Absence of the prion protein homologue Doppel causes male sterility

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The agent that causes prion diseases is thought to be identical with PrP^{Sc}, a conformer of the normal prion protein PrP^C. PrP^C-deficient mice do not exhibit major pathologies, perhaps because they express a protein termed Dpl, which shares significant biochemical and structural homology with PrP^C. To investigate the physiological function of Dpl, we generated mice harbouring a homozygous disruption of the *Prnd* gene that encodes Dpl. Dpl deficiency did not interfere with embryonic and postnatal development, but resulted in male sterility. Dpl protein was expressed at late stages of spermiogenesis, and spermatids of Dpl mutants were reduced in numbers, immobile, malformed and unable to fertilize oocytes *in vitro*. Mechanical dissection of the zona pellucida partially restored *in vitro* fertilization. We conclude that Dpl regulates male fertility by controlling several aspects of male gametogenesis and sperm–egg interaction.

Keywords: Doppel/knockout mice/prion protein/spermatogenesis

Introduction

PrP^{Sc}, which is derived by a poorly understood post-translational mechanism from the normal host-encoded protease-sensitive PrP^C (Oesch *et al.*, 1985; Basler *et al.*, 1986), is a major component of the infectious agent that causes transmissible spongiform encephalopathies (TSEs) (Prusiner, 1982). Prion diseases are fatal neurodegenerative disorders that occur naturally in humans and in a variety of animals. A wealth of evidence indicates that PrP^C is essential for the development of prion disease (Aguzzi *et al.*, 2001). Most importantly, mice lacking PrP^C are resistant to experimental scrapie inoculation (Bueler *et al.*, 1993), and familial cases of human TSEs are

characterized by mutations of *Prnp*, the gene that encodes PrP^C (Prusiner, 1998).

Sequence analysis of a cosmid containing *Prnp* revealed that 16 kb downstream of the murine *Prnp* gene, an open reading frame (ORF) is present that encodes a protein, which shares significant homology with PrP^C (Moore *et al.*, 1999). The new protein of 179 residues was christened Dpl for 'downstream of the *Prnp* locus' (or 'doppel', German for 'double') (Moore *et al.*, 1999). The *Prnd* gene coding for Dpl is evolutionarily conserved from humans to sheep and cattle, suggesting an important function of Dpl (Tranulis *et al.*, 2001). Dpl has a lower molecular weight than PrP^C because it lacks the N-terminally located octameric repeats and transmembrane region present in PrP^C. The predicted Dpl protein showed roughly 25% identity with the carboxy-proximal two thirds of PrP^C, and both PrP^C and Dpl are GPI-anchored membrane proteins (Moore *et al.*, 1999; Silverman *et al.*, 2000). Dpl may contain three α helices like PrP^C and two disulfide bridges between the second and third helix, and the structure of Dpl has significant similarity to PrP^C (Lu *et al.*, 2000; Silverman *et al.*, 2000; Mo *et al.*, 2001). Dpl mRNA is expressed at high levels in testes, less in other peripheral organs and at very low levels in brain of adult wild-type mice. However, significant *Prnd* mRNA was detected during embryogenesis (Li *et al.*, 2000b).

The Dpl protein resembles an N-terminally truncated PrP^C protein lacking the octamer repeats. The latter version of PrP^C is capable of PrP^{Sc} propagation (Flechsigg *et al.*, 2000). We have previously investigated the role of Dpl in prion pathogenesis using a neural grafting paradigm. Embryonic stem (ES) cells carrying a homozygous null mutation of the *Prnd* locus, but a normal *Prnp* locus, were found to undergo normal neurogenic differentiation and were capable of giving rise to all neural cell lineages when transplanted into host brains. After inoculation with scrapie prions, Dpl-deficient neural grafts showed spongiosis, gliosis and unimpaired accumulation of PrP^{Sc} and infectivity similar to wild-type neuroectodermal grafts (Behrens *et al.*, 2001). Four polymorphisms in the human *Prnd* gene were detected, but no strong linkage disequilibrium was found between any of these polymorphisms and human prion diseases (Mead *et al.*, 2000; Peoc'h *et al.*, 2000). Taken together, these findings suggest that Dpl is dispensable for prion disease progression and generation of PrP^{Sc}.

Although it was not realized at the time, Dpl was first identified accidentally by generating knock-out mice lacking PrP^C. To elucidate the physiological function of PrP^C, several mouse lines with targeted disruptions of *Prnp* were independently generated. All mutant mouse lines lacked significant portions of the *Prnp* ORF and did not produce PrP^C protein, but

showed two strikingly different phenotypes. Zürich *Prnp*^{0/0} and Edinburgh *Prnp*^{-/-} (named after their cities of origin, Zürich and Edinburgh, respectively), were generated by replacing parts of the *Prnp* ORF with a neomycin phosphotransferase gene and showed only minor electrophysiological and circadian rhythm defects (Bueler *et al.*, 1992; Collinge *et al.*, 1994; Manson *et al.*, 1994; Tobler *et al.*, 1996). In contrast, Nsgsk *Prnp*^{-/-} (which were generated in Nagasaki) developed cerebellar Purkinje cell degeneration causing late-onset ataxia. In the Nsgsk mice, not only the ORF but also 0.9 kb of intron 2, the 5' non-coding region and 0.45 kb of the 3' non-coding region of *Prnp* were deleted (Sakaguchi *et al.*, 1996). Subsequently, the observations made in the Nsgsk mice were independently confirmed in two additional *Prnp* knockout lines, Zürich II and Rcm0, in which the *Prnp* ORF and its flanking regions were replaced by a *lox* sequence and an HPRT cassette, respectively (Moore *et al.*, 1999; Rossi *et al.*, 2001). This enigma was solved through the realization that in the brain of ataxic but not of healthy *Prnp*-mutant mice, *Prnd* mRNA was upregulated (Moore *et al.*, 1999; Li *et al.*, 2000a). An intergenic splicing event places the Dpl locus under the control of the *Prnp* promoter, probably due to the deletion of the *Prnp* intron 2 sequence including its splicing acceptor (Moore *et al.*, 1999). This intergenic splicing event could also be detected at very low levels in wild-type mice, but was greatly enhanced by the absence of the intron 2 splice acceptor (Moore *et al.*, 1999; Behrens and Aguzzi, 2002). The *Prnp* promoter, but not the *Prnd* promoter, is highly active in neuronal cells (Moore *et al.*, 1999; Li *et al.*, 2000a,b; Rossi *et al.*, 2001). Therefore, *Prnd* transcription off the *Prnp* promoter results in overproduction of Dpl in the brain (Moore *et al.*, 1999; Li *et al.*, 2000a; Rossi *et al.*, 2001). Further experiments have demonstrated an inverse correlation between the mRNA levels of *Prnd* and the onset of ataxia. Disease progression was accelerated by increasing *Prnd* levels, supporting the notion that ectopic Dpl expression, but not functional loss of PrP^C, may be responsible for neuronal degeneration in ataxic *Prnp*-deficient mice (Rossi *et al.*, 2001). Interestingly, Nsgsk and Zürich II *Prnp*^{0/0} mice were rescued from neuronal degeneration by introduction of a *Prnp* transgene (Nishida *et al.*, 1999; Moore *et al.*, 2001; Rossi *et al.*, 2001) suggesting that PrP^C can antagonize Dpl neurotoxicity and that the absence of PrP^C is necessary for Dpl to induce cell death. Therefore, the structural similarity between PrP^C and Dpl argues for a similar biological function, but the genetic data demonstrate that they can also have antagonistic function in the regulation of neuronal cell death.

To clarify the physiological function of the Dpl protein, we have generated mice with a homozygous targeted disruption of the *Prnd* gene. Whereas females lacking Dpl were viable and fertile, males without Dpl were sterile. We found that, in the absence of Dpl, spermatids were malformed and showed a defect in acrosome function, which resulted in the inability to achieve oocyte fertilization. Therefore, mice lacking *Prnd* identify Dpl as a critical regulator of male gametogenesis.

Results

Generation of *Prnd*-mutant mice

We addressed the physiological role of Dpl through targeted inactivation of the *Prnd* gene in the mouse. The complete ORF encoding Dpl was replaced by a neomycin resistance gene in ES cells, as described previously (Behrens *et al.*, 2001), and mutant mice were generated (Figure 1A). Heterozygous mice carrying the mutated *Prnd* allele (*Prnd*^{neo}) were intercrossed to obtain homozygous mutant mice lacking Dpl. Both male and female homozygous *Prnd*^{neo/neo} mice were born with Mendelian frequencies (data not shown) and displayed normal growth and survival, demonstrating the absence of obvious detrimental effects of Dpl deficiency on development and growth. To ascertain the successful functional

Fig. 1. Generation of mice lacking Dpl. (A) Schematic representation of the targeting strategy to generate ES cells harbouring a homozygous mutation of *Prnd*. Exons of the *Prnd* gene are represented by rectangles, thin lines represent intronic regions of the *Prnd* locus. The Dpl ORF is represented as a dark gray rectangle. The neomycin resistance gene (PGK-Neo) and the diphtheria toxin α gene (DT α) are represented by light gray rectangles, while loxP sites are shown as black triangles. (B) Northern analysis of total testes RNA from wild-type, *Prnd*^{neo/+} and *Prnd*^{neo/neo} mice, hybridized with a probe encompassing the *Prnd* and *Prnp* ORF. (C) *Prnd*^{+/+}, *Prnd*^{neo/+} and *Prnd*^{neo/neo} males were mated to wild-type females and copulation plugs were recorded in the morning after mating (*Prnd*^{+/+}, *n* = 1; *Prnd*^{neo/+}, *n* = 1; *Prnd*^{neo/neo}, *n* = 2). (D) Epididymal sperm number of *Prnd*^{+/+} and *Prnd*^{neo/neo} males (*Prnd*^{+/+}, *n* = 2; *Prnd*^{neo/neo}, *n* = 2). (E) Motility of epididymal sperm isolated from *Prnd*^{+/+} and *Prnd*^{neo/neo} males (*Prnd*^{+/+}, *n* = 2; *Prnd*^{neo/neo}, *n* = 2). Bars in (D) and (E) represent the standard error of the mean. (F) Similar number of copulation plugs in Doppel expressing and deficient mice, indicative of sexual activity similar to that of controls.

inactivation of the *Prnd* locus, northern blot analysis was performed. *Prnd* mRNA was detected in the testes of *Prnd*^{+/+} and *Prnd*^{+/^{neo}} mice, but was absent in homozygous mutant animals confirming that the *Prnd*^{neo} allele was null (Figure 1B). The levels of *Prnp* mRNA, which is also expressed in testes (Shaked et al., 1999), was unaffected by the absence or presence of Dpl (Figure 1B). Similarly, Dpl protein was present in the testes of *Prnd*^{+/+} and *Prnd*^{+/^{neo}} mice, but was absent in homozygous mutants, whereas PrPC protein was detected in all samples (Figure 1C). As *Prnd*^{neo/neo} mice showed no apparent gross morphological or behavioural abnormalities, we investigated the development of the hematopoietic system in Dpl mutant mice. *Prnd* mRNA was found to be expressed in the spleen, suggesting a potential role for Dpl in lymphoid cells (Li et al., 2000b). However, the immune system appeared to be normally developed. CD4⁺ and CD8⁺ T cell subsets as well as B220⁺ B cells in peripheral blood revealed no obvious alterations. B-lymphocyte development in bone marrow, as analyzed by anti-B220, anti-CD19 and anti-CD25 antibodies, was also unaffected. Spleen cell suspensions showed no abnormalities in marginal zone B cells (CD21^{high}CD23⁻) and follicular B cells (CD21^{med}CD23^{high}) in *Prnd*^{neo/neo} mice (data not shown).

Absence of Dpl causes male sterility

Female *Prnd*^{neo/neo} mice were fertile and yielded litter sizes similar to those of wild-type. In contrast, we noted that male *Prnd*^{neo/neo} mice were infertile. Breeding of 13 *Prnd*^{neo/neo} mice with wild-type females resulted in only a single litter, whereas all tested *Prnd*^{+/+} and *Prnd*^{+/^{neo}} littermate controls were fertile and yielded multiple litters (Table I). Whereas wild-type males produced an average litter size of 5.7 offspring, corresponding to a fertility rate of 100%, Dpl-deficient males were dramatically less fertile (0.7%) (Table I). To determine whether the absence of Dpl caused a defect in breeding and copulation behaviour, *Prnd*^{neo/neo}, *Prnd*^{+/^{neo}} and *Prnd*^{+/+} males were mated to hormone-primed females. *Prnd*^{neo/neo} males showed normal mating behavior: their sexual activity was similar to that of controls as revealed by a normal number of copulation plugs (Figure 1D). To clarify the basis of the infertility, we studied the mature sperm of *Prnd*^{neo/neo} males. The number of spermatozoa in the cauda epididymis of *Prnd*^{neo/neo} males was reduced to 50% of wild-type controls (Figure 1E). Importantly, also the motility of mutant sperm was significantly decreased (Figure 1F). We concluded that the sterility of Dpl-mutant males is not due to behavioural abnormalities, but may be due to a spermatogenesis defect.

Table I. Breeding performance and fertility of *Prnd*^{neo/neo} and control mice

Genotype of males	<i>Prnd</i> ^{+/+}	<i>Prnd</i> ^{+/^{neo}}	<i>Prnd</i> ^{neo/neo}
Number of males mated	5	4	13
Number of females mated	8	8	21
Number of litters	8	19	1
Average litter size	5.7	5.6	0.04
Fertility rate (%)	100	98	0.7

Normal testes development without Dpl

There was no reproducible difference in the average testicular weight between *Prnd*^{neo/neo} mice (185.9 ± 27.1 mg; n = 4) and *Prnd*^{+/^{neo}} littermate controls (175.9 ± 27.8 mg; n = 4). The overall histological appearance of mutant testes was normal (Figure 2A), as were brain, colon, heart, kidney, liver, lung, spleen and thymus (data not shown). Histological examination revealed normal distribution and numbers of spermatogonia and spermatocytes, and vimentin staining confirmed the presence of Sertoli cells (data not shown).

To detect more subtle defects in male gametogenesis, the expression of genes that characterize various stages of sperm development was investigated by RT-PCR. The mRNA levels of BMP-8, Hox1.4, protamine 1 and 2 and several other marker genes analyzed was similar in *Prnd*^{+/+}, *Prnd*^{+/^{neo}} and *Prnd*^{neo/neo} mice (Figure 2B). Therefore, expression of maturation stage-specific genes in testes appears to be normal despite the absence of Dpl. Moreover, TUNEL staining did not reveal differences in the number of apoptotic cells between mutant and control testes (data not shown).

Abnormal spermiogenesis in Dpl-deficient testes

We next investigated the expression pattern of Dpl in testes using immunohistochemistry. Dpl protein was detected in the luminal aspect of wild-type seminiferous tubules (Figure 3A and C; indicated by arrows), but not in

Fig. 2. Normal histology and gene expression in *Prnd*^{neo/neo} testes. (A) Histological analysis of *Prnd*^{+/+} and *Prnd*^{neo/neo} testes. (B) RT-PCR analysis of total testes RNA from wild-type, *Prnd*^{neo/+} and *Prnd*^{neo/neo} mice. Reverse transcription reaction was performed with (+RT) and without (-RT) the addition of the reverse transcriptase to control for DNA contamination of the RNA preparation.

Fig. 3. Dpl is expressed in spermatids. Histological analysis of wild-type (A and C) and *Prnd*^{neo/neo} testes (B and D). Expression varies among individual seminiferous tubuli, a finding suggestive of stage-specific expression. Arrowheads in (A) and (C) indicate Dpl immunoreactivity.

Fig. 4. Dpl is required for late stages of spermatogenesis. Semi-thin sections of *Prnd*^{+/+} (A and C) and *Prnd*^{neo/neo} testes (B and D) were stained with Toluidine blue. Arrows in (A) and (B) indicate round spermatids. Arrows in (C) and (D) indicate elongating spermatids, arrowheads in (D) show early stages of the elongation phase.

testes of Dpl mutant mice (Figure 3B and D). Both round and elongated spermatids were strongly immunoreactive for Dpl (Figure 3C). To analyze spermiogenesis in the absence of Dpl in greater detail, we examined Toluidine blue-stained semithin sections by light microscopy. The initial Golgi phases of acrosome formation did not require Dpl, since the development of round spermatids was normal (Figure 4A and B). However, the transformation of

round spermatids into testicular spermatozoa was abnormal in Dpl-deficient testes. Whereas wild-type spermatids were undergoing synchronous differentiation, the number of spermatozoa was reduced in *Prnd*^{neo/neo} testes, and the regional separation of spermiogenic differentiation stages was disturbed. Mutant spermatids and more differentiated spermatozoa were detectable in close proximity, indicating a partial blockade of spermiogenesis in the absence of Dpl (Figure 4C and D). Differentiated spermatozoa at late stages of spermiogenesis were present at reduced numbers in mutant seminiferous tubules, in agreement with the reduced numbers of mature sperm found in the cauda epididymis. These findings suggested a role for Dpl in spermiogenesis.

The absence of Dpl causes sperm malformations

We next investigated whether the absence of Dpl had any effect on mature sperm. The spermatozoa from mutant males showed several structural abnormalities. In *Prnd*^{neo/neo} spermatozoa, the sperm head was disoriented with respect to the flagellum, and often the flagellum appeared to fold back towards the sperm head instead of being straight and elongated (Figure 5A and B). However, the composition of the mitochondrial sheet was normal in spermatozoa from *Prnd*^{neo/neo} males as shown by mitochondrial staining using mitotracker (Figure 5C and D). Moreover, Acridine orange staining did not reveal impaired chromosomal condensation (data not shown). *Prnd*^{neo/neo} sperm heads were also severely malformed and lacked a discernible well-developed acrosome (Figure 5E–H). As the acrosome is essential for sperm–egg interaction, this defect could explain the sterility of *Prnd*-deficient males.

Table II. In IVF experiments, Dpl-deficient sperms could only fertilize eggs upon mechanical removal of the zona pellucida (ZP)

Genotype of sperm	<i>Prnd</i> ^{+/+}	<i>Prnd</i> ^{neo/+}	<i>Prnd</i> ^{neo/neo}
2-cell stage (cells with intact ZP)	51/66 (77%)	Not performed	0/118 (0%)
2-cell stage (cells with dissected ZP)	31/39 (80%)	74/60 (78%)	54/180 (30%)

Fig. 5. Spermatozoa from Dpl-deficient mice are malformed. (A and B) Photographs of bright field images of spermatozoa isolated from *Prnd*^{+/+} (A) and *Prnd*^{neo/neo} mice (B). (C and D) Spermatozoa isolated from *Prnd*^{+/+} (C) and *Prnd*^{neo/neo} mice (D) were stained with mitochondrial tracker to detect mitochondria (green) and with the DNA stain Hoechst (blue). (E–H) Photographs of bright field images of sperm heads from *Prnd*^{+/+} (E) and *Prnd*^{neo/neo} mice (F–H).

Dpl is required for acrosome function

To gain further insight into the defect caused by the absence of Dpl and to validate the role of Dpl in acrosome function, *in vitro* fertilization (IVF) experiments were performed. After IVF, the percentage of 2-cell embryos recovered in the control group was 77%, but spermatozoa isolated from *Prnd*^{neo/neo} males were unable to fertilize wild-type oocytes (Table II). Spermatozoa from *Prnd*^{neo/neo} males occasionally stuck to, but never penetrated, the zona pellucida. In a further set of experiments, we performed a partial zona dissection (PDZ) to test whether the acrosomal reaction could be triggered in spermatozoa from homozygous mutants. The zona pellucida was partially dissected and IVF was performed with sperm suspension from *Prnd*^{neo/neo} males. Out of 180 PDZ oocytes fertilized with *Prnd*^{neo/neo} spermatozoa, after 24 h, 39 zygotes had progressed to 2-cell stage embryos and 15 zygotes displayed a prominent second polar body, indicative of a fertilization rate of 30% (Table II). Spermatozoa isolated from wild-type males achieved a significantly higher percentage (80%) of fertilization. These data indicated that *Prnd*^{neo/neo} spermatozoa are capable of oocyte fertilization, albeit at a lower frequency than controls, but that they cannot overcome the barrier imposed by the zona pellucida.

Discussion

In this study, we have investigated the biological function of the prion protein homologue Dpl by targeted disruption

of its coding gene *Prnd*. Whereas females lacking Dpl are normal, Dpl-deficient male mice revealed an essential function of Dpl in spermiogenesis and acrosome biogenesis. A number of gene inactivation studies have identified gene products involved in male fertility, but in most cases female reproduction was unaffected, presumably because spermatogenesis and oogenesis are controlled by largely non-redundant genetic programmes (Grootegeod *et al.*, 2000; Wassarman and Litscher, 2001). It has previously been observed that a significant amount of PrP^C is expressed in mature spermatozoa. The PrP protein found in testes was truncated in its C-terminus in the vicinity of residue 200 (Shaked *et al.*, 1999). A protective role for PrP against copper toxicity has been proposed since sperm cells originating from *Prnp*-deficient mice were significantly more susceptible to high copper concentrations than sperm from wild-type mice. However, male *Prnp*^{0/0} knock-out mice are not sterile and produce normal litter sizes. As we have shown here, PrP expressed in testes is not capable of compensating for the absence of Dpl, suggesting at least partially non-redundant functions of these two proteins. Also, PrP^C protein levels are comparable in testes and a number of other organs from *Prnd*^{neo/neo} and control mice (data not shown). However, it cannot be excluded that Dpl masks a minor function of PrP^C in testes development or spermiogenesis, and that males lacking both PrP^C and Dpl function would display a more severe defect than *Prnd*^{neo/neo} single mutant mice.

Spermatozoa are formed from spermatogonial stem cells by a tightly orchestrated process that can be divided into three sequential phases of cell proliferation and differentiation (Grootegeod *et al.*, 2000). First, there is an extensive multiplication and proliferation of spermatogonial stem cells to produce an optimal number of spermatogonia, which give rise to primary spermatocytes, and also to maintain a pool of stem cells. Secondly, the primary spermatocytes undergo a lengthy meiotic prophase, followed by the first meiotic division resulting in the formation of two secondary spermatocytes, each undergoing the second meiotic phase to produce four haploid round spermatids. Finally, there is a gradual remodelling of the nuclear and cellular components of round spermatids during transformation into sperm cells by a process referred to as spermiogenesis (Abou-Haila and Tulsiani, 2000; Grootegeod *et al.*, 2000).

The initial phases of spermatogenesis appear to proceed normally in the absence of Dpl, since the production of round spermatids is unaffected in *Prnd*^{neo/neo} mice. The elongation of the spermatid characterizes the late stages of spermiogenesis when the sperm cell flattens as the chromatin condenses and the acrosome becomes compact.

The spermatids are in close association with the Sertoli cells until spermatozoa are fully differentiated. Spermatozoa then detach from Sertoli cells, and at this point, the spermatozoon becomes an isolated cell characterized on the basis of its electron-dense nucleus and fully developed acrosome (Abou-Haila and Tulsiani, 2000). Dpl protein expression is confined to round and elongated spermatids, and the presence of Dpl appears to be required for the correct development of the sperm head and the acrosome.

It is worth noting that mature spermatozoa from *Prnd*^{neo/neo} males also display reduced motility, although the flagella of *Prnd*^{neo/neo} sperm developed normally as judged by transmission electron microscopy, and the mitochondrial sheath was correctly configured. At the onset of sperm elongation, the acrosomic vesicle undergoes a series of highly complex morphological changes that shape the sperm cell. Failure of acrosome biogenesis can result in sperm malformations (Abou-Haila and Tulsiani, 2000; Kang-Decker *et al.*, 2001). At present, the molecular mechanism of Dpl-regulated acrosome development is unclear. Dpl may be present on the acrosomic vesicles through its GPI-anchor, and possibly directly participate in acrosome morphogenesis. Alternatively, Dpl may regulate acrosome formation in a more indirect way. We have also observed that sperm isolated from *Prnd*^{neo/neo} mice is greatly impaired in the sperm-egg interaction, and that *Prnd*^{neo/neo} spermatozoa fail to trigger the acrosomal reaction. Oligosaccharides have been strongly implicated as being responsible for both sperm binding and signalling for the acrosome reaction, but the composition and structure of the essential carbohydrate moieties remain controversial (Wassarman and Litscher, 2001). Dpl is a highly glycosylated protein that has been shown to be mainly located outside of the plasma membrane (Moore *et al.*, 1999; Silverman *et al.*, 2000). It is plausible, but not yet proven, that Dpl present on the sperm plasma membrane is directly involved in the sperm-egg interaction.

A better characterization of the genetic factors of male infertility in humans is required to improve the precision of diagnosis and counselling for couples, and to arrive at new methods of contraception targeting the male (Grootegeod *et al.*, 2000). Therefore, the identification of genetic causes of male infertility has practical clinical relevance. It will be interesting to determine whether Dpl mutations are present in human patients suffering from infertility. Finally, with the need to develop new methods of contraception, in particular methods targeting the male, inhibition of Dpl function may provide a novel target for contraceptive intervention.

Materials and methods

Targeting the *Prnd* locus in ES cells

The genomic *Prnd* locus was cloned from genomic DNA of TC-1 ES cells by PCR. In the targeting vector, the *Prnd* ORF was replaced with a floxed neomycin resistance selection cassette. A diptheria toxin (*DTα*) gene was inserted for selection against random integrants. Gene targeting was performed as described (Behrens *et al.*, 2001).

Histological analysis and TUNEL assay

Tissues were fixed in 4% formaldehyde, dehydrated, embedded in paraffin and sectioned (5 µm). Sections were stained with Harris

haematoxylin and eosin (Sigma). TUNEL stains were performed as described (Behrens *et al.*, 1999). For immunohistochemistry, tissue sections were fixed with acetone, air-dried and blocked in 2% normal donkey serum (Jackson ImmunoResearch Laboratories). Anti-Dpl antibody (G-20; sc-16863, Santa Cruz, CA) diluted 1:20 in 2% normal donkey serum was used as primary antibody. Donkey-anti-goat AP (Jackson) diluted 1:80 in 5% normal donkey serum was used as a secondary antibody. Sections were counterstained with new fuchsin.

Northern and western blot analysis

Mice were killed and RNA isolation, protein isolation and northern and western blots were performed as described (Behrens *et al.*, 1999). The *Prnd* and *Prnp* ORFs were used as probes for northern blot analysis. For western blot analysis, we used a polyclonal anti-Dpl antiserum (kindly provided by Dr Hermann M. Schätzl).

FACS analysis

Two- and three-colour fluorescence-activated cell sorting (FACS) analyses were performed on a FACS Calibur (Becton Dickinson, CA) as described recently (Heppner *et al.*, 2001). Bone marrow cells were prepared by flushing the femur with 5 ml ice-cold FACS buffer (phosphate-buffered saline, 2% fetal calf serum, 10 mM EDTA pH 8, 15.3 mM NaN₃). Spleen cell suspensions were obtained by meshing total spleen through a 40 µm nylon cell strainer (Falcon). Mouse blood was obtained retro-orbitally. The following anti-mouse antibodies were used (all PharMingen, Europe): peridinin chlorophyll-a protein (PerCP)-labelled anti-B220, fluorescein isothiocyanate (FITC)-labelled anti-CD21, phycoerythrin (PE)-labelled anti-CD19, FITC-labelled anti-CD25, PE-labelled anti-CD23, PE-labelled anti-CD8 and FITC-labelled anti-CD4. Lysis of red blood cells was performed with FACS lysing solution (Becton Dickinson) according to the manufacturer's protocol. Living cells were gated using a combination of forward scatter and side scatter. Data were acquired with CellQuest software (Becton Dickinson); analyses were performed with WinMDI (Version 2.8, available at <http://facs.scripps.edu>).

RT-PCR

Total testes RNA from wild-type, *Prnd*^{neo/+} and *Prnd*^{neo/neo} mice was reverse transcribed using the Geneamp kit (Roche) according to the manufacturer's instructions. The primer sequences used for PCR amplification are available upon request.

Semen processing and examination

Testis and epididymis were dissected from both sides of each mouse and the weight was measured. The cauda epididymis was excised and immediately punctured in 500 µl human tubal fluid (HTF) medium at 37°C. After 5 min, a 6 µl drop of medium with dispersed sperm cells was placed on a warmed glass slide and covered with a coverslip (24 × 24 mm). Progressive motility was estimated by phase-contrast microscopy at a magnification of ×200 (Olympus BX50, UplanFI 20x/0.50 Ph1). The sperm concentration was measured 30 min after puncturing the epididymis, using an improved Neubauer hemocytometer counting chamber.

Sperm viability was determined 5 min after puncturing the epididymis by dual DNA staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probes Europe, Leiden, NL) using SYBR-14 in combination with propidium iodide (PI). The SYBR-14 (component A) was diluted with anhydrous dimethyl sulfoxide (DMSO) 1:50 (SYBR-14 working solution), while PI (component B) was used in the original concentration. One microlitre of the SYBR-14 working solution was added to 100 µl of semen. After incubation of 5 min at room temperature, 1 µl of component B was added. Five minutes later, 5 µl of stained semen were placed on a slide, covered with a coverslip (24 × 24 mm) and examined using fluorescence microscopy (Olympus BX50, UPlanApo 40x/0.85, FITC filter, high pressure Hg-lamp). Different sequences of fluorescence-stained spermatozoa were monitored by connecting a video camera (SANYO VCC-2972) to the microscope. At least 200 sperm cells were counted on the screen and the percentage of green fluorescing spermatozoa was defined as viability.

IVF and PZD

Cumulus masses of super ovulated B6D2F1 females were collected in HTF medium with 300 iU hyaluronidase/ml. The cumulus-free oocytes were introduced into a drop of 0.3 M sucrose solution (to increase the perivitelline space) but without bovine serum albumin (BSA; to attach the oocytes on the dish surface). The zona pellucida was partially dissected by a single downward motion with a 30G needle. The attached oocytes

were then removed from the dish surface by adding medium with 4% BSA. PDZ oocytes were washed gently in HTF medium and transferred into a 200 µl fertilization drop. For IVF, 60 µl of sperm suspension isolated from the epididymis (after 1 h culture for capacitation) were added.

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