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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*. This paper describes 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.

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*a*). 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

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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 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 a 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.* 2003*a*, 2003*b*) 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. This paper describes 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 4×10^8 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 (4×10^8 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 1×10^7 pfu/50 µl virus, GFP was detected throughout the cytoplasm of the SC and a patent central lumen 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 orphological 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 Sdmg-1 (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 Sdmg-1-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 2004*b*) 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 a result that is in agreement with previous reports (Batias *et al.* 2000). Viral infection that resulted in a gross disturbance in the organisation of spermatogenesis was associated with disruption in the pattern of expression of Gja1 (Fig. 4B) although the protein was still readily detectable associated with SC-GC junctions tubules containing intra-epithelial vacuoles (Fig. 4C-F). In controls, espin was localised in basal ES associated with the BTB (Fig. 4A, arrowheads) and within in the apical compartment was localised at ES between SC and elongating spermatids (Fig. 4A, ES). 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 injections. 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 been increased 4 days after infection (Fig. 5B). In testes injected with 4×10^8 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 (uninjected) testes (Fig. 5D), but were clearly increased in testes injected with 4×10^8 pfu recovered 7 days (Fig. 5E) but not 3 days (not shown) after infection. In few tubules disruption of the pertibular myoid cell layer

(Fig. 5E and F) appeared to be associated with invasion of MPO-positive cells into basal compartment of the tubule.

Discusson

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.* 2003*a*, 2003*b*) 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.* (2003*a*) 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 4×10^8 pfu/testis. This resulted detection of GFP in all SC, identified as such based on their location in 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 3×10^{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 2×10^7 viral particles were injected directly into the lumen of seminiferous tubules. Ikawa *et al.* (2002) injected ~1×10^8 viral particles expressing CMV-LacZ into mouse testes with ~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/Sl^d* 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 4×10^8 pfu/testis this would equate to a MOI of ~ 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 4×10^8 pfu/testis of caused alterations in testis function all further studies were conducted with lower concentrations of adenovirus (1×10^5–1×10^7 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 ß-galactosidase (LacZ; R. Hooley, unpublished observations 2005). 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.* 2003*a*). 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.* (2003*a*, 2003*b*) observed SC-specific expression of EGFP and human γ-tubulin in adult rat testes following injection of 1.4×10^9–2.75×10^{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 paper 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 (Kerr *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 are 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 preleptotene 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 (1.3×10^{10}–1.66×10^{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 minimal essential medium containing 10% FBS and penicillin–streptomycin at 37°C in 5% CO_2 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 nM Tris–HCI (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 antisera (Autogen Bioclear 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, Johnston & Johnston Medical Ltd., Livingston, UK.) 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 Axiovert 100M 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 $CoCl_2$ (VWR International, Lutterworth, Leics, UK) made up in dH_2O. 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 Ominslide 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 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/Tris buffered saline (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.

Acknowledgments

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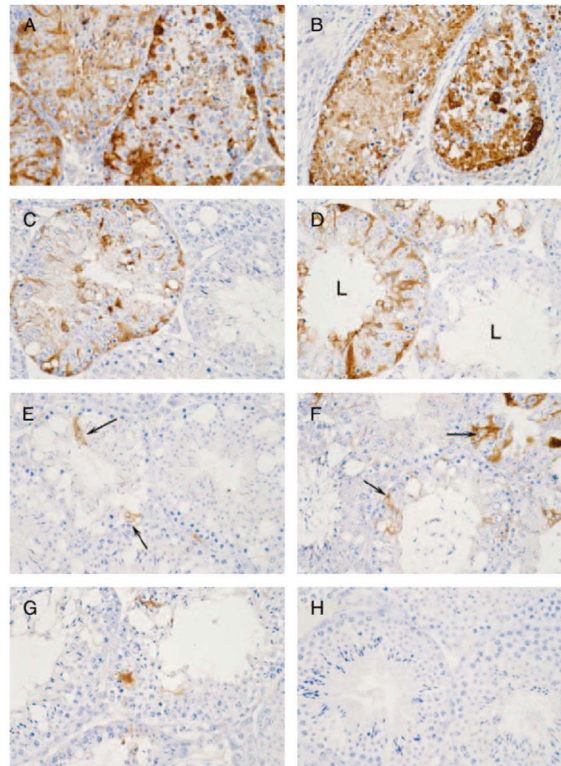
Figure 1.
Expression of GFP was only detected in SC following intratesticular injection via the efferent ductules. Testes were recovered 4 days after injection except for those in (A) (3 days) and (B) (7 days). The doses of virus given per testis were (A and B) 4×10^8 pfu, (C and D) 1×10^7 pfu, (E and F) 1×10^6 pfu, (G and H) 1×10^5 pfu. The uninjected contralateral testis from the same animal as the testis in (A) is shown as a control in (H). Images are $40 \times$ magnification and the scale bar represents 50 μm.
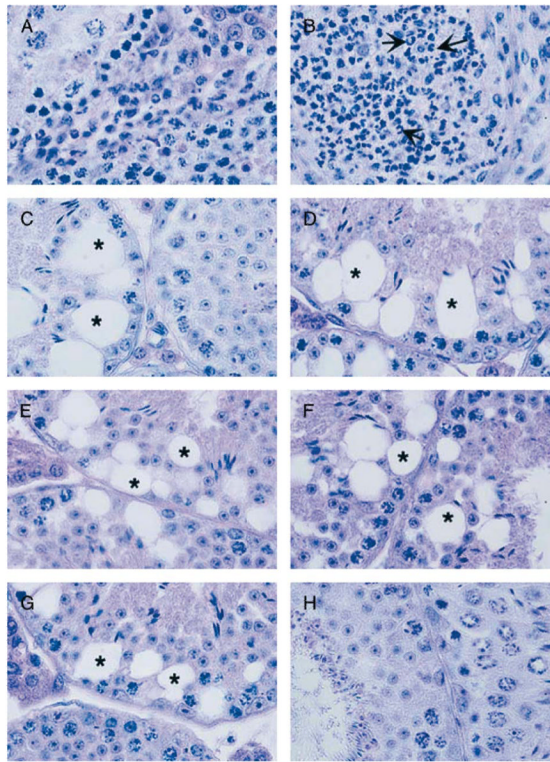
Figure 2.
Intra-testicular injection of adenovirus resulted in disturbances in testicular architecture associated with GC loss and development of vacuoles. Testes were collected 4 days after infection except for those in (A) and (H) (3 days) and (B) (7 days). The doses of virus given per testis were (A and B) 4×10^8 pfu, (C and D) 1×10^7 pfu, (E and F) 1×10^6 pfu, (G and H) 1×10^5 pfu. Arrows in (B) indicate potential immune cells that have invaded the tubule. The asterisks in (C-G) are located in the lumens of intra-epithelial vacuoles. The control contralateral testis from the same animal as the testis in (A) is shown in (H). Images are 100×magnification and the scale bar represents 20 μm.
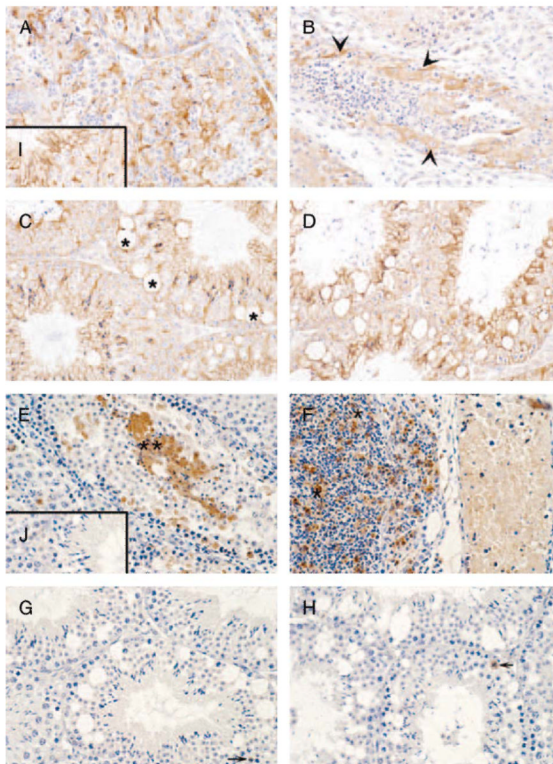
Figure 3.
Sertoli cell architecture and evidence of DNA fragmentation. Immunolocalisation of Sdmg-1 (SC-specific protein) or detection of DNA fragmentation (TUNEL staining) in testes infected with Ad-CMV-GFP at (A, B, E, F) 4×10^8 pfu/testis or (C, D, G, H) 1×10^7 pfu. The untreated contralateral testis from the same animal as the testis in (A) and (E) are shown inset (I) and (J). Note that Sdmg-1 was immunolocalised to SC cytoplasm in all samples even those in which tubule architecture was grossly disrupted (e.g. A, B, arrowheads). In tubules containing intra-epithelial vacuoles (asterisks) immunostaining could still be detected extending across the full width of the tubule from the base to the lumen. Images 40× magnification, scale bar 50 µm.
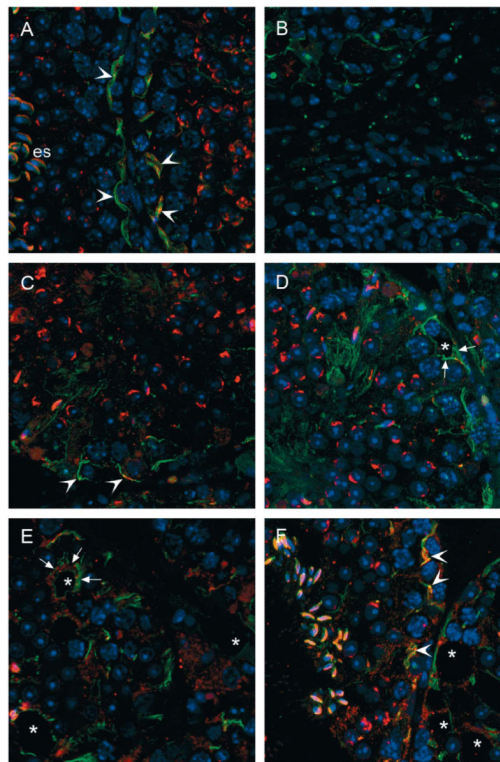
Figure 4.
Junctional complement of tubules following adenoviral injection. Fluorescent immunohistochemistry for espin (green) and Gja1 (red). In control testes (A) espin was localised to basal (arrowheads) and apical ES. Gja1 was localised to gap junctions close to the basal ES (arrowheads) and between SC and GCs throughout the seminiferous epithelium. (B) Seven days after injection of 4×10^8 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 1×10^5 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.
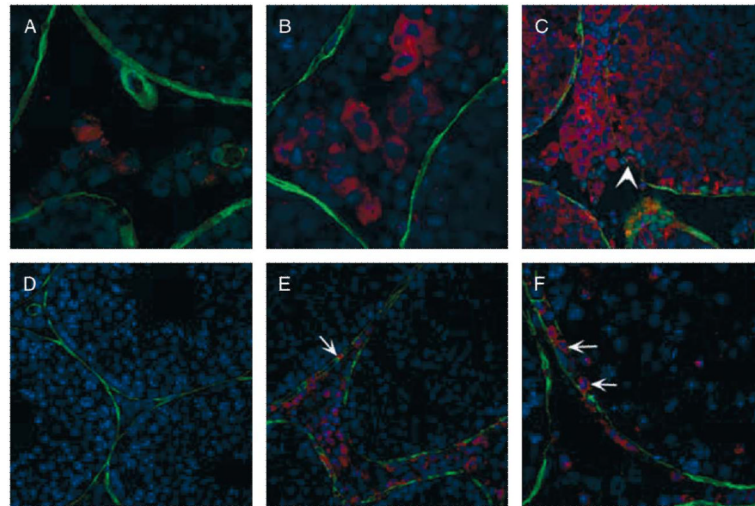
Figure 5.
Immune cell complement within the testis and evidence of intra-tubule invasion. CD68 positive macrophages (red staining, A-C) were detected in the interstitial compartment of (A) control (B, C) and injected testes. The number of interstitial CD68 positive cells appeared increased in some of the testes injected with adenovirus even when an intact layer of peritubular (smooth muscle actin positive) cells was present (compare A and B). Intra-tubular macrophages were only detected in the testis where tubule integrity had been severely compromised (arrowhead) 3 days after injection of 4×10^8 pfu virus (C). MPO positive neutrophils (red staining E, F) were not detected in control, uninjected, testes (D) but were strikingly elevated in the interstitium of a testis injected 7 days previously with 4×10^8 pfu virus (E). In a few samples disruption of the peritubular cell layer appeared to be associated with local migration of MPO-positive cells into the seminiferous epithelium (E, F arrows). Note that in all sections the peritubular cell layer was identified by immunostaining for smooth muscle actin (green) and sections were counterstained with DAPI (blue) that localised to all cell nuclei. Magnifications: A, B 80×, C, D, E, F 40×.

Table 1

Details of testicular injections

Dose of virus	Code number	% Fill
4×10^8 GFP (3d)	2026	60
4×10^8 GFP (7d)	2028	80
1×10^7 GFP (4d)	2232	70
1×10^7 GFP (4d)	2236	90
1×10^6 GFP (4d)	2217	80
1×10^6 GFP (4d)	2745	60
1×10^6 GFP (4d)	2749	80
1×10^5 GFP (4d)	2982	90
1×10^5 GFP (4d)	2994	70
1×10^5 GFP (4d)	3049	65
1×10^5 GFP (4d)	3053	75
1×10^6 LacZ (4d)	2737	85
1×10^6 LacZ (2d)	2741	50
1×10^5 empty (4d)	2986	95
1×10^5 empty (4d)	2990	55
1×10^5 empty (4d)	3057	10

Table 2

Antibodies used for immunohistochemistry

Antigen	Dilution	Species raised in	Source
GFP	1:1000	Rabbit	Molecular probes
Sdmg-1	1:1000	Rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
CD68	1:50	Mouse	DAKO
Gja1	1:100	Rabbit	Zymed
Espin	1:30	Mouse	Transduction laboratories
MPO	Pre-diluted	Rabbit	Abcam
SMA	1:500	Mouse	Sigma