Testicular Development Involves the Spatiotemporal Control of PDGFs and PDGF Receptors Gene Expression and Action

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Abstract. Platelet-derived growth factors (PDGFs) are growth-regulatory molecules that stimulate chemotaxis, proliferation and metabolism primarily of cells of mesenchymal origin. In this study, we found high levels of PDGFs and PDGFs receptors (PDGFRs) mRNAs, and specific immunostaining for the corresponding proteins in the rat testis. PDGFs and PDGFRs expression was shown to be developmentally regulated and tissue specific. Expression of PDGFs and PDGFRs genes was observed in whole testis RNA 2 d before birth, increased through postnatal day 5 and fell to low levels in adult. The predominant cell population expressing transcripts of the PDGFs and PDGFRs genes during prenatal and early postnatal periods were Sertoli cells and peritubular myoid cells (PMC) or their precursors, respectively, while in adult animals PDGFs and PDGFRs were confined in Leydig cells. We also found that early postnatal Sertoli cells produce PDGF-like substances and that this production is inhibited dose dependently by follicle-stimulating hormone (FSH). The expression of PDGFRs by PMC and of PDGFs by Sertoli cells corresponds in temporal sequence to the developmental period of PMC proliferation and migration from the interstitium to the peritubulum. Moreover, we observed that all the PDGF isoforms and the medium conditioned by early postnatal Sertoli cells show a strong chemotactic activity for PMC which is inhibited by anti-PDGF antibodies. These data indicate that, through the spatiotemporal pattern of PDGF ligands and receptors expression, PDGF may play a role in testicular development and homeostasis.

TESTICULAR development involves a complex combination of cell proliferation, hypertrophy, migration, differentiation, and apoptosis which proceeds within strict temporal and anatomical constraints. These processes imply intercellular communication, rather than relying entirely on intracellular programming and hormonal control (Skinner, 1991). The testis is composed of both somatic and germinal cell populations which are distributed in two main compartments: the interstitial and the tubular compartments. The interstitium is composed of Leydig cells, connective tissue, endothelial elements, and along with the intertubular blood vessels, macrophages and cells with fibroblast-like appearance. The seminiferous tubule is composed of germ cells, Sertoli cells, and peritubular myoid cells (PMC).[1] Sertoli cells are of the epithelial cell type; they form the tubule and provide structural and nutritional support for the developing germinal cells. Peritubular cells are a mesenchymal cell type; they surround the Sertoli cells and form the exterior wall of the seminiferous tubule.

Pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), are essential for optimal germ cells and somatic cells growth and differentiation, as well as for the initiation and maintenance of spermatogenesis, however, they do not directly contribute in the intercellular signaling within the tissue. Therefore, the importance of paracrine or autocrine factors, such as growth factors may be fundamental in the development of the testis (Bellve and Zheng, 1989; Mullaney and Skinner, 1991; Spiteri-Grech and Nieschlag, 1993; Lamb, 1993).

One of the substances that have marked effects on cellular differentiation and growth is platelet-derived growth factor (PDGF). PDGF is a major mitogen for cells of mesenchimal origin that is widely expressed in normal and transformed cells (Heldin and Westermark, 1990; Heldin, 1992).

PDGF is composed of two polypeptide chains named A-chain and B-chain, respectively. These two chains, en-

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1. *Abbreviations used in this paper*: FSH, follicle-stimulating hormone; PDGF, platelet-derived growth factor; PMC, peritubular myoid cells.

coded by homologous but distinct genes, can combine in three disulfide-linked dimers, AA, AB, and BB, with a molecular weight of ~30,000 (Antoniades, 1991). The PDGF isoforms exert their biological actions via binding to cell surface receptors which belong to the protein tyrosine kinase family of receptors (Williams, 1989). Two receptor subunits have been identified, the α subunit which can bind both the PDGF A-chain and PDGF B-chain, and the β subunit, which can bind only the PDGF B-chain. These subunits, upon ligand binding, dimerize to form three high affinity–binding sites for the dimeric PDGF ligand: an αα-receptor, an αβ-receptor, and a ββ-receptor (Heldin et al., 1989). Due to the binding specificities of the α and β subunit, PDGF-AA can only bind to αα-receptors, PDGF-AB can bind to αα- and αβ-receptors, and PDGF-BB can bind to all three (Seifert et al., 1989). Apart from being a mitogen, PDGF appears to serve other important roles as well. PDGF is a potent chemoattractant for a number of cell types both in vitro and in vivo and activates the early transcription of otherwise quiescent genes, several of which encode potent cytokines and others of which are proto-oncogenes (Deuel, 1987). These diverse responses to PDGF differ among cell types as a complex function of the isoforms and receptors of PDGF, whose expression is further regulated by tissue specificity, cellular phenotypic states, and time of developmental appearance.

The possibility that the PDGF system may play an important role during mammalian development was suggested by the observation that PDGF and PDGFR mRNA transcripts are coexpressed in preimplantation mouse blastocysts and early embryos (Mercola et al., 1988; Rappolee et al., 1988), in a variety of mesenchymal cells in the mid-gestation embryo (Mercola et al., 1990; Orr-Urtreger and Lonai, 1992*a*; Schatteman et al., 1992) in the developing and adult central nervous system (Sasahara et al., 1991; Yeh et al., 1991, 1993) and in the kidney glomerulus (Alpers et al., 1992). Ideas concerning the functions of PDGF in vivo are primarily based on the expression patterns of PDGF ligands and receptors and their changes under physiological and pathological conditions.

Recent evidences have shown that PDGF is a member of the locally produced growth factors that mediate testicular cell–cell interactions. In particular, it has been found that purified adult rat Leydig cells produce PDGF-like molecules and bind PDGF through specific receptors (Gnessi et al., 1992), and that rat PMC are induced to express functionally active PDGFRs in response to cell culturing (Gnessi et al., 1993). Moreover, it has been reported that PDGF-BB exerts a stimulatory effect on adult Leydig cells steroidogenesis in vitro (Risbridger, 1993; Loveland et al., 1993).

In the present studies, we have examined the in vivo and in vitro occurrence of PDGF and its receptors in the testis. The time course of appearance of PDGFs and PDGFRs transcripts, their characteristic cellular expression in the testicular tissue during development, the ability of early postnatal Sertoli cells to secrete a PDGF-like substance under FSH control, the autocrine modulatory effect of PDGF on adult Leydig cells testosterone production, and the migratory effect of PDGFs and of PDGF-like substances produced by Sertoli cells on PMC, strongly suggest a previously undescribed role of the PDGF system in the developmental regulation of the testicular function.

Materials and Methods

Tissue and Cell Preparation

Male Sprague Dawley rats (Charles River Italia, Calco, Italy) were used in all experiments. Decapsulated testes from fetuses (2 d before birth), from rats of different ages (1, 5, 15, 60, and 300 days old), or brain from adult animals, were weighed and kept in liquid nitrogen until RNA preparation or fixed in Bouin's fluid until immunohistochemistry.

Sertoli cells–enriched cultures were prepared from 5, 10, and 15-d-old animals by digesting decapsulated testes with trypsin (0.25%, 30 min, 32°C; Difco Laboratories, Detroit, MI), collagenase (0.1%, 30 min, 32°C; Boehringer Mannheim GmbHm, Mannheim, Germany), and hyaluronidase (0.1%, 30 min, 32°C; Sigma Chemical Co., St. Louis, MO), as previously described (Jannini et al., 1994). All cultures were maintained in basal Eagle's Medium with Hank's salt (HBME) buffered with Hepes (20 mM, pH 7.4; Sigma) and supplemented with nonessential amino acids (0.1 mM; Flow Laboratories, ICN Biomedical, Milan, Italy) and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, 0.05 mg/ml gentamicin sulfate; Flow Laboratories). The cells were plated and incubated in 5% CO_2, 95% air at 32°C. 48 h later, the Sertoli cells were treated with a hypotonic solution (20 mM Tris HCl, pH 7.4) for 3 min to remove germ cell contaminant. The purity of cell cultures was assessed, after 2 d of culture, by staining with alkaline phosphatase for PMC and by phase-contrast microscopy observation for germ cells contamination. The Sertoli cell–enriched preparation from 15-d-old animals was more than 95% pure, with the major contaminant being germ cells. Sertoli cells from 5- and 10-d-old rats showed a lower degree of purity, ranging between 80–90%, with the major contaminant being germ cells and PMC.

Leydig cells were prepared from 15-d-old and 60-d-old rats and purified by Percoll gradient centrifugation as described previously (Gnessi et al., 1992). The percentage of Leydig cells in the final preparations, as established by staining for 3β-hydroxysteroid dehydrogenase activity, ranged between 85–90%. The Leydig cells were either freshly purified for RNA extraction or incubated in 5% CO_2, 95% air at 37°C in HBME for 24 h for immunocytochemistry. PMC were prepared from 5- and 15-d-old rats and purified by Percoll gradient centrifugation as described (Gnessi et al., 1993). The cells were used either immediately or plated and incubated at 37°C in 5% CO_2-95% air in HBME. Microscopic examination of the cells using alkaline phosphatase staining, revealed a positive reaction in more than 94% of the cells indicating their purity as PMC.

For the organ cultures, decapsulated testis fragments from 3-d-old animals were briefly rinsed in medium and placed on small platforms (Cyclopore, Falcon, Lincoln Park, NJ). The platforms were bathed in 1 ml of medium, so that a very thin film of medium covered the surface of the tissue. HBME, supplemented with nonessential amino acids, sodium pyruvate, glutamine, and containing penicillin and streptomycin was used as described (Jannini et al., 1993). Organ cultures were incubated at 32°C in 5% CO2, 95% air. The testis fragments were fixed directly on the platform in Bouin's fluid.

When required, the Sertoli cells or organ cultures were treated with the indicated doses of ovine FSH-20 (National Hormone and Pituitary Program, NIDDK, lot n. AFP-7028D, Bethesda, MD).

RNA Isolation and Northern Blot Analysis

Total RNA from testis, brain, and purified cells was prepared by using RNAzol B (Cinna/Biotecx Laboratories, Houston, TX). The RNA samples were separated on 1% formaldehyde-agarose gel, blotted on Nytran (Schleicher & Schuell, Keene, NH) and probed with random primer ^{32}P-labeled cDNAs. The following cDNA probes were used: 650 bp SacII–StuI fragment of the human PDGF-A cDNA, sharing 84% homology with the rat cDNA sequence, and the 462-bp SacII–PvuII fragment of the human PDGF-B cDNA, sharing 81% homology with rat cDNA sequence, excised from plasmids pMMTPDGFA and pMMTPDGFB, respectively, kindly provided by Dr. Stuart Aaronson (Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD); full-length coding region of the rat PDGFR α-subunit and β-subunit cDNAs (a generous gift from Dr. Michael Peck, F. Hoffman-La Roche, LTD, Basel, Switzerland). Hybridization in QuikHib solution (Stratagene, La Jolla, CA) and high stringency washing were carried out as suggested by the

manufacturer. To verify the loading of RNA samples on Northern blots, the filters were hybridized with a β-actin probe. Autoradiograms were scanned, processed and analyzed using the NIH Image 1.51 program, and specific signals were normalized to the signal of β-actin mRNA per lane. Multiple dilution of the samples, and multiple film exposure times gave comparable results.

Immunostaining

For immunohistochemistry, fixed testes or organ cultures were dehydrated in ethanol and embebbed in paraffin. Immunostaining was carried out on 5-μm thick sections of the tissues or organ cultures. For immunocytochemistry the cells, isolated and cultured as described, were plated in 8-well LabTek slides (Nunc Inc., Naperville, IL). At the end of the incubation times the cultures were washed in PBS and immediately fixed with ice cold ethanol for 5 min. Immunostaining was performed by the streptavidin-biotin immunoperoxidase method, using a commercial kit (Zymed Laboratories Inc., San Francisco, CA). The deparaffinized sections or the cell preparations were incubated overnight in a moist chamber at 4°C with 1:100 dilution of the primary antibodies.

The following antisera were used: affinity-purified polyclonal rabbit anti–PDGF-BB and anti–PDGF-AA antibodies (Genzyme, Cambridge, MA); PDGFR-7 and PDGFR-3, rabbit polyclonal antisera to the PDGF receptor α- and β-subunit, respectively (provided by Dr. Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden). PDGFR-7 was generated against a synthetic peptide covering amino acids 1066-1084 of the COOH-terminal region of human PDGF receptor α-subunit and does not cross-react with the PDGF receptor β-subunit. It recognizes both human and rat PDGF receptor α-subunit. PDGFR-3 was raised against a synthetic peptide corresponding to amino acids 981-994 of the mouse PDGF β-receptor. It recognizes rat PDGF receptor β-subunit. PDGFR-7 and PDGFR-3 were affinity purified on columns with immobilized synthetic peptides (kindly provided by Dr. Carl-Henrik Heldin) against which the antisera were raised (Hermanson et al., 1992; Eccleston et al., 1993). All the antibodies react specifically with the respective antigens in immunoprecipitation and Western blotting experiments (Claesson-Welsh et al., 1989; Han et al., 1993; Eccleston et al., 1993). In control experiments for specificity, the antibodies were blocked by incubation overnight at 4°C with excess of the corresponding peptides. Slides were developed using amino-ethylcarbazole (AEC) as chromogenic substrate which is converted by the peroxidase into a red to brownish-red precipitate at the sites of antigen localization in the tissue. The preparations were counterstained with hematoxylin, dehydrated, cleared, and mounted.

PDGF Radioreceptor Assay

PDGF-like immunoreactivity in the Sertoli cells supernatants was measured by means of a standard simultaneous competition radioreceptor assay (RRA) (Gnessi et al., 1992). Purified PMC from 15-d-old animals were plated at 2×10^5 cells/well in 24-well tray (Becton Dickinson Labware, Lincoln Park, NJ) in HBME. After 4 d of culture the cells were rinsed with HBME, 0.1% BSA, and exposed to 100,000 cpm of [^{125}I]PDGF-BB (Amersham International plc, Amersham, Buckinghamshire, UK; specific activity, 1,000 Ci/mmol) and increasing concentrations of unlabeled PDGF-BB (from 0.15 to 50 ng/ml) or test samples for 60 min at 24°C. Then the cells were washed three times with cold binding medium and lysed in 1% Triton X-100, 10% glycerol, 20 mM Hepes, pH 7.4. Radioactivity was determined in a γ spectrometer. The PDGF receptor competing activity of the conditioned media was converted into PDGF equivalents (ng/ml). To allow comparison among different samples, the PDGF equivalents were normalized to the protein content of the cells from which the supernatants were taken, determined by the protein microassay (Bio-Rad Laboratories GmbH, München, Germany) using bovine serum albumin as standard (Bradford, 1976), after cells lysis via freeze thaw and protein solubilization. Since the PMC in culture express both the PDGFR α- and β-subunit (Gnessi et al., 1993), and the α subunit recognizes both PDGF A- and B-chain with the same affinity (Seifert et al., 1989; Gnessi et al., 1993), this RRA detects all classes of PDGF dimers.

Chemotaxis Assay

PMC chemotaxis was assayed by a modification of Boyden's chamber method using a 48-well micro chemotaxis chamber (Neuro Probe, Inc., Bethesda, MD) (Falk et al., 1980). 25 μl of HBME 0.1% BSA containing PDGF-BB, PDGF-AA, PDGF-AB (Boehringer Mannheim GmbH), or conditioned media to be tested, were placed in the lower wells of the microchamber plates. Medium alone served as a negative control. A 25 × 80 mm, 5-μm pores, polyvinylpirrolidine-free polycarbonate filter (Nucleopore Co., Pleasanton, CA), separated the lower wells from the upper wells containing 40 μl of the cell suspension, which consisted of freshly isolated PMC from 5-d-old or 15-d-old animals or PMC from 15-d-old animals trypsinized after 4 d of culture, suspended in HBME 0.1% BSA to a final concentration of 10^6 cells/ml. The assay was incubated at 37°C in 5% CO_2 for 4 h. PMC on the upper side of the filter were scraped off. Then, PMC, which migrated to the lower side of the filter, were fixed and stained with hematoxylin. Chemotactic activity was expressed as the average number of cells (± SEM) migrated to the lower surface of the filter in five random high power (×400) microscopic fields (HPF) in an area representing a well. All experiments were performed at least two times in triplicate. In experiments to determine the effect of an antibody on PMC migration, the antibody was incubated at 37°C for 1 h with the corresponding test substance or the conditioned media before the start of the assay. The antisera used were anti–PDGF-BB, anti–PDGF-AA (Genzyme), and a polyclonal rabbit anti–PDGF-AB antiserum (R & D Systems, Inc., Minneapolis, MN). In separate studies, checkerboard analysis was performed as previously described (Zigmond and Hirsch, 1973), to determine if PDGFs stimulated directed migration (chemotaxis) or increased random locomotion (chemokinesis).

Statistical Analysis

The significance of the results was determined by using the Student's t test on data from 2 to 3 separate experiments. Statistical differences were considered significant at $P < 0.05$. Data are presented as the mean ± SEM.

Results

Expression of PDGF Ligands and Receptors mRNAs during Testicular Development

Expression of PDGF A-chain, PDGF B-chain, and PDGFR α- and β-subunit mRNAs were evaluated by Northern blot hybridization. Total RNA was isolated from prenatal (day 20 of gestation), newborn (day 1), prepubertal (day 5), early pubertal (day 15), adult (day 60), and aged (day 300) rat testis. All forms of PDGF and PDGFR mRNAs were detected in prenatal testis, increased from day 1 to day 5 postnatal, and then decreased to extremely low levels in pubertal and adult tissue (Fig. 1 *A*). The PDGFR α-subunit and PDGF A-chain genes were preferentially expressed over the PDGFR β-subunit and PDGF B-chain genes.

Although it was difficult to discriminate between the 2.3-kb and 2.9-kb transcripts, three PDGF A-chain mRNA species of 1.7, 2.3, and 2.9 kb were revealed. PDGF-A mRNA was detected in prenatal testis and in 15-d-old testis primarily as 1.7 kb and 2.3 kb transcripts. In day 1 and day 5 postnatal testis all three PDGF-A mRNA transcripts were expressed. Expression of the 3.3-kb transcript for PDGF B-chain followed the same pattern of appearance of the PDGF A-chain mRNA. Hybridization of cDNAs encoding the rat PDGFR α- and β-subunit were observed, with RNA species of ~6.7 kb and 5.6 kb, respectively. The highest levels of PDGFR α- and β-subunit mRNAs expression were also evident between day 1 and day 5 postnatal and progressively decreased with age. The relative levels of expression of the PDGF chains and PDGFR subunits were estimated by densitometric scanning followed by normalization with respect to the control β-actin mRNA signal (Fig. 1 *B*). In the case of PDGF A-chain, the 1.7, 2.3, and 2.9 transcripts were scanned. A comparison of the strengths of the bands of mRNA confirmed a progres-

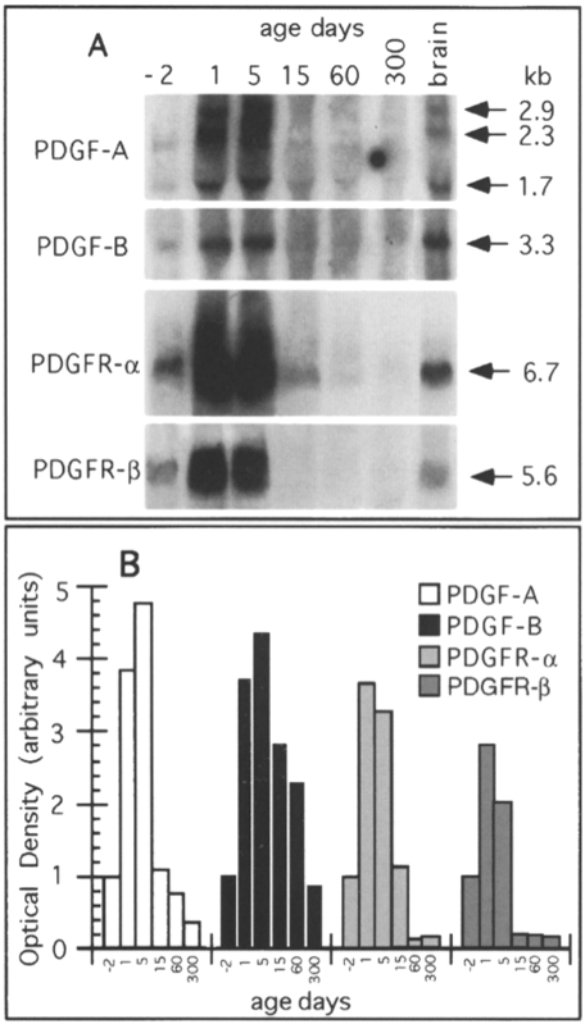


Figure 1. Northern analysis of PDGF A-chain, B-chain, and PDGFR α-subunit, and β-subunit mRNA transcripts in extracts from rat testis obtained at prenatal day 2 and 1, 5, 15, 60, and 300 d postnatal and adult brain. (*A*) Each lane contained 20 μg of total RNA from the indicated samples, isolated and analyzed as described in Materials and Methods. The sizes of the transcripts were estimated from the positions of 28-S and 18-S ribosomal RNA bands. Data presented are a representative example of three experiments which gave comparable results. (*B*) The histograms show a quantitative representation of hybridization obtained by densitometric scanning of the autoradiogram of the Northern blot of the testicular RNA extracts shown in *A*. Results are expressed in arbitrary densitometric units after normalization to the signal obtained when the blot was rehybridized with the β-actin probe (not shown). The prenatal day 2 data have been set to one for each of the four transcripts compared.

sive increase in PDGFRs and PDGFs steady state mRNA levels up to postnatal day 5, followed by a sharp decline with aging. The abundance of PDGFR α-subunit transcript in 1-d-old rat testis was 3.7-fold higher than in the prenatal testis and 1.12-fold higher than in 5-d-old testis. Similarly, the PDGFR β-subunit RNA in 1-d-old testis was 2.8-fold higher than in the prenatal testis, and 1.4-fold higher than in 5-d-old testis. The PDGF A-chain gene from 5-d-old tissue was 1.2-fold higher, and 4.7-fold higher compared to 1-d-old and prenatal rat testis, respectively. The PDGF B-chain gene from 5-d-old tissue was 1.17-, and 4.35-fold higher compared to newborn and prenatal testis. The prepubertal testis and particularly the adult has germ cells as the predominant cell type. Therefore the decline of the mRNAs relative abundance could be due to both germ cell accumulation as well as decreased expression. However the subsequent data support a decreased expression.

To determine which of the various cell types expressed PDGFR subunits, and PDGF chains mRNAs, total RNA was prepared from Sertoli cells, Leydig cells, and PMC isolated from testes at different stages of development, including prepubertal, early pubertal, and adult animals (Fig. 2). Sertoli cells expressed PDGF-A and PDGF-B mRNAs only during the prepubertal period. No PDGFR α- and β-subunit mRNAs were detected in early pubertal Sertoli cells RNA. PMC showed a distinct pattern of PDGFs and PDGFRs gene expression. Both PDGFR α- and β-subunit mRNAs were visible in freshly isolated prepubertal PMC; the expression dropped to almost nondetectable levels when mRNA was extracted, immediately after isolation, from cells obtained from 15-d-old animals. On the other hand, the PMC from 15-d-old animals, showed an increasing content of both PDGFR α- and β-subunit transcripts when cultured for 4 d. No signs of expression of the PDGF A-chain and PDGF-B chain genes were detected in any of the experimental groups.

Adult Leydig cells expressed mRNAs for PDGF A- and B-chain as well as for the PDGFR α- and β-subunit. On the contrary, early pubertal Leydig cells did not show measurable amounts of mRNA for either PDGF chains or PDGFR subunits.

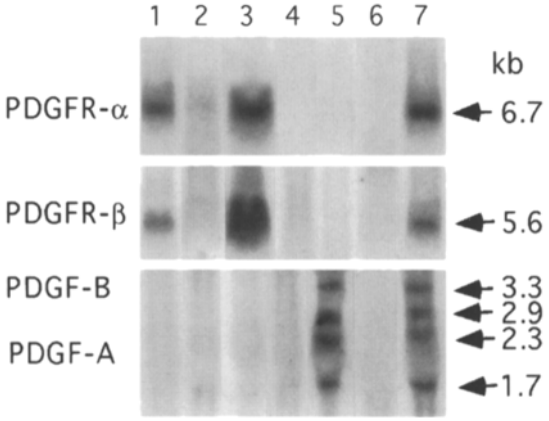


Figure 2. Northern blot analysis of RNA from various cell types to determine the site and the time of PDGF A-chain, B-chain, and PDGFR α-subunit, and β-subunit expression in rat testis. 20 μg of total RNA were loaded into each lane, with the exception of lane 7, into which 10 μg of total RNA were loaded. Freshly isolated PMC from 5-d-old animals (lane *1*), freshly isolated PMC from 15-d-old animals (lane *2*), PMC from 15-d-old animals after 4 d of culture (lane *3*), isolated day 15 Sertoli cells (lane *4*), isolated day 5 Sertoli cells (lane *5*), purified early pubertal Leydig cells (lane *6*), and purified adult Leydig cells (lane *7*). The sizes of transcripts were estimated from the positions of 28-S and 18-S ribosomal RNA bands. Data presented are a representative example of three experiments which gave comparable results.

Developmental Expression of PDGF Ligands and Receptors Proteins in the Testis and in Purified Gonadal Cells

To determine if the testicular cells translate the PDGFs and PDGFRs mRNAs, and to examine the specific sites of distribution of PDGF-AA and PDGF-BB and of PDGFR α- and β-subunit, sections of day 20 rat fetal testis and from 5-d-old, 15-d-old, and adult rat testis were reacted with anti–PDGF-AA, anti–PDGF-BB, anti–PDGFR α-subunit, and anti–PDGFR β-subunit antibodies. In the fetal testis, a positive immunoreactivity to PDGF-AA and PDGF-BB was detected within the testicular cords (Fig. 3, *A* and *C*), while staining for PDGFR α- and β-subunit was evident in cells scattered in the tissue between the testicular cords (Fig. 3, *E* and *G*). A similar distribution pattern was observed in 5-d-old testis, with intense staining for PDGF-AA and PDGF-BB localized within the tubule in the cytoplasm of the Sertoli cells (Fig. 4, *A* and *C*) and PDGFR

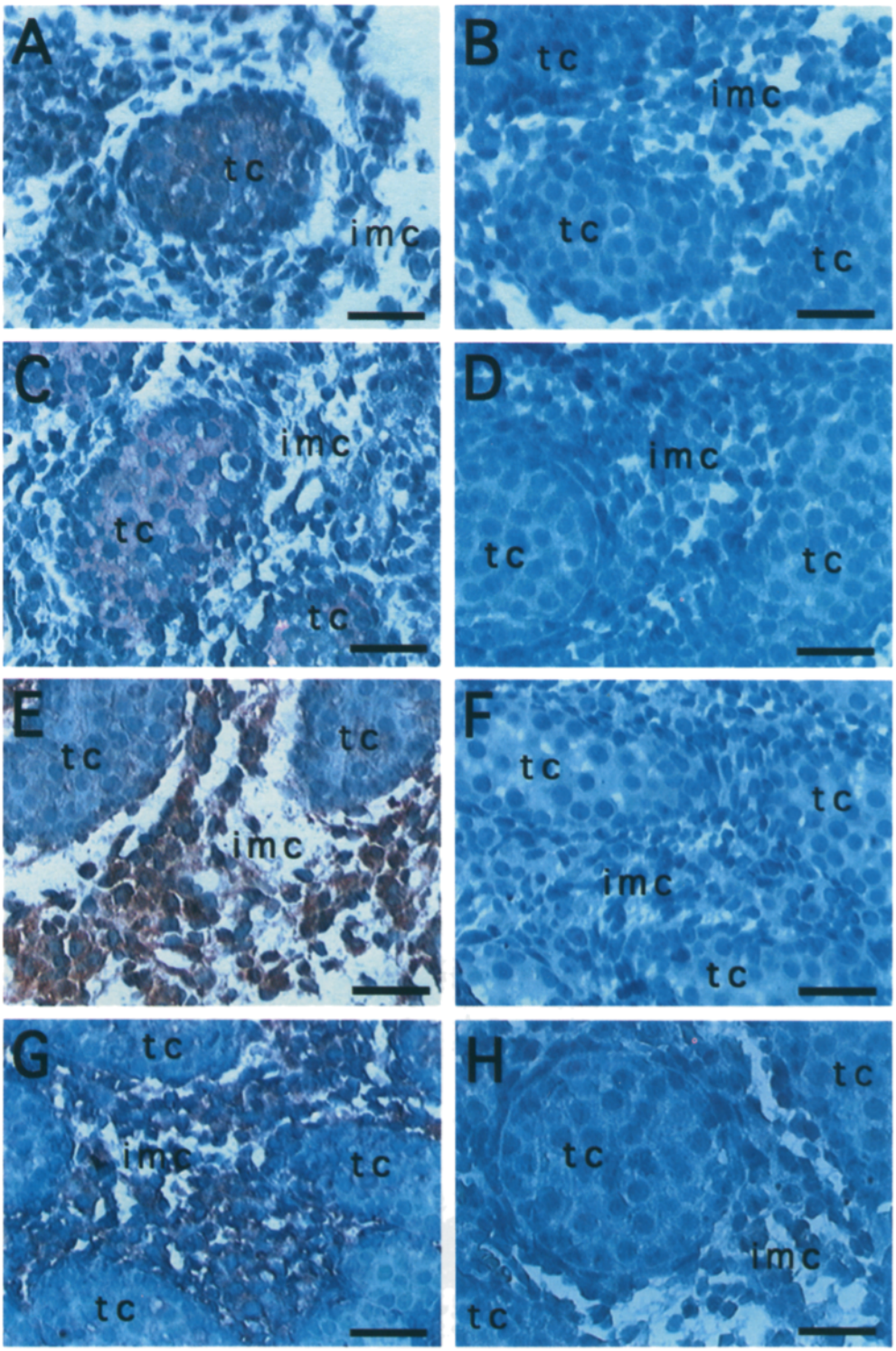
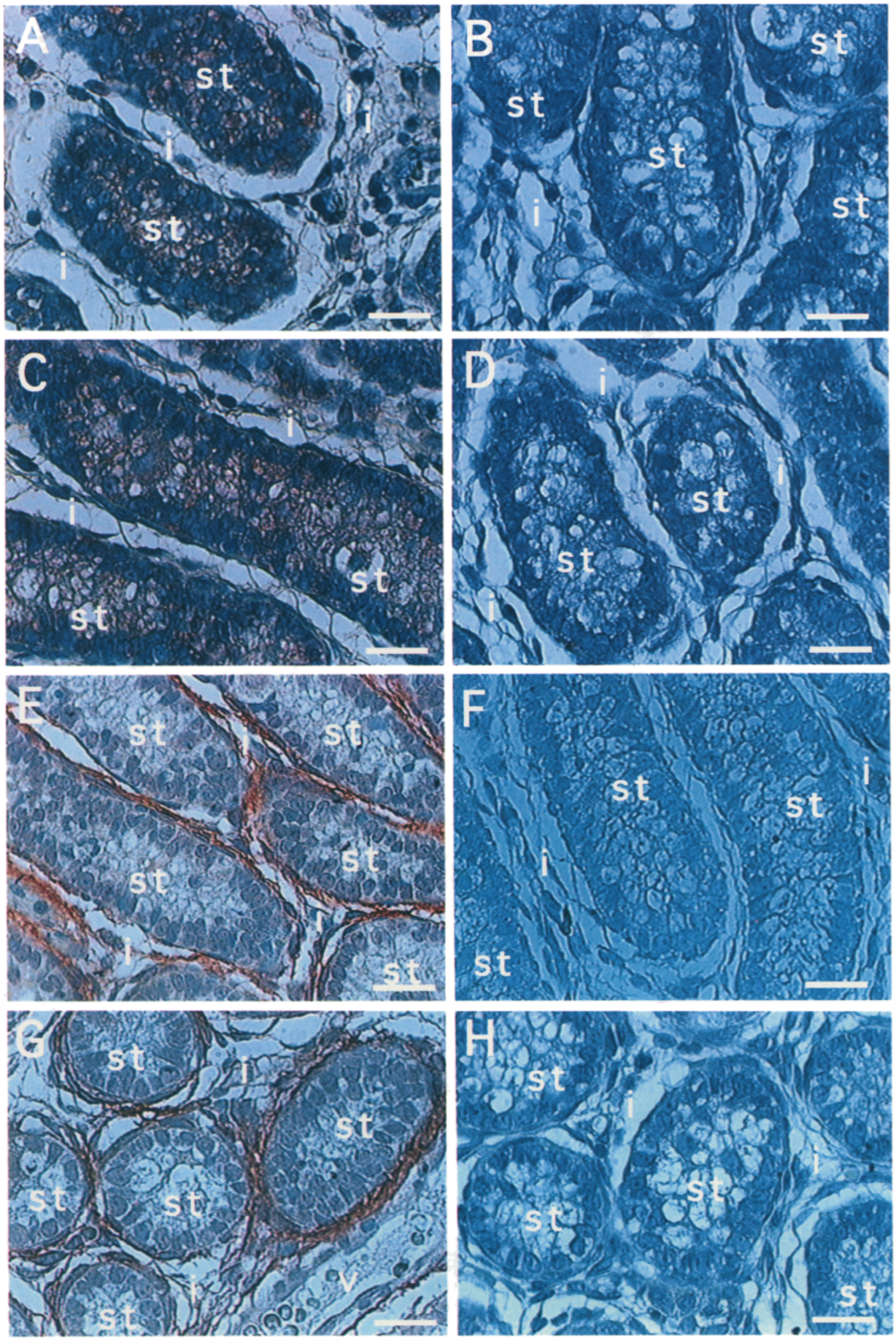


Figure 3. Immunohistochemical localization of PDGF-AA, PDGF-BB, and of PDGFR α- and β-subunit in day 20 fetal rat testis. The seminiferous cords are intensely stained using antibody against PDGF AA (*A*); with anti–PDGF-BB antibody a positive reaction is visible in the same area (*C*). Numerous positively stained cells for both PDGFR α- (*E*) and β-subunit (*G*) are identified among the cells in the space between the seminiferous cords. If sections are stained with anti–PDGF-AA, anti–PDGF-BB, anti–PDGFR-α, and anti–PDGFR-β antibodies preincubated for 12 h at 4°C with the corresponding immunizing peptides then all staining is blocked (*B*, *D*, *F*, and *H*). The sections were studied immunohistochemically using the streptavidin-biotin immunoperoxidase method and the AEC chromogen as described in Materials and Methods. *tc*, testicular cord; *imc*, intercordal mesenchymal cells. Bars, 50 μm.



α- and β-subunit immunoreactivity confined in the peritubular cells (Fig. 4, *E* and *G*). In the testis from 15-d-old rats only minimal positive staining for PDGF-AA and PDGF-BB in the Sertoli cells and for PDGFR α- and β-subunit in PMC was observed (data not shown). In the adult testis, the Leydig cells were the only cellular component that showed positive reaction for both the PDGF homodimers (Fig. 5, *A* and *C*) and the PDGFR α- and β-subunit (Fig. 5, *E* and *G*). Panels *B*, *D*, *F*, and *H* of the Figs. 3, 4, and 5 show the control staining of the sections from prenatal, 5-d-old, and adult testis when the anti–PDGF-AA, anti–PDGF-BB, anti–PDGFR α-subunit, and anti–PDGFR β-subunit antibodies were used after preincubation with excess of the synthetic peptides to which they were raised. All the controls remained unstained demonstrating the specificity of the immunoreaction.

To characterize further the immunohistochemical findings, immunocytochemical studies were conducted on purified Leydig cells, Sertoli cells, and PMC at various ages (Fig. 6). PMC from 5-d-old rats showed positive immunostaining for PDGFR α- and β-subunit (Fig. 6, *A* and *B*), but not for PDGF-AA and PDGF-BB (Fig. 6, *C* and *D*). Purified PMC from 15-d-old rats after 24 h of culture did not stain with any one of the anti-PDGFs and anti-PDGFRs antibodies (data not shown). However, a strong immunostaining was observed when the cells were reacted with the anti-PDGFR α- and β-subunit antibodies after 4 d in culture (Fig. 6, *G* and *H*). The same cells were negative for PDGF-AA and PDGF-BB (Fig. 6, *E* and *F*).

Positive immunostaining for PDGF-AA and PDGF-BB and for the PDGFR subunits was readily detectable in adult Leydig cells (Fig. 6, *I–L*). Early pubertal Leydig cells did not stain with any of the PDGFs and PDGFRs antibodies employed (data not shown). These findings agree with and extend the immunohistochemical observations in the intact tissue, and are consistent with previous observations of PDGF-like material production and PDGFRs expression by adult rat Leydig cells in culture (Gnessi et al., 1992). Sertoli cells from 5-d-old animals showed positive immunostaining for either PDGF-AA or PDGF-BB (Fig. 6, *M* and *N*), while no reaction was observed with anti-PDGFR α- and β-subunit antibodies (Fig. 6, *O* and *P*). Sertoli cells from early pubertal rats did not react with any of the antisera used (data not shown). Incubation of the antibodies with excess of the corresponding immunizing peptides abolished labeling, demonstrating the specificity of the reaction (inserts of Fig. 6). These results are in good agreement with the evidences of the Northern blots of the RNA extracted from the purified gonadal cells (Fig. 2).

PDGF-like Molecules Production by Sertoli Cells and Effect of the Treatment with FSH

The striking difference in PDGF B-chain and A-chain transcripts and proteins expression between prepubertal and adult Sertoli cells was paralleled by a similar difference in the secretion of PDGF-like molecules. Culture medium conditioned by the same prepubertal, and early pubertal Sertoli cells isolates from which RNA was extracted and analyzed by RNA blot hybridization was examined for its PDGF-like content in the RRA. Medium conditioned by prepubertal rat Sertoli cells contained measurable amounts of PDGF-like activity, while early pubertal Sertoli cells did not (Fig. 7 *A*). The PDGF-like immunoreactivity was 30 ± 5 ng/ml in the conditioned medium of 5-d-old Sertoli cells, decreased to 5 ± 2 ng/ml in the conditioned medium from 10-d-old Sertoli cells, and then disappeared in older animals. As shown in the insert of Fig. 7 *A*, an internal standard curve constructed by adding increasing concentrations of PDGF in the presence of test substance behaved equivalently to the standard curve, and serial dilutions of the medium conditioned by Sertoli cells from 5-d-old animals showed parallel displacement to the standard, indicating the absence of components that might interfere with PDGF binding.

The secretion of PDGF competitor was decreased in a dose-dependent manner by FSH (Fig. 7 *B*). The levels of PDGF-like molecules ranged from 27 ± 5 ng/ml in the absence of FSH to 7 ± 1 ng/ml after 24 h of treatment with 100 ng/ml of FSH.

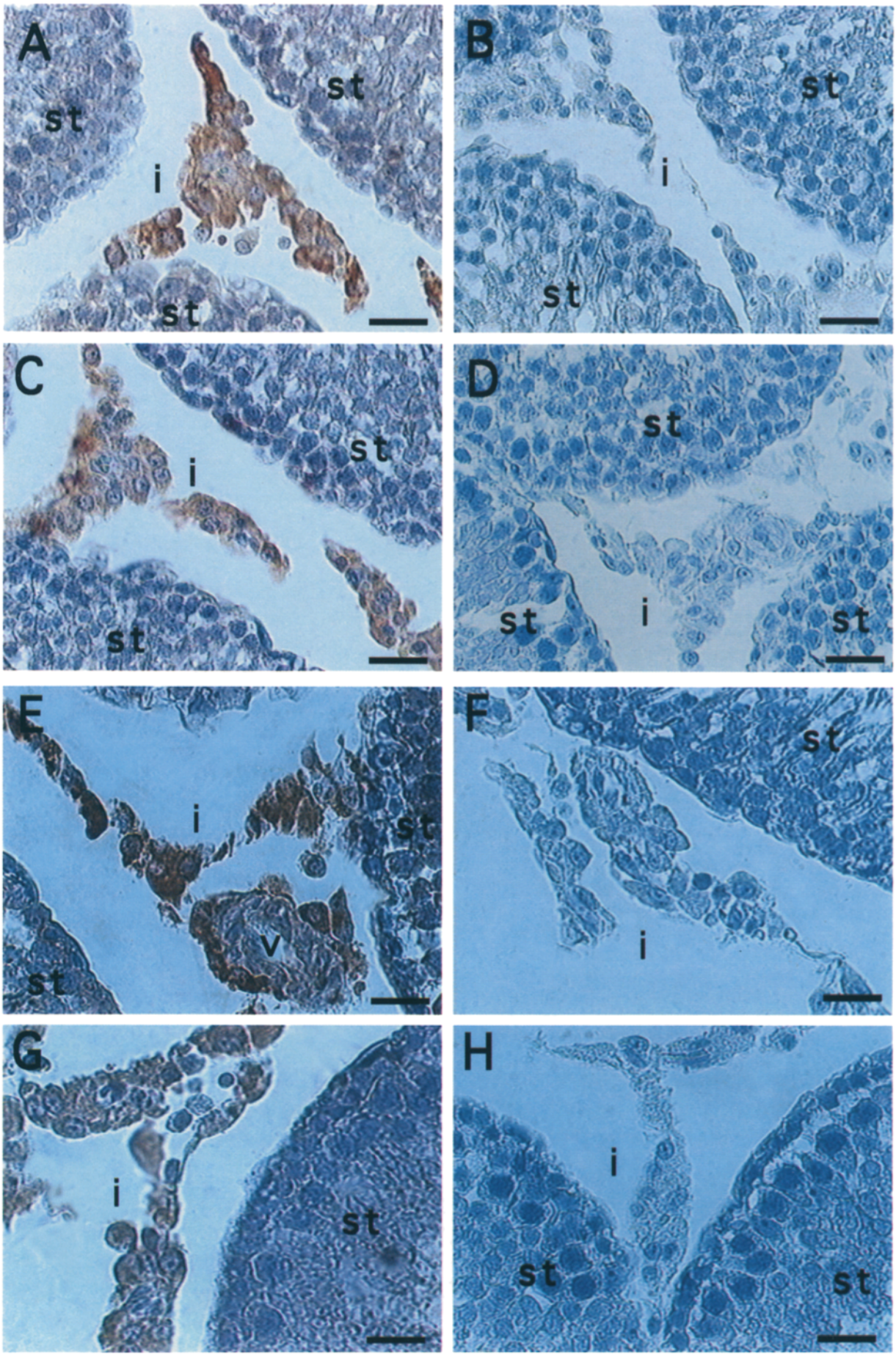
Effect of FSH Treatment on PDGF and PDGFR Immunostaining in Testicular Organ Culture

To verify the effect of FSH on the PDGF expression by Sertoli cells in a more physiological condition, we carried out immunohistochemical studies on organ cultures from 3-d-old rat testis in basal conditions and after 24 h of treatment with FSH. Analogously to the results obtained with the testicular fragments from 5-d-old animals, a positive immunostaining for PDGF-AA (Fig. 8 *A*) and PDGF-BB (Fig. 8 *B*) was observed within the tubules. On the contrary, a positive reaction for the PDGFR α- and β-subunit was localized in the intertubular tissue (Fig. 8, *C* and *F*). The 24-h treatment with 100 ng/ml FSH abolished the immunoreactivity for both PDGF-AA and PDGF-BB within the tubules (Fig. 8, *B* and *E*), confirming the results obtained with the RRA of the Sertoli cells conditioned media. No changes in the staining for the PDGFR α- and β-subunit after treatment with FSH was observed (data not shown).

Chemotactic Response of PMC to PDGFs

PDGF has been shown to be a potent chemotactic agent for fibroblasts, smooth muscle cells, and phagocytic cells (Deuel, 1987). It is conceivable that the ability to stimulate chemotaxis plays an important role in the fulfilment of the in vivo function of PDGF in tissue repair, and in embryo-

Figure 4. Immunohistochemical localization with anti–PDGF-AA, anti–PDGF-BB, anti–PDGFR α-subunit, and anti–PDGFR β-subunit antibodies in 5-d-old rat testis. Cells positive for PDGF-AA (*A*) and PDGF-BB (*C*) are readily observed within the tubules, whereas cells positive for PDGFR α-subunit (*E*) and β-subunit (*G*) are evident in the cell layer encircling the seminiferous tubules. Negative immunostaining is noted when anti–PDGF-AA, anti–PDGF-BB, anti–PDGFR α-subunit, and anti–PDGFR β-subunit antibodies are preabsorbed with the corresponding immunizing peptides (*B*, *D*, *F*, and *H*). Note the absence of positive staining in the blood vessel in *G*. Tissue sections were studied immunohistochemically as described in Materials and Methods. *st*, seminiferous tubule; *i*, intertubular space; *v*, blood vessel. Bars, 50 μm.



nal development, as well as pathological processes, such as formation of atherosclerotic plaques (Ross, 1993). We therefore investigated the three isoforms of PDGF with regard to their ability to induce directed migration of PMC.

PDGF-BB, PDGF-AB, and PDGF-AA stimulated migration of freshly purified PMC from 5-d-old rats (Fig. 9 *A*) and of PMC purified from 15-d-old animals that had cultured for 4 d (Fig. 9 *B*) but not of PMC from 15-d-old animals immediately after isolation (data not shown). This lack of response of freshly isolated 15-d-old PMC is in line with previous studies that have indicated that PMC from early pubertal animals express functional receptors for PDGF upon tissue culturing (Gnessi et al., 1993). A linear dose response of PMC migration toward increasing concentrations of the three PDGF isoforms was observed up to 25 ng/ml of the attractants followed by a decline at higher concentrations. This pattern of response is typical for chemotactic substances. At low concentrations of chemoattractant, no cell movement occurs. At higher concentrations, the cell is able to distinguish a difference in the concentration gradient of substance, and thus moves in a directed fashion. At still higher concentrations, the gradient is no longer distinguishable and the cell does not move toward the chemoattractant. Directionality of migration (chemotaxis) was established by analyzing the response of PMC when the same concentrations of the test substances were placed both in the upper and lower wells of the chemotactic chamber. In this condition, no migration above background was present (data not shown). The PDGF dimers showed a different chemotactic potency. PDGF-BB was the most active, achieving cell migration of ~8.5-fold over control at 25 ng/ml. The PDGF-BB effect was about one third higher compared to PDGF-AB, and twice that of PDGF-AA. Since the chemotactic behavior of the PMC from 5-d-old animals was equivalent to that of the PMC from 15-d-old animals after 4 d of culture, we used this latter cell preparation in the subsequent experiments.

Polyclonal anti–PDGF-BB, AA, and AB antibodies at 10 μg/ml completely inhibited the PMC migration induced by the most active concentration gradient of the corresponding antigens, demonstrating the specificity of the effect (Fig. 9 *C*).

Migratory Activity of Sertoli Cells Conditioned Media on PMC and Effect of anti-PDGFs Antibodies and of FSH Treatment

Sertoli cells from prepubertal animals express mRNAs for the PDGF A-chain and PDGF B-chain and secrete PDGF-like material. Concomitantly, PMC express transcripts for the PDGFR α- and β-subunit and the mRNAs are translated in the corresponding proteins.

Since we observed a chemotactic response to all the PDGF isoforms by PMC, and the Sertoli cells in culture secrete PDGF-like substances, we examined whether PMC respond chemotactically to the Sertoli cells conditioned medium (Fig. 10). Chemotactic activity was detected in conditioned medium from cultured prepubertal Sertoli cells, but not in the medium conditioned by Sertoli cells from 15-d-old animals (Fig. 10 *A*). Conditioned medium from prepubertal Sertoli cells enhanced PMC migration in a concentration-gradient–dependent fashion (Fig. 10 *A*). The PDGF-like concentration, measured by RRA, in the undiluted conditioned medium used for these experiments, was 27 ng/ml. The highest activity was obtained at a dose of 100% and was around 72% of that observed with 25 ng/ml of standard PDGF-BB. This lower activity, if compared with a similar amount of standard PDGF-BB, could be due to the fact that the PDGF-like content in the Sertoli cells supernatant is a sum of the three PDGF isoforms that have differing chemotactic potencies. The pattern of the chemotactic activity of the medium following dilution was similar to that described for the synthetic substances (Fig. 10 *A*).

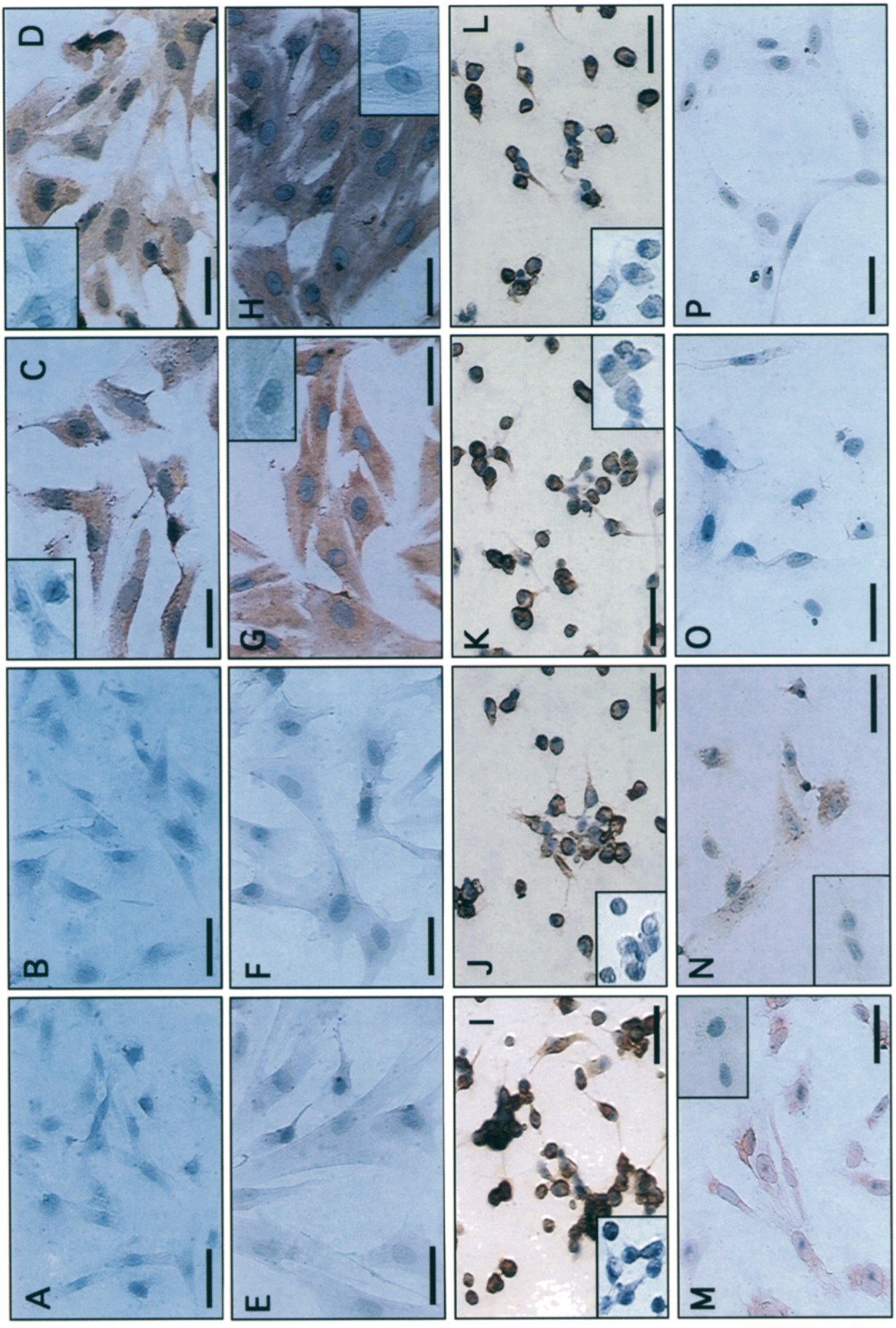
Using anti-PDGF antibodies, we evaluated whether the efficacious migratory activity of the medium conditioned by the prepubertal Sertoli cells was due to PDGF. Anti–PDGF-AA, anti–PDGF-BB, and anti–PDGF-AB antibodies inhibited the chemotactic effect of the conditioned medium by 66, 83, and 78%, respectively, suggesting that this potent activity was mainly due to PDGF. In accordance with the inhibitory effect of FSH on the immunoreactive PDGF production by the Sertoli cells, no chemotactic response was observed to the prepubertal Sertoli cells supernatant after 24 h of treatment with FSH (Fig. 10 *B*). These data indicate that Sertoli cells from 5-d-old animals secrete an efficacious migration factor for PMC, probably PDGF in nature, whose production can be modulated by FSH, and thus that there may be a paracrine mechanism for inducing PMC migration.

Discussion

Testicular development is an highly coordinated process which requires a precise temporal regulation of growth and differentiation of somatic and germ cell elements and imply a number of cell–cell interactions accomplished by locally produced growth and differentiation factors.

This report demonstrates that PDGF and PDGFR expression are temporally controlled in the testis and that the pattern of the PDGF system expression could be required for prenatal and postnatal organ development and maintenance of homeostasis. Transcripts from the genes expressing PDGF A-chain, PDGF B-chain, and PDGFR α- and β-subunit are first detected in RNA extracted from testis 2 d before birth, rise to high levels at postnatal day 1, remain high through the first 5 postnatal days and decline to much lower levels in 15-d-old and 60-d-old animals to

Figure 5. Immunohistochemical localization of PDGF-AA, PDGF-BB, PDGFR α-subunit, and PDGFR β-subunit in 60-d-old rat testis. The Leydig cells located in the intertubular space show strong immunoreactivity for PDGF-AA (*A*), PDGF-BB (*C*), PDGFR α-subunit (*E*), and PDGFR β-subunit (*G*), while the seminiferous tubules are negative. Note the absence of positive staining in the blood vessel in *E*. Immunoreaction is absent when the anti–PDGF-AA, anti–PDGF-BB, anti–PDGFR α-subunit, and anti–PDGFR β-subunit antibodies are preabsorbed with the corresponding immunizing peptides before analysis (*B*, *D*, *F*, and *H*). For methodological details see Materials and Methods. *st*, seminiferous tubule; *i*, intertubular space; *v*, blood vessel. Bars, 50 μm.



nearly disappear in older animals, suggesting that the expression of the PDGFs and PDGFRs genes is under developmental control in this tissue. The age-related difference in PDGFR α-subunit gene expression is consistent with previous observations by Loveland et al. (1993) which reported the highest levels of a transcript encoding the PDGFR α-subunit in RNA prepared from testes of 5-d-old animals followed by a decline in relative abundance with increasing age. These findings have been extended by our results, in that the expression of the PDGFR α-subunit is also detected prenatally and at postnatal day 1.

When the localized expression of the PDGFRs subunits and their ligands are compared, total RNA prepared from purified prepubertal, and early pubertal Sertoli cells and PMC as well as from prepubertal and adult Leydig cells revealed that in adult animals the expression of the PDGF A- and B-chain, and of PDGFR α- and β-subunit mRNAs is confined in Leydig cells, whereas before puberty PDGFR α- and β-subunit transcripts are visible in PMC and PDGF A- and B-chain transcripts are evident in Sertoli cells. The prominent expression of PDGFR α-subunit mRNA in the Leydig cells of the adult testis is consistent with the results of a previous study (Loveland et al., 1993). In the same study, Loveland and colleagues (1993), following Northern blot analysis of PDGFR α-subunit mRNA in total RNA prepared from isolated day 20 Sertoli cells, reported a faint signal. We were not able to reproduce this finding, however, despite this reported apparent mRNA expression, significant expression of functional PDGFRs by Sertoli cells is unlikely based on the lack of binding of [^{125}I]PDGF-BB on purified 5-d-old and 15-d-old Sertoli cells (Gnessi, L., unpublished results) and on the subsequent immunohistochemical data.

The immunohistochemistry confirms the Northern blot findings and demonstrates that in the prenatal, early postnatal, and prepubertal testis the PDGFs and the PDGFRs are distributed in the Sertoli cells and peritubular cells, respectively, while in the adult PDGFs and PDGFRs positive immunoreactivity is limited to the Leydig cells. Thus, the mRNAs and corresponding proteins for PDGFs and PDGFRs are localized in separate but adjacent cell types during prenatal and early postnatal testicular development and are confined in the Leydig cells in the adult. Accordingly, recent studies, using in situ hybridization, have described the location of the PDGFR β-subunit and α-subunit mRNA in the mesenchyme of the gonads of 12.5- and 14.5-d mouse embryos, respectively (Shinbrot et al., 1994; Orr-Urtreger et al., 1992*b*). Additional in situ hybridization studies, conducted in older mouse embryos, have revealed the PDGFR β-subunit mRNA expression clearly localized in the mesenchyme cells of the testis between the testicular cords (Shinbrot, E., personal communication).

The immunolocalization in the intact tissues has been further reinforced by the experiments performed on purified cells. Primary Sertoli cell cultures from 5-d-old animals showed a positive immunostaining for PDGF-AA and PDGF-BB but not for PDGFR α- and β-subunit, while in cells from 15-d-old animals no staining for any of the antisera used was observed. Adult Leydig cells were positive for all the PDGF and PDGFR subunits whereas early pubertal cells were negative. The PMC from 5-d-old animals were intensively positive for PDGFR α- and β-subunit but not for PDGF-AA and PDGF-BB. Previous studies have indicated that PMC from 15-d-old animals express PDGFRs upon cell culturing (Gnessi et al., 1993). The results obtained here confirm this finding revealing a strong immunoreactivity for PDGFR α- and β-subunit in PMC from 15-d-old rats after 96 h of culture but not in freshly isolated cells.

To correlate PDGF gene expression, immunohistochemical localization and protein production, the ability of cultured Sertoli cells to secrete PDGF was evaluated. The RRA of the secreted proteins indicates that Sertoli cells from 5-d-old but not from 15-d-old testicular tissue are able to produce PDGF-like substances. Although our assay system cannot distinguish between the various PDGF isoforms, the evidences obtained with immunohistochemistry and Northern analysis suggest that prepubertal Sertoli cells can produce all three the PDGF isoforms.

Interestingly, in vitro FSH treatment was found to significantly decrease the PDGF-like substance secretion by Sertoli cells from 5-d-old animals, suggesting that the PDGF expression is hormone dependent. This result has been further reinforced by the immunohistochemical experiments on the organ cultures in which, the treatment with FSH produced a marked reduction of the intratubular staining for PDGF-AA and PDGF-BB. Do these experimental manipulations parallel what happens in vivo? Previous studies suggested that PDGF is expressed primarily by mesenchymal cell types (Ross et al., 1986). The Sertoli cell is believed to be derived from undifferentiated mesenchymal cells in the gonadal ridge during fetal gonad development (Pelliniemi et al., 1993). Therefore, the Sertoli cell displays mesenchymal characteristics during its early development. The decrease in PDGF expression may be correlated to the mesenchymal to epithelial transformation related with Sertoli cell differentiation which is driven by FSH. Moreover, the response of the Sertoli cells to FSH is entirely dependent on the age of the animal. In the rat, the FSH receptor can be measured from 17.5 d of gestation,

Figure 6. Immunocytochemical localization of PDGF-AA, PDGF-BB, PDGFR α subunit, and PDGFR β-subunit in PMC, Leydig cells and Sertoli cells. PMC isolated from 5-d-old testis, after 24 h of culture, are negative for PDGF-AA (*A*) and PDGF-BB (*B*), whereas the cells show a positive staining for PDGFR α- (*C*) and β-subunit (*D*). PMC from 15-d-old animals, after 96 h of culture, are negative for PDGF-AA (*E*), and PDGF-BB (*F*), while a strong immunoreactivity can be observed for PDGFR α- (*G*) and β-subunit (*H*). Leydig cells from adult animals are positively stained with anti–PDGF-AA (*I*), anti–PDGF-BB (*J*), anti–PDGFR α-subunit (*K*), and anti–PDGFR β-subunit (*L*) antibodies. Sertoli cells isolate from 5-d-old animals, show positive immunoreactivity for PDGF-AA (*M*), and PDGF-BB (*N*), whereas the cells are negative for PDGFR α-subunit (*O*) and PDGFR β-subunit (*P*). The cells were cultured and processed for histochemistry as outlined in Materials and Methods. The inserts in the panels of the figure show the negative controls which consisted in staining of the cells with the respective antibodies preincubated for 12 h at 4°C with the corresponding immunizing peptides. All the controls are negative. Bars, 50 μm.

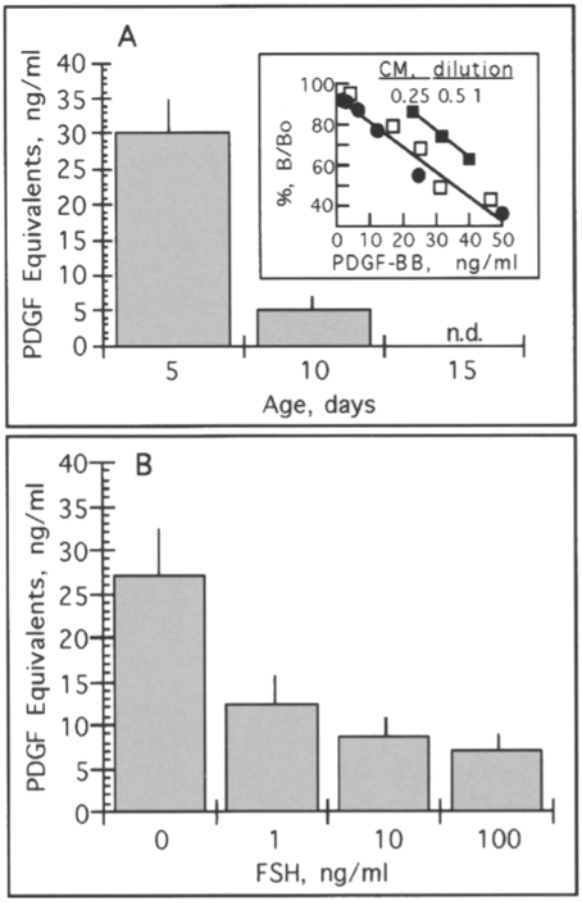


Figure 7. Production of PDGF receptor-competing activity by Sertoli cells isolated from testes at different stages of development and effect of the treatment with FSH. (*A*) Medium conditioned by purified Sertoli cells from 5-, 10-, and 15-d-old rat testes, collected after 24 h of culture, was assayed for PDGF competitor activity in the RRA (ND = not detectable). (*B*) Assay of PDGF competitor activity in medium conditioned by Sertoli cells from 5-d-old animals after 24 h of treatment with various concentrations of FSH. Results are expressed as equivalents of PDGF (ng/ml) adjusted for the protein content of the cells in the respective cultures. Each bar represents the mean ± SEM of triplicate experiments. In *A*, the PDGF equivalents production by 10-d-old cells was significantly lower compared to 5-d-old cells ($P < 0.01$). In *B*, FSH treatment significantly decreased the PDGF equivalents production vs control ($P < 0.05$ at 1 ng/ml and $P < 0.01$ at concentrations of 10 ng/ml and 100 ng/ml). The insert of *A* shows the standard curve of the PDGF competition assay (●), serial dilutions of conditioned medium from cultures of Sertoli cells from 5-d-old animals (■), and internal standard curve constructed with PDGF-BB in the presence of conditioned medium from Sertoli cells (□). For the internal standard curve, PDGF-BB (1.5–50 ng/ml) was added to the medium conditioned by the Sertoli cells after 24 h of culture (*CM*, conditioned medium). For methodological details, see Materials and Methods. B/Bo, bound to free ratio. Each point represents the mean of two determinations with the SEM less than 5%.

remains very low through 19.5 d of fetal life, and its concentration begins to rise sharply from days 2–10 after birth (Warren et al., 1984). FSH secretion starts around day 21 of fetal life (Huhtaniemi, 1994), hence the testis should be responsive to FSH as soon as it reaches the circulation. However, the fetal testis does not respond to FSH stimulation with clearly increased cAMP production (Picon and Gangnerau, 1980; Eskola et al., 1993). The situation changes after birth, when in the rat there is a dramatic increase in FSH stimulated cAMP production that begins after birth with a maximal stimulation evident at 9–15 d (Heindle et al., 1977; van Sickle et al., 1981). Therefore, both FSH-induced Sertoli cell differentiation, and the temporal pattern of Sertoli cell responsiveness to FSH in terms of cAMP production may result in a suppression of PDGF secretion and correlates with the developmental data shown in Figs. 1 and 2. It is worth noting that a similar effect of FSH on the expression of TGFβ2 by prepubertal Sertoli cells has been reported (Mullaney and Skinner, 1993).

Sertoli cells start to synthesize PDGF-A and PDGF-B at least as early as fetal day 20. Synthesis is present at very high levels between 2 d before birth and 5 d postnatal and the same is apparent for PDGFR α- and β-subunit in the cells localized in the interstitial tissue.

The high levels of expression of the PDGFRs by the peritubular cells corresponds with the timing of recruitment of this cell type from the undifferentiated intertubular stromal-fibroblast population (Bressler and Ross, 1972), their higher proliferation index (Palombi et al., 1992), and their ability to secrete extracellular matrix components to form in cooperation with the Sertoli cells the basal membrane of the tubule (Pelliniemi et al., 1984), while the PDGFRs expression decreases concomitantly with the acquisition of the tubule to contract spontaneously (Kormano and Hovatta, 1972; Worley et al., 1984). In other words, it seems that the peritubular cells cease to express PDGFRs as soon as they shift from a synthetic to a contractile phenotype which corresponds with both morphological and functional maturation of PMC. In this respect it appears likely that the PMC behavior may be homologous to what previously described for smooth muscle cells in other systems. For example vascular smooth muscle cells, dependent on age, may exhibit either a contractile or a synthetic phenotype (Ross, 1993). In the fetus and in the young evolving organism, vascular smooth muscle cells are in the synthetic state, in that they are capable of expressing genes for a number of growth regulatory molecules and cytokines, can respond to growth factors by expressing appropriate receptors, and can synthesize extracellular matrix. In contrast, in the adult, when cells are in contractile phenotype, they respond to agents that induce either constriction or relaxation. Accordingly, it has been reported that vascular smooth muscle cells express PDGF receptors as they modulate from a contractile to a synthetic phenotype (Sjölund et al., 1988). The intimate mechanism of regulation of PDGFRs expression on PMC in vivo is not known. Testicular peritubular cells contain androgen receptors (Verhoeven, 1979) and require androgens for their maturation (Bressler and Ross, 1972), thus, it is an interesting possibility, which remains to be investigated, that the PDGFRs levels of the PMC, as suggested for smooth muscle cells from porcine uterus (Terracio et al., 1988), are steroid hormone dependent.

The importance of Sertoli cell–myoid cell communications in seminiferous tubule formation has been emphasized (Dym, 1994). Cell migration and proliferation are

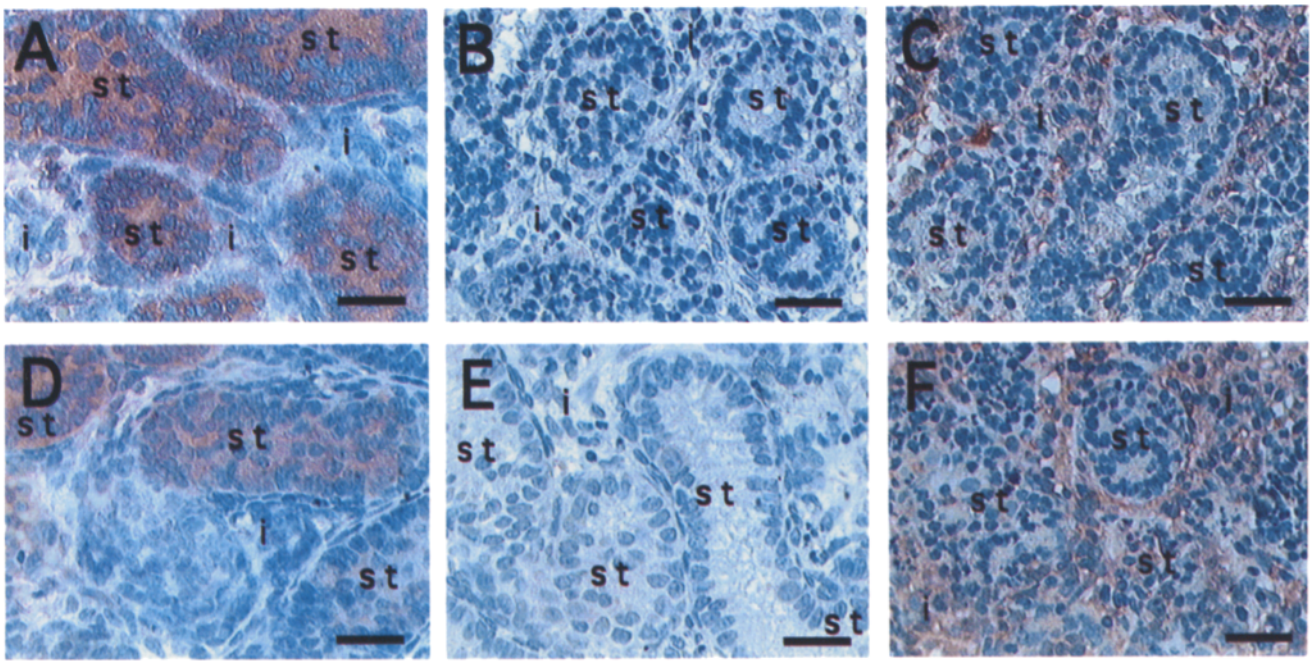


Figure 8. Immunohistochemical staining for PDGF-AA (*A*), PDGF-BB (*D*), and for PDGFR α- (*C*) and β-subunit (*F*) in 3-d-old testicular organ culture and effect of the FSH treatment on the PDGF-AA (*B*) and PDGF-BB (*E*) immunoreactivity. The tubules are intensely stained using both anti–PDGF-AA (*A*) and anti–PDGF-BB antibodies (*D*). The treatment of the organ cultures with 100 ng/ml FSH for 24 h, completely abolishes the staining for both PDGF-AA (*B*) and PDGF-BB (*E*). A positive immunostaining for PDGFR α-subunit (*C*) and PDGFR β-subunit (*F*) is evident in the intertubular space. Tissue sections were treated for immunohistochemistry as described in Materials and Methods. The negative controls obtained as described in Fig. 3 and Materials and Methods were negative (not shown). *st,* seminiferous tubule; *i*, intertubular space. Bars, 50 μm.

critical events in this process. Both environmental interactions, mediated primarily through the extracellular matrix, and local regulatory interactions, mediated by the numerous secretory products produced by both cell types, have been implicated in the morphogenetic cascade resulting in the formation of the seminiferous tubule. Examples of environmental and regulatory interactions responsible for the migration of the peritubular cells precursors toward the tubule have been described. The appearance of laminin on the surface of the Sertoli cells has been shown to be required to permit the peritubular cells to adhere and subsequently to migrate on Sertoli cell surfaces (Tung and Fritz, 1994), and it has been reported that TGFβ may potentially act as a chemotactic agent for the peritubular cell (Skinner and Moses, 1989). Additional experimental data suggest that in vivo the Sertoli cells produce a factor that attracts the myoid cells. Seminiferous cords formed on a Matrigel substrate when returned to the testis attract, within 24 h, the mesenchymal cells which appear to surround the cords in a manner similar to the PMC relationship to the seminiferous tubules (Dym, 1994). PDGF is a potent chemotactic agent for a number of cell types (Deuel et al., 1982; Seppä et al., 1982; Hosanng et al., 1989; Barnes and Hevey, 1990; Siegbahn et al., 1990) and is the strongest chemoattractant for arterial smooth muscle cells described to date (Grotendorst et al., 1981, 1982; Koyama et al., 1992; Bornfeldt et al., 1994). The findings presented here indicate that PMC show migratory responses to PDGF. The chemotactic activity of PDGF is evident in freshly isolated PMC from 5-d-old animals and in PMC purified from 15-d-old animals after 4 d in culture, that is more than the time required for cells of this age to express functional PDGFRs in culture. The chemotactic response of PMC to PDGF occurs rapidly, with significant migration observed within 4 h. By comparison, PMC division does not begin until 48 h after the exposure to PDGF (Gnessi et al., 1993). We found that all three the PDGF isoforms are chemoattractants for PMC although with different potencies, with PDGF-BB being the most active followed by PDGF-AB and PDGF-AA. The observed lower maximal effect of PDGF-AA, compared to PDGF-AB and PDGF-BB may result from the lower number of binding sites for PDGF-AA on these cells (Gnessi et al., 1993). Since during the early stages of testicular development the Sertoli cells are the primary source of PDGF-like molecules and PMC express PDGFRs, our findings support the view that Sertoli cells may direct the development of their neighboring precursors of the mature PMC population via PDGF. The mitoattractant property of PDGF suggests that Sertoli cell secretion of this growth factor may be ideally suited to chemotactically attract and mitogenically stimulate peritubular cells in proximity of the tubule and thus that Sertoli cell expression of PDGF may be important in testicular morphogenesis. Accordingly, we found that PMC show a migratory response to conditioned medium from early postnatal but not early pubertal Sertoli cells. PMC migration to the Sertoli cells conditioned medium was inhibited by anti-PDGFs antibodies, suggesting that the chemotactic activity may be due to PDGF. Furthermore, in line with the ability of FSH to reduce the se-

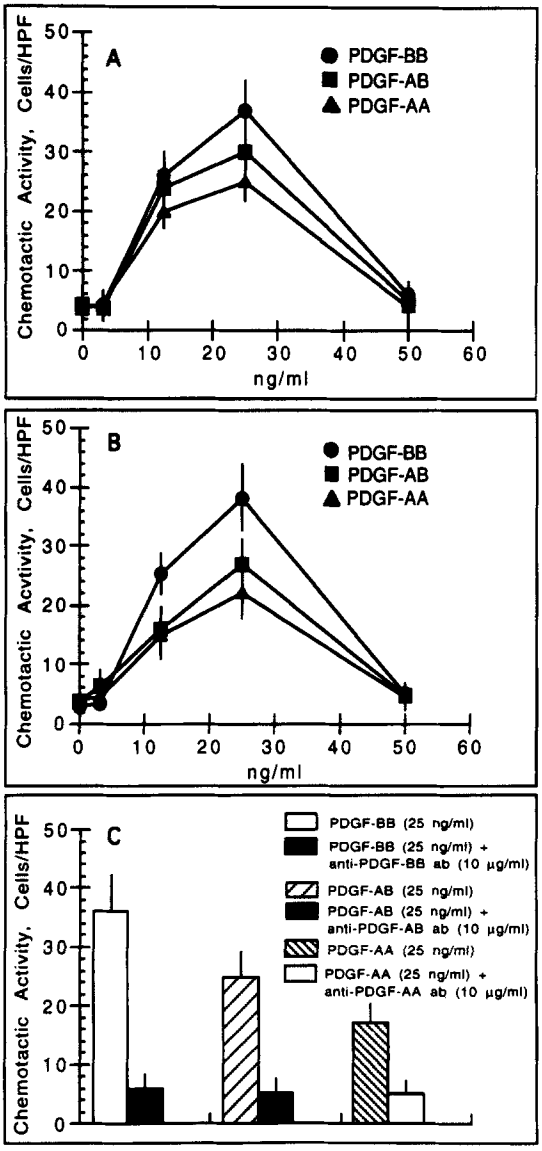


Figure 9. PMC migration to the three PDGF isoforms and inhibition by the corresponding antibodies. *A* shows the chemotactic behavior to various concentrations of PDGF-AA, PDGF-AB, and PDGF-BB, of PMC from 5-d-old animals immediately after isolation. *B* shows the chemotactic behavior of PMC obtained from 15-d-old animals trypsinized after 96 h of culture to different concentrations of the three PDGF isoforms. In *C*, PMC from 15-d-old animals were harvested after 96 h of culture and analyzed for chemotaxis toward PDGF-BB, PDGF-AB, and PDGF-AA at the concentrations indicated or to the same concentrations of the PDGF isoforms preincubated for 1 h at 37°C with the corresponding antibodies. Chemotactic activities were assayed in triplicate as described in Materials and Methods. Results are expressed as the means ± SEM for cell numbers observed per HPF.

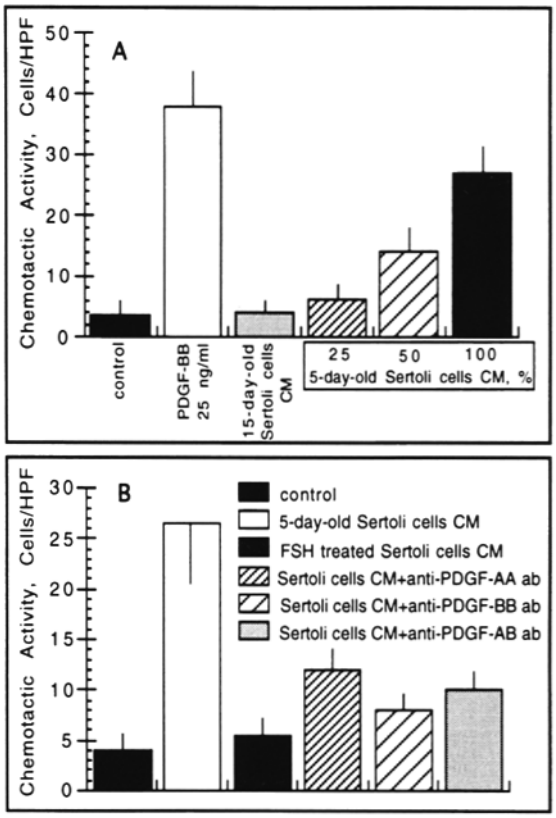


Figure 10. PMC chemotaxis toward Sertoli cells conditioned medium, Sertoli cells conditioned medium treated with anti-PDGFs antibodies, and medium conditioned by Sertoli cells after 24 h of treatment with 100 ng/ml of FSH. (*A*) PMC suspension was placed in the upper wells and medium alone (*negative control*), medium containing 25 ng/ml PDGF-BB (*positive control*), conditioned medium from Sertoli cells isolated from 15-d-old animals collected after 24 h of culture, and various concentrations of conditioned medium from Sertoli cells isolated from 5-d-old animals collected after 24 h of culture, were placed in the lower wells of a chemotactic chamber. *B* shows the effect of FSH treatment, and of anti–PDGF-AA, -BB, and -AB, antibodies on the chemotactic activity of conditioned medium from Sertoli cells isolated from 5-d-old animals (*CM*, conditioned medium). The antibodies were used at a concentration of 10 µg/ml. Chemotaxis was assayed in triplicate as described in Materials and Methods. Values are means ± SEM for cell numbers observed per HPF. In *A*, PDGF-BB vs control $P < 0.01$; 50% and 100% Sertoli cells conditioned medium vs control, $P < 0.05$ and $P < 0.01$, respectively. In *B*, the significance of the difference of PMC migration toward PDGF-BB vs control is $P < 0.01$. There is no statistical significance vs control in the number of cells migrated when the conditioned medium from 15-d-old Sertoli cells was tested. A P value < 0.01 for all the antibodies treated and the FSH-treated Sertoli cells conditioned medium vs the positive control is calculated.

cretion of PDGF-like substances by Sertoli cells in culture, the conditioned medium from the FSH-treated cells displays a significantly weakened chemotactic effect. These results suggest that Sertoli cells may secrete PDGF-like substances that attract the PDGF responsive intertubular mesenchymal cells which will form the mature PMC population. Interestingly, PMC increase the production of extracellular matrix components in response to PDGF (Gnessi et al., 1993). Again all these events require a strict spatiotemporal control of the PDGFs and PDGFRs expression and action, and emphasize the importance of the epithelial-mesenchymal interaction in the development of the testis. Topographically similar interactions characterize numerous classical developmental models (Gilbert, 1991), and have been described for both PDGF-A/PDGFR-α and PDGF-B/PDGFR-β (Orr-Urtreger and Lonai, 1992*a*;

Holmgren et al., 1991) during the development of various organs.

Our results suggest that the control of PDGF secretion by Sertoli cells may be at least in part due to FSH. Further possibilities emerged from studies on the Wilm's tumor suppressor gene (WT1), which has been identified as a candidate gene involved in urogenital development. WT1 encodes a zinc finger protein with four zinc fingers which acts as a transcriptional regulator (Call et al., 1990; Gessler et al., 1990). WT1 gene is expressed in only a very limited set of tissues in the developing mammalian fetus, but principally in the kidney and the gonads. The gene is expressed in the genital ridge of both males and females before the formation of an overt testis or ovary. As the gonads develop, its expression is restricted to the Sertoli cells of the testis and granulosa cells of the ovary (Pelletier et al., 1991). The WT1 protein has been shown to interact with the PDGF-A promoter region acting as a transcriptional activator as well as a repressor (Wang et al., 1992; Wang et al., 1993). This difference occurs by RNA editing that is developmentally regulated in rat testis (Sharma et al., 1994). WT1 mRNA levels in the testis increase steadily after birth, reaching their highest expression during the first week after birth and decreasing slightly as the animal matures (Pelletier et al., 1991). The ability of WT1 to modulate the PDGF A-chain gene expression and the regulation of WT1 expression during development of the gonads coupled with our findings on the pattern of testicular expression of PDGF A-chain, suggest that WT1 may be involved in the control of PDGF production in the testis. Future studies to determine the exact role of WT1 in the control of the expression of PDGF will help to define the role of this gene during testicular development.

Maintenance of homeostasis and control of local environment are critical factors involved in the prevention of disease. It has been reported that aberrant expression of PDGF or PDGFR is likely to be involved in the stimulation of growth of certain tumors (Heldin, 1992). Furthermore, overactivity of PDGF could also be part of the development of certain nonmalignant disorders involving an excess of cell proliferation (Martinet et al., 1987; Antoniades et al., 1990; Ross, 1993; Sano et al., 1993; Qu et al., 1994). The mitotic clock, while definitive under physiological conditions, is corruptible by alterations in the environment. For example, continued exposure of oligodendrocyte type 2 astrocyte precursors to a mixture of PDGF and FGF results in extensive proliferation in the absence of differentiation (Bogler et al., 1990). Growth factors can influence the timing with which differentiation takes place, and thus determine the size of a given mature cell population. It is interesting to note that hypercellularity, matrix expansion, and tubular sclerosis are often observed in testicular biopsies of infertile males (de Kretser et al., 1975; Salomon and Hedinger, 1982; Pöllanen et al., 1985).

PDGF has been proposed to be one of the growth factors that drive proliferation during normal development and in various pathological conditions. Different experimental approaches have been taken to confirm the hypothesized roles of PDGF in vivo. It has been reported that Patch (Ph) mice, which show dominant pigmentation defects and recessive embryonic lethality (Grüneberg and Truslove, 1960) with defects in a number of mesenchymal lineages and in neural crest derivatives (Morrison-Graham et al., 1992; Orr-Urtreger et al., 1992*b*; Schatteman et al., 1992), carry a deletion of the PDGF α-receptor (Smith et al., 1991; Stephenson et al., 1991). However, Ph represent a large deletion, and thus it is uncertain whether the phenotype is attributable to the absence of the α receptor alone or of several genes in addition to the α receptor; besides, since the Ph/Ph embryos die well before birth, they do not shed any light on the role of the PDGF/PDGFR system in late development and after birth.

More recently, PDGF-B (Levéen et al., 1994) and PDGF β-receptor (Soriano, 1994) deficient mice have been generated. In both studies the animals show kidney glomerular defects, hemorrages, anemia, and thrombocytopenia. Some of the more severe defects observed in the PDGF-B mutants, such as hypertrabeculation of the heart and dilation of the arteries, do not appear in the β-receptor mutant, probably as a consequence of the partial overlapping functions of the PDGFs and PDGFRs forms.

There is no mention of testicular problems in the PDGF-B and PDGF β-receptor mutant mice, but no attention has been paid to the reproductive systems of these animals (Betsholtz, C., personal communication). The most conspicuous phenotype of PDGF-B and PDGFR-β mutants was the absence, or significant paucity of mesangial cells of the kidney glomerulus. Interestingly, there are a number of similarities between mesangial cells and PMC. Both originate from the mesenchime and exhibit muscle-like properties. Mesangial cells and PMC respond mitogenically to PDGF and the responses to the different PDGF isoforms vary, with PDGF-BB and PDGF-AB being more mitogenic than PDGF-AA (Floege et al., 1991; Gnessi et al., 1993). Besides being mitogenic, PDGF also stimulates several other functions of mesangial cells and PMC, including directed migration (Barnes and Hevey, 1990), the production of extracellular matrix components (Doi et al., 1992; Gnessi et al., 1993), and contraction (Mene et al., 1987; Tung and Fritz, 1991). Thus, taking into account the evidences presented here, a closer look to the reproductive system of the mutant animals coupled with a comparison of the individual knockout phenotypes for PDGF-B, PDGF-A, PDGFR-α, and PDGFR-β and combinations thereof could help elucidate the roles of PDGF and its receptors during gonadal development in vivo. Studies examining the testicular organogenesis in PDGF mutant mice are currently in progress.

In conclusion, based on our results and on other recent findings and as a framework for future studies, a tentative and simplified model of the developmental regulation that could be exerted by the PDGF system in the rat testis is presented. This model includes the following main steps: (*a*) in the prenatal and early postnatal period the Sertoli cells produce PDGF-like molecules. These molecules bind to and activate PDGFRs which are expressed during the same time period by the neighboring intertubular precursors of the PMC. (*b*) In response to the PDGF-like molecules action the intertubular cells precursors of the mature PMC may be chemotactically attracted to the peritubulum and induced to divide. (*c*) Concomitantly to the acquisition of the Sertoli cells to respond to the FSH with a clearcut cAMP increase, the PDGF secretion by Sertoli cells drops, while the PMC as part of their differentiation program

modulate from a synthetic to a contractile phenotype losing the ability to express PDGFRs on their surface. (*d*) In adult life, Leydig cells start to produce PDGF. PDGF binds to and activates the PDGFRs on the Leydig cell itself modulating in an autocrine way the testosterone production.

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