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Method

Lentiviral vector transduction of spermatozoa as a tool for the study of early development

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ABSTRACT

Spermatozoa and lentiviruses are two of nature's most efficient gene delivery vehicles. Both can be genetically modified and used independently for the generation of transgenic animals or gene transfer/therapy of inherited disorders. Here we show that mature spermatozoa can be directly transduced with various pseudotyped lentiviral vectors and used in in vitro fertilisation studies. Lentiviral vectors encoding Green Fluorescent Protein (GFP) were shown to be efficiently processed and expressed in sperm. When these transduced sperm were used in in vitro fertilisation studies, GFP expression was observed in arising blastocysts. This simple technique of directly transducing spermatozoa has potential to be a powerful tool for the study of early and pre-implantation development and could be used as a technique in transgenic development and vertical viral transmission studies.

1. Introduction

Many genetic processes that occur in the early embryo could be much more easily studied if male germ cells rather than female could be utilised for genetic modification. The accessibility and easy availability of spermatozoa make them attractive candidates for use in such studies. However, although sperm themselves are ideally suited to gene transfer, introducing genetic material into sperm has proven less straightforward [1–3].

Lentiviruses are one of nature's most efficient gene delivery vehicles. They have been used successfully to treat various human genetic disorders (acquired and inherited) [4–7], as tools for research into gene function and regulation/knock down, and more recently as vehicles for reprogramming of differentiated somatic cells to an embryonic state. As such, the genetic payload engineered into these lentiviral vectors has ranged from short interfering RNAs, to transcription factors and microRNAs [8–10].

To generate safe gene transfer vectors the lentiviral genome is modified by removing non-essential genes encoding most of the viral accessory and regulatory genes, and splitting the genome of the virus into separate transcriptional units (trans complementing for expression of viral structural and some regulatory components and cis acting for vector development encoding the transgene/reporter construct and packaging signal). An additional safety mechanism ensuring that only the transgene is expressed in transduced cells, is provided by deletion of enhancer elements present in the 3′ Long Terminal Repeat (LTR) of the vector [11,12]. Reporter genes or the genetic payload can be engineered into these vectors. Commonly utilised promoters driving the reporter genes or genetic payload include Phosphoglycerate kinase promoter (PGK), Elongation factor 1 alpha promoters (EF-1) and Cytomegalovirus

Abbreviations: GFP, Green Fluorescent Protein; LTR, Long Terminal Repeat; PGK, Phosphoglycerate kinase promoter; EF-1, Elongation factor 1 alpha promoter; CMV, Cytomegalovirus promoter; UCOE, ubiquitous chromatin opening element promoter; VSV-g, vesicular stomatitis virus; 293T, Human embryonic kidney cells; 7-AAD, 7-Aminoactinomycin D; IVF, in vitro fertilisation; AZT, azidodeoxythimidine

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promoter (CMV). Recently, the use of methylation resistant promoters such as ubiquitous chromatin opening element promoter (lacking in enhancer activity) (UCOE) has been successfully utilised in gene transfer protocols, whilst maintaining relative expression levels of reporter and increasing the safety profile of the vectors [13,14]. The resulting self-inactivating lentiviral vectors can also be readily pseudotyped with diverse variety of viral envelope proteins, thus altering the natural tropism of the vectors for various cell types. The envelope most commonly utilised in lentiviral transduction protocols is the G-glycoprotein that originates from the vesicular stomatitis virus (VSV-g). VSV-g-pseudotyped lentiviral vectors have been shown to transduce a wide variety of somatic cells.

We thus hypothesised that spermatozoa, though terminally differentiated haploid cells, might still function as targets for lentiviral vector transduction, and therefore enable the exploitation of such genetically modified spermatozoa as tools for the study of early development.

2. Methods

2.1. Pig and mouse spermatozoa

Boar spermatozoa were generously donated by the Pig Improvement Company, UK. Swim up population of B6/CBA1 mouse sperm was obtained following cervical dislocation of the animal and excision of the epididymis and vas deferens. All procedures involving animals were carried according to the regulations set out by the Home Office, Animals in Scientific Procedures Act (PPL 70/6320).

2.2. Generation of pseudotyped lentiviral vectors

Human embryonic kidney cells (293T), 293T cells stably expressing murine or porcine membrane-bound Stem Cell Factor (293T-M-mbSCF or 293T-P-mbSCF respectively) was used as packaging cells to generate various pseudotyped lentiviral vectors. Murine and porcine membrane bound Stem Cell Factor were isolated from the testis of both species by RT-PCR amplification and insertion into a TA Cloning vector (Invitrogen). The isolated SCF cDNA fragments were then sub-cloned into pRep8 (Invitrogen) expression vectors. Establishment of 293T cells over-expressing these membrane-bound Stem Cell Factors was done as we described previously for human SCF [15], resulting in the murine and porcine constructs 293T-M-mbSCF and 293T-P-mbSCF, respectively. Briefly, 15 μg of vector plasmid (pRRL.ppt.hPGK.eGFP.WPRE.SIN18) [11] or pHR'SINcPPT-UCOE-E [13], 10 μg of packaging construct plasmid (pΔ8.74) and 5 μg envelope plasmid-VSV-G or 10 μg envelope plasmid-Ecotropic, GALV-© or RD114 [16] was used in a calcium phosphate transfection mix (Invitrogen) and transfected in a 10 cm tissue culture plate in the presence of 25 μM chloroquine (Sigma). The transfection mix was removed 18 h later and cells washed twice in complete media. 8 ml of fresh medium was added to the plate and viral laden supernatants harvested 24 h later, centrifuged at low speed (1200 rpm for 5 min), filtered through a 0.45μ filter and frozen at −80 °C. A second round of harvest was carried out by replacing the old media with 8 ml of fresh medium and incubation carried out a further 24 h after which the supernatant was again cleared by low speed centrifugation, filtered through a 0.45μ filter and frozen at −80 °C [17].

Lentiviral particles were concentrated using polybrene and chondroitin sulphate method [18] and viral pellet resuspended (one hundredth of the original volume) in capacitating medium. Lentiviral particles were titered on 293T cells or NIH 3T3 cells depending on the envelope utilised and analysed by flow cytometry [17,19]. Concentrated lentiviral vector particles ranged from 10^8 to 10^9 infectious particles per ml.

2.3. Trans methionine-^{35}S radiolabeling and immunoprecipitation of GFP from lentivirally transduced spermatozoa

Porcine spermatozoa were used to determine *de novo* protein synthesis. One ml of sperm (10^8) cells were washed twice in PBS and sperm pellet resuspended in 780 μl of capacitating [20] media containing amino acids (lacking methionine) with penicillin and streptomycin. 200 μl of VSV-g pseudotyped viral supernatant was added to the mixture. The media was supplemented with 20 μl (30 μCi/mL) Trans methionine-^{35}S label (NEN) and incubation carried out for 4 h, at 37 °C and 5% CO_2 in atmosphere. Sperm were then washed twice in cold PBS and lysed using RIPA buffer (containing protease inhibitors). Total lysates (normalised to protein concentrations of 2 mg/ml protein) were either run on a 10% denaturing polyacrylamide gel or immunoprecipitated using a rabbit polyclonal GFP antibody (1:100 dilution) (Abcam, ab290). Gels were dried down and exposed to radioactive film for 2 weeks.

2.4. Lentiviral transduction of spermatozoa

Porcine sperm transduction was carried by initially spinning 1 ml of sperm (1×10^8 sperm cells). The sperm pellet was resuspended in 100 μl capacitating medium (adapted from bovine sperm capacitating medium) [20] and 200 μl of viral supernatant (multiplicity of infection, MOI = 1) and incubated for 48 h at 37 °C and 5% CO_2 in atmosphere. GFP analysis was done by flow cytometry and was performed by 7-Aminoactinomycin D (7-AAD) exclusion of dead cells. Transduced cells were compared to mock population.

Mouse spermatozoa were obtained following cervical dislocation of an adult male mouse. Both epididymis (including the vas deferens) were carefully excised and placed in one well of a 4 well embryo culture plate (Nunc) containing 200 μl Krebs ringer solution (Sigma) supplemented fresh with 0.2 mM Calcium Chloride, 3 mg/ml BSA and 25 mM Sodium Bicarbonate. A 30 g needle was used to puncture various parts of the epididymis and along the vas deferens to allow sperm to swim out. 200 μl of viral supernatant was added to the plate as well and incubated for 3 h at 32 °C and 5% CO_2 in atmosphere. Control (mock) experiments were done in the same way except viral supernatant was omitted and replaced with a further 200 μl Krebs ringer solution. Mock and transduced swim up population of sperm was centrifuged, supernatant discarded and sperm pellet resuspended in 100 μl Krebs–Ringer solution. Transduced sperm were then either visualised by confocal microscopy (Zeiss-510 inverted microscope, 40× lens) or used in IVF studies (murine study only).

2.5. In vitro fertilisation (IVF) studies

Standard mouse IVF and embryo transfer techniques were utilised. Briefly, B6CBF1 19 day old female mice were superovulated using 0.1U PMSG and HCG (Intervet). Five mice were used per IVF cycle (a total of 3 independent experiments). Collected eggs (between 10 and 20) were placed in HTF buffer for IVF with lentivirally-transduced sperm. IVF was allowed to proceed for 4–5 h after which presumptive zygotes were washed (in four drops in succession) in HTF buffer and incubation carried out overnight. The next day, the developing embryos were placed in KSOM containing amino acid supplements and the IVF was allowed to proceed for 4 days and embryos imaged by confocal epifluorescence imaging (Leica SP5). Images were obtained using a 40× lens utilising identical settings for mock and transduced samples.

2.6. Genomic DNA extraction, PCR and LAM-PCR analysis from sperm

DNA was extracted from transduced and mock sperm samples (Qiagen). 50 ng DNA was used as template for PCR detection of the Woodchuck Post Regulatory Element Sequence (WPRE) encoded within the lentiviral vector. The primers used were:

WPRE-F 5′-A C T G T G T T T G C T G A C G C A A C-3′ and

WPRE-R 5′-C A A C A C C A C G G A A T T G T C A G-3′

The cycling conditions were: an initial denaturation step at 94 °C for 2 min, followed by 34 cycles at 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min. A final cycle was carried out with the extension step of 72 °C extended to 10 min. 20% of PCR products were resolved on a 2% agarose gel and a 174 bp product indicated the amplified WPRE DNA from the lentiviral vectors.

LAM-PCR was performed as previously described [21].

3. Results

3.1. Gene expression in porcine spermatozoa

To establish that mature spermatozoa are capable of *de novo* protein synthesis we performed radioisotope labelling of actively synthesised proteins and immunoprecipitation studies. When pig sperm were cultured in methionine-free medium supplemented with ^{35}S labelled methionine, under conditions favouring capacitation [20], newly synthesised proteins could be detected by autoradiography following polyacrylamide gel electrophoresis (Fig. 1A). This observation was consistent with previous findings that mature spermatozoa are thought to translate nuclear encoded genes by mitochondrial type ribosomes contained in sperm [20].

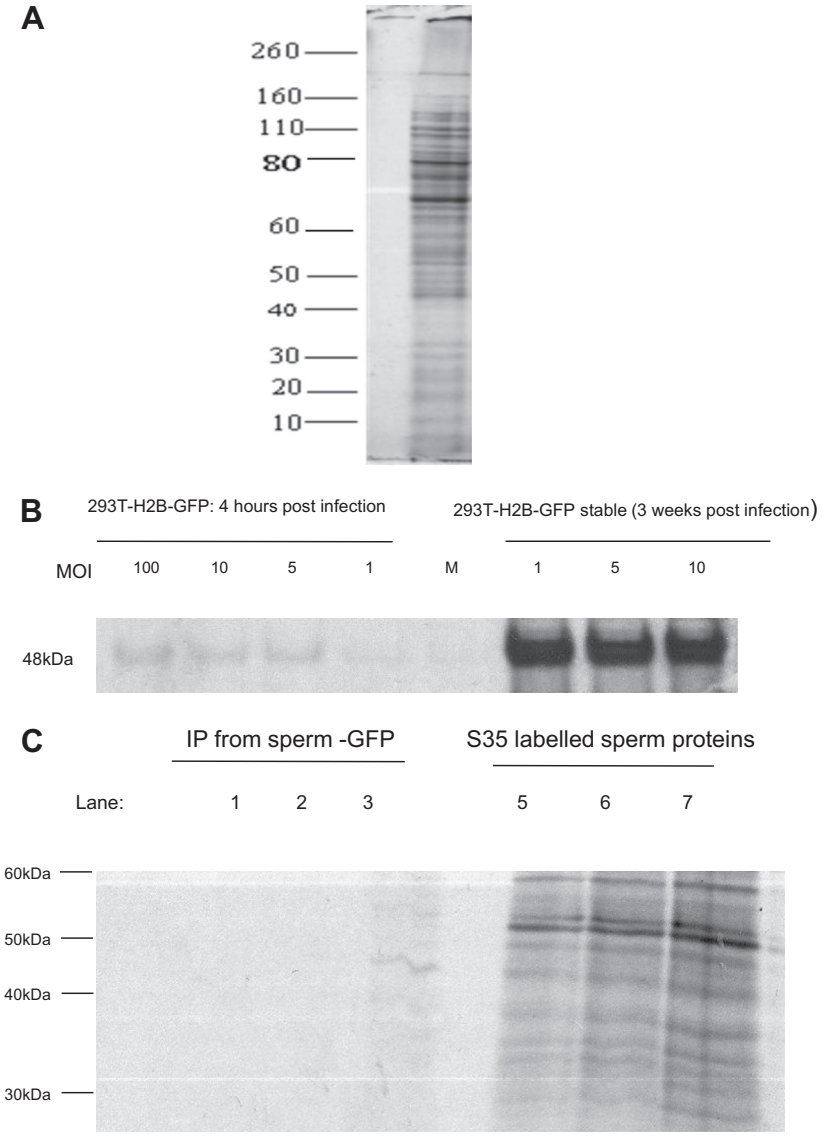


Fig. 1. *De novo* protein synthesis and GFP precipitation from vector transduced spermatozoa. (A) Newly synthesised proteins could be detected following culture of porcine sperm with radioactively labelled methionine. Gel blots were dried to completion onto filter paper, exposed to an X-ray film for 2 weeks and X-ray film developed using an automatic developer. (B) 293T cells were incubated with VSV-g pseudotyped lentivectors (with histone 2b-gfp as the reporter) at MOIs ranging from 1–100 in the presence of ^{35}S labelled methionine for 4 h, GFP could be immunoprecipitated quantitatively with an anti GFP antibody. On the other hand, 293T cells that had been transduced with same vector and propagated for 3 weeks in culture and then subjected to radiolabeling (4 h) and immunoprecipitation with antibody to GFP also revealed the 48 kDa histone2b-gfp marker but did not differ quantitatively. (C) In spermatozoa, GFP could also be immunoprecipitated as seen in lane 3. Mock transduced and protein G beads only (no antibody) controls are in lanes 1 and 2 respectively. Total ^{35}S radiolabelled proteins (from mock, agarose-beads only and vector incubated lysates) prior to immunoprecipitation are represented in lanes 5–7 respectively. Exposure of blots to an auto radiography film was carried out for 2 weeks.

Furthermore, when the VSV-g pseudotyped lentiviral vector encoding Histone 2B-fused Green Fluorescent Protein (H2B-GFP) (Fig. 2A top panel) was used to transduce 293T cells and porcine spermatozoa, newly synthesised GFP could be immunoprecipitated from both types of transduced cells (Fig. 1B and C, lane 3).

3.2. Lentiviral transduction efficiencies in porcine spermatozoa

Using this vector (Fig. 2A top panel), the transduction efficiency on porcine spermatozoa was established by flow cytometry. GFP expression was determined in combination with 7-AAD staining

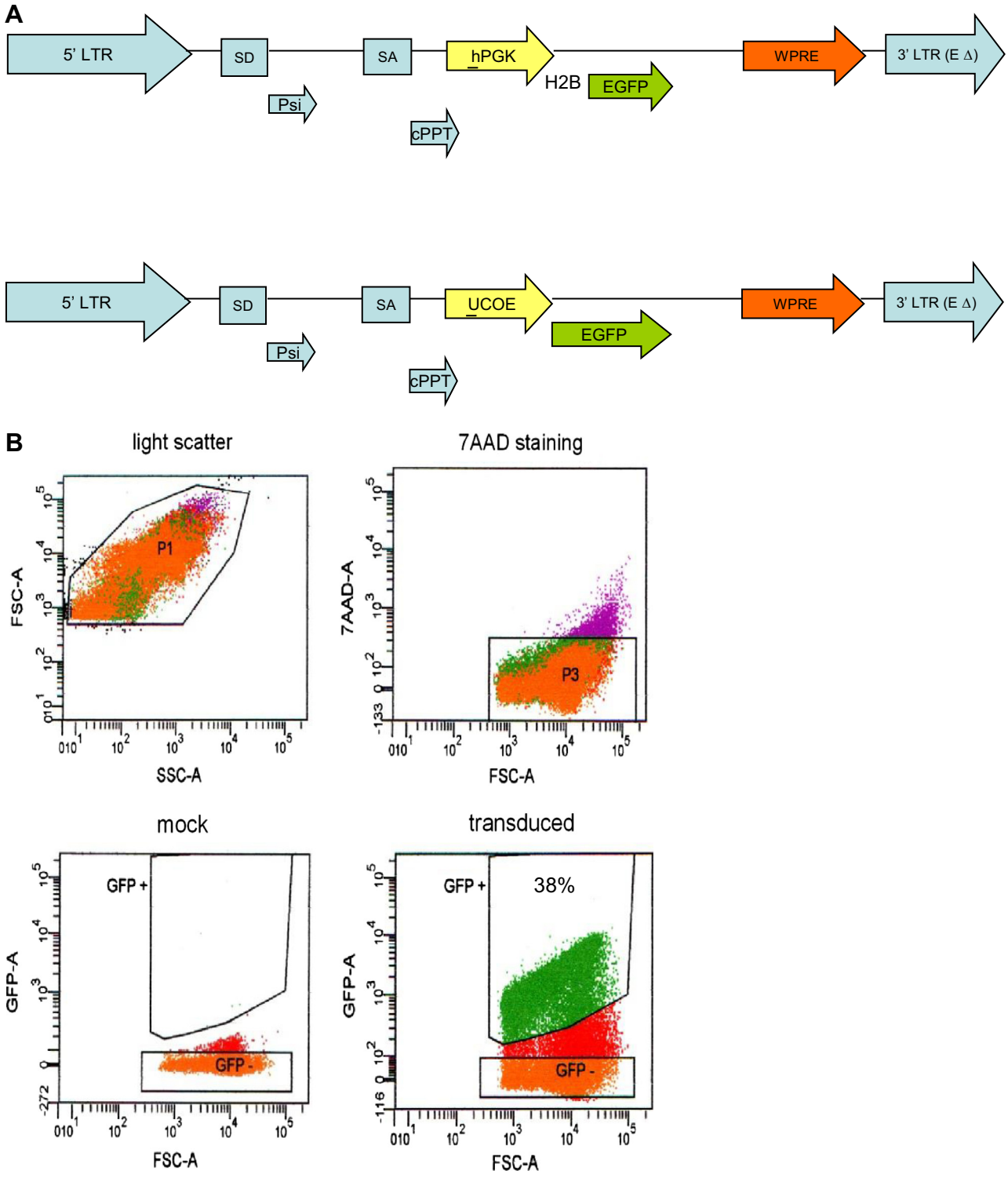


Fig. 2. Schematic map of the lentiviral vectors utilised in this study. (A) This vector is self-inactivating in transduced cells due to deletions of the enhancer region in the 3′ LTR. The reporter gene, GFP is driven by a housekeeping promoter PGK (top panel) and UCOE (bottom panel). (B) Flow cytometry analysis of porcine transduced spermatozoa. Porcine spermatozoa were incubated with a pSCF-VSV-g pseudotyped lentiviral vectors (Pgk promoter) and 7-AAD staining to exclude dead cells. (Top left panel) Mock/untransduced sperm light scatter analysis. (Top right panel) 7-AAD staining for exclusion of dead cells. (Bottom left panel) Untransduced (mock) live spermatozoa with no GFP expression. (Bottom right panel) Live and transduced spermatozoa showing 38% of spermatozoa expressing GFP.

to exclude dead cells. Up to 38% GFP-positive spermatozoa were detected (Fig. 2B, bottom right panel).

Our laboratory has previously demonstrated that retroviral transduction of somatic cells can be enhanced [22], or specifically targeted [15] by engineering retroviral packaging cells to express (in their plasma membrane) a ligand for a receptor present on the surface of the target cells. Retroviruses produced from such engineered packaging cells incorporate the ligand into their surface, resulting in particles with enhanced binding activity on target cells [17]. When utilised in conjunction with a ubiquitously transducing envelope, such as VSV-g, these engineered virus particles demonstrate significantly enhanced transduction efficiencies [17].

Spermatozoa have been reported to express c-kit [23], the receptor for stem cell factor (SCF) ligand. We confirmed this finding by immunostaining porcine spermatozoa and demonstrating distinct c-kit expression on the acrosome (Fig. 3A). Consequently, VSV-g-pseudotyped lentivirus particles produced in packaging cells expressing porcine membrane-bound SCF (pSCF-VSV-g) showed enhanced levels (about 3.5-fold) of transduction on porcine spermatozoa compared to lentivirus pseudotyped with VSV-g alone (Fig. 3B). Similar results were also obtained on transduction of murine spermatozoa using lentiviruses displaying murine mbSCF [24].

We then tested the use of the enhancerless UCOE promoter to drive GFP expression from vectors (Fig. 2A, bottom panel) in lentivirally-transduced porcine spermatozoa. Transduction efficiencies comparable to those obtained using vectors employing the PGK promoter were observed. Notably though, the range of expression levels from UCOE was significantly reduced in comparison to PGK, and the peak level of expression was approximately an order of magnitude lower (Fig. 3C).

Lentiviral particles pseudotyped with various types of retroviral envelopes, such as (chimaeric)Gibbon-Ape Leukaemia Virus envelope (cGALV) or Feline Immunodeficiency Virus envelope, (RD114) could also be used to infect porcine spermatozoa with varying levels of efficiency (data not shown).

3.3. Validation of GFP expression in spermatozoa

To validate that the fluorescence observed in porcine spermatozoa was due to lentiviral encoded, *de novo* GFP synthesis, we performed experiments to block the expression of GFP, either with the HIV reverse transcriptase inhibitor, azidodeoxy thimidine (AZT) (Fig. 4B), or by transfection (Escort V, Sigma) with silencing RNA to GFP (siRNA-GFP) (Ambion) (Fig. 4A). When sperm were incubated with 1 μM AZT or 10 nM siRNA-GFP 2 h prior to the addition of the lentiviral vector, GFP expression, as determined by flow cytometry, was inhibited by about 50% compared to untreated or scrambled siRNA controls (Fig. 4A and B). Furthermore, when porcine spermatozoa were incubated with lentiviral vectors at 18 °C for 24 h, GFP expression was reduced by at least 3-fold compared to incubations carried out at 32 °C (Fig. 4C),

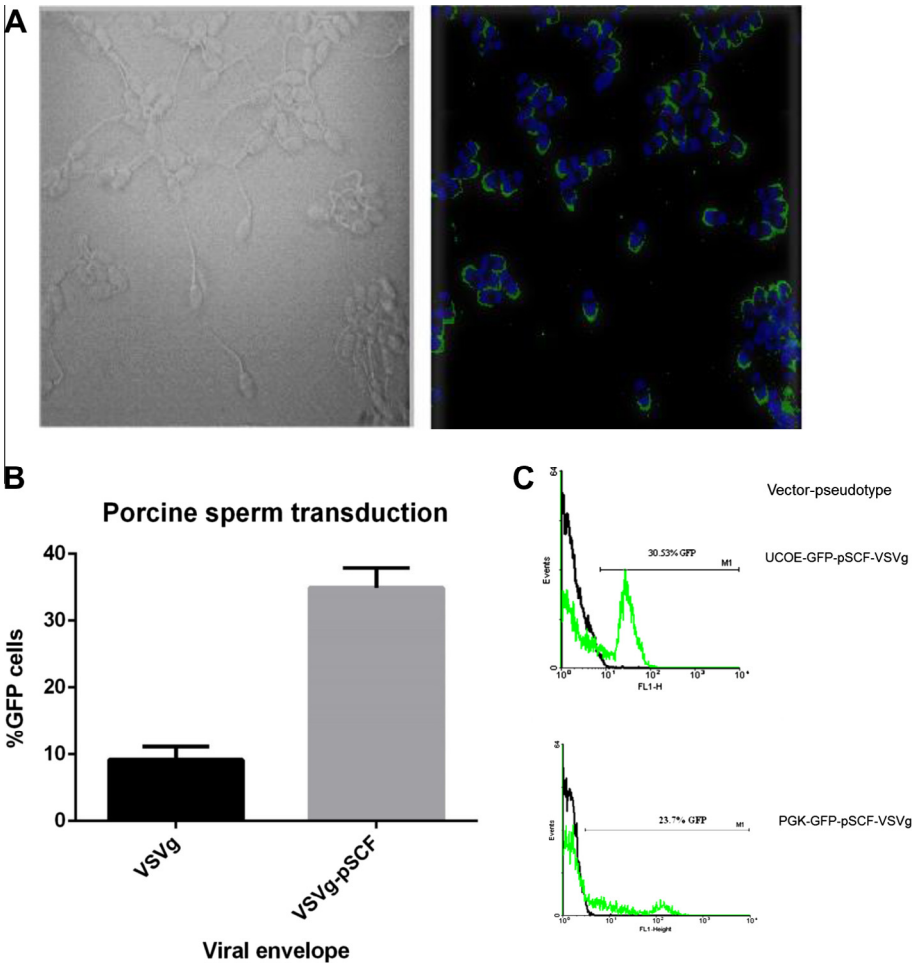


Fig. 3. Lentiviral transduction efficiencies in mature spermatozoa. Porcine spermatozoa were cytospun onto microscope slides and stained with anti c-kit-FITC (Santa Cruz) conjugated antibody. (A) Spermatozoa expressing c-kit on the acrosome region. (B) Enhanced transduction of efficiency of spermatozoa was observed when pSCF was used in conjunction with VSV-g envelope compared to VSV-g alone, as analysed by flow cytometry. (C) Comparison of UCOE and PGK promoters on transduction efficiencies and expression levels in sperm at an MOI of 10.

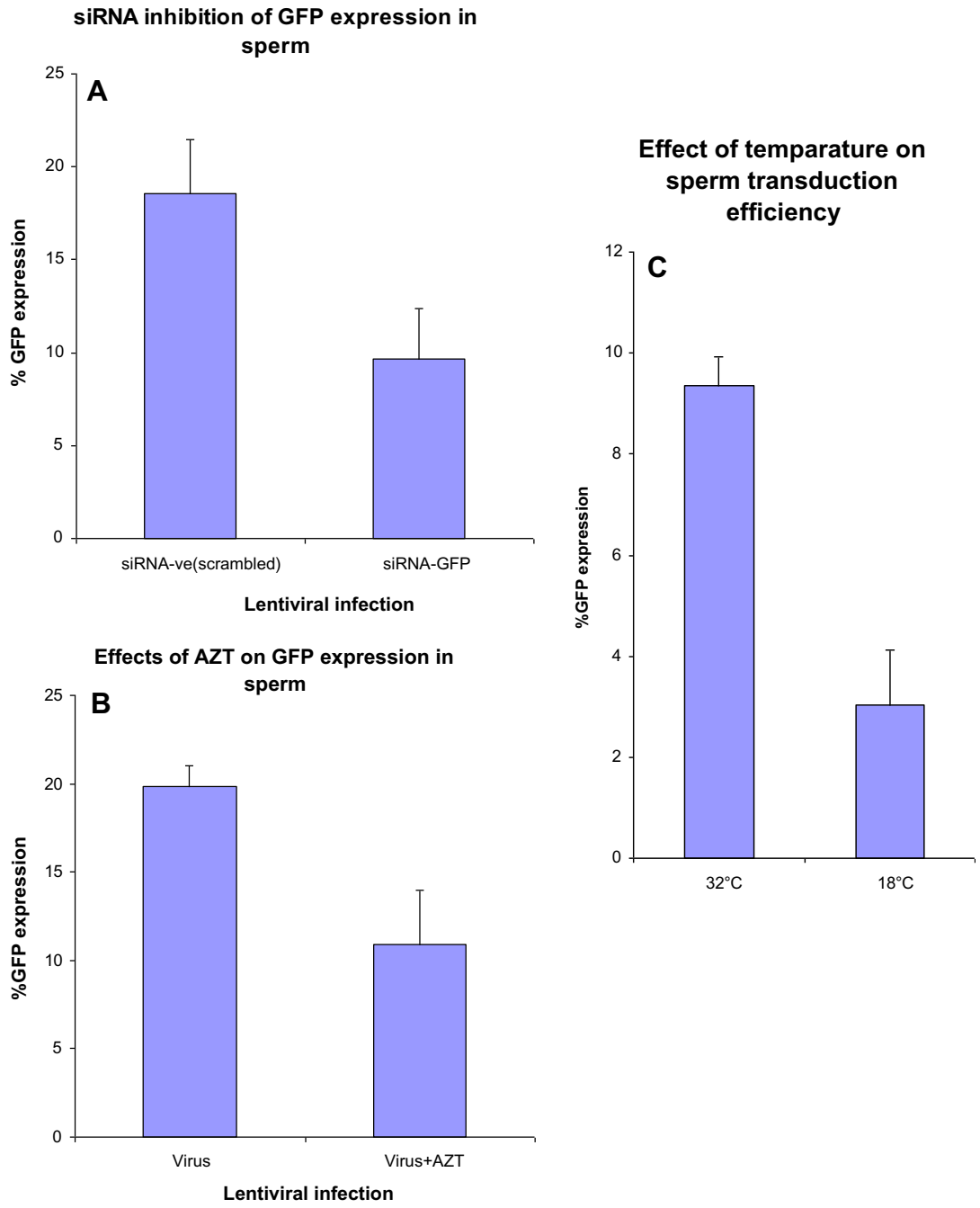
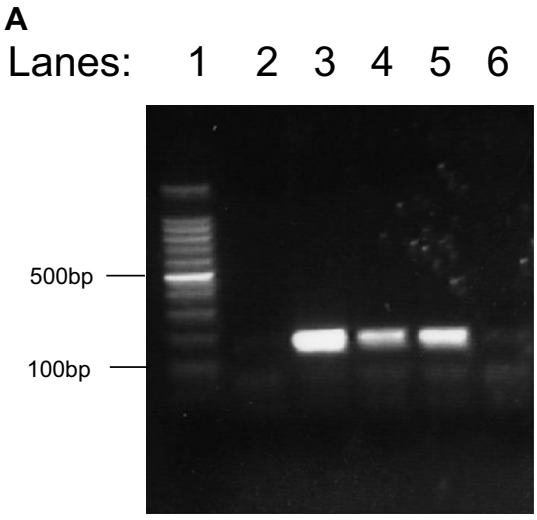


Fig. 4. Validation of GFP expression in spermatozoa. (A)Porcine spermatozoa were transfected with si RNA to GFP or scrambled/control siRNA (10 nM) using Escort V (Sigma) in triplicate. Two hours later, VSV-g-pSCF pseudotyped lentiviral vectors were added to the siRNA transfected cells and incubation carried out for a further 24 h. GFP expression was then compared by flow cytometery. (B) Porcine spermatozoa were initially incubated with 1 μM AZT in triplicate. Again 2 h later, VSV-g-pSCF pseudotyped lentiviral vectors were added to the sperm culture and GFP expression determined by flow cytomtery 24 h later. (C) Porcine spermatozoa were incubated with a VSV-g-pSCF pseudotyped lentiviral vector at two different temperatures. GFP expression was again determined 24 h later by flow cytometry.

demonstrating that the GFP expression in mature spermatozoa was dependent on lentiviral transduction.

Lentivirally transduced porcine spermatozoa were further analysed for the presence of the lentiviral vector genome in sperm. This was done by PCR amplification of the WPRE sequence encoded within the lentiviral vector genome from lentivirally transduced porcine sperm genomic DNA. The expected 174 bp PCR fragment was successfully amplified (Fig. 5A).

We then investigated whether, following in vitro transduction, the lentiviral vector genome had integrated into the spermatozoon DNA. This was determined by performing linear-amplification-mediated polymerase chain reaction (LAM-PCR) [21] on DNA from transduced pig spermatozoa. From this analysis we found evidence for at least 2 integration sites, one of which appeared to be unique to the pig genome. The first unique integration sequence was located in the X-chromosome (Fig. 5B). The other integration site was mapped imperfectly to the porcine homologue of the human (and chimp) Rh D gene. Two other identifiable LAM–PCR sequences indicated the presence of episomal (non-integrated) or pre-integration complex forms of the provirus. Despite several attempts, no integration sites could be detected in murine spermatozoa. However, routine detection of pre-integration complexes or episomal forms was observed.



B

MH1 CO5 seq.
Pig DNA sequence from clone CH242-144C16 on chromosome X, complete sequence

```
Sperm   73     CAGGAAGTCAAACCACAGAGAGAGGGAGGGAGGGAGGTGTCGGAGAAATT   122
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Genome 70946   CAGGAAGTCAAACCACAGAGAGAGGGAGGGAGGGAGGTGTCGGAGAAATT   70995

Sperm   123    CCTTTTCGGCAGAGATTTGAAGCGTCTTGTTATATAACAGGATCCGTCTC   172
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Genome 70996   CCTTTTCGGCAGAGATTTGAAGCGTCTTGTTATATAACAGGATCCGTCTC   71045

Sperm   173    CACTGCAGCGTGATGCAAGAAGCTGCAACAGAACTGAAACACGGGACTTG   222
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Genome 71056   CACTGCAGCGTGATGCAAGAAGCTGCAACAGAACTGAAACACGGGACTTG   71195

Sperm   223    TGTGTCCCCTGTCCCTGCAGTGGGGAGTCCTCCCAGGCGTTGTTTGCAGG   272
               |||||||||||||||||||||||||||||||||||| ||||||||||||
Genome 71116   TGTGTCCCCTGTCCCTGCAGTGGGGAGTCCTCCCAGGTGTTGTTTGCAGG   71145

Sperm   273    CC                                                  274
               ||
Genome 71146   CC                                                  71147
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MH2 D05 seq.
Pig DNA sequence from clone CH242-144C16 on chromosome X, complete sequence

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Sperm   72     CCTGCAAACAACGCCTGGGAGGACTCCCCACTGCAGGGACAGGGGACACA   121
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Genome 71147   CCTGCAAACAACACCTGGGAGGACTCCCCACTGCAGGGACAGGGGACACA   71098

Sperm   122    CAAGTCCCGTGTTTCAGTTCTGTTGCAGCTTCTTGCATCACGCTGCAGTG   171
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Genome 71097   CAAGTCCCGTGTTTCAGTTCTGTTGCAGCTTCTTGCATCACGCTGCAGTG   71048

Sperm   170    GAGACGGATCCTGTTATATAACAAGACGCTTCAAATCTCTGCCGAAAAGG   221
               ||||||||||||||||||||||||||||||||||||||||||||||||||
Genome 71047   GAGACGGATCCTGTTATATAACAAGACGCTTCAAATCTCTGCCGAAAAGG   70998

Sperm   222    AATTTCTCCGACACCTCCCTCCCTCCCTCTCTC                   254
               |||||||||||||||||||||||||||||||||
Genome 70967   AATTTCTCCGACACCTCCCTCCCTCCCTCTCTC                   70935
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Fig. 5. Lentiviral vector detection and integration of vectors into porcine spermatozoon genome. (A) Detection of WPRE sequence from lentivirally transduced spermatozoa by PCR of sperm genomic DNA. Lanes 1: 100 bp Molecular weight marker-Promega, 2: water control (no template DNA), 3: Genomic DNA from 293T cells stably transduced lentiviral vectors, 4: Genomic DNA from porcine sperm transduced with lentiviral vectors (VSV-g only) for 48 h, 5: Genomic DNA from porcine sperm transduced with lentiviral vectors (pSCF-VSV-g) for 48 h, 6: Mock (untransduced control) sperm DNA. (B) Following LAM-PCR, amplified products were cloned and sequenced. The first unique sequence, integrated to the x-chromosome of the porcine genome. The alignments shown were from two colonies containing similar integration sites.

3.4. The use of lentivirally transduced spermatozoa in early mouse embryonic development

To further determine whether lentivirally transduced spermatozoa could be used for gene transfer to developing embryos; IVF experiments were carried out in mice. "Swim up" populations of mouse spermatozoa from the epididymis and vas deferens were obtained from 8–12 week old male mice and incubated at 32 °C with mSCF-VSV-g pseudotyped lentiviral vectors for 3 h prior to sperm imaging or IVF. Following lentiviral transduction, fluorescent mouse spermatozoa could also be visualised by confocal imaging (Fig. 6A, bottom two panels). Using standard IVF protocols, transduced sperm gave rise to transgenic blastocysts as shown in Fig. 6D and E. This figure is from the 3rd experiment carried out.

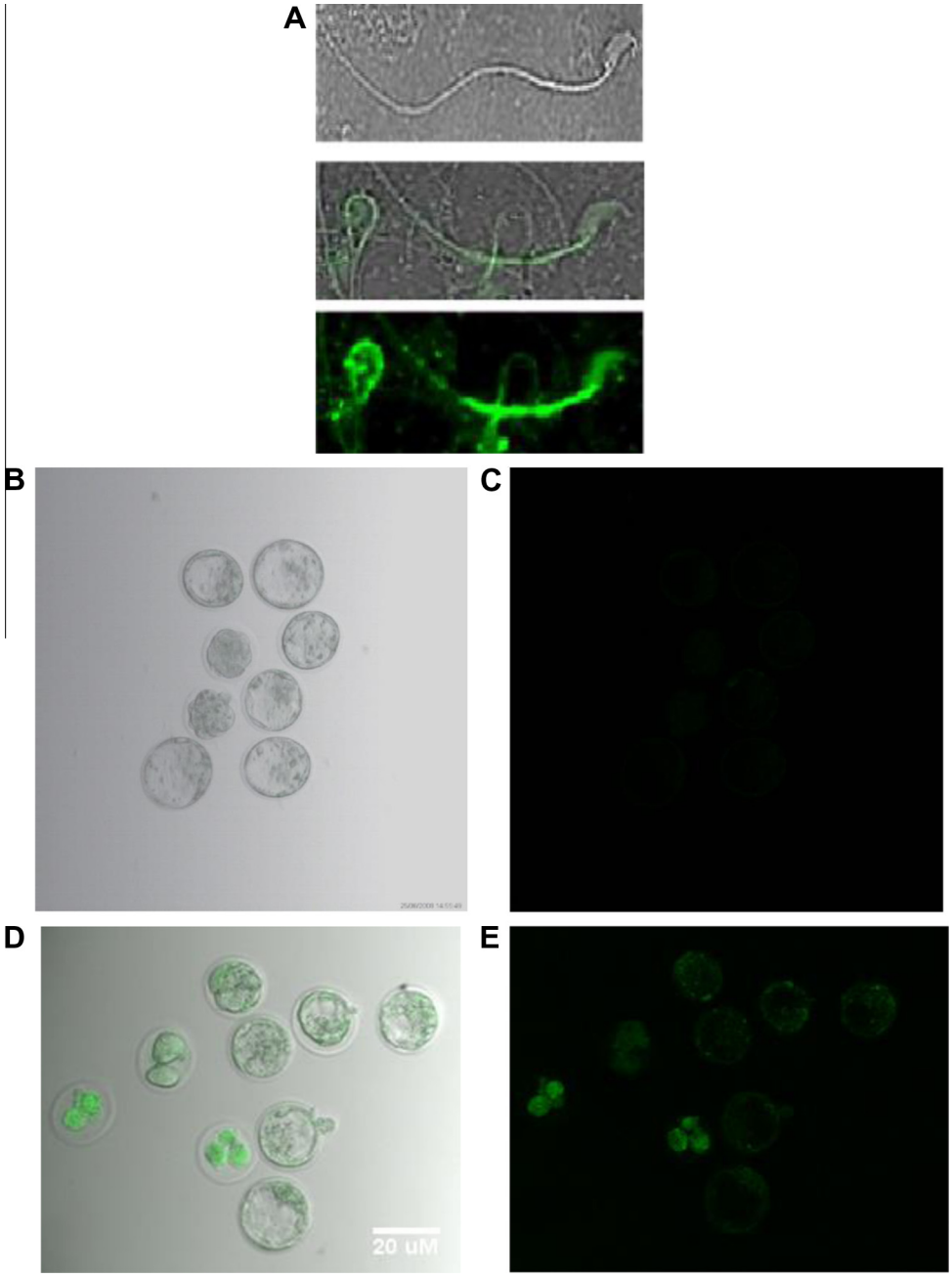

Fig. 6. Transgenic mouse blastocysts obtained following lentiviral transduction of mature spermatozoa. (A) Murine spermatozoa were incubated for 3 h with mSCF-VSV-g-pseudotyped lentiviral vector and subjected to confocal imaging. Middle panel: with transmitted light and bottom panel, without transmitted light showing intense GFP expression, particularly in the mid-piece and in the head. The top panel of this image was sperm not expressing GFP (untransduced), which was an image from a different field of view from the same chamber imaged using identical settings. GFP expression could only be observed using the 40× magnification. (B) Blastocysts obtained from mock transduced spermatozoa with transmitted light. (C) As in B without the transmitted light. (D) Fluorescent blastocysts obtained from lentivirally transduced spermatozoa, with transmitted light. (E) As in D without the transmitted light. All images were obtained using identical settings for fluorescence detection, in controls (mock) and transduced sperm/blastocysts.

4. Discussion

In this report, we have shown that mature spermatozoa can be transduced by pseudotyped lentiviral vectors and that these transduced spermatozoa can then be utilised efficiently for the study of development. GFP expression in sperm was confirmed and validated by biochemical and molecular analyses.

This study has raised some fascinating biological questions, such as the mode of viral entry, the frequency with which these vectors integrate within the zygote genome and the mechanism by which lentiviral encoded gene products are translated into protein. Binding of lentiviral vectors to spermatozoa, internalisation and completion of reverse transcription in sperm appears to be sufficient for transgene expression within sperm and blastocysts. The acrosome matrix of sperm may well facilitate the import of the lentiviral vector to the nucleus [25] as it contains proteins that have endosomal like properties [26] and are pH regulated [26,27]. Mature spermatozoa are thought to translate nuclear encoded genes by mitochondrial type ribosomes contained in sperm [20]. A similar mechanism of translation from exogenously introduced vectors may be operating for transgenes delivered into sperm.

Interestingly, consistent with our previous observations on c-kit+ haemopoietic cells [21], increased transduction efficiencies on sperm resulted from the use of lentiviral particles displaying membrane-bound stem cell factor. The SCF-mediated binding to c-kit on spermatozoa, may also have a significant impact in capacitating sperm [28,29].

As seen previously in somatic cells, it was also observed that utilising a promoter lacking an enhancer (UCOE) significantly changed the level and pattern of GFP expression, whilst maintaining transduction efficiency. Given the random nature of integration of lentiviral vectors, careful consideration must also be given to the choice of promoter utilised [30].

Integration of viral vectors in porcine spermatozoa was confirmed by LAM-PCR analysis. However, no integration sites in the mouse genome were detected following lentiviral transduction of murine spermatozoa. Internal bands indicating the presence of episomes or pre-integration complexes were routinely obtained suggesting that GFP expression in mouse spermatozoa probably derived from these. This observation is, perhaps, unsurprising given the short duration of incubation (3 h) of sperm with the viral vector. The viability of murine sperm was found to decrease significantly after incubation periods longer than 3hrs, rendering them unsuitable for IVF (data not shown). Nevertheless, murine spermatozoa incubated with virus for short periods (containing the lentiviral vector episome or pre-integration complexes) were still able to transmit the GFP gene to developing embryos via fertilisation. Such a pattern would be consistent with a hypothesis that integration may be occurring soon after the cleavage of the first polar body in the presumptive zygote (and completion of meiosis), following sperm penetration of the oocyte.

This technique of transducing spermatozoa with SCF-displaying lentiviruses should therefore be particularly useful for the establishment of large-animal transgenics, where spermatozoa are readily obtained and are stable for longer periods in culture, thereby facilitating integration of the vector into the genome of the spermatozoon. The relatively simple strategy described in this report could be further enhanced and scaled up by flow-sorting of GFP-positive spermatozoa and their use in artificial insemination techniques routinely performed on farm animals.

Our findings imply that transducing spermatozoa directly with pseudotyped lentiviral vectors would be a very powerful tool, not only for the study of early embryonic development, sperm physiology, transgenesis and fertilisation processes, vertical viral gene transmission [31–33] and epigenetics studies, but also in the development of large-animal transgenics for production of therapeutically important proteins.

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