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Evidence for the Presence of Angiogenin in Human Testis

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ABSTRACT: We have reported the expression and possible roles of angiogenin, a potent angiogenic factor, in human female reproductive organs. In this study, we investigated the expression of angiogenin in the human testis, a male reproductive organ. Western blot analysis showed the presence of angiogenin in the human testis, with a single band of the same size as recombinant human angiogenin. Immunohistochemical study and in situ hybridization showed that the angiogenin protein and messenger RNA (mRNA) localized in peritubular myoid cells (PTMCs) and vascular endothelial and smooth muscle cells. PTMCs are known to play various roles in the testes concerned with spermatogenesis, transport of spermatozoa, structural support to the seminiferous tubules, and mediation of Sertoli cell function. The specific localization of angiogenin in PTMCs suggests that angiogenin plays physiologic roles in the human testis.

Key words: Immunohistochemistry, in situ hybridization.

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Human angiogenin, a 14.1-kd polypeptide that was initially isolated from supernatants from colon carcinoma (Fett et al, 1985), has been shown to be present in various body fluids, including the serum of humans (Shapiro et al, 1987) and other mammals (Bond et al, 1993), human follicular fluid (Koga et al, 2000), and bovine milk (Maes et al, 1988). Angiogenin is a potent angiogenic factor, as has been shown in several animal models such as the chicken chorioallantoic membrane and the rabbit cornea (Fett et al, 1985). Another line of evidence, the expression of the angiogenin gene in rat tissue that was not temporally related to vascular development (Weiner et al, 1987), and the widespread expression of angiogenin in different human cells (Moenner et al, 1994) may imply as yet undescribed biologic functions.

We have recently demonstrated the presence of angiogenin in the human ovary (Koga et al, 2000) and the human endometrium (Koga et al, 2001) in addition to various regulators for its production, which include gonadotropins, ovarian hormones, and oxygen tension. However, to our knowledge, investigations concerning the presence of angiogenin in male reproductive organs have not been conducted, while other angiogenic factors, such as vascular endothelial growth factor (VEGF) (Ergun et al, 1997), endocrine gland–derived VEGF (EG-VEGF) (LeCouter et al, 2001), basic fibroblast growth factor (bFGF) (Mullaney and Skinner, 1992), and hepatocyte growth factor (HGF) (Catizone et al, 1999), have been shown to be present in rat or human testes. In this study, we demonstrated the presence of angiogenin in human testes and its localization in peritubular myoid cells (PTMCs).

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Materials and Methods

Human Testes

Tissue specimens of human testes were obtained from 6 patients (age range, 65–72) who underwent surgical castration for the treatment of prostate cancer. All of the patients had normal spermatogenesis, which was confirmed by testicular weights (8.4 ± 0.23 g/1 testicle, median ± SD), preoperative serum testosterone levels (555 ± 97.5 ng/dL, median ± SD), and hematoxylin-eosin staining that showed the presence of mature spermatids in the seminiferous epithelium. No histologic abnormality was proven to be present by light microscopy. The testes were decapsulated, washed in phosphate-buffered saline (PBS), and cut into several pieces. Some pieces of the tissue were snap-frozen in liquid nitrogen and stored at −80°C for protein extractions. The other pieces were fixed overnight in 4%(wt/vol) paraformaldehyde (PFA) at 4°C for immunohistochemistry and in situ hybridization. For immunohistochemistry, the PFA-fixed tissue was immersed in OCT compound (Tissue Tek, Elkhart, Ind) and snap-frozen in dry ice–precooled acetone. For in situ hybridization,

the PFA-fixed tissue was dehydrated with a series of ethanol washes and embedded in paraffin.

The experimental procedures were approved by the Institutional Review Board, and signed informed consent was obtained from each man.

Western Blotting

Testicular tissue was homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.5% Igepal, and 50 mM dithiothreitol and was diluted to 1 mg total protein per milliliter. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) in parallel lanes with recombinant human angiogenin (Genzyme/Techne, Minneapolis, Minn). Proteins were blotted onto a nitrocellulose membrane and incubated with an anti-human angiogenin goat antibody (1:500; Genzyme/Techne) as a primary antibody and an anti-goat horseradish peroxidase antibody (1:1000; Santa Cruz Biotechnology, Calif) as a secondary antibody. Immune complexes were visualized by the ECL Western blotting system (Amersham, Little Chalfont, United Kingdom).

Immunohistochemistry

Cryostat sections (6 μm) cut from frozen specimen were mounted on poly-L-lysine–treated slides. Sections were treated with 0.3% hydrogen peroxide for 30 minutes to eliminate endogenous peroxidase. After being blocked with 1.5% rabbit serum, the sections were incubated with anti-human angiogenin goat antibody (1:50; Genzyme/Techne) for 30 minutes at room temperature. For the negative control, the sections were incubated with anti-angiogenin antibody that was preabsorbed overnight in the presence of excessive amounts of recombinant human angiogenin. The sections were then incubated with biotinylated rabbit anti-goat immunoglobulin G (IgG), followed by avidin peroxidase, using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif). The chromogenic reaction was carried out with diaminobenzidine (Vector Laboratories). All sections were counterstained with hematoxylin and evaluated under a light microscope.

In Situ Hybridization

To prepare digoxigenin (DIG)-labeled riboprobes, the PCRII-TOPO (Invitrogen, Carlsbad, Calif) vector containing 402-bp angiogenin complementary DNA (cDNA) (Kurachi et al, 1985) was linearized with an appropriate restriction enzyme. The probes were synthesized using in vitro transcription with a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

In situ hybridization was performed using an ISHR Starting kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The paraffin-embedded specimens were sliced (6 μm thick). These sections were mounted on poly-L-lysine–treated slides, deparaffinized, and rehydrated. They were further digested with 5 mg/mL proteinase K for 10 minutes at room temperature, treated with 0.25 (vol/vol) acetic anhydride, and then subjected to treatment with prehybridization solution (50% formamide, 2× SCC [1× SCC is 0.15 M NaCl, 0.015 M sodium, and citrate]) for 30 minutes at 42°C. The probe was diluted to 0.5 μg/mL in hybridization buffer (50% formamide, 2× SCC, 1 μg/μL transfer RNA [tRNA], 1 μg/μL salmon sperm DNA, 1 μg/μL bovine serum albumin [BSA], and 10% dextran sulfate). Hybridization was carried out by applying the diluted probe to each slide section (20 μL). Each section was covered with a cover glass and incubated in a humidified chamber overnight at 42°C. Slides were washed in washing solution (50% formamide and 2× SCC) at 42°C for 20 minutes each, treated with RNase for 30 minutes at 37°C, and washed 3 times in 0.1× SCC for 20 minutes each at 42°C.

After being blocked with blocking solution, the sections were incubated with an anti-DIG, alkaline phosphatase–conjugated antibody (1:500; Roche) for 1 hour at room temperature, washed 3 times in PBST (PBS containing 0.1% Tween 20 [vol/vol]) for 30 minutes each, and rinsed 3 times in NTMT (100 mM Tris-HCl [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl, and 0.1% Tween 20 [vol/vol]) for 30 minutes each. Color development was carried out by overlaying them with nitroblue tetrazolium/5-bromo-4-choloro-3-indolyl phosphate (NBT-BCIP; Roche), and they were incubated overnight in a humidified container in the dark. All sections were counterstained with methyl green and evaluated under a light microscope. Sense probe hybridization was used as a control for background levels.

Results

Expression of Angiogenin Protein in the Testis

Western blotting analysis of the samples of the testicular tissue lysate showed a band of the same size as recombinant human angiogenin (Figure 1). The apparent molecular weight of the band was approximately 14 kd, which corresponds with the reported size of angiogenin (Fett et al, 1985). No corresponding band was detected when a nonimmune IgG or a preabsorbed antibody was used instead of the primary antibody for angiogenin (data not shown).

Figure 2 demonstrates the presence of immunoreactive angiogenin in the human testis. Angiogenin protein was stained strongly in testicular PTMCs, vascular endothelial cells, and smooth muscle cells (Figure 2B through D). Both cytoplasm and nucleus appeared to be stained because of the presence of angiogenin. Angiogenin protein was not observed in Sertoli cells, Leydig cells, germ cells, and spermatozoa. No staining was seen when a preabsorbed antibody was used as a primary antibody for angiogenin (Figure 2A).

Expression of Angiogenin mRNA in the Testis

Angiogenin mRNA was detected in PTMCs, vascular endothelial cells, and smooth muscle cells using in situ hybridization (Figure 3B and C). The expression of angiogenin mRNA was not observed in Sertoli cells, Leydig cells, germ cells, and spermatozoa. No specific hybridization products were observed when using the sense riboprobes (Figure 3A).

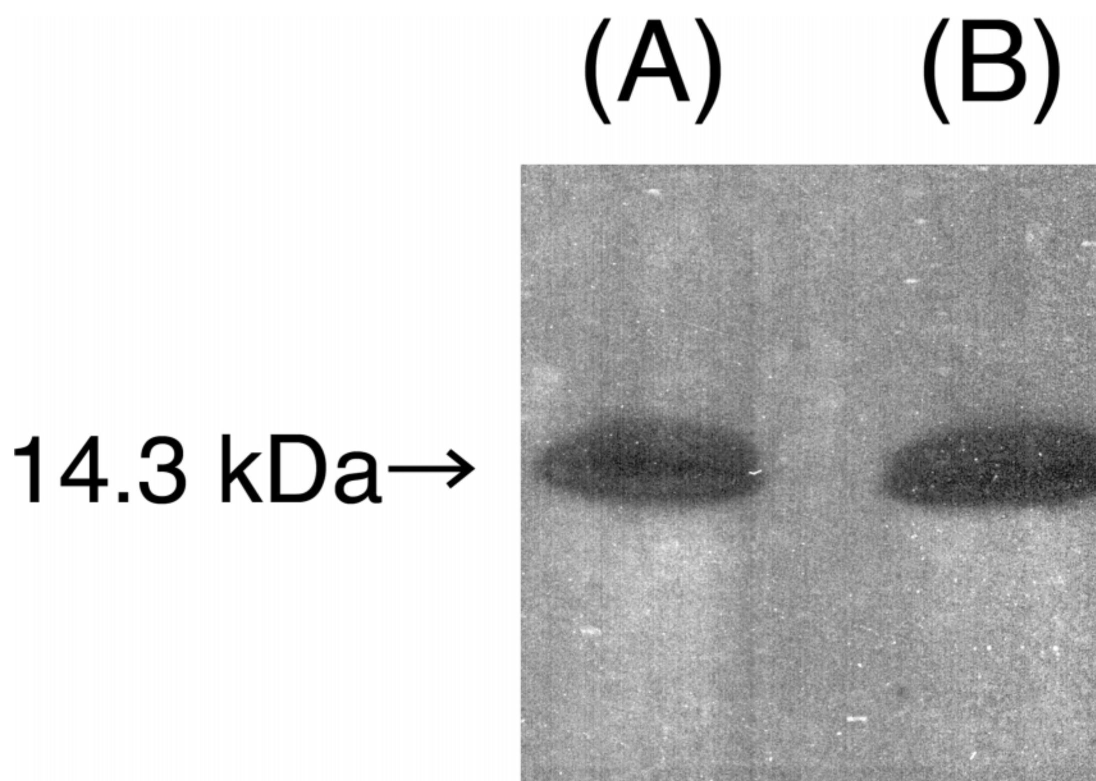

Figure 1. Western blot analysis of angiogenin in human testicular tissue. Recombinant human angiogenin **(A)** and human testicular tissue lysate **(B)** were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Discussion

In the present study, we demonstrated the presence of angiogenin in the human testis. Both angiogenin protein and mRNA were localized to PTMCs, vascular endothelial cells, and smooth muscle cells. In view of its well-known angiogenic activity, together with a recent report that demonstrated its association with the neovascularization of prostate cancer (Majumder et al, 2003), angiogenin localized to the testicular vessel wall could be involved with angiogenesis in the testis.

An unexpected finding is the localization of angiogenin to PTMCs. In this regard, several angiogenic factors observed in the testis are present in cells unrelated to blood vessels. For example, immunoreactivity tests for VEGF are positive in Leydig and Sertoli cells but not in blood vessels (Ergun et al, 1997). EG-VEGF transcripts are restricted to Leydig cells (LeCouter et al, 2001) in the human testis. In the rat testis, bFGF is expressed in PTMCs and Sertoli cells (Mullaney and Skinner, 1992), while HGF expression is exclusively localized to PTMCs (Catizone et al, 1999). These findings suggest that angiogenic factors present in the testis have pleiotropic roles in addition to their proangiogenic activities.

Angiogenin has been recently associated with properties such as ribosomal RNA synthesis (Li et al, 1997) and transcription (Xu et al, 2003) and innate immune activity (Hooper et al, 2003). These findings also suggest that angiogenin plays various roles in the human testis.

PTMCs, members of the mesenchymal cell family, are located in the space outside the seminiferous tubule. PTMCs are the most prominent component of the seminiferous tubule lamina propria and provide structural support for the tubules. Additionally, PTMCs show contractile movement, which is responsible for the peritubular contractility that results in the forward propulsion of tubular fluid and spermatozoa. Dysfunction of PTMCs seems to be involved in certain testicular disorders such as cryptorchidism (Francavilla et al, 1979) and the Sertoli cell–only syndrome (Salomon and Hedinger, 1982). Another important function of PTMCs is to produce various bioactive substances. To date, fibronectin (Tung et al, 1984), proteoglycan (Skinner and Fritz, 1985a), type I and type IV collagens, peritubular factor that modulates Sertoli cell function (PmodS) (Skinner and Fritz, 1985b), transforming growth factor beta (TGF-β) (Skinner and Moses, 1989), insulin-like growth factor I (IGF-I) (Cailleau et al, 1990), and activin-A (de Winter et al, 1994) have been reported to be produced by PTMCs. Some of these substances are known to enhance Sertoli cell func-

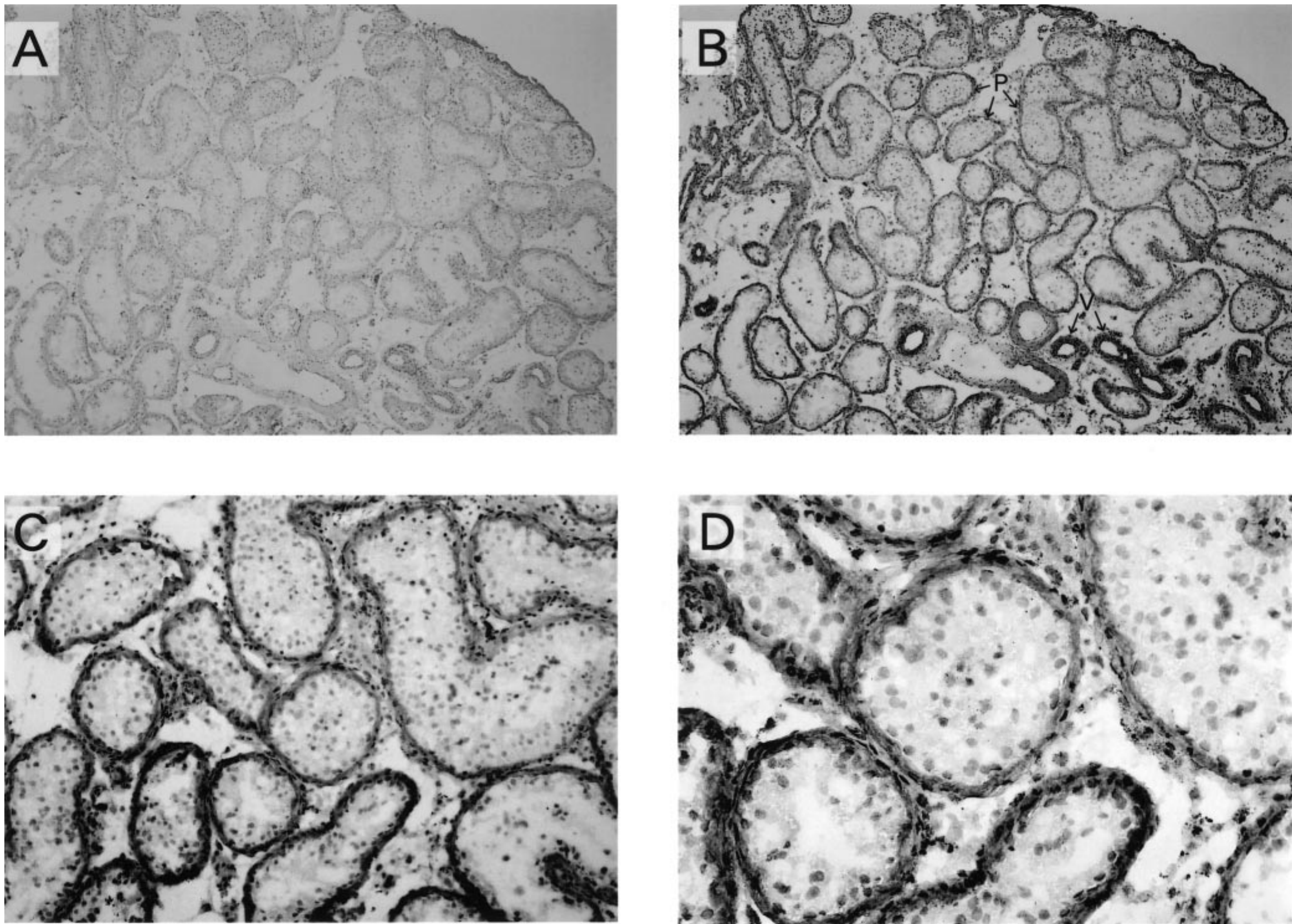

Figure 2. Immunohistochemistry of angiogenin in the human testis. Sections were immunostained with anti-human angiogenin goat antibody **(B–D)** or preabsorbed antibody **(A)**. Angiogenin protein was strongly expressed in testicular peritubular myoid cells, vascular endothelial cells, and smooth muscle cells. Magnification: **(A, B)** 40×, **(C)** 100×, and **(D)** 200×. P indicates peritubular myoid cells; V, vascular endothelial cells. The section represents 6 samples stained with the same procedure.

tions, thus implying that PTMCs are involved in the regulation of spermatogenesis. At any rate, the present study added angiogenin to the list of factors produced by PTMCs.

Angiogenin is known to stimulate the proliferation of vascular smooth muscle cells (VSMCs) (Heath et al, 1989; Xu et al, 2001). PTMCs resemble smooth muscle cells in their ultrastructural features (Fawcett et al, 1969). Moreover, both cells express cytoskeletal markers in common, such as α-isoactin, F-actin, myosin, and desmin (Toyama, 1977). Especially, α-smooth muscle isoactin is expressed selectively in VSMCs and PTMCs in the rat testis (Tung and Fritz, 1990) and monkey testis (Schlatt et al, 1993), indicating that VSMCs and PTMCs have similar cellular properties. In addition, the reaction between angiogenin and actin, a known binding protein of angiogenin, has been suggested to be an essential step in angiogenin-induced angiogenesis (Hu et al, 1993). In this context, because PTMCs are a major cellular component in the stroma surrounding the seminiferous tubules, angiogenin might exert its effects on PTMCs in an autocrine fashion and thereby modulate the structure and function of the seminiferous tubules.

In summary, the present study demonstrates the presence of angiogenin in the human testis. In light of its localization in PTMCs, cells with well-known roles in spermatogenesis, sperm transport, and formation of the seminiferous tubules, the present results suggest a pivotal function for angiogenin in the testis.

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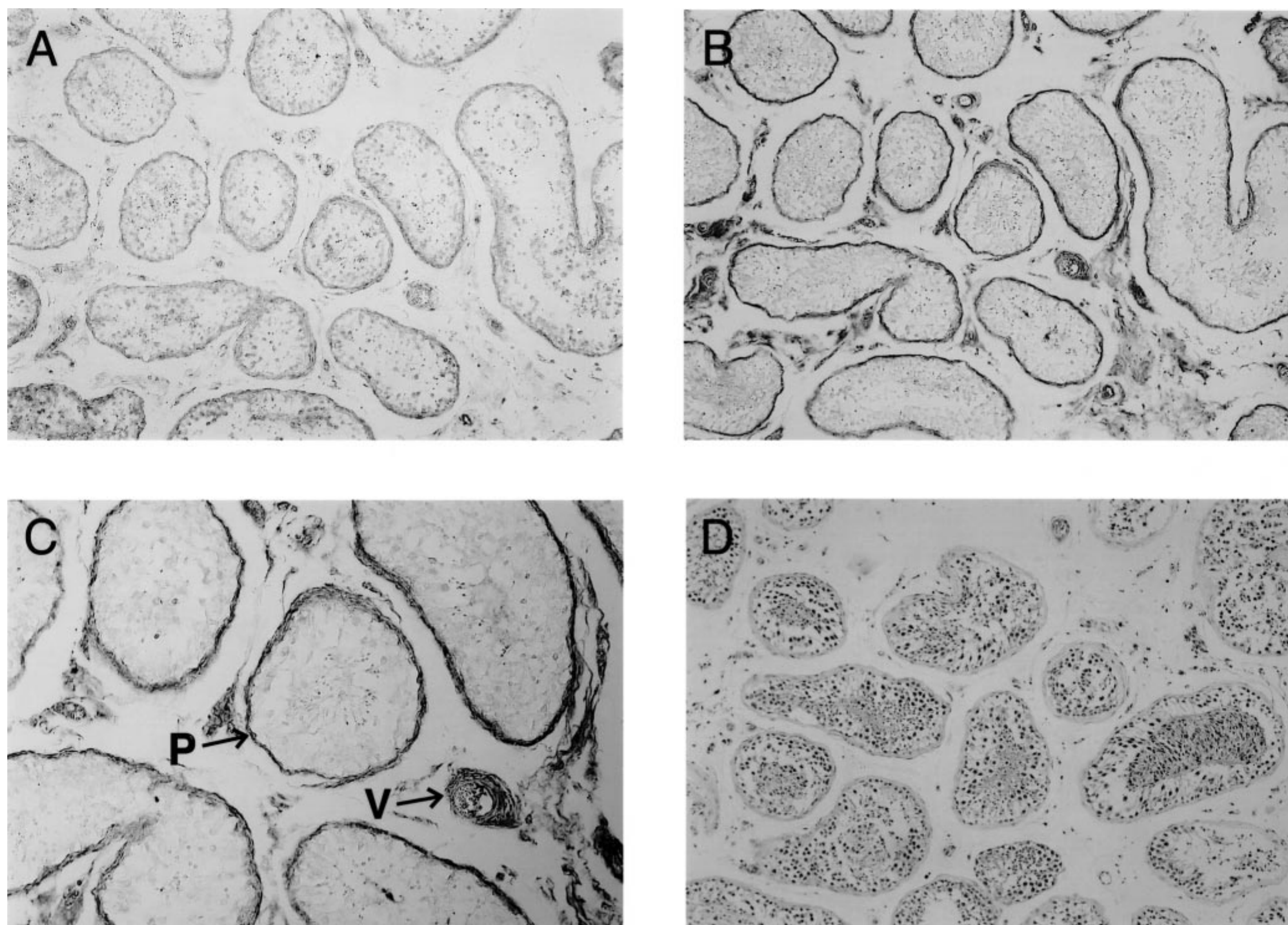

Figure 3. In situ hybridization for angiogenin in the human testis. Slide sections were hybridized with digoxigenin (DIG)-labeled antisense **(B, C)** or sense **(A)** riboprobes. Angiogenin messenger RNA (mRNA) is strongly expressed in testicular peritubular myoid cells, vascular endothelial cells, and smooth muscle cells. **(D)** shows hematoxylin-eosin staining of the same section, which shows the presence of mature spermatids in the seminiferous epithelium. Magnification: **(A, B, D)** 40×, **(C)** 100×. P indicates peritubular myoid cells; V, vascular endothelial cells. The section represents 6 samples stained with the same procedure.

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