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Andrea S. Cupp
University of Nebraska-Lincoln, acupp2@unl.edu

Jannette M. Dufour
Washington State University, Pullman

Grace Kim
Washington State University, Pullman

Michael K. Skinner
Washington State University, Pullman

Kwan Hee Kim
Washington State University, Pullman

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Action of Retinoids on Embryonic and Early Postnatal Testis Development

ANDREA S. CUPP, JANNETTE M. DUFOUR, GRACE KIM, MICHAEL K. SKINNER, AND KWAN HEE KIM

Center for Reproductive Biology, Department of Genetics and Cell Biology and Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4231

ABSTRACT

The current study investigates the hypothesis that retinoids have a role in embryonic testis development. The action of retinoids on testis development and the expression of retinoic acid receptors (RARα, RARβ, RARγ) were examined. In embryonic day 13 (E13; plug date = E0) testis organ cultures an RAR-selective agonist and all-*trans* retinoic acid completely inhibited seminiferous cord formation. In contrast, an RARα-selective antagonist had no effect. RT-PCR demonstrated that RARα messenger RNA (mRNA) was expressed at all developmental time points evaluated, which included embryonic day 14 (E14) through postnatal day 30 (P30). Expression of RARβ mRNA was present at E15 through P2, whereas RARγ mRNA was expressed at E18 through P2. Cellular localization of receptors by immunohistochemistry indicated that RARα was localized to the interstitium at E18 and to the seminiferous cords by P0. RARβ and RARγ were detected in both interstitium and cords at E16 and by E18 were mainly expressed in the cords. At P0 RARβ and RARγ were localized to the germ cell populations. To examine retinoid actions, the growth of P0 testis cultures were investigated. Interestingly, retinol and retinoic acid did not inhibit growth of P0 testis cultures but did inhibit the action of growth stimulators. Retinoic acid inhibited FSH, EGF, and 10% calf serum stimulated growth in P0 testis cultures. The hypothesis tested was that the inhibitory effects of retinoids on P0 testis growth may be mediated through the growth inhibitor, transforming growth factor-β (TGFβ). The action of retinoids on TGFβ mRNA expression was examined in P0 testis cultures. Retinoic acid stimulated TGFβ3 mRNA expression within 24 h and increased expression of TGFβ1 and TGFβ2 after 72 h. Retinol increased expression of TGFβ1 and TGFβ2 but not TGFβ3 after 72 h of treatment. These observations indicate that retinoic acid can influence seminiferous cord formation and testis growth. The inhibitory actions of retinoids may in part be mediated through increased expression of TGFβ isoforms. (*Endocrinology* **140:** 2343–2352, 1999)

THERE ARE TWO critical processes that occur in the embryonic testis to ensure successful testis development. The first is the process of seminiferous cord formation that occurs at embryonic 13.5 (E13.5) (E0 = plug date) in the rat. The formation of cords involves Sertoli cell mesenchymal to epithelial transition (1, 2) and an aggregation of Sertoli and germ cells within the differentiating gonad. Migration of cells (presumably pre-peritubular cells) from the adjacent mesonephros to surround the Sertoli and germ cell aggregates completes the compartmentalization of the testis into seminiferous cords and interstitium (3, 4). Seminiferous cord formation is a crucial process because this is the first morphological indicator of sex determination in most mammals. Seminiferous cord formation has been determined to be gonadotropin independent (5). Therefore, cord formation and testis development must rely on factors produced by cells within the developing testis. Few factors have been determined to influence seminiferous cord formation. Both integrin subunit α 6 (6) and lectin (7) have been demonstrated to be involved in the early steps of cell aggregation leading to cord formation.

Retinoic acid is also a factor that has been determined to effect seminiferous cord formation during embryonic testis development (8). Treatment of testis organ cultures with retinoic acid at high concentrations (9) disrupt formation of the basement membrane and perturb the formation of seminiferous cords. Cellular localization of the messenger RNA (mRNA) encoding receptors for retinoic acid have been investigated in the whole embryo (10). Retinoic acid receptor (RAR)α transcripts were shown to be expressed ubiquitously in the gonads after cord formation in mice. Expression of RARβ transcript was restricted to the proximal mesenchyme of the genital tubercle, close to the urogenital sinus (10). The RARγ expression was absent from the proximal mesenchyme of the genital tubercle and present in the distal tip. These results suggest a potential role for RARs in embryonic testis development.

After seminiferous cord formation, a second process occurs that involves a sex-specific increase in growth of the testis. All populations of cells within the testis proliferate after seminiferous cord formation, and by E15, the testis is twice the size of the ovary from the same age animals (11). This process of embryonic testis growth is critical because adequate numbers of somatic cells are necessary to support spermatogenesis in the adult (12). Much of embryonic testis growth occurs before the acquisition of gonadotropin receptors (13) and may be attributed to paracrine factors produced locally in the testis. Recently TGFα has been shown to be important for embryonic testis growth subsequent to cord formation (14). Other potential regulators of embryonic testis growth are basic FGF (15), FGF-8 (16), and TGFβ (17, 18, 19, 20), which are all produced by cells within the embryonic testis after cord formation.

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Address all correspondence and requests for reprints to: Michael K. Skinner, Center for Reproductive Biology, Department of Genetics and Cell Biology, Washington State University, Pullman, Washington 99164-4231. E-mail: skinner@mail.wsu.edu.

Retinoic acid has been reported to interact with TGFβ to affect cell proliferation and differentiation in other tissues (21). In the prostate, retinoic acid has been demonstrated to inhibit cell growth and proliferation through the stimulation of TGFβ expression (22). Therefore, retinoids have the potential to regulate differentiation of the testis (seminiferous cord formation) as well as embryonic testis growth. The objective of the current study was to investigate the action of retinoids and expression of RARs at two periods during testis development. The first critical period was during testis morphogenesis (cord formation) around E13. The second period was just after birth at P0 when cells are mitotically active. The hypothesis tested was that retinoids are critical for both seminiferous cord formation and subsequent embryonic testis growth.

Materials and Methods

Organ cultures

Timed pregnant Sprague Dawley rats were obtained from Charles River (Wilmington, MA). Plug date was considered to be E0. Embryonic day 13 (E13) gonads were dissected out with the mesonephros. The organs were cultured in drops of medium on Millicell CM filters (Millipore Corp., Bedford, MA) floating on the surface of 0.4 ml of CMRL 1066 media (Gibco BRL, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μg/ml) and transferrin (10 μg/ml). Antibodies and factors were added directly to the culture medium. The medium was changed every day. E13 gonad + meso- nephros were typically kept for 3 days by which point cords were well developed. Images of whole organs were obtained by an image analysis system (Pixera, Pixera Corp., Los Gatos, CA) (22).

Genomic DNA isolation and PCR for SRY

To determine the sex of E13 embryos PCR for SRY was conducted on each embryo. Embryonic tails were collected to isolate genomic DNA by standard procedures. Briefly, the tissue was homogenized through a 25-gauge needle in digestion buffer (100 mM NaCl; 10 mM Tris, pH 8; 25 mM EDTA; 0.5% SDS), and treated 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 1/10 volume 7.5 M ammonium acetate and 3 volumes cold ethanol and collected by centrifugation at 4 C for 30 min after an hour incubation at −80 C. Pellets were dried and resuspended in 10 μl distilled H_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 bp (23).

Testicular cell culture and growth assay

To generate a testicular culture from P0 testis, the tunica was removed and the testis digested with 0.125% trypsin, 0.1% EDTA, and 0.02 mg/ml DNase in 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. The pellet was resuspended and either used in growth assays immediately or placed in 100-mm plates in F12 media supplemented with 10% bovine calf serum until confluent (approximately 2 days). For growth assays cells were plated at a 25% confluence in 24-well plates and allowed to settle overnight in DMEM media without thymidine. Media was replaced the next day and cells were treated for 24 h with different hormones or growth factors. Media was removed after the 24-h treatment period and media containing tritiated thymidine (10 μCi/ml) was placed on cells for 5–6 h. After 5–6 h media was discarded and cells were either frozen or processed using the tritiated thymidine assay. Briefly, solution of 0.5 M NaH_2P0_43 (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_2P0_4 buffer the filters were dried, placed in counting vials with 5 ml of scintillation fluid and counted. The remaining sonicate was used for DNA assays to normalize number of cells per well (22).

DNA assay

To determine the DNA content of each well of P0 testis cultures, the remaining sonicate from the growth assay was combined with 100 μl ethidium bromide buffer (EBB, 20 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5). DNA content 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 (22).

RNA isolation and RT-PCR

Total RNA was obtained using Tri Reagent (Sigma Chemical Co.). Briefly, tissue or cells were lysed in Tri Reagent (1 ml/50–100 mg tissue or 1 ml/100 mm of culture plate). After adding 0.2 ml chloroform/ml Tri Reagent, the mixture was centrifuged at 12,000 × *g* for 15 min at 4 C, the colorless upper aqueous phase was transferred to a fresh tube, and 0.5 ml isopropanol/ml Tri Reagent was added to pellet the RNA. RT was performed using MMLV-reverse transcriptase under standard conditions. RT-PCR was performed at 55 C annealing temperature for 30 cycles. The primer sequences and procedures were from previously reported experiments (24). The primer sequences are as follows: RARα: 5′ CAGATGCACAACGCTGGC 3′ and 5 ′CCGACTGTCCGCTTAGAG 3′; RARβ: 5′ATGCTGGCTTCGGTCCTC 3′ and 5′ CTGCAGCAGTGGTGACTG 3′; RARγ: 5′ GTGGAGACCGAATGGACC 3′ and 5′ GACAGGGATGAACACAGG 3′.

Quantitative RT-PCR

Quantitative RT-PCR (QRT-PCR) procedures were performed as previously published (22). Briefly, total RNA (1 μg) was reverse transcribed using the specific 3′ primers. Plasmid DNA's containing subclones of interest were used to generate standard curves from 1 ng/μl to 10 pg/μl each containing 10 ng/μl Bluescript carrier DNA. Identical 10-μl aliquots of each sample or standard were used for PCR amplification. At least 0.25 μCi of ^{32}P-labeled dCTP was included in each sample during amplification. Specific PCR products were quantitated on 4–5% polyacrylamide gels. The gels were exposed to a phosphor screen for 8–24 h, followed by quantification of specific bands on a PhosphorImager (Molecular Dynamics, Inc.) and analyzed with Image Quant. 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. Optimal cycle number for amplification was determined for each assay to achieve maximum sensitivity while maintaining linearity. The sensitivity of each quantitative PCR assay is below 1 fg with intraassay variabilities of 6–15%. Primers used for the QRT-PCR were as follows: TGFβ1, 5′,5′-TCG ATT TTG ACG TCA CTG GAG TTG T-3′ and 3′,5′-GGG GTG GCC ATG AGG AGC AGG-3′; TGFβ2, 5′-5′-CCG CCC ACT TTC TAC AGA CCC-3′ and 3′, 5′-GCG CTG GGT GGG AGA TGT TAA-3′; TGFβ3, 5 prime 5′ TGC CCA ACC CGA GCT CTA AGC G-3′, 3′,5′-CCT TTG AAT TTG ATC TCC A-3′; cyclophilin, 5′,5′-ACA CGC CAT AAT GGC ACT GG-3′ and 3′,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 (22). The tissue sections (3 μm) were deparaffinized, rehydrated, microwaved, and blocked in 10% goat serum for 15 min at room temperature. Immunohistochemistry was performed as described previously (25, 26). The RARα antibody was an anti-RARα peptide antibody (Santa Cruz Biotechnology, Inc. (SCB), Santa Cruz, CA) raised against amino acids 443–462 (CSPSLSPSSNRSSPATHSP) of human RARα (which is 100% homologous to rat

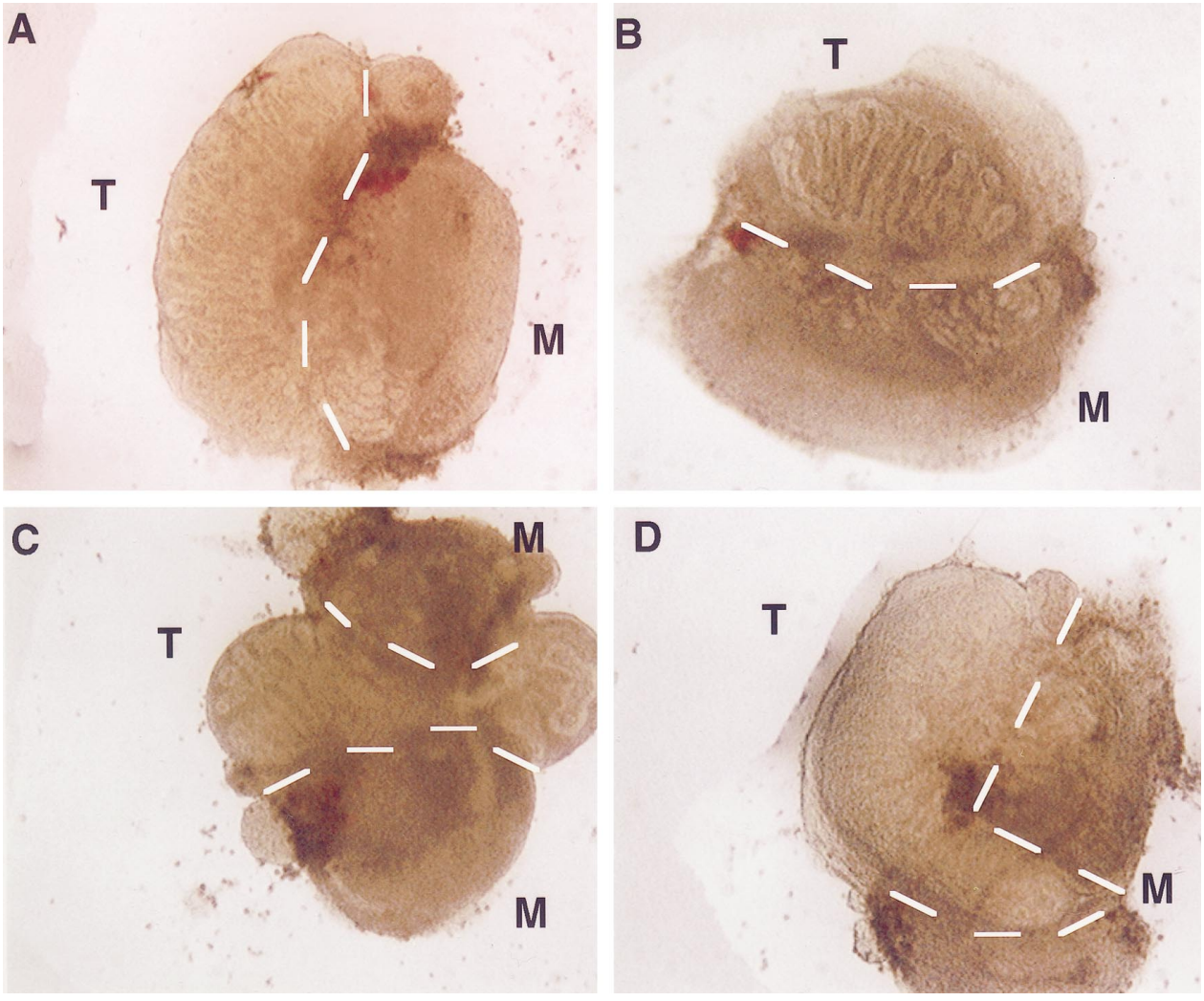

FIG. 1. E13 testis organ cultures treated with (A) ethanol control or with (B)-(D) 0.7 μM retinol. The retinol treatments (B), (C), and (D) demonstrate the variability of seminiferous cord formation with this treatment. Organ cultures were treated daily at the time of media changes with 0.7 μM retinol or with ethanol control. These are representative images from 24 testis pairs (n = 24; 24 treated, 24 controls). M, Mesonephros; T, testis. Magnification, 80×.

RARα, unpublished data). The RARβ antibody was an anti-RARβ peptide antibody (SCB) raised against amino acids 430–447 (SISPSSVENSGVSQSPLVQ) of human RARβ. The RARγ antibody was an anti-RARγ peptide antibody (SCB) raised against amino acids 436–454 (CSSEDEVPGGQGKGGLKSPA) of human RARγ. The RARα antibody was diluted 1:200 in 10% goat serum, and he RARβ and γ primary antibodies were diluted 1:50 in 10% goat serum. As a negative control, serial sections were put through the same procedure without any primary antibody. Additional negative control sections were incubated with 50×–100× excess of synthetic immunizing peptide and the anti-RARα, anti-RARβ, or anti-RARγ antibody. The biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA) was diluted 1:300. The secondary antibody was detected by using the histo stain-SP kit (Zymed Laboratories, Inc., South San Francisco, CA) and immunohistochemical images were digitized with a slide scanner (Sprint Scan, Polaroid, Cambridge, MA).

Immunoblotting

Soluble proteins from six P0 testes were prepared as previously described (26). The testes were homogenized and lysed in 1 ml of lysis buffer (50 mM Tris-HCI, [pH 7.5], 250 mM NaCl, 0.1% Triton X-100, 50 mM NaF, 5 mM EDTA) containing a cocktail of proteinase inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml Aprotinin, 10 μg/ml leupeptin). Protein concentration was determined by the method of Bradford (27) with BSA as the standard.

Fifty (RARα) or one hundred (RARβ and γ) micrograms of protein were loaded on 8.5% SDS-polyacrylamide gels and subjected to electrophoresis. This was repeated three times. The proteins were transferred to an Immobilon-P membrane (Millipore Corp.) to perform Western blot analysis. The membranes were blocked with 5% blotto (Carnation, Los Angeles, CA) in PBS for 1 h at room temperature and then incubated with the appropriate primary antibody at a dilution of 1:200 in PBS/Tween-20 for 1 h. This was followed by incubation with horseradish peroxidase-conjugated antirabbit IgG secondary antibody at a dilution of 1:2500 in PBS/Tween-20 for 30 min. The proteins were detected by the Enhanced Chemiluminescence (ECL) Western blotting system (Amersham Corp., Arlington Heights, IL) (27).

Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute, Inc., Cary, NC). All values are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using the Dunnett's test for comparison to controls and using the Tukey-Kramer honesty difference test for multiple comparisons. Statistical difference was confirmed at $P < 0.05$.

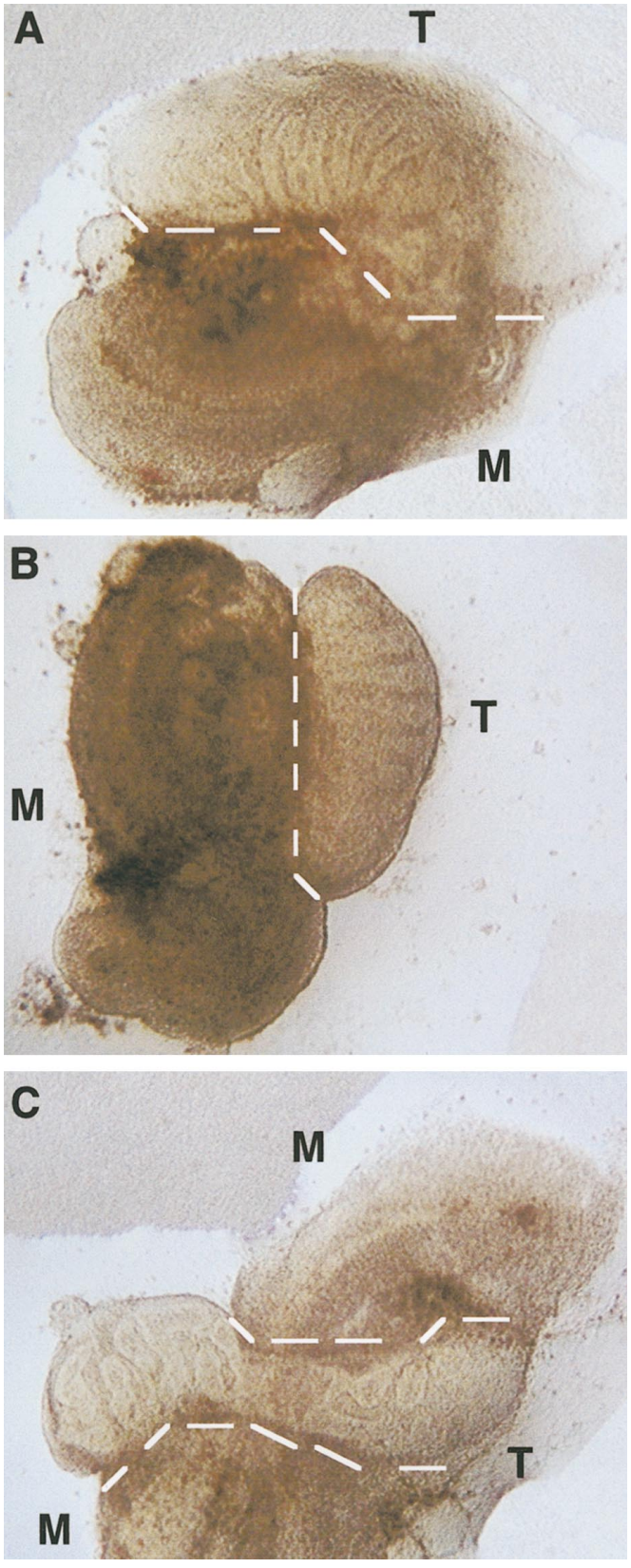

FIG. 2. E13 testis organ cultures treated with (A) ethanol control or with (B)-(C) 0.1 μM all-*trans* retinoic acid The all-*trans* retinoic acid treated organ cultures (B) and (C) demonstrate the variability of seminiferous cord formation with this treatment. Organ cultures were treated daily at the time of media changes with 0.1 μM all-*trans* retinoic acid or with ethanol control. These are representative images from 36 testis pairs (n = 36, 36 treated, 36 were controls). Magnification, 80×.

Results

Effect of retinoids and retinoid antagonists on seminiferous cord formation

Embryonic day 13 (E13) testis organ cultures were used to determine the effects of retinoids, retinoid agonists, and retinoid antagonists on seminiferous cord formation. E13 testis with mesonephros were cultured and placed on floating filters. One of each testis pair was treated with retinoids or retinoid antagonists while the other served as a control. Embryonic testis organ cultures were treated daily for 3 days, at which time the control testis formed seminiferous cords. Retinol at a dose of 0.35 μM did not effect seminiferous cord formation or embryonic testis organ culture morphology (data not shown). However, when this dose was increased to 0.70 μM, there was an increase in seminiferous cord disruption but not a complete inhibition of seminiferous cords (Fig. 1, B–D). Higher concentrations of retinol may be necessary to cause seminiferous cord disruption. This confirms that the retinoic acid metabolite of retinol is likely the effective form of retinoid. To examine the effects of retinoic acid on seminiferous cord formation the E13 testis organ cultures were treated with 0.1 μM or 1 μM all *trans*-retinoic acid. The 0.1 μM dose of retinoic acid (Fig. 2, B and C) did not affect seminiferous cord formation, but the 1 μM concentration completely inhibited cord formation in E13 testis organ cultures (Fig. 3B). Therefore, these data confirm previous reports (8) and provide novel data that demonstrate that retinoic acid can perturb formation of seminiferous cords in E13 testis organ cultures in a dose-dependent manner.

To extend the results of the previous experiments (8), a specific RAR agonist (28) and RARα antagonist (28) were used to treat E13 testis organ cultures. The RAR specific agonist when treated at 0.1 μM perturbed cord formation (Fig. 3D) to a greater extent than either retinol or retinoic acid treated at similar doses. In contrast, a specific antagonist to RARα did not have any effect on seminiferous cord formation in E13 testis organ cultures (data not shown). These novel results demonstrate that the RAR may be important for early testis differentiation and excessive amounts of retinoic acid are disruptive to testis morphogenesis.

Expression of mRNA for RARs during testis development

To determine expression of mRNAs for RARs during testis development, RT-PCR for RARα, RARβ, and RARγ was conducted in E14 through P30 testis sections. Expression of mRNA for RARα (Fig. 4A) was present in the testis during developmental periods between E14 and P30. Expression of mRNA for RARβ (Fig. 4B) and RARγ (Fig. 4C) appeared to be more transiently expressed during testis development. Expression of mRNA for RARβ (Fig. 4B) was detected in testis from rats at E15, E18-P2, and then at P10. The expression of mRNA for RARγ (Fig. 4C) was present in testis from E18 through P2 (similar to RARβ) and then at P5 and P30. These observations suggest that the mRNAs for receptors of

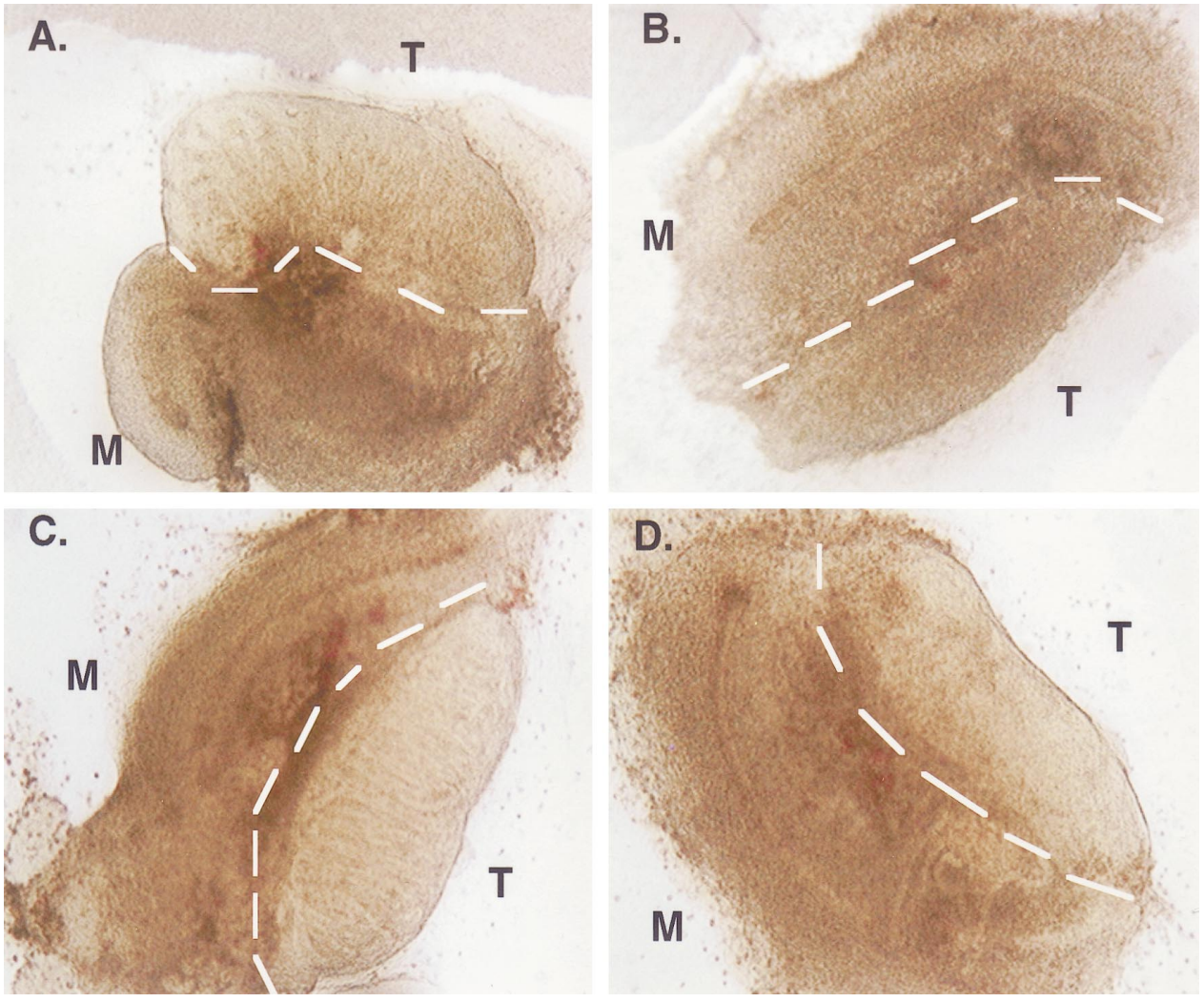

FIG. 3. E13 testis organ cultures treated with (A) ethanol control, (B) 1 μM all-*trans* retinoic acid, (C) ethanol control or (D) 0.1 μM RAR agonist (CH57). Organ cultures were treated daily at the time of media changes with 0.1 μM RAR agonist, 1 μM all-*trans* retinoic acid or with ethanol control. These are representative images from 14 testis pairs (n = 14; 14 treated, 14 controls). Magnification, 80×.

retinoic acid are present during embryonic development and that expression of mRNAs for RARβ and RARγ appear to be developmentally regulated during testis development.

Protein expression and cellular localization of RARs during embryonic testis development

Antibody specificities for anti-RARα, RARβ, and RARγ were determined on proteins isolated from P0 testis by Western blot analysis. Two bands (54 and 50 kDa) were detected for RARα (Fig. 5). One band was detected (55 kDa) for both RARβ and RARγ. RARβ and RARγ also had a minor band detected at approximately 45 kDa (data not shown). These results are consistent with previously published results for the receptors in mouse and human (29–31).

The cellular localization of RAR protein expression was examined by immunohistochemistry for RARα, RARβ, and RARγ using testis sections from E14, E16, E18, and P0 testis (Fig. 6, A–O). Expression of RARα protein in E16 testis was variable (Fig. 6B). Some sections had low signal at the edge of the seminiferous cords, while others had greater staining within the interstitium. By E18, cells within the interstitium stained positive for RARα including cells surrounding the seminiferous cords that are presumed to be peritubular cells (Fig. 6C). Low levels of staining were detected in selected cells within the cords. At P0, germ cells within the cords stained positive for RARα (Fig. 6D).

Expression of protein for RARβ was detected in both interstitial cells and cells within the cords at E16 (Fig. 6G). In contrast, at E18 only cells within the cords stained positive for RARβ (Fig. 6M). The positive staining was in both Sertoli and germ cells. By P0, positive staining for RARβ was detected in the germ cells of the seminiferous cords (Fig. 6, I–J). The Sertoli cells had little or no positive staining for RARβ at P0. The expression and cellular localization of protein for RARγ was similar to that of RARβ at E16, E18, and P0 (Fig. 6, L–O). At E16, both the interstitium and cords stained positive for RARγ, whereas at E18 only the cells within the seminiferous cords stained positive for RARγ. At P0, the highest level of expression for RARγ was detected in the germ cells. Therefore, by P0 of testis development expression

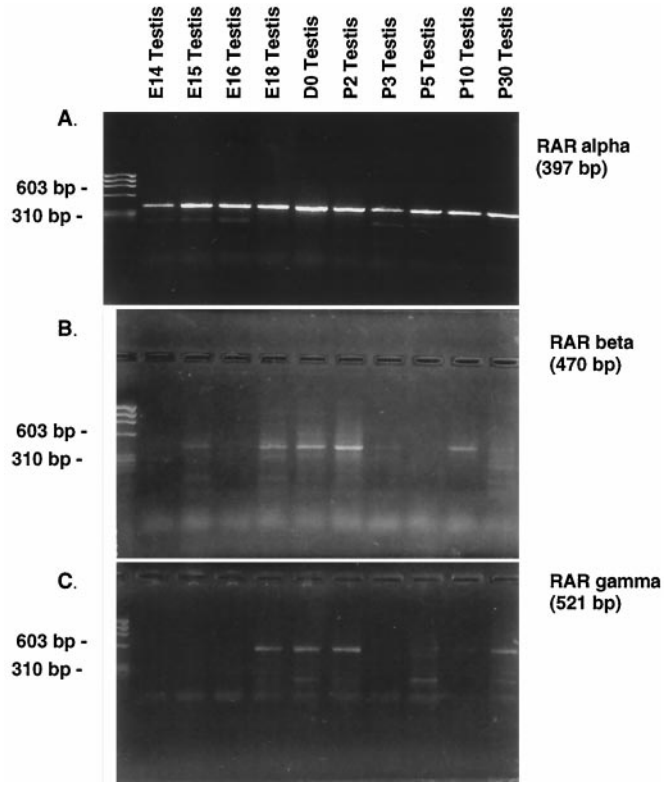

FIG. 4. RT-PCR for (A) RARα, (B) RARβ, and (C) RARγ mRNA expression from E14-P30 during testis development. These data are representative of two different PCR reactions for each time period. Sizes of expected PCR fragments are: RARα = 397 bp; RARβ = 470 bp; RARγ = 521 bp.

of RARα, RARβ, and RARγ was present within the germ cell population.

Effect of retinoids on early testis growth

The effect of retinoic acid and retinol on whole P0 testis growth was examined with testicular cultures from P0 rats. FSH, EGF, and 10% calf serum were used as positive controls because all of these reagents stimulate growth of P0 testis cultures (Fig. 7). Interestingly, retinol or retinoic acid treatment alone had no effect on growth of whole P0 testis cultures (Fig. 7, A and B). Retinol and retinoic acid inhibited EGF (Fig. 7A) and 10% calf serum stimulated growth (Fig. 7B). In addition, retinoic acid inhibited FSH stimulated growth (Fig. 7A). Thus, the current study demonstrates that retinoids influence the ability of FSH, EGF, and 10% calf serum to stimulate whole P0 testis growth.

Effects of retinoids on expression of mRNA for TGFβ

The mechanism of how retinoids may regulate cell growth in P0 testis cultures was investigated by measuring expression of TGFβ isoforms through QRT-PCR. A representative autoradiogram of a QRT-PCR gel is shown in Fig. 8. Previous results (20) have demonstrated that TGFβ inhibits EGF and 10% calf serum stimulated growth in P0 testis cultures. Testis cell cultures from P0 rats were treated with retinol (0.35 μM) or retinoic acid (0.1 μM), and mRNA was collected after 24 and 72 h of treatment. Retinol at 0.35 μm did not induce

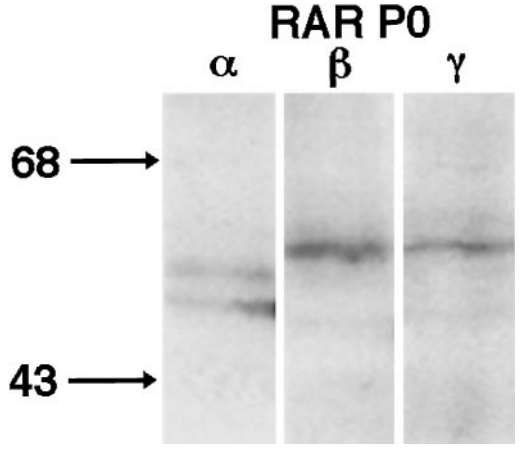

FIG. 5. Western blot analysis with RARα, RARβ, and RARγ antibodies in P0 testis extracts. The bands for RARα are approximately 54 and 50 kDa, whereas the band for RARβ and RARγ is approximately 55 kDa. Molecular markers for 43 kDa and 68 kDa are shown on the *left*.

changes in expression of mRNA for any TGFβ isoforms after 24 h of treatment (Fig. 9C). In contrast, retinoic acid stimulated TGFβ3 mRNA levels after 24 h of treatment. Interestingly, retinol increased expression of TGFβ1 and TGFβ2 after 72 h of treatment. This was in contrast to retinoic acid, which increased expression of mRNA for all three TGFβ isoforms after seventy two hours of treatment (Fig. 9, A–C).

FSH did not affect expression of any TGFβ isoforms. FSH given in combination with retinol appeared to suppress the stimulatory effects of retinol on TGFβ1 and TGFβ2 after 72 h of treatment. In contrast, FSH given in combination with retinoic acid suppressed the stimulatory effects of retinoic acid on mRNA expression for TGFβ1, but not TGFβ2 or TGFβ3 after 72 h of treatment. Interestingly, FSH treatment in combination with retinoic acid also suppressed retinoic acid induced expression of TGFβ3 after 24 h of treatment (Fig. 9). These observations suggest that retinoic acid increases expression of TGFβ isoforms that have previously been shown to inhibit cellular proliferation and growth. FSH when administered in combination with retinol or retinoic acid is capable of suppressing retinoid increased mRNA expression of specific TGFβ isoforms after 24 and 72 h of treatment. Therefore, retinoid inhibition of P0 testis growth is likely through indirect actions on the expression of TGFβ isoforms.

Discussion

Retinoic acid is one of the few factors that has been determined to perturb seminiferous cord formation in E13 testis organ cultures at high doses (8). This disruption of cord formation was proposed to occur due to inhibition of laminin production or production of factors that form the basement membrane. Excess retinoic acid may also disrupt events associated with Sertoli cell mesenchymal to epithelial cell transition that occurs early in testis development. Retinoic acid is required for normal morphogenesis of the embryo and cannot be synthesized *de novo* (32). However, detrimental effects have been observed when retinoic acid is present at concentrations higher than optimal levels (33). The range of

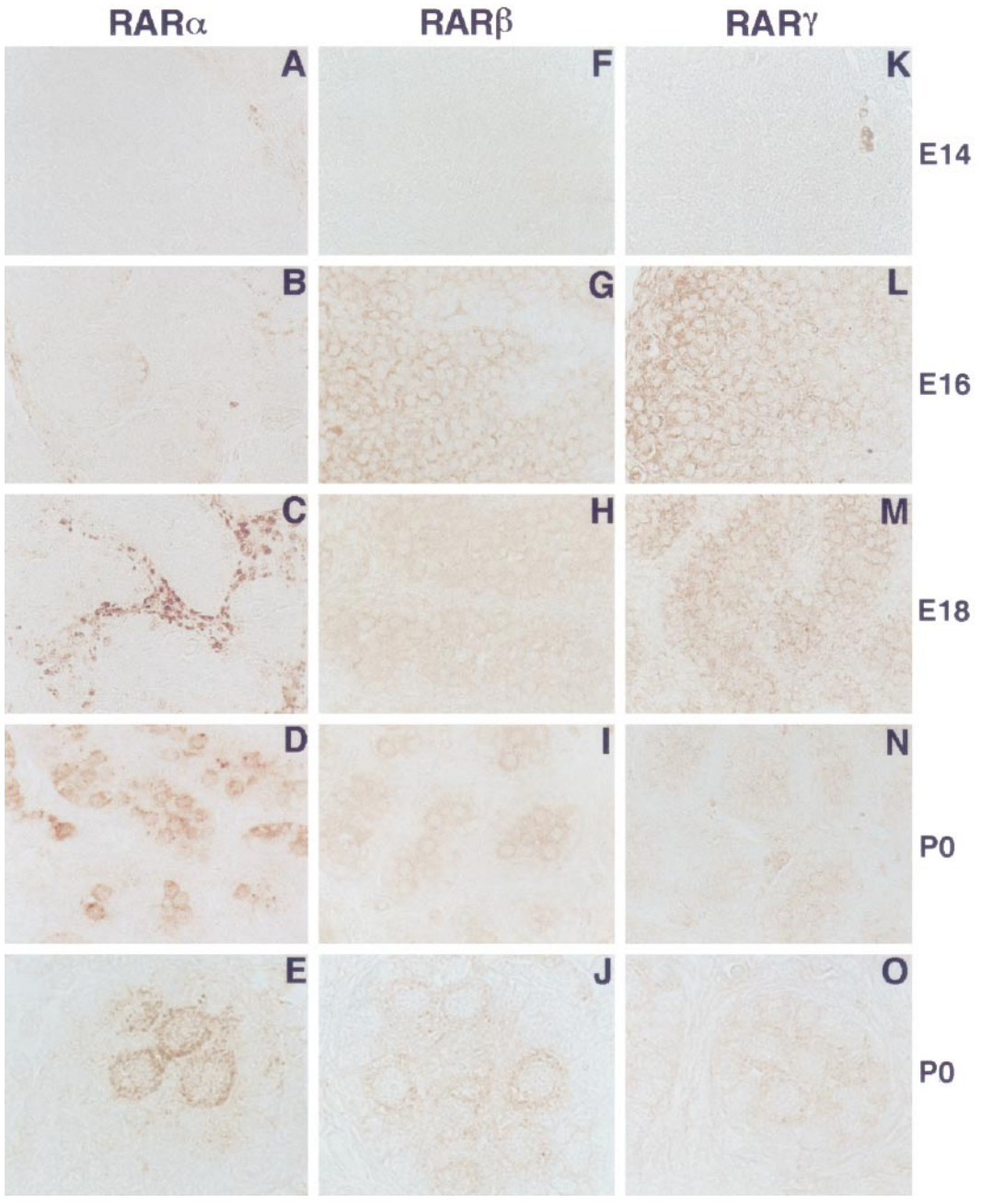

FIG. 6. Immunohistochemistry for RARα at (A) E14, (B) E16, (C) E18, and (D, E) P0; RARβ at (F) E14, (G) E16, and (H) E18 and I, J P0; RARγ at (K) E14, (L) E16, (M) E18, and (N, O) P0. Magnification (A–D, E–I, K–N), 400×; E, J, O, 1,000×. This experiment was repeated three times for each developmental time point.

retinoic acid previously reported in embryos is 20 nM to 150 nM (34, 35). Receptors for retinoic acid have not been localized to specific cells within the embryonic testis. In whole embryos, mRNA for RARα, RARβ, and RARγ have been localized to the gonad around the time of cord formation (10).

The current study was designed to determine the cellular expression of the retinoic acid receptors and action of retinoids during embryonic testis development. While 1 μM RA has been demonstrated to inhibit seminiferous cord formation, the actions of retinol, lower doses of RA, and specific RAR selective agonist on seminiferous cord formation have not been evaluated. Observations confirm previous research as well as demonstrate novel data on the dose dependent effects of retinol, all-*trans* retinoic acid and a RAR-selective agonist on seminiferous cord formation. All-*trans* retinol is a circulating form of retinoid in the bloodstream and can be converted to either all-*trans* retinoic acid or to 9-cis retinoic acid in the tissue (36). All-*trans* retinoic acid binds preferentially to the RARs. In contrast, 9-cis retinoic acid binds to and activates both RAR and RXR (36). In the present study, all forms of retinoids caused disruption or disorganization of seminiferous cord formation. All-*trans* retinoic acid at concentrations of 1 μM and 0.1 μM RAR-selective agonist had the greatest effect on inhibition of cord formation. This infor-

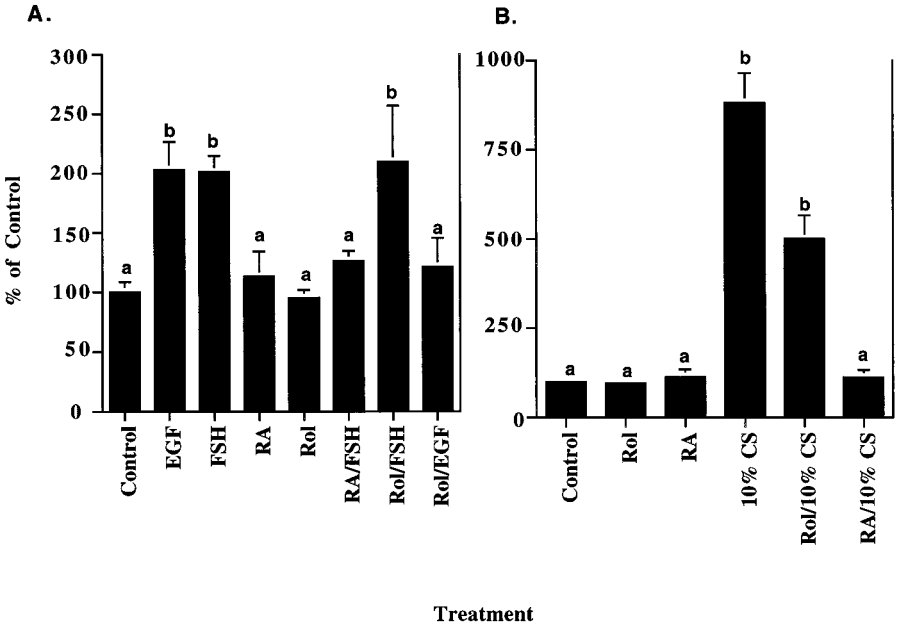

FIG. 7. Effects of retinol and all-*trans* retinoic acid on (A) EGF and FSH stimulated and (B) 10% calf serum stimulated P0 testis growth. Results are presented as percentage of control and represent three to four individual experiments in triplicate. *Different superscript letters* for each mean represent a statistical differences at $P < 0.05$.

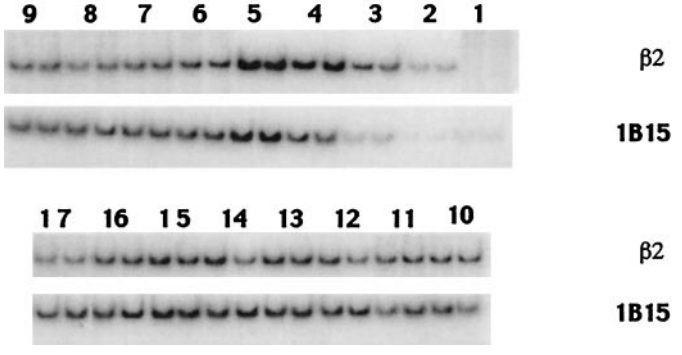

FIG. 8. Autoradiogram of a representative QRT-PCR gel for TGFβ2. Lanes 1–5 represent TGFβ2 standard or cyclophilin at 0.5 pg, 50 pg, 500 pg, and 5 ng. Lanes 6 through 17 represent P0 testis samples.

mation is important because the RAR selective agonist demonstrates that seminiferous cord formation disruption may be through the RAR and not RXR. It is not surprising that retinol did not have as dramatic effect on cord formation because conversion of retinol is necessary to produce retinoic acid. Therefore, it was necessary to increase the amount of retinol added to the organ cultures to elicit a similar effects as either all-*trans* retinoic acid or the RAR-selective agonist.

The current study used RT-PCR and immunohistochemistry to determine the expression patterns and localization of RARs. RT-PCR demonstrated that expression of RARα mRNA was present during all developmental periods evaluated (E14-P30). RARα mRNA was the only RAR mRNA present around the time of seminiferous cord formation. Therefore, any action of retinoids may be elicited through RARα at this developmental period. However, RARα protein was not expressed in E14 testis and did not appear until E16. This may be due to a translational control that has been reported previously in adult testis (26). This suggests that RARs may not participate in the normal process of seminiferous cord formation. Previous observations have also demonstrated that retinoids are capable of up regulating the expression of RARα (37, 38). This may provide a potential explanation of how treatment of retinoic acid could inhibit seminiferous cord formation in E13 testis organ cultures. Further investigation is necessary to determine whether RARα expression can be up-regulated in E13 organ cultures. Cellular localization of RARα protein in the testis was demonstrated to be in the interstitium at E16 and E18. By P0 of testis development, RARα protein was in the germ cells. Therefore, RARα may be important after E16 to regulate the growth and differentiation of the interstitial and germ cells. The phosphorylation state and expression of the protein are both important to determine if RARα is capable of binding retinoic acid in the embryonic and postnatal testis. Previous reports have demonstrated that posttranslational modification can influence the activity of RARα in several different tissues (37).

In contrast to RARα, the mRNA for both RARβ and RARγ were transiently expressed during testis development. This suggests that there is potential regulation of these two receptors during testis development. RARβ mRNA is present at E15 while both RARβ and γ are present in the embryonic testis from E18 through P2. This is a time during testis development when germ cell populations have undergone mitotic arrest and have stopped cell division (39). The expression of mRNA for RARβ present at E15 occurs before protein expression at or around E16 of testis development. There is a discrepancy in the first appearance of mRNA for RARγ (E18) and the appearance of RARγ protein at E16. One possible explanation is that mRNA expression for RARγ occurs before E14. By P0 of testis development, all receptors for retinoic acid are localized to the germ cell population. This cellular localization of the RARs suggests a potential regulation of germ cell differentiation and proliferation within the perinatal developing testis.

The effects of retinoids on cell growth was examined in the current study with a mixed population of testicular cells from P0 rats. Retinoids alone did not influence cell

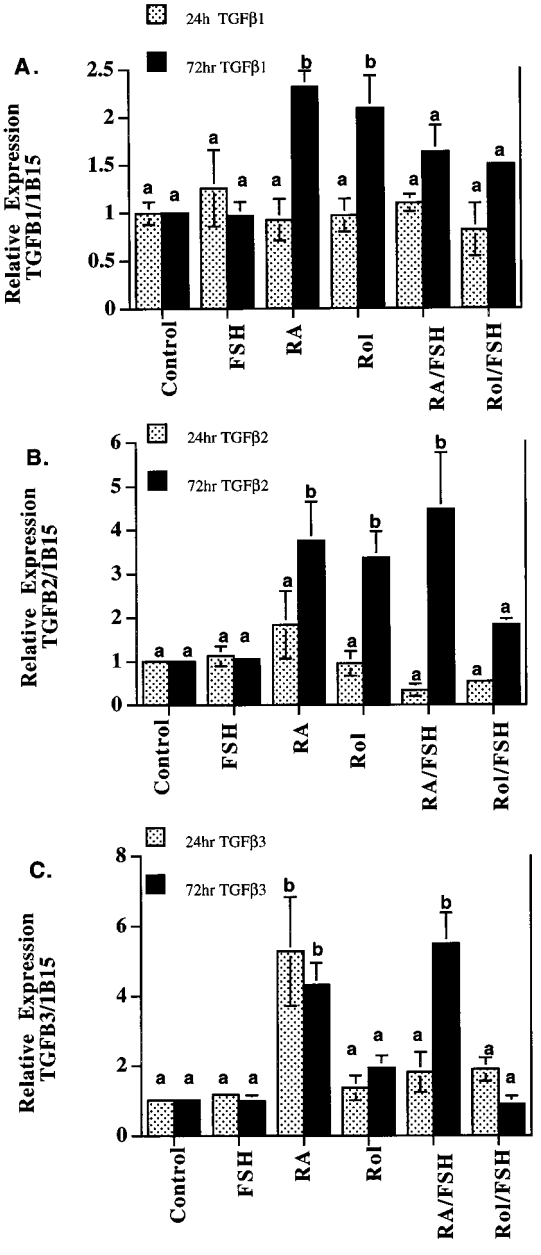

FIG. 9. Effects of retinoic acid on relative amounts of mRNA for (A) TGFβ1, (B) TGFβ2, and (C) TGFβ3 after 24 or 72 h of treatment. Amounts of mRNA for TGFβ isoforms were normalized to cyclophilin (1B15) and expressed relative to controls. These data represent three individual experiments assayed in duplicate. *Different superscript letters* for each mean represent a statistical differences at $P < 0.05$.

growth. However, retinol and all-*trans* retinoic acid inhibited thymidine incorporation in EGF and 10% calf serum stimulated cells. In addition, retinoic acid inhibited FSH stimulated growth. It is interesting that retinoid treatment alone did not inhibit growth. At P0, germ cells *in vivo* are the only cell population within the testis that is not actively proliferating. Because receptors for RARs are present in germ cells at P0, retinoids may contribute to the growth arrest of germ cells. Further treatment of P0 testis cultures with retinoids may not have inhibitory effects on this cell population. However, stimulation of P0 testis cells by FSH, EGF, and 10% calf serum may allow for progression of the cell cycle in germ cells when in culture. These growth stimulators may cause the germ cells to resume mitosis and allow for subsequent inhibition of germ cell proliferation by retinoid treatment.

The inhibition of cell growth by retinoids is not novel to the testis. Retinoic acid has been observed to prevent cell growth in several other tissues. In the prostate, retinoic acid inhibits cellular growth and proliferation by stimulating expression of mRNA and protein for all three isoforms of TGFβ (21). In addition, a monoclonal neutralizing antibody to all isoforms of TGFβ blocked the ability of retinoic acid to inhibit growth (21). Therefore, it was proposed that retinoic acid caused the inhibition of growth through increased or altered expression of TGFβ isoforms in P0 testis.

Retinoic acid increased expression of mRNA for TGFβ3 within 24 h. After 72 h, TGFβ1, TGFβ2, and TGFβ3 mRNA expression was also elevated in retinoic acid treated cultures. These results are similar to those demonstrated previously in the prostate (21). In the prostate, up-regulation of mRNA for TGFβ2 and TGFβ3 was greater and earlier than subsequent increases in mRNA for TGFβ1 by retinoic acid (20). In the testis TGFβ1 inhibits testis growth in embryonic and P0 testis cultures (20). Therefore, regulation of cellular proliferation by retinoic acid is potentially mediated through the expression of specific TGFβ isoforms which in turn cause inhibition of cellular proliferation.

Interestingly, retinol did not have similar effects on expression of TGFβ isoforms as retinoic acid. Retinol did not increase expression of any TGFβ isoforms after 24 h. However, after 72 h of treatment retinol increased expression of TGFβ1 and TGFβ2, but did not effect mRNA expression of TGFβ3. These differences are presumably do to conversion of retinol into both all-*trans* and 9-cis retinoic acid which act at both RAR (all-*trans* and 9-cis) and RXR (9-cis).

FSH did not stimulate expression of mRNA for any TGFβ isoform. This supports previous reports that FSH stimulation of P0 testis does not influence expression of TGFβ isoforms (20). FSH treatment in combination with retinoic acid appeared to inhibit the ability of retinoic acid to stimulate TGFβ1 and TGFβ3 isoform expression. After 72 h, expression of TGFβ1 was suppressed in a retinoic acid and FSH combined treatment when compared with retinoic acid treatment alone. In addition, the expression of TGFβ3 was also altered when retinoic acid was given in combination with FSH. Therefore, FSH may alter the ability of retinoic acid to stimulate expression of TGFβ isoforms in P0 testis cultures.

Knockout mice lacking RARs demonstrate that retinoids are important for testis development. RARα knockout mice are sterile due to defective spermatogenesis (40). RARγ knockouts have problems associated with secondary sex glands, which is not associated with testis development but may alter viability of sperm (41). However, no problems have been detected in embryonic testis development in these knockouts. The redundant nature of the RARs may allow for compensation to occur in mice lacking one of the RAR genes or retinoic acid may only be important in later testis development.

In conclusion, the novel results of the current study demonstrate that retinoic acid or an RAR-specific agonist can

influence the process of seminiferous cord formation. The potential absence of RAR isoforms at E14 and the presence of RAR isoforms after E16 in testis development suggests that retinoic acid is not necessary for seminiferous cord formation. Because seminiferous cord formation is disrupted by high doses of retinoic acid and RAR specific agonists the lack of RARs may be a protective mechanism to ensure successful testis development. The primary function of retinoic acid may be to allow for cell differentiation and growth in the interstitium and germ cells after E16. The localization of the RARs in P0 testis is interesting because all receptors are present within the germ cell population. This suggests that retinoic acid may be critical to germ cell development. At P0, the germ cells in the testis are in mitotic arrest, and retinoic acid may be involved in initiating this process to allow for germ cell differentiation. The current study also presents novel information on potential mechanisms for retinoid regulation of testis cell growth. The mechanism for retinoid regulation of cell growth is proposed to be through increased expression of TGFβ isoforms. Therefore, retinoids appear to be important during perinatal testis development to regulate cellular growth and differentiation.

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