

Role of Transforming Growth Factor- α and the Epidermal Growth Factor Receptor in Embryonic Rat Testis Development¹

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ABSTRACT

Embryonic testis development requires the morphogenesis of cords and growth of all cell populations to allow organ formation. It is anticipated that coordination of the growth and differentiation of various cell types involves locally produced growth factors. The current study was an investigation of the hypothesis that transforming growth factor- α (TGF- α) is involved in regulating embryonic testis growth. TGF- α has previously been shown to function in the postnatal testis. TGF- α and other members of the epidermal growth factor (EGF) family act through the epidermal growth factor receptor (EGFR) to stimulate cell proliferation and tissue morphogenesis. To understand the potential actions of TGF- α in the embryonic testis, general cell proliferation was investigated. Characterization of cell proliferation in the rat testis throughout embryonic and postnatal development indicated that each cell type has a distinct pattern of proliferation. Germ cell growth was transiently suppressed around birth. Interstitial cell growth was high embryonically and decreased to low levels around birth. A low level of Sertoli cell proliferation was observed at the onset of testis cord formation. Sertoli cell proliferation in early embryonic development was low; the levels were high later in embryonic development and remained high until the onset of puberty. Both TGF- α and the EGFR were shown to be expressed in the embryonic and postnatal rat and mouse testis. Perturbation of TGF- α function using neutralizing antibodies to TGF- α on testis organ cultures dramatically inhibited the growth of both embryonic and neonatal testis. TGF- α antibodies had no effect on cord formation. The TGF- α antibody was found to be specific for TGF- α in Western blots when compared to EGF and heregulin. Testis growth was also inhibited by perturbation of EGFR signaling using an EGFR kinase inhibitor. Therefore, TGF- α appears to influence embryonic testis growth but not morphogenesis (i.e., cord formation). Treatment of embryonic testis organ cultures with exogenous TGF- α also perturbed development, leading to an increased proliferation of unorganized cells. Testis from EGFR and TGF- α knockout mice were analyzed for testis morphology. TGF- α knockout mice had no alterations in testis phenotype, while EGFR knockout mice had a transient decrease in the relative amount of interstitial cells before birth. Observations suggest that there may be alternate or compensatory factors that allow testis growth to occur in the apparent absence of TGF- α actions in the mutant mice. In summary, the results obtained suggest that TGF- α is an important factor in the regulation of embryonic

testis growth, but other factors will also be involved in the process.

INTRODUCTION

The development of the testis requires coordination of the growth and differentiation of several cell types. The testis is formed initially from an undifferentiated bipotential gonad (i.e., embryonic Day [e]13 in the rat). At this stage it is indistinguishable from the ovary and possesses no morphological organization. The first sign of male development is the differentiation of precursor Sertoli cells, which then aggregate with germ cells and undergo a transition from mesenchyme to epithelia to form cords (i.e., e13.5 to e14 in the rat) [1, 2]. The testis cords remain solid until a lumen forms after the onset of puberty (i.e., postnatal Day 10 in the rat) to convert them into tubules (i.e., postnatal Days 14 to 18 in the rat). The seminiferous tubules are the site of spermatogenesis, in which spermatogonia develop into spermatozoa in close interaction with the Sertoli cells that make up the walls of the tubule [3–5]. Surrounding the Sertoli cells is a layer of peritubular myoid cells that are responsible for contraction of the tubule [6–8] and regulation of differentiated function [9, 10]. In the interstitial space between the tubules are Leydig cells, stromal fibroblasts, vascular and lymphatic endothelial cells, and macrophages [9].

Dramatic growth occurs in the testis during embryonic and early postnatal development [11]. One of the earliest male-specific characteristics of the testis after the formation of cords is rapid growth. This extensive growth does not occur in the ovary [12]. The peak of Sertoli cell proliferation occurs in the late embryo at e20–21 just before birth [13]. This proliferation in the embryonic and early postnatal period is crucial for establishing the mature adult size of the testis since no Sertoli cell proliferation occurs after initiation of puberty [14, 15]. In addition, this proliferation is necessary for reproductive function, since a sufficient population of Sertoli cells is necessary for adequate sperm production in the adult [13, 16, 17]. The majority of information about testicular growth regulation has concerned postnatal growth or occasionally late embryonic growth [11, 13–19]. Several growth factors have been identified in the postnatal testis and are postulated to be involved in the regulation of puberty and spermatogenesis [9, 20, 21]. The current study was an investigation of growth in the embryonic testis.

One family of growth factors that may play a role in embryonic testis growth is the epidermal growth factor (EGF) family. There are multiple related ligands in this family, including EGF, transforming growth factor α (TGF- α), amphiregulin, heparin-binding EGF, crypto, betacellulin, and heregulin [22–24]. The receptors include the epidermal growth factor receptor (EGFR/HER1/erbB1), HER2/neu, HER3, and HER4 [25, 26]. The EGFR family of receptors function by ligand-dependent dimerization and

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activation of the tyrosine kinase in the cytoplasmic domain [27, 28]. EGF and its related ligands are capable of inducing proliferation, differentiation, and migration in many different cell types [29]. The EGF family, and particularly the heregulin receptors, have been associated with stimulation of proliferation and malignant transformation. In addition, these factors are known to stimulate growth and morphogenesis in several developing systems including the kidney, lung, mammary gland, and microvascular endothelial cells [30–36].

EGF and the related ligand transforming growth factor α (TGF- α) are expressed in the postnatal testis [37–42]. TGF- α and EGF have various effects on the proliferation, differentiation, and migration of Sertoli, Leydig, and peritubular cells [37, 39, 43]. Although they have not been as well characterized, other members of the EGF/TGF- α family are also found in the testis [26, 44, 45]. Since the TGF- α /EGF family of ligands have the ability to stimulate both growth and morphogenesis, they have the potential to play a critical role in early testis development. The current experiments were designed to investigate the hypothesis that the TGF- α family of growth factors has a role in embryonic testis development.

MATERIALS AND METHODS

Dissections and Organ Cultures

Timed-pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Plug date was considered to be embryonic Day 0 (e0). For e13 dissections, gonads were dissected out with the mesonephros; for later-stage dissections, testes alone were dissected. The organs were cultured at 37°C in 5% CO₂ in drops of medium on Millicell CM filters (Millipore, Bedford MA) floating on the surface of 0.5–1 ml of CMRL 1066 medium (Gibco BRL, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μ g/ml), and transferrin (10 μ g/ml). Antibodies and factors were added directly to the culture medium. The medium was changed every 1 or 2 days. Cultures of e13 gonads + mesonephros were incubated for 3 days, by which point cords were well developed; e14 testis cultures were incubated for 4 days. For e18 and postnatal Day 0 (0d) organ cultures, the testes were cultured as fragments (halves of e18 testes and eighths of 0d testes), since entire testes were too large to culture intact without considerable necrosis in the centers. The tunica was removed from 0d testis before culturing. The testes collected for morphological analysis prior to cell culture were fixed and processed immediately after microdissection or were obtained from whole embryos that had been fixed prior to microdissection. All procedures were approved by the appropriate University Animal Care committees.

Testis Suspension and Reaggregation

To generate a testicular suspension from 0d testis, the tunica was removed and the testes were digested with 0.125% trypsin, 0.1% EDTA, and 0.02 mg/ml 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 pellet was resuspended in a small volume (10 μ l from 6–7 pooled testes) and contained a single-cell suspension according to visual inspection under a microscope. Drops of 2 μ l of this sus-

pension were cultured and allowed to reaggregate on floating filters as for intact organs.

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

To determine the sex of e13 gonads, 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 precipitated by adding 1/10 volume 7.5 M NH₄Ac 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 μ l H₂O. PCR was performed by using 1 μ l of genomic DNA with the consensus SRY primers 5' CGGGATCCATGTCAAGCGCC CCATGAATGCATT-TATG 3' and 5' GCGGAATTCACCTTAGCCCTCCGAT-GAGGCTGA TAT 3', producing a PCR product of 240 base pairs (bp). PCR was performed using an annealing temperature of 55°C for 30 cycles.

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

RNA for RT-PCR was obtained by freezing samples on dry ice and then using the Tri Reagent (Sigma Chemical Co., St. Louis, MO) for RNA isolation. Reverse transcription was performed using murine Moloney leukemia virus-RT under standard conditions. RT-PCR was performed for EGFR at 60°C annealing temperature for 30 cycles and for TGF- α at 65°C for 5 cycles followed by 55°C for 30 cycles. The EGFR primer sequences were 5'TGCGpTCTCTTG-CCGGAATGTCAG3' and 5'GCAGTGGGGGCCGTCA-ATGTAGT3', producing a PCR product of 205 bp. The TGF- α primer sequences were 5'ACCCCTTATCACACA-GTTTT3' and 5'CCCTGGCTGTCTC ATTATCACCTG 3', producing a PCR product of 380 bp. The identity of the PCR products was confirmed by restriction digests.

Embedding, Histology, and Immunocytochemistry for Proliferating Cell Nuclear Antigen (PCNA)

Tissues were fixed in Histochoice (Amresco, Solon, OH) and embedded in paraffin or in JB4 (Polysciences, Warrington, PA) according to standard procedures. Sections were stained with hematoxylin and eosin (paraffin sections) or with toluidine blue (JB4 sections) according to standard procedures. Immunocytochemistry for PCNA was performed according to standard procedures. Briefly, 7- μ m sections were deparaffinized and rehydrated, quenched in 20% methanol/3% hydrogen peroxide, and blocked in 5% serum for several hours at room temperature before incubation with primary antibody monoclonal anti-PCNA clone PC10 (Sigma) at 1:500 dilution overnight at 4°C. Secondary antibody (biotinylated sheep anti-mouse biotin from Amersham Pharmacia Biotech, Piscataway, NJ) was detected by using the Vectastain kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine. Slides were counterstained lightly with hematoxylin to visualize the tissue.

Labeling indices for the percentage of each cell type stained for PCNA were calculated by counting random regions on at least 3 different sections from 3 different ex-

periments. The total number of cells counted in specific regions ranged from 155 to 396 Sertoli cells, 80 to 396 germ cells, 338 to 862 interstitial cells, and 139 to 306 peritubular cells. Counts were performed by two different people independently for comparison, and data were combined. Data are presented as the mean \pm SEM to assess the percentage of stained cells. The data from the two individuals generally compared as shown by the reproducibility of the data.

DNA Assay

To assay the DNA content of organs, each organ was sonicated in 100 μ l 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 [37]. Briefly, 0.25 nM ethidium bromide and 100 units/ml heparin in EBB were added to each sample; samples were 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 μ g to 6 μ g DNA.

Imaging and Area Quantification

Images of whole organs were obtained by using a Lumina digital scanning camera (Leaf Systems, Southborough, MA). Area was calculated by using Adobe Photoshop (Mountain View, CA). To determine the relative percentage of interstitium in testis, representative sections were imaged and analyzed using Adobe Photoshop. The outline of each cord was traced and the area within each was calculated, as was the total area. The percentage of interstitium was calculated as area cords/total area.

Western Blotting

Western blotting was performed according to standard procedures. Protein samples were electrophoresed on a 8–25% Phast gradient gel (Phast System; Pharmacia, Alameda, CA) and transferred to nitrocellulose by using the PhastTransfer semidry electrophoretic transfer unit (Pharmacia). After blocking overnight in 5% dry milk/1% glycine/0.05% Nonidet P-40 (NP40) in 50 mM Tris-HCl (pH 7.4)/0.15 M NaCl (TBS), the primary antibodies against TGF- α were incubated with the blots for 4 h. After three washes in TBS/NP40 and one in TBS, the blots were incubated with secondary antibody anti-sheep conjugated to horseradish peroxidase (Sigma) at 1:3000 for 1 h. After three washes in TBS/NP40, one in TBS, and three in water, detection was carried out using the ECL system (Amersham).

Reagents

The antibodies against TGF- α (S574 raised against rat TGF- α and S509 raised against human TGF- α) were obtained from East Acres Biological (Southbridge, MA). These TGF- α antibodies have been shown to be neutralizing polyclonal antibodies and to cross-react with mouse, rat, human, and bovine TGF- α and not to cross-react with EGF, amphiregulin, TGF- β , or fibroblast growth factor (FGF) on Western blots (East Acres Biological). The EGFR inhibitor AG1478 was obtained from Calbiochem (La Jolla, CA). AG1478 is a highly selective competitive inhibitor of ATP binding to the EGFR. The *in vitro* IC₅₀ for EGFR is 3 nM, while the IC₅₀ for HER2 and the platelet-derived

growth factor receptor are greater than 100 μ M. TGF- α and recombinant human heregulin- α were obtained from R&D Systems (Minneapolis, MN). TGF- α knockout mice were provided generously by Dr. Gerald Cunha (University of California at San Francisco [UCSF], San Francisco, CA), who maintains a colony of mice previously developed [19]. EGFR knockout mice were provided generously by Zena Werb and Rik Derynk (UCSF) and by Terry Magnuson (Case Western University, Cleveland, OH).

Statistical Analysis

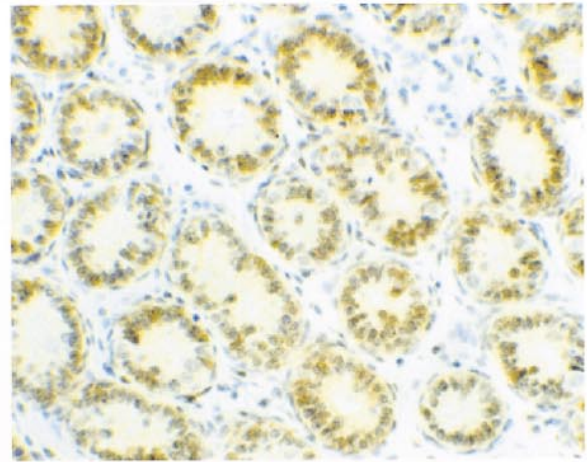
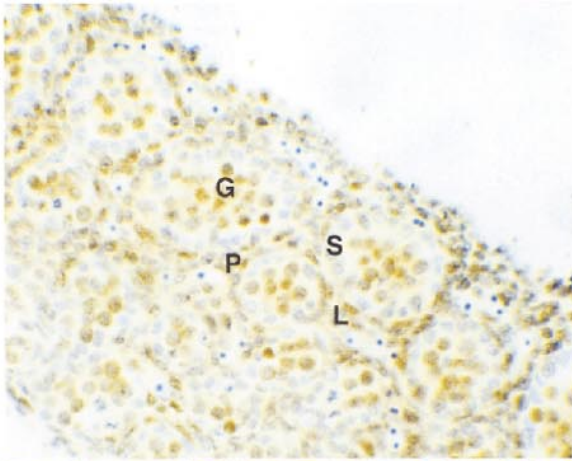
All the data were obtained from a minimum of three different experiments unless otherwise stated. Each data point was converted to a mean and SEM from multiple experiments determined as indicated in the figure legends. Data were analyzed by a Student's *t*-test or ANOVA as indicated in the figure legends. Different superscript letters denote a statistical difference.

RESULTS

Proliferation Pattern in Developing Testis

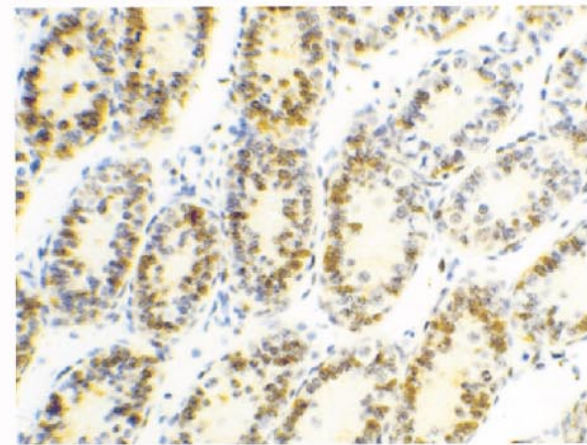
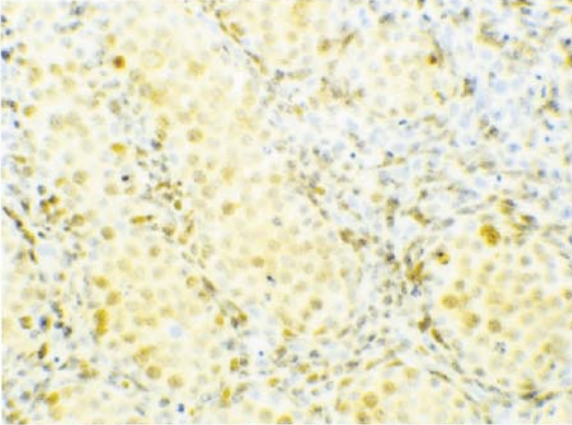
Investigation of the effects of a particular factor on growth in the developing testis requires an understanding of the overall pattern of growth of different cell types at various stages of development. The cell populations that are proliferating provide potential targets for the locally produced growth factors (e.g., TGF- α). The pattern of cell proliferation in the early rat testis was determined by using immunocytochemistry for PCNA (Fig. 1). PCNA is an auxiliary protein of DNA polymerase that is required for DNA replication during S-phase and therefore is expressed only in proliferating cells [46–48]. The earliest time examined was e14, which is the earliest point at which the testis is distinguishable morphologically from the ovary, just after cords have formed (Fig. 1A). Additional time points were examined through late embryonic and early postnatal development up to the early pubertal period of 20 days postnatal (20d). The pattern of PCNA staining changed during embryonic and early postnatal development (Fig. 1). At e14 (Fig. 1A), many cells in both the cords and the interstitium expressed PCNA, although many of the Sertoli nuclei that were lined up at the edges of the cords were unstained. By e16 (Fig. 1B), all cells in the cords were stained, as were the peritubular cells surrounding the cords. The interstitium contained both PCNA-positive and PCNA-negative cells. The pattern at e18 (Fig. 1C) was similar except that more germ cells in the centers of the cords did not stain. This high percentage of PCNA-positive cells throughout the late embryonic testis is consistent with the rapid growth of the organ. At birth (0d; Fig. 1D) there was little staining of interstitial, peritubular, or germ cells, but almost all Sertoli cells were stained. By 5d (Fig. 1E), the cells in the cords have proliferated so that the cords occupy much more of the area of the testis than in the embryonic testis. The Sertoli cells were still stained heavily for PCNA, and some germ cells had begun expressing PCNA again. At 10d (Fig. 1F), some Sertoli cells had left the cell cycle; at 20d, the Sertoli cells did not stain (data not shown). Similar results were obtained with the adult 60d testis (data not shown). Various spermatogenic cells were stained, including spermatogonia at the outside edges of the tubules and more centrally located spermatocytes. In addition, meiotic figures were visible. A high magnification of 10d testis is presented

A.
e14



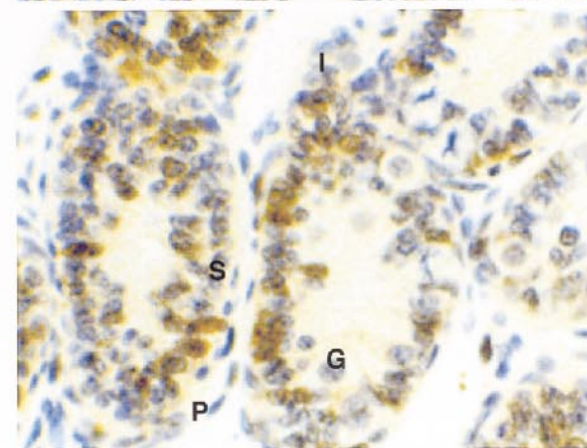
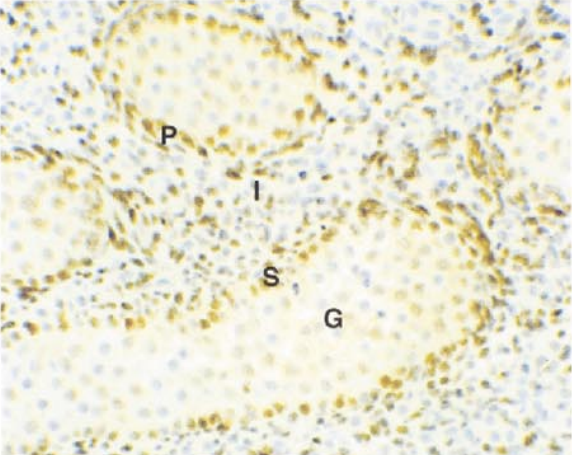
E.
5d

B.
e16



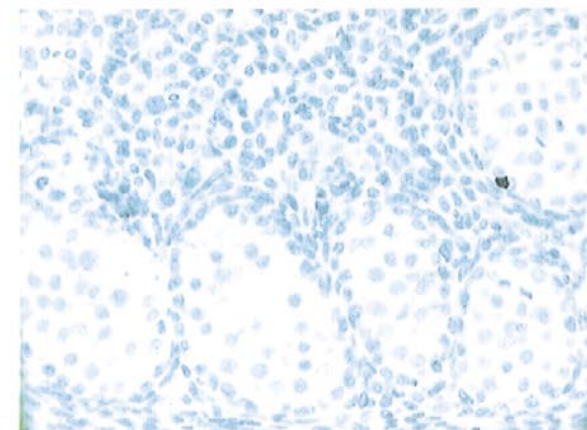
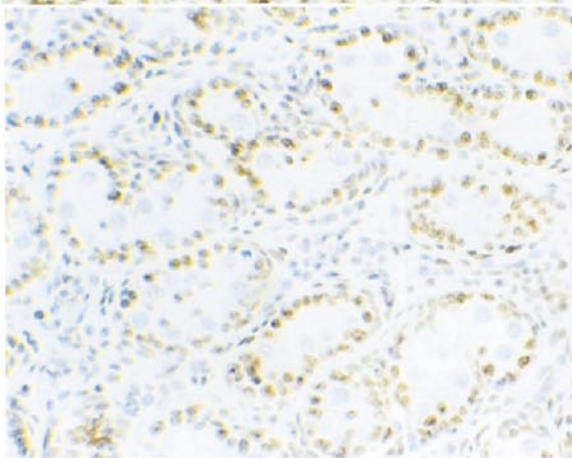
F.
10d

C.
e18



G.
10d

D.
0d



H.
e18

in Figure 1G to show specific cell populations. A control background stain with nonimmune IgG is shown in Figure 1H.

Labeling indices were calculated at ages e14, e18, 0d, 5d, and 10d to extend the observations with a calculation of the nuclear PCNA staining pattern and ratio of cells stained (Fig. 2). Proliferation decreased in germ cells by e18, was minimal at 0d, and returned to high levels by 5d postnatal. The interstitium proliferated from the initiation of testis development through the late embryo and decreased substantially in the early postnatal period. Sertoli cells did not appear to be proliferating at the time of cord formation (e14) but proliferated extensively through the rest of embryonic and early postnatal development until puberty. The decrease of Sertoli cell proliferation at the onset of puberty has been previously documented [14]. Peritubular proliferation was high until after birth and then decreased dramatically. Observations suggest that all the testis cell populations provide potential targets for locally produced growth factors (e.g., TGF- α).

Expression of TGF- α and EGFR in the Embryonic Testis

Both TGF- α and EGFR are expressed in the postnatal testis. To investigate their presence in the embryonic rat testis, RNA was isolated from testes of various ages and RT-PCR was performed. RT-PCR for TGF- α and EGFR (Fig. 3) demonstrated that both genes were expressed throughout embryonic testis development at e14, e18, and 0d. Similar results were obtained from e14 and 0d mouse testis RNA (data not shown).

Immunocytochemistry was performed to confirm the expression of the TGF- α and EGFR proteins. TGF- α and EGFR immunocytochemistry utilized sections from 0d rat testis. The TGF- α and EGFR were primarily localized to the interstitial cells with lower levels of staining in the cords (Fig. 4). Preabsorption of the TGF- α antibody with excess TGF- α eliminated the staining detected in the immunocytochemistry (data not shown). Observations suggested that interstitial cells and to a lesser extent the cords in the 0d testis express the TGF- α and EGFR proteins and supported the mRNA data presented in Figure 3. In addition, the specificity of the TGF- α antibody was indicated by the immunocytochemistry control.

Functional Perturbation of Embryonic Testis Development with TGF- α -Neutralizing Antibody

To investigate whether TGF- α is important for stimulating the growth of the embryonic testis, organ cultures of the embryonic testis were established. Growing the testes in organ culture allowed direct perturbation of their development. Testes from e14 embryos grew during 4 days in culture and maintained their cord structure. Testes from e13 embryos cultured with the mesonephros developed cords over 3 days in culture (Fig. 5A). The top part of the micrograph (marked "T") is the testis connected to the lower

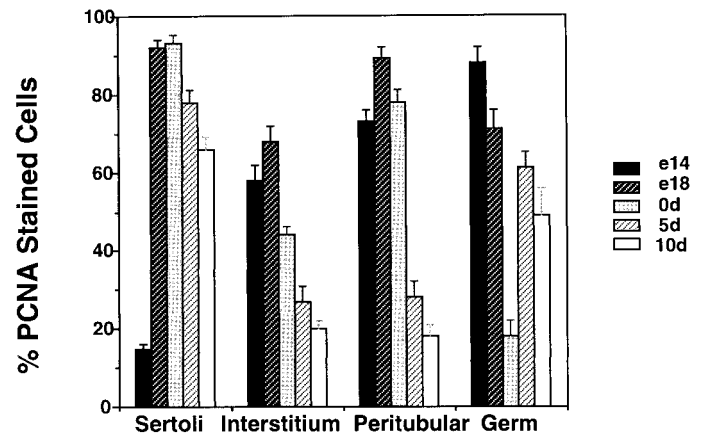


FIG. 2. Labeling indices of the percentage of PCNA-positive cells for each cell type at various stages of development. Labeling indices were calculated by counting stained and unstained cells in random regions of replicate slides from at least 3 different sections in 3 different experiments. Data are presented as the mean \pm SEM.

FIG. 1. PCNA immunocytochemistry in the developing rat testis; (G) high magnification of F. PCNA-positive cells are brown while negative cells are purple from hematoxylin counterstain. (H) A negative control e18 hematoxylin stain. Representative cells: P, peritubular cell; S, Sertoli cell; G, germ cell; L or I, interstitial cell. (G) Two different stages of germ cell are labeled (spermatogonia and spermatocyte). $\times 200$ except for G, which is $\times 400$. Representative of at least three different experiments done in replicate.

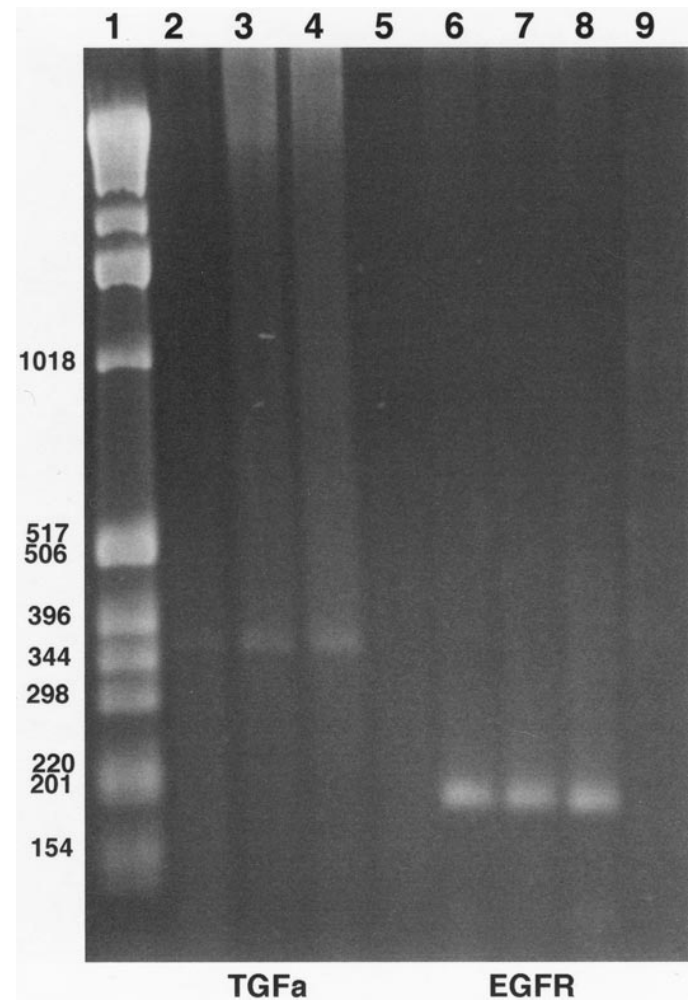


FIG. 3. Expression of TGF- α and EGFR mRNA by RT-PCR in the developing testis. Lanes 1–5, TGF α ; lanes 6–9, EGFR. Individual lanes: 1, molecular weight markers; 2, e14; 3, e18; 4, 0d; 5, no template; 6, e14; 7, e18; 8, 0d; 9, no template. The sizes of the PCR products were 380 bp for TGF- α and 205 bp for EGFR. Representative of at least 3 different experiments.

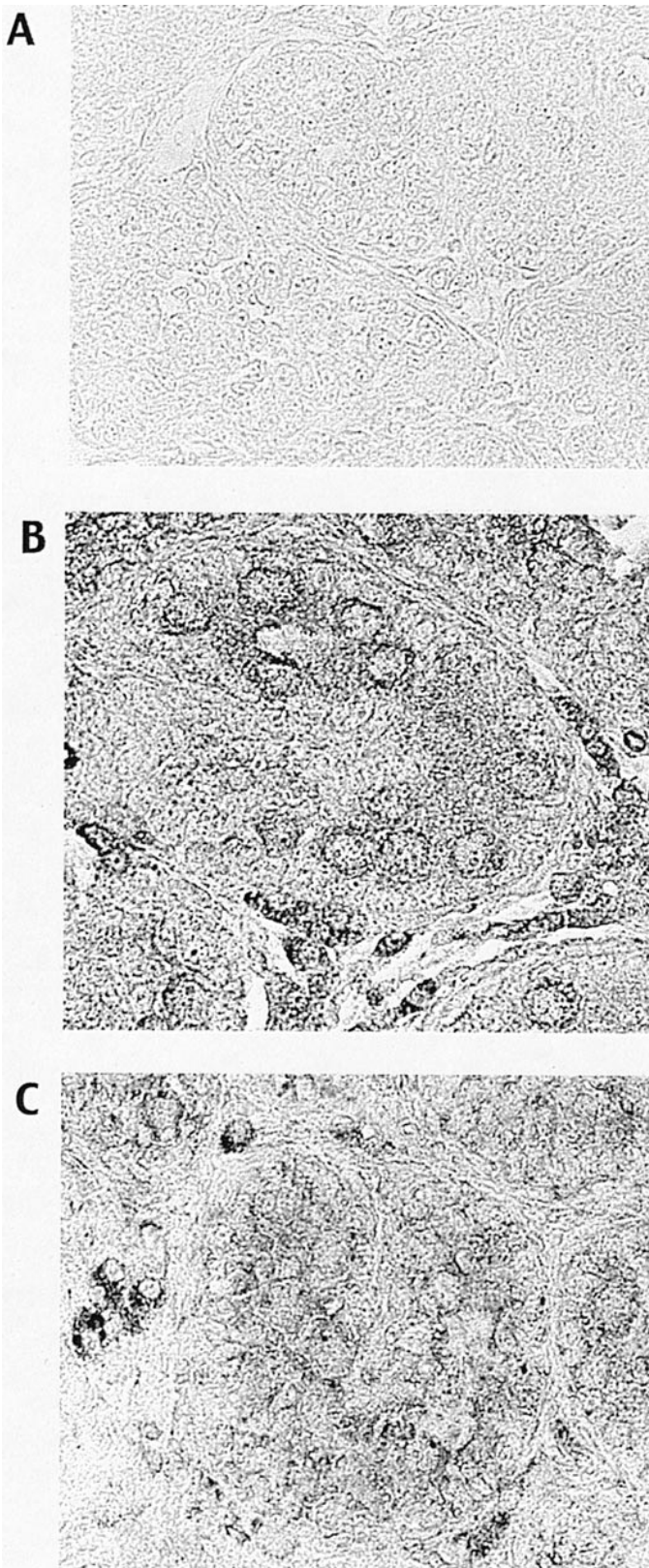


FIG. 4. TGF- α (B), EGFR (C), and control nonimmune IgG (A) immunocytochemistry on 0d testis sections. TGF- α - and EGFR-positive cells are dark grey/black. $\times 100$ (published at 93%). Representative of at least 2 different experiments.

darkly shaded mesonephros (Fig. 5B). The ability to observe the cords and their lighter appearance were likely due to the comparatively less dense cell population in the cords than in the interstitium.

Two different neutralizing antibodies to TGF- α were used to treat embryonic testes placed in culture at e14 when cords were already present. These TGF- α antibodies have previously been shown not to cross-react with other EGF-like factors such as EGF and amphiregulin. In addition, these are neutralizing antibodies. The antibodies to TGF- α (i.e., IgG fraction) caused dramatic inhibition of growth compared to the control nonimmune IgG (Fig. 5). The cords were not disrupted by the TGF- α antibody (Fig. 5C,D). Various titers of TGF- α antibody (i.e., IgG fraction) were used, and the optimal titer (1:8000 dilution) is presented. The lighter-colored cord structures in the tissue are indicated with arrows (Fig. 5C, D). As suggested above, the lighter staining in the cords was likely due to a decreased number of nuclei and cells. These are isolated testes devoid of mesonephros in comparison to those in Figures 5A and 5B. Therefore, the morphology of the testis in Figures 5B and 5D are different owing to absence of the mesonephros and manipulation of the tissue in Figure 5B. The effect on total tissue or cell growth was quantified by determining the DNA content of organs as an estimate of cell numbers. DNA content was determined at the time of initial dissection and after 4 days in culture with nonimmune IgG or anti-TGF- α antibody (Fig. 6A). There was a 2.5-fold increase in DNA content in the untreated testes or in testes treated with nonimmune IgG. In contrast, there was almost no growth in the TGF- α antibody-treated organs. A second independent measure of cell growth was performed by video imaging of the organs into a computer followed by quantification of the area of the images (Fig. 6B). Two independently raised antibodies to TGF- α gave the same inhibition of testis growth (data not shown).

PCNA immunocytochemistry was performed to examine the general proliferation of cells in the TGF- α antibody-treated (i.e., IgG fraction) organ cultures (data not shown). There was less PCNA staining, indicating fewer proliferating cells in the testis treated with TGF- α antibody. After 4 days in culture the PCNA expression in both the cords and the interstitium was quantified. Labeling indices revealed a significant decrease in the percentage of proliferating Sertoli cells and interstitial cells (Fig. 7).

Examination of the Role of TGF- α at the Initiation of Testis Development

TGF- α appears to be important in stimulating the growth of the testis from the earliest point at which the testis is distinguishable from an ovary. Inhibiting TGF- α at this stage did not disrupt testis cords. This observation suggests that TGF- α is not necessary for the maintenance of testis cords. To investigate whether TGF- α is required for the formation of cords, organ cultures of embryonic gonads with mesonephros were established early on e13 before cords had formed. Organ cultures of e13 testes and mesonephros were treated with the antibody (i.e., IgG fraction) to TGF- α to determine whether inhibiting TGF- α at this stage would interfere with cord formation (Fig. 8). The antibody to TGF- α was functional in these cultures, since inhibition of growth was observed. However, there was no effect on cord formation. There was no delay in the timing of the appearance of cords (examined every 12 h) (Fig. 8).

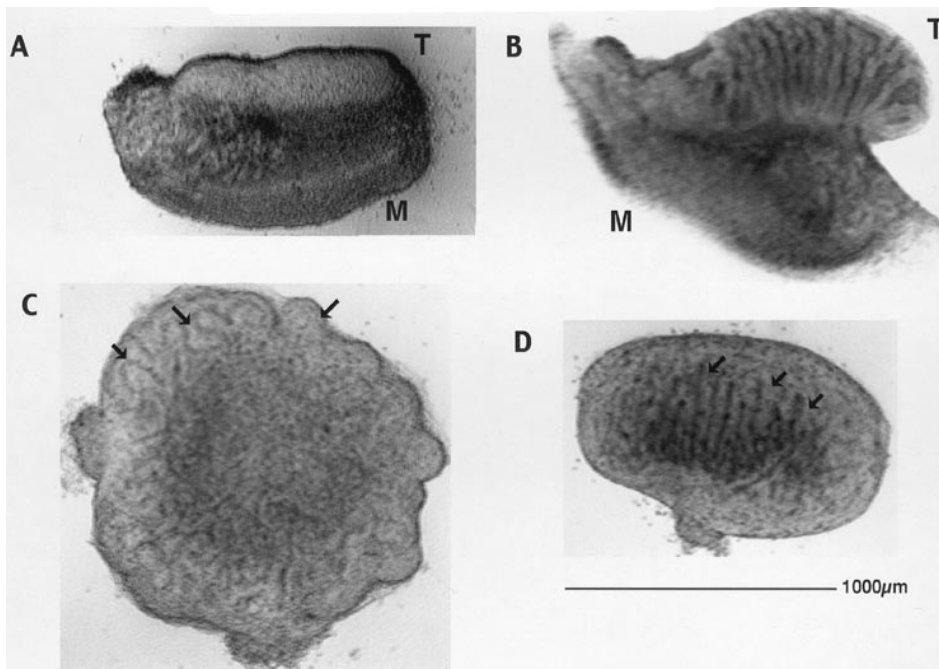


FIG. 5. E13 testis + mesonephros organ cultures after 1 day (A) and after 3 days (B) in culture. The upper structure (T) is the testis attached to the lower, dark-colored mesonephros (M). Representative of more than 10 different experiments. The diameter of the organ was approximately 1000 μ m. E14 testis organ cultures treated with control nonimmune IgG (C) or with anti-TGF- α IgG antibody S574 (D) after 4 days in culture. Representative cords are marked by arrows placed on the lighter-colored structures and are shown in C and D. This is a microdissected testis devoid of mesonephros with some manipulation in comparison to that shown in A and B. Representative of 10 experiments.

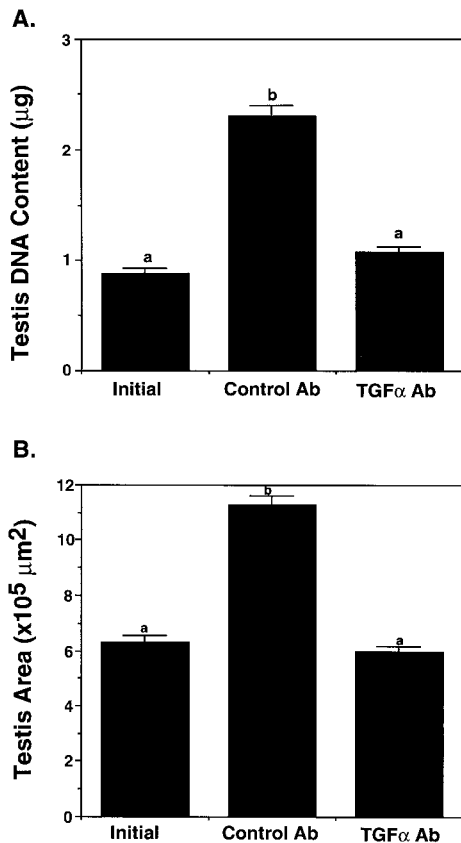


FIG. 6. Quantitation of e14 testis organ cultures treated with control non-immune IgG or with anti-TGF- α IgG antibody S574 for 4 days in culture. (A) DNA content (μ g) was measured by ethidium bromide fluorometry on extracts of testis organ cultures. Data are presented as the mean \pm SEM for 3 separate experiments with n = 9 (Initial), n = 21 (Control Ab), and n = 22 (TGF- α Ab). (B) Area (μm^2) was measured using NIH Image after 1 day and after 4 days in culture. Data are presented as the mean \pm SEM for 3 separate experiments with n = 13 (Initial = 1 day), n = 23 (Control Ab), and n = 24 (TGF- α Ab). Different superscripts designate statistically significant difference ($P < 0.01$) as determined by ANOVA.

Investigation of the Role of TGF- α Later in Embryonic and Perinatal Development

In order to determine whether TGF- α is required for testis growth in perinatal development, testis organ cultures at later embryonic ages were treated with an antibody to TGF- α and assayed for DNA content as a measure of cell number. There was still dramatic inhibition of testis growth at e18 by the antibody to TGF- α (Fig. 9A). The control organs treated with nonimmune IgG exhibited only about a 1.5-fold increase in DNA content after 6 days in culture instead of the 2.5-fold increase seen with e14 testis. The antibody to TGF- α abolished all growth. The smaller increase in size of the control organs may reflect a decreased growth and/or a limitation on growth in the organ culture system. The 0d testes grew less, and growth was inhibited partially by

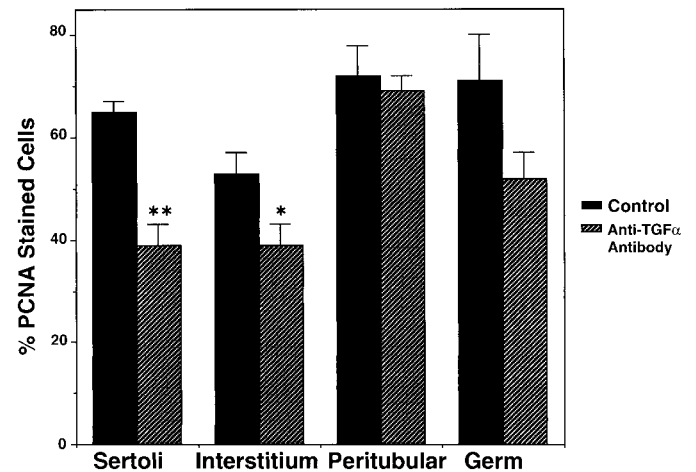


FIG. 7. Labeling index for the percentage of each cell type positive for PCNA on e14 testis organ cultures treated with control nonimmune or anti-TGF- α antibody for 4 days. Labeling indices were calculated by counting stained and unstained cells in random regions of at least 3 different sections from 3 different experiments. ** Statistically significant difference from control ($P < 0.001$); * statistically significant difference from control ($P < 0.02$) as determined by a *t*-test.

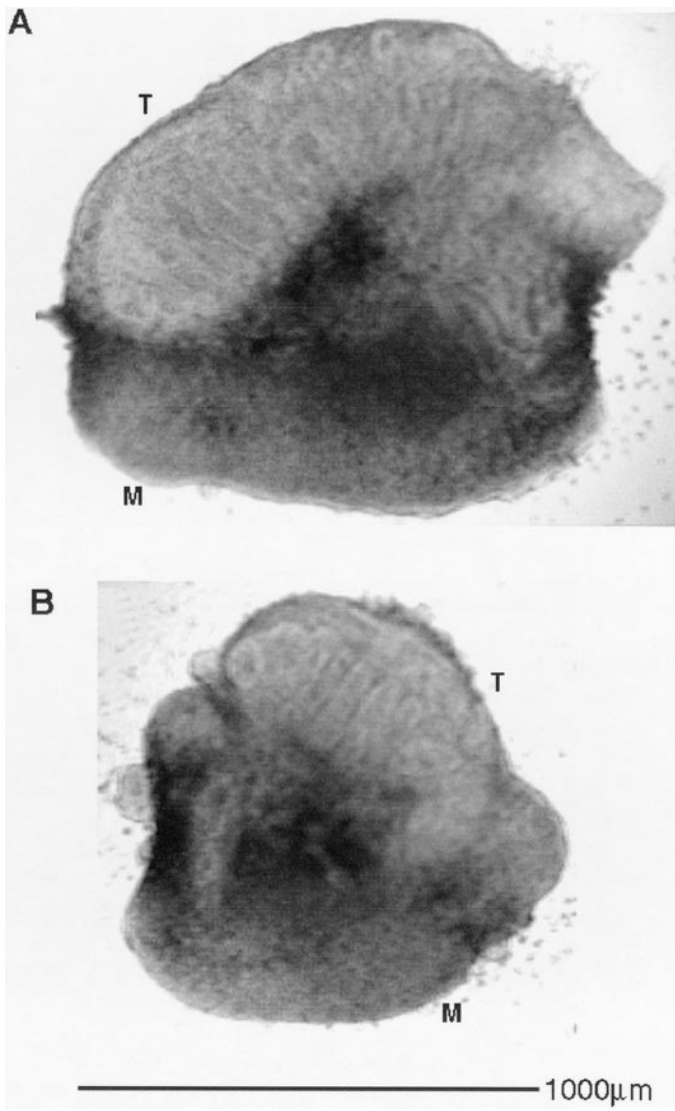


FIG. 8. E13 testis + mesonephros organ cultures treated with control nonimmune IgG or anti-TGF- α IgG antibody S574 for 3 days. (A) Control nonimmune IgG; (B) anti-TGF- α IgG antibody. T, testis; M, mesonephros. A and B are the same magnification. Representative of 4 different experiments.

the antibody to TGF- α (Fig. 9B). Observations suggest that TGF- α is important for testis growth throughout embryonic development and in early postnatal development.

Perturbation of the EGFR by a Tyrophostin Inhibitor

To confirm the importance of TGF- α in testis growth, an independent approach of perturbing the EGFR signaling

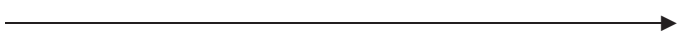
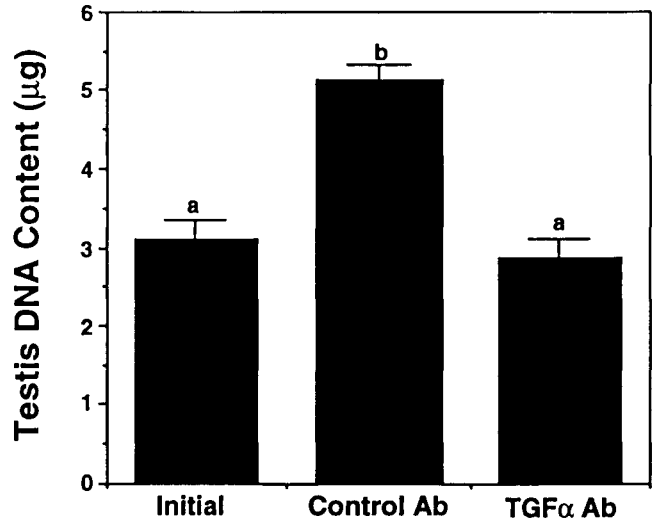
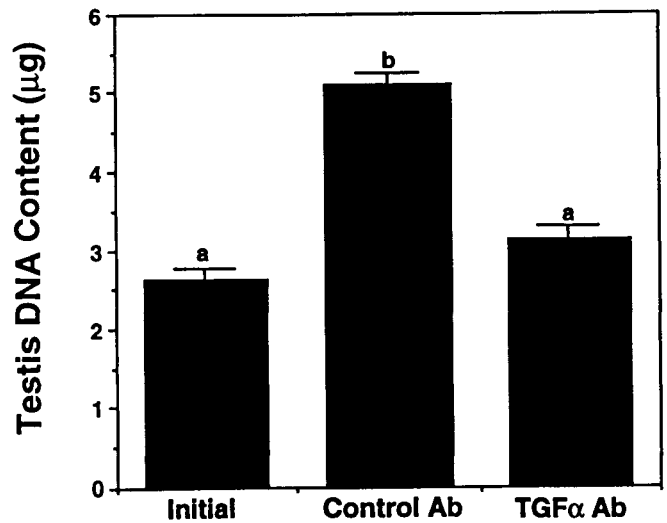


FIG. 9. Effects of control nonimmune IgG or anti-TGF- α antibody S574 on the growth of e18 (A) and 0d (B) testis. DNA content (μg) was determined at the time of dissection (initial) or after 4 days in culture. (A) Mean \pm SEM for 2 separate experiments with $n = 22$ for all conditions. (B) Mean \pm SEM for 3 separate experiments with $n = 28$ (initial) and $n = 29$ (control Ab and anti-TGF- α antibody) using 1/8 of testis. (C) Effects of EGFR tyrosine phosphorylation inhibitor AG1478 (160 nM) or control solvent dimethyl sulfoxide (DMSO) on the growth of e14 testis cultured for 4 days; mean \pm SEM for 3 separate experiments with $n = 13$ (initial), $n = 14$ (DMSO), and $n = 15$ (AG1478). Different superscripts designate statistically significant differences ($P < 0.01$) as determined by ANOVA.

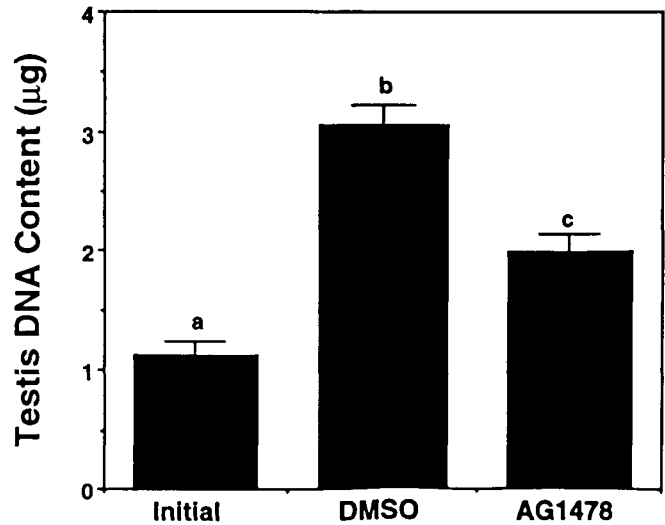
A. E18 TESTIS



B. 0 DAY TESTIS



C. E14 TESTIS



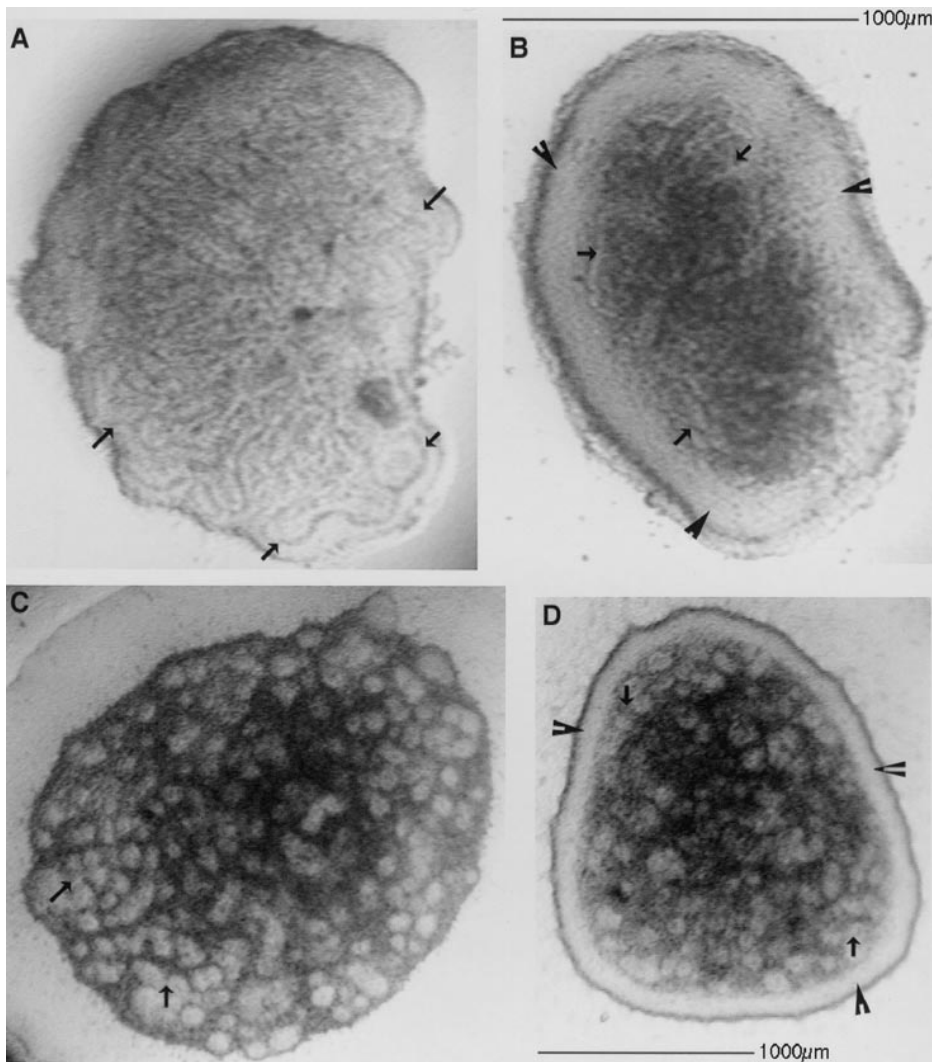


FIG. 10. Effects of exogenous TGF- α (50 ng/ml) on testis cultures. **A,B**) E14 testis cultured for 4 days in the absence (**A**) or presence (**B**) of TGF- α (40 ng/ml). Representative of 5 different experiments. (**C,D**) Dissociation-reaggregation cultures of 0d testis in the absence (**C**) or presence (**D**) of TGF- α (100 ng/ml). Representative of 2 different experiments. Representative cords are marked by arrows. The ring of unorganized tissue around the edge in **B** and **D** is marked by arrowheads.

pathway was used. The action of the EGFR was blocked with use of a highly selective tyrosine kinase inhibitor of the tyrphostin family known as AG1478 [49, 50]. Treatment of e14 testes in organ culture with 160 nM AG1478 resulted in a striking inhibition of growth (Fig. 9C). Treatment with 80 nM AG1478 was found to be less effective, although it resulted in some inhibition of growth (data not shown). Similar growth inhibition was apparent when 0d testis cultures were treated with AG1478 (data not shown). Observations suggest that the TGF- α receptor EGFR is required for optimal embryonic testis growth.

Effect of Exogenous TGF- α on Testis Organ Cultures

If TGF- α is important in promoting the proliferation of cells in the embryonic testis, exogenous TGF- α may influence testis growth. Testis organ cultures at e14 were treated with 50 ng/ml of TGF- α and then stained for PCNA to determine whether there was an increase in the number of proliferating cells. A 25-ng/ml and a 100-ng/ml dose of TGF- α gave results similar to those with the 50-ng/ml dose (data not shown). Addition of TGF- α to the culture medium caused a ring of unorganized tissue around the edge of the organ (Fig. 10A, B). Immunocytochemistry for PCNA demonstrated that almost all the cells in the outer ring were proliferating (data not shown). This suggests that cells in the embryonic testis are capable of responding to exoge-

neous TGF- α by inducing additional proliferation. The same effect was seen with reaggregated cultures of a cell suspension from a 0d testis. When a 0d testis was dissociated to single cells and the cells were allowed to reaggregate on a floating filter, cords reformed in the aggregate (Fig. 10C). When these cultures were treated with exogenous TGF- α , a ring of unorganized cells developed around the edge just as in the intact testis organ cultures (Fig. 10C, D).

Examination of Testis from EGFR and TGF- α Knockout Mice

The data with the neutralizing antibodies suggested that TGF- α is critical for growth of the early embryonic testis. Since TGF- α functions through the EGFR, it was possible that EGFR knockout mice might show a defect in testis growth. Testis from EGFR knockout mice were examined at several ages starting with e13 (the equivalent of e15 in the rat). Despite dramatic effects on other organs and on the overall size of the EGFR knockout mice [51–53], the testes were grossly normal (Fig. 11). There was apparently no decrease in size in the early embryonic testis compared to that in wild-type littermates. Later in development there was some decrease in size, but it was less than the overall decrease in size of the animals (data not shown). It appears

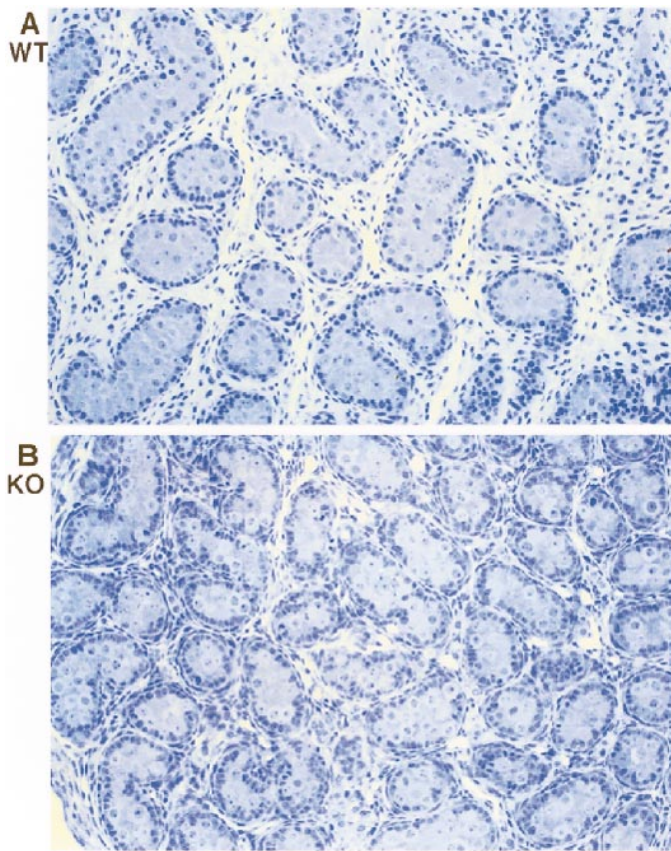


FIG. 11. **A**) E18 wild-type (WT) and **B**) e18 knockout (KO) mice testes. JB4 sections were stained with toluidine blue. $\times 200$ (published at 80%). Data are representative of more than 10 sections from different testes.

that the testis is able to compensate for the lack of the EGFR.

When sections through the testes were examined, there appeared to be a subtle effect on the growth of the interstitium in the EGFR knockout mice. Less interstitium was present in the knockout mice than in the wild-type littermates (Fig. 11). Quantitation of this effect revealed that at e18 there was approximately a 20% decrease in the percentage of interstitium in the EGFR knockout testis (Fig. 12). The difference in the area of the interstitium observed was found to be due to a decrease in cell number through quantitation of cell numbers in selected sections (data not shown). In the 0d testis, the difference between the area of the interstitium in the wild-type and knockout testis was not significant (data not shown). Therefore, the EGFR knockout testis phenotype is a transient decrease in interstitial cell growth late in embryonic development.

In contrast, testes from TGF- α knockout mice appeared normal at the developmental stages examined in comparison to normal littermates (data not shown). Although the EGFR knockout phenotype supports a role for EGF family ligands in the developing testis, combined data suggest that alternate and/or compensating TGF- α -related ligands and/or EGFR-related receptors appear to be present.

TGF- α Antibody Specificity

The presence of TGF- α antibody resulted in complete inhibition of testis growth. This neutralizing TGF- α antibody has previously been shown to not cross-react with EGF, amphiregulin, TGF- β , or FGF. The inability of the

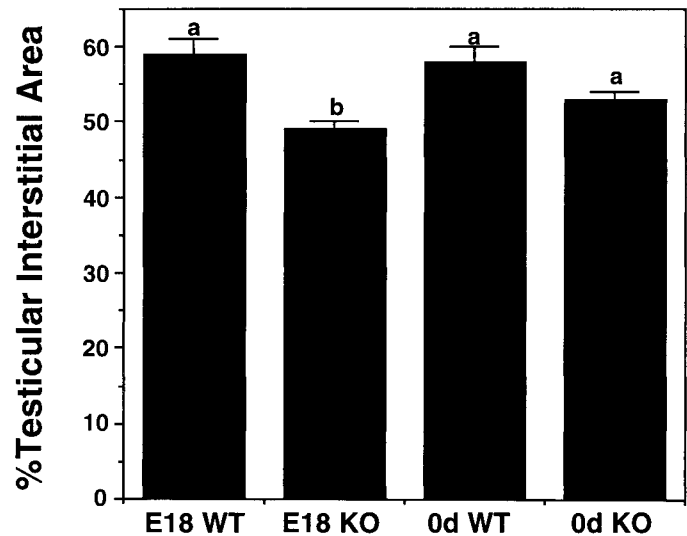


FIG. 12. Quantitation of percentage interstitium in EGFR knockout and wild-type testes from e18 and 0d mice. Computer analysis of the area of the interstitium was performed by subtracting the combined areas of the cords from the total area of each testis section. Data are presented as the mean \pm SEM; numbers of different testes analyzed were $n = 11$ (e18 WT), $n = 16$ (e18 KO), $n = 9$ (0d WT), and $n = 8$ (0d KO). The e18 mice were from two independently produced knockout mouse lines. Different superscripts designate statistically significant difference ($P < 0.01$) as determined by ANOVA.

antibody to react with other EGF-like ligands such as EGF or amphiregulin suggests a high degree of specificity; however, reactivity with another EGF-like ligand, heregulin, has not been shown. To further examine whether the antibodies to TGF- α are able to recognize related ligands, a Western blot was performed. The antibodies to TGF- α were found to react only with TGF- α and not with either EGF or heregulin- α (Fig. 13). A Western blot of Day 0 testis showed the 25-kDa precursor and 6-kDa mature forms of the TGF- α protein (data not shown). Therefore, the TGF- α antibodies appear specific with the factors tested thus far.

DISCUSSION

The results provide information about the control of growth during embryonic testis development. Immunocytochemistry for the proliferation marker PCNA confirmed the dramatic growth of the embryonic testis. Each cell type had its own temporal pattern of proliferation. During late embryonic development, almost all somatic cells were proliferating both in the cords and in the interstitium. The number of proliferating cells decreased first in the interstitium. A high percentage of proliferating cells continued to be found in the Sertoli cell population through early postnatal development, decreasing by 10 days at the onset of pubertal testis development. Germ cell proliferation was extensive at e14 and e16, decreased by e18, and then stopped until the early postnatal period, at which point it increased through 20d development. Therefore, all the testis cell types provide targets for locally produced growth factors such as TGF- α .

These observations based on PCNA as a marker of proliferating cells confirmed earlier experiments in which mitotic figures were quantified [14] and the incorporation of tritiated thymidine was examined [13, 15]. The only difference from the previous data is that the experiments demonstrated mitotic arrest of the germ cells by e18 [13, 54]. In the previous thymidine incorporation experiments, e18

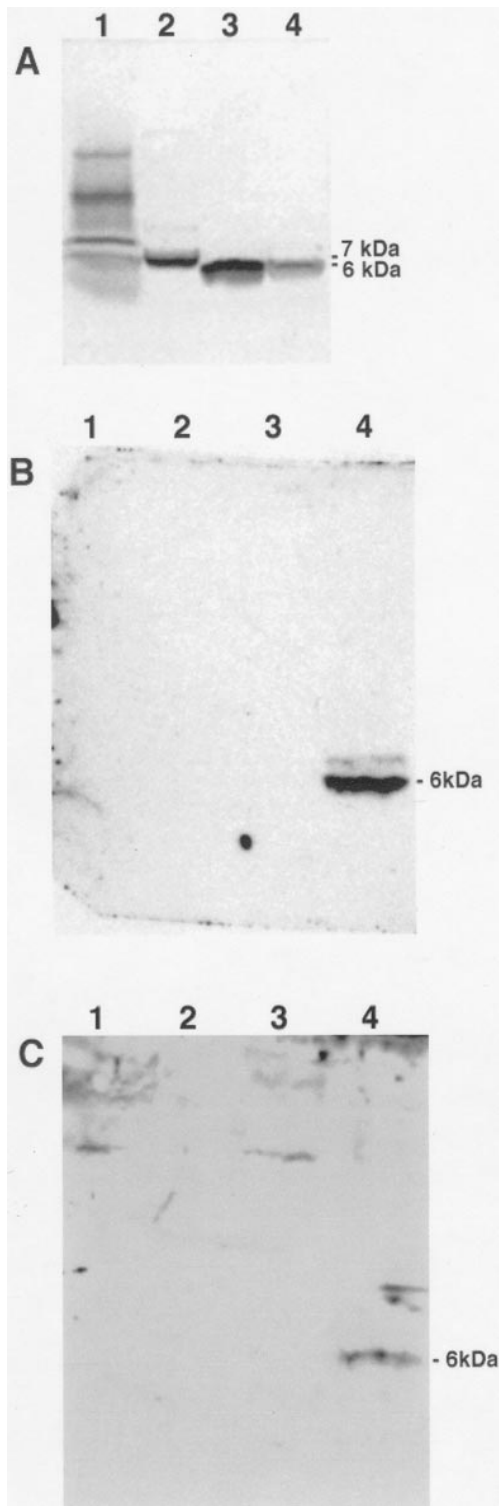


FIG. 13. Western blot with anti-TGF- α antibodies S574 and S509 of recombinant TGF- α , EGF, and heregulin- α (300 ng each). **A)** SDS gel stained with Coomassie Blue. **B)** Western blot with anti-TGF- α antibody S509. **C)** Western blot with anti-TGF- α antibody S574. **A,B,C)** Lanes: 1, molecular weight markers; 2, heregulin- α ; 3, EGF; 4, TGF- α . TGF- α and EGF are 6 kDa and heregulin- α is 7 kDa.

germ cells did not stain positive, whereas in the current study, e18 germ cells stained positive for PCNA. A limitation to the use of PCNA is that the protein is retained by the cell for a period after cell proliferation. Therefore, PCNA is a marker of general cell growth, and some PCNA

likely remains after mitotic arrest in male germ cells. A similar observation has been made for female germ cells, since there is PCNA staining in the oocyte while it is in meiotic arrest [55]. PCNA staining has been shown to correlate extremely well with cell proliferation, but it is also involved in DNA repair [47, 55–58]. Increased levels of DNA repair in germ cells could also explain the persistence of PCNA at e18. Therefore, the PCNA data presented confirm the previous studies, and the difference at e18 is likely a technical limitation to the use of PCNA. The extensive cell proliferation suggests a need for stimulators of growth in the embryonic testis. This dramatic growth is sex-specific, since the ovary grows very little during late embryonic development [12]. In addition, the differences in the timing of proliferation of the various cell types suggests the need for the regulation of growth through local growth factors and receptors such as TGF- α and EGFR.

TGF- α was found to be important in stimulating the growth of the embryonic testis. The growth of e14 testis in organ culture was inhibited by either of two neutralizing antibodies to TGF- α . Growth was measured by changes in DNA content of the organs and was confirmed by measuring the area of the organ. Quantitation by area is less accurate since it is a two-dimensional measurement of a three-dimensional tissue. However, it was still apparent that the antibodies to TGF- α effectively inhibited the growth of e14 testis in organ culture. The neutralizing antibodies to TGF- α and the EGFR inhibitor were still able to inhibit the growth of late embryonic (e18) and perinatal (0d) testis. This suggests that TGF- α ligand is important for growth throughout early testis development. Immunocytochemistry for PCNA in the organ cultures demonstrated that growth of both Sertoli and interstitial cells is inhibited by the antibodies to TGF- α . These observations suggest that TGF- α is important for promoting growth of both cell types. However, the current experiments do not rule out possible indirect effects through various cell-cell interactions. The exact location of TGF- α expression and action remain to be determined.

Testis growth was also inhibited by the tyrophostin AG1478, which is a specific inhibitor of the EGFR tyrosine kinase. AG1478 is selective for the EGFR and does not inhibit other growth factor receptor tyrosine kinases including the related HER2 kinase at the concentrations used in these experiments [49]. Inhibition of growth of the embryonic testis by AG1478, therefore, provides further evidence for the importance of TGF- α -induced signaling in stimulating testis growth.

Despite dramatic effects on embryonic testis growth, neutralizing antibodies to TGF- α did not disrupt cord formation in e13 testis. TGF- α and EGF have been implicated in morphogenetic events in other systems involving tube formation and branching such as the kidney, lung, mammary gland, and endothelial cells [30–36]. Organ cultures of early e13 testis and mesonephros are susceptible to the inhibition of cord formation by serum [1, 59]. This demonstrates the possibility of perturbing cord formation in testis organ cultures. The lack of an effect of the TGF- α antibodies on the development of cords in the testis suggests that TGF- α is not necessary for testis cord formation. If TGF- α does play a role in testis cord formation, then other factors must be able to compensate in the organ cultures.

Exogenous TGF- α was found to affect testis organ cultures, implying that the cells are capable of responding to TGF- α . There was not an overall increase in the size or DNA content of the organ but an increase in disorganized

cells around the outside of the testis. One reason could be that TGF- α stimulates proliferation to such an extent that the cells can no longer maintain the cord structure; another could be an overstimulation of growth of the interstitium such that it grows out beyond the cords. PCNA immunocytochemistry of the organs treated with TGF- α revealed an increase in the number of proliferating cells around the outside of the testis, such that almost all the cells in this region expressed PCNA. These observations support the interpretation that TGF- α treatment preferentially increased the proliferation of the more accessible cells on the outside of the organs. Maintenance of the cord structure is clearly compatible with some proliferation, since there is extensive PCNA staining in the cords throughout late embryonic and early postnatal development. However, it is possible that an increased rate of proliferation causes a disruption of the structure.

In contrast to the extensive inhibition of testis growth by neutralizing TGF- α antibodies or inhibiting signaling through the EGFR, the lack of EGFR in knockout mice did not cause a significant inhibition of testis growth. The EGFR knockout mice did show a dramatic inhibition of growth of other organs and of the whole animal [51–53]. There was a decrease in the percentage of the interstitium in late embryonic (e18) testis, suggesting that the growth of the interstitium was slightly inhibited or delayed. This effect was observed in two different EGFR knockout strains. Interstitial volume and cell numbers were transiently decreased. Observations suggest that the interstitial cells may be more dependent on the presence of the EGFR for growth and less able to compensate for its absence than Sertoli cells.

There has been no evidence that TGF- α is capable of interacting directly with any other related receptors. Previously, the TGF- α antibodies were shown to not cross-react with EGF, amphiregulin, TGF- β , or FGF. The inability to react with EGF suggests a high degree of specificity, but reactivity to heregulin had not been examined. Western blotting of both antibodies against recombinant human EGF and heregulin- α failed to detect any cross reactivity. Although the antibodies used appear specific, further investigation is required to determine whether unidentified TGF- α -related ligands may be recognized by the antibodies and function normally in the stimulation of testis growth.

The observation that TGF- α is important for stimulating the growth of the embryonic testis is significant because of the importance of embryonic cell proliferation for adult testis size and fertility. FSH is the most active agent found to promote testis growth and particularly Sertoli cell proliferation. FSH *in vitro* can stimulate Sertoli cell proliferation, and removal of FSH *in vivo* causes a dramatic decrease in Sertoli proliferation in e18 testis [16, 17]. However, the earliest detectable FSH in the pituitary and the earliest detectable FSH receptors in the testis are in e17 rats [11]. Other factors must be responsible for promoting the growth of the earlier testis development. These factors presumably are produced locally, since testis growth is maintained in organ culture. The current data imply that TGF- α is capable of performing this function in the early embryonic testis. In addition, it is not known what local testicular factors might be involved in the response to FSH in the late embryonic testis. β -Endorphin produced by Leydig cells has been implicated in modulating the response to FSH, since blocking its action increases the stimulation of e20 Sertoli cell proliferation by FSH [17]. It is possible that TGF- α is involved in the local mediation of FSH action, since the

TGF- α family appears to be important for testis growth in the late embryo.

The involvement of other proteins such as basic FGF and activin has been suggested in testis growth at particular times in development. Basic FGF is expressed embryonically by both germ cells and Leydig cells beginning around e16, so it is a potential mediator of local testis cell interactions in the late embryo [60]. FGF increases the survival of Sertoli cells in culture, but there are conflicting data on whether it increases proliferation [61, 62]. Activin can enhance the proliferation of postnatal Sertoli cells, and activin receptor knockouts show decreased testis size and a delay in fertility [18, 63]. However, activin actually inhibits the growth of e14 testis in organ culture [64]. The data suggest that any promotion of growth by activin occurs postnatally, but it may be a negative regulator of embryonic testis growth. TGF- α is therefore one of the first factors implicated thus far in the local promotion of early embryonic testis growth.

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