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## Genetic Vasectomy—Overexpression of Prm1-EGFP Fusion Protein in Elongating Spermatids Causes Dominant Male Sterility in Mice

Sabine Haueter<sup>1</sup>, Miyuri Kawasumi<sup>2</sup>, Igor Asner<sup>1</sup>, Urszula Brykczynska<sup>3</sup>, Paolo Cinelli<sup>1</sup>, Stefan Moisyadi<sup>2</sup>, Kurt Bürki<sup>1</sup>, Antoine H.F.M. Peters<sup>3</sup>, and Pawel Pelczar<sup>1,\*</sup>

<sup>1</sup>Institute of Laboratory Animal Science, University of Zurich, Switzerland <sup>2</sup>Institute for Biogenesis Research, University of Hawaii, Hawaii <sup>3</sup>Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

### Summary

Transgenic mice are vital tools in both basic and applied research. Unfortunately, the transgenesis process as well as many other assisted reproductive techniques involving embryo transfer rely on vasectomized males to induce pseudopregnancy in surrogate mothers. Vasectomy is a surgical procedure associated with moderate pain and must be carried out under full anaesthesia by qualified personnel. Eliminating the need for vasectomy would be beneficial from the economic and animal welfare point of view. Our aim was to develop a transgene-based alternative to the surgical vasectomy procedure. We generated several transgenic mouse lines expressing a Protamine-1 (Prm1) EGFP fusion protein under the transcriptional and translational regulatory control of *Prm1*. Male mice from lines showing moderate transgene expression were fully fertile whereas strong overexpression of the Prm1-EGFP fusion protein resulted in complete and dominant male sterility without affecting the ability to mate and to produce copulatory plugs. Sterility was due to impaired spermatid maturation affecting sperm viability and motility. Furthermore, sperm having high Prm1-EGFP levels failed to support preimplantation embryonic development following Intracytoplasmic Sperm Injection (ICSI). The “genetic vasectomy system” was further improved by genetically linking the dominant male sterility to ubiquitous EGFP expression in the soma as an easy phenotypic marker enabling rapid genotyping of transgenic males and females. This double transgenic approach represents a reliable and cost-effective “genetic vasectomy” procedure making the conventional surgical vasectomy methodology obsolete.

### Keywords

vasectomy; transgenesis; protamine; spermatozoa; fertilization

### INTRODUCTION

Virtually all techniques used to generate transgenic and knock-out mice rely on Embryo Transfer (ET) as a means of producing animals from embryos manipulated in vitro. Strain

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\*Correspondence to: Pawel Pelczar, Institute of Laboratory Animal Science, University of Zurich, Sternwartstrasse 6, CH-8091 Zurich, Switzerland. pawel.pelczar@ltk.uzh.ch.

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rederivation by ET is also the most frequent method of eliminating disease pathogens and parasites from laboratory strains of mice. Presently, the ET procedure requires the preparation of pseudopregnant females by mating them with vasectomized males. Only pseudopregnant foster mothers can subsequently implant graft embryos and until now, mating of females with vasectomized males is the only reliable way of inducing pseudopregnancy in mice. The vasectomy procedure is painful and must be carried out by trained personnel with the animal receiving full anesthesia followed by a recovery period and analgesic regime. Eliminate of the need for vasectomy would not only simplify the ET process but is also highly desirable from the animal welfare point of view. One possible strategy would be to use male mice that are capable of copulation but are unable to produce offspring due to a genetic defect in sperm maturation.

In a variety of species including mammals, the genome in male germ cells undergoes a major remodeling of its chromatin during the final stages of spermatogenesis (Rousseaux *et al.*, 2008). In somatic cells, the genome is packaged in a nucleosomal configuration consisting of DNA wrapped around octamers of different histone proteins. In elongating spermatids, nucleosomes become successively replaced by transition proteins and protamines. Protamines are small highly basic proteins that are required for packaging of the DNA into a compacted state. In mouse, *Prm1* and *Prm2* serve crucial and nonredundant functions in the nuclear reorganization of elongating spermatids during the final stages of sperm development. Remarkably, heterozygosity for either the *Prm1* or *Prm2* allele impairs spermatid nuclear condensation and induces male sterility (Cho *et al.*, 2001). Furthermore, injection of sperm from *Prm2* heterozygous males into the cytoplasm of an oocyte does not support embryonic development (Cho *et al.*, 2003). These data therefore suggest that proper levels of Prm1 and Prm2 proteins are required for effective remodeling of the spermatid nucleus during spermiogenesis. Additional studies showed that premature overexpression of mouse *Prm1* can also cause dominant male sterility. The *Prm1* gene is transcribed in round spermatocytes, while the resulting mRNA is translated about four days later in late elongating spermatids (Peschon *et al.*, 1987). This translational delay is brought about by regulatory elements residing in the 3'UTR of the *Prm1* mRNA (Zhong *et al.*, 2001). Previous studies have shown that premature translation of Prm1 in round spermatids, brought about by replacement of the *Prm1* 3'UTR with a heterologous 3'UTR, leads to defects in spermatid maturation and results in the absence of sperm and subsequent male sterility (Lee *et al.*, 1995) albeit the penetrance of the phenotype is not complete in all genetic backgrounds.

Transgene-based approaches to induce dominant males sterility using *Prm1* could hold great promise as possible genetic alternatives for surgical vasectomy. Here we describe the development of a transgenic mouse strain that can efficiently replace the vasectomy procedure. Transgenic overexpression of a Prm1-EGFP fusion protein induces dominant male-sterility with full penetrance. In addition we have generated strains where male sterility is genetically linked to ubiquitous EGFP expression in the soma making it easy to identify transgenic animals without marking and taking of biopsies. This double transgenic approach represents a reliable and cost-effective procedure, outperforming the classical surgical vasectomy methodology.

## MATERIALS AND METHODS

### Mouse Strains

C57BL/6 x DBA/2 (B6D2F2) hybrid mice were obtained from our own breeding colony. CD-1 mice were obtained from Charles River Laboratories, Germany. All animal were housed in accordance with Swiss Federal Animal Protection Law. All experiments were

carried out in accordance with Swiss Federal Guidelines under the auspices of the animal experiment licence granted by the Cantonal veterinary authority (Zurich licence 42/2005).

## DNA

The mouse *Prm1* locus including the promoter as well as the full length coding sequence was amplified by PCR from C57BL/6J genomic DNA using following primers: (5'Prm1-5'GTCTAGTAATGTCCAACACCTCCC3', 3'Prm1-5'AGATCTGTACAGGTGGCTTGG3'). The fragment was cloned in vector pGEM-T-easy (Invitrogen). The complete EGFP open reading frame was inserted just upstream of the *Prm1* STOP codon using inverted PCR and the following primers (Prm1CDS-rev-5'GTATTTTTTACACCTTATGGTGTATG3', Prm1UTR-fwd-5'TAGATGCACAGAATAGCAAGTCC3' to yield plasmid pGEM-Prm1-EGFP.

## Generation of Transgenic Mice

pGEM-Prm1-EGFP was released from vector sequences using *AseI* and *PvuII*. The resulting 2.7 kb fragment was purified and microinjected into B6D2F1 zygotes. Transgenic founder were identified by performing PCR on genomic DNA isolated from tail biopsies using the following primers (GFP290-5'-5'GCACGACTTCTTCAAGTCCGCCATGCC3', GFP290-3'-5'GCGGATCTTGAAGTTCACCTTGATGCC3').

## Vasectomies

Nontransgenic littermates were vasectomized and used as a control group in mating experiments involving genetically vasectomized transgenic males. Vasectomy was carried out on males between the age of six to eight weeks. The vasectomized males were allowed to recover for a period of two weeks prior to subsequent mating.

## Mating/Plug Assays

Test male mice of 10–15 weeks were housed with two wild-type (wt) CD1 females (Charles River Laboratories) for a test period of five days followed by a two-day rest period during which the male and the females were separated into their own cages. Each test group consisted of three males aged 10–15 weeks at the beginning of the experiment. During the test period, females were checked every morning for the appearance of a vaginal plug. Plugged females were removed and replaced by fresh females. The plugged females were maintained and housed individually in order to score for pregnancy. The complete experiment for every male consisted of eight test rounds carried out over an eight-week period.

## RNA Extraction

Following dissection, the testes were halved and preserved in 2 ml of RNA later (Ambion-Applied Biosystems). For each sample, half a testis was transferred in 600 ml RLT buffer containing 143 mM β-Mercaptoethanol (Qiagen) and homogenized using a Miller Mill MM 300 (Retsch). Two homogenization rounds of 2 min at 20Hz were performed. The total RNA from the homogenized testes was then extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The total RNA of each testis was eluted in 50 ml RNase-free water (Qiagen). The quality of each sample was assessed using a Bioanalyzer Nano Chip (Agilent), and the RNA concentration measured by spectrophotometry on a NanoDrop ND 1000 (Thermo Scientific).

## Reverse Transcription

for each line, the total RNA was reversed transcribed into cDNA. 2 μl Oligo d(T)<sup>12–18</sup> Primer (Invitrogen), 2 μl 10 mM dNTP Mix (Invitrogen), were added to 500 ng total RNA

and RNase-free water (Qiagen) was added to each sample to obtain a final volume of 26  $\mu$ l. After a 5-min denaturation step at 65°C, 8  $\mu$ l 5XFirst Strand Buffer (Invitrogen), 2  $\mu$ l 0.1  $\mu$ M DTT (Invitrogen), 2  $\mu$ l RNAsin RNase Inhibitor 40U/ $\mu$ l (Promega), and 2  $\mu$ l Superscript III Reverse Transcriptase 200U/ $\mu$ l (Invitrogen), were added to each sample. For the negative controls 2  $\mu$ l RNase-free water were added instead of Superscript. The reverse transcription was carried out for 2 h at 50°C and 15 min at 70°C.

### Real-Time PCR

The expression levels of the *Prm1* gene and the Prm1-EGFP fusion gene were assessed by Real-Time PCR. Two different Real-Time PCR experiments were performed using different sets of primers. For the Prm1 Real-Time PCR, the forward and the backwards primers were located in the *Prm1* gene (Prm1\_FWD: 5'CCAGCACCAT GGCCAGATAC3', and Prm1\_BKD: 5'GATGTGGCGAGAT GCTCTTG3'). For the Prm1-EGFP Real-Time PCR the forward primer was located in the *Prm1* gene (Prm1\_FWD: 5'CCAGCACCATGGCCAGATAC3'), and the backward primer was located in the EGFP gene (EGFP\_BKD: 5'GGGTGTCGCCCTCGAACTTCACCTCGGCG3'). In both experiments Real-time PCR reactions of the  $\beta$ -Actin gene were performed for normalization purposes. The primers for the  $\beta$ -Actin were: Actin-FWD (5'CATCCAGG CTGTGCTGTCCCTGTATGC3') and Actin-BKD (5'GATC TTCATGGTGCTAGGAGCCAGAGC3'). For both Real-Time PCR reactions, the samples were prepared as follows: 2  $\mu$ l cDNA, 10  $\mu$ l Quantitect SYBR Green PCR MasterMix, 1  $\mu$ l Forward primer 10  $\mu$ M, 1  $\mu$ l Backwards primer 10  $\mu$ M, and 6  $\mu$ l Rnase-Free water (Qiagen). The Real-Time PCR reactions were performed on a Rotor-Gene Real-Time PCR machine (Corbett Research). The amplification program of the Prm1 fragment consisted in a 10 min denaturation phase at 94°C followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The amplification program of the Prm1-EGFP fusion gene fragment consisted in a 10 min denaturation phase at 94°C followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s.

### Western Blotting

Testes were homogenized with RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl). Protein concentration was determined with the BCA-Method (Pierce). Samples (10  $\mu$ g of protein per lane) were subjected to SDS-PAGE and blotted onto PVDF membrane (Millipore) at 100 V for 1.5 h at 4°C. Immunodetection was performed with rabbit antibodies against GFP (Abcam: ab290) used at a 1/5,000 dilution, followed by incubation with horseradish peroxidase conjugated anti rabbit antibodies (Santa Cruz Biotechnology; sc-2030) at a 1/5,000 dilution. Chemiluminescent visualization was performed as recommended by the supplier of the chemiluminescence blotting kit (Roche Diagnostics).

### Histology and Microscopic Analysis

Testes and epididymi of mature transgenic males and their control siblings were dissected and fixed in 3% Paraformaldehyde in PBS for 24 h at 4°C. After washing with PBS, tissues were incubated in 30% sucrose (w/v in water) for 24 h at 4°C. Subsequently, tissues were embedded in OCT embedding matrix and frozen on dry ice. Ten micrometer cryosections were generated using the Microm HM560 and subsequently embedded into Vectashield (Vector) mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). Confocal microscopy was performed using the LSM510 META system (Zeiss).

## Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) was carried out essentially as described (Kimura and Yanagimachi, 1995). Metaphase II (MII) Oocytes were collected from superovulated 8- to 12-week-old B6D2F1 females induced by intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG), followed by injection of 5 IU human chorionic gonadotrophin (hCG) 48 h later. Matured oocytes were collected from the ampullary region of oviducts 13–15 h after hCG injection. Oocytes were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (359 units/mg solid) in HEPES-CZB medium, rinsed and kept at room temperature (26°C) in fresh HEPES-CZB medium before sperm injection. Epididymal spermatozoa were collected from 8- to 12-week-old males by “swim-up” procedure in HEPES-CZB medium. In the case of the line *tg38* where no motile spermatozoa were observed, spermatozoa were released by dissociating the dense sperm mass in HEPES-CZB medium. Spermatozoa were collected and placed in a 20  $\mu$ l droplet containing a 12% (w/v) polyvinylpyrrolidone (PVP; Mr 360,000) prepared in HEPES-CZB. A single spermatozoon was drawn, tail first, into the injection pipette in such a way that its neck was at the opening of the pipette. The head was separated from the tail by applying a few piezo-pulses to the neck region. The tail-less spermatozoon was injected into an oocyte.

## Embryo Culture

All oocytes used for ICSI experiments were cultured for 5 days in M16 medium (Sigma) at 37°C under 5% CO<sub>2</sub> in air. Oocyte activation as judged by appearance well-developed pronuclei and a distinct second polar body as well as subsequent embryonic development was followed with daily observations.

## RESULTS

### Overexpression of Prm1-EGFP Causes Dominant Male Sterility

Our approach aimed at impairing the maturation and production of spermatozoa by interfering with the histone-to-protamine exchange during the final stages of spermatogenesis. Towards this goal, we set out to create a dominant-negative variant of the Prm1 protein that would interfere with either proper DNA condensation during sperm maturation and/or decondensation following fertilization. We used the *Prm1* genomic locus as a template to generate a transgenic construct containing the regulatory elements of the mouse *Prm1* gene (promoter and 3'UTR) to selectively overexpress a fusion protein of Prm1 and EGFP in elongating spermatids (see Fig. 1). We inserted the EGFP coding sequence following the last amino acid coding triplet of the Prm1 protein. The resulting construct was linearized and injected into pronuclei of B6D2F1 oocytes resulting in a total of seven transgenic F0 founders, three females and four males respectively. All the attempts to breed the four male founders failed despite repeated successful mating as demonstrated by appearance of copulatory plugs. The three female founders bred successfully with B6D2F1 male mice giving rise to three transgenic lines, *Prm1-EGFP tg36* (*tg36*), *Prm1-EGFP tg37* (*tg37*), and *Prm1-EGFP tg38* (*tg38*). Regular breeding showed that hemizygous males from the *tg36* and *tg37* lines could breed successfully while males of line *tg38* consistently failed to produce offspring.

The sterility of the line *tg38* was tested in more detail. We assessed the mating performance of hemizygous *tg37* and *tg38* males as well as of normal and vasectomized males that served as positive and negative controls respectively (see Fig. 2). These analyses showed that though males from all genotypes could efficiently produce copulatory plugs (Fig. 2A), only normal nontransgenic littermates as well as *tg37* hemizygous mice could impregnate their partners (Fig. 2B) and produce normal size litters (Fig. 2C).

## Dominant Sterility Correlates With Prm1-EGFP Expression Levels

Different transgenic lines generated by pronuclear microinjection frequently show different levels of transgene expression due to position effects. We investigated whether lines *tg37* and *tg38* express different mRNA and protein levels of the *Prm1-EGFP* transgene. Quantitative real time PCR performed on total RNA isolated from testes of three-month-old male mice showed no difference in total levels of Prm1 message between *tg37*, *tg38*, and wild-type mice (Fig. 3A) indicating that any differences in Prm1EGFP transcript level were masked by much higher levels of the endogenous Prm1 message. However, comparison of Prm1-EGFP RNA levels between the two transgenic lines showed that Prm1-EGFP transcripts levels were roughly 7-fold higher in *tg38* males compared with *tg37* males (Fig. 3B). This difference correlated with approximately 30-fold higher levels of the fusion protein in whole testis extracts as detected in western blot analyses using an anti-EGFP antibody (Fig. 3C). It appears therefore that the higher levels of Prm1-EGFP expression correlate with and are most likely the cause of the dominant sterility phenotype observed in line *tg38*.

## Overexpression of Prm1-EGFP Induces Spermiation Defects During Spermiogenesis

To determine the possible mechanism underlying the sterility of line *tg38*, we performed morphological and histological analysis of testes and epididymi. The overall architecture and the tubular structures of both organs was normal in both transgenic lines (Figs. 4A–C and 5). In general, EGFP levels were higher in testes of *tg38* versus *tg37* males (Fig. 4A), consistent with the Prm1-EGFP protein levels measured in testis extracts (Fig. 3C). In caput epididymi, EGFP levels were comparable between the two strains; in caudal epididymi; however, EGFP levels were greatly reduced in *tg38* versus *tg37* males (Fig. 4A). Histological analyses revealed that the overall lower EGFP levels in caudal epididymi of *tg38* males correlated with a strongly reduced number of spermatozoa (Fig. 4B,C). Nevertheless, individual spermatozoa of *tg38* males show a much greater EGFP fluorescence when compared to those of *tg37* (Fig. 4C). Sperm quantification analyses of epididymal tissues confirmed that while the number of spermatozoa seemed comparable in the epididymi of both wt and *tg37* males, they were strongly reduced in both caudal and caput epididymi of *tg38* males (Fig. 4D).

These data suggest that a high level of Prm1-EGFP expression does not necessarily impair the development of spermatids but would affect sperm viability.

Timely translation of the Prm1 message is crucial for proper spermatid development (Zhong *et al.*, 2001). In transgenic approaches, translation of Prm1 at the time of its transcription affects the morphogenesis of spermatid heads in early elongating spermatids and causes a failure to release spermatids from the seminiferous epithelium. In more highly expressing transgenic animals, premature translation of Prm1 caused a complete spermatogenic arrest at the round spermatid stage (Lee *et al.*, 1995). In light of these findings, we analyzed the timing of Prm1-EGFP expression in *tg37* and *tg38* lines. In both lines, EGFP signal is weakly detectable in elongating spermatids at Stage XII of the seminiferous epithelium (see Fig. 5). EGFP expression increases from Stage I onwards, mimicking expression of endogenous Prm1 protein. These data indicate that the long *Prm1-EGFP* transcript is properly subjected to translational repression via its 3' UTR, even in the *tg38* line. Quantitative imaging analyses revealed higher levels of Prm1-EGFP in *tg38* versus *tg37* elongated spermatids (e.g. at stage VI and IX). Moreover, the release of elongated spermatids into the lumen of the seminiferous tubules was partially impaired in *tg38* males but not in *tg37* males as indicated by the presence of highly condensed EGFP-positive nuclei at all stages of the seminiferous epithelium (arrow heads in Fig. 5B). In summary, Prm1-EGFP overexpression does not fully block spermiogenesis as observed in transgenic animals

prematurely expressing Prm1. Instead, it affects the development of elongated spermatids in a more subtle manner.

### Overexpression of Prm1-EGFP Impairs Developmental Potential of Spermatozoa

To test the developmental potency of *tg38* spermatozoa, we performed in vitro fertilization (IVF). Unlike spermatozoa of wild-type and *tg37* mice, those of *tg38* males failed to fertilize oocytes since they were completely immotile. To ascertain whether the *tg38* spermatozoa are in principle capable of fertilizing an oocyte and supporting early embryonic development, we performed ICSI experiments using sperm of wild-type and both transgenic lines (Table 1). The spermatozoa of wild-type and *tg37* mice were able to efficiently activate oocytes and support early embryo development at similar rates. In marked contrast, *tg38* spermatozoa failed in the majority of attempts to activate the oocyte and to support embryonic development.

### Linking Dominant Sterility to Full-Body EGFP as a Phenotypic Marker

The nature of the Prm1-EGFP based male sterility requires that the transgene is transmitted in crosses between hemizygous transgenic females and wild-type males. Such breeding scheme requires conventional genotyping since only 50% of the progeny carries the *Prm1-EGFP* transgene. To simplify the breeding and to eliminate the need for taking biopsies, we generated transgenic lines where the *Prm1-EGFP* transgene is genetically linked to the *CAG-EGFP* transgene that is strongly expressed in all mouse tissues (Okabe *et al.*, 1997) and thus can serve as an easy phenotypic marker. Genetic linking was achieved by coinjection of linearized plasmid DNA of the *Prm1-EGFP* and *CAG-EGFP* transgenes. We obtained several transgenic founders containing both transgenes. One of the female double-transgenic founders gave rise to the *Prm1-EGFP*<sup>tg1432</sup> (*tg1432*) mouse line in which the dominant male sterility reliably cosegregated with full-body EGFP expression (Fig. 6A). Currently, line *tg1432* has been backcrossed for 5 generations using wt B6D2F1 males and yielding over 200 transgenic-sterile male offspring. The sterility trait persisted in subsequent generation as indicated by persistent conception failure. (data not shown) as well as low epididymal sperm counts observed in subsequent generations (Fig. 6B). In all generations, full-body EGFP expression cosegregated with *Prm1-EGFP* mediated male sterility (Fig. 6A, C).

## DISCUSSION

Proper remodeling of chromatin states during spermiogenesis is essential for male fertility. Previous studies indicated that particularly the timing of expression and proper gene dosage of Protamine 1 and 2 are essential (Cho *et al.*, 2001; Zhong *et al.*, 2001). Here we show that increased expression of a Prm1-EGFP fusion protein at the time of remodeling induces dominant male sterility. *Prm1-EGFP*-mediated expression does not result in premature translation of the message indicating that the long transgene is subjected to proper translational control mediated by the *Prm1* 3' UTR. Prm1-EGFP overexpression is associated with defects in spermiation, impaired motility and increased lethality of spermatozoa in the epididymis. Furthermore, Prm1-EGFP positive spermatozoa do not support early pre-implantation development suggesting that high levels of the fusion protein impairs the remodeling of the paternal genome during spermiogenesis and/or during the decondensation of the sperm head and formation of the male pronucleus in the one-cell embryo. Extensive experiments are required to elucidate the changes in chromatin states and nuclear organization in Prm1-EGFP bearing spermatozoa that underlie their inability to support early embryogenesis. Nonetheless, regardless of its mode of action, the dominant male-sterility caused by the overexpression of Prm1-EGFP can serve as a reliable and simple replacement of the surgical vasectomy procedure.

Mutations resulting in male sterility have been previously described and most of them can be assigned to genes controlling DNA recombination, DNA repair or differentiation of the male reproductive tract or the male germline (Bellve, 1979; Brugmans *et al.*, 2007; Jaroudi *et al.*, 2007; Matzuk *et al.*, 2002). Unfortunately, these mutations are usually associated with decreased viability, susceptibility to cancer or other diseases. In addition, strains harboring such mutations are not good candidates for vasectomy replacement since these recessive traits require breeding and reliable genotyping of heterozygous (fertile) versus homozygous (sterile) male mutant mice. Although dominant mutations causing male sterility have also been described, also none of them appear to be ideally suited for the purpose of replacing surgically vasectomized males in a laboratory environment. Such mutants include the Dominant spermiogenesis defect (*Dspd*) which results from a reciprocal translocation between chromosomes 14 and 7 (Kai *et al.*, 2004) as well as two randomly generated insertional mutations named Lacking vigorous sperm (*Lvs*) (Magram *et al.*, 1991) and Dominant male sterility (*Dms*) (Meng *et al.*, 2002), respectively. Since the genes causing the sterility have not been identified, it is impossible to reproduce the phenotype in other strains. Dominant male-sterility has also been described in mice overexpressing transgenes. For example, ubiquitous overexpression of the Retinoic acid receptor alpha (*Rara*) causes dominant male-sterility due to severe malformation of the epithelium of the reproductive tract (Costa *et al.*, 1997). Unfortunately, the perturbation of the epithelium also prevents the formation of the ejaculate. Hence, sterile males fail to produce a copulatory plug upon mating making the identification of pseudopregnant females virtually impossible. Overexpression of the Retinits pigmentosa GTPase regulator (*Rpgr*) protein leads also to male infertility due to defects in flagellum assembly (Brunner *et al.*, 2008). Here the transgenic mice and their F1 offspring did show dominant male sterility. Instability of the transgenic locus in subsequent generations (Brunner *et al.*, 2008), however, raises doubts about using the *Rpgr* overexpressing mice as vasectomy replacements. In conclusion, none of these mutant or transgenic mouse models display dominant sterility with full penetrance in combination with a normal mating behavior as exhibited by the *Prm1-EGFP tg38* and *tg1432* lines.

Additional advantage of using *Prm1-EGFP* transgene as vasectomy replacement is the genetic linkage of the trait to a phenotypical marker in the form of ubiquitous EGFP expression. This strategy removes the burden of identification of transgenic offspring by standard genotyping methods. To maintain the double transgenic *Prm1-EGFP; CAG-EGFP tg1432* strain, hemizygous transgenic females need to be bred to non-transgenic males. Whereas 50% of the offspring will be transgenic, the remaining males and females will be wild-type. These wild-type mice can be used for other purposes such as foster mothers, sentinels or breeding studs. The versatility in applications of the double transgenic *Prm1-EGFP; CAG-EGFP* mice will thus guarantee its efficient usage in common transgenesis practice. Furthermore, the simplicity of using *Prm1-EGFP* transgenes as means for inducing dominant male sterility suggests that the method could be extended to other relevant livestock or pet species where uncontrolled breeding of males needs to be prevented.

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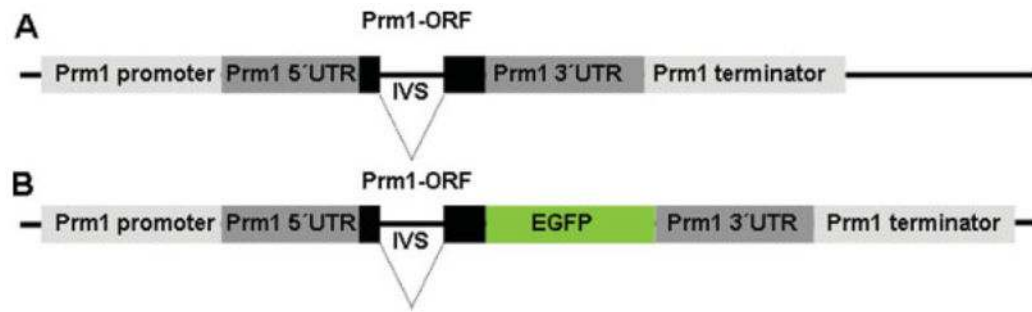
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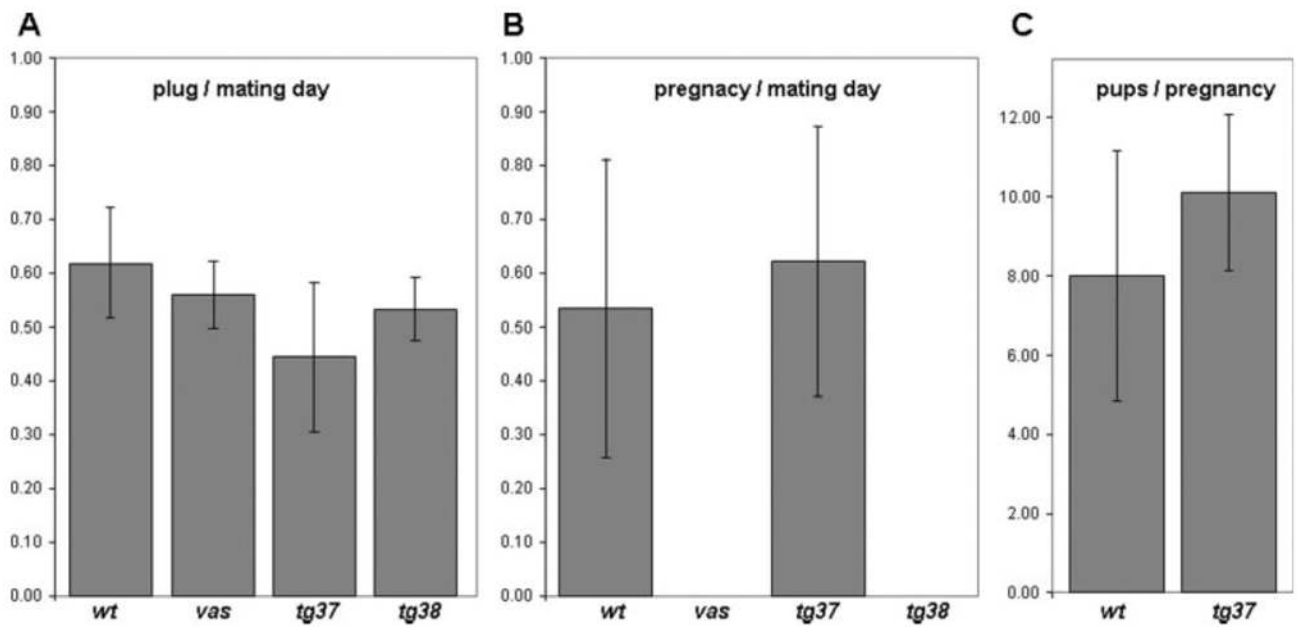


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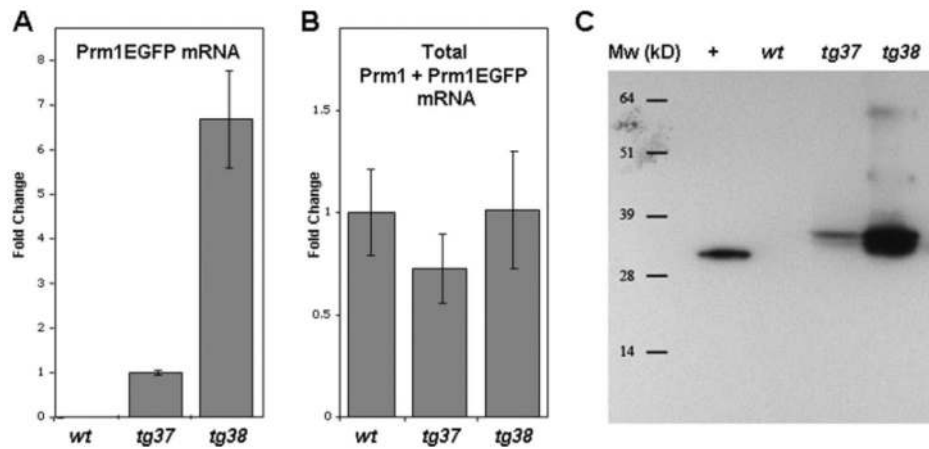
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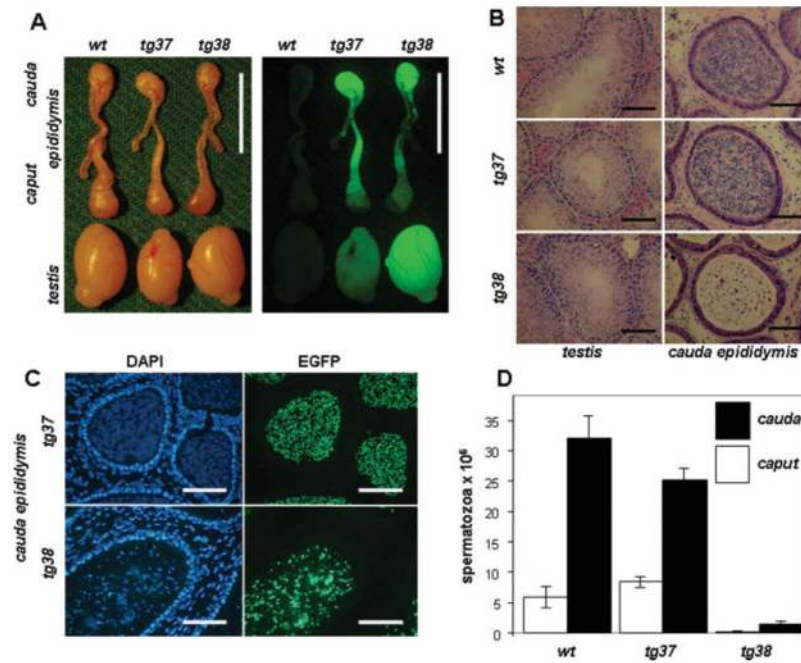
**FIG. 1.** Schematic diagram of the endogenous mouse *Prm1* locus (A) and of the *Prm1-EGFP* transgene (B). Promoter and transcribed elements: untranslated regions (UTR), protein coding region (ORF) and introns (IVS) are indicated. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]



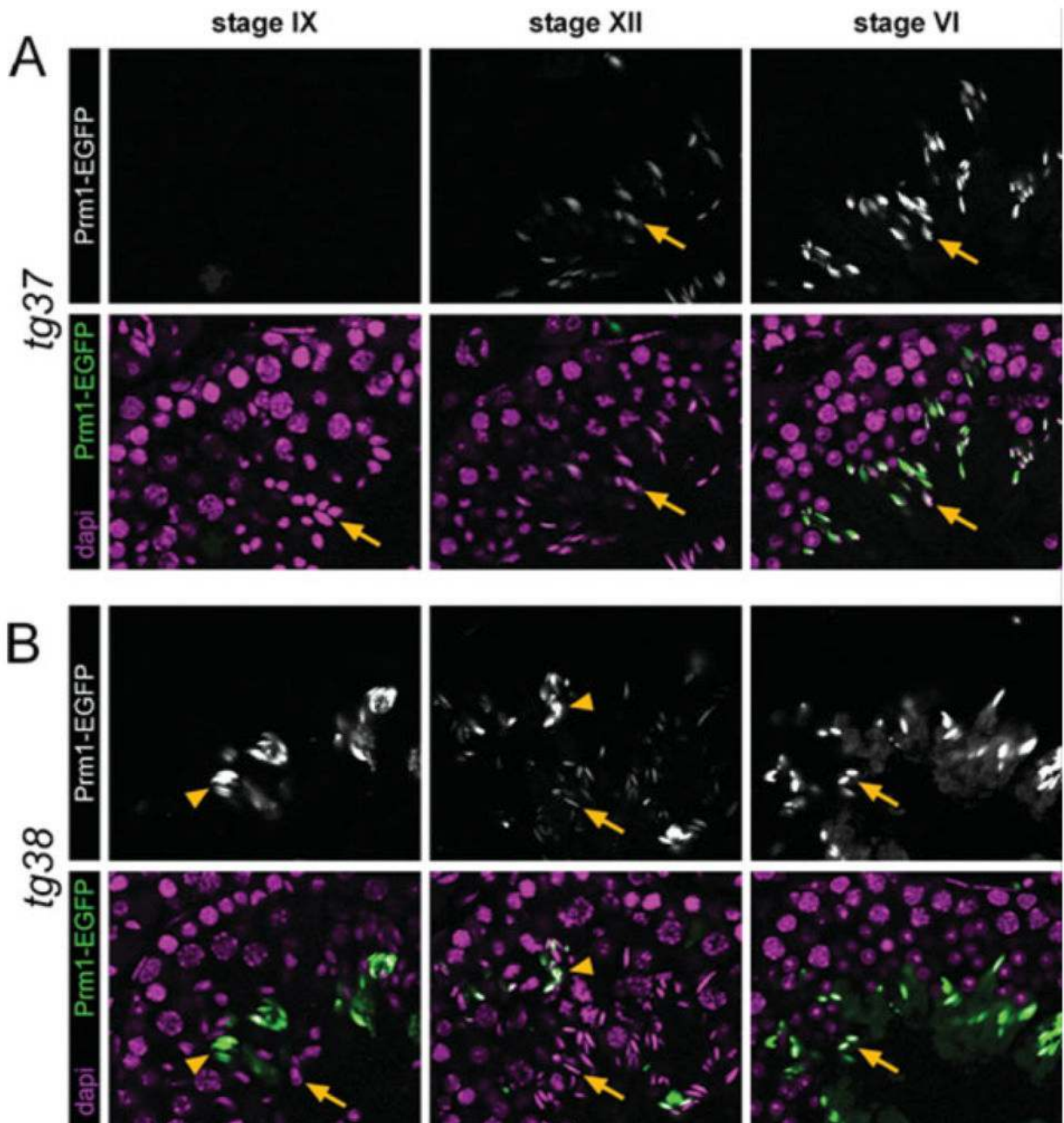
**FIG. 2.** Reproductive performance of *tg37* and *tg38* transgenic lines. **A:** Daily plugging frequencies of transgenic and control males as assessed over a period of 30 days (average and standard deviation;  $n = 3$  per genotype). Control values were obtained from normal and vasectomized wild-type littermates. **B:** Daily conception frequencies of transgenic and control males as assessed over a period of 30 days (average and standard deviation;  $n = 3$  per genotype). **C:** Average litter sizes of the first litter produced by females impregnated by *wt* and *tg37* males.

**FIG. 3.**

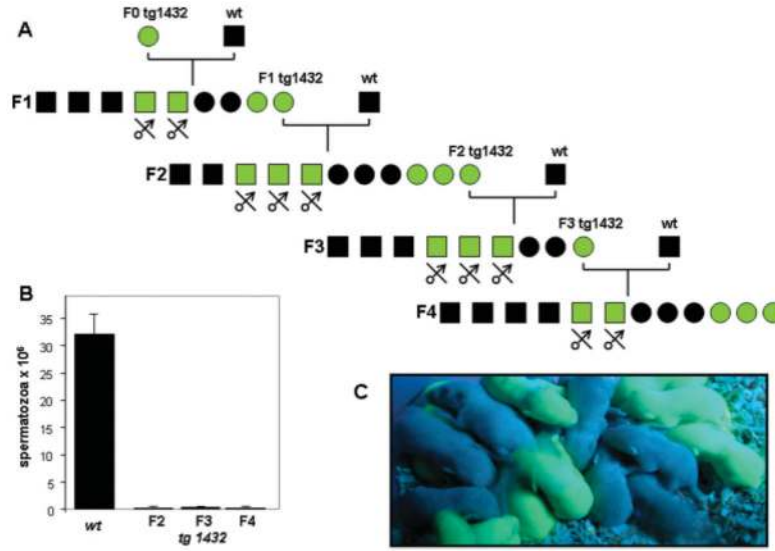
Prm1-EGFP expression analysis in lines tg37 and tg38. **A:** Real time RT-PCR quantification of Prm1-EGFP mRNA in testis RNA of wild-type (wt) and transgenic (*tg37* and *tg38*) male mice. **B:** Real time RT-PCR quantification of total Prm1 mRNA in testis RNA of wild-type (wt) and transgenic (*tg37* and *tg38*) male mice. The primer pair used detects both the endogenous *Prm1* mRNA as well as the transgene-derived Prm1-EGFP RNA. **C:** Detection of Prm1-EGFP fusion protein in total protein extracts from testes of adult mice. Equal amounts of total protein extract (10  $\mu$ g) were separated on 10% SDS-PAGE gel blotted onto PVDF membrane and probed with an anti-EGFP antibody. Lanes: (Mw) molecular weight marker, (+) EGFP positive control, (wt) wild-type extract, (*tg37*) *tg37* extract, (*tg38*) *tg38* extract.

**FIG. 4.**

**A:** EGFP expression in reproductive organs of wild-type (wt) and transgenic lines *tg37* and *tg38*. Prm1-EGFP is more strongly expressed in testes and caput epididymi of *tg38* males compared to *tg37* males. Prm1-EGFP expression is strongly reduced in caudal epididymi of *tg38* males but not of *tg37* males. Scale bar 1 cm. **B:** Structure of testis and cauda epididymis in wt, *tg37*, and *tg38* mice. HA staining reveal that all structures are normal; however, the numbers of spermatozoa in the epididymi of *tg38* are greatly reduced. Scale bar 100  $\mu$ m. **C:** Prm1-EGFP expression in cross sections of caput and caudal epididymi of *tg37* and *tg38* males. Caudal epididymi of *tg38* males harbor reduced numbers of EGFP positive spermatozoa; however, EGFP fluorescence of individual *tg38* spermatozoa is much stronger than that of *tg37*. Scale bar 100  $\mu$ m. **D:** Sperm counts in caput and caudal epididymi of wild-type (wt) and transgenic *tg37* and *tg38* male mice (average and standard deviation;  $n = 3$  per genotype).



**FIG. 5.** Prm1-EGFP is strongly overexpressed in elongating and elongated spermatids of *tg38* males and induces spermiation defects. Fluorescent microscopy analyses of DAPI staining and EGFP fluorescence in testis sections of (A) *tg37* and (B) *tg38* males. Representative images of stage IX, XII, and VI are provided. Arrows points to elongating (Stage XII) and elongated (Stages VI and IX) spermatids. Arrowheads point to highly condensed elongated spermatids that originate from a previous spermatogenic cycle.



**FIG. 6.** Transgenic line *tg1432*—linking dominant male sterility to full-body EGFP expression. **A:** Part of the pedigree tree for line *tg1432*. Males and females are indicated by squares and circles, respectively. Green fluorescence caused by expression of the *CAG-EGFP* transgene is indicated. In all cases dominant male sterility was linked to full-body EGFP expression indicating tight genetic linkage of *CAG-EGFP* and *Prm1-EGFP* transgenes. **B:** Sperm counts in caudal epididymi of wild-type (*wt*) and transgenic *tg1432* male mice in generations F2, F3, and F4, respectively (average and standard deviation;  $n = 3$  per genotype). **C:** Typical litter resulting from a backcross of a hemizygous *tg1432* female with a wild-type male showing EGFP positive and negative offspring.

**Table 1**

Developmental Capacities of Spermatozoa Following ICSI

Sperm strain	Oocytes injected (experiments)	Oocytes surviving (%) injected	Embryo development				
			2 Pronuclei $t = 6$ h (%) surviving	2-cell $t = 22$ h (%) surviving	Morula $t = 76$ h (%) surviving	Blastocyst $t = 120$ h (%) surviving	
wt-BDF1	68 (2)	54 (79.4)	54 (100)	52 (96.3)	42 (77.8)	33 (61.1)	
tg37	86 (3)	69 (80.2)	66 (95.7)	66 (95.7)	47 (68.1)	29 (42.0)	
tg38	67 (2)	62 (92.5)	11 (17.7)	9 (14.5)	4 (6.5)	2 (3.2)	

BDF1 oocytes were isolated at the metaphase II stage and injected with sperm heads of the indicated strain. The embryos were cultured for 5 days and their development was scored daily at times indicated.