**In-vivo gene transfer induces transgene expression in cells and secretions of the mouse cauda epididymis**

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**ABSTRACT:** Mouse cauda epididymis were in-vivo transfected using the lipid FuGENE 6 as gene vector. Two gene constructions were employed: the p-GeneGRIP which codifies for the Green Fluorescent Protein (GFP) and the pSEAP-control that expresses an alkaline phosphatase as a secretion. Transfection was detected by fluorescence and appeared in the nucleus and cytoplasm of epithelial cells. Transfection was observed in 39.70% of cells after 2 days and in 31.77% after 7 days, and then diminished progressively. Moreover, the presence of the transgene in the DNA isolated from treated epididymides was observed by polymerase chain reaction. GFP gene expression occurred in large areas of the cauda epididymis and it was observed exclusively in the cytoplasm of epithelial cells. GFP gene expression occurred during 2 weeks after gene injection and occupied 32.24, 29.98 and 22.37% of the area of the tubules when analyzed 2, 7 and 15 days after gene injection. The cauda was also analyzed in toto and showed similar results. The use of the pSEAP-control gene showed that cauda epididymis secretions can also be modified by the transfection procedure. A significant increase of alkaline phosphatase activity appeared in the epididymal fluids 7 days after gene injection. These results indicate that transfection procedures could be an important tool in the future to study epididymal physiology or to change the fertilizing ability of spermatozoa.

**Key words:** epididymis / gene transfer / lipid vectors / transfection

**Introduction**

From the first transfection experiments developed in the early 1960s, experimentation in this area has become widespread (Mulligan, 1993; Ledley, 1995). Most transfections have been undertaken under in-vitro conditions, but in-vivo procedures have also been used as a potentially important method for gene therapy (Ledley, 1995; Anderson, 1998). Recombinant viruses, liposomes, lipids or polymeric nanoparticles have been employed to deliver foreign genes into cell and tissues in culture (Mulligan, 1993; Hart, 2005; Vasir and Labhasetwar, 2006). Furthermore, in some cases naked DNA has also been employed successfully (Wang et al., 1995; Danko et al., 1997; Huguet and Esponda, 1998). For the in-vivo methods, foreign genes has been delivered using gene injections into organs or arteries, and recently the electroporation procedure has also been employed as a method for in-vivo gene transfer (Bigey et al., 2002). The epididymis and in particular its secretions promote these well-known (Bedford, 1979; Cooper, 1986; Kirchoff et al., 1997). On the other hand, the cauda epididymis also has an important role as a reservoir of spermatozoa (Bedford, 1979). Therefore, the possibility of modifying the physiology of the epithelial cells lining the cauda, and its secretions, would be an important goal for changing some characteristics of the spermatozoa stored in the epididymis. Transfections of the genital tract have been principally developed in the female tract (Daftary and Taylor, 2003). The in-vitro transfection of endometrial cell lines have been extensively performed (Telgmann and Gellersen, 1998; Waidehra et al., 2006; Bogess et al., 2006). On the other hand, in-vivo transfections have been developed in the normal (Charnock-Jones et al., 1997; Rellosio and Esponda, 2000; Sato, 2005) and in the pregnant mouse uterus (Kimura et al., 2005; Koyama et al., 2006). In the human uterus, Daftary and Taylor (2001) developed a liposome-mediated ex-vivo gene transfer. In the oviduct, successful in-vivo transfections have been performed using DNA (Rellosio and Esponda, 1998, 2000) and RNA (Rios et al., 2002).

In the male genital tract, epididymal cultured cell lines have been transfected in vitro on several occasions (Kirchoff et al., 2004; Dufresne et al., 2005; Yu et al., 2006). Recently, using an electroporation...
procedure, the rat epididymis was in-vivo transfected and the results showed that an efficient transfection occurred (Kirby et al., 2004).

On the other hand, the vas deferens of the mouse has been in-vivo transfected using a direct injection of DNA–Liposome complexes (Valenzuela et al., 2002). Human vas deferens, collected from vasectomy, has also been transfected using in-vitro methods (Esponda et al., 2004).

In the present report we show a single method to produce an in-vivo transfection of the epithelial cells in the mouse cauda epididymis. We investigated the efficiency of the method by analyzing the percentage of transfected cells, the extension of transgene expression and the production of a transgenic protein in the secretions of the cauda epididymis.

Materials and Methods

Animals

Adult male mice (CD1 strain) bred in our colony were used. They were sexually mature and maintained in a cycle of 12 h light:12 h dark, at constant temperature and with food and water ad libitum. Animals were killed by ether anesthesia and in all cases the epididymis was removed in PBS buffer. The animal protocol used was in accordance with the law 1201/2005 on Animal Protection of Spain, the European Union Agreement about Vertebrate Animal Protection (3/18/1986), the NIH Guide and the CSIC (Spain) ethical Committee for the care and use of laboratory animals.

Reporter genes and constructs

Two vectors were employed: (a) The commercial plasmid pGeneGrip (GTS, CA, USA) that encodes the green fluorescent protein (GFP) under the control of the hCMV IE promoter/enhancer. This plasmid is rhodamine labeled. (b) The secreted alkaline phosphatase added and (pSEAP)-control (Clontech, Palo Alto, CA, USA), a reporter system that uses a secreted form of human placental alkaline phosphatase as a reporter protein. The pSEAP-control gene expresses a protein that is stable to higher temperatures (65°C), so it can be easily differentiated from the endogenous alkaline phosphatase activity.

DNA–lipid mixture and in-vivo gene transfer

The lipid FuGENE 6 (Roche, Mannheim, Germany) was employed as gene vector (Selingier et al., 2005; Arnold et al., 2006). DNA and FuGENE were mixed in Dulbecco’s Medium such that 125–200 ng DNA was mixed with 1 μl of the commercial lipid. This mixture was resuspended in 50 μl of medium. Animals were anesthetized using an intraperitoneal injection of Rompun and Ketolar (Parke-Davis, Madrid, Spain): 4 mg of Ketolar and 0.35 mg of Rompun in 1 ml of PBS were employed, and 21 μl/g of weight was injected. After a simple surgical incision, the left epididymis was exposed and, using a glass microcapillary needle (Huguet and Esponda, 1998) filled with the DNA/Liposome mixture, 1–2 μl of this solution was gently injected into the lumen of the distal region of the cauda epididymis. The number of males injected with pGeneGrip was 38. Injected animals were killed 2, 7, 15 and 30 days after gene injections. From the 38 males injected two of them died soon after injection, then 36 males were analyzed for transfection. Control animals were three mice that were not injected with gene constructions and another three that were injected with the FuGENE 6 solution prepared without DNA.

Analysis of pGeneGrip transfection and GFP expression

Treated and control cauda epididymis were dissected under a microscope, divided in small pieces and treated as follows: (a) Some samples were placed in PBS buffer in a slide and gently squashed with a coverslip. These samples were directly analyzed in vivo by fluorescence microscopy. In this way some groups or free epithelial cells were observed under the microscope. (b) Other pieces were fixed in 1% paraformaldehyde in PBS for 1 h. After exhaustive washings they were sectioned using a Frico cut E Cryostat (Reichert-Jung, Wien, Austria). Sections of about 10 μm were mounted on poly-L-lysine treated slides. For observations, samples were mounted using a PBS solution containing Glycerol 10%, Vectashield 1% and 10 mM Hoechst 33342 (Sigma, St. Louis, MO, USA). Vectashield (Vector Labs, Burlingame, CA, USA) was employed to avoid quenching of the fluorescence and the Hoechst 33342 was used for DNA staining. (c) Some small pieces of fixed cauda tubules were mounted in excavated slides and observed using low magnification objectives. Slides were observed and photographed in a Nikon fluorescence microscope or captured using a microscope equipped with a CCD system (200A Polytronics, Emsworth, UK).

To analyze transfection efficiency, 200–500 squashed cells from each time period were analyzed in several randomly selected fields. The number of cells was demonstrated by the blue fluorescence of the nuclei and transfected cells were identified by the red fluorescence of the rhodamine. To check the efficiency of GFP gene transfection, we applied the QWin program from Leica Microsystems (Wetzlar, Germany). We employed 25 sections from each time period. The program measures both the total epithelial area and the fluorescent regions in each section, and calculates the percentage of these regions.

Analysis of the GFP transgene by polymerase chain reaction

Total DNA was obtained from animals treated for 7 days and from control epididymides. Minced tissues were incubated for 2 h at 65°C in 12.5 mM Tris, 25 mM EDTA, 37.5 mM NaCl, 1% SDS, 20 mM DTT and 0.2 mg/ml proteinase K, pH 8.0. The samples were extracted twice with 1:1 phenol/chloroform and the DNA was precipitated with cold ethanol. The amount and quality of the DNA was assessed on agarose gels and the samples were stored frozen until use. For polymerase chain reaction (PCR) detection of the foreign gene, 1 μl of each sample (50–100 ng DNA) was used as template for amplification. The primers and conditions for detecting a 411 bp fragment of the GFP gene were as previously described (Carballada et al., 2000). The primers sequences were: 5’-GGG CAG ATT GTG TGG ACA GGT AAT GTG TG-3’ and 5’-GCT TTT CAA GAT ACC CAG ATC ATA TG-3’. The PCR conditions were 35 cycles of 30 s/95°C, 30 s/55°C and 30 s/72°C. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide and examined and photographed under UV light.

Detection of pSEAP-control

A pool of fluids collected from the cauda epididymides from injected and from non-injected animals (controls) were used. Two different experiments were done: in the first, 10 injected males and 10 controls were used. In the second, we employed seven injected and five non-injected males. Fluid collection was done 7 days after the injection of the foreign gene. The cauda epididymis of each animal was collected after surgery, and minced in 250 μl of PBS. Samples from each experiment were pooled and spun down (480 G) to separate spermatozoa and tissues. Furthermore, samples from four injected and four control animals were employed for the analysis of the cytosal fraction. Cauda epididymides
were carefully minced in PBS, centrifuged at 500 x g during 5 min and fractions of the pellet were employed. The Cytosol/Particulate Rapid Separation Kit (BioVision Research Products, CA, USA) was used according to the manufacturer instructions. To analyze the pSEAP-control, samples were incubated 30 min at 65°C and then assayed with the Great Escape Genetic Reporter System Kit (Clontech, Palo Alto, CA, USA) and measured in Relative Luminiscent Units (RLU) using a turner TD-2020 light meter.

Results

Analysis of transfection

Transfection was recognized by the presence of the red fluorescence of the Rhodamine molecule associated with the p-GeneGrip plasmid. After gene injection the cauda epididymis showed numerous red fluorescence spots, indicating the presence of the gene construction. Red fluorescent points appeared located in the epithelial cells, in both the cytoplasm and the nucleus (Fig. 1a and b). Large areas of the cauda epididymis appeared transfected and sometimes, in a single section, several regions showed fluorescence. When squashed cells from the non-fixed material were analyzed, the location of the gene construction was particularly noted. In this case, fluorescence appeared principally located in the cytoplasm, and a few fluorescent dots were observed into the nucleus (Fig. 1d and e). The analysis of the positive cells numbers were done using these preparations and showed that 39.70% of cells appeared fluorescent after 2 days, 31.77% after 7 days, 18.33% after 15 days and 7.54% after 30 days (Table I). Control preparations did not show red fluorescence (Fig. 1c).

Expression of the GFP gene

The green fluorescence of the GFP gene construction was clearly observed at all time periods analyzed. The most intense fluorescence was observed 2 days after treatment, and in some cases all tubules in a section appeared fluorescent (Fig. 1f). A decreasing fluorescence was seen 7 and 15 days after injection. Tubules analyzed 30 days after gene injection show a low green fluorescence. The fluorescence was clearly located in the cytoplasm and the nuclei appeared to be free of fluorescence (Fig. 1g and h). Green fluorescence appeared diffuse in the cytoplasm and as dense granules (Fig. 1i), showing the characteristics described for other GFP transfected cells (Olson et al., 1995; Kendall and Badminton, 1998). Fluorescence images showed that transfected cells are principal cells which are the most numerous in the epididymal epithelium. A similar observation was made in the rat epididymis after GFP gene transfer by electroporation (Kirby et al., 2004). The observation of complete (in toto) cauda epididymis showed that some regions were fluorescent and that others were not so bright (Fig. 1j and k). The fluorescence appeared restricted to the cytoplasm of epithelial cells (Fig. 1j). Control preparations of complete tubules did not show the fluorescent characteristics (Fig. 1k). The QWin Program, used to measure the percentage of fluorescent areas, showed that the percentage varied depending on the time periods after gene injection. Epididymidis collected 2 days after the gene injection showed fluorescence in the 32.24% of the epithelium. Then 29.98% appeared fluorescent after 7 days; 22.37% after 15 days and the 3.55% after 30 days (Table I). The PCR method confirmed the presence of the transgene in the DNA of the treated cells and the negative reaction of the control (Fig. 2).

Expression of pSEAP-control gene

The analysis done with the luminometer to detect pSEAP-control gene expression clearly showed a significant increase in alkaline phosphatase in the fluid collected from the treated epididymides. The increase was observed in the results of the two experiments: in experiment 1 the RLU of the test sample was 1110.43 compared with 91.08 in the control, and in experiment 2 the test sample showed a value of 522.03 RLU and the control was 40.44 RLU (see Fig. 3). Analysis of the cytosol fraction showed a RLU of 445.12 in treated animals and of 88.18 in the controls.

Discussion

Our results on epididymal transfection show that the epithelial cells of this organ can be easily transfected in vivo after a gene injection. In the case of the mouse vas deferens a similar situation occurred (Valenzuela et al., 2002). This is not surprising because when liposomes or lipids are employed, they form complexes with the DNA and these complexes are subsequently endocyted and incorporated into the cytoplasm of the transfected cell (Straubinger et al., 1998; Higashi and Sunamoto, 1995). Nevertheless, for expression of the recombinant DNA to occur, it is mandatory for it to be exported to the nucleus. In the case of the cauda epididymis, the red rhodamine fluorescence was located in a large percentage of epithelial cells, attaining a maximum of 39.70% of cells 2 days after gene injection. The transfected probe also appeared in several nuclei and the expression of the transgene was observed in a large extent of the treated epididymal tubules (32.24% of the epididymal area 2 days after gene injection). In the case of the mouse vas deferens, transfected cells have also been observed exclusively in the epithelium and attained a maximum of 13.3% (Valenzuela et al., 2002). In human vas deferens, 14.7% of cells were transfected after employing an in vitro method (Esporda et al., 2004). Furthermore, in our study, the results of PCR ensured the presence of the transgene in the DNA of the transfected cells.

The green fluorescence of the treated epididymides and the lack of reaction in the control experiments indicate that the foreign genes are expressed in the epithelial cells. The large number and morphology of transfected cells indicated that they are principal cells, which have a prismatic form and are the most common in the epididymal epithelium. On several occasions, it has been shown that transfection performed in different organs exclusively affects the epithelium. This has been observed in the case of the trachea and bronchi (Katkin et al., 1995; Fortunati et al., 1996), alveolar epithelium (Katkin et al., 1995) and in the female (Chamock-Jones et al., 1997; Rellos and Esponda, 2000) and male genital tracts (Esporda et al., 2004).

Recently electroporation methods have been employed to in vivo transfect the initial segment of the rat epididymis in order to analyze the expression of some genes and the influence on testicular fluids (Kirby et al., 2004; Yang et al., 2006). In these reports, GFP gene expression was observed for 1 week after electroporation and the GFP expression pattern was very similar to our observations, showing a green fluorescence occupying the whole cytoplasm of epithelial principal cells. The liposome mediated gene transfer method
Figure 1 (a–e) Analysis of transfection of the cauda epididymis.
(a) and (b) the red fluorescence of the pGeneGrip construction is clearly observed in cryostat sections of the epididymal epithelial cells 2 days (a) or 7 days (b) after gene injection. Transfection occurs in the epithelium. Connective tissues (c) and basal cells (indicated by arrows) do not show fluorescence. (c) control. A cryostat section of a epididymis from a non-injected animal show a negative red fluorescence. In all figures, nuclei fluoresce in blue due to DAPI staining. S shows spermatozoa nuclei. (d) and (e) a group of non-fixed epithelial cells from an epididymis collected two days after gene injection is observed under phase contrast (d) and under fluorescence (e). Numerous fluorescence dots appeared in these cells. Nuclei are indicated (arrows). (f–h) GFP expression in cryostat sections of the treated epididymides. (f) shows a cauda collected 2 days after gene injection. (g–h) a cauda collected 15 days after injection observed after DAPI staining (g) and green fluorescence (h). The green fluorescence appears in the cytoplasm of the epithelial cells, and its intensity is higher in some areas of the epithelium (indicated by arrows in f) while some areas are not positives (n). In (h), the green fluorescence of GFP appears diffuse and as intense dots in the cytoplasm. Nuclei do not fluoresce as indicated by an asterisk in h (The same nuclei are also indicated in g). (i, j and k) show cauda tubules observed in toto: (i) tissue collected 2 days after gene injection; (j) 7 days after injection, the intense green fluorescence is observed in some regions (arrows), but others show a slight fluorescence (*). (k) shows a control section from an epididymis injected with FuGENE alone after 7 days. No green fluorescence appears. Scale bars in figures a, b, c, d, e, g, h and i = 10 μm; in figures f, j and k = 20 μm.
used here appears as an alternative procedure to electroporation and shows longer times of gene expression.

Transgene expression occurred only in some areas of the epithelium along the epididymis. The maximum expression was observed 2 days after gene injection and the 32.24% of the epididymal area appeared fluorescent. Later, the fluorescence decreased and 1 month after injection a very low expression occurred. In the case of the rat epididymis, the *in-vivo* transfection using electroporation results in a striking expression of different transgenes during the first days after treatment (Kirby *et al.*, 2004). In the human vas deferens after *in-vitro* transfection, 40% of samples show transgene expression in 9.86% of the epithelial area (Esponda *et al.*, 2004). The fact that not the whole extent of the epididymal tubule shows expression of the transfected gene could be related to different causes such as the rate of RNA transcription, translation and stability, the half-life of the transfected DNA, the cell cycle stage or mitotic activity (Ledley and Ledley, 1994). Furthermore, it has been shown that secretion cycles could also affect transfection efficiency (Relloso and Esponda, 2000). Regarding the percentages of *in-vivo* transfected cells that express the gene, it has been shown that they are very variable. In some cases, a low number of cells appeared modified, such as in the respiratory epithelium (Grub *et al.*, 1994) or muscle (Acsadi *et al.*, 1991) in which only 1–5% of cells expressed the foreign DNA. In other cases, percentages are higher such as in the mouse vas deferens (Valenzuela *et al.*, 2002). Nevertheless, in other cases, such as the muscle (Danko *et al.*, 1997) uterus (Charnock-Jones *et al.*, 1997) and liver (Sullivan *et al.*, 1997), percentages reach the 70% of cells.

The GFP green fluorescence indicates that the presence of foreign DNA and its expression decreases 2 weeks after gene injection into the cauda epididymis. The persistence of GFP expression for more than 2 weeks in the cauda epididymis indicated that this organ could be a significant target tissue for the introduction of foreign genes. Transfections developed *in vivo* in different tissues have shown that the period of transgene expression has sometimes been very brief (Leibiger *et al.*, 1991; Sullivan *et al.*, 1997), but in other cases periods as prolonged as 3 months in the vas deferens (Valenzuela *et al.*, 2002) or 19 months in the arterial wall (Nabel *et al.*, 1990) have occurred. The cause of this variation depends on

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### Table I Results of transfection experiments using the p-GeneGRIP gene construction

<table>
<thead>
<tr>
<th></th>
<th>2 days</th>
<th>7 days</th>
<th>15 days</th>
<th>30 days</th>
<th>Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>9</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>% Transfected cells</td>
<td>39.70</td>
<td>31.77</td>
<td>18.33</td>
<td>7.54</td>
<td>3</td>
</tr>
<tr>
<td>% Area expressing GFP (1)</td>
<td>32.24</td>
<td>29.98</td>
<td>22.37</td>
<td>3.55</td>
<td>0.9</td>
</tr>
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*Represents the percentages of GFP fluorescent areas calculated by the QWin Program.

*Controls are represented by no injected animals (a) and animals injected only with FuGENE 6 (b).

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**Figure 2** PCR detection of GFP sequences in the transfected epididymis.

Lane 1 shows the molecular weight markers (the 394 Kda band is indicated). Lane 2 is the positive control using the pGeneGrip plasmid as template. Lane 3 shows the PCR product after using the DNA from a non-transfected epididymis. Lane 4 is the DNA from a pool of transfected samples. The band of 411 bp is observed in lanes 2 and 4.

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**Figure 3** The results of the luminometer measurements of epididymal fluids from pSEAP- (shaded bars) and control- (black bars) treated animals are shown.

A significant positive reaction appears in the fluids collected from treated males of experiments 1 and 2 (RLU: Relative Luminiscent Units).
the foreign gene (half-life of the DNA; rate of transcription translation and stability of the RNA) and on the tissue (cell cycle stage, mitotic activity, etc.). It is well-known that in the epididymis, the epithelium has a low mitotic activity (Sun and Flickinger, 1982) and this could be the probable cause of the persistence of foreign gene expression in these epithelial cells.

The use of the pSEAP-control gene construction indicated that epididymal secretions can also be modified by the transfection procedure. The pSEAP-control gene has been previously used for in vivo transfection of the mouse vas deferens (Valenzuela et al., 2002) and showed a good efficiency. The modification of epididymal secretions would be an important tool regarding the fertilizing ability of spermatozoa contained in the cauda epididymis. It is well-known that epididymal secreted proteins are related to the sperm maturation phenomena and to the acquisition of sperm fertilizing ability and motility (Bedford, 1979; Cooper, 1986). Various macromolecules secreted by the epididymal epithelium interact with the sperm plasma membrane and some of them participate in the sperm/oocyte (or sperm/ovocyte envelopes) interactions during fertilization (Cooper, 1986). In the future, the possibility to use some particular genes, antisense genes or some siRNAs may provide a way of modifying secretions and thus the fertilizing capacity of the spermatozoa stored in the cauda epididymis.

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