Retrovirus-mediated *in vitro* gene transfer into chicken male germ line cells

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Abstract

Chicken testicular cells, including spermatogonia, transplanted into the testes of recipient cockerels sterilized by repeated γ -irradiation repopulate the seminiferous epithelium and resume the exogenous spermatogenesis. This procedure could be used to introduce genetic modifications into the male germ line and generate transgenic chickens. In this study, we present a successful retroviral infection of chicken testicular cells and consequent transduction of the retroviral vector into the sperm of recipient cockerels. A vesicular stomatitis virus glycoprotein G-pseudotyped recombinant retroviral vector, carrying the enhanced green fluorescent protein reporter gene was applied to the short-term culture of dispersed testicular cells. The efficiency of infection and the viability of infected cells were analyzed by flow cytometry. No significant CpG methylation was detected in the infected testicular cells, suggesting that epigenetic silencing events do not play a role at this stage of germ line development. After transplantation into sterilized recipient cockerels, these retrovirus-infected testicular cells restored exogenous spermatogenesis within 9 weeks with approximately the same efficiency as non-infected cells. Transduction of the reporter gene encoding the green fluorescent protein was detected in the sperms of recipient cockerels with restored spermatogenesis. Our data demonstrate that, similarly as in mouse and rat, the transplantation of retrovirus-infected spermatogonia provides an efficient system to introduce genes into the chicken male germ line.

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Introduction

The availability of genetic models created through transgenesis and gene targeting is the major advantage of using the mouse over all other experimental animals. Due to the lack of immortal and pluripotent embryonic stem (ES) cells in species other than the mouse, efficient gene transfer and gene knockout technology is not feasible. In the chicken, particularly, this task is further complicated by specific features of the reproductive system, which make the newly fertilized zygote relatively inaccessible and difficult to handle.

Because of limited success in cultivation and genetic modification of chicken ES cells (Pain *et al.* 1996, Petite *et al.* 2004), the current methods of chicken transgenesis (Sang 2004) concentrate on blastodermal cells of stage X embryo (Koo *et al.* 2004, Kwon *et al.* 2004, McGrew

et al. 2004, Lillico *et al.* 2007) and primordial germ cells (PGCs; Vick *et al.* 1993, Naito *et al.* 1999, van de Lavoie *et al.* 2006). Spermatogonial stem cells (SSCs) might serve as an alternative to ES cells provided that they could be genetically modified *in vitro* and transplanted into recipient cockerels. This approach of germ line gene transfer could eliminate the background of mosaic animals and produce hemizygotously transgenic animals in F1 generation.

Mouse SSCs are seldomly found among freshly explanted testicular cells, but expand *in vivo* in a functional transplantation assay (Ogawa *et al.* 2000) as well as *in vitro* (Nagano *et al.* 2003) and can be infected with a replication-defective ecotropic retroviral vector (Nagano *et al.* 2000). Growth conditions essential for self-renewal and expansion of SSCs in the mouse and even in rats have been elaborated (Kanatsu-Shinohara

et al. 2003, Kubota *et al.* 2004, Hamra *et al.* 2005 respectively). In such a way, genetically modified mouse SSCs restore fertility after long-term *in vitro* culture (Kanatsu-Shinohara *et al.* 2003, 2004, Kubota *et al.* 2004). In contrast, avian spermatogenesis is poorly understood (Thurston & Korn 2000) and SSCs as well as other germ cells of the seminiferous epithelium have yet to be well characterized in birds. Recently, we have demonstrated that the transfer of dispersed testicular cells from fertile donor cockerels resulted in the colonization of infertile recipient testes with final production of viable and fertilization-competent spermatozoa (Trefil *et al.* 2006). Therefore, efficient gene delivery into the crude mix of testicular cells may target the rare SSCs and pave the way to chicken transgenesis. The exact requirements for the maintenance of chicken SSCs in culture are not yet known. Under such circumstances, infection with a high-titer virus vector seems to be the only choice for gene delivery because virus entry needs only a short time to displace the SSCs from their protected niche in the seminiferous tubule. In addition, infection with retrovirus in comparison with transfection, lipofection, or electroporation techniques minimizes the harsh chemical or physical influences leading to the decrease in the transplantation capacity of SSCs. Initially, replication-competent avian sarcoma and leukosis virus (ASLV)-derived and reticuloendotheliosis virus-derived vectors (Salter *et al.* 1987, Bosselman *et al.* 1989) were used, and since that time, retroviral vectors have become a common gene transfer vehicle for chicken cells. Recent studies show that replication-defective retroviral vectors can be used to introduce foreign genes into the avian germ line and that the major approaches to avian transgenesis have been made through retroviral and lentiviral vectors (Kwon *et al.* 2004, McGrew *et al.* 2004, Lillico *et al.* 2007).

In this report, we demonstrate that chicken testicular cells can be efficiently infected *in vitro* with a replication-defective reporter retrovirus vector pantropized by vesicular stomatitis virus envelope glycoprotein (VSV-G). All cell types present in the mix of testicular cells express the transduced reporter enhanced green fluorescent protein (EGFP) after *in vitro* cultivation and freshly explanted infected testicular cells restore spermatogenesis in sterilized recipient cockerels. The presence of the transduced reporter gene in the sperm of transplanted recipients suggests that the application of these techniques might be useful for transgenesis in chicken.

Materials and Methods

Animals

Inbred Black Minor (BM; genotype ii, EE, b/b) and White Leghorn (WL; genotype II) cockerels at the age of 32 weeks were used as donors and recipients of testicular

cells respectively, as successful transplantation of testicular cells from BM into WL cockerels was demonstrated previously in our chicken germ cell transplantation model (Trefil *et al.* 2003, 2006). Three males of the same genetic origin as recipients were retained as positive controls and other three males were subjected to the same sterilizing treatment as recipient males but kept untransplanted as negative controls. All animals used in the experiments were obtained from the Experimental Animal Farm of the Institute of Molecular Genetics (Prague). Only animals of standard weight for these breeds and known fertility were used in the experiments. Cockerels were kept in individual cages (4200 cm²) fitted with perches under standard husbandry conditions. Feed and water were provided *ad libitum* and a 12 h light:12 h darkness photoperiod was arranged. Experimental animals were killed by cervical dislocation and decapitation. All experiments were performed in accordance with Czech legal requirements for animal handling.

Irradiation treatment

The radiation treatment unit Theratron T1000 (Theratronics, Kanata, Canada) was used to irradiate testes of recipient WL cockerels according to the protocol described previously by Trefil *et al.* (2003).

Construction of the retroviral vector

The retroviral vector was assembled by insertion of the EGFP coding sequence from the pEGFP plasmid into the pLPCX vector designed for retroviral gene delivery and expression (both plasmids obtained from BD Biosciences Clontech, Palo Alto, CA, USA). The pEGFP was digested with EcoRI and HindIII and the 791 bp fragment was ligated into the multiple cloning site of pLPCX cleaved with EcoRI and HindIII. To eliminate the puromycin resistance gene and the internal P_{CMVIE} promoter, the resulting vector was partially digested with AgeI, the single cut fragment was isolated and digested with XhoI. The overhangs were removed by treatment with mung bean nuclease and the 5658 bp fragment was isolated and self-ligated. The resulting pLG vector (Fig. 1) was used for virus production. The 5' LTR of the pLG vector is derived from Moloney murine sarcoma virus (MoMuSV), whereas the 3' LTR comes from Moloney murine leukemia virus (MoMuLV).

GP-293 cell culture, virus propagation, and fluorescence microscopy

The HEK 293-based cell line GP-293 (purchased from BD Biosciences Clontech) was maintained in F-12 and MEM-D mixed in the ratio of 1:1 (Sigma) supplemented

Figure 1 Schematic structure of the reporter retrovirus. The construct is based on the parental vector pLPCX, in which the internal P_{CMVIE} promoter was deleted and puromycin resistance gene is replaced by the EGFP gene. The positions of HindIII and EcoRI sites used for the EGFP cloning are depicted. Not in the appropriate scale.

with 5% calf serum and antibiotics in 3% CO_2 atmosphere at 37 °C.

For virus production, the GP-293 cells were cultivated on a 150 mm Petri dish. The cells were calcium phosphate co-transfected with 50 µg pLG and 10 µg pVSV-G (BD Biosciences Clontech). The medium containing the virus was collected 24, 36, and 48 h after transfection. The virus stock was clarified by centrifugation (150 *g* at 4 °C for 10 min). The supernatant was centrifuged in the SW28 rotor (Beckman Instruments, Fullerton, CA, USA) at 73 000 *g* at 4 °C for 2 h to pellet the virus. After centrifugation, the supernatant was aspirated and the pelleted virus was resuspended in a small volume of cultivation medium and stored in aliquots at –80 °C. The virus titer was determined by infecting chicken embryo fibroblasts with diluted virus stock and counting GFP-positive cell clusters in the infected culture under a fluorescence microscope (Olympus IX 51) with excitation wavelength 488 nm and emission wavelength 507 nm. Typically, virus stocks containing 5×10^7 infection units (IU) per ml were obtained.

Retrovirus infection of testicular cells

Dispersed testicular cells from BM male donors were prepared using a two-step enzymatic digestion as described by Bastos *et al.* (2005). Testicular cell cultures were infected with concentrated virus stocks at the multiplicity of infection (MOI) as high as 90. Infections were carried out in a small volume of media for 60–120 min at 41 °C in 5% CO_2 atmosphere. Afterwards, complete cultivation medium was added to the normal volume without removing the virus. Infected testicular cells were either used for further cultivation

and expression analysis or transferred immediately into sterilized recipient cockerels.

Flow cytometry and classification of cell ploidy

The infected suspension of testicular cells was subjected to fluorescence activated cell analysis (FACS) using a FACSVantage SE apparatus (BD Biosciences) at 48 and 120 h post-infection. We assessed the proportion of EGFP-positive cells, measured the ploidy classes of testicular cells by staining with Hoechst 33342 (H42; Sigma) and verified their viability by counterstaining with propidium iodide (PI; 2 µg/ml).

Cells were harvested using trypsin/EDTA solution (0.05% trypsin in 0.5 mM EDTA) and resuspended in HBBS. For detection of the cell ploidy, testicular cells were further stained with H42 at a final concentration of 10 µM for 30 min at room temperature. H42 and PI were excited by u.v. helium/neon laser 50 mW (Coherent Enterprise, Santa Paula, CA, USA). H42 blue was collected using a combination of 485/58 nm long pass and 505 nm short pass filters in front of the first detector. PI and H42 red fluorescences were detected with a 660 nm/20 nm band pass filter in front of the second detector. GFP was excited with a second 488 nm laser and fluorescence was collected with a 530/30 nm band pass filter.

Genomic DNA analysis

Genomic DNA was extracted from 5-day-old culture of testicular cell mixture and from ejaculates of recipient cockerels after transplantation of infected donor testicular cells using a genomic DNA purification kit (DNeasy Tissue Kit, Qiagen) or using a standard process of phenol purification. The N-terminal primer (5'-TGACCCT-GAAGTTCATCTGCA-3') and the C-terminal primer (5'-ACGAAGTCCAGCAGGACCATGT-3') correspond to the pEGFP nucleotide sequences that yield a 546 bp DNA fragment. Each reaction mixture contained 1 µg genomic DNA extract, 50 pmol of each primer, 0.2 mM dNTP, 5 µl complete 10× PCR buffer, 2.5 U Taq polymerase (Top Bio), and the reaction volume was adjusted to 50 µl with ddH₂O. The samples were denatured at 95 °C for 5 min and then subjected to 36 cycles of PCR amplification. Each cycle was as follows: denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

Bisulfite cytosine methylation analysis

DNA samples from virus-infected testicular cells were isolated by phenol–chloroform extraction and digested by HindIII restriction endonuclease. Bisulfite treatment of DNA was performed according to Hájková *et al.* (2002). We amplified the 5' LTR of integrated proviruses as a 525 bp DNA fragment encompassing 21 CpGs from

bisulfite-treated DNA by nested PCR: forward external primer (A) 5'-GAATAGATGGAATAGTTGAATATGGG-3' (nucleotides -342 to -316 upstream to the transcription start), forward internal primer (B) 5'-GGGTAAATAGGATATTTGTGGTAAGTAG-3' (nucleotides -319 to -290 upstream to the transcription start), reverse internal primer (C) 5'-AACCCCCAAATAAAAAACCC-3' (nucleotides 133–153 downstream to the transcription start), and reverse external primer (D) 5'-CCTAAACAAAATCTCCAAATCC-3' (nucleotides 160–183 downstream to the transcription start). The first round of PCR contained ~25 ng DNA. Taq polymerase (Takara, Shiga, Japan) was used following manufacturer's recommendation with 5 µM MgCl₂, 0.9 M betaine, and 0.9% dimethylsulfoxide. Conditions for PCR were as follows: 95 °C for 1 min, 58 °C for 2 min, and 72 °C for 1 min (40 cycles). The second round of PCR started with 1 µl of the first-round PCR product and the PCR conditions were identical. PCR products were subsequently cloned using the pGEM-T vector cloning system (Promega). Individual PCR clones were sequenced using the AmpliTaq FS L Big Dye Terminator sequencing kit (Applied Biosystems, Norwalk, CT, USA) with universal pUC/M13 forward and reverse primers. Only PCR clones with at least 95% conversion of Cs outside CpGs were taken into account. Only PCR clones containing the hybrid MoMuSV/MoMuLV LTR sequence were used for further analysis in order to discern between reversely transcribed and integrated proviral DNA and original plasmid DNA persisting in the infected cells.

Transplantation of retroviral-infected testicular cells

Approximately, 300 µl of the dispersed virus-infected testicular cells with densities varying from 10⁵ to 10⁶ cell/ml were injected into both testes of anesthetized recipient cockerels WL. The injection needle was inserted through the tunica albuginea directly into the testes and donor cell suspension was applied. Recipient cockerels were anesthetized by i.m. injection of 15 mg/kg ketamine (Narkamon, Spofa, Czech Republic) and 4 mg/kg xylazine (Rometar, Spofa, Czech Republic) prior to transfers.

Sperm quality assessment

The repopulation of the seminiferous tubules and the onset of spermatogenesis in the testes of recipient WL cockerels were assessed by light microscopy. Ejaculates from transplanted and control cockerels were collected at 1-week-interval using the conventional abdominal massage technique (Burrows & Quinn 1937). Progressive sperm motility was assessed and rated in the five-grade scale, where a rating of five means very good motility and a rating of one is a very poor motility score. Sperm

concentration was determined after centrifugation in microcapillaries (Bonitz 1970).

Statistical analysis

The best regression equation for the adjustment of the time course of spermatozoa concentration from recipient males transplanted with adult dispersed testicular cells was achieved using a linear model with polynomial interpolation (Meloun *et al.* 1994). Comparison between the regression equations was performed using the Barlett and Chow test.

Results

Infection of dispersed testicular cells *in vitro* with a reporter retrovirus

We have developed an experimental system to determine whether chicken SSCs could be infected with a pantropic retrovirus *in vitro* after being displaced from their protected niche in the seminiferous epithelium. The basic features of this system are: (i) non-replicative character of the virus vector, which makes it a suitable model for approaching transgenesis in chicken; (ii) transduction of the EGFP reporter gene, which serves for simple detection of retroviral expression; (iii) pantropic character of infectious virus particles that can enter cells without any receptor restrictions due to the VSV-G (Emi *et al.* 1991); and (iv) differences between DNA sequences of 5' and 3' LTRs, which make it possible to discern the integrated proviruses versus episomal DNA of the cloned retrovirus. The schematic structure of the reporter retrovirus cloned in pLG construct is shown in Fig. 1. The infectious retrovirus was propagated in GP293 packaging cells lacking any envelope sequence in their helper retroviral construct. After co-transfection of pLG together with a vector expressing VSV-G, we obtained stocks of infectious virus, which was concentrated 50 times by ultracentrifugation. The titer of the concentrated viral stocks was determined by infection of chicken embryo fibroblasts and typically reached 10⁷–5 × 10⁷ IU/ml.

The testicular cell suspension was infected by the retroviral vector at the MOI reaching 90 infectious virus particles per cell. We followed the EGFP fluorescence in *in vitro* cultivated testicular cells from five donor cockerels for 5 days with the first inspection 8 h post-infection. A substantial fraction of cells emitted green light at that time. The percentage of EGFP-positive cells increased during the *in vitro* cultivation, with gradual overgrowth of EGFP-positive colonies (Fig. 2A and B). We counted the percentage of EGFP-positive cells and assessed the transduction efficiency by flow cytometry analysis 48 h after infection. We were able to identify at least 20% of EGFP-positive cells in the whole testicular cell suspension. After 5 days of cultivation, we were able

Figure 2 Four-day-old testicular cell culture 48 h after infection with the EGFP-transducing retroviral vector. Images from fluorescence microscopy (A) and from phase contrast with low level of fluorescence (B) illustrate the percentage of EGFP-positive cells (magnification 200 $\times$).

to detect $\sim 37\%$ EGFP-positive cells (Fig. 3C). The viability of these suspension cells was very high as measured by the PI exclusion. As much as 99.7% of cells from the infected culture of testicular cells were PI-negative. This clearly demonstrates that EGFP expression is non-toxic for chicken testicular cells. Using H42 fluorescence, cell cycles of 2-day (A) and 5-day (B) cell cultures were analyzed.

During the second day of cultivation of testicular cells (Fig. 3A), we were able to detect 20% of diploid, 16% of tetraploid, and 44% of haploid cells. After the fifth day, however, the character of the cell populations changed (Fig. 3B). The haploid population represented by spermatides was no longer detected. On the contrary, the proportion of diploid cells increased significantly up to 85%.

In 20% (2-day culture) or 37% (5-day culture) of EGFP-expressing testicular cells (Fig. 3C), that were stained with H42, we were able to identify changes mainly in the number of diploid cells. In the 2-day culture, it was possible to identify various classes of ploidy (Fig. 3E),

Figure 3 EGFP expression and ploidy classification in populations of infected testicular cells. Using the H42 dye, the ploidy of (A) 2-day and (B) 5-day cell cultures was analyzed by flow cytometry. (C) The percentage of EGFP-positive cells in the entire population of infected testicular cells (the gray part of the graph represents 5-day culture and the white part represents 2-day culture). The ploidy of selected EGFP-positive cells after (E) 2-day and (D) 5-day culture was classified separately by flow cytometry and H42 staining. (F) The image of cells FACS-sorted by H42 2 days after infection from phase contrast with low level of fluorescence.

while in the 5-day culture, we were able to detect mainly diploid cells (Fig. 3D). When the fraction of haploid cells from the infected 2-day culture stained with H42 was sorted by FACS, we have observed round cells representing the stage of round spermatids. EGFP-positive cells were found among these cells (Fig. 3F), but the percentage is clearly lower in comparison with the primary testicular cells.

CpG methylation of the retroviral promoter region in cultivated testicular cells

To analyze the CpG methylation of integrated proviruses, we performed the bisulfite sequencing of proviral DNA in testicular cells cultivated *in vitro* for 5 days. We focused on the 5' LTR, which contains 21 CpG dinucleotides scattered along the whole LTR, with

Figure 4 CpG methylation of the retroviral promoter region in cultivated testicular cells. We performed bisulfite sequencing to analyze CpG methylation of the integrated proviruses. We have focused our attention to the 5' LTR, which contains 21 CpG dinucleotides scattered along the entire LTR with a majority of them concentrated in a circa 100 bp region around the transcription start. Localization of CpG dinucleotides (vertical bars) and PCR primers (arrows). Methylated CpGs are depicted by filled circles, non-methylated CpGs by empty circles.

a majority of them concentrated in a circa 100 bp region around the transcription start. In the 19 analyzed clones of the PCR product, we have not found any densely methylated sequence pointing to transcriptionally inactive silenced provirus. The level of CpG methylation was extremely low; only 2% out of 240 CpGs analyzed were methylated (Fig. 4). None of the 21 CpGs analyzed was found to be methylated with a strikingly higher frequency than others and no non-CpG methylation was detected. These results are in agreement with our previous observation that retroviral sequences integrated in chicken cells are methylated with low efficiency even after prolonged cultivation (Jana Blažková, unpublished results) and correspond with the transcriptionally active state of proviruses. Among the majority of hybrid MoMuSV/MoMuLV LTR sequences indicating reversely transcribed and integrated proviral DNA, we have found the MoMuSV LTR sequences as well. This demonstrates the persistence of plasmid DNA in the retroviral vector preparations and in the infected testicular cells.

Production of spermatozoa from the recipient testis

Seven WL males were transplanted with dispersed infected testicular cells from five adult BM donors. Three males of the same genetic origin as recipients were retained as positive controls and other three males were subjected to the same sterilizing treatment as recipient males but kept untransplanted as negative controls. Two of the WL recipients restored spermatogenesis within 2–3 months after transplantation and started to produce ejaculates.

A small volume of semen was first collected 7 weeks after transplantation. Sperm concentration increased gradually but with high variation and reached 500×10^6 sperm/ml at 23 week post-

transplantation (Fig. 5). The control group of irradiated but non-transplanted WL cockerels failed to produce any semen during the same period of time. One of the three positive control WL males transplanted with non-infected testicular cells restored spermatogenesis and produced ejaculates 2 months after transplantation.

The presence of the viral vector in spermatozoa of recipient males transplanted with retrovirus-infected testicular donor cells was demonstrated by PCR of the specific EGFP DNA sequence (Fig. 6) and determination the nucleotide sequence of this PCR product (data not shown). This EGFP was not detected in spermatozoa produced by recipients transplanted with non-infected testicular cells.

Discussion

Efficient transplantation of donor testicular cells was widely described in mammals (Brinster & Avarbock 1994, Ogawa *et al.* 2000). Trefil *et al.* (2006) have shown previously that this transplantation in chickens resulted in

Figure 5 The time course of spermatozoa production in one WL recipient transplanted with dispersed testicular cells from adult BM donors (A). Only recipient cockerels with restored spermatogenesis were included into this calculation. Regression lines were constructed to demonstrate the rate of increase in sperm concentration.

Figure 6 PCR analysis of the EGFP gene in spermatozoa isolated from a recipient cockerel to whom retrovirus-infected testicular cells carrying EGFP (lanes 3 and 4) were transplanted and PCR analysis of retrovirus-infected testicular cells (lane 6). The expected size of the amplified band of the EGFP gene was 546 bp, which corresponds with the 100 bp marker of molecular size (lanes 1 and 10). For positive (lanes 2 and 9) and negative (lanes 5, 7, and 8) controls, plasmid DNA (pEGFP) and genomic DNA isolated from non-infected spermatozoa (lane 5) and non-infected cells (lane 7) were used. Lane 8, the negative PCR control without DNA.

the re-colonization of the seminiferous epithelium with partial resumption of spermatogenesis and production of viable and fertilization-competent spermatozoa. In this report, we have continued our studies and observed the functional recovery of exogenous spermatogenesis after transplantation of *in vitro* infected testicular cell mixture. We have clearly demonstrated that infection with a high-titer reporter retrovirus can be achieved during short-term *in vitro* cultivation of donor testicular cells. Furthermore, these cells can be transplanted into sterilized recipients without any substantial loss of their capacity to repopulate recipient testes and resume spermatogenesis. Finally, we have detected the reporter DNA sequences in ejaculates of transplanted recipient cockerels, which suggest that this procedure might be a promising way to produce transgenic chickens.

Two factors are obviously important for retrovirus infection of the chicken male germ line cells. First, a pantropic VSV-G-pseudotyped retrovirus vector was used in our experiments to overcome the strict requirement for specific receptors. ASLV-derived replication-defective vectors could be used for this purpose as well; however, the sensitivity of particular chicken breeds to individual ASLV subgroups (A–E, J; Barnard *et al.* 2006) should be tested. Expression of *Tva* and *Tvb* but not *Tvc* receptor genes was shown in the testes (Elleder *et al.* 2005), but their expression in spermatogonia remains to be characterized. Secondly, the recovery of spermatogenesis by donor cells can be established only from rare SSCs. This cell population has not yet been unequivocally identified in chicken and, as a consequence, the precise number of stem spermatogonia transferred into each male remained unknown. To target them, a reasonable titer of retrovirus vector is necessary. We have used the titer of 5×10^7 IU/ml VSV-pseudotyped reporter vector, which was applied at MOI reaching 90. Even at this MOI, only a fraction of infected testicular cells was EGFP-positive, but the colony proliferation and overgrowth of these cells is an evidence that undifferentiated cells with the potential to repopulate recipients' testes are targeted by virus infection. The retrovirus titer in our study was significantly higher than

those used in the previous study in mice (Nagano *et al.* 2001), which was clearly beneficial for the increase in number of infected testicular cells.

Two out of seven (28.6%) cockerels transplanted with infected testicular cells produced sperm after 2–3 months. This efficiency is comparable with animals transplanted in parallel directly with uninfected testicular cells, but a bit lower in comparison with our previous transplantation experiments (Trefil *et al.* 2006), where the efficiency reached 50%. Small numbers of birds used in these experiments might cause such differences. Clearly, in addition to the transient removal from their specific microenvironment, the survival and maintenance of proliferation capacity of germ line stem cells might be further affected by infection of the virus that is equipped with the toxic VSV-G envelope. Our partial success in re-colonization of testes in sterilized recipients evidences the retrovirus-mediated transduction of a reporter gene into male germ line stem cells. Further examination of the progeny of these recipients will show whether this approach may be of use in chicken transgenesis.

Of particular importance in retrovirus gene delivery experiments is the long-term expression of the transduced gene. After integration, proviruses are often subjected to epigenetic silencing by CpG methylation and histone modifications. Transcription of MLV and MLV-derived vectors is efficiently suppressed in embryonic carcinoma cells, an *in vitro* model of pluripotent stem cells. CpG methylation probably plays a role in epigenetic silencing of retroviruses infecting heterologous hosts. For example, Rous sarcoma virus (RSV) proviruses are often densely methylated after infection of rodent cells (Hejnar *et al.* 1996) and RSV LTR-driven transcription is more sensitive to CpG methylation in mammalian than in chicken cells (Hejnar *et al.* 1999). We have not detected any significant CpG methylation within the LTR of our vector integrated into testicular cells, which were cultivated in parallel with cells transplanted in recipient cockerels. The non-methylated status of the integrated vector correlates with expression of the transduced EGFP marker gene. We have not followed the EGFP expression in recipients' testes or in ejaculates after resumption of spermatogenesis, but the analogous study in mouse (Nagano *et al.* 2000) demonstrated that the β -galactosidase activity in frequent spermatogenic colonies derived from transplanted, retrovirus-infected germ line stem cells. In future studies, we will check the F1 progeny of fertile recipient cockerels for the presence of integrated retroviral vector to see if we are able to produce transgenic chicks and detect the expression of EGFP in the tissues of positive animals. In the case of silenced proviral copies, we plan to continue experiments with modified LTRs containing sequences blocking CpG methylation, for example, core sequences of CpG island (Hejnar *et al.* 2001).

To date, there is no evidence of a successful infection of chicken testicular cells that would have led to the production of transgenic spermatozoa. The optimized irradiation protocol (Trefil *et al.* 2003) eliminating the original acceptor germ cells enabled us to perform the efficient transplantation of dispersed testicular cells (Trefil *et al.* 2006). In this report, we have demonstrated that retrovirus infection of these testicular cells before transplantation could be used for gene delivery into the male germ line and for transgenesis in chicken.

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References

- Barnard RJ, Elleder D & Young JA 2006 Avian sarcoma and leukosis virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. *Virology* **344** 25–29.
- Bastos H, Lassalle B, Chicheportiche A, Rioul L, Testart J, Allemand I & Fouchet P 2005 Flow cytometric characterization of viable meiotic and post meiotic cells by Hoechst 33 342 in mouse spermatogenesis. *Cytometry* **65A** 40–49.
- Bonitz W 1970 Die Ermittlung des Spermazellvolumens in Ejakulaten von Hähnen mit Hilfe der Mikro-Kapillarmethod. *Archiv für Geflügelzucht und Kleintierkunde* **19** 193–201.
- Bosselman RA, Hsu RY, Boggs T, Hu S, Bruszewski J & Ou S 1989 Germ line transmission of exogenous genes in the chicken. *Science* **243** 533–535.
- Brinster RL & Avarbock MR 1994 Germ line transmission of donor haplotype following spermatogonial transplantation. *PNAS* **91** 11303–11307.
- Burrows WH & Quinn JP 1937 Collection of spermatozoa from domestic fowl and turkey. *Poultry Science* **16** 19–24.
- Elleder D, Stepanets V, Melder DC, Šenigl F, Geryk J, Pajer P, Plachý J, Hejnar J, Svoboda J & Federspiel M 2005 The receptor for the subgroup C avian sarcoma and leukosis viruses, Tvc, is related to mammalian butyrophilins, members of the immunoglobulin superfamily. *Journal of Virology* **79** 10408–10419.
- Emi N, Friedemann T & Yee JK 1991 Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *Journal of Virology* **65** 1202–1207.
- Hájková P, El-Maarri O, Engemann S, Oswald J, Olek A & Walter J 2002 DNA-methylation analysis by the bisulfite-assisted genomic sequencing method. *Methods in Molecular Biology* **200** 143–154.
- Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE & Garbers DL 2005 Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. *PNAS* **102** 17430–17435.
- Hejnar J, Svoboda J, Geryk J, Fincham VJ & Hák R 1996 High rate of morphological reversion in tumor cell line H-19 associated with permanent transcriptional suppression of the LTR, v-src, LTR provirus. *Cell Growth and Differentiation* **5** 277–285.
- Hejnar J, Plachý J, Geryk J, Machoň O, Trejbalová K, Guntaka RV & Svoboda J 1999 Inhibition of the Rous sarcoma virus long terminal repeat-driven transcription by *in vitro* methylation: different sensitivity in permissive chicken cells versus mammalian cells. *Virology* **255** 171–181.
- Hejnar J, Hájková P, Plachý J, Elleder D, Stepanets V & Svoboda J 2001 CpG island protects Rous sarcoma virus-derived vectors integrated into nonpermissive cells from DNA methylation and transcriptional suppression. *PNAS* **98** 565–569.
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogbura A, Toyokuni S & Shinohara T 2003 Long-term proliferation in culture and germ line transmission of mouse male germline stem cells. *Biology of Reproduction* **69** 612–616.
- Kanatsu-Shinohara M, Toyokuni S & Shinohara T 2004 Transgenic mice produced by retroviral transduction of male germ line stem cells *in vivo*. *Biology of Reproduction* **71** 1202–1207.
- Koo BC, Kwon MS, Choi BR, Lee HT, Choi HJ, Kim J-H, Kim N-H, Jeon I, Chang W & Kim T 2004 Retrovirus-mediated gene transfer and expression of EGFP in chicken. *Molecular Reproduction and Development* **68** 429–434.
- Kubota H, Avarbock MR & Brinster RL 2004 Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *PNAS* **101** 16489–16494.
- Kwon MS, Koo BC, Choi BR, Lee HT, Kim YH, Ryu W-S, Shim H, Kim J-H, Kim N-H & Kim T 2004 Development of transgenic chickens expressing enhanced green fluorescent protein. *Biochemical and Biophysical Research Communications* **320** 442–448.
- van de Lavoie MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Cerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME & Etches RJ 2006 Germ line transmission of genetically modified primordial germ cells. *Nature* **44** 766–769.
- Lillico SC, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA & Sang HM 2007 Oviduct-specific expression of two therapeutic proteins in transgenic hens. *PNAS* **104** 1771–1776.
- McGrew MJ, Sherman A, Ellard FM, Lillico SG, Gilhooley HJ, Kingsman AJ, Mitrophanous KA & Sang H 2004 Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO Reports* **5** 728–733.
- Meloun M, Miličty J & Forina M 1994 PC-Aided regression and related methods, In *Chemometrics for Analytical Chemistry*, 2nd edn. Chichester: Ellis Horwood.
- Nagano M, Shinohara T, Avarbock MR & Brinster RL 2000 Retrovirus-mediated gene delivery into male germ line stem cells. *FEBS Letters* **475** 7–10.
- Nagano M, Brinster CJ, Orwig KE, Ryu B-E, Avarbock MR & Brinster RL 2001 Transgenic mouse produced by retroviral transduction of male germ-line stem cells. *PNAS* **99** 13090–13095.
- Nagano M, Ryu BY, Brinster CJ, Avarbock MR & Brinster RL 2003 Maintenance of mouse male germ line stem cells *in vitro*. *Biology of Reproduction* **68** 2207–2214.
- Naito M, Matsubara Y, Harumi T, Tagami T, Kagami H, Sakurai M & Kuwana T 1999 Differentiation of donor primordial germ cells into functional gametes in the gonads of mixed-sex germline chimeric chickens produced by transfer of primordial germ cells isolated from embryonic blood. *Journal of Reproduction and Fertility* **117** 291–298.
- Ogawa T, Dobrinski I, Avarbock MR & Brinster RL 2000 Transplantation of male germ line stem cells restores fertility in infertile mice. *Nature Medicine* **6** 29–34.
- Pain B, Clark ME, Shen M, Nakazawa H, Sakurai M, Samarut J & Etches RJ 1996 Long-term *in vitro* culture and characterization of avian embryonic stem cells with multiple morphogenetic potentialities. *Development* **122** 2339–2348.

- Petitte JN, Liu G & Yang Z** 2004 Avian pluripotent stem cells. *Mechanisms of Development* **121** 1159–1168.
- Salter DW, Smith EJ, Hughes SH, Wright SE & Crittenden LB** 1987 Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology* **157** 236–240.
- Sang H** 2004 Prospects for transgenesis in the chick. *Mechanisms of Development* **121** 1179–1186.
- Thurston RJ & Korn N** 2000 Spermiogenesis in commercial poultry species: anatomy and control. *Poultry Science* **79** 1650–1668.
- Trefil P, Polak J, Poplstein M, Mikus M, Kotrbova A & Rozinek J** 2003 Preparation of fowl testes as recipient organs to germ-line chimeras by means of γ -radiation. *British Poultry Science* **44** 643–650.
- Trefil P, Mičáková A, Mucksová J, Hejnar J, Bakst MR, Kalina J, Poplštejn M & Brillard J-P** 2006 Restoration of spermatogenesis and male fertility by transplantation of dispersed testicular cells in the chicken. *Biology of Reproduction* **75** 575–581.
- Vick L, Li Z & Simkiss K** 1993 Transgenic birds from transformed primordial germ cells. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **251** 179–182.

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