Intra-testicular injection of adenoviral constructs results in Sertoli cell-specific gene expression and disruption of the seminiferous epithelium

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Abstract

Spermatogenesis is a complex process that cannot be modelled in vitro. The somatic Sertoli cells (SCs) within the seminiferous tubules perform a key role in supporting maturation of germ cells (GCs). Progress has been made in determining what aspects of SC function are critical to maintenance of fertility by developing rodent models based on the Cre/LoxP system; however, this is time-consuming and is only applicable to mice. The aim of the present study was to establish methods for direct injection of adenoviral vectors containing shRNA constructs into the testis as a way of inducing target-selective knock-down in vivo. We describe here a series of experiments using adenovirus expressing a green fluorescent protein (GFP) transgene. Injection via the efferent ductules resulted in SC-specific expression of GFP; expression levels paralleled the amount of infective viral particles injected. At the highest doses of virus seminiferous tubule architecture were grossly disturbed and immune cell invasion noted. At lower concentrations, the expression of GFP was variable/negligible, the seminiferous tubule lumen was maintained but stage-dependent GC loss and development of numerous basal vacuoles was observed. These resembled intercellular dilations of SC junctional complexes previously described in rats and may be a consequence of disturbances in SC function due to interaction of the viral particles with the coxsackie/adenovirus receptor that is a component of the junctional complexes within the blood testis barrier. In conclusion, intra-testicular injection of adenoviral vectors disturbs SC function in vivo and future work will therefore focus on the use of lentiviral delivery systems.

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Introduction

Germ cell (GC) maturation takes place within the seminiferous tubules of the testis and involves the transformation of a small round diploid cell (the spermatogonium) into mature haploid spermatozoa, a process that is dependent upon support from the somatic Sertoli cells (SCs; Cooke & Saunders 2002). A close association between the SC and GC is ensured by the formation of junctional complexes including adherens, gap and tight junctions between the cell types (reviewed by Lee & Cheng 2004). In addition, the seminiferous epithelium is divided into two compartments by virtue of the existence of the blood testis barrier (BTB) that is characterised by highly specialised tight junctions between neighbouring SC (Mruk & Cheng 2004). The BTB ensures that the ad-luminal compartment, where the post-meiotic GCs reside, is functionally separated from the basal compartment and this protects the GC from being recognised by the immune cells found within the interstitial compartment that surrounds the seminiferous tubules (Fijak & Meinhardt 2006). The full process of spermatogenesis cannot be modelled in vitro.

Our appreciation of the critical role played by SC in mediating the functional maturation of GC has been enhanced by the analysis of mice with SC-specific gene deletions achieved by cross-breeding animals expressing Cre recombinase under the control of promoters controlling SC-specific genes such as anti-Mullerian hormone (Lecureuil et al. 2002) with those transgenic for a gene with exon(s) flanked by LoxP sites. This strategy was used to generate mice with a SC-specific deletion of androgen receptor and these mice were found to be infertile because GC maturation failed during meiosis (De Gendt et al. 2004). The disadvantage of this approach is that every gene of interest requires the generation of a separate line of transgenic animals expressing Cre recombinase under the control of promoters controlling SC-specific genes such as anti-Mullerian hormone (Lecureuil et al. 2002) with those transgenic for a gene with exon(s) flanked by LoxP sites. This strategy was used to generate mice with a SC-specific deletion of androgen receptor and these mice were found to be infertile because GC maturation failed during meiosis (De Gendt et al. 2004). The disadvantage of this approach is that every gene of interest requires the generation of a separate line of transgenic mice expressing a floxed gene in which normal expression of the gene is maintained. This is time-consuming, involves a large number of animals, is currently only applicable to studies in mice, and in some cases the introduction of the LoxP sites results in generation of a hypomorphic allele (Holdcraft & Braun 2004). With array analyses of testes from genetically modified mice now leading to the identification of increasing numbers of gene products that may play a role in regulation of fertility (Denolet et al. 2006), we need to consider alternative
approaches to investigate the importance of SC-specific gene expression in vivo. Wilkinson and co-workers (Maiti et al. 1996, Sutton et al. 1998) identified and cloned the promoter elements responsible for SC-specific expression of the Rhox5 gene and used this to drive expression of constructs expressing a short interfering (si) RNA directed against the Wilms tumour (WT-1; Rao et al. 2006). Target-specific knock-down of WT-1 was achieved and an impact on fertility demonstrated confirming the power of RNA interference as a means of cell-specific knock-down of gene expression in SC. We therefore set out to explore whether we could manipulate SC-specific gene expression by introducing an shRNA transgene directly into the testis using an intra-testicular injection of an adenoviral construct.

We considered this approach to have merit for a number of reasons. First, intra-testicular injection via the efferent ductules or rete testis has been used for many years as a means of transferring germ stem cells from a donor testis into the seminiferous epithelium of a recipient (Ogawa et al. 1997, 2000) and members of our team had successfully performed this technique. Second, previous studies have reported the use of adenoviral vectors to introduce transgenes into the testes of adult rats (Blanchard & Boekelheide 1997, Scobey et al. 2001, Fleming et al. 2003a, 2003b) and mice (Kanatsu-Shinohara et al. 2002). In the rat, expression of the transgene was confined to SC and persisted for up to 10 days after injection (Blanchard & Boekelheide 1997, Scobey et al. 2001). In mice, injection of an adenovirus containing the Steel gene encoding the kit ligand via the efferent ductules was able to partially rescue spermatogenesis in infertile Steel mutant mice (Kanatsu-Shinohara et al. 2002).

As we did not expect all SC to be infected with adenovirus we decided to use constructs co-expressing green fluorescent protein (GFP) with the specific shRNAs so that we could mark those cells where specific knock-down should have occurred. We describe a pilot study that used constructs expressing GFP alone that aimed to establish a robust methodology. We elected to introduce the adenoviral particles via the efferent ductules as this route has been used for introduction of GCs into the seminiferous tubule lumen (Nagano et al. 1999, Rilianawati et al. 2003) and avoided the potential for inadvertent introduction into the interstitium. The results obtained were at odds with some previous reports and demonstrated that adenoviral infection can cause alterations in SC function resulting in disturbances in seminiferous tubule structure and GC survival.

Results

Injection of adenovirus via the efferent ductules results in SC-specific expression of a GFP reporter gene

The adenoviral construct expressing GFP was tested in vitro and shown to result in 100% expression with no cell death in the SK11 mouse SC line (Sneddon et al. 2005) using a multiplicity of infection (MOI) of 50 (not shown) although with higher concentrations of virus (MOI 100, 200) a negative impact on cell morphology/survival was observed. Following intra-testicular injection of the same construct via the efferent ductules no gross change in the appearance of the testis or size of injected testes compared with the contralateral (uninjected) control was noted except for the single testis injected with 4 × 10⁸ pfu (plaque forming units) adenovirus 7 days previously that was smaller than the contralateral control. The expression of GFP was detected exclusively in the cytoplasm of SC (Fig. 1) and GFP was never immunolocalised to GCs. At the highest concentrations of virus (4 × 10⁸ pfu/50 μl), protein expression was readily detected at 3 days after injection and expression persisted at 7 days (Fig. 1A and B). In these testes, there was gross disruption of tubule structure, intense expression of protein and the lumen of the tubules was no longer apparent. In testes injected with ≤1 × 10⁷ pfu/50 μl virus, GFP was detected throughout the cytoplasm of the SC and a patent central lumen was present (Fig. 1D and F). Four days after infection the...
amount of protein detected in individual SC varied in intensity even between adjacent cells and when concentrations of virus below 1 × 10^7 pfu/50 μl were used very few SC were immunopositive for GFP (Fig. 1E–H).

**Infection of SC with adenovirus results in GC loss and formation of intra-epithelial vacuoles**

Examination of haematoxylin and eosin (H and E) stained sections (Fig. 2) revealed disturbances in testicular architecture and/or reduced GC complement with the most severe disruption in those testes injected with the highest concentrations of virus. In testes previously injected with 4 × 10^8 pfu that were recovered 7 days after injection (Fig. 2B) groups of cells that appeared to have a morphology resembling immune cells were detected (arrows) but in all other samples no abnormal immune cell invasion was apparent on H and E and this was therefore investigated further using specific immunohistochemistry (see below). In the testes injected with lower concentrations of virus (maximum amounts 1 × 10^7–10^5 pfu), the interstitial and seminiferous compartments were maintained. However, even in these testes, the integrity of the seminiferous epithelium in a proportion of the tubules was disturbed with the striking appearance of large empty ‘vacuoles’ (asterisks Fig. 2C–G). Tubules all contained a clearly defined lumen and GCs with the appearance of round and elongate spermatids were present.

A detailed analysis of tubules injected with 1 × 10^6 or 1 × 10^7 pfu viral per testis was carried out in order to determine whether there was evidence of stage-dependent GC loss. Vacuoles and reduced GC complement were easily observed in tubules at stages II–VI and IX–XI, and were particularly striking at stages V and VI. Based on morphological appearance the GC subpopulation that was most obviously reduced was that of the spermatocytes. Although a substantial number of tubules at stages VII and VIII were observed in the sections from infected testes, all appeared to have a full GC complement.

**SC architecture, DNA damage and junctional complexes**

Immunostaining for the SC-specific protein SDMG1 (Best et al. 2008) revealed that functional SC were present in all samples and the testes could be divided into two groups based on whether the tubules still contained an identifiable lumen or not. In line with results from H and E the highest concentrations of virus clearly caused gross disruption to the tissue although notably even at 7 days post-infection SDMG1-positive cells were still clearly organised into a ‘tubule’ structure (Fig. 3B). In the testes containing ‘vacuoles’ (Fig. 3C and D), the SC cytoplasm still extended from the periphery of the tubules to the lumen and appeared to encircle the gaps in the epithelium.

Testes injected with 4 × 10^8 pfu viral and collected 3 or 7 days after infection contained numerous TUNEL-positive cells within the structures resembling collapsed seminiferous tubules (Fig. 3E and F). Occasional cells with faint immunopositive staining were detected in sections from testes collected 4 days after infection with 1 × 10^5 (Fig. 3G and H) to 1 × 10^7 pfu/testis and were similar in number to those detected in control (uninfected) testes.

Post-meiotic GCs develop in a distinct functional compartment within the seminiferous epithelium that is maintained by junctional complexes between SC within the BTB additional structural support for GCs being provided by junctions between SC and GC. In order to determine whether viral infection resulted in a gross disturbance of junction complex sections were costained with antibodies directed against espin and gap junction protein, α1 (GJA1). Espin is an actin linker protein associated with the ectoplasmic specialisations (ES), a form of adherens junction (reviewed by Lee & Cheng 2004b) that contributes to the BTB (basal ES) and
associations between elongating spermatids and SC (apical ES). GJA1 is an essential component of gap junctions that form between SC, between Leydig cells and between spermatogonia/spermatocytes and SC; mice with a SC-specific KO of GJA1 are infertile (Brehm et al. 2007). In sections from controls GJA1 was detected in the basal area of the seminiferous tubules in close association with espin (Fig. 4A, arrowheads) and between GCs and SC throughout the epithelium. The expression of espin was readily detectable in testes injected with adenovirus although in some tubules the pattern of expression appeared abnormal (Fig. 4B–D). In tubules with intra-epithelial vacuoles, espin and GJA1 could both be detected around their periphery (asterisks, Fig. 4D and F arrows) and were also localised at the base of the tubules in a region consistent with the existence of a BTB (Fig. 4F, arrowheads).

**Immune cell populations**

Sections were stained with antibodies directed against myeloperoxidase (MPO, neutrophil marker, Lysiak et al. 2001) or CD68 (macrophage marker, Holness & Simmons 1993) in order to determine whether immune cell populations in the testis were altered by viral infections. Consistent with reports that the testis contains a population of resident macrophages CD68-positive cells were detected in the interstitial compartment of both infected and uninfected testes (Fig. 5A and B). Immunostaining with smooth muscle actin (SMA) revealed that in testicular tissues with an intact peritubular cell layer macrophages were confined to the interstitium even though in some sections, there was some indication that numbers might have increased 4 days after infection (Fig. 5B). In testes injected with $4 \times 10^8$ pfu the SMA-positive cell layer was disrupted at 4 days post-injection and CD68-positive cells had infiltrated the seminiferous tubules (Fig. 5C).

MPO-positive neutrophils were not detected in control (uninfected) testes (Fig. 5D), but were clearly increased in testes injected with $4 \times 10^8$ pfu recovered 7 days (Fig. 5E) but not 3 days (not shown) after infection. In few tubules disruption of the peritubular myoid cell layer (Fig. 5 E and F) appeared to be associated with invasion of MPO-positive cells into basal compartment of the tubule.

**Discussion**

In the present study, we detected SC-specific expression of GFP following intra-testicular injection of an adenoviral vector containing a GFP transgene. The viral vector used was a disabled form of the human type 5 adenovirus. The prime receptor for human adenoviruses is the coxsackie/adenovirus receptor (CXADR) a transmembrane protein, whose availability is the primary determinant of a cell’s susceptibility to adenoviral infection (Bergelson et al. 1997). The expression of CXADR has been detected in isolated populations of SC and GC from rats (Wang et al. 2007) and the protein has been immunolocalised to the BTB as well as ES within the adult rat seminiferous tubule (Wang et al. 2007). Following in vivo injection of adenovirus via the efferent ductules, GFP was only ever detected in SC and no expression in GC occurred a result that was in agreement
with the findings in previous studies in rats (Blanchard & Boekelheide 1997, Scobey et al. 2001, Fleming et al. 2003a, 2003b) and mice (Kanatsu-Shinohara et al. 2002).

Prior to intra-testicular injection the impact of the Ad-CMV-GFP stock on SC cell survival was tested in vitro using an immortalised mouse SC (SK11, Sneddon et al. 2005). These cells were readily infected with the Ad-CMV-GFP and expression of the transgene in the cytoplasmic compartment occurred in >50% of cells at MOI of 50 pfu; identical results were obtained using a viral construct containing LacZ in place of GFP (not shown). Fleming et al. (2003a) used MOIs of 10 or 100 pfu/cell adenovirus to investigate over expression of γ-tubulin transgenes 24 or 48 h after infection of TM4 immortalised mouse SC and reported a seven to tenfold increase in protein expression above control values. Blanchard et al. reported that infection of primary cultures of rat GC and SC using a LacZ transgene linked to a nuclear targeting signal resulted in SC-specific expression that persisted for at least 20 days without obvious cell death.

In the present study, dramatic responses to adenoviral infection were observed 3, 4 and 7 days after infection. The testes could be separated into two groups based upon their responses to infection. In the first set of experiments, testes were injected with a maximum of $4 \times 10^8$ pfu/testis. This resulted detection of GFP in all SC, identified as such based on their location in

**Figure 4** Junctional complement of tubules following adenoviral injection. Fluorescent immunohistochemistry for espin (green) and GJA1 (red). In control testes (A) espin was localised to basal (arrowheads) and apical ES. GJA1 was localised to gap junctions close to the basal ES (arrowheads) and between SC and GCs throughout the seminiferous epithelium. (B) Seven days after injection of $4 \times 10^8$ pfu adenovirus espin could still be detected localised between cells but GJA1 was barely detectable. In testes recovered 3 days after a similar dose of virus (C) disturbances in the localisation of espin were apparent but GJA1 was readily detectable. Vacuoles within the seminiferous epithelium of testes injected with $1 \times 10^9$ pfu adenovirus (E, F, code 3053) are identified by asterisks (*); in some sections espin was clearly localised around the periphery of the vacuoles (arrows in D, E). Sections were counterstained with DAPI nuclear marker (blue). All 80× magnification.
tubule-like structures, but also in a gross disturbance in testicular architecture with loss of seminiferous tubule integrity 7 days after introduction of the virus. Additional analysis revealed evidence of hypoxia (not shown), increased rates of apoptosis (including expression of caspase 3 and TUNEL-positive staining), with more immune cells in the interstitium and invasion of neutrophils and macrophages into the seminiferous tubules as early as 3 days after infection. The rapid disruption of seminiferous epithelium integrity and increased immune cell invasion post-infection was not anticipated based on previous studies that had reported a minimal immune cell response when viral particles were injected via routes that avoided contact with interstitial cell populations. For example, Blanchard & Boekelheide (1997) reported an inflammatory response that began 10 days and lasted around 8 days after injection of a maximum of $3 \times 10^{10}$ LacZ-adenoviral particles into the rete testis of an adult rat. The inflammatory event was marked by lymphocyte and plasma cell infiltration into the interstitium, and chromatin fragmentation in cells of the seminiferous epithelium but was shorter than that associated with injections into the interstitium. Scobey et al. (2001) did not record an inflammatory response in rats when $2 \times 10^7$ viral particles were injected directly into the lumen of seminiferous tubules. Ikawa et al. (2002) injected $\sim 1 \times 10^8$ viral particles expressing CMV-LacZ into mouse testes with $\sim 70\%$ fill and observed a substantial reduction in testis size when they were recovered 2 months later; testicular morphology was not investigated but strong expression of the transgene was noted. Direct comparisons between our experiments and these studies are difficult as two were conducted in rats and in all three cases the viral concentrations used are quoted as ‘viral particles’. In our studies, all concentrations of virus were based on the number of viral particles capable of cell infection, i.e. pfu, and it is important to note that the number of infective particles will depend upon the purification methods used and may only represent a fraction of the total number of particles. A more relevant comparison is with the study performed by Kanatsu-Shinohara et al. (2002), after they injected $\sim 6 \times 10^3$ pfu adenovirus containing a construct expressing Kit ligand into testes of Sl/Sld mice they noted an occasional local inflammatory reaction, however, 10 weeks after injection three out of the four mice had partial restoration of spermatogenesis confirming restoration of SC function.

A recent paper reported that there are $\sim 3 \times 10^6$ SC in the adult mouse testis (Tan et al. 2005) and therefore in the present study if viral infection was evenly distributed following injection of $4 \times 10^8$ pfu/testis this would equate to a MOI of $\sim 100$/SC. Monitoring of the injections using dye allowed us to estimate that the percentage of tubules that were filled and this was 50 to 90% suggesting that the MOI could be as high as 200/SC and this may explain some of the disturbances observed in testicular function.

Even at the highest concentrations of virus we used,
although testis size was reduced 7 days after infection, in all other experiments where tissue was recovered up to 4 days after infection there was no obvious impact on testis size and in every case changes in testicular morphology were confined to the testis that was injected directly with virus. Following the observation that injection with $4 \times 10^8$ pfu/testis of caused alterations in testis function all further studies were conducted with lower concentrations of adenovirus ($1 \times 10^5$–$1 \times 10^7$ pfu/testis) with the expectation that the highest MOI/SC would be <30 assuming that more than 30% of the cells were infected. In these experiments, the seminiferous tubules maintained a distinct lumen and the expression of GFP was SC specific but highly variable in intensity. Even at the lowest dose, i.e. even in the absence of detectable GFP expression, GC complement was reduced in some tubules and ‘vacuoles’ had appeared within the seminiferous epithelium only 4 days after introduction of the adenovirus although there was no evidence of an inflammatory response. GC loss and the incidence of vacuoles were both cell type and stage dependent and were independent of the presence of the GFP transgene as similar responses were seen following injection of viral particles lacking an insert (Rad60) or expressing β-galactosidase (LacZ; R Hooley, unpublished observations). Differential susceptibility of SC to adenoviral infection has been reported in rats although variable results have been obtained with testes with SC in tubules at stage II–VI predicted to be the primary targets in one study (Blanchard & Boekelheide 1997) while the majority of infection appeared to be between stages VII and XIV in another (Fleming et al. 2003a). In the present study, the only tubules that were identified as lacking vacuoles were those in stages VII and VIII. Four days previously, when the testes were initially infected, these tubules would have been in stages I–III; the worst effected were V and VI, which would have been stages XI and XII of the previous cycle. Fleming et al. (2003a, 2003b) observed SC-specific expression of EGFP and human γ-tubulin in adult rat testes following injection of $1.4 \times 10^9$–$2.75 \times 10^{10}$ pfu adenovirus/testis via the rete testis and recorded appreciable disturbances in testicular histology as early as 48 h after injection. Some of the images in their study show testicular sections with ‘gaps’ in the epithelium similar to the ‘vacuoles’ we observed in our sections.

The disturbances we observed within the seminiferous epithelium resembled those described following a variety of testicular insults including exposure to toxicants (Creasy et al. 1987), hypophysectomy (Ghosh et al. 1991), transient scrotal heat stress (Paul et al. 2008) and acute withdrawal of androgens following destruction of Leydig cells with ethane dimethane sulphonate (Kert et al. 1993). We therefore considered it likely that viral infection of the SC per se was responsible for the changes we observed. It is notable that CXADR is reported to be associated with tight junctions that form part of the BTB (Cohen et al. 2001).

Wang & Cheng (2007) have proposed that cell adhesion molecules of the immunoglobulin superfamily including CXADR facilitate transendothelial migration of leukocytes at sites of inflammation as well as migration of pre-leptotene spermatocytes from the basal to the adluminal compartment of the seminiferous epithelium. CXADR can form homodimers and it has been reported that during viral infection the adenoviral fibre protein competitively inhibits CXADR–CXADR interactions resulting in disturbances in junctional complexes (Wang & Cheng 2007). This may be what happens in the seminiferous epithelium after adenoviral infection with a resultant disturbance in tight junctions but no impact on other junctional components such as the basal ES typified by expression of espin. Seminiferous tubule fluid is produced by the SC and although junctional integrity may have been partially compromised by adenoviral infection the BTB appears to remain functional because seminiferous tubules still contained a normal lumen regardless of the presence of intra-epithelial vacuoles. The reduction in GC complement in the affected tubules could occur either because SC metabolism is altered as a result of activation of second messenger signalling involving Src kinase family members following binding to CXADR (Wang et al. 2007) and/or because migration of pre/leptotene spermatocytes from the basal to the adluminal compartment is impaired.

In conclusion, data from the present study suggest that adenoviral vectors are not suitable as vehicles for delivery of shRNAs into SC in vivo. Lentiviral infection of isolated testicular germ stem cells has been demonstrated using both rats and mice (Hamra et al. 2002, Nagano et al. 2002), however, following injection of a lentivirus into the seminiferous tubules of adult mice two studies have demonstrated efficient SC-specific expression of LacZ (Ikawa et al. 2002) and GFP (Wang et al. 2008) respectively without any disturbances in testicular morphology or evidence of GC expression. Pseudotyping of lentiviral vectors with the vesicular stomatitis virus envelope G protein enables virus particles to bind to phospholipids in the membrane of target cells rather than relying on specific receptor binding (Naldini et al. 1996), this may be less deleterious to the infected cell and explain why there was no adverse effect on spermatogenesis. Therefore, as a follow up to the present study we propose to switch to using lentiviral vectors in order to introduce target specific shRNAs into SC in vivo.

Materials and Methods

Preparation of adenoviral stocks

An adenoviral construct expressing GFP under the control of a CMV promoter element (Ad-CMV-GFP) was purchased from Vector Biolabs, (Philadelphia, PA, USA). High titre stocks ($1.3 \times 10^{10}$–1.66 $\times 10^{10}$ pfu/ml viral particles were prepared by infection of HEK293 cells at five to seven viral particles per cell (known as the MOI) according to the standard methods
(Graham et al. 1977). Briefly, HEK293 cells were maintained in MEM containing 10% FBS and penicillin–streptomycin at 37 °C in 5% CO₂ until the cells showed a cytopathic response; virus was released from the cells by three cycles of freeze thawing. Viral particles were purified using a Vivascience AdenoPack column (Generon House, Eton Wick, UK), buffer exchanged into 8 volumes of 2.5% glycerol, 20 mM Tris–HCl (pH 8) and then concentrated. The viral titre, in pfu/ml, was quantified on HEK293 cells using an Adeno-X rapid titre kit (BD Bioscience, Oxford, UK) with 1:500 rabbit anti-adenovirus Serotype 5 hexon antiserum (Autogen Bioceol UL Ltd, Calne, UK).

**Intra-testicular injection of adenovirus**

Mice (C57Bl/6) were housed at 22/23 °C, 50% humidity in a 12 h light: 12 h darkness photoperiod with food and water provided. All experimental procedures were performed under a UK Home Office licence following ethical approval from the University of Edinburgh. The procedure used was essentially that of Ogawa et al. (1997, 2000) and was based on introduction of fluid via the efferent ductules. All surgical procedures were carried out under aseptic conditions. Anaesthesia was induced with ketamine/medetomidine and long-acting analgesia was achieved with buprenorphine. Following surgery, atipamezole was given to rapidly reverse the sedative effects of medetomidine. The lower abdomen was shaved and swabbed with chlorhexidine (Sigma). A small incision was made in the lower abdominal and muscle wall slightly to the left of the midline. The left testis and epididymis were delivered onto a sterile, moist swab and kept moist at all times with sterile saline. Under a dissecting microscope, the efferent ducts were identified and isolated. A hypodermic needle was used to make a small incision in an efferent duct, close to the rete testis, which was then cannulated with a glass microneedle syringe loaded with viral particles. Gentle, steady pressure was applied to ensure the seminiferous tubules were filled and this was monitored by the addition of trypan blue (0.08% w/v) to the solution of viral particles. Dye was observed in up to 95% of the surface seminiferous tubules; full details of viral doses and % fill in each case are given in Table 1. The testis was carefully replaced into the body cavity, which was closed with both internal and external vicryl (Ethicon) sutures. The animal was kept warm and observed frequently during the post-operative period. The right testis was left undisturbed as a paired control for the injected testis; animals were killed, both testes recovered 3, 4 or 7 days after surgery, fixed separately in Bouins for 6 h then transferred into 70% ethanol prior to processing into paraffin wax.

**Analysis of testicular architecture**

General histology was determined by staining thin sections (5 μl) with H and E. Immunohistochemistry was performed according to the standard methods (De Gendt et al. 2004, Anderson et al. 2007) using the antibodies and dilutions detailed in Table 2. Sections stained with H and E or 3,3’-diaminobenzidine (DAB) were examined using an Olympus Provis microscope (Olympus, Optical Co., London, UK) and photographed using a Canon EOS 30D camera (Canon Europe, Amsterdam, The Netherlands). Sections stained with fluorescent-tagged secondary antibodies were viewed and photographed on a Zeiss LSM 510 Meta Axiocamt00M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

**TUNEL assay**

Sections were stained using the in situ apoptosis detection (TUNEL) assay to detect single stranded DNA consistent with DNA breaks (Gavrieli et al. 1992) as follows. Sections were blocked in 3% hydrogen peroxide (v/v) in methanol for 30 min, washed twice, for 5 min each, in PBS then placed on ice-cold trays to cool before addition of 50 μl/section of TdT/Dig-11-dUTP reaction mixture. The reaction mixture consists of 400 U/ml terminal d-transferase (TdT, Roche) and 5 μl/ml 1 mM digoxigenin-11-deoxy-uridine-5’-triphosphate (Dig-11-dUTP, Roche) diluted in reaction buffer (30 mM TRIS/HCl (pH 7.2; Sigma), 140 mM sodium cacodylate (VWR International) and 1.5 mM CoCl₂ (VWR International, Lutterworth, Leics, UK) made up in dH₂O. Reaction buffer was sealed on the slide under a GelBond (Cambrex, Rockland, ME, USA) coverslip with cow gum/hexane, and heated to 37 °C for 30 min on a Hybaid Omnislide to enable incorporation of dUTP by the TdT enzyme. The slides were washed twice for 5 min in PBS to remove the reaction mixture, and then blocked with normal rabbit serum (NRS, dissolved 1 in 5 in PBS) for 10 min at room temperature in humidified conditions. Blocking

<table>
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<td>Rabbit</td>
<td>Molecular probes (MRC HGU, Edinburgh)</td>
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<tr>
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**Table 1 Details of testicular injections.**

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<td>1 × 10⁵ GFP (4d)</td>
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<td>1 × 10⁵ GFP (4d)</td>
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<tr>
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<tr>
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<tr>
<td>1 × 10⁵ LacZ (2d)</td>
<td>2741</td>
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<tr>
<td>1 × 10⁶ empty (4d)</td>
<td>3057</td>
<td>10</td>
</tr>
</tbody>
</table>

Provis microscope (Olympus, Optical Co., London, UK) and photographed using a Canon EOS 30D camera (Canon Europe, Amsterdam, The Netherlands). Sections stained with fluorescent-tagged secondary antibodies were viewed and photographed on a Zeiss LSM 510 Meta Axiocamt00M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).
buffer was replaced with sheep anti-DIG primary antibody (Roche) diluted 1:100 in NRS/PBS, which was incubated for 90 min at room temperature in humidified conditions. Sections were washed twice with PBS, 5 min each, and then incubated for 30 min with rabbit anti-sheep biotinylated antibody (Vector, Peterbourgh, UK) diluted in NRS/TBS (1 in 5) at room temperature. After incubation with the secondary antibody slides were washed in TBS twice for 5 min. The TUNEL staining was visualised with ABC-HRP and DAB.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


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