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Expression and Action of Transforming Growth Factor Beta (TGFβ1, TGFβ2, and TGFβ3) during Embryonic Rat Testis Development[1]

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ABSTRACT

The objective of the current study was to determine the role of transforming growth factor beta (TGFβ) during seminiferous cord formation and embryonic testis development. The expression pattern of mRNA for TGFβ isoforms was evaluated during testis development through a quantitative reverse transcription-polymerase chain reaction (QRT-PCR) procedure. Expression of mRNA for TGFβ1 was highest at postnatal day 0 (P0) and P10. In contrast, TGFβ2 was high at embryonic day 15 (E15), declined at E16, and showed a transient increase at P0 through P3 of testis development. Interestingly, expression of mRNA for TGFβ3 was high during embryonic development and then declined after P3. Immunohistochemical localization of TGFβ1 and TGFβ2 demonstrated expression in Sertoli cells at E14 and in the seminiferous cords at P0. Selective interstitial cells expressed high concentrations of TGFβ1 and TGFβ2 in P0 testis. TGFβ3 was expressed in selective cells at the junction of the E14 testis and mesonephros. The cells expressing TGFβ3 in the testis appeared to be preperitubular cells that resided around the seminiferous cords. TGFβ3 was localized to gonocytes in P0 testis. TGFβ1 was found to have no influence on seminiferous cord formation in embryonic organ cultures of E13 testis. In contrast, growth of both E13 and E14 embryonic organ cultures was inhibited by TGFβ1 and resulted in reduced testis size (40% of controls) with fewer cords present. A P0 testis cell culture and thymidine incorporation assay were used to directly examine the effects of recombinant TGFβ1. TGFβ1 alone had no influence on thymidine incorporation in P0 testis cell cultures when compared to controls. Interestingly, TGFβ1 inhibited epidermal growth factor (EGF), and 10% calf serum stimulated P0 testis cell growth but not FSH-stimulated growth. Therefore, TGFβ1 appears to inhibit testis growth in both the embryonic and early postnatal periods. The hormonal regulation of TGFβ expression was measured using P0 testis cell cultures and a QRT-PCR procedure for each TGFβ isoform. High concentrations of EGF stimulated expression of mRNA for TGFβ1 after 24 h but suppressed expression of TGFβ3. In contrast, there was no effect of FSH on TGFβ isoform expression. In summary, TGFβ regulates embryonic and P0 testis growth through inhibiting the actions of positive growth factors such as EGF. In addition, EGF but not FSH appears to regulate TGFβ isoform expression. Combined observations from the present study demonstrate that TGFβ isoforms are differentially expressed and appear to be regulators of testis growth during the embryonic and early postnatal periods.

INTRODUCTION

Seminiferous cord formation is one of the first critical events in testis development, occurring at embryonic day 13.5 (E13.5; E0 is the date a vaginal copulation plug was detected) of gestation in the rat [1]. Both aggregation of pre-Sertoli and germ cells within the differentiating gonad and cellular migration of pre-peritubular cells from the adjoining mesonephros [1–4] must occur in order for seminiferous cords to form. After seminiferous cord formation, all cell populations within the testis proliferate, and by E15 the testis is twice the size of the ovary from animals of the same age [5]. Growth and proliferation of cells within the testis are necessary to allow for adequate numbers of somatic cells to support adult germ cell maturation and function. [6]. All somatic cell populations proliferate during embryonic testis development. In contrast, the germ cell population undergoes mitotic arrest around E17-E18 and by postnatal day 5 (P5) resumes cellular proliferation [7]. Early growth of the embryonic testis is presumed to be independent of gonadotropins. Specific binding of gonadotropin receptors has been detected around E16, but it is unknown if these receptors have the ability to elicit a response at this time [8]. Therefore, much of the growth associated with the testis during early embryonic testis development must be due to locally produced paracrine growth factors.

Transforming growth factor betas (TGFβs) are critical for growth regulation and development of many different cell types within an organism. Of the five TGFβ isoforms identified, three [9–11] are present in mammals (TGFβ1, TGFβ2, TGFβ3). Each of the TGFβ isoforms is encoded by a unique gene, each on a different chromosome. The primary functions of the TGFβ isoforms are to enhance formation of the extracellular matrix and inhibit proliferation of most cells (for reviews see [9, 12]). Inhibition of growth by TGFβ occurs through an arrest of the cell cycle in late G1 phase and may require interactions with retinoblastoma and cyclin or cyclin-dependent kinases. The effects of TGFβs are elicited by activation of two types of membrane receptors containing serine/threonine kinase activity [12, 13], but all TGFβ isoforms bind and signal primarily through the TGFβ receptor II.

Gene knockout and overexpression experiments with TGFβ have demonstrated that precise regulation of each isoform is essential for survival. TGFβ1 knockouts are phenotypically normal until approximately 3 wk after birth and then develop a severe wasting syndrome [14, 15]. Reproductive traits and organs within TGFβ1 isoform knockout mice have not been extensively studied. However, there are significant deviations from normal Mendelian ratios, resulting in decreased offspring for both heterozygotes and homozygotes carrying the allele with the TGFβ1 gene disruption. Thus, TGFβ1 may be important in reproductive function or embryonic development.

Both TGFβ1 and TGFβ2 have been localized to the somatic cells in early embryonic testis [16–18], with receptor localization within the germ cells [19]. Before birth, the TGFβ2 isoform is also detected within the germ cells [18]. The primary functions of TGFβ isoforms during embryonic testis development are regulation of steroidogenesis within Leydig cells [20] and potential regulation of germ cell ap-

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optosis [19]. Since TGFβ isoforms are present in somatic cells of the testis during early embryonic development, their presence may be necessary for cell-cell interactions that occur during the morphological process of cord formation or embryonic testis growth.

In the current study, the expression of TGFβ isoforms during embryonic testis development was examined. In addition, two critical time points were evaluated to determine the actions of TGFβ during testis development. The first was E13-E14, when seminiferous cord formation occurs. The second time was P0, when cells of the testis are actively proliferating. The hypothesis tested was that TGFβ1, TGFβ2, and TGFβ3 have critical roles in testis development and are necessary for normal cell-cell interactions during the process of seminiferous cord formation and embryonic testis growth.

MATERIALS AND METHODS

Organ Cultures

Timed pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). For E13 dissections, gonads were dissected out with the mesonephros, and for later-stage dissections testes alone were dissected. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford, MA) floating on the surface of 0.4 ml of CMRL 1066 medium (Gibco BRL, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μg/ml), and transferrin (10 μg/ml). Antibodies and factors were added directly to the culture medium. The medium was changed every day. E13 gonad + mesonephros cultures were typically kept for 3 days, by which time point cords are well developed. E14 testes were kept for 3 days, and cords were formed before dissection and organ culture.

Genomic DNA Isolation and Polymerase Chain Reaction (PCR) for SRY

To determine the sex of E13 gonads, PCR for SRY was examined. Embryonic tails were collected to make 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 EDTA, 0.5% SDS) and digested with proteinase K (0.15 mg/ml) for at least 4 h at 60°C. The samples were then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol. The DNA was then precipitated by adding a 1/10 volume of 7.5 M NH_4Ac and 3 volumes cold ethanol, and incubating at −80°C for 1 h before centrifugation at 4°C for 30 min. Pellets were dried and resuspended in 10 μl dH_2O. PCR was performed using 1 μl of genomic DNA with primers to SRY. The sequences of the SRY primers are: 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) [21].

P0 Testis Culture and Growth Assay

To generate a testicular culture from P0 testis, the tunica was removed, and the testis was digested with 0.125% trypsin, 0.1% EDTA, and 0.02 mg/ml deoxyribonuclease (DNase) in Hanks' Balanced Salt Solution (HBSS) for 15 min 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 by resuspending, spinning for 2 min, and removing the supernatant. The remaining cell pellet was resuspended and used immediately in growth assays or plated in 100-mm plates in F12 medium supplemented with 10% bovine calf serum until confluent (approximately two days). Cells were plated at a 25% confluency in 24-well plates and allowed to settle overnight in Dulbecco's Modified Eagle's medium (DMEM) without thymidine. Medium was replaced the next day, and cells were treated for 24 h with different hormones or growth factors. Media were removed after the 24-h treatment period, and medium containing tritiated thymidine (10 μCi/ml) was placed on cells and allowed to remain for 6 h. After 6 h, media were discarded, and cells were either frozen or processed using the following tritiated thymidine assay. A solution of 0.5 M NaH_2PO_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 three washes with the NaH_2PO_4 buffer (vacuum applied after each wash) the filters were dried, placed in counting vials with 5 ml of scintillation fluid, and counted. The remaining sonicate was then used for DNA assays to normalize number of cells (DNA) per well [22].

DNA Assay

To assay the DNA content of organs, each organ was sonicated in 100 μl ethidium bromide buffer (EBB; 20 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.5) and stored at −20°C. DNA content then was determined fluorometrically with ethidium bromide as previously described [22]. Briefly, 0.25 nM ethidium bromide and 100 U/ml heparin in EBB were added to each sample, vortexed, and incubated for 15 min at room temperature. Fluorescent emission was measured and quantified by using a standard curve with calf thymus DNA from 0.5 μg to 6 μg DNA. For growth assays, the above procedure was used on remaining sonicate from the tritiated thymidine procedure [22].

Imaging and Area Analysis

Images of whole organs were obtained by using an image analysis system (Pixera; Pixera Corp., Los Gatos, CA; [22]). Areas were quantified using the NIH image program. Previous data correlated DNA concentrations of testis organ cultures with the area imaged by the NIH image program [23]. Each testis (without mesonephros) was outlined three times, and the areas of these outlines were averaged to obtain accurate area measurements. The averages for the control testis organ cultures were set to 100%, and the area of a treated testis was calculated as a percentage of its paired control. Approximately 18 testis pairs for the E14 testis organ cultures (three experiments with 6 testis pairs per experiment) and 36 testis pairs for the E13 testis organ cultures (4 experiments with 6 testis pairs per experiment) were imaged for area quantifications. Areas for each age were averaged and presented as a percentage of their respective controls.

RNA Isolation and Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

RNA for RT-PCR was extracted from tissue using Tri Reagent (Sigma, St. Louis, MO) for RNA isolation. RT and quantitative RT-PCR (QRT-PCR) procedures were utilized as previously published [22]. Briefly, for QRT-PCR, total RNA (1 μg) was reverse-transcribed using the specific 3′-

primers of interest. Carrier DNA (Bluescript plasmid; Stratagene, La Jolla, CA) was added to a final concentration of 10 ng/μl. Plasmid DNAs containing standard subclones of interest were used to generate standard curves from 1 ng/μl (10^{-15} g/μl) to 10 pg/μl (10×10^{-9} g/μl), each containing 10 ng/μl Bluescript carrier DNA. Identical 10-μl aliquots of each sample and standard were used for PCR amplification. By this design it was possible to simultaneously assay 5 known standard concentrations and 40 unknown samples for each gene. At least 0.25 μCi of ^{32}P-labeled dCTP was included in each sample during amplification. Specific PCR products were quantitated by electrophoresing all samples on 4–5% polyacrylamide gels, simultaneously exposing the gels to a phosphor screen for 8–24 h, and then quantifying specific bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT samples. Equivalent steady-state mRNA levels for each gene were determined by comparing each sample to the appropriate standard curve. All gene expression data were normalized for 1B15 (cyclophilin) mRNA. Cyclophilin is constitutively expressed in the testis until the pachytene spermatocytes are present [24]. Since pachytene spermatocytes first appear around P17–18 of age, measurements at any age after this point should be evaluated with this limitation considered. The optimal cycle number for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e., logarithmic phase of PCR reactions). The sensitivity of each quantitative PCR assay is below 1 fg, with intra-assay variabilities of 6.0–15%. Primers used for the QRT-PCR are as follows: TGFβ1, 5 prime: 5′-TCG ATT TTG ACG TCA CTG GAG TTG T-3′, 3 prime: 5′-GGG GTG GCC ATG AGG AGC AGG-3′; TGFβ2, 5 prime: 5′-CCG CCC ACT TTC TAC AGA CCC-3′, 3 prime: 5′-GCG CTG GGT GGG AGA TGT TAA-3′; TGFβ3, 5 prime: 5′ TGC CCA ACC CGA GCT CTA AGC G-3′, 3 prime: 5′ CCT TTG AAT TTG ATC TCC A-3′; cyclophilin, 5 prime: 5′ ACA CGC CAT AAT GGC ACT GG-3′, 3 prime: 5′-ATT TGC CAT GGA CAA GAT GCC-3′ [22].

Embedding, Histology, and Immunohistochemistry

Tissues were fixed in Histochoice (Amresco, Solon, OH) and embedded in paraffin according to standard procedures [25, 26]. Sections were stained with hematoxylin and eosin according to standard procedures [25, 26]. Briefly, 3-μm sections were deparaffinized and rehydrated, microwaved (15 min), and blocked in 10% serum for 30 min at room temperature. Immunocytochemistry was performed as previously described [22, 25]. The TGFβ1 primary antibody was an anti-TGFβ1 peptide antibody (Santa Cruz Biotechnology [SCB], Santa Cruz, CA) raised against amino acids 328–353 of human TGFβ1 (which is 100% homologous to mouse TGFβ1). The TGFβ2 primary antibody was an anti-TGFβ2 peptide antibody (SCB) raised against amino acids 352–377 of human TGFβ2. The TGFβ3 primary antibody was an anti-TGFβ3 peptide antibody (SCB) raised against amino acids 350–375 of human TGFβ3. The TGFβ1 and TGFβ3 antibodies were diluted 1:50 in 10% goat serum; the TGFβ2 primary antibody was diluted 1:1200 in 10% goat serum. The biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlington, CA) was diluted 1:300. Two negative controls were conducted for each TGFβ isoform. The first negative control involved a serial section of testis tissue treated as described above, but with no primary TGFβ antibody. The second negative control involved incubation of each primary antibody with 50- to 100-fold excess of each respective TGFβ protein before application to the tissue; then these tissues were treated as described above. The secondary antibody was detected by using the histo stain-sp kit (Zymed Laboratories, South San Francisco, CA), and immunohistochemical images were digitized with a slide scanner (Sprint Scan, Polaroid, Cambridge, MA).

Statistical Analysis

All data were analyzed by a 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 Dunnett's test for comparison to controls and using the Tukey-Kramer honestly significant difference test for multiple comparisons. Statistical difference was confirmed at $p < 0.05$.

RESULTS

Characterization of the QRT-PCR Procedure for TGFβ

Preliminary studies were performed to establish a reproducible and accurate QRT-PCR procedure [22]. The linearity of generating a PCR product relative to cycle number was determined [22]. Thirty cycles was selected as an appropriate cycle number for TGFβ1, 33 cycles for TGFβ2, 35 cycles for TGFβ3, and 25 cycles for cyclophilin [22]. Comparison of standard DNA with unknown samples was conducted to determine parallel displacement. Plasmid DNA with subcloned PCR product and cDNA produced from unknown RNA samples were run in parallel. PCR products of diluted plasmid DNA and diluted unknown cDNA were compared. These two curves were parallel with each growth factor, indicating that the QRT-PCR assay could be used to quantitate mRNA levels. Minimal variability of this assay was demonstrated previously [22].

Developmental Regulation of TGFβ Expression during Testis Development

Experiments were designed to investigate the changes in expression of TGFβ1, TGFβ2, and TGFβ3 from E15 through postnatal day 30 (P30) of testis development. Whole testes were removed from rats aged E15, E16, E18, P0, P3, P4, P5, P10, P20, and P30, and the mRNA expression of TGFβ was measured by QRT-PCR [22]. The expression of TGFβ isoforms was quantitated using a standard curve for each isoform and was normalized by the expression of the gene cyclophilin, which is constitutively expressed through P10 of testis development [22], for each sample. The amount of mRNA for TGFβ1 was low early in embryonic development (E15, $p < 0.05$); then increased at E16 ($p < 0.05$), birth (P0, $p < 0.05$), and prepuberty (P10, $p < 0.05$); and decreased after puberty and into adulthood (P20-P30, $p < 0.05$) (Fig. 1A). In contrast to TGFβ1 expression, mRNA for TGFβ2 was elevated early in embryonic development (E15, $p < 0.05$) and then decreased during the late embryonic period (E16, E18). TGFβ2 transiently increased during the early postnatal period (P0-P4) and decreased during the pubertal and adult stages of testis development (P5-P30, $p < 0.05$) (Fig. 1B). Expression patterns for TGFβ3 were the most dramatic, with higher concentrations of TGFβ3 during embryonic testis development (E16-P0) and significantly lower concentrations during the

postnatal, pubertal, and adult periods (P3-P30, $p < 0.05$) (Fig. 1C). In a subsequent study, E14 mRNA was analyzed, and levels of TGFβ isoforms were consistent with those of E15 (data not shown). Each of the TGFβ isoforms had a different pattern of expression during embryonic testis development. This suggests that these TGFβ isoforms are differentially regulated. The reduction in message for all TGFβ isoforms after puberty (P20–30) may be a dilution effect of increased number of germ cells in the testis due to the onset of spermatogenesis, or due to the increased amount of cyclophilin produced by pachytene spermatocytes at these developmental stages [24].

Localization of TGFβ during Embryonic Testis Development

Immunohistochemistry was conducted on testis sections from E14 and P0 rats to determine expression of TGFβ1, TGFβ2, and TGFβ3 (Fig. 2, A–H). Sections were evaluated and cell types were determined by staining of serial sections for cell-specific antibodies. Negative controls for each antibody were used to determine positively stained cells. Excess TGFβ protein abolished staining for TGFβs 1, 2, and 3 antibodies for the sections evaluated (data not shown). At E14 there appeared to be more widespread staining of TGFβ1. Positive staining for TGFβ1 was present in Sertoli cells and surrounding gonocytes. However, even at higher magnification it was not conclusive whether gonocytes themselves were stained or the Sertoli cells surrounding the gonocytes were positive for TGFβ1 (Fig. 2C). At P0, TGFβ1 was expressed in Sertoli cells surrounding gonocytes, in gonocytes, and in some interstitial cells (Fig. 2D). Staining for TGFβ2 was observed to be specific for Sertoli cells at E14 (Fig. 2E). At P0, TGFβ2 was expressed at low levels in Sertoli cells, and high levels of expression were observed in selective interstitial cells (Fig. 2F). In contrast, TGFβ3 was expressed in cells bordering the E14 mesonephros and testis (Fig. 2G). The intensely stained cells in E14 testis appeared to be pre-peritubular and to reside around seminiferous cords. Single cells of the testis interstitium also stained for TGFβ3 at E14. Lower-intensity staining was observed both in mesonephric ducts of the mesonephros and in specific cells of the testis (Fig. 3, A–D). This pattern of TGFβ3 may provide a marker for migrating cells from the mesonephros. In contrast to TGFβ3 expression at E14, TGFβ3 was expressed in gonocytes in the P0 testis (Fig. 2H). Cellular localization of TGFβ isoforms appeared to be distinct, with changing cellular localization during embryonic and early postnatal testis development. These data complement the QRT-PCR data and confirm the expression of the TGFβ proteins.

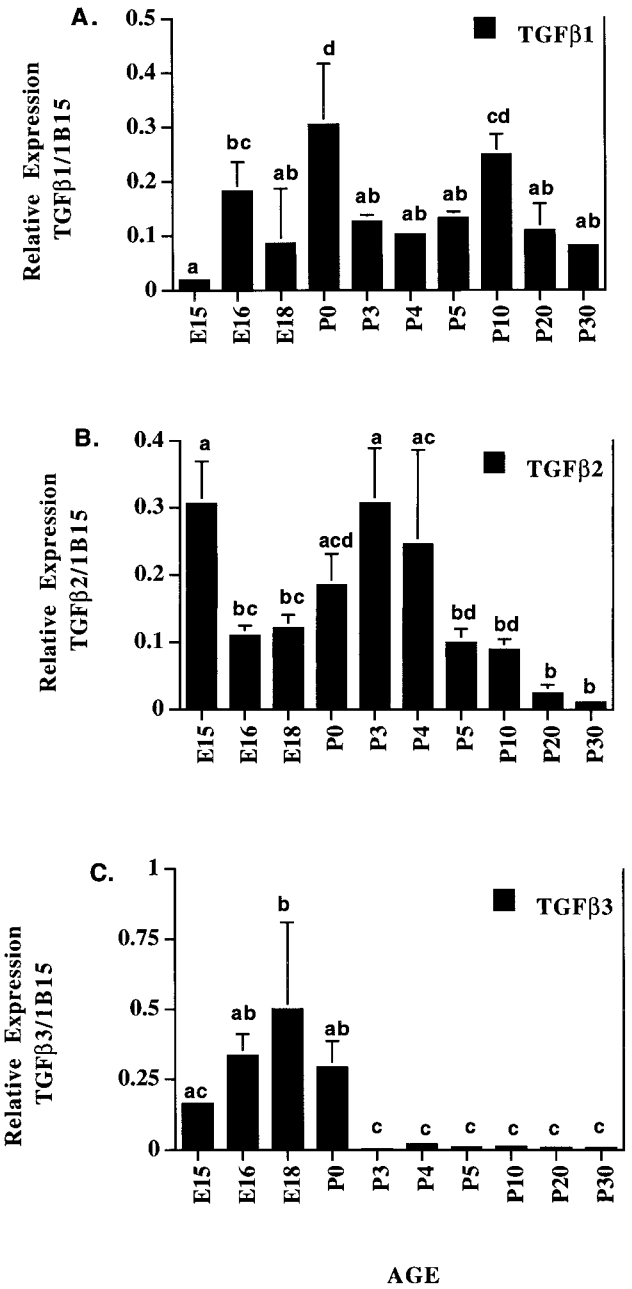

FIG. 1. Relative expression of mRNA for **A**) TGFβ1, **B**) TGFβ2, and **C**) TGFβ3 during E15 though P30 of testis development. Expression of TGFβ isoforms is normalized for amount of cyclophilin. The mean + SEM are presented for each time period during which RNA was evaluated. Three to six pooled tissue samples were assayed in duplicate for each developmental time period in a minimum of three pooled different experiments. Different letters for each developmental time represent statistical differences at $p < 0.05$.

TGFβ1 Regulation of Embryonic Seminiferous Cord Formation and Growth

The effects of TGFβ1 on seminiferous cord formation were investigated in E13 testis organ cultures. E13 testes with mesonephroi were cultured on floating filters in the presence or absence of 40 ng/ml recombinant TGFβ1 for a 3-day period. The testis organ cultures were routinely treated each day with daily changes of media. The control organ cultures formed seminiferous cords by the third day of culture. The dose of 40 ng/ml of TGFβ1 was used because it had been determined previously [22, 23] to be the most effective dose to inhibit cellular growth in cell culture. Others have demonstrated that 10 ng/ml of TGFβ1 can also inhibit cell growth in embryonic testis germ cells [19]. In the current study, TGFβ1 did not inhibit seminiferous cord formation (Fig. 4, A and B) but did inhibit growth of the E13 testis organ cultures. The number of seminiferous cords formed in the TGFβ1-treated testis was reduced, but this was due to the overall reduced size of the testis organ culture.

To determine the effects of TGFβ on growth of testis organ cultures, the embryonic testes were cultured in the presence or absence of 40 ng/ml of TGFβ1. TGFβ1 inhibited growth of the E14 testis organ cultures in a manner similar to that of the E13 testis organ cultures (Fig. 4, C

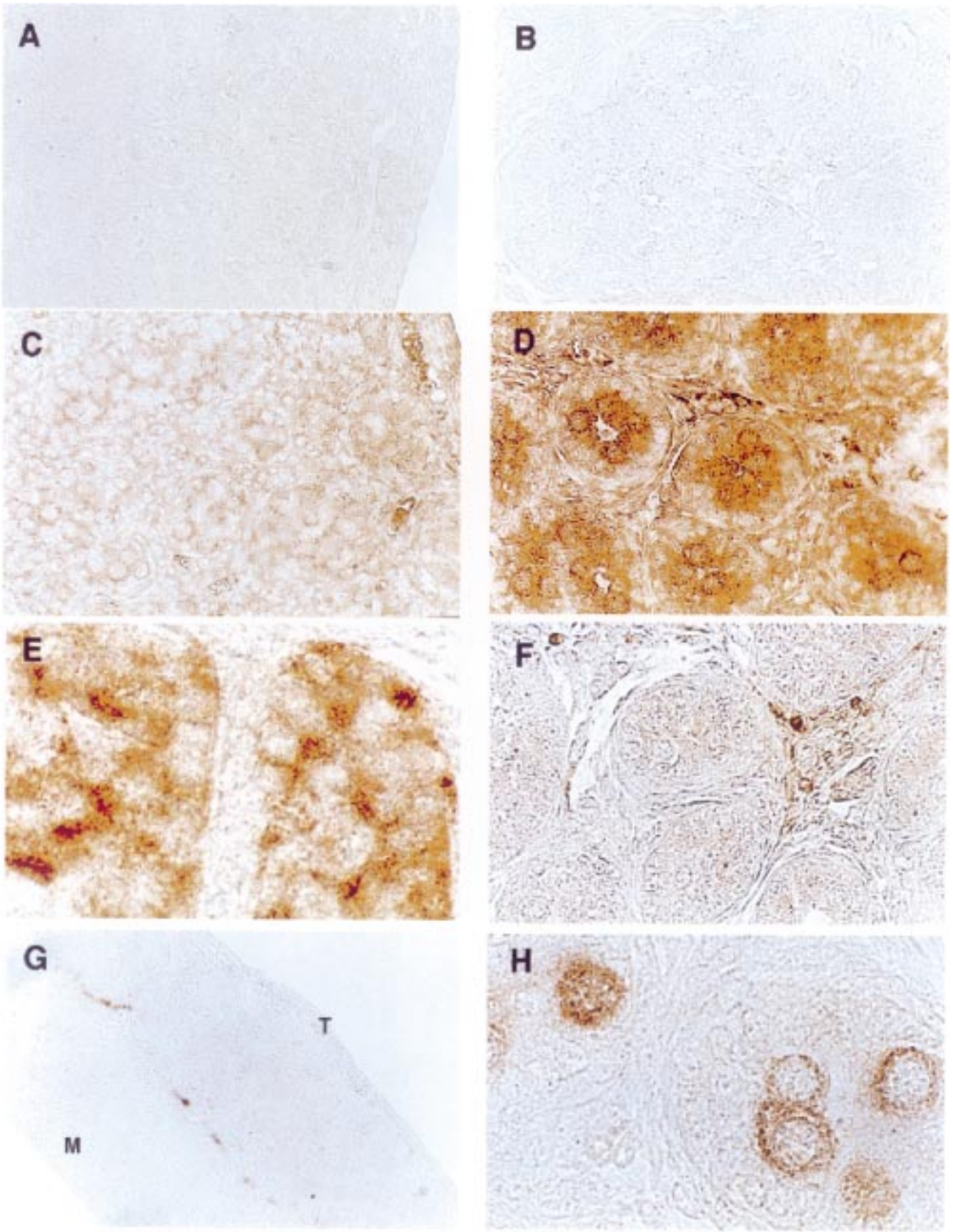

FIG. 2. Immunohistochemistry for control sections at **A**) E14 and **B**) P0; TGFβ1 at **C**) E14 and **D**) P0; TGFβ 2 at **E**) E14 and **F**) P0; and TGFβ3 at **G**) E14 and **H**) P0. Immunohistochemistry was conducted three separate times for each developmental time period. Controls were samples not treated with primary antibody (**A, B**) and incubation of excess (50–100×) TGFβ protein to suppress signal. ×400: **A, B, E, F**; ×200: **G, C, H**; ×1000. Reproduced at 93%. M, Mesonephros; T, testis.

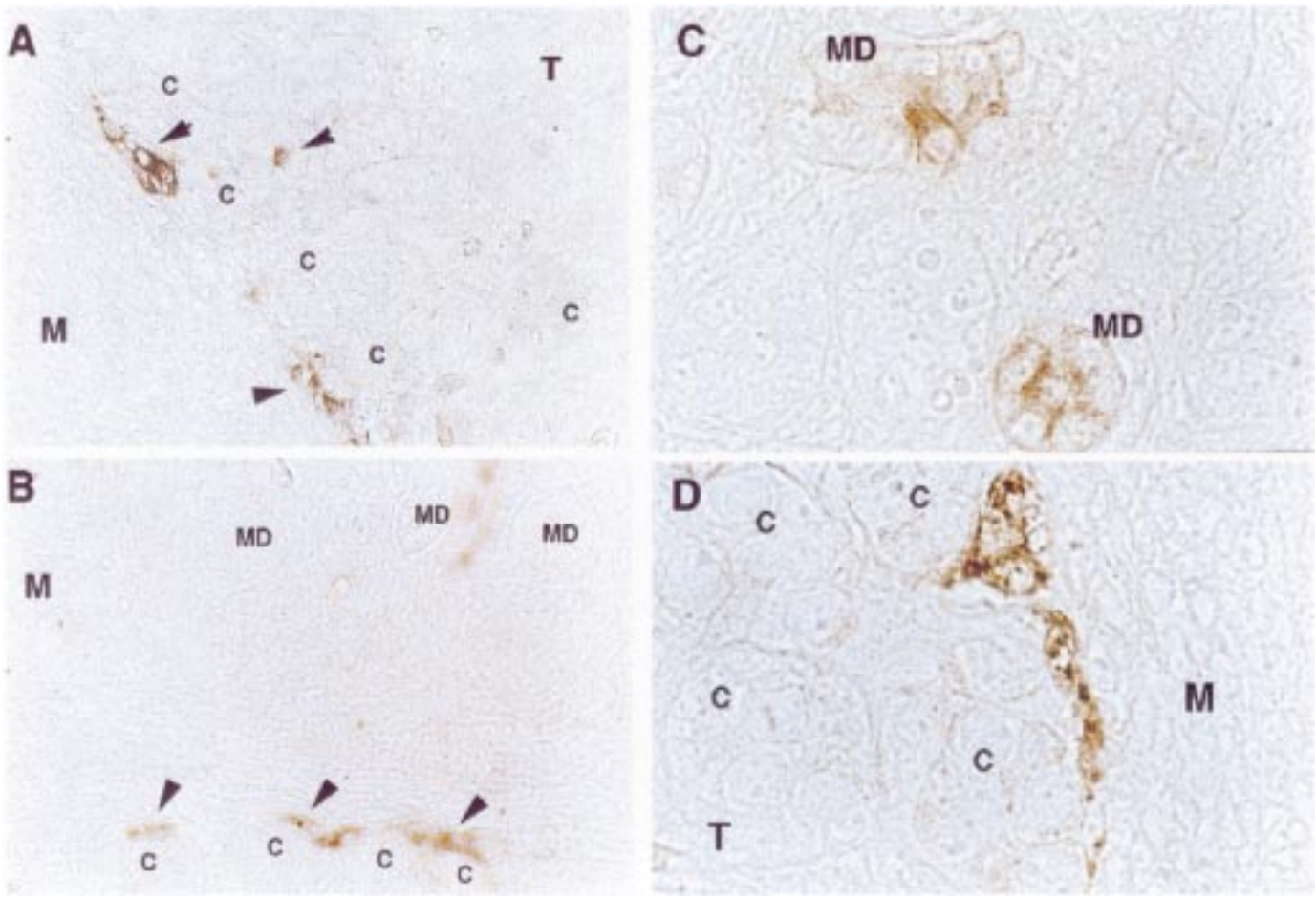

FIG. 3. Immunohistochemistry for TGFβ3 in E14 testis sections. **A–B** represent two different testis sections (×400). Arrows point to positive stained cells for TGFβ3 surrounding seminiferous cords. **C–D**) Higher magnification of mesonephros (**C**) and testis (**D**) at ×1000. Reproduced at 93%. M, Mesonephros; T, testis; MD, mesonephric duct; C, seminiferous cord.

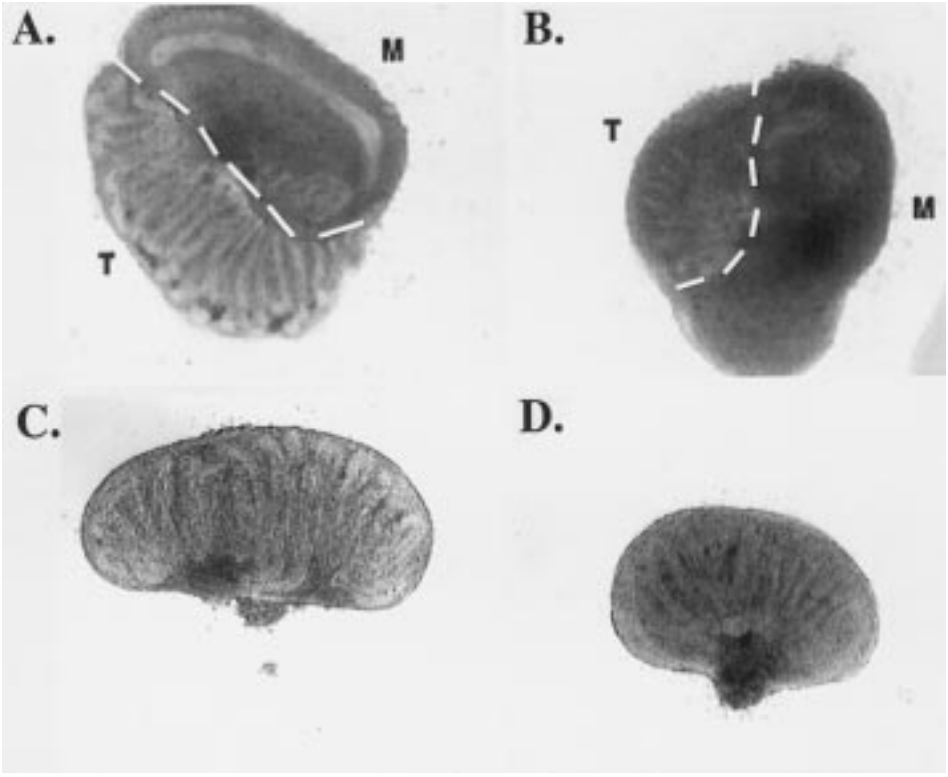

FIG. 4. **A**) Control E13 testis organ cultures plus mesonephros, **B**) E13 testis organ cultures plus mesonephros treated with 40 ng/ml TGFβ1, **C**) control E14 testis organ culture, **D**) E14 testis organ culture treated with 40 ng/ml of TGFβ1. Organ cultures were either not treated (controls) or treated daily with 40 ng/ml of TGFβ1 at the time of the daily change in medium for a 3-day period. These are representative of E13 organ cultures from 36 pairs of testes treated (n = 36), and E14 testis organ cultures from 18 pairs of testes treated (n = 18; one control and one treated per pair). T, Testis; M, mesonephros. Dashed lines separate testis and mesonephros. ×80 (reproduced at 88%).

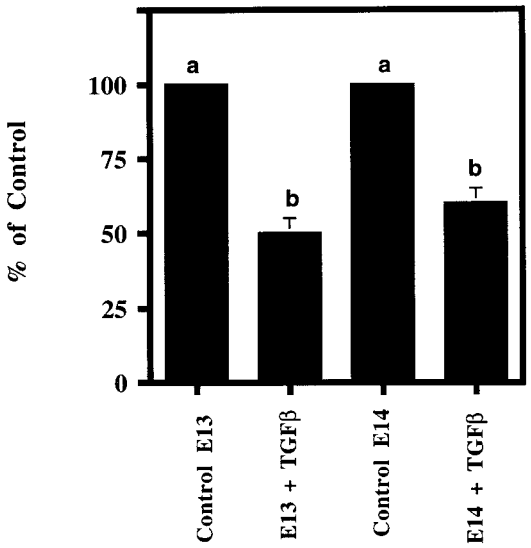

FIG. 5. Area of testis expressed as percentage of control for E13 and E14 testis organ cultures either treated with TGFβ or controls. The mean + SEM are presented for each developmental age for E13 (n = 36; 4 replicates with 6 testis pairs per experiment) and E14 testis (n = 18; 3 replicates with 6 testis pairs per experiment). Different letters for each mean represent statistical differences at $p < 0.05$.

and D). The areas of the cultured testes were analyzed, and a significant reduction in testis size was observed in E13 and E14 cultured testes when compared to matched controls (approximately 40–50% reduction, $p < 0.05$; Fig. 5). Previously, the reduced area has been shown to correlate to a reduced DNA content in the organ (data not shown). Therefore, TGFβ1 does not alter seminiferous cord formation but can inhibit testis growth, as demonstrated by the decreased testis area in TGFβ1-treated E13 and E14 testis organ cultures.

TGFβ1 Regulation of P0 Testis Cell Growth

The effects of TGFβ1 on P0 testis growth were investigated through a tritiated thymidine incorporation assay to access cellular growth. Data were normalized to the amount of DNA for each sample. Mixed populations of P0 testis cultures were treated with the most effective dose of the growth factors FSH (25 ng/ml), EGF (50 ng/ml), and 10% calf serum. All treatments stimulated tritiated thymidine incorporation in growth assays conducted on P0 testis cultures (Fig. 6). P0 testis cell cultures were treated with TGFβ alone or with positive regulators of P0 testis growth—FSH, EGF, and 10% calf serum. TGFβ1 (40 ng/ml) treatment alone at the most effective dose for growth inhibition did not significantly alter thymidine incorporation into P0 testis cultures when compared to controls. However, TGFβ1 inhibited ($p < 0.05$) EGF- and 10% calf serum-stimulated thymidine incorporation into P0 testis cultures ($p < 0.05$; Fig. 5). Interestingly, TGFβ1 did not modulate FSH-stimulated growth in P0 testis cultures. Therefore, TGFβ1 can inhibit growth factor-stimulated growth in P0 testis cultures (i.e., EGF and 10% calf serum) and may be a potential regulator of testis growth during this perinatal period.

Hormonal Regulation of TGFβ Expression

The effects of positive stimulators of growth (i.e., EGF and FSH) on expression of TGFβ1, TGFβ2, and TGFβ3 were examined (Fig. 7) in P0 testis cultures using QRT-PCR. All values were normalized with cyclophilin and expressed as relative expression compared with that in control cultures. P0 testis cultures were treated with FSH (25 ng/ml) and EGF (100 ng/ml), and RNA was collected after 24 h of treatment. EGF significantly stimulated expression of TGFβ1 ($p < 0.05$) and reduced expression of TGFβ3 ($p < 0.05$) after a 24-h period relative to controls (Fig. 7). There was no effect of treatment of P0 testis cultures with FSH (25–50 ng/ml) on expression of TGFβ isoforms 24 h after stimulation. Therefore, these data demonstrate that EGF may directly regulate expression of mRNA for TGFβ1 and TGFβ3 in P0 testis cultures. This regulation may be part of a negative feedback loop between EGF and TGFβ to modulate testis cell growth and differentiation.

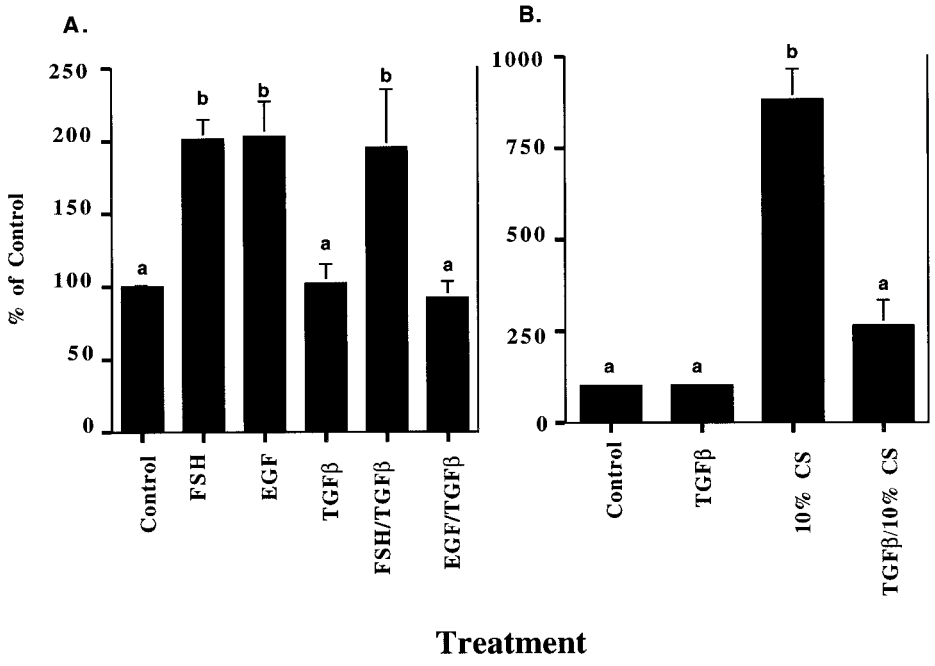

FIG. 6. Effect of TGFβ1 on cell growth in P0 testis suspensions. P0 testes were stimulated with FSH (25 ng/ml), EGF (50 ng/ml), or 10% calf serum alone or with TGFβ1 (40 ng/ml). Data are the combined results of four independent experiments conducted in triplicate. Values have been normalized for DNA/well and are expressed as percentage of controls with controls set to 100%. Different letters for each mean represent statistical differences at $p < 0.05$.

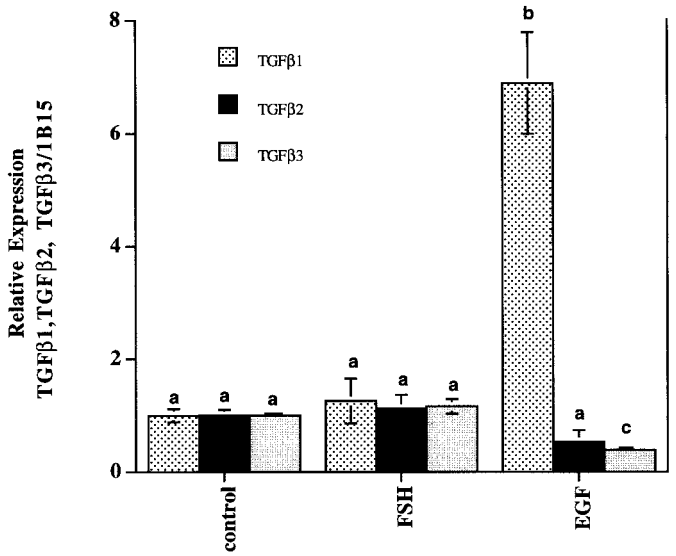

FIG. 7. Relative amounts of mRNA for TGFβ1, TGFβ2, and TGFβ3 after 24 h of P0 testis cultures with no treatment (control) or treatment with FSH (25 ng/ml) or EGF (100 ng/ml). Data are combined results of three different experiments conducted in duplicate. Messenger RNA levels for each isoform have been normalized to cyclophilin and are relative to controls for each experiment. Different superscript letters for each represent statistical differences at $p < 0.05$.

DISCUSSION

The current study was designed to investigate the action and expression of TGFβ during embryonic testis development. Previously, three isoforms of TGFβ (TGFβ1, TGFβ2, and TGFβ3) have been determined to be present in the postnatal testis [9–12]. In addition, TGFβ1 and TGFβ2 have been identified in the embryonic testis by immunohistochemistry [16–18]. Localization of TGFβ1 and TGFβ2 in the current study was similar to that in data previously reported. In previous studies, TGFβ1 was found to be positive in Sertoli cells, but not in gonocytes, in E14.5 testis. In the current study, E14 testis sections also stained positive for TGFβ1 in Sertoli cells. There was also staining that surrounded gonocytes at a higher magnification of these testis sections, but it was unclear as to whether TGFβ1 was present in the gonocytes. At P0, TGFβ1 positive staining was present in Sertoli cells, surrounding gonocytes, and in selected interstitial cells. No previous reports have described TGFβ1 staining in P0 testis. Previously, TGFβ1 has been demonstrated to be present in Leydig cells at E16.5, P4, and P20. The antibodies used in the present study for TGFβ1 were different from those used in previous studies. Antibody differences may explain these slight localization differences for TGFβ1. Positive staining for TGFβ2 was present at E14 in Sertoli cells, a finding that agrees with previously published data. In addition, at P0 there were faint staining for TGFβ2 in Sertoli cells and high levels of staining in selected cells of the interstitium. In addition, the current study also localized expression of TGFβ3 during embryonic testis development, which previously has not been reported. TGFβ3 was expressed in cells surrounding seminiferous cords at the testis-mesonephros interface at E14. Mesonephric ducts within the mesonephros and individual interstitial cells within the gonad also expressed TGFβ3 at E14. This pattern of expression of TGFβ3 suggests potential cellular interactions between mesonephric and testicular cells. Previously, it had been shown that cells migrate from the mesonephros into the testis to participate in seminiferous cord formation. It appears that pre-peritubular cells and selected interstitial cells stain for TGFβ3 at E14. Therefore, pre-peritubular cells may express TGFβ3 to induce migration or differentiation resulting in seminiferous cord formation. Interestingly, only cells surrounding cords adjacent to the mesonephros stained for TGFβ3. Further investigation is necessary to determine the potential role of TGFβ3 in mesonephric cell migration into the testis. Expression of TGFβ3 in the P0 testis appeared to be in Sertoli cells with high concentrations localized in gonocytes. Therefore, TGFβ3 may regulate two distinct developmental processes at different times during testis development.

To extend these data, expression of mRNA for TGFβ isoforms were also evaluated in the current study. Expression patterns of TGFβ1, TGFβ2, and TGFβ3 are unique during testis development and do not appear to have overlapping expression patterns. Early in embryonic testis development (i.e., E15), only TGFβ2 expression was high while the concentrations of mRNA for TGFβ1 and TGFβ3 were relatively low. There were similar results with E14 testis (data not shown). In addition, expression of TGFβ1 was significantly higher at P0 and P10 while expression of TGFβ2 was highest at E15 and P3. Interestingly, TGFβ3 has the most distinctive expression pattern, with highest concentrations observed during embryonic testis development. This pattern may indicate an important role of TGFβ3 during embryonic testis development, or it may have been due to the dilution effect of spermatogenesis during the postnatal period. TGFβ1 has been reported to be present in germ cells and Sertoli cells during the postnatal period, and there was no dramatic change in expression of mRNA between the embryonic and postnatal periods. It is not known what the expression pattern of TGFβ3 is during the postnatal period; therefore, if it is present in the somatic cells, a decrease in expression of this gene would be observed.

Actions of TGFβ during embryonic testis development are thought to be primarily on gonocytes (E14), and by E16.5 on both Leydig cells and gonocytes [19]. Receptors for TGFβ have been localized to gonocytes and Leydig cells at these developmental time periods. TGFβ1 has been identified as a regulator of Leydig cell steroidogenesis, and it can inhibit LH-stimulated testosterone production [20] in E16.5 testis cultures. Both TGFβ1 and TGFβ2 have been demonstrated to induce gonocyte apoptosis around E14 of embryonic testis development [19]. Therefore, these results suggest that paracrine and/or autocrine interactions occur between cells that produce TGFβ1 and TGFβ2, to regulate both Leydig and gonocyte functions in the embryonic testis.

The potential action of TGFβ on seminiferous cord formation has not been previously reported and is addressed in the current study. Treatment of E13 testis organ cultures did not affect seminiferous cord formation in the present study. However, growth of the embryonic testis organ cultures was inhibited as measured by testis area. A reduction in seminiferous cord numbers was observed in E13 testis organ cultures, but this was determined to occur because of a reduced overall size of the testis. This inhibition of growth was further demonstrated in E14 testis organ cultures treated with TGFβ1, in which a 40% reduction in testis size compared to that of controls was observed. Previous reports have demonstrated a similar effect on gonocyte numbers with TGFβ1 and TGFβ2 treatment of E13.5 testis organ cultures. This reduction was demonstrated to occur through an increase in gonocyte apoptosis [19], and

no data were included on effects of TGFβ on overall testis size. In the current study, a 40% reduction in testis size in both E13 and E14 testis organ cultures was observed with TGFβ1. A similar effect would be hypothesized with TGFβ2. It seems unlikely that a 40% reduction in the size of the testis would occur because of the inhibition of one cell population alone. The dose of TGFβ1 used in these experiments (40 ng/ml) was greater than in the experiments previously reported (10 ng/ml) [19]. Additionally, our experiments were conducted for 3 days, with TGFβ1 treatments each day. Thus, by the last day of treatment, the Leydig cells may have obtained receptors for TGFβ, causing a reduction in cellular proliferation within this cell type. This may explain the dramatic reduction in testis size within the testis organ cultures in the current study. Therefore, regulation of expression of TGFβ1 may be important during embryonic testis development to initially regulate germ cell numbers and later to potentially regulate somatic cell growth.

TGFβ1 is capable of inhibiting early postnatal testis growth as well as embryonic testis growth. In the current study, TGFβ1 inhibited EGF- and 10% calf serum-stimulated growth in P0 testis cultures. The P0 testis cultures have a mixed population of cells, and these data must be interpreted carefully. Both gonocytes and Leydig cells, but not Sertoli cells, have receptors for TGFβ at this time during testis development. Treatment of P0 testis cultures with FSH stimulates tritiated thymidine incorporation, presumably through stimulation of growth of Sertoli cells, which contain FSH receptors. TGFβ1 did not inhibit FSH-stimulated growth but did inhibit EGF- and 10% calf serum-stimulated growth. Therefore, most of the effects of TGFβ1 on inhibition of P0 testis may be elicited through inhibition of interstitial and/or gonocyte cell growth.

The TGFβs have been reported to stop the cell cycle in numerous cell types [13, 27] late in G1 when the cell becomes committed to enter S phase. Previous literature has demonstrated that gonocytes undergo mitotic arrest around E17-E18 and resume mitosis after P5 [28]. TGFβ1 and TGFβ2 may contribute to the regulation of this mitotic arrest since they are both highly expressed around P0. Growth stimulators such as EGF and FSH must either stimulate the expression of genes that promote progression to the S phase of the cell cycle, or inhibit expression of genes that halt cell cycle progression. Therefore, in the present study we hypothesized that EGF and FSH may inhibit mRNA expression of the TGFβ isoforms. EGF did suppress expression of TGFβ3 in P0 testis 24 h after stimulation. However, most of the data collected in the present study did not support our hypothesis. FSH stimulation had no effect on expression of TGFβ isoforms. EGF stimulated expression of TGFβ1 and suppressed expression of mRNA for TGFβ3. These observation are interesting since FSH does not appear to regulate TGFβ isoforms while EGF appears to regulate them differentially. Previous data suggest overlapping roles for the TGFβ isoforms in the regulation of cell growth and proliferation; however, the current study suggests a unique regulation of each TGFβ isoform by growth factors.

Interestingly, FSH stimulation of P0 testis was not inhibited by TGFβ. In addition, FSH treatment did not influence expression of TGFβ isoforms. Potential interactions have been reported between TGFβ1 and LH in the fetal rat testis. TGFβ1 has been demonstrated to inhibit LH-induced cAMP production and LH-induced testosterone production in dispersed fetal testis (E16.5) [20]. However, no studies have demonstrated interactions between FSH and TGFβ isoforms. The results of the current study suggest that there is no direct regulation of FSH on expression of TGFβ isoforms in cultures of P0 testis. This may be due to the fact that Sertoli cells do not appear to contain receptors for TGFβ isoforms. Therefore, TGFβ isoforms are incapable of directly regulating Sertoli cell specific growth. FSH may also stimulate the expression of factors that stimulate growth such as TGFα.

It is also interesting that the growth factor EGF can stimulate expression of a growth-inhibitory factor such as TGFβ1 while suppressing TGFβ3. This differential regulation of the two isoforms of TGFβ may be due to the different cell types that produce each factor. At P0, TGFβ1 is localized to three different cell types while TGFβ3 is in gonocytes. EGF may differentially regulate these cell types. Increased expression of TGFβ1 in response to EGF would demonstrate a feedback loop between these growth factors. High concentrations of EGF may act to stimulate TGFβ1, which in turn suppresses EGF-stimulated growth.

The novel results of the current study demonstrate that TGFβ isoforms effect embryonic testis growth through the regulation of locally produced growth factors. The unique expression patterns and cellular localization of the TGFβ isoforms during embryonic testis development support a role for each isoform in the regulation of cellular growth and differentiation. TGFβ regulates embryonic testis growth through inhibition of growth stimulators such as EGF. In contrast, EGF regulates expression of mRNA for TGFβ isoforms. Combined observations suggest that the interactions of paracrine factors such as TGFβs and EGF allow for optimal cellular growth and differentiation during embryonic testis development.

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