultures by 26% when compared with their paired control (Figure 4A through C). Therefore, these results further substantiate previous results that the main action of TGFα during embryonic testis development is regulation of testis growth (cellular proliferation). TGFα does not appear to influence the morphological events that result in seminiferous cord formation.

Effect of TGFα on Perinatal Testis Growth

A series of experiments were conducted to determine the effects of TGFα on perinatal testis growth (PD0). This stage of development was selected due to the elevated amount of mRNA for TGFα (Figure 1A). At this stage of testis development, primarily somatic cells are proliferating, whereas there is little to no proliferation of germ cells (Orth, 1982; Levine et al, 2000b). Previous experiments with tritiated thymidine indicated that FSH, EGF, and 10% calf serum were positive stimulators of PD0 testis culture growth (Cupp et al, 1999a,b). In the present study, TGFα stimulated thymidine incorporation at the 50 and 100 ng/mL doses, whereas the 25 ng/mL dose of TGFα did not differ from controls (Figure 5). Ten percent calf serum was used as a positive control in all of the growth assay experiments conducted. Preliminary studies suggested that a combination of FSH and TGFα are not additive (data not shown), however, effects on dose curves and effective concentrations remain to be determined. Observations demonstrate that TGFα can regulate PD0 testis growth.





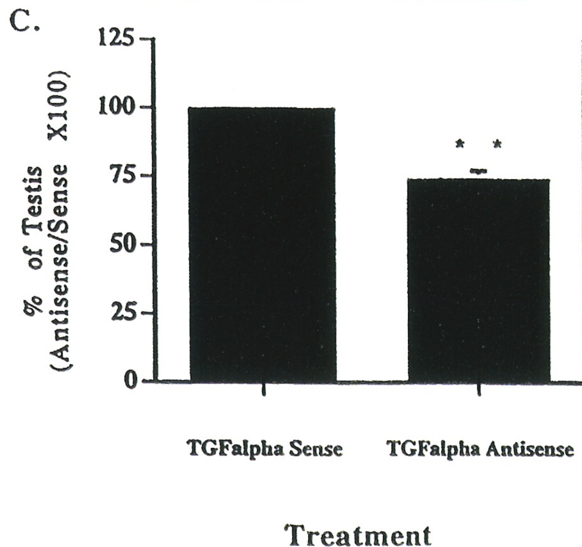


Figure 4. Effects of ED13 testis organ cultures treated with **(A)** sense oligonucleotides to TGFα or **(B)** antisense oligonucleotides to TGFα. **(C)** Percentage area of testis when TGFα antisense-treated testis was compared with TGFα sense-treated testis pair. Organ cultures were treated every 10–12 hours with a 5 μM concentration of oligonucleotide. These images are representative of 36 testis pairs. T indicates testis; M, mesonephros. **Denotes treatment of TGFα antisense oligonucleotides was different, $P < .01$, than TGFα sense-treated testes.

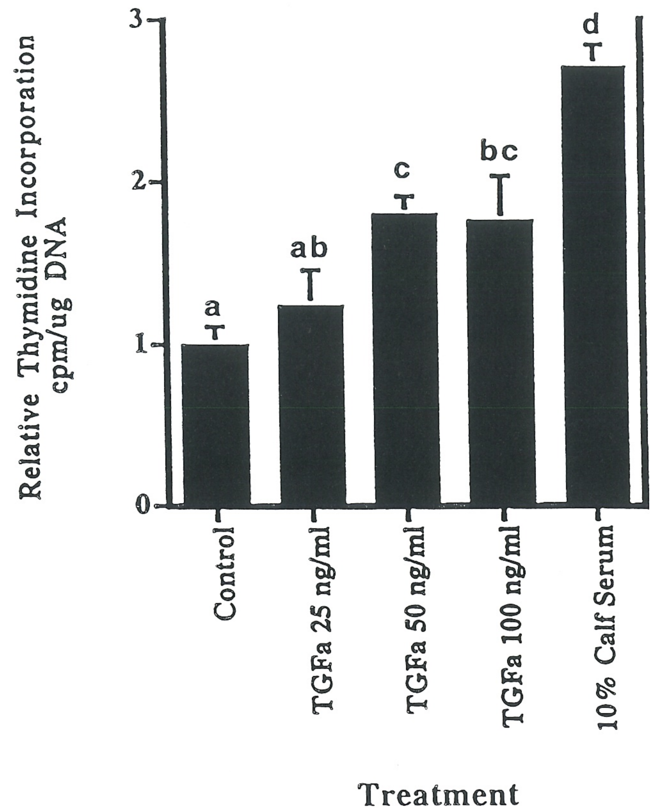


Figure 5. Effects of dose of TGFα on PD0 testis growth as measured by tritiated thymidine incorporation into DNA. Results are presented as relative to control and represent at least 4 different individual experiments in triplicate. Ten percent calf serum served as the positive control.

Effect of Hormones on Expression of mRNA for TGFα and EGFR

PD0 testis cultures were treated with FSH, EGF, and testosterone to determine whether these regulatory agents influence TGFα and EGFR mRNA expression. After a 24-hour hormone stimulation the PD0 testis cultures were collected for RNA isolation and the QRT-PCR was used to determine the amount of TGFα and EGFR mRNA. A minimum of 3 different experiments in replicate were performed. Cyclophilin mRNA expression was used to normalize the data and to assess the quality of the RNA collected. FSH (50 ng/mL) and testosterone (10^{-7} M) did not affect expression of mRNA for TGFα (Figure 6A). However, EGF (50 ng/mL) inhibited mRNA expression of TGFα by more than 50% (Figure 6A). FSH stimulated an increase in expression of EGFR ($P < .05$) and testosterone also tended ($P < .07$) to increase expression of mRNA for EGFR (Figure 6B). In contrast, treatment with EGF did not appear to affect expression of mRNA for TGFα (Figure 6B). Therefore, it appears that FSH, and to a lesser degree testosterone, can regulate expression of mRNA for EGFR, whereas EGF/TGFα can negatively influence TGFα mRNA levels.

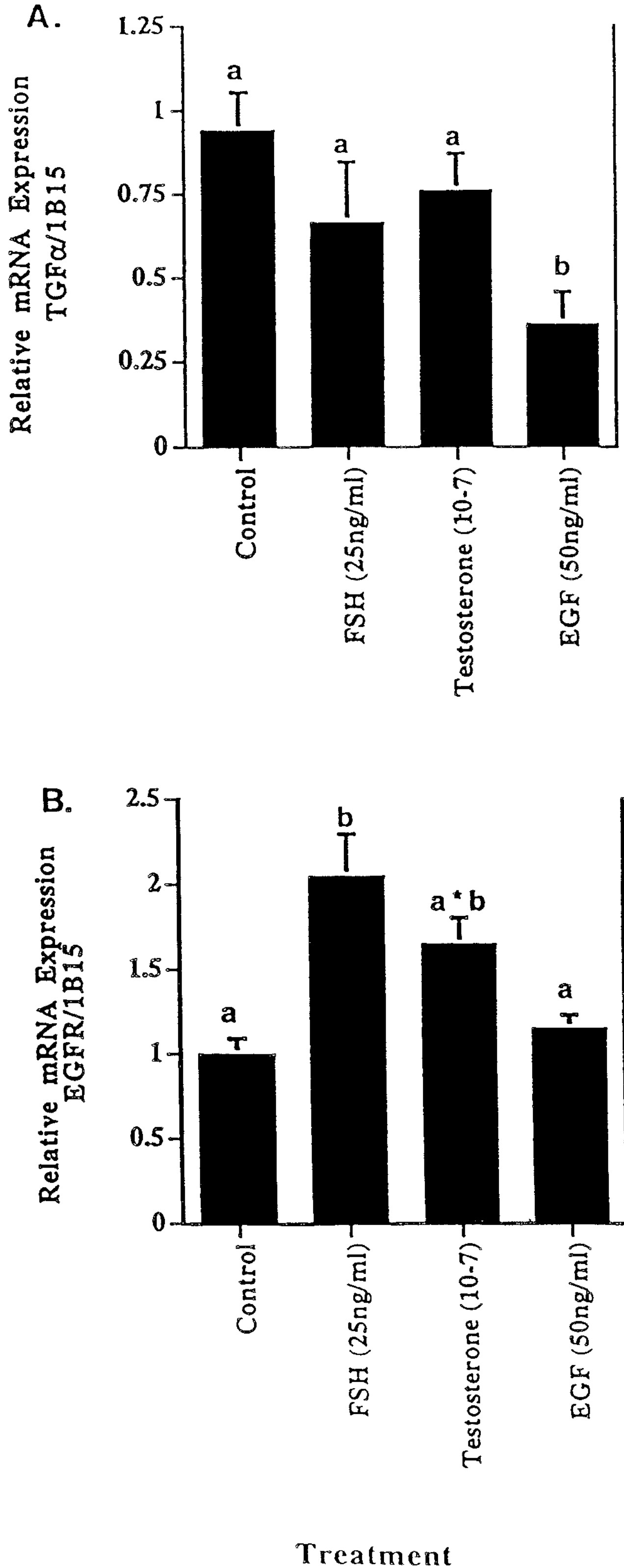


Figure 6. Effects of FSH, testosterone, and EGF on relative amounts of mRNA for **(A)** TGFα and **(B)** EGFR in PD0 testis cultures. Amounts of mRNA for TGFα and EGFR were normalized to cyclophilin (1B15) and expressed relative to controls. These data represent 3 individual experiments assayed in duplicate. Different letters for means represent statistical differences at $P < .05$.

Discussion

Proliferation of cells within the testis is critical in order to have an adequate complement of germ cells and somatic cells to maintain normal adult spermatogenesis (Orth, 1982; Magre and Jost, 1991; McClaren and Southee 1997; Schmahl et al, 2000). Proliferation of Sertoli cells is particularly crucial because a finite number of germ cells can be supported by each Sertoli cell. The greatest amount of proliferation of somatic and germ cells occurs after cord formation from ED16 to birth (Levine et al, 2000b). Many growth factors have been identified that may potentially regulate embryonic testis proliferation. Recently, TGFα and its receptor, EGFR, have been demonstrated to be important for embryonic testis growth occurring just after cord formation at ED14–ED16 (Levine et al, 2000b). TGFα is one of the few growth factors, other than TGFβ (Olaso et al, 1998; Cupp et al, 1999b) and activin (Boitani et al, 1995; Matzuk et al, 1995), that have been demonstrated to regulate testis growth just after cord formation (ED14). Therefore, TGFα may be an important paracrine growth factor necessary to stimulate early proliferation of somatic and germ cells in the embryonic testis. The objective of the present study was to further delineate the expression, actions, and regulation of TGFα and EGFR. The hypothesis for these experiments was that TGFα is an important growth factor during testis development in the regulation of cellular proliferation.

Previous studies have demonstrated the pattern of proliferation of different cell types in the testis. Peritubular myoid and interstitial cells have the greatest level of proliferation from ED14 through ED18 of testis development, whereas Sertoli cell proliferation is greatest from ED18 to PD0 (Levine et al, 2000b). The current study evaluates the expression pattern of both TGFα and EGFR mRNA from ED15 to PD5 of testis development. The expression pattern of TGFα mRNA fluctuated at the developmental time points measured. The differences in expression of TGFα and EGFR mRNA suggests that TGFα expression changes during testis development, whereas EGFR expression is relatively constant. The highest level of TGFα mRNA expression was detected at PD0, with the lowest levels being detected at ED16 and PD4. At PD0, TGFα appeared in interstitial cells to a lesser extent than in Sertoli cells, and there was a distinct expression in germ cells that was not present prior to this developmental period. Both an increased number of cells expressing TGFα and an enhanced expression by the different cells may be an explanation for the increased level of TGFα mRNA expression at PD0.

In contrast to TGFα, EGFR mRNA was relatively constant after ED15 during testis development. Retrospective

experiments measured the expression of both TGFα and EGFR at ED14 and found that the expression levels were similar to those of each at ED15. It is intriguing that the expression of EGFR dramatically increases at ED16 and subsequently maintains a constant level of expression. It is at ED15–ED16 in testis development that receptors for the gonadotropins FSH and luteinizing hormone appear within the testis. FSH in the current study was found to stimulate the expression of mRNA for EGFR at PD0. Therefore, the speculation is made that FSH actions may increase the expression of mRNA for EGFR to allow for enhanced TGFα action on testis growth. Further experiments are necessary to correlate FSH action with alterations in expression of EGFR, but the results from the present study provide a foundation for these potential interactions.

Throughout testis development EGFR protein expression appears to be in multiple cell types and is not localized to one specific cell type at any period in development. The localization of EGFR may be due to its dual role as receptor for both EGF and TGFα. The expression pattern of mRNA for EGFR was negligible at ED15, and this result was surprising because there was a distinct detection of EGFR protein using immunohistochemistry. These differences may stem from an uncoupling of EGFR mRNA expression and protein synthesis. Messenger RNA expression for EGFR may have been greater prior to ED14, which led to detectable protein production in the ED14 testis sections. Further studies are required to understand this early stage of testis development. Only the isolated testes were measured and quantitated for EGFR mRNA concentrations in the current study. EGFR expression was also detected in the wolffian duct of the mesonephros (Figure 2). This result was expected, because EGF and its receptor, EGFR, have been previously demonstrated to be important in development of the wolffian duct and later reproductive tract of the male (Gupta, 1996).

After ED16 the protein expression of TGFα, similar to EGFR, was found in many different cell types of the testis. It is at this time in development (ED16–ED18) that somatic and germ cell proliferation is at its greatest. Increased expression of TGFα and EGFR may be necessary at this time to promote cellular proliferation and ensure normal testis size. The actions of TGFα on testis growth were further demonstrated in the current study by the inhibitory actions of TGFα antisense oligonucleotides on ED13 testis organ cultures. These results were similar to previous data in which TGFα antibodies and EGFR antagonists inhibited the growth of ED13 testis organ cultures, but did not affect morphology of the testis (cord formation; Levine et al, 2000b). In addition to the effects of TGFα on embryonic testis growth, the current study also demonstrates that TGFα has stimulatory actions on PD0 testis cultures. Other reports have also demonstrated that TGFβ (a known antagonist of TGFα) can inhibit growth in the ED13 organ (Olaso et al, 1998; Cupp et al, 1999b) and PD0 testis cultures (Cupp et al, 1999b). TGFα appears to be an important factor at this period of testis development to support a high level of cellular proliferation and growth.

Hormones such as FSH and testosterone have been demonstrated to influence cellular proliferation in the testis. The hypothesis was tested that FSH and testosterone influence testis growth through a stimulation of EGFR and TGFα expression. It is interesting that FSH and, to some extent, testosterone, increased expression of mRNA for EGFR in PD0 testis cultures. Therefore, it is possible that FSH may in part elicit its growth effects on the testis through increasing available sites for TGFα and EGF to bind and stimulate growth. The dramatic increase in mRNA for EGFR after ED16 suggests that one or more critical regulators of EGFR mRNA expression appears at this time in testis development. Because the FSH receptor is present around ED16 (Warren et al, 1984) and FSH appears at ED18, FSH could be a potential regulator of the onset of EGFR expression.

Unlike EGFR expression, FSH and testosterone did not affect expression of mRNA for TGFα. Only EGF treatment altered mRNA expression of TGFα by reducing the message for TGFα in PD0 testis. These results suggest that high levels of EGF/TGFα may be inhibitory to the expression of mRNA for TGFα. Because both EGF and TGFα bind to the same receptor, these 2 ligands likely compensate for each other during testis development. The TGFα knockout mouse does not appear to have a major testis phenotype (Mann et al, 1993), however, the knockout mouse for EGFR has been demonstrated to have altered testis development (Levine et al, 2000b) with reductions in area of interstitium at ED18. The compensatory actions of other members of the large EGF family of ligands and receptor isoforms is likely a major factor in why no major phenotypes are observed in testis development in the TGFα or EGFR knockout mice.

The current study demonstrates the changes in expression and cellular localization of both TGFα and EGFR in the embryonic and perinatal testis. In addition, results substantiate previous data that TGFα is a growth factor present early in testis development, with its primary function being to influence cellular proliferation and not morphogenesis. Furthermore, regulatory agents such as FSH, testosterone, and EGF may influence the expression of both TGFα and EGFR in order to alter the ability of TGFα to act within the testis at critical stages of development. Proliferation of the testis during the embryonic and early postnatal stages of testis development is crucial to normal testis function and successful spermatogenesis. Without the appropriate complement of Sertoli, germ, and

interstitial cells the testis cannot function at its optimum level. Therefore, TGFα is likely one of numerous growth factors involved in cellular proliferation occurring during embryonic and perinatal testis development.

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