

# Leydig Cell Loss and Spermatogenic Arrest in Platelet-derived Growth Factor (PDGF)-A-deficient Mice

Lucio Gnessi,\* Sabrina Basciani,\* Stefania Mariani,\* Mario Arizzi,\* Giovanni Spera,\* Chiayeng Wang,<sup>‡</sup> Cecilia Bondjers,<sup>§</sup> Linda Karlsson,<sup>§</sup> and Christer Betsholtz<sup>§</sup>

\*Department of Medical Pathophysiology, University of Rome "La Sapienza", 00161 Roma, Italy; <sup>‡</sup>Center for Molecular Biology of Oral Diseases, University of Illinois at Chicago, Chicago, Illinois 60612-7213; and <sup>§</sup>Department of Medical Biochemistry, University of Göteborg, SE 405 30 Göteborg, Sweden

**Abstract.** Platelet-derived growth factor (PDGF)-A-deficient male mice were found to develop progressive reduction of testicular size, Leydig cells loss, and spermatogenic arrest. In normal mice, the PDGF-A and PDGF-R $\alpha$  expression pattern showed positive cells in the seminiferous epithelium and in interstitial mesenchymal cells, respectively. The testicular defects seen in PDGF-A<sup>-/-</sup> mice, combined with the normal developmental expression of PDGF-A and PDGF-R $\alpha$ , indicate that through an epithelial-mesenchymal signal-

ing, the PDGF-A gene is essential for the development of the Leydig cell lineage. These findings suggest that PDGF-A may play a role in the cascade of genes involved in male gonad differentiation. The Leydig cell loss and the spermatogenic impairment in the mutant mice are reminiscent of cases of testicular failure in man.

**Key words:** PDGF-A • gene targeting • Leydig cell • spermatogenesis • testis

## Introduction

The genes involved in the determination of the gonads comprise the Wilms' tumor suppressor gene WT1, the orphan nuclear receptor SF-1 and the high mobility group family member SOX9 (Parker et al., 1999). After determination, the differentiation of the testis depends on the testis-determining factor SRY (Goodfellow and Lovell-Badge, 1993). SRY induces the Sertoli cells to differentiate and to secrete the Mullerian inhibiting substance, which suppresses the development of the female reproductive tract (Gustafson and Donahoe, 1994). Later, under the influence of Sertoli cells, the Leydig cells develop (Ge et al., 1996). The ontogenesis of Leydig cells involves two distinct cell generations (McLaren, 1998). The fetal Leydig cells originate prenatally and produce androgens required for the masculinization during fetal and neonatal life. They regress thereafter and are substituted during puberty by the adult Leydig cells, which supply the testosterone necessary for completion of spermatogenesis and maintenance of male reproductive function. Adult Leydig cells do not derive from preexisting fetal cells but from undifferentiated mesenchymal stem cells (McLaren, 1998). The factors that reg-

ulate the commitment and early differentiation of stem cells to adult Leydig cell lineage are unknown.

PDGF-A, a high-affinity ligand for the receptor tyrosine kinase PDGF-R $\alpha$ , is required for embryonic and postnatal development (Betsholtz and Raines, 1997). PDGF-A-deficient animals develop lung emphysema secondary to the failure of alveolar septation (Boström et al., 1996), defective oligodendrogenesis (Calver et al., 1998; Fruttiger et al., 1999), and skin and hair defects (Karlsson et al., 1999). Previous studies have revealed that PDGF-A may play a role in testicular development (Gnessi et al., 1995) and that Leydig cells express PDGF-R $\alpha$  (Gnessi et al., 1992; Loveland et al., 1995). Here we report the testicular phenotype of PDGF-A-deficient mouse generated by targeted gene disruption. From this study, we conclude that PDGF-A is an essential factor for adult Leydig cells development.

## Materials and Methods

### Animals

PDGF-A null mutant mice, heterozygous, and wild-type (WT)<sup>1</sup> litter-

Address correspondence to Lucio Gnessi, Dipartimento di Fisiopatologia Medica, Policlinico Umberto I, Università di Roma "La Sapienza", 00161 Roma, Italy. Tel.: 39 06 49970509. Fax: 39 06 4461450. E-mail: l.gnessi@caspur.it

<sup>1</sup>Abbreviations used in this paper: BrdU, bromodeoxyuridine; LH, luteinizing hormone; PAS, periodic acid-Schiff; PMC, peritubular myoid cells; RLF, relaxin-like factor; TUNEL, TdT-mediated dUTP biotin nick end labeling; WT, wild-type.

mates were obtained from heterozygous crosses all bred as 129Ola/C57 B16 hybrids (Boström et al., 1996). Transgenic mice carrying the reporter gene  $\beta$ -galactosidase (*lacZ*) under control of a 6-kb regulatory domain of the murine PDGF-R $\alpha$  promoter were generated as previously described (Reinertsen et al., 1997).

### In Situ Hybridization

Nonradioactive in situ hybridization using PDGF-A and PDGF-R $\alpha$  probes was performed as described (Boström et al., 1996).

### Histology, Immunohistochemistry, and *lacZ* Staining

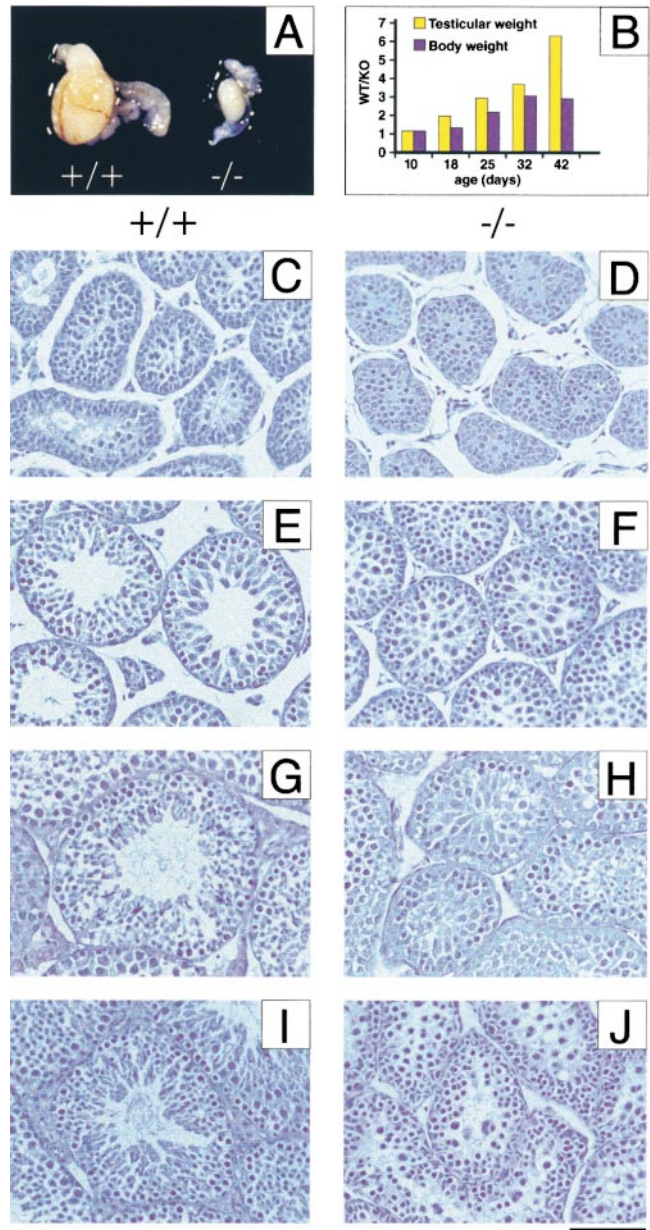
Testes to be stained by periodic acid-Schiff (PAS)/haematoxylin, bromodeoxyuridine (BrdU), TdT-mediated dUTP biotin nick end labeling (TUNEL), PDGF-A, PDGF-R $\alpha$ , relaxin-like actor (RLF), and GATA-1 immunohistochemistry were fixed in 4% buffered paraformaldehyde and paraffin embedded. The reactions were carried out on 5- $\mu$ m sections. For routine histology, sections were stained by PAS/haematoxylin according to standard protocols. For BrdU labeling, BrdU (Sigma-Aldrich) was injected intraperitoneally (100 mg/g body mass). Injected animals were killed 2 h later and the fixed testes were processed as described (Karlsson et al., 1999). Apoptotic cells were detected by TUNEL using the ApopTag Plus in situ detection kit (Oncor) according to the instructions of the manufacturer. Sertoli cells were detected using a GATA-1-specific antibody (Santa Cruz Biotechnology); Leydig cells were detected using a rat anti-RLF antibody (generously provided by Professor Richard Ivell, Institute of Hormone and Fertility Research, Hamburg, Germany); PDGF-A and PDGF-R $\alpha$ -positive cells were detected using rabbit anti-PDGF-A (RDI Inc.) and rat anti-PDGF-R $\alpha$  (PharMingen) antibodies. The antibodies (anti-GATA1, anti-PDGF-A, and anti-PDGF-R $\alpha$  at 1:100 dilution; anti-RLF at 1:1,000 dilution) were incubated overnight at 4°C. The avidin-biotin immunoperoxidase system with 3-amino-9-ethylcarbazole as chromogen was used to visualize bound antibodies (Histostain-Plus kit; Zymed Laboratories). For *lacZ* staining, transgenic animals were fixed and stained with X-gal for *lacZ* activity, and 10- $\mu$ m cryostat testicular sections were treated as described (Reinertsen et al., 1997).

### Hormone Assays

Serum testosterone and luteinizing hormone (LH) levels were determined by radioimmunoassays using DiaSorin and Amersham assay systems according to the manufacturers' instructions.

## Results and Discussion

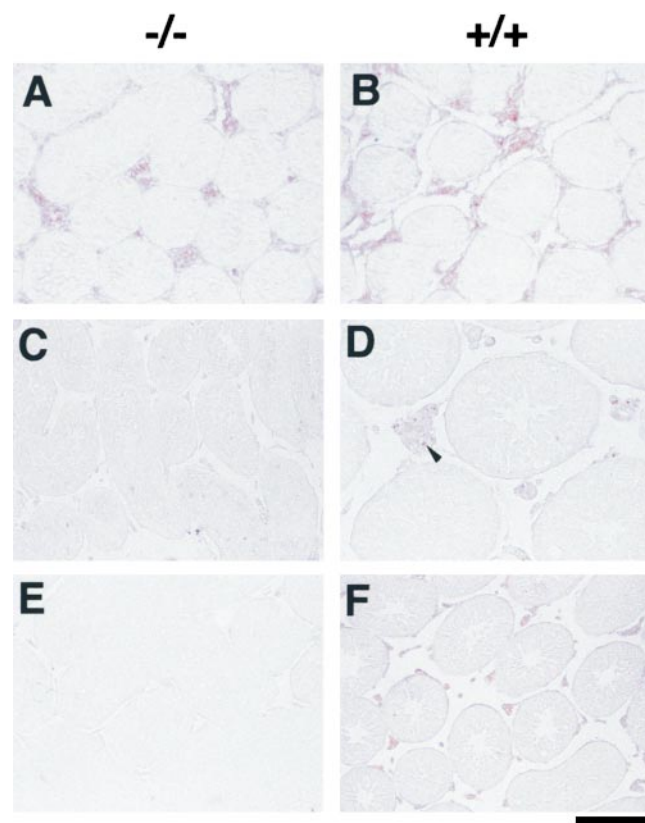
Newborn PDGF-A $^{-/-}$  mice are outwardly normal but fail to thrive after birth. Most die within a few days, but some individuals survive for as long as 6 wk, eventually dying of pulmonary failure. Due to the lethal phenotype of the homozygous mutants, our analysis was restricted to male mice recovered between postnatal day 10 (P10) and P42, killed before the first signs of respiratory failure were apparent. The heterozygous mice were phenotypically indistinguishable from WT. Surviving null mice weighed less than WT littermates and their growth curve was slower. PDGF-A $^{-/-}$  males had no defects in the external genitalia and testicular descent appeared normal. The testes were reduced in size (Fig. 1 A), however, the testicular size reduction in the mutants was more pronounced than the average reduction of the body size, and this feature increased dramatically with age (Fig. 1 B). Testis histology revealed profound differences between PDGF-A null mice and WT (Fig. 1, C–J). At P10,  $^{-/-}$  animals showed absence of tubular lumen whose formation was starting in the controls; no significant difference was observed in tubular diameter and germ cells number. Sertoli cells, interstitial cells, peritubular myoid cells (PMC) and extracellular matrix layers appeared intact as well (Fig. 1, C and D).



**Figure 1.** Morphology and histology of the testis of PDGF-A-deficient and control mice. (A) Gross analysis of the testis and epididymis of 32-d-old PDGF-A $^{-/-}$  (right) and control littermate (left) mice. (B) WT/PDGF-A-knockout (KO) testicular weights and WT/KO body weight ratios at different ages. The numbers (n) of mice used were: 10 d, WT (2), KO (1); 18 d, WT (4), KO (4); 25 d, WT (4), KO (2); 32 d, WT (2), KO (1); 42 days, WT (2), KO (1). PAS/haematoxylin stained sections of WT testis (C, E, G, and I) compared with mutant testis (D, F, H, and J) at P10 (C and D), P18 (E and F), P32 (G and H), and P42 (I and J) photographed at the same magnification. Starting from P18 the tubular diameter of  $^{-/-}$  animals is greatly reduced. Note the progressive loss of interstitial cells and the spermatogenic arrest in the mutants. Spermatids are completely absent from the seminiferous epithelium of P32 and P42  $^{-/-}$  gonads (H and J). At P42 the PDGF-A null testis shows a complete lack of Leydig cells and tubular hypotrophy; the tubules contain multinucleated giant cells. Bar, 50  $\mu$ m.

At P18, the mutant testes had reduced seminiferous tubule diameter and poor lumen formation but, likewise control littermates, spermatogenesis had proceeded up to pachytene spermatocytes; Sertoli cells and PMC were normal. However, the null mice showed an initial reduction in number of morphologically recognizable Leydig cells, scattered in the interstitium among small undifferentiated spindle-shaped mesenchymal cells of fibroblastic appearance (Fig. 1, E and F). At P25, round spermatids were frequently found in the tubules of  $+/+$  animals and the interstitium was filled with Leydig cells; no spermatids and a further reduction of the interstitial cells population were observed in the mutants (data not shown). At P32 and P42 all stages of the spermatogenic cycle and abundant Leydig cells were seen in control testis (Fig. 1, G and I). In  $-/-$  P32 testis, the tubular diameter was greatly reduced and a spermatocytic arrest with further loss of Leydig cells was observed (Fig. 1 H). P42  $-/-$  testis displayed a dramatic decrease of spermatocytes, adluminal multinucleated giant cells, and an almost complete absence of Leydig cells (Fig. 1 J). Immunostaining of P10-P42  $-/-$  testicular sections with an antibody against the Sertoli cell-specific gene GATA-1 (Yomogida et al., 1994) confirmed the presence of Sertoli cells in the null testis, indicating that the lack of mature forms of germ cells in PDGF-A  $-/-$  mice did not result from a loss of the supporting cells (data not shown). We also performed an immunohistochemical analysis of RLF, which is a specific marker for the mature forms of fetal as well as adult Leydig cells (Balvers et al., 1998). Anti-RLF staining identified mature fetal Leydig cells in both mutant and WT P10 testis (Fig. 2, A and B). At P18, concomitantly with the involution of fetal Leydig cells and with the first appearance of the adult Leydig cells progenitors, no RLF was expressed in  $-/-$  testis while sporadic punctate staining was seen in the interstitium of  $+/+$  testis (Fig. 2, C and D). This trend increased at P25. Here there was no staining in the few interstitial cells of the KO testis, while the majority of the interstitial cells of the WT testis exhibited RLF-specific staining (Fig. 2, E and F). By day 32 and onward, a more homogeneous cytoplasmic distribution of RLF positivity within the interstitial cells of the WT animals was observed while, concomitantly with the progressive depletion of the interstitium, RLF staining in the KO testis was absent (data not shown). This RLF staining pattern indicates that the mutant prepubertal testis contains mature fetal Leydig cells and confirms the absence of the adult Leydig cell population in older  $-/-$  testis.

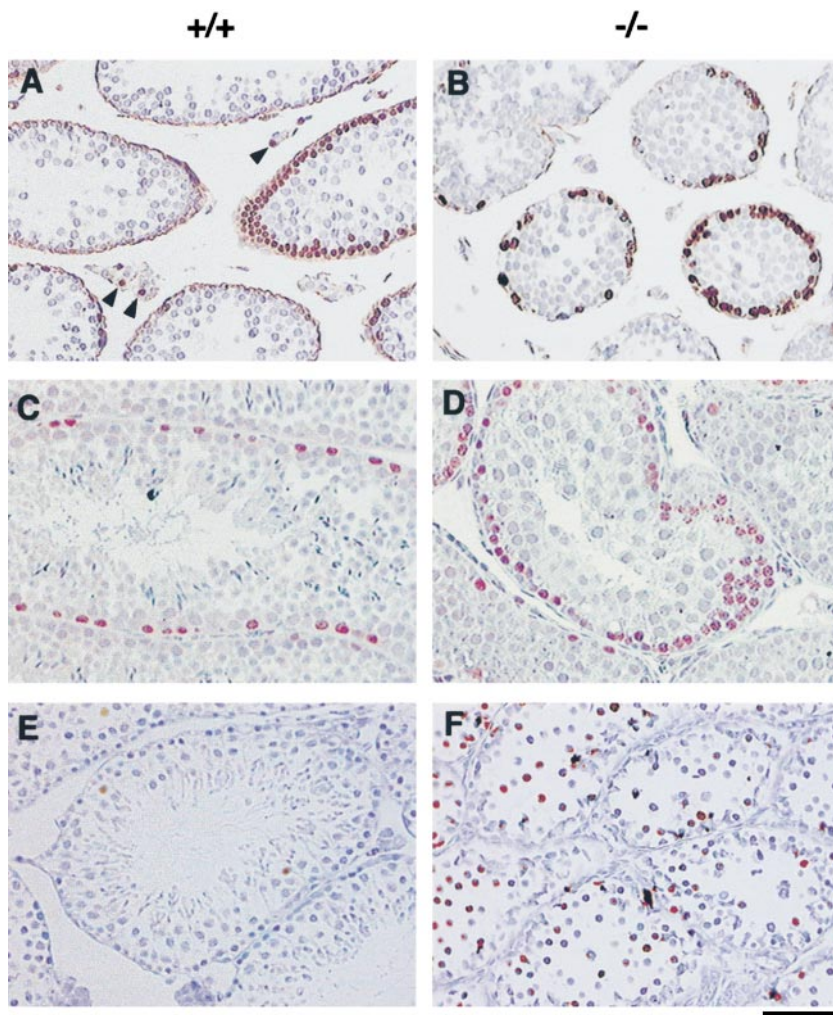
To analyze whether the reduced size of the PDGF-A mutant testis and its histologic appearance could be due to decreased cell proliferation and/or increased apoptosis, BrdU and TUNEL labeling were performed. At P18, P25, P32, and P42 there was no difference in BrdU incorporation of the actively proliferating germ cells between WT and mutant testes (Fig. 3, A-D; data not shown). During the third week of postnatal life, the stem cell precursors of the adult Leydig cell proliferate (Ge et al., 1996). Accordingly, BrdU staining was observed in some interstitial cells of P18 WT animals (Fig. 3 A); on the contrary, no BrdU labeling was seen in P18 PDGF-A  $-/-$  littermates (Fig. 3 B). Regarding TUNEL, few apoptotic cells were seen in the tubules of both WT and  $-/-$  siblings between P10 and



**Figure 2.** Immunohistochemical localization of RLF in the testes of PDGF-A mutant and WT mice. A comparable frequency of RLF immunostaining is observed in the interstitial cells of both WT and homozygous mutants at P10 (A and B). Clear punctate RLF-specific staining (arrowhead) is visible in the interstitium of WT testis on day 18 (D), and a more homogeneous RLF signal is seen at P25 (F). No staining can be observed in the interstitial compartment of P18 and P25 KO testes (C and E). Bar: (A, B, and D) 50  $\mu\text{m}$ ; (C, E, and F) 100  $\mu\text{m}$ .

P32 (data not shown). However, at P42 a large number of spermatocytes undergoing apoptosis was observed in null mice compared with control (Fig. 3, E and F). No evidence for apoptosis-mediated death of other testicular cell types was seen. Thus, an arrest of differentiation followed by increased apoptosis seem to be responsible for the seminiferous tubules aspect in the PDGF-A  $-/-$  testes. The lack of BrdU incorporation and the absence of TUNEL labeling in the interstitium of  $-/-$  testis, indicate that the paucity of Leydig cells might be due to a proliferative arrest. Thus, we speculate that in the PDGF-A  $-/-$  testis the normal pubertal replacement of the fetal Leydig cells with the adult population of cells is deranged because of a deficiency in the commitment of the adult Leydig cell precursors to proliferate and perhaps to differentiate.

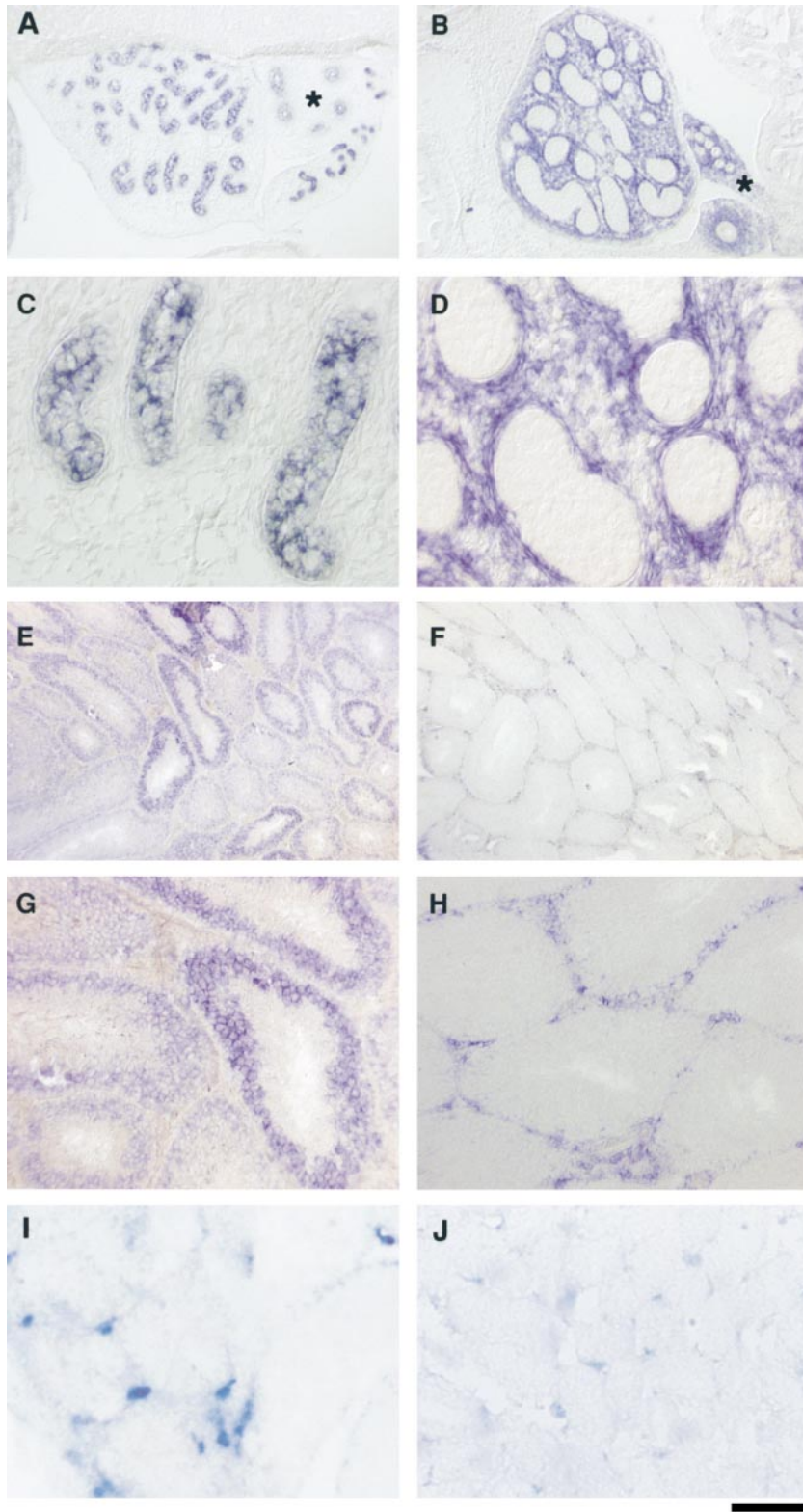
Such a model requires the knowledge of the localization of PDGF-A and its receptor in normal testis. In situ hybridization for PDGF-A and PDGF-R $\alpha$  in embryonic-day-17.5 (E17.5)-P30 WT testis showed the mRNAs expression in seminiferous epithelium and interstitial mesenchymal cells, respectively (Fig. 4, A-H). At E17.5 the PDGF-A and PDGF-R $\alpha$  labeling was rather homoge-



**Figure 3.** BrdU (A–D) and TUNEL (E and F) labeling in PDGF-A  $+/+$  and  $-/-$  testes. A comparable frequency of heavy BrdU labeling is noted in the proliferating spermatogonia in many seminiferous tubules of both WT and homozygous mutants at P18 (A and B) and P32 (C and D). Only in P18 WT testis, some interstitial cells (arrowheads) are labeled by BrdU (A). TUNEL staining in the seminiferous tubules of WT (E) and PDGF-A  $-/-$  testis (F) at P42. There is a significant increase of apoptotic cells (brown) in the tubules of mutant testis (F). TUNEL-labeled cells were counted in ten random tubule sections from the testes of one  $-/-$  and a control littermate mouse at P42. Results of this count are  $1.5 \pm 0.6$  TUNEL-positive cells per tubule for  $+/+$  mouse and  $11 \pm 2.1$  TUNEL-positive cells per tubule for  $-/-$  mouse. Bar: (A, B, E, and F) 50  $\mu\text{m}$ ; (C and D) 25  $\mu\text{m}$ .

neous. Later, some PDGF-A-positive tubules and distinct populations of PDGF-R $\alpha$ -positive interstitial cells could be seen. PDGF-R $\alpha$  expression was moreover localized using transgenic mice carrying the reporter gene  $\beta$ -galactosidase (*lacZ*) under control of the murine PDGF-R $\alpha$  promoter. According to the in situ data, *lacZ* staining was found in the interstitium (Fig. 4, I and J). The immunohistochemistry for PDGF-A confirmed the intratubular expression of the growth factor which, in agreement with previous results (Gnessi et al., 1992, 1995; Loveland et al., 1995), was mainly localized in the cytoplasm of the Sertoli cells (Fig. 5). At P18, although with different intensities, all the tubules were stained (Fig. 5 A). At P42, in line with the in situ findings, only the Sertoli cells in some tubules, corresponding to spermatogenic stages IX–X (Russell et al., 1990), were positive (Fig. 5 B). Altogether, these results indicate that the PDGF-A expression in Sertoli cells depends on the stage of maturation of the associated germ cells. At P42, a weak signal was also found in some interstitial cells (Fig. 5 B), which is consistent with the reported production of PDGF-A by adult Leydig cells in vitro (Gnessi et al., 1992, 1995; Loveland et al., 1995). Concerning PDGF-R $\alpha$ , in prepubertal animals the immunostaining was localized in the interstitial cells of both  $+/+$  and  $-/-$

testis, although in the latter the interstitial cells were less in number (Fig. 5, C and D). In older animals, the WT testis continued to show PDGF-R $\alpha$ -positive cells between the tubules (Fig. 5 E), while the null testis showed a steadily decreasing number of positive cells, culminating in their complete absence at P42 (Fig. 5 F). The comparison between the testicular defects found in  $-/-$  mice and the normal expression pattern of PDGF-A and its receptor, indicates that paracrine PDGF-A/PDGF-R $\alpha$  signaling constitutes a critical part of the epithelial-mesenchymal interaction, essential for adult Leydig cells development. PDGF-A does not influence the fetal generation of Leydig cells, as suggested by both the normal adrogenization and RLF-positive interstitial immunohistochemical staining of prepubertal  $-/-$  animals. Due to the lack of specific markers, we could not produce direct evidence that among the interstitial PDGF-R $\alpha$ -positive cells are indeed the precursors of the adult Leydig cells, but the spatiotemporal distribution of PDGF-R $\alpha$ -interstitial cells in normal mice seems to suggest this possibility. It is also reasonable to predict that a cell type specifically lost in PDGF-A  $-/-$  mice should carry the receptor for PDGF-A, and should locate close to the source of the ligand, as is the case for the PDGF-R $\alpha$ -positive interstitial mesenchymal cells

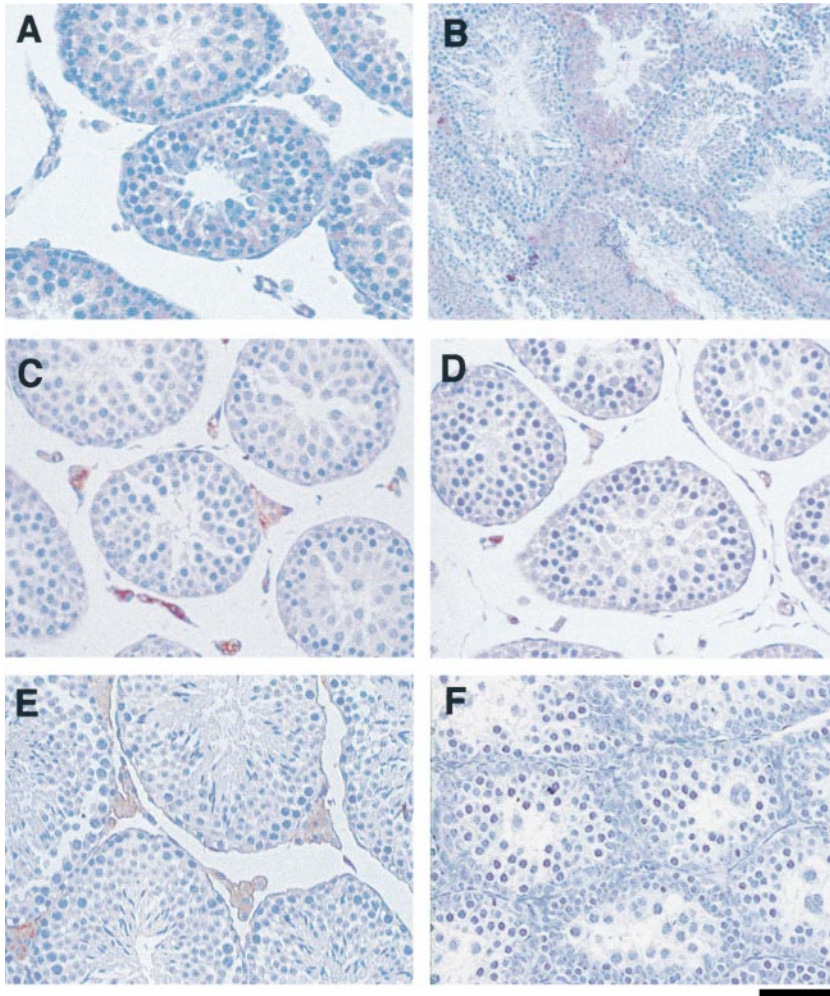


**Figure 4.** In situ hybridization for PDGF-A (A, C, E, and G) and PDGF-R $\alpha$  (B, D, F, and H) and expression of PDGF-R $\alpha$ -*lacZ* transgene (I and J) in the WT testis during development. At E17.5 PDGF-A expression is seen in the tubular epithelium in the testis and in the epididymis (asterisk), but not in the interstitial mesenchyme (A, low magnification view; C, high magnification view of the testicular section shown in A). PDGF-R $\alpha$  expression is reciprocal [B, low magnification view of the testis and epididymis (asterisk); D, high magnification view of the testicular section shown in B]; it is seen in the interstitial mesenchyme but not in the epithelium. The strongest PDGF-R $\alpha$  expression is observed in cells adjacent to the seminiferous tubules. PDGF-A expression is still seen in testicular tubules at P30, but at levels varying between different tubules (E, low magnification view; G, high magnification view of the testicular section in E) and PDGF-R $\alpha$  is still expressed in interstitial cells at this time (F, low magnification view; H, high magnification view of the testicular section in F). PDGF-R $\alpha$  promoter activity was detected in the interstitium of P10 (I) and P32 (J) testis as displayed by the *lacZ* expression. Bar: (A, B, E, F, and J) 250  $\mu$ m; (C and D) 50  $\mu$ m; (G, H, and I) 100  $\mu$ m.

described. However, given the chemotactic activity of PDGF-A (Heldin and Westermark, 1999), we cannot exclude that, similarly to what is described for other PDGF-dependent developmental processes (Boström et al., 1996; Lindahl et al., 1997a,b; Calver et al., 1998), PDGF-A might influence the long-range migration of Leydig stem cells,

thus preventing their arrival in the gonads from primordial germ layer source besides proliferation and differentiation.

Regardless of whether PDGF-A influences the migration of the Leydig cells precursors or their proliferation/differentiation, the main consequence of the lack of replacement of the fetal Leydig cells with adult cells would



**Figure 5.** Immunohistochemical localization of PDGF-A in +/+ (A and B) testis and comparison of the localization of PDGF-R $\alpha$  in +/+ (C and E) and -/- (D and F) testis. PDGF-A staining is localized in the seminiferous epithelium of all the testicular tubules at P18 (A) and in a subset of tubules (spermatogenic stages IX-X) and some interstitial cells at P42 (B). At P18, PDGF-R $\alpha$  expression is seen in the interstitial cells of +/+ (C) and -/- (D) testis. At P42, PDGF-R $\alpha$  is still localized in the interstitial cells of the WT testis (E). Note the lack of positive staining in the P42 -/- testis which is in line with the Leydig cells loss (F). Bar: (A, C, D, E, and F) 50  $\mu$ m; (B) 100  $\mu$ m.

be a progressive reduction of testosterone, followed by spermatogenic arrest and germ cells degeneration. The testicular phenotype of the null animals strongly indicates that this is the case. In -/- testis the initiation of spermatogenesis, which depends mainly on follicle-stimulating hormone, occurs normally, while its testosterone-dependent subsequent completion and maintenance (Sharpe, 1994) are lacking. Indeed, serum testosterone levels were similar in prepubertal/early pubertal (P10-P18) WT ( $1.79 \pm 0.7$  ng/ml;  $n = 5$ ) and mutant mice ( $2.0 \pm 0.89$  ng/ml;  $n = 3$ ), while in -/- older animals, circulating testosterone was not detectable, confirming that the spermatogenic arrest and germ line apoptotic regression in PDGF-A $^{-/-}$  animals are mediated by androgen deficiency.

The plasma levels of luteinizing hormone (LH) were also measured. They were similar in -/- ( $1.6 \pm 0.4$  ng/ml;  $n = 4$ ) and +/+ ( $1.4 \pm 0.5$  ng/ml;  $n = 6$ ) animals between P10 and P25. In agreement with the testosterone reduction, in a null P42 animal the levels of LH (7.5 ng/ml) were higher than in control littermates ( $3.8 \pm 0.3$  ng/ml;  $n = 4$ ). These data indicate that in PDGF-A null animals, the fate of the Leydig cells cannot be ascribed to an LH deficiency.

In conclusion, understanding of Leydig cell development is still incomplete. Although LH, androgens and IGF-1 are recognized important elements required for the

completion of Leydig cell maturation, they cannot be the factors that regulate the commitment of stem cells to adult Leydig cell lineage (Benton et al., 1995). In fact, initial proliferation of stem cells can occur when LH is absent (Teerds et al., 1989), and animals with androgen insensitivity (Murphy et al., 1994) or IGF-1 gene deletion (Baker et al., 1996) develop Leydig cells, suggesting that a separate factor regulates the earliest stage of adult Leydig cell evolution. Our findings indicate that PDGF-A may be this factor, and suggest that adult Leydig cells arise from PDGF-R $\alpha$  progenitors.

The pivotal role played by PDGF-A in adult Leydig cells development suggests that PDGF-A may be a potential target for the master genes involved in testicular organogenesis. In this respect, it is worth mentioning that WT1, whose spatio-temporal Sertoli cells expression profile (Pelletier et al., 1991; Mundlos et al., 1993; Rackley et al., 1993; Del Rio-Tsonis et al., 1996) is virtually superimposable on that of PDGF-A, can either repress or activate the PDGF-A gene (Wang et al., 1992, 1993a, 1993b; Gashler et al., 1992), and has been involved in posttranscriptional processing within the Sertoli cells (Larsson et al., 1995). The dramatic testicular phenotype of the PDGF-A $^{-/-}$  animals is also of interest because recent studies have reported detection of PDGF-A and PDGF-R $\alpha$  in the

human testis (Basciani, S., L. Gnessi, M. Arizzi, N. Rucci, S. Mariani, S. Ulisse, E.A. Jannini, G. Spera. 1999. Proceedings of the 81st Annual Meeting of the Endocrine Society, San Diego, CA. Abst. P2-P81). These considerations, coupled with our findings, may furnish a new approach for the understanding of the WT1-mediated mechanisms involved in testicular development and perhaps of WT1-dependent urogenital abnormalities and tumorigenesis (Hastie, 1994; Reddy and Licht, 1996; Menke et al., 1998). Moreover, a local impairment of the PDGF-A/PDGF-R $\alpha$  system may provide a new conceptual framework for the comprehension of some forms of Leydig cells hypoplasia, with high LH, low testosterone, and no mutations of the LH receptor gene (Zenteno et al., 1999), and of cases of spermatogenic failure, which are probably due to intratesticular testosterone deficiency in man (Sharpe, 1994).

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## References

Baker, J., M.P. Hardy, J. Zhou, C. Bondy, F. Lupu, A.R. Bellve, and A. Efstratiadis. 1996. Effect of an IGF1 gene null mutation on mouse reproduction. *Mol. Endocrinol.* 10:903-918.

Balvers, M., A.-N. Spiess, R. Domagalski, N. Hunt, E. Kilic, A.K. Mukhopadhyay, E. Hanks, H.M. Charlton, and R. Ivell. 1998. Relaxin-like factor expression as a marker of differentiation in the mouse testis and ovary. *Endocrinology.* 139:2960-2970.

Benton, L., L.-X. Shan, and M.P. Hardy. 1995. Differentiation of adult Leydig cells. *J. Steroid Biochem. Mol. Biol.* 53:61-68.

Betsholtz, C., and E.W. Raines. 1997. Platelet-derived growth factor: a key regulator of connective tissue cells in embryogenesis and pathogenesis. *Kidney Int.* 51:1361-1369.

Boström, H., K. Willetts, M. Pekny, P. Leveen, P. Lindahl, H. Hedstrand, M. Pekna, M. Hellstrom, S. Gebre-Medhin, M. Schalling, et al. 1996. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell.* 85:863-873.

Calver, A.R., A.C. Hall, W.P. Yu, F.S. Walsh, J.K. Heath, C. Betsholtz, and W.D. Richardson. 1998. Oligodendrocyte population dynamics and the role of PDGF in vivo. *Neuron.* 20:869-882.

Del Rio-Tsonis, K., L. Covarrubias, J. Kent, N.D. Hastie, and P.A. Tsonis. 1996. Regulation of the Wilms' tumor gene during spermatogenesis. *Dev. Dyn.* 207:372-381.

Fruittiger, M., L. Karlsson, A.C. Hall, A. Abramsson, A.R. Calver, H. Bostrom, K. Willetts, C.-H. Bertold, J.K. Heath, C. Betsholtz, and W.D. Richardson. 1999. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development.* 126:457-467.

Gashler, A.L., D.T. Bonthron, S.L. Madden, F.J.J. Rauscher 3rd, T. Collins, and V.P. Sukhatme. 1992. Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms' tumor suppressor WT1. *Proc. Natl. Acad. Sci. USA.* 89:10984-10988.

Ge, R.-S., L.-X. Shan, and M.P. Hardy. 1996. Pubertal development of Leydig cells. In *The Leydig cell*. A. Payne, M.P. Hardy, and L.D. Russell, editors. Cache River Press, Vienna, IL. 159-173.

Gnessi, L., A. Emidi, D. Farini, S. Scarpa, A. Modesti, T. Ciampani, L. Silvestroni, and G. Spera. 1992. Rat Leydig cells bind platelet-derived growth factor through specific receptors and produce platelet-derived growth factor-like molecules. *Endocrinology.* 130:2219-2224.

Gnessi, L., A. Emidi, E.A. Jannini, E. Carosa, M. Maroder, M. Arizzi, S. Ulisse, and G. Spera. 1995. Testicular development involves the spatiotemporal control of PDGFs and PDGF receptors gene expression and action. *J. Cell Biol.* 131:1105-1121.

Goodfellow, P.N., and R. Lovell-Badge. 1993. SRY and sex determination in mammals. *Annu. Rev. Genet.* 27:71-92.

Gustafson, M.L., and P.K. Donahoe. 1994. Male sex determination: current concepts of male sexual differentiation. *Annu. Rev. Med.* 45:505-524.

Hastie, N.D. 1994. The genetics of Wilms' tumor a case of disrupted development. *Annu. Rev. Genet.* 28:523-558.

Heldin, C.-H., and B. Westermark. 1999. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol. Rev.* 79:1283-1316.

Karlsson, L., C. Bondjers, and C. Betsholtz. 1999. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. *Development.* 126:2611-2621.

Larsson, S.H., J.P. Charlier, K. Miyagawa, D. Engelkamp, M. Rassoulzadegan, A. Ross, F. Cuzin, V. van Heyningen, and H.D. Hastie. 1995. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. *Cell.* 81:391-401.

Lindahl, P., B. Johansson, P. Leveen, and C. Betsholtz. 1997a. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science.* 277:242-245.

Lindahl, P., Karlsson L., M. Hellstrom, S. Gebre-Medhin, K. Willetts, J.K. Heath, and C. Betsholtz. 1997b. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. *Development.* 124:3943-3953.

Loveland, K.L., K. Zlati, A. Stein-Oakley, G. Risbridger, and D.M. de Kretser. 1995. Platelet-derived growth factor ligand and receptor subunit mRNA in the Sertoli and Leydig cells of the rat testis. *Mol. Cell. Endocrinol.* 108:155-159.

McLaren, A. 1998. Gonad development: assembling the mammalian testis. *Curr. Biol.* 8:R175-R177.

Menke, A.L., A.J. van der Eb, and A.G. Jochemsen. 1998. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? *Int. Rev. Cytol.* 181:151-212.

Mundlos, S., J. Pelletier, A. Darveau, M. Bachmann, A. Winterpacht, and B. Zabel. 1993. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development.* 119:1329-1341.

Murphy, L., I.A. Jeffcoate, and P.J. O'Shaughnessy. 1994. Abnormal Leydig cell development at puberty in the androgen-resistant Tfm mouse. *Endocrinology.* 135:1372-1377.

Parker, K.L., A. Shendl, and B.P. Schimmer. 1999. Gene interactions in gonadal development. *Annu. Rev. Physiol.* 61:417-433.

Pelletier, J., M. Schalling, A.J. Buckler, A. Rogers, D.A. Haber, and D. Housman. 1991. Expression of the Wilms' tumor gene WT1 in the murine urogenital system. *Genes Dev.* 5:1345-1356.

Rackley, R.R., A.M. Flenniken, N.P. Kuriyan, P.M. Kessler, M.H. Stoler, and B.R. Williams. 1993. Expression of the Wilms' tumor suppressor gene WT1 during mouse embryogenesis. *Cell Growth Differ.* 4:1023-1031.

Reddy, J.C., and J.D. Licht. 1996. The WT1 Wilms' tumor suppressor gene: how much do we really know? *Biochim. Biophys. Acta.* 1287:1-28.

Reinertsen, K.K., R.T. Bronson, C.D. Stiles, and C. Wang. 1997. Temporal and spatial specificity of PDGF  $\alpha$  receptor promoter in transgenic mice. *Gene Expr.* 6:301-314.

Russell, L.D., R.A. Ettl, A.P. Sinha-Hikim, and E.D. Clegg. 1990. Histological and Histopathological Evaluation of the Testis. Cache River Press, Clearwater, FL. 286 pp.

Sharpe, R.M. 1994. Regulation of spermatogenesis. In *The Physiology of Reproduction*. E. Knobil, and J.D. Neill, editors. Raven Press, New York. 1363-1434.

Teerds, K.J., D.G. de Rooij, F.F.G. Rommerts, R. van den Hurk, and C.J.G. Wensing. 1989. Stimulation of the proliferation and differentiation of Leydig cells precursors after the destruction of existing Leydig cells with ethane dimethyl sulphonate (EDS) can take place in the absence of LH. *J. Androl.* 10:472-477.

Wang, Z.Y., S.L. Madden, T.F. Deuel, and F.J. Rauscher III. 1992. The Wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene. *J. Biol. Chem.* 267:21999-22002.

Wang, Z.Y., Q.-Q. Qiu, and T.F. Deuel. 1993a. The Wilms' tumor gene product WT1 activates or suppresses transcription through separate functional domains. *J. Biol. Chem.* 268:9172-9175.

Wang, Y., Q.-Q. Qiu, K.T. Enger, and T.F. Deuel. 1993b. A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. *Proc. Natl. Acad. Sci. USA.* 90:8896-8900.

Yomogida, K., H. Ohtani, H. Harigae, E. Ito, Y. Nishimune, J.D. Engel, and M. Yamamoto. 1994. Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. *Development.* 120:1759-1766.

Zenteno, J.C., P. Canto, S. Kofman-Alfaro, and J.P. Mendez. 1999. Evidence for genetic heterogeneity in male pseudohermaphroditism due to Leydig cell hypoplasia. *J. Clin. Endocrinol. Metab.* 84:3803-3806.