Development 134, 3815-3825 (2007) doi:10.1242/dev.003467

# *PU.1* (*Sfpi1*), a pleiotropic regulator expressed from the first embryonic stages with a crucial function in germinal progenitors

**Virginie Olive[1,2,*], Nicole Wagner[1,2,*], Susan Chan[3], Philippe Kastner[3], Christine Vannetti[1,2], François Cuzin[1,2] and Minoo Rassoulzadegan[1,2,†]**

In the adult mammalian testis, spermatogenic differentiation starts from a minute population of spermatogonial stem cells (SSCs). SSCs are generated after birth from the fetal gonocytes, themselves derived from the primordial germ cells (PGCs), which are specified during the first days after implantation. Transcriptome profiling of purified preparations evidenced the preferential accumulation in SSCs of transcripts of *PU.1* (*Sfpi1*), a regulatory gene previously identified in hematopoietic progenitors. In situ immunolabeling and RNA determination showed a complex pattern of expression in the adult testis, first in SSCs and early spermatogonia followed by de novo expression in pachytene spermatocytes. Spermatogenesis in a null mutant (*PU.1*$^{G/G}$) was arrested at the prenatal stage, with reduced numbers of gonocytes owing to a defect in proliferation already noticeable at E12.5. Transcripts of several germinal markers, including vasa (*Mvh*, *Ddx4*), *Oct4* (*Pou5f1*), *Dazl* and *Taf4b*, were detected, whereas stella (*PGC7*, *Dppa3*) was not. Germ cells of *PU.1*$^{G/G}$ newborn testes grafted in nude mice did not initiate the postnatal replicative stage, whereas grafts of their wild-type littermates underwent complete spermatogenesis. During embryonic development, *PU.1* transcription was initiated as early as the blastocyst stage, with a generalized expression at E6.5 in the embryonic ectoderm. *PU.1* therefore appears to play a determinant role in at least two distinct lineages and, given its wide range of expression, possibly in other stem cells.

**KEY WORDS: Spermatogonial stem cells, Mouse development, Blastocyst, Spermatogenesis, Transcriptome**

## INTRODUCTION

Male germinal differentiation in mammals comprises two distinct periods delimited by birth (reviewed by Hogan et al., 1994). During embryonic development, the primordial germ cells (PGCs) specified in the epiblast at embryonic day (E) 7.5 divide and colonize the gonadal ridge, where they become associated with somatic cells in the cord structures characteristic of the fetal testis. At the end of prenatal development, the germ cells (gonocytes) stop dividing. The spermatogonial stem cells (SSCs), which resume proliferation during the first days after birth (de Rooij and Grootegoed, 1998), were functionally defined by their ability to reconstitute a functional seminiferous epithelium after transplantation into a sterile host (Brinster, 2002; Brinster and Zimmermann, 1994). The cyclic process of spermatogenesis initiated from SSCs will thereafter be maintained throughout the whole of adult life.

The two critical turning points in this process are the specification of PGCs in the early embryo and the appearance of dividing SSCs shortly after birth. Genes expressed at the earliest stages of specification of the germ line have been identified recently. They include fragilis (*Ifitm3*) (Saitou et al., 2002; Tanaka et al., 2005), stella (*PGC7*, *Dppa3*) (Bortvin et al., 2004; Nakamura et al., 2007; Payer et al., 2003; Saitou et al., 2002; Sato et al., 2002) and *Blimp1* (*Prdm1*) (Ohinata et al., 2005; Saitou et al., 2005; Vincent et al., 2005). PGCs appear first in the proximal epiblast, in a region in which fragilis is upregulated at E6.5. This restricted PGC population (approximately 40 cells) expresses *Blimp1* (E7.25) and thereafter (E7.5) stella. After birth, only a limited number of genes crucial for establishment and self-renewal of SSCs have been identified. Requirement for the *Ret* receptor was postulated based on the requirement for its ligand GDNF (Meng et al., 2000), and a partial requirement for *Bcl6b* was described recently (Oatley et al., 2006).

Our starting point was the establishment of a transcriptome profile of purified preparations of SSCs, on the assumption that crucial genes would appear among those expressed at elevated levels in the stem cells. Work on adult SSCs is made difficult by their small number. We previously reported an efficient procedure, in which a truncated version of the *Stra8* promoter is used to express in SSCs a neutral heterologous surface protein including two domains of the human CD4 antigen (huCD4) (Giuili et al., 2002). Once fractionated by immunomagnetic sorting, the huCD4-positive fraction is homogeneous for the expression of known SSC markers and highly efficient in the colonization of a sterile recipient. Hybridization on DNA arrays provided a shortlist of transcripts upregulated in the purified fraction. Interestingly, the list included *PU.1* (*Sfpi1*), a member of the Ets family, which has been extensively studied for its important regulatory functions in the renewal of progenitor cells and in early differentiation, so far exclusively in the various lineages of hematopoietic differentiation (reviewed by Lloberas et al., 1999; Metcalf et al., 2006). The availability of a null mutation generated by insertion of the green fluorescent protein (GFP) reporter gene in the first exon (Back et al., 2004), allowed us to demonstrate here a strict requirement for *PU.1* in the fetal differentiation of the male germ line and to show promoter activity starting at the earliest embryonic stages.

[1]Inserm U636, F-06108, Nice, France. [2]Université de Nice-Sophia Antipolis, Laboratoire de Génétique du Développement Normal et Pathologique, F-06108 Nice, France. [3]Institut de Biologie Moléculaire et Cellulaire CNRS-INSERM-ULP, Illkirch, F-67404, France.

*These authors contributed equally to this work
†Author for correspondence (e-mail: minoo@unice.fr)

**Accepted 7 August 2007**

## MATERIALS AND METHODS

### Cell culture

129Sv ES cells were grown at 37°C, 5% $CO_2$, 95% humidity in dishes coated with a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts. Cells were kept in Dulbecco's modified Eagle's medium supplemented with 15% FCS, 100 IU/ml penicillin/streptomycin, 100 IU/ml non-essential amino acids, 0.1 mM 2-mercaptoethanol and 1000 IU/ml LIF.

### Transgenic mice

A truncated version of the mouse *Stra8* promoter was used as described previously (Giuili et al., 2002) to direct expression to the surface of SSCs of a fusion protein including two domains of the human CD4 protein (Stra8-CD4HAglo mice). Progeny were genotyped by PCR amplification of tail DNA using primers hCD4F and hCD4R (Table 1). *PU.1*$^{G/+}$ and *PU.1*$^{G/G}$ mice (Back et al., 2004) were genotyped by PCR with primers VP102 (wild-type exon 1), VP104 (mutated exon 1) and WF189 (intron 1) (Table 1). Amplified fragments were 650 bp for the *PU.1*$^{+}$ allele and 900 bp for *PU.1*$^{G}$. Families were maintained in the C57BL/6×DBA/2 F1 genetic background. Investigations were conducted in accordance with French and European rules for the care and use of laboratory animals.

### Embryonic staging and dissections

The day of appearance of a vaginal plug was taken as E0.5. Plug-positive females were sacrificed by cervical dislocation for collecting blastocysts, E6.5, E12.5, E15.5 and E17.5 embryos. E6.5 and older embryos were genotyped by PCR with primers VP102, VP104, and WF189 (Table 1). E12.5, E17.5 and E18.5 embryos were dissected for collection of the embryonic and fetal gonads.

### Immunomagnetic cell-sorting

Total germ cells were prepared as described previously (Vidal et al., 2001). Immunomagnetic isolation of huCD4-positive cells from total testicular cells of Stra8-CD4HAglo mice was performed using the CD4-positive Isolation Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. For each SSC preparation, 50 transgenic males were used for a final yield of $2\times10^6$ cells corresponding to the expected number of 20,000 stem cells per testis (de Rooij and Grootegoed, 1998).

### Purification of spermatids and pachytene spermatocytes

Total germ cells prepared from 20 mice were loaded in the Beckman (Palo Alto, CA, USA) elutriation rotor JE-5.0, at a flow rate of 7 ml/minute and a constant speed of 2000 rpm. Cells were collected in 11 fractions of 400 ml each, obtained by changing the flow rate from 7 to 50 ml/minute at constant speed. Fraction purity was checked by microscopic analysis after Hoechst 33253 staining.

### RNA preparation

Total RNA was extracted from cell fractions using Trizol (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Ten to 20 μg of stem cell RNA was obtained per SSC preparation. Quality of RNA was checked by agarose gel electrophoresis and using an Agilent bioanalyser.

### Histology and immunohistochemistry

Histology and immunohistochemistry analyses were performed according to published protocols (Wagner et al., 2005). Briefly, the samples were fixed overnight at 4°C in paraformaldehyde (3% in PBS) and embedded in paraffin. Routine histological examination was performed after Hematoxylin staining. Sections (3 μm) were incubated (16 hours, 4°C) with primary antibodies against PU.1 (T-21, sc-352, Santa Cruz Biotechnology, Santa Cruz, CA), PCNA (PC10, sc-56, Santa Cruz Biotechnology), each diluted 1:100 in PBS, 0.1% Triton X-100, 3% BSA. Antigen detection was performed with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA), followed by incubation with peroxidase-coupled streptavidin (Sigma, St Louis, MO). Visualization was achieved with DAB substrate (SK-4100, Vector Laboratories) (Wagner et al., 2006). For PCNA staining, the subsequent antigen detection was performed using the M.O.M. Kit (PK-2200, Vector laboratories). ES cells were stained with the PU.1 antibody as described for U2OS cells (Wagner et al., 2005). Omission of the first antibody served as a negative control; additionally, blocking peptide against PU.1 (sc-352P, Santa Cruz Biotechnology) was used to confirm the specificity of staining.

### Tunel labeling of apoptotic cells

Apoptotic cells were detected by Tunel staining of paraffin sections using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) as described previously (Wagner et al., 2005). Three sections from three different animals were analyzed.

### Confocal microscopy

Distribution of GFP fluorescence within the embryo was determined by confocal microscopy with a Leica CLSM microscope equipped with an argon-krypton ion laser emitting light at 488 and 514 nm and producing an excitation

**Table 1. Oligonucleotide primers**

| Amplified sequence | Forward (F) | Reverse (R) |
|---|---|---|
| *PU.1*$^{+}$ (genomic) | GGCCTACAGGAAGAGCAAGCCTGGCA | ATTCGTTACAAATGCAAGCTAAA |
| *PU.1*$^{G}$ (genomic) | AGGGGCGGTGAGGGAAAACCCTTCCA | ATTCGTTACAAATGCAAGCTAAA |
| *PU.1* (RNA) | TGGGAGGGTTTTCCCTCACC | TGCTGTCCTTCATGTCGCCG |
| *huCD4* | CCTCAGTGCAATGTAGGAGTC | CTCATCAGATGCATATTCTGC |
| *Stra8* | TCACAGCCTCAAAGTGGCAGG | GCAACAGAGTGGAGGAG |
| *Gapdh* | CACCACCAACTGCTTAGCC | CGGATACATTGGGGGTAGG |
| *Kit* | CAACAGCAATGGCCTCACGAGT | GTGGTCACCTTTGCTCTGCTC |
| *Sycp1* | ATGGAGAAGCAAAAGCCCTTC | ATGTTTTCCCGACTGGACATTG |
| *Prm1* | GGCCAGATACCGATGC | CCTTAGCAGGCTCCTG |
| β-actin | AAGGCCAACCGTGAAAAGAT | GTGGTACGACCAGAGGCATAC |
| *Cd81* | GGGCATCTACATTCTCATTGC | GTATGGTGGTAGTCAGTGTGG |
| *Cd97* | CTCGGAGAAGGAATTGATCAC | CTTCTGAGTCTCCCATCTGTG |
| *Nptn* | GAATTGTCACCAGTGAAGAGG | CATCATAGCATCCTGCCCTTC |
| *Fos* | CAGCCTTTCCTACTACCATTC | CTGACACGGTCTTCACCATTC |
| *Cdc42* | GGCAAGAGGATTATGACAGAC | CAGCAGTCTCTGGAGTAATAG |
| *Cd14* | GGAAGCCAGAGAACACCATCG | GCAGGGCTCCGAATAGAATCC |
| *Lyn* | GAGAGAAGGCTTCATCCCCAG | GTCTTCACAGCCACCTTTGTG |
| *Nek7* | GAGCATCCTGTCTCTTGGATG | GACTCTTCGAGAATGCATGTG |
| stella | AAAGCTAACCCTAAACCCCGG | TGGCTCACTGTCCCGTTCA |
| vasa | CCAAAAGTGACATATATACCC | TTGGTTGATCAGTTCTCGAG |
| *Plzf* | CCACCTTCGCTCACATACAG | TGTGGCTCTTGAGTGTGCTC |
| *Taf4b* | CAGACAGCCAAAGTCAAGCA | TGGAGGTCACATGATTCACAC |
| *Oct4* | TGAGGCTACAGGACACCTTTC | GTGCCAAAGTGGGGACCT |
| *Dazl* | GCTGATATTTTGCCCAATGAA | ATGCTTCGGTCCACAGACTT |

Sequences are shown 5′ to 3′.

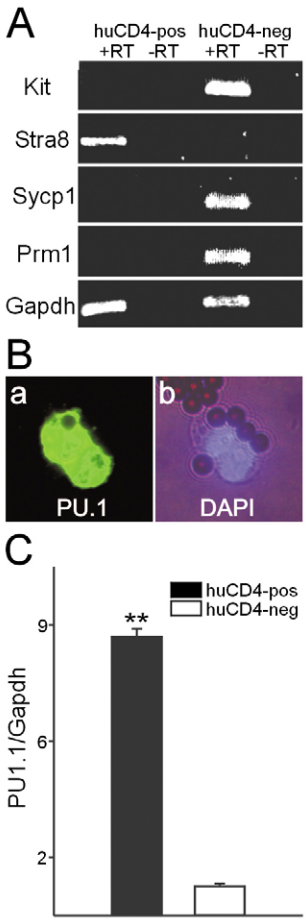


**Fig. 1. Mouse *PU.1* (*Sfpi1*) expression in the huCD4-positive SSC fraction.** (**A**) Germ cell markers in the purified spermatogonial stem cell fraction. RT-PCR assays performed on RNA of huCD4$^+$ cells and of the bulk of differentiated germ cells (huCD4$^-$ fraction). *Stra8* is expressed in both differentiated and stem spermatogonia (Oulad-Abelghani et al., 1996). *Kit* is expressed in differentiated spermatogonia, but absent from stem cells (Shinohara et al., 2000). The synaptonemal complex protein 1 gene (*Sycp1*) is expressed in pachytene spermatocytes (Meuwaissen et al., 1992). *Prm1* (protamine 1) is expressed in haploid germ cells (Peschon et al., 1987). *Gapdh* served as a control. (**B**) Immunodetection of the PU.1 protein in huCD4$^+$ cells (**a**) and DAPI staining (**b**). Note that after immunomagnetic sorting, the cells are still attached to the beads. (**C**) Quantitative RT-PCR assay of *PU.1* RNA in the bulk of differentiated germ cells (huCD4$^-$ cells) and in huCD4$^+$ cells. Normalization was performed relative to *Gapdh* RNA.

wavelength of 515 and 580 nm, and with a UV laser (excitation 352 nm, emission 405 nm). A series of optical sections of approximately 0.95 μm was recorded through the *z*-axis, using an objective of 43× and a zoom of 1.99.

### RT-PCR analysis

DNase I-treated total RNA (1 μg) was reverse-transcribed using the M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCR amplification was performed with the Ready Mix Red Taq PCR Mix (Sigma) using the oligonucleotide primers shown in Table 1.

### Microarray data analysis

RNA preparations were analyzed by in vitro amplification and hybridization to DNA microarrays containing about 14,000 genes (Mouse Genome 430A 2.0, Affymetrix, Santa Clara, CA, USA). Absolute and comparison analyses of the mouse oligonucleotide arrays were conducted using the statistics-based Affymetrix software MAS-5.0 (GeneChip Software) with the default settings. We used the MAS5 algorithm (Affymetrix) to determine the 'present' and 'absent' calls for each probe set. Quantitative expression values were obtained with the robust microarray analysis (RMA) algorithm, which uses a method based on quantile normalization to calculate an expression value for each probe set. The complete microarray data are available in the NCBI GEO repository (http://www.ncbi.nlm.nih.gov/geo/).

### Quantitative real-time PCR

cDNA samples from SSC and total fractions were analyzed by real-time PCR using the qPCR MasterMix Plus for SYBR Green (Eurogentec, Seraing, Belgium) and an ABI Prism 7000 sequence detector according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Amplification was performed by applying the comparative Ct method, as described in the manufacturer's protocol. Sequences of oligonucleotide primers are shown in Table 1.

### BrdU incorporation

Pregnant female mice received 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU, Roche Molecular Biochemicals) by intraperitoneal injection and were sacrificed 5 hours later. Paraffin sections were stained by immunolabeling with anti-BrdU antibody (Roche Molecular Biochemicals) as described above.

### Testis transplantation

The donor testes were dissected at E17-19. For transplantation, the recipient testis (6-week-old ICR nu/nu mice, Charles River, Wilmington, MA) was exteriorized through a midline abdominal incision after anaesthetizing the recipient mouse. Using fine forceps, a small cut was made in the tunica albuginea, and the testis graft inserted into the testicular parenchyma. One week to 1 month after transplantation, the recipient testis with the transplant was embedded in paraffin and histological sections were cut at 8-μm intervals from each testis piece and stained with Hematoxylin and Eosin for microscopic examination.

## RESULTS

### Preparation and validation of SSC fractions

SSCs were prepared by immunomagnetic sorting with anti-huCD4 antibodies from males of the Stra8-CD4 transgenic family. In these families (Giuili et al., 2002), a neutral surface marker is expressed under control of a truncated version (411 bp) of the promoter of the *Stra8* gene. Unlike the wild-type promoter, the truncated version directs transgene expression to a minor population of premeiotic cells, positively identified as SSCs on the basis of expression of marker genes and of their high efficiency of establishment upon transplantation. Among other markers routinely used to ascertain the purity of the fractions (expression of cyclin A2, but not A1, of integrins β1 and α6), the two most characteristic complementary criteria are shown in Fig. 1A, namely expression of the endogenous *Stra8* gene, characteristic of both SSCs and differentiated spermatogonia (Oulad-Abdelghani et al., 1996), and absence of *Kit* transcripts, which are expressed only in differentiated spermatogonia (Shinohara et al., 2000). Contamination by cells engaged in the germinal differentiation process was minimal, as shown by the negative results registered for *Sycp1*, which is characteristic of the pachytene spermatocytes (Meuwissen et al., 1992) and *Prm1*, a marker of spermatids (Peschon et al., 1987). Possible contamination by postmeiotic cells was in addition limited by starting the purification process from young males (3-4 weeks), in which the first wave of spermatogenesis would have just passed the second meiotic division (Bellve et al., 1977). Yields were ~20,000 cells per testis, in agreement with previous estimates (de Rooij and Grootegoed, 1998).

**Table 2. Transcripts preferentially expressed in huCD4-positive cells**

| Probe set number* | Gene† | Symbol† | Fold change (log2)‡ |
|---|---|---|---|
| 1422903_at | lymphocyte antigen 86 | *Ly86* | 4.08 |
| 1419627_s_at | C-type lectin domain family 4, member n | *Clec4n* | 3.82 |
| 1448148_at | granulin | *Grn* | 3.80 |
| 1448617_at | CD53 antigen | *Cd53* | 3.74 |
| 1450241_a_at | ecotropic viral integration site 2a | *Evi2a* | 3.61 |
| 1454268_a_at | cytochrome b-245, alpha polypeptide | *Cyba* | 3.54 |
| 1451941_a_at | Fc receptor, IgG, low affinity IIb | *Fcgr2b* | 3.52 |
| 1448891_at | macrophage scavenger receptor 2 | *Msr2* | 3.38 |
| 1417963_at | phospholipid transfer protein | *Pltp* | 3.30 |
| 1431724_a_at | purinergic receptor P2Y, G-protein coupled 12 | *P2ry12* | 3.26 |
| 1449556_at | histocompatibility 2, T region locus 23 | *H2-T23* | 3.25 |
| 1425951_a_at | C-type lectin domain family 4, member n | *Clec4n* | 3.20 |
| 1426454_at | Rho, GDP dissociation inhibitor (GDI) beta | *Arhgdib* | 3.18 |
| 1450678_at | integrin beta 2 | *Itgb2* | 3.01 |
| 1425519_a_at | CD74 antigen | *Cd74* | 2.97 |
| 1427076_at | macrophage expressed gene 1 | *Mpeg1* | 2.94 |
| 1449918_at | CD209g antigen | *Cd209g* | 2.86 |
| 1419482_at | complement component 3a receptor 1 | *C3ar1* | 2.84 |
| 1416326_at | cysteine-rich protein 1 (intestinal) | *Crip1* | 2.79 |
| 1425214_at | pyrimidinergic receptor P2Y, G-protein coupled, 6 | *P2ry6* | 2.72 |
| 1423805_at | disabled homolog 2 (*Drosophila*) | *Dab2* | 2.70 |
| 1449971_a_at | CD209f antigen | *Cd209f* | 2.54 |
| 1452544_x_at | histocompatibility 2, D region locus 1 | *H2-D1* | 2.47 |
| 1435476_a_at | Fc receptor, IgG, low affinity IIb | *Fcgr2b* | 2.46 |
| 1451386_at | biliverdin reductase B [flavin reductase (NADPH)] | *Blvrb* | 2.43 |
| 1419043_a_at | interferon inducible GTPase 1 | *Iigp1* | 2.42 |
| 1427329_a_at | immunoglobulin heavy chain 6 (heavy chain of IgM) | *Igh-6* | 2.41 |
| 1423266_at | RIKEN cDNA 2810405K02 gene | *2810405K02Rik* | 2.37 |
| 1450919_at | membrane protein, palmitoylated | *Mpp1* | 2.31 |
| 1416246_a_at | coronin, actin binding protein 1A | *Coro1a* | 2.29 |
| 1427301_at | CD48 antigen | *Cd48* | 2.28 |
| 1452231_x_at | interferon activated gene 203 | *Ifi203* | 2.19 |
| 1417268_at | CD14 antigen | *Cd14* | 2.18 |
| 1448160_at | lymphocyte cytosolic protein 1 | *Lcp1* | 2.13 |
| 1424542_at | S100 calcium binding protein A4 | *S100a4* | 2.07 |
| 1419027_s_at | glycolipid transfer protein | *Gltp* | 2.06 |
| 1417869_s_at | cathepsin Z | *Ctsz* | 2.06 |
| 1416816_at | NIMA (never in mitosis gene a)-related expressed kinase 7 | *Nek7* | 2.05 |
| 1448944_at | neuropilin 1 | *Nrp1* | 1.96 |
| 1419144_at | CD163 antigen | *Cd163* | 1.95 |
| 1448276_at | tetraspanin 4 | *Tspan4* | 1.92 |
| 1435375_at | cDNA sequence BC052328 | BC052328 | 1.88 |
| 1426918_at | integrin beta 1 (fibronectin receptor beta) | *Itgb1* | 1.88 |
| 1451683_x_at | similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor | *LOC547343* | 1.86 |
| 1417491_at | cathepsin B | *Ctsb* | 1.84 |
| 1434036_at | metastasis suppressor 1 | *Mtss1* | 1.81 |
| 1448748_at | pleckstrin | *Plek* | 1.77 |
| 1419282_at | chemokine (C-C motif) ligand 12 | *Ccl12* | 1.77 |
| 1416985_at | signal-regulatory protein alpha | *Sirpa* | 1.77 |
| 1455269_a_at | coronin, actin binding protein 1A | *Coro1a* | 1.77 |
| 1423166_at | CD36 antigen | *Cd36* | 1.72 |
| 1423570_at | ATP-binding cassette, sub-family G (WHITE), member 1 | *Abcg1* | 1.70 |
| 1418641_at | lymphocyte cytosolic protein 2 | *Lcp2* | 1.65 |
| 1419120_at | lymphoblastomic leukemia | *Lyl1* | 1.63 |
| 1423653_at | ATPase, Na+/K+ transporting, alpha 1 polypeptide | *Atp1a1* | 1.61 |
| 1417162_at | transmembrane BAX inhibitor motif containing 1 | *Tmbim1* | 1.52 |
| 1450040_at | tissue inhibitor of metalloproteinase 2 | *Timp2* | 1.51 |
| 1448239_at | heme oxygenase (decycling) 1 | *Hmox1* | 1.50 |
| 1455393_at | – | – | 1.49 |
| 1417676_a_at | protein tyrosine phosphatase, receptor type, O | *Ptpro* | 1.46 |
| 1417561_at | apolipoprotein C-I | *Apoc1* | 1.44 |
| 1420464_s_at | paired-Ig-like receptor A1 | *Pira1* | 1.44 |
| **1418747_at** | **SFFV proviral integration 1** | ***Sfpi1*** | **1.40** |
| 1420911_a_at | milk fat globule-EGF factor 8 protein | *Mfge8* | 1.38 |
| 1428340_s_at | ATPase type 13A2 | *Atp13a2* | 1.36 |
| 1450107_a_at | renin binding protein | *Renbp* | 1.34 |
| 1452169_a_at | diacylglycerol kinase zeta | *Dgkz* | 1.25 |
| 1416897_at | poly (ADP-ribose) polymerase family, member 9 | *Parp9* | 1.14 |
| 1428369_s_at | Rho GTPase activating protein 21 | *Arhgap21* | 1.14 |
| 1448797_at | ELK3, member of ETS oncogene family | *Elk3* | 1.14 |

Table continued on next page.

**Table 2. Continued**

| Probe set number* | Gene† | Symbol† | Fold change (log2)‡ |
|---|---|---|---|
| 1428369_s_at | Rho GTPase activating protein 21 | *Arhgap21* | 1.14 |
| 1448797_at | ELK3, member of ETS oncogene family | *Elk3* | 1.14 |
| 1415892_at | sphingosine phosphate lyase 1 | *Sgpl1* | 1.14 |
| 1423757_x_at | insulin-like growth factor binding protein 4 | *Igfbp4* | 1.14 |
| 1424778_at | DNA segment, Chr 10, University of California at Los Angeles 1 | *Reep3* | 1.13 |
| 1424394_at | selenoprotein M | *Selm* | 1.12 |
| 1451041_at | Rho-associated coiled-coil containing protein kinase 2 | *Rock2* | 1.11 |
| 1423153_x_at | complement component factor h | *Cfh* | 1.11 |
| 1450026_a_at | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2 | *B3gnt2* | 1.09 |
| 1426663_s_at | solute carrier family 45, member 3 | *Slc45a3* | 1.08 |
| 1425609_at | neutrophil cytosolic factor 1 | *Ncf1* | 1.07 |
| 1426376_at | receptor accessory protein 5 | *Reep5* | 1.07 |
| 1450105_at | – | – | 1.07 |
| 1448957_at | recombining binding protein suppressor of hairless (*Drosophila*) | *Rbpsuh* | 1.05 |
| 1418394_a_at | CD97 antigen | *Cd97* | 1.04 |
| 1424769_s_at | caldesmon 1 | *Cald1* | 1.01 |

*Probe set number relative to the Mouse Genome 430A 2.0 (Affymetrix).
†Official gene name and symbol according to NCBI.
‡Fold change (log2) between huCD4-positive and -negative samples.

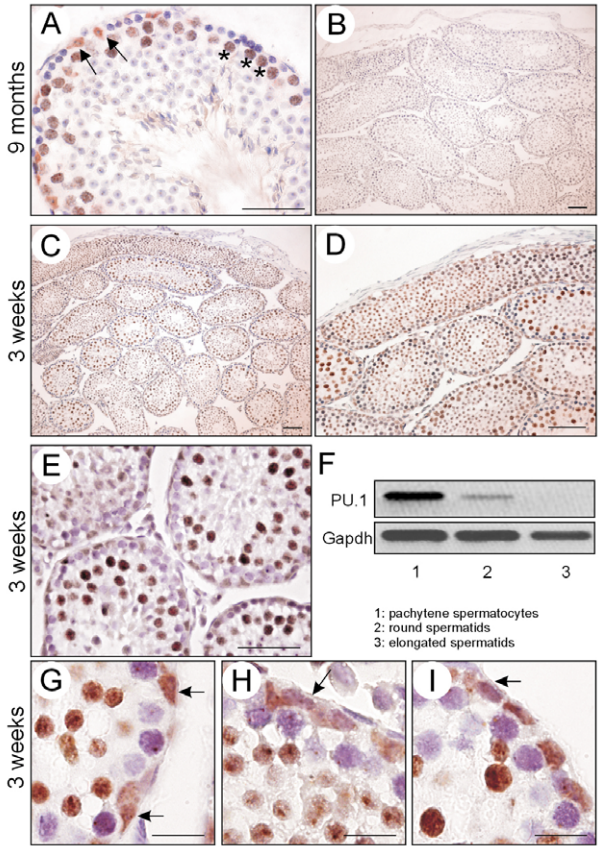


**Fig. 2. A complex pattern of *PU.1* expression in the mouse testis.** (**A**) Immunodetection of the PU.1 protein in the adult (9-month-old) testis, in a fraction of spermatogonia (arrows) and in meiotic spermatocytes (asterisks). (**B**) A blocking peptide against PU.1 antibody was used to confirm the specifity of staining. (**C-E**) Expression of PU.1 in meiotic cells during the first synchroneous wave of germinal differentiation (3-week-old testis). (**F**) RT-PCR assay of *PU.1* RNA in fractions enriched by elutriation (⩾90%) in pachytene spermatocytes (lane 1) and elongated and round spermatids (lanes 2 and 3, respectively). (**G-I**) Expression in single (G), paired (H) and aligned (I) cells (arrows) in the peripheral compartment of the seminiferous tubule ($A_{single}$, $A_{paired}$ and $A_{aligned}$ spermatogonia). Scale bars: 50 μm in A-E; 25 μm in G-I.

## A shortlist of 'increased in SSCs' transcripts includes *PU.1* (*Sfpi1*), a pleiotropic regulator of hematopoiesis

Three independent preparations of 20-40 μg SSC RNA were analyzed by hybridization on DNA microarrays (14,000 genes, Affymetrix Mouse Genome 430A 2.0). In order to identify genes selectively expressed in SSCs, we selected genes that were detected as 'present' in all three huCD4$^+$ samples and 'absent' in all three huCD4$^-$ samples. We further imposed that the measured detection values exhibit at least a 2-fold difference between huCD4$^+$ and huCD4$^-$ samples, with a *P*-value (Student's *t*-test) of less than 0.05.

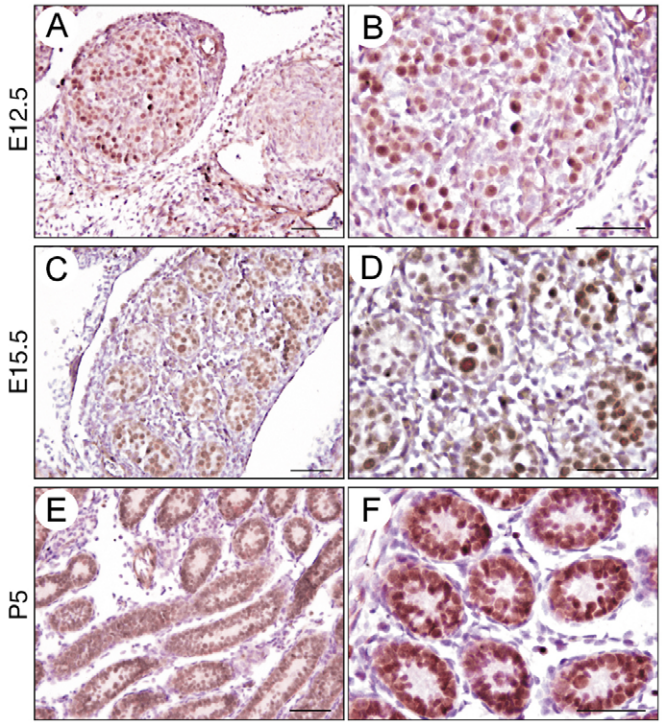


**Fig. 3. Immunodetection of PU.1 in the fetal and neonate mouse testis.** Immunodetection was performed at E12.5 (**A**,**B**), E15.5 (**C**,**D**) and at postnatal day 5 (**E**,**F**). Scale bars: 50 μm.

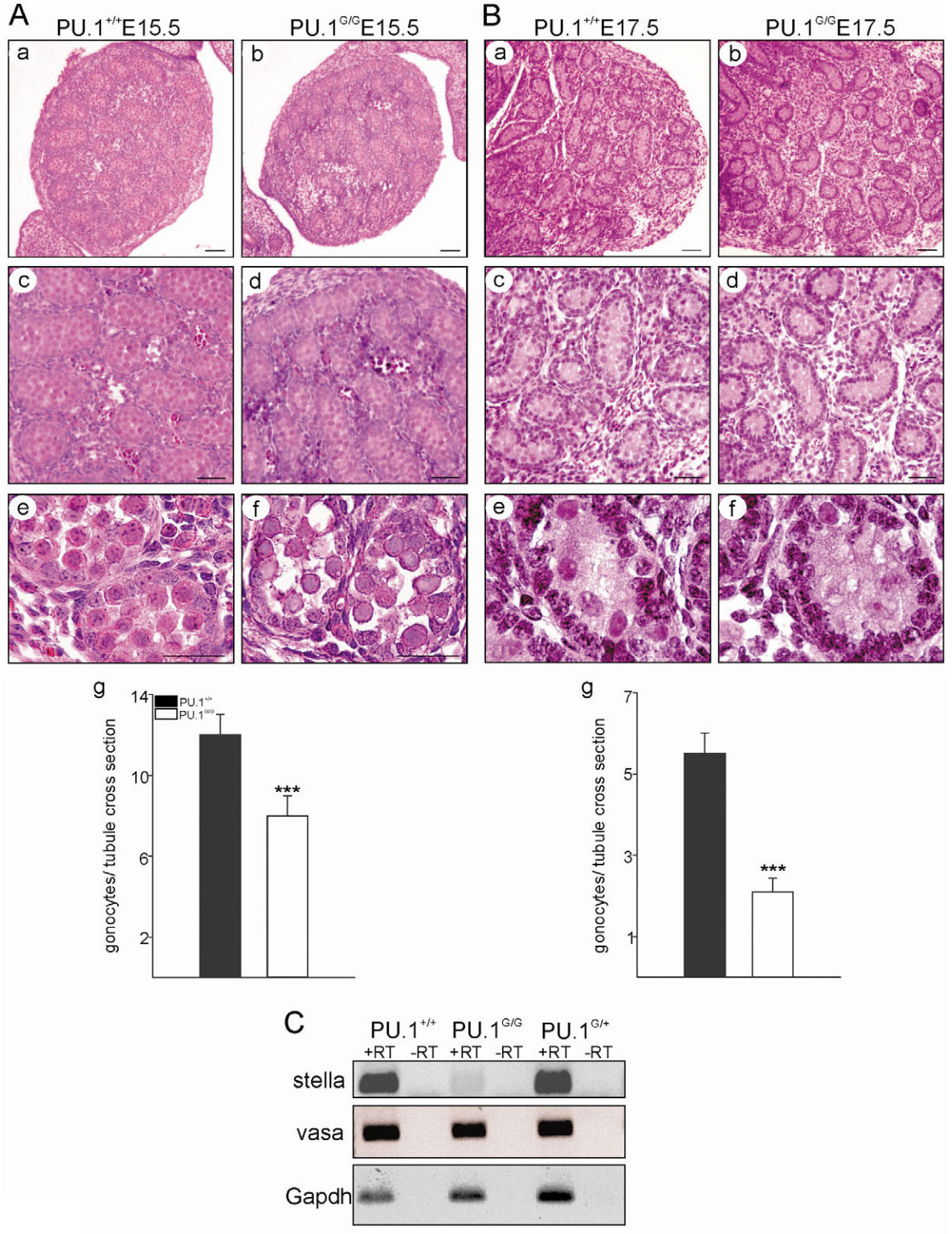


**Fig. 4. Abnormal testicular development of the *PU.1*-negative mouse mutant.** (**Aa-g**,**Ba-g**) Testis sections of *PU.1*$^{+/+}$ (a,c,e) and *PU.1*$^{G/G}$ (b,d,f) littermates at E15.5 (A) and E17.5 (B). (Ag,Bg) The average number of gonocytes per transverse tubule section, for both developmental times. (**C**) RT-PCR assay for vasa and stella expression performed on RNA prepared from E18.5 *PU.1*$^{G/G}$, *PU.1*$^{G/+}$ and *PU.1*$^{+/+}$ fetal testis. Scale bars: 50 μm.

These combined criteria led to a list of 84 genes (Table 2). In addition, to estimate the 'background' of non-specific genes within that list, we performed similar analyses between permuted combinations of samples, in which two samples had been switched between the positive and negative groups. In each case, less then ten genes were found, suggesting that most of the selected genes are indeed differentially expressed between SSCs and non-SSCs. Differential expression of four genes randomly taken in the list

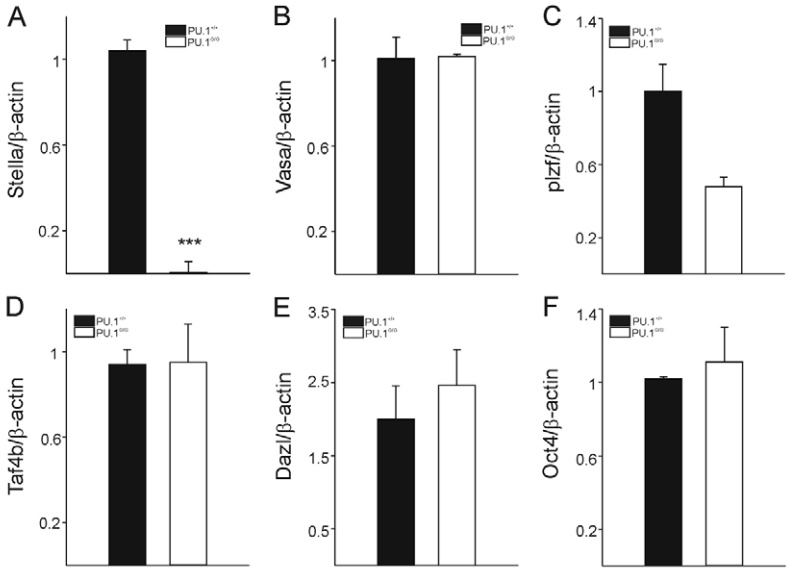


**Fig. 5. Expression of early germinal markers in the fetal *PU.1*$^{G/G}$ mouse testis.** (**A-F**) Quantitative RT-PCR determination at E18.5 relative to β-actin RNA. Oligonucleotide primers are listed in Table 1.

(*Cd14*, *Cd97*, *Nek7*, *Sfpi1*) was also confirmed by quantitative RT-PCR (data not shown). We also noted that a number of genes, although excluded from this most stringent list because of low but significant expression in at least one of the huCD4$^{-}$ samples, showed higher expression levels in the positive fraction. RT-PCR assays of five transcripts in this category (*Cd81*, *Nptn*, *Fos*, *Cdc42*, *Lyn*) again showed a differential expression in the two fractions (data not shown).

Several genes encoding transcription factors were present among the genes in the most stringent shortlist: *Lyl1* encoding a bHLH factor (Capron et al., 2006), *Elk3* encoding an Ets-domain factor (Chung et al., 2006) and *Rbpsuh* (*Rbpj*) encoding the transcriptional mediator of Notch signaling (Vasyutina et al., 2007).

Although all of these genes might play important regulatory roles in controlling SSC gene expression, we were especially intrigued by the presence of *PU.1* (*Sfpi1*). This finding warranted further analysis because the published literature (reviewed by Lloberas et al., 1999; Metcalf et al., 2006) consistently refers to *PU.1* as a transcription regulator that acts exclusively in the progenitors of blood cells. Both *PU.1* RNA and the protein were unequivocally detected in huCD4$^{+}$ cells by quantitative RT-PCR and by immunolabeling (Fig. 1B,C).

## *PU.1* expression in the adult testis

Immunodetection of PU.1 protein in adult testis sections revealed a complex pattern. As expected from analysis of the huCD4$^{+}$ fraction, the protein was detected in a small number of spermatogonia in the most peripheral region of the tubule (Fig. 2A). Among these cells, the paired ($A_{paired}$) and aligned ($A_{aligned}$) cells characteristic of the early spermatogonial stages (de Rooij and Grootegoed, 1998) were clearly identified (Fig. 2G-I). Whereas the bulk of differentiated spermatogonia were negative, expression of the protein was also evidenced in meiotic spermatocytes (Fig. 2A, asterisk). This finding was somewhat unexpected, a contamination by pachytene spermatocytes of the huCD4$^{+}$ fraction being made unlikely by the absence of the characteristic meiotic transcripts *Sycp1* (Fig. 1A) and *Ccna2* (cyclin A2, not shown) (see Giuili et al., 2002). Meiotic expression was nevertheless confirmed by in situ analysis in the testis of 3-week-old males, in which the first synchronous wave of spermatogenesis leads to the accumulation of meiotic cells, which were uniformly and strongly labeled by anti-PU.1 immunostaining (Fig. 2C-E). This was further confirmed by PCR assays performed on elutriation-purified spermatocytes (Fig. 2F). The quantitatively minor representation of pachytene spermatocytes in the total huCD4$^{-}$ fraction accounts for the initial detection of higher levels of *PU.1* transcripts in huCD4$^{+}$ cells.

## PU.1 activity during development is required in germinal differentiation

In the embryonic testis, PU.1 was detected in the germinal compartment, at least from E12.5 onward (Fig. 3). Further studies took advantage of a mouse mutant, *PU.1*$^{G}$, generated by insertion of a GFP cassette into the first exon of the gene to provide both a reporter for promoter activity and a null mutation (Back et al., 2004). *PU.1*$^{G/G}$ homozygotes die shortly after birth and heterozygotes are healthy and fertile. Embryos of crosses between heterozygotes were individually genotyped and testis development was analyzed in the *PU.1*$^{G/G}$ progeny, with their *G/+* and *+/+* littermates used as controls. Histological analysis of homozygotes at E15.5 and E17.5 seemed at first to indicate that the testis was normally constituted, with a myoid cell layer surrounding internal cells in a structure similar to the testis cords of the wild type (Fig. 4A,B). The number of gonocytes per section was, however, smaller than in the controls. Among a series of genes characteristic of prenatal germinal differentiation, RT-PCR analysis detected transcripts of *Oct4* (Yoshimizu et al., 1999), *Dazl* (Lin and Page, 2005), *Taf4b* (Falender et al., 2005) and vasa (Tanaka et al., 2000), but expression of *Plzf* (*Zbtb16*) (Buaas et al., 2004; Costoya et al., 2004) and stella (Saitou et al., 2002) was strongly reduced or absent in the null mutant (Fig. 4C and Fig. 5).

The defect in spermatogenesis of the *PU.1*$^{G/G}$ mutant was related to a decrease in cell proliferation, as evidenced by decreased BrdU incorporation during development (Fig. 6). The number of germ cells incorporating BrdU was significantly decreased at E12.5 and E13.5. By contrast, BrdU incorporation in the somatic component of the testis cords (essentially Sertoli cells) was decreased to a lesser extent and only at later times, an observation that will be addressed in future studies. At postnatal stages, a functional defect of *PU.1*$^{G/G}$ testes was evidenced by testis graft experiments, an approach made necessary by the early death of the mutant homozygotes. Testes dissected shortly before birth (E18.5) were grafted under the tunica albuginea of recipient

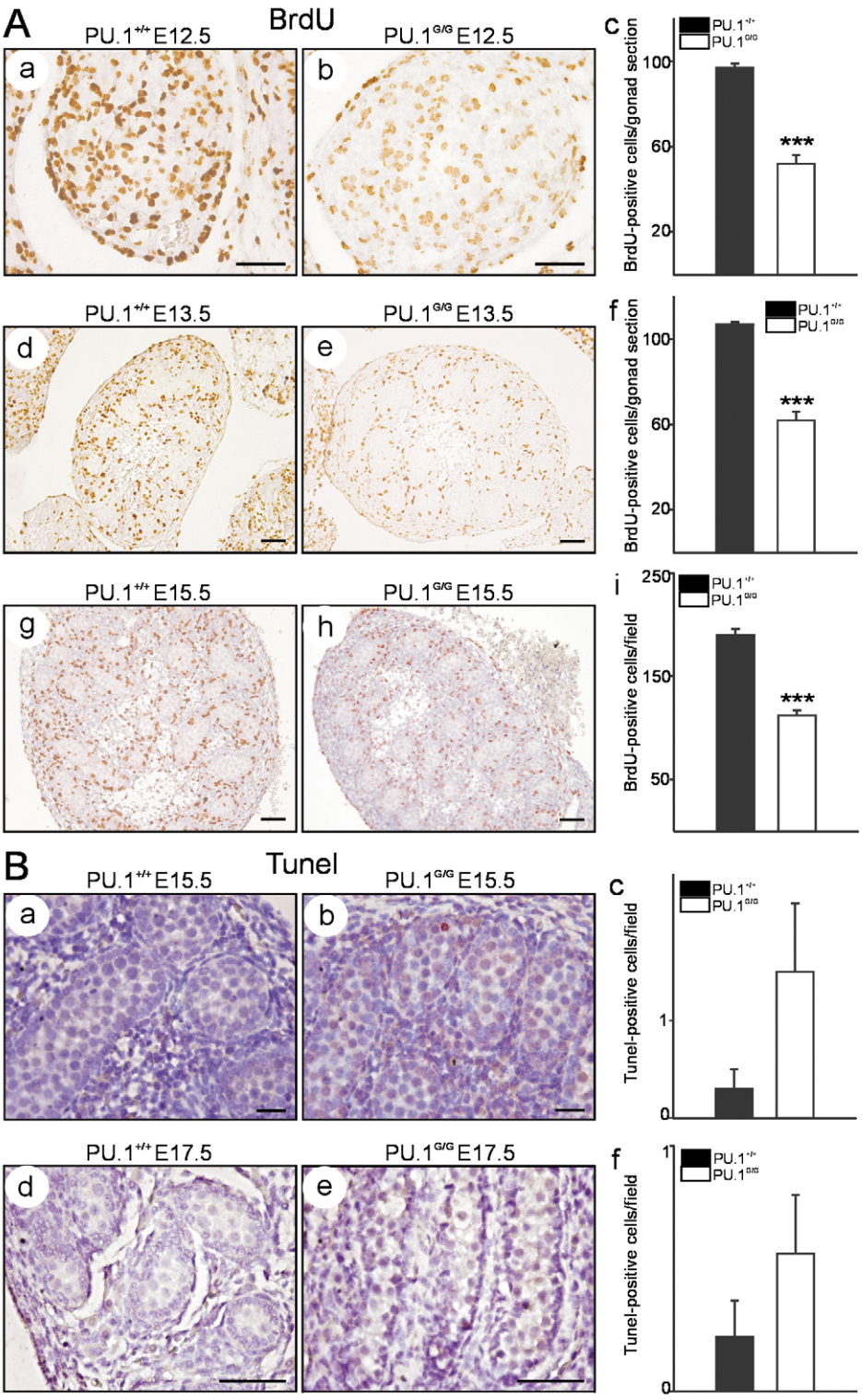


**Fig. 6. Decreased proliferation and absence of apoptosis of male germ cells in the *PU.1*-negative mouse mutant.** (**Aa-i**) BrdU incorporation was monitored by immunolabeling after 5-hour pulses at E12.5, E13.5 and E15.5 in *PU.1*$^{G/G}$ homozygotes (b,e,h) and their *PU.1*$^{+/+}$ littermates (a,d,g). (c,f,i) Quantitation by counts of positive cells in whole-gonad sections (including germ cells and somatic cells). The faint staining of the mutant cells counted as positive at E12.5 may reflect either lower rates of replication or repair activity. Note that the label in the mutant testis at E13.5 and E15.5 is restricted to the peripheral somatic cells. (**Ba-f**) Tunel assay for apoptotic cells was performed on E15.5 (a,b) and E17.5 (d,e) embryos. (c,f) Quantitation of Tunel-positive cells. Scale bars: 50 μm.

nude mice. Spermatogenesis is known to proceed normally in such grafts up to the production of functional sperm (Honaramooz et al., 2002). Grafts of the *PU.1*$^{G/+}$ testes showed, after 1 week, a strong proliferative activity as evidenced by PCNA immunolabeling (Fig. 7Ae,g), leading, after 1 month, to the development of a structurally normal seminiferous epithelium (Fig. 7Ba,c). By contrast, grafts of their *PU.1*$^{G/G}$ littermates showed, 1 week after transplantation, only a limited proliferative activity of Sertoli cells in the periphery of the tubules (Fig. 7f,h), which were essentially empty after 2 weeks (not shown) and 4 weeks (Fig. 7Bb,d). Thus, not only was the number of gonocytes reduced owing to a decreased proliferation rate, but the surviving germ cells were unable to enter a normal differentiation pathway.

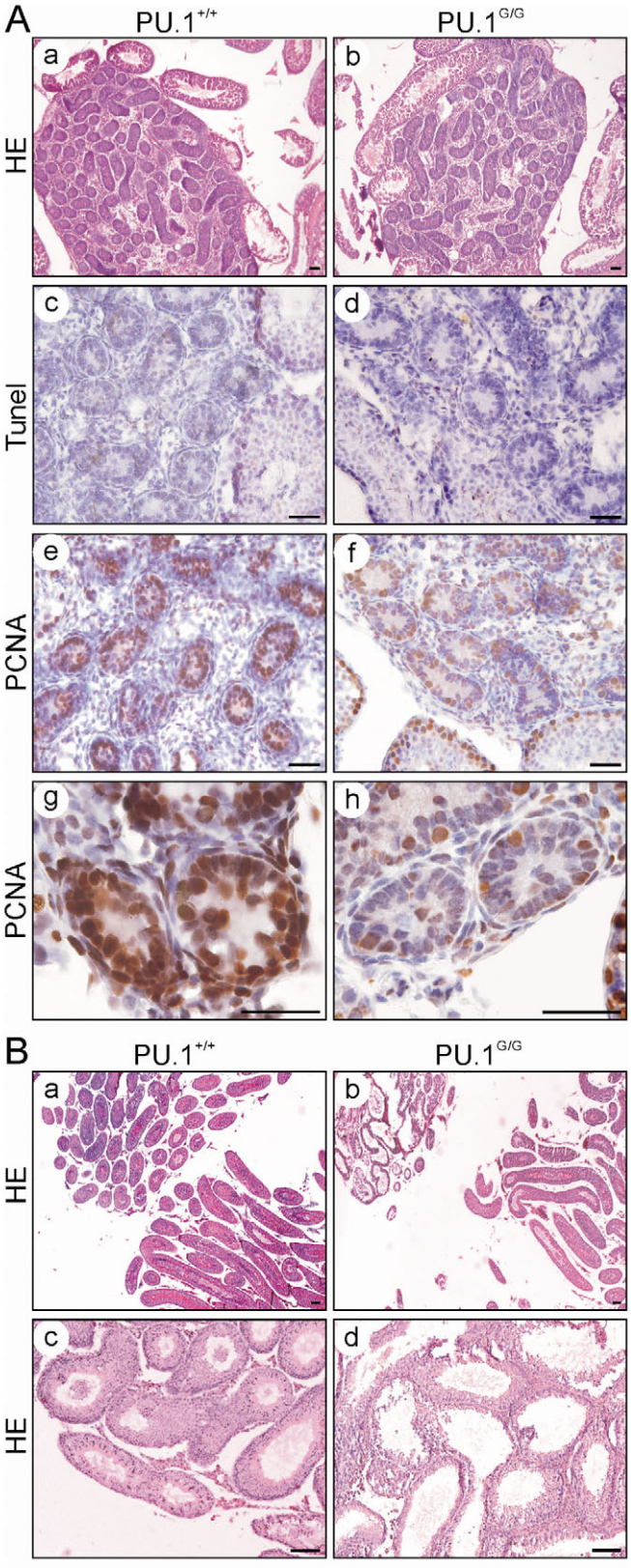


**Fig. 7. Arrest of spermatogenesis in the *PU.1* mutant gonad.** (**Aa-h**) Wild-type E18.5 testes (a,c,e,g) and testes of their *PU.1*$^{G/G}$ littermates (b,d,f,h) were dissected and grafted under the tunica albuginea of recipient nude mice. Histological analysis and PCNA immunostaining were performed 1 week after transplantation. The contorted shape of the peripheral labeled nuclei in h is a known characteristic of Sertoli cells. (**Ba-d**) The same analysis 4 weeks after grafting. At low magnification, the limit of the graft (upper left) and the host tissue (lower right) can be seen in a and b, corresponding in b to that between empty tubules and normal spermatogenesis. Scale bars: 200 μm in Aa,Ab,Ba,Bb; 50 μm in all other panels.

### *PU.1* expression in early embryonic stages and embryonic stem cells

Although not absolutely required for spermatogenesis (Bortvin et al., 2004), stella is the earliest marker of embryonic differentiation; it is already expressed in the blastocyst and later restricted to the germinal lineage (Saitou et al., 2002; Sato et al., 2002) (our unpublished results). The correlation between *PU.1* and stella expression at the late fetal stage led us to check whether, as is stella itself, *PU.1* is a marker of early embryonic development. Confocal microscopy detected GFP fluorescence in *PU.1*$^{G/+}$ heterozygotes as early as the blastocyst stage (E3.5), at which the inner cell mass showed heterogeneous but strong labeling (Fig. 8A). ES cells were accordingly checked and found positive by immunolabeling with anti-PU.1 antibodies (Fig. 8B). In the E6.5 postimplantation embryo (Fig. 8C), GFP fluorescence indicated extensive expression in the epiblast and extraembryonic ectoderm, with a marked concentration in the general area of the node, which was maintained during the following days in a variety of structures. Although both genes are expressed at the same early stage, we could exclude the possibility that the PU.1 protein directly controls stella transcription, as transcripts of the latter were clearly detected in the early *PU.1*$^{G/G}$ embryo (E10.5, data not shown). The pattern of *PU.1* expression is in fact distinct and more extensive than that of stella, *Blimp1* and fragilis, currently considered as the relevant markers of germ line specification and similarly expressed at very early stages (Saitou et al., 2002).

## DISCUSSION

Previous work underlined the pleiotropic roles of the PU.1 protein, which exerts distinct functions in the progenitors of the various hematopoietic lineages (reviewed by Lloberas et al., 1999; Metcalf et al., 2006). The picture must now be expanded to include other classes of stem cells, at least the SSCs of the adult mammalian testis and embryonic PGCs. The list of progenitors under PU.1 control might not yet be complete, considering the widespread expression of the gene during embryogenesis. Considering the lineages for which sufficient data exist, namely the lymphopoietic and erythropoietic progenitors (Back et al., 2004; Iwasaki et al., 2005) and now the germinal lineage, a common picture seems to be emerging, in which the gene would act in the maintenance of cells in the undifferentiated state.

In the germinal differentiation process, our initial finding was a regulated expression of *PU.1* RNA in the adult testis, first in SSCs and early spermatogonia, and later in meiotic spermatocytes. The availability of the null mutant *PU.1*$^{G}$ was initially thought to provide the right tool to unravel its role in these cell types. It turned out, however, that expression of the locus starts at a much earlier stage of germinal differentiation – in fact, at the time of the first specification of germ cells and even before. Furthermore, although the structure of the fetal testis appeared at least grossly normal, it became clear that proliferation of the germ cells was strongly

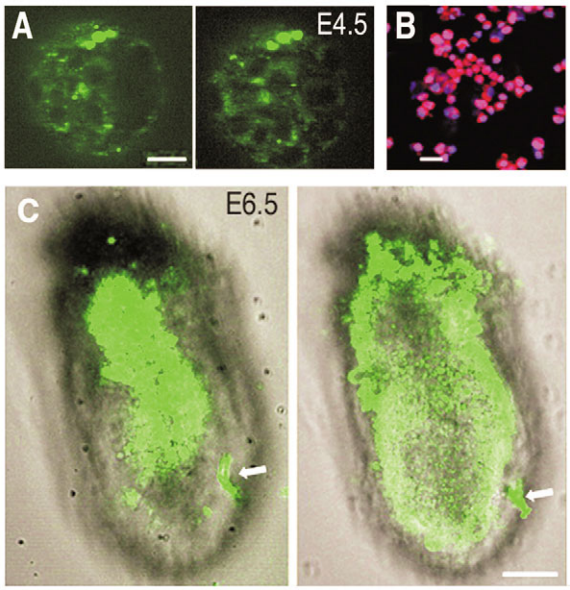


**Fig. 8. *PU.1* promoter expression in the early mouse embryo and ES cells.** (**A**) GFP fluorescence in *PU.1*$^{G/+}$ heterozygote E3.5 blastocyst determined by confocal microscopy. (**B**) Immunodetection of the PU.1 protein in ES cells. (**C**) Confocal microscopy detection of GFP fluorescence in two optical sections of the same E6.5 *PU.1*$^{G/+}$ postimplantation embryo (left, tangential upper section; right, middle section). Arrow shows the area of the node that displays a marked concentration of GFP-positive cells in an as yet undefined structure. Images shown in A and C are representative of those from a series of 20-30 embryos from three litters. Scale bars: 20 μm in A,B; 80 μm in C.

affected, resulting in a much reduced number of gonocytes at the end of fetal development. The remaining cells were in fact unable to enter the normal differentiation pathway, the lack of recognizable germinal progression in the grafted testes defining an absolute requirement for *PU.1* expression in the fetal differentiation of germ cells.

A relationship between *PU.1* and stella expression had been considered because of the concomitant expression of the genes at very early developmental stages and by the absence of stella RNA in the fetal testis of the *PU.1*-null mutants. A direct transcriptional regulation of stella by the PU.1 protein was, however, excluded by the observation that the gene is expressed in the early embryo (E7.5) of the *PU.1*-negative mutant. It must also be remembered that abrogation of stella expression does not affect germinal differentiation (Bortvin et al., 2004), and we also noted that the widespread expression of *PU.1* in the postimplantation embryo does not correspond to that of stella, which is rapidly restricted to the germinal lineage.

The finding that PU.1 is required for the embryonic differentiation of germ cells leaves open the question of its role in the adult testis. An analysis would require a conditional mutation in which gene expression would be arrested exclusively after birth. An intriguing and complex pattern, with expression in SSCs and possibly early spermatogonia, extinguished in differentiated spermatogonia and starting again in meiotic cells, might actually suggest distinct activities in these different cell types. Altogether, a relatively extended domain of expression, starting at the earliest stages of development and required in at least two independent lineages, suggests a common function in the maintenance of embryonic and adult stem cells and in the control of their differentiation. Given the multiplicity of protein variants encoded by the *PU.1* gene (Lloberas et al., 1999), it remains to be determined whether they exert distinct functions in different lineages.

We thank Yan Fantei-Caujolles and Sandra Kanani for expert handling of databases; Jean Claude Hammou for pathological expertise; Christelle Thibault for the generation of microarray data; Cedric Mathews for confocal microscopy analysis; and K. D. Wagner for critical reading of the manuscript. V.O. was supported by scholarships from Région PACA-Inserm and from Association pour la Recherche sur le Cancer, France. N.W. is a fellow of Fondation de France and was supported financially by A. Schedl. This work was made possible by a grant of Ligue Nationale Contre le Cancer as Equipe Labellisée and by grants from Ministère de la Recherche to M.R. and from Inserm (ACI FNS2001) to F.C.

## References

**Back, J., Dierich, A., Bronn, C., Kastner, P. and Chan, S.** (2004). PU.1 determines the self-renewal capacity of erythroid progenitor cells. *Blood* **103**, 3615-3623.

**Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnagar, Y. M. and Dym, M.** (1977). Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J. Cell Biol.* **74**, 68-85.

**Bortvin, A., Goodheart, M., Liao, M. and Page, D. C.** (2004). Dppa3/Pgc7/stella is a maternal factor and is not required for germ cell specification in mice. *BMC Dev. Biol.* **4**, 2.

**Brinster, R. L.** (2002). Germline stem cell transplantation and transgenesis. *Science* **296**, 2174-2176.

**Brinster, R. L. and Zimmermann, J. W.** (1994). Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11298-11302.

**Buaas, F. W., Kirsh, A. L., Sharma, M., McLean, D. J., Morris, J. L., Griswold, M. D., de Rooij, D. G. and Braun, R. E.** (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nat. Genet.* **36**, 647-652.

**Capron, C., Lecluse, Y., Kaushik, A. L., Foudi, A., Lacout, C., Sekkai, D., Godin, I., Albagli, O., Poullion, I., Svinartchouk, F. et al.** (2006). The SCL relative LYL-1 is required for fetal and adult hematopoietic stem cell function and B-cell differentiation. *Blood* **107**, 4678-4686.

**Chung, S. W., Chen, Y. H., Yet, S. F., Layne, M. D. and Perrella, M. A.** (2006). Endotoxin-induced down-regulation of Elk-3 facilitates heme oxygenase-1 induction in macrophages. *J. Immunol.* **176**, 2414-2420.

**Costoya, J. A., Hobbs, R. M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K. E., Wolgemuth, D. J. and Pandolfi, P. P.** (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat. Genet.* **36**, 653-659.

**de Rooij, D. G. and Grootegoed, J. A.** (1998). Spermatogonial stem cells. *Curr. Opin. Cell Biol.* **10**, 694-701.

**Falender, A. E., Freiman, R. N., Geles, K. G., Lo, K. C., Hwang, K., Lamb, D. J., Morris, P. L., Tjian, R. and Richards, J. S.** (2005). Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. *Genes Dev.* **19**, 794-803.

**Giuili, G., Tomljenovic, A., Labrecque, N., Oulad-Abdelghani, M., Rassoulzadegan, M. and Cuzin, F.** (2002). Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. *EMBO Rep.* **3**, 753-759.

**Hogan, B., Costantini, F. and Lacy, L.** (1994). *Manipulating the Mouse Embryo – A Laboratory Manual* (2nd edn). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

**Honaramooz, A., Snedaker, A., Boiani, M., Scholer, H., Dobrinski, I. and Schlatt, S.** (2002). Sperm from neonatal mammalian testes grafted in mice. *Nature* **418**, 778-781.

**Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E. A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T. et al.** (2005). Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* **106**, 1590-1600.

**Lin, Y. and Page, D. C.** (2005). Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. *Dev. Biol.* **288**, 309-316.

**Lloberas, J., Soler, C. and Celada, A.** (1999). The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. *Immunol. Today* **20**, 184-189.

**Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M. et al.** (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**, 1489-1493.

**Metcalf, D., Dakic, A., Mifsud, S., Di Rago, L., Wu, L. and Nutt, S.** (2006). Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. *Proc. Natl. Acad. Sci. USA* **103**, 1486-1491.

**Meuwissen, R. L. J., Offenberg, H. H., Dietrich, A. J. J., Riesewijk, A., van Iersel, M. and Heyting, C.** (1992). A coiled-coiled protein specific for synapsed regions of meiotic prophase chromosomes. *EMBO J.* **11**, 5091-5100.

**Nakamura, T., Arai, Y., Umehara, H., Masuhara, M., Kimura, T., Taniguchi,**

**H., Sekimoto, T., Ikawa, M., Yoneda, Y., Okabe, M. et al.** (2007). PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat. Cell Biol.* **9**, 64-71.
**Oatley, J. M., Avarbock, M. R., Telaranta, A. I., Fearon, D. T. and Brinster, R. L.** (2006). Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc. Natl. Acad. Sci. USA* **103**, 9524-9529.
**Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A. et al.** (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**, 207-213.
**Oulad-Abdelghani, M., Bouillet, P., Decimo, D., Gansmuller, A., Heyberger, S., Dolle, P., Bronner, S., Lutz, Y. and Chambon, P.** (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *J. Cell Biol.* **135**, 469-477.
**Payer, B., Saitou, M., Barton, S. C., Thresher, R., Dixon, J. P., Zahn, D., Colledge, W. H., Carlton, M. B., Nakano, T. and Surani, M. A.** (2003). Stella is a maternal effect gene required for normal early development in mice. *Curr. Biol.* **13**, 2110-2117.
**Peschon, J. J., Behringer, R. R., Brinster, R. L. and Palmiter, R. D.** (1987). Spermatid-specific expression of protamine 1 in transgenic mice. *Proc. Natl. Acad. Sci. USA* **84**, 5316-5319.
**Saitou, M., Barton, S. C. and Surani, M. A.** (2002). A molecular programme for the specification of germ cell fate in mice. *Nature* **418**, 293-300.
**Saitou, M., Payer, B., O'Carroll, D., Ohinata, Y. and Surani, M. A.** (2005). Blimp1 and the emergence of the germ line during development in the mouse. *Cell Cycle* **4**, 1736-1740.
**Sato, M., Kimura, T., Kurokawa, K., Fujita, Y., Abe, K., Masuhara, M., Yasunaga, T., Ryo, A., Yamamoto, M. and Nakano, T.** (2002). Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. *Mech. Dev.* **113**, 91-94.
**Shinohara, T., Orwig, K. E., Avarbock, M. R. and Brinster, R. L.** (2000). Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc. Natl. Acad. Sci. USA* **97**, 8346-8351.
**Tanaka, S. S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R., Yokoyama, M. and Noce, T.** (2000). The mouse homolog of Drosophila Vasa is required for the development of male germ cells. *Genes Dev.* **14**, 841-853.
**Tanaka, S. S., Yamaguchi, Y. L., Tsoi, B., Lickert, H. and Tam, P. P.** (2005). IFITM/Mil/fragilis family proteins IFITM1 and IFITM3 play distinct roles in mouse primordial germ cell homing and repulsion. *Dev. Cell* **9**, 745-756.
**Vasyutina, E., Lenhard, D. C., Wende, H., Erdmann, B., Epstein, J. A. and Birchmeier, C.** (2007). RBP-J (Rbpsuh) is essential to maintain muscle progenitor cells and to generate satellite cells. *Proc. Natl. Acad. Sci. USA* **104**, 4443-4448.
**Vidal, F., Lopez, P., Lopez-Fernandez, L. A., Ranc, F., Scimeca, J. C., Cuzin, F. and Rassoulzadegan, M.** (2001). Gene trap analysis of germ cell signaling to Sertoli cells: NGF-TrkA mediated induction of Fra1 and Fos by post-meiotic germ cells. *J. Cell Sci.* **114**, 435-443.
**Vincent, S. D., Dunn, N. R., Sciammas, R., Shapiro-Shalef, M., Davis, M. M., Calame, K., Bikoff, E. K. and Robertson, E. J.** (2005). The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development* **132**, 1315-1325.
**Wagner, N., Wagner, K. D., Theres, H., Englert, C., Schedl, A. and Scholz, H.** (2005). Coronary vessel development requires activation of the TrkB neurotrophin receptor by the Wilms' tumor transcription factor Wt1.*Genes Dev*. **21**, 2631-2642.
**Wagner, N., Wagner, K. D., Scholz, H., Kirschner, K. M. and Schedl, A.** (2006). Intermediate filament protein nestin is expressed in developing kidney and heart and might be regulated by the Wilms' tumor suppressor Wt1. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **291**, R779-R787.
**Yoshimizu, T., Sugiyama, N., De Felice, M., Yeom, Y. I., Ohbo, K., Masuko, K., Obinata, M., Abe, K., Scholer, H. R. and Matsui, Y.** (1999). Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. *Dev. Growth Differ.* **41**, 675-684.