Gene Transfer in Ovarian Cancer Cells: A Comparison between Retroviral and Lentiviral Vectors[1]

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ABSTRACT

Local gene therapy could be a therapeutic option for ovarian carcinoma, a life-threatening malignancy, because of disease containment within the peritoneal cavity in most patients. Lentiviral vectors, which are potentially capable of stable transgene expression, may be useful to vehicle therapeutic molecules requiring long-term production in these tumors. To investigate this concept, we used lentiviral vectors to deliver the enhanced green fluorescent protein (*EGFP*) gene to ovarian cancer cells. Their efficiency of gene transfer was compared with that of a retroviral vector carrying the same envelope.

In vitro, both vectors infected ovarian cancer cells with comparable efficiency under standard culture conditions; however, the lentiviral vector was much more efficient in transducing growth-arrested cells when compared with the retroviral vector. Gene transfer was fully neutralized by an anti-VSV-G antibody, and *in vitro* stability was similar.

In vivo, the lentiviral vector delivered the transgene 10-fold more efficiently to ovarian cancer cells growing i.p. in SCID mice, as evaluated by real-time PCR analysis of the tumors. Confocal microscopy analysis of tumor sections showed a dramatic difference at the level of transgene expression, because abundant EGFP+ cells were detected only in mice receiving the lentiviral vector. Quantitative analysis by flow cytometry confirmed this and indicated 0.05 and 5.6% EGFP+ tumor cells after administration of the retroviral and lentiviral vector, respectively. Injection of *ex vivo* transduced tumor cells, sorted for EGFP expression, indicated that the lentiviral vector was considerably more resistant to *in vivo* silencing in comparison with the retroviral vector. Finally, multiple administrations of a murine IFN-α_1-lentiviral vector to ovarian carcinoma-bearing mice significantly prolonged the animals' survival, indicating the therapeutic efficacy of this approach. These findings indicate that lentiviral vectors deserve attention in the design of future gene therapy approaches to ovarian cancer aimed at achieving long-term expression of therapeutic genes.

INTRODUCTION

Ovarian carcinoma is a leading cause of death attributable to gynecologic malignancies both in Europe and North America (1). Because of the lack of effective screening strategies and the possible absence of symptoms with early-stage disease, ~70% of patients present with advanced disease. Furthermore, advances in surgical techniques and conventional chemotherapy have not impacted significantly on the survival of ovarian cancer patients (2), and indeed the long-term survival for patients with advanced disease does not exceed

30%. In view of this, novel therapies for ovarian cancer are required, including gene therapy. Ovarian carcinoma is an appropriate disease for gene therapy because nearly 90% of women have disease contained within the peritoneal cavity at diagnosis. This allows for relatively easy delivery of viral vectors to the cavity through i.p. injections. Furthermore, the confinement of the tumor within the peritoneal cavity might also add a safety factor, because the potential for the transfer of the transgene to normal tissues may be decreased, compared with i.v. administration of the genetic material (3). The list of uses for potential therapeutic genes is increasing with time; however, some categories can be identified and include molecular chemotherapy, mutation compensation, immunotherapy, and angiogenesis inhibition (4).

Initial gene therapy approaches for ovarian carcinoma used recombinant retroviruses (5–7); recombinant adenoviral vectors have also been used as alternative vectors to accomplish gene delivery to tumor cells (3, 8, 9). The outcome of the currently available clinical trials indicates that gene transfer to the peritoneal cavity in ovarian cancer patients is feasible and safe (10, 11).

Although a variety of systems have been used to achieve transgene expression in ovarian cancer cells, there is a lack of studies on lentiviral vectors, which might be particularly useful for long-term expression of therapeutic genes in the tumor microenvironment. Lentiviral vectors, while sharing many features with retroviral vectors derived from oncogenic retroviruses, are also able to transduce some resting cells *in vivo*. This feature is theoretically interesting for gene transfer in cancer cells because all solid tumors contain a certain fraction of cells that are within the G_0 stage of arrest (12). In addition, lentiviral vectors may also deliver the transgene to normal cells, which could contribute to the therapeutic effect.

In particular, lentiviral vectors could be used to deliver antiangiogenic factors to the tumor microenvironment. With respect to this application, although some reports indicate that systemic production of antiangiogenic factors by normal cells (including muscle and liver cells) may suffice for achieving significant antitumor effects (reviewed in Ref. 13), their predominant production in the tumor microenvironment, as achieved by local administration of the vector, could prevent unforeseen consequences of systemic inhibition of angiogenesis or other side effects. Additionally, they would be particularly useful in delivering angiostatic cytokines and chemokines and in obtaining regional control of selected malignancies.

On the basis of these considerations, we set out to evaluate the efficiency of lentiviral vector-mediated gene transfer in ovarian cancer cells, compared with retroviral vectors, and to establish whether they could be useful in transferring a reporter gene directly *in vivo* by i.p. injection of the vector in a xenograft tumor model. Our findings indicate that lentiviral vectors are efficient for gene transfer *in vivo* in ovarian cancer cells; furthermore, delivery of the *IFN-α_1* gene showed the therapeutic efficacy of this approach. This information will be useful in designing novel gene therapy approaches to ovarian cancer.

MATERIALS AND METHODS

Plasmids. The LESN vector, a derivative of the LXSN retroviral vector carrying the gene for EGFP[4] driven by the Mo-MLV LTR, as well as a neomycin resistance gene (*neoR*; Fig. 1), was used as the transfer vector in this study (14). The Mo-MLV Gag-Pol expression construct gag-polgpt, which harbors the Mo-MLV *gag* and *pol* genes under the control of the Mo-MLV LTR, and a SV40 polyadenylation signal (15), was used to generate retroviral vector particles. This construct lacks ψ packaging sequences as a consequence of a 134-bp deletion between the Mo-MLV LTR and *gag* gene. A third plasmid, termed HCMV-G, expressing the VSV-G protein under the transcriptional control of the cytomegalovirus promoter, encoded the envelope of MLV and SIV vectors (16). The SIV vector used in this study was generated as described previously (17) by exploiting the transient three-plasmid vector-packaging system based on 293T cells. The ViGΔBH construct, expressing the EGFP reporter gene from an internal SFFV retroviral promoter, was used as the transfer vector. The genomic RNA was packaged in lentiviral particles produced by transfection of Sgpsyn, a plasmid carrying a synthetic gene coding for SIVmac239 Gag and Pol, and pseudotyped with VSV-G (18). In a set of experiments aimed at investigating long-term transgene expression *in vivo*, a retroviral vector expressing EGFP from the SFFV retroviral promoter and devoid of the *neoR* cassette, termed SFβ91-EGFP, was generated and used to transduce ovarian cancer cells (19). In gene therapy experiments, the *EGFP* gene in the ViGΔBH construct was replaced with that of murine IFN-α_1, obtained from plasmid LMuIFNα_1SN (20) by standard cloning protocols.

Cell Culture and Transfections. 293A and 293T human kidney cell lines were obtained from Quantum Biotechnologies Inc. (Montreal, Canada) and American Type Culture Collection, respectively, and grown in DMEM supplemented with 10% FCS (Life Technologies, Inc., Gaithersburg, MD) and 1% L-glutamine. 293A and 293T cells were used for titration experiments and as a packaging cell line, respectively. The day before transfection, 5.0×10^6 293T cells were seeded in 75-cm^2 tissue culture flasks. The cultures were transfected with plasmid DNA using a calcium phosphate precipitation technique (21). IGROV-1 and OVCAR-3 cells were grown in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine.

Transduction of Cells with Retroviral Vectors. Infectious particles were generated by transfection of 293T cells with 6 μg of the Mo-MLV Gag-Pol expression construct pgag-polgpt, along with 12 μg of LESN, and 0.3 μg of the VSV-G expression construct. The lentiviral vectors were generated by transfection of 293T cells with 10 μg each of ViGΔBH or its derivatives and Sgpsyn plasmids and 0.3 μg of the VSV-G expression construct. Fresh medium was added to the cultures 12–18 h before the supernatant was collected and passaged though 0.45 μm pore size filters. To determine viral titer, serial dilutions of the filtered supernatants were layered over 293A target cells that had been seeded into 96-well culture plates the day before infection at 4×10^3 cells/well. After 6 h at 37°C, the supernatant was replaced with fresh medium; 72 h later, EGFP expression was detected by epifluorescence microscopy, and the titer was calculated and expressed as TU/ml of supernatant.

Transduction of ovarian cancer cells was performed by using cell-free supernatant. To this end, variable volumes of supernatant, containing identical amounts of vector particles, were incubated with 8×10^4 target cells in 6-well plates for 6 h at 37°C. In a set of experiments, to arrest IGROV-1 cells in the G_1-S-phase of the cell cycle, cells were seeded as above in the presence of aphidicolin (0.35 μg/ml; Sigma Chemical Co., St. Louis, MO). To determine the effect of aphidicolin on the proliferation of these cells, [^{3}H]thymidine was added at a concentration of 10 μCi/ml on days 2 and 3 after plating the cells. The incorporation of [^{3}H]thymidine was assayed 1 day later; aphidicolin at 0.35 μg/ml completely blocked IGROV-1 proliferation. Seventy-two h after transduction, cells were fixed with PBS-1% formaldehyde and analyzed for EGFP expression either by FACS or under the epifluorescence microscope.

For *in vivo* experiments, the retroviral and lentiviral vectors carrying the VSV-G envelope were concentrated by ultracentrifugation in a Beckman
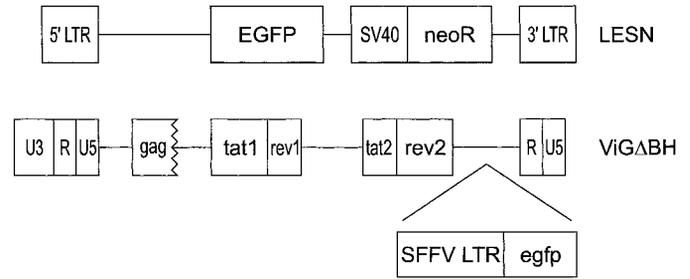
[4] The abbreviations used are: EGFP, enhanced green fluorescent protein; Mo-MLV, Moloney murine leukemia virus; HSV-TK, herpes simplex virus-thymidine kinase; LTR, long terminal repeat; VSV-G, protein G of vesicular stomatitis virus; SIV, simian immunodeficiency virus; SFFV, spleen focus-forming virus; TU, transducing unit(s); SCID, severe combined immunodeficiency; MOI, multiplicity of infection; MFI, mean fluorescence intensity; TAMRA, 6-carboxytetramethylrhodamine; FACS, fluorescence-activated cell sorter.

Fig. 1. Schematic representation of the vectors used. The retroviral vector, termed LESN, carries the *EGFP* and the neomycin resistance genes (*neoR*) driven by the MLV-LTR and an internal SV40 promoter, respectively (14). The ViGΔBH lentiviral vector is a SIV-based lentiviral vector described in detail elsewhere (17), which carries an *EGFP* gene expressed from an internal SFFV LTR.

centrifuge in a SW41 rotor at $50,000 \times g$ (25,000 rpm) at 4°C for 90 min and resuspended in PBS at 6×10^6 TU/ml.

Neutralization Experiments. Neutralization experiments of the MLV/VSV-G pseudotypes were conducted by minor changes of a protocol reported previously (22). Briefly, IGROV-1 cells were plated in 24-well plates at 20,000 cells/well. One day later, 1-ml aliquots of retroviral vector-containing supernatants were incubated for 1 h at 37°C either as such, or in the presence of a 1:20 dilution of a rabbit anti-VSV-G antiserum (Access Biomedical, La Jolla, CA) or control rabbit pre-immune serum, before their addition to the target cells. Seventy-two h after transduction, EGFP$^+$ cells were scored by observation under the epifluorescence microscope.

***In Vivo* Gene Transfer Studies.** SCID mice (6–8 weeks of age) were purchased from Charles River (Wilmington, MA) and allowed to acclimatize to local conditions for 1 week. Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, Dec. 12, 1987). Logarithmically growing human IGROV-1 or OVCAR-3 cells were harvested and resuspended in PBS at a density of 5×10^7 cells/ml. One hundred μl of this cell suspension were then injected i.p. into mice. After 3, 6, and 9 days, animals were injected i.p. with 3×10^6 TU in 0.5 ml of PBS. Tumor growth was monitored by daily observation. Two weeks after the last injection, mice were sacrificed, and tumors were analyzed by H&E staining, or they underwent further analysis. In gene therapy experiments, groups of 6 SCID mice received an i.p. injection of 5×10^6 IGROV-1 cells. On day 2, 4, 6, and 8 after tumor implantation, they were injected with a lentiviral vector encoding murine IFN-α_1. Each mouse received 0.4 μg of p24 vector equivalents per injection in 0.5 ml, which was estimated to correspond to 4×10^7 TU. The viral p24 antigen concentration in the vector stocks was determined by HIV-1 p24 Core profile ELISA (NEN Life Science Products, Boston, MA). Control animals received an equivalent volume of saline or equivalent amounts of the lentiviral vector encoding EGFP. Animals were observed closely, and survival studies were then performed.

Confocal Laser Scanning Microscopy. Frozen tumor masses embedded in OCT were sliced (5–6-μm thick) and deposited on poly-L-lysine-coated glass slides before undergoing either staining with H&E or fixation with paraformaldehyde 1% in PBS for 15 min. Slides were then washed three times with PBS and then incubated for 1 h at room temperature with a saturating solution consisting of 5% goat serum, 1% BSA, and 0.1% Triton-X in PBS. After saturation, the slides were incubated with 1:100 dilution of mouse antihuman nucleoporin p62 (Transduction Laboratories, Lexington, KY) and 1:200 dilution of rabbit anti-GFP (Molecular Probes, Eugene, OR) in PBS-0.1% Triton X-100 for 1 h. After three washes with PBS-0.1% Triton X-100, the slides were incubated with a 1:500 dilution of Alexa 594-conjugated antimouse secondary antibody (red signal) and a 1:500 Alexa 488-conjugated antirabbit secondary antibody (green signal). At this point, slides were washed three times with PBS and mounted before being analyzed by laser scanning microscopy. Confocal laser scanning microscopy was carried out with a Zeiss LSM 510 microscope using Argon (488 nm) and Helium-Neon (543 nm) laser sources. Images of tumors were taken at an optical section <5.8 μm using a $\times$40 objective (NA 1.3) with the zoom set at $\times$1 (512 $\times$ 512 pixels). Laser intensity, pinhole aperture, and photomultiplier parameters were standardized and applied to all scans, enabling direct comparison of signals obtained in different samples.

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Cytofluorimetric Analysis. EGFP-expressing, vector-transduced ovarian cancer cells were analyzed on an EPICS-Elite cytofluorimeter (Coulter, Fullerton, CA). At different times after infection, cells were pelleted, washed, and fixed with PBS-1% formaldehyde. Mock-transduced cell lines served as the negative control for EGFP expression analysis. In a set of experiments, tumor cells recovered from SCID mice were labeled with an anti-HLA-ABC-PE antibody (Dako, Glostrup, Denmark), and EGFP expression was determined on both the HLA$^+$ and HLA$^-$ cell populations. The MFI was calculated by the formula MFI = $\log_{10}$(mean × 10) × (1024/4). The ratio 1024/4 represents the ratio between the number of channels (parameter resolution) and the number of histogram decades.

Estimation of Transduction Efficiency by Real-Time PCR. Genomic DNA was extracted from tumor cells using the Easy DNA kit (Invitrogen, Groningen, the Netherlands). The EGFP copy number per μg genomic DNA was estimated in duplicate using the EGFP734p real-time PCR assay (23). This method measures the accumulation of PCR products by a fluorescence detector system and allows for quantification of the amount of amplified PCR products in the log phase of the reaction. The primers for EGFP734p assay were EGFP553f (5′-ATC ATG GCC GAC AAG CAG AAG AAC-3′) and EGFP810r (5′-GTA CAG CTC GTC CAT GCC GAG AGT-3′). The fluorogenic probe used in this system was EGFP734p (5′-VIC-CAG GAC CAT GTG ATG GCG CTT CTC GT-TAMRA-3′); this sequence was located between those recognized by the PCR primers. The fluorochromes VIC and TAMRA served as reporter and quencher, respectively. Each PCR was performed in a 25-μl reaction mixture containing 12.5 μl of 2× Taq Man Universal PCR master Mix (PE Applied Biosystems, Foster City, CA), 300 nM each primer, and 100 nM fluorogenic probe. Amplifications were carried out in a 96-well reaction plate (PE Applied Biosystems) in a spectrofluorimetric thermal cycler (ABI PRISM 7700 Sequence Detector; PE Applied Biosystems); after the initial denaturation (2 min at 95°C), amplification was performed with 45 cycles of 15 s at 95°C and 60 s at 60°C. Each sample was run in duplicate. A threshold cycle (C_t) for each duplicate was calculated by determining the point at which the fluorescence exceeded a threshold limit (10-fold the SD of the baseline), and the mean of the two duplicates was used to calculate the EGFP copy number in the samples. To quantify EGFP, a reference curve was prepared by amplifying serial dilutions of DNA extracted from EGFP$^+$ IGROV-1 cells, which contained one EGFP copy/cell; the C_t values were plotted against the input DNA, and a standard reference curve was obtained. The C_t values of the experimental samples were plotted against the reference curve, and the EGFP copy number was then estimated. The conversion factor of 6.6 pg of DNA/diploid cell was used to express EGFP copy number/μg DNA. To minimize the influence of DNA quality and to normalize the samples for cell equivalents, a second real-time PCR assay was performed targeting the 18S ribosomal DNA gene. Primers/probe combinations used in this assay were the following: rDNA343f (5′-CCA TCG AAC GTC TGC CCT A-3′), rDNA409r (5′-TCA CCC GTC GTC ACC ATG), and the probe rDNA370p (5′-FAM-CGA TGG TGG TCG CCG TGC CTA-TAMRA-3′). PCR conditions were the same as those used for EGFP quantification. A reference curve to quantify 18S ribosomal DNA was generated by amplifying serial dilutions of DNA extracted from EGFP$^+$ cells, as performed for the EGFP standard curve. Appropriate negative controls (DNA from parental IGROV-1 cells and distilled water) were included in each set of experiments. To determine the efficiency of *in vivo* gene transfer, three independent experiments for each vector were performed.

Statistical Analysis. Data were managed using the Statgraphics software. The significance of the *in vitro* and *in vivo* differences in gene transfer efficiency in ovarian cancer cells between retroviral and lentiviral vectors were determined by Student's *t* test (two-tailed). Statistical significance was set at $P < 0.05$. Kaplan-Meier survival curves were analyzed with the Mantel-Haenszel test.

RESULTS

***In Vitro* Gene Transfer by Retroviral and Lentiviral Vectors in Ovarian Cancer Cells.** Retroviral and lentiviral vectors were generated by a triple transfection procedure of 293T cells and used to transduce the human ovarian cancer cell line IGROV-1 *in vitro*. Preliminary titration experiments of the different vectors on 293A cells indicated titers of

2×10^6 and 4×10^5 TU/ml for the lentiviral and retroviral vector, respectively. For transduction experiments with ovarian cancer cells, identical amounts of vector particles were layered on IGROV-1 cells using a MOI of 2. In three independent experiments, we detected on average 14.3% ± 4.3% and 7.1% ± 1.7% EGFP$^+$ IGROV-1 cells after transduction by the lentiviral and retroviral vectors, respectively (Fig. 2A). Thus, the lentiviral vector was only slightly more efficient in delivering the transgene to proliferating ovarian cancer cells ($P = 0.29$, Student's *t* test). In view of the planned *in vivo* exploitation of the vectors, a setting where tumor cells may be partially quiescent (12), we evaluated how the two vectors performed on growth-arrested cells. As expected, because of the different biological features of the two vectors, aphidicolin treatment of IGROV-1 cells, which blocks cell proliferation, reduced gene transfer by the retroviral vector by >80%; yet it did not impair gene transfer by the lentiviral vector, which has a less stringent requirement for actively dividing target cells (Fig. 2A).

To confirm that gene transfer was VSV-G dependent, we performed a neutralization experiment by adding an anti-VSV-G polyclonal rabbit serum (22) to the vector-containing supernatants. This experiment showed that it was possible to reduce the EGFP$^+$ fraction by >95%, compared with figures obtained after incubation of the retroviral and lentiviral vectors with control serum (Fig. 2B). The neutralization effect was specific because gene transfer efficiency of a similar vector carrying the A-MLV Env in place of VSV-G was unchanged (Fig. 2B).

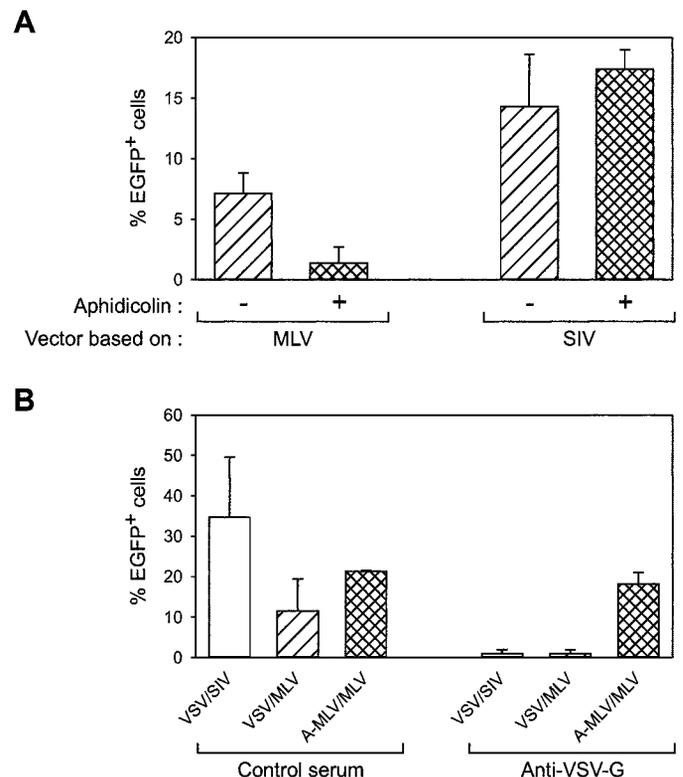Finally, we compared the stability of these vectors *in vitro* by

Fig. 2. *In vitro* gene transfer studies. *A,* flow cytometric analysis of EGFP expression in IGROV-1 cells transduced *in vitro* by the retroviral (*MLV*) or the lentiviral (*SIV*) vectors with a MOI of 2. The vectors were produced by transient transfections of 293T cells, and infection was performed either in the absence (−) or in the presence (+) of aphidicolin at 0.35 μg/ml to block cell proliferation. The average values of three independent experiments are reported; *bars,* SD. *B,* neutralization of retroviral and lentiviral vectors carrying the VSV-G envelope by an anti-VSV-G antiserum. Retroviral (*MLV*) and lentiviral (*SIV*) particles mounting either VSV-G or A-MLV envelopes were incubated for 1 h at 37°C with a 1:20 dilution of either a rabbit anti-VSV-G antiserum (22) or a control preimmune rabbit serum used at the same dilution. The supernatants were subsequently used to infect IGROV-1 cells with a MOI of 4. The percentages of EGFP$^+$ cells of two independent experiments are reported; *bars,* SD.

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incubating aliquots of the vector-containing supernatants at 37°C for various intervals and assaying the infectivity of the viral particles on IGROV-1 cells (data not shown); the outcome of this experiment clearly indicated that their half-life *in vitro* was similar (~2.5 h).

Efficiency of *in Vivo* Gene Delivery by Retroviral and Lentiviral Vectors: Transgene Detection and Vector Biodistribution. To determine whether these vectors could be used to transfer the reporter gene *in vivo* in ovarian cancer cells, SCID mice, previously injected i.p. with 5×10^6 IGROV-1 cells, received 3×10^6 TU of each vector 2, 4, and 6 days later. Twenty days after the beginning of the experiment, the animals were sacrificed, and the tumors were analyzed for transgene presence and expression.

We first determined the presence of vector sequences in the tumor DNA by a real-time PCR assay targeting the *EGFP* gene. To make sure that the amount of proviral DNA in the tumor sample, calculated by the real-time PCR assay, correlated with the number of infected cells, we first determined the correlation between percentage of EGFP^{+} cells by flow cytometric analysis and proviral DNA copy number by Southern blot analysis. We found that, as long as the EGFP[+] cell population is <20%, on average transduced cells carry one integrated copy of provirus/cell (data not shown). Within these limits, quantification of proviral DNA in infected cells can be used to estimate the efficiency of gene transfer.

The outcome of real-time PCR analysis in one representative experiment is shown in Fig. 3. The kinetics of the reaction showed that the threshold cycle (C_t) of tumor DNA from a mouse injected with the lentiviral vector, which represents the PCR cycle at which fluorescence exceeds baseline, was lower (C_t, 23) than the one obtained from amplification of tumor DNA from a mouse that received the retroviral vector (C_t, 29), indicating a higher *EGFP* gene content of the former samples (Fig. 3A). Molecular analysis disclosed that on average $0.6 \pm 0.3\%$ and $6.1 \pm 1.1\%$ of the tumor cells carried proviral DNA sequences after administration of the retroviral and lentiviral vectors, respectively (Table 1); the difference in the efficiency of gene delivery *in vivo* by the two vectors was statistically significant ($P < 0.005$).

The real-time PCR assay was also used to investigate the biodistribution of the vectors after their i.p. injection; we analyzed the liver, the spleen, the lungs, and the kidneys of the injected mice and compared each PCR value obtained with those generated by the corresponding organs of nontreated mice that were used as negative controls. This analysis indicated the presence of the transgene in some cells of the liver and the spleen of the animals injected with the lentiviral vector (0.1–1% and 0.8–2%, respectively); on the other hand, the lungs and the kidneys of these mice yielded background values in PCR analysis, and all of the tissues of the retroviral vector-injected mice were also negative for *EGFP* sequences.

Analysis of Transgene Expression in Tumors. Preliminary staining with H&E disclosed a similar histology of all tumors, including both transduced and nontransduced tumors; IGROV-1 cells grew in SCID mice as solid tumors with limited infiltration of inflammatory or
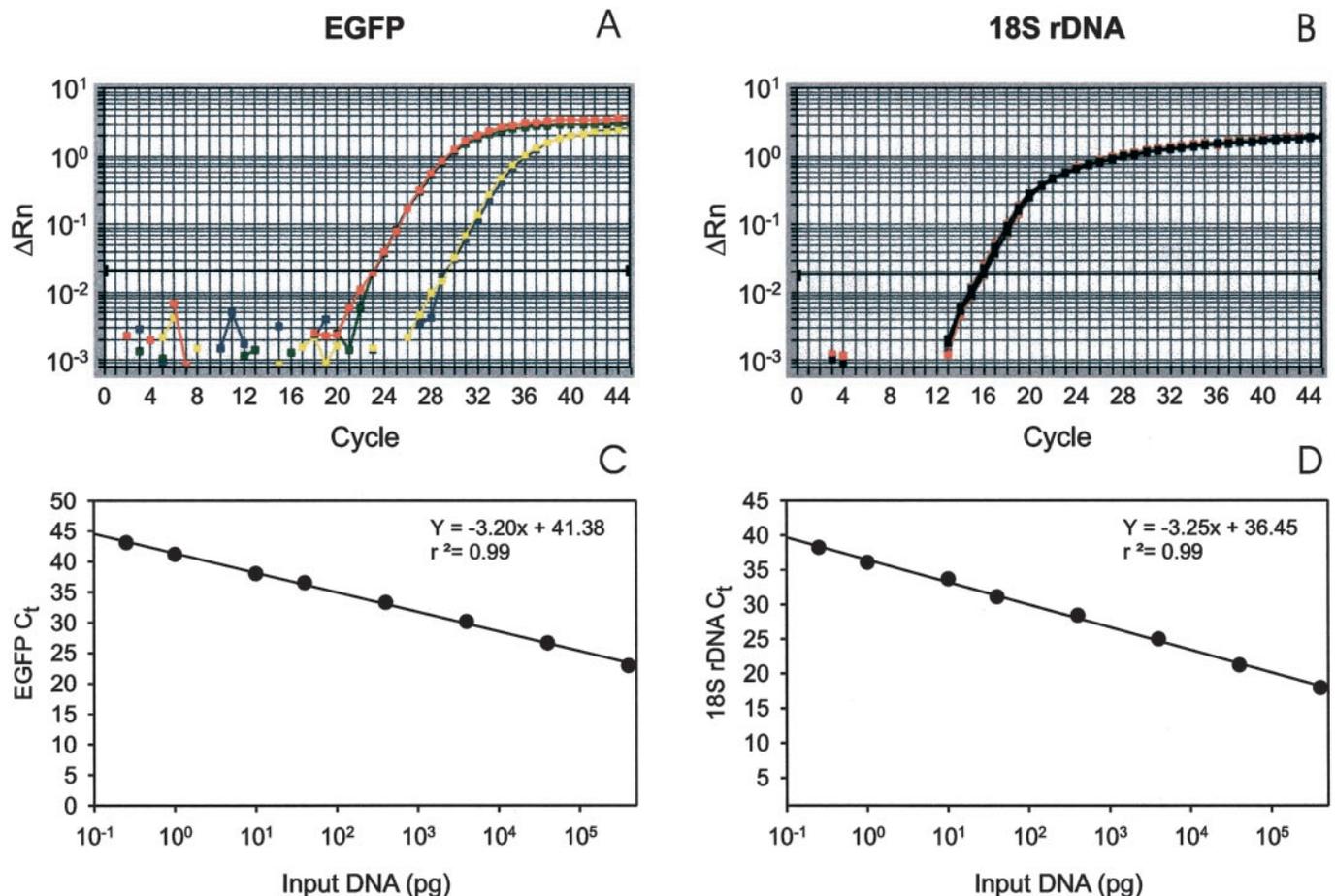

Fig. 3. Quantification of vector DNA by real-time PCR. Analysis was performed on genomic DNA obtained from tumors injected with retroviral and lentiviral vectors with primers targeting the EGFP and the 18S ribosomal DNA genes. One representative experiment is shown. Ten-fold serial dilutions of genomic DNA from IGROV-1 EGFP^{+} cells (ranging from 6×10^5 pg to 6 pg) and appropriate negative controls (DNA from parental IGROV-1 cells for EGFP and H_2O for 18S DNA) were amplified for *EGFP* (A) and 18 S DNA (B) genes. For each dilution, fluorescent emission (ΔRn) is plotted against the cycle number of PCR. In *A*, amplification plots of DNA obtained from tumors transduced with the retroviral and lentiviral vectors are shown in *yellow* and *red*, respectively. Standard curves were constructed for both *EGFP* (C) and 18S DNA (D) genes by plotting the input target DNA against the threshold cycle (C_t). Each dilution was run in duplicate; mean C_t values (●) are shown. The reaction efficiency (*Y*) and the coefficient of correlation (r^2) are indicated in the *box*.

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Table 1 *Efficiency of in vivo EGFP gene transfer by retroviral and lentiviral vectors in tumor-bearing SCID mice[a]*

	Vector based on			
	MLV % EGFP$^+$ cells		SIV % EGFP$^+$ cells	
SCID no.	PCR	FACS	PCR	FACS
1	0.9	0.1	6.3	5.8
2	0.9	0.2	6.7	6.5
3	0.2	0	5.3	3.6
4	0.9	0.1	4.7	4.1
5	0.2	0	6.6	6.3
6	0.6	0	8.2	8.0
7	0.5	0	5.9	5.7
8	ND	ND	5.1	4.7
Mean ± SD	0.6 ± 0.3[b]	0.05 ± 0.07[c]	6.1 ± 1.1[b]	5.6 ± 1.4[c]

[a] SCID mice were injected i.p. with 5×10^6 IGROV-1 cells. Two, 4, and 6 days later, they received 3×10^6 TU of each vector i.p. Twenty days after the beginning of the experiment, the animals were sacrificed, and the tumors were analyzed for transgene presence and expression. The percentages of cells carrying the transgene, according to real-time PCR analysis, are listed in the PCR columns; the percentages of cells expressing the transgene, according to cytofluorimetric analysis, are listed in the FACS columns. ND, not determined.

[b] Statistically significant differences ($P < 0.005$) according to the Student t test in the percentage of cells carrying the transgene between retroviral vector-transduced and lentiviral vector-transduced cells.

[c] Statistically significant differences ($P < 0.005$) according to the Student t test in the percentage of EGFP$^+$ cells at cytofluorimetric analysis between retroviral vector-transduced and lentiviral vector-transduced cells.

other host cells (Fig. 4*A*). After confocal laser scanning analysis, only occasional EGFP$^+$ cells could be detected in the tumor sections obtained from animals receiving the retroviral vector (data not shown). In marked contrast, a diffuse presence of EGFP$^+$ cells was detected in the tumors injected with the lentiviral vector (Fig. 4, *B–D*). Their morphology suggested that they were tumor cells, thus indicating that gene delivery to host cells in this system did not frequently occur, as was directly shown by flow cytometric analysis (see below).

To quantify transgene expression, IGROV-1 cells were analyzed for EGFP expression by flow cytometry. FACS analysis on tumor cells freshly recovered from mice injected with the retroviral vector evidenced a tiny fraction of EGFP$^+$ cells (on average, 0.05 ± 0.07%; Table 1), in agreement with the outcome of confocal microscopy analysis. To check whether this small EGFP$^+$ cell subset carried an integrated and functional provirus, we cultivated tumor cells *in vitro* for 3 weeks in the presence of G418. This selection, attributable to the presence of the neomycin resistance gene in the retroviral vector, resulted in the generation of G418-resistant cell lines in 7/8 LESN-injected animals, which expressed the *EGFP* gene at high levels (not shown). As expected, no G418-resistant cell lines were recovered from 4 control animals that received IGROV-1 cells but were not injected with the retroviral vector-containing supernatant (not shown). These analysis confirmed that it was feasible to use retroviral vectors to transduce the *EGFP* gene in ovarian cancer cells *in vivo*; however, gene transfer efficiency was very low.

In marked contrast, after injection of the lentiviral vector, a sizeable fraction of EGFP$^+$ IGROV-1 cells was detected at FACS analysis (Fig. 5). Although the tumor sample also contained a small subset of murine cells, negative for HLA class I gene expression, the totality of the EGFP$^+$ cells coexpressed the human marker and were, therefore, tumor cells (Fig. 5). On average, we obtained 5.6 ± 1.4% EGFP$^+$ cells after injection of the lentiviral vector in tumor-bearing SCID

Fig. 4. Histology of IGROV-1 tumors in SCID mice and EGFP expression by confocal microscopy analysis. Tumor sections were in part stained with H&E (*A*) or fixed and subjected to indirect immunofluorescence (*B–D*). Fixed sections were stained with mouse antihuman nucleoporin p62 and rabbit anti-GFP, followed by Alexa 594-conjugated goat antimouse and Alexa 488-conjugated goat antirabbit secondary antibodies before being analyzed by confocal microscopy. Staining with H&E disclosed that IGROV-1 cells grew in SCID mice as solid tumors with limited infiltration of inflammatory or other host cells (*A*). In confocal laser scanning images of tumor sections, the *green* signals correspond to GFP (*B*), whereas *red* signals correspond to the nuclear staining determined by Nup62 (*C*). *Yellow* signals generated in the overlay (*D*) show the colocalization of GFP and Nup62, highlighting the diffuse presence of GFP$^+$ cells in the tumors injected with the lentiviral vector.
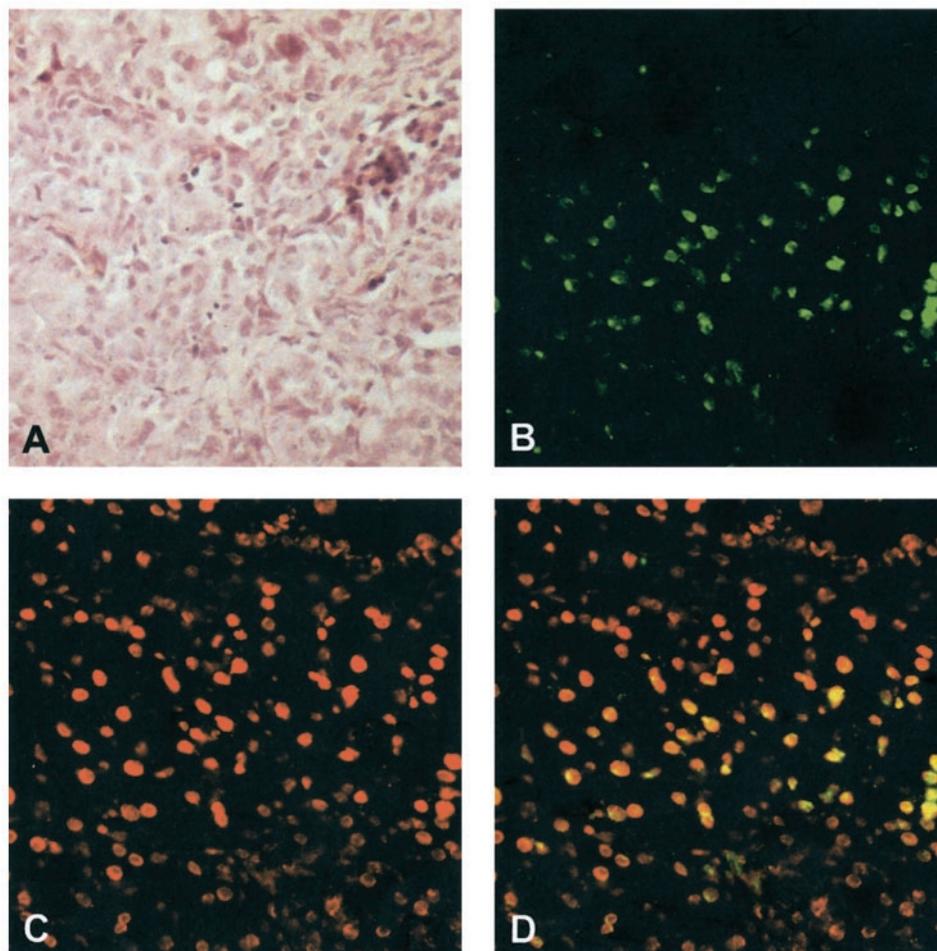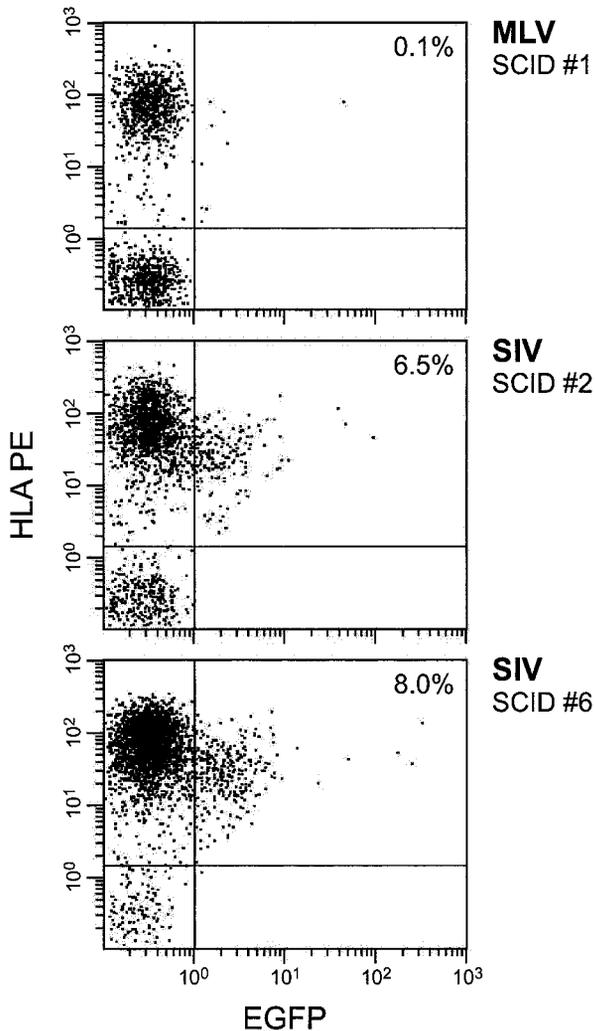
Fig. 5. Quantitative analysis of EGFP expression in tumors by flow cytometric analysis performed on single cell suspensions obtained through the mechanical dissociation of the tumors. Cells were incubated with a phycoerythrin-labeled anti-HLA class I antibody and analyzed for both HLA class I and EGFP expression on an EPICS-Elite cytofluorimeter. The percentage of EGFP-expressing cells is indicated. The panels show the representative analysis of one retroviral vector (*MLV*)-transduced and two lentiviral vector (*SIV*)-transduced tumors.

esized that this discrepancy might in part depend on a differential susceptibility of the two vectors to transcriptional silencing. To investigate this, we transduced IGROV-1 cells *in vitro* with either the retroviral or the lentiviral vector and sorted the EGFP$^+$ fraction by FACS to establish cell populations expressing the transgene in the large majority of the cells (Fig. 6). Subsequently, the EGFP$^+$ cells were injected i.p. in SCID mice and allowed tumors to form. To quantify EGFP expression within tumors, we stained tumor cells with an anti-human HLA antibody and analyzed EGFP expression in tumors by flow cytometry, comparing it with preimplantation levels. As shown in Fig. 6, IGROV-1 cells transduced by the retroviral vector expressed EGFP at high levels (MFI, 575) in the large majority of cells (99%) before implantation; on the other hand, EGFP expression was strongly down-regulated *in vivo* when compared with preimplantation levels, because only 12% of the tumor cells expressed EGFP at a reduced intensity (MFI, 399). This phenomenon probably reflects *in vivo* silencing of the retroviral vector-encoded gene, as already reported by others in different systems (24, 25). Interestingly, however, the IGROV-1 cells transduced by the lentiviral vector were more resistant to silencing; in fact, EGFP was expressed by 90% of the cells at very high levels (MFI, 726) before implantation and as many as 54.2% of the cells with MFI, 539 in tumors (Fig. 6). Real-time PCR analysis of genomic DNA extracted from the transduced cells before implantation and from the tumors yielded similar figures (Table 2), indicating that proviral DNA was stably integrated in all of the samples analyzed with comparable copy numbers, and that the transgene loss did not account for the strongly reduced EGFP expression detected in tumors formed by the Mo-MLV-vector-transduced IGROV-1 cells. Finally, to rule out whether the small differences in vector promoter and the presence in the retroviral vector of the *neoR* gene (Fig. 1), which may also act as a transcriptional silencer (26), could account for differences in EGFP expression patterns, we transduced IGROV-1 cells with a retroviral vector that carried the same SFFV promoter as the lentiviral vector and lacked the neoR cassette (19); injection of sorted EGFP$^+$ IGROV-1 cells in SCID mice was followed by a marked reduction in EGFP expression in tumors (Fig. 6). These findings indicate that reduced susceptibility to transcriptional silencing might also contribute to the observed improvement in EGFP expression after administration of the lentiviral vector in tumor-bearing mice.

Effect of Lentiviral Vector-mediated IFN-α_1 Gene Transfer on Survival in an Ovarian Cancer Model. To assess the therapeutic efficacy of lentiviral vector i.p. administration, we used a murine IFN-α_1-expressing lentiviral vector to deliver this cytokine to ovarian cancer-bearing SCID mice. Our previous studies have indicated that IFN-α_1 delivered by retroviral vectors has strong antitumor effects in a breast cancer model, at least partially because of its antiangiogenic activity (20). Furthermore, preliminary experiments indicated that the *in vivo* growth of IGROV-1 cells producing this cytokine was dramatically impaired (data not shown), suggesting that IFN-α_1 may represent a good therapeutic gene in this model. *In vitro* experiments demonstrated that lentiviral vector-transduced ovarian cancer cells released detectable amounts of IFN-α_1, as determined by Western blotting analysis (data not shown). For *in vivo* experiments, concentrated vector aliquots (0.4 μg of p24 vector equivalents/injection) were injected on days 2, 4, 6, and 8 i.p. in SCID mice that had received 5×10^6 IGROV-1 ovarian cancer cells by the same route on day 0. Control animals received an equivalent volume of saline or equivalent amounts of the lentiviral vector encoding EGFP. Production of the cytokine *in vivo* was demonstrated by analyzing the expression of the sensitive IFN-regulated Ly-6c marker on murine splenocytes after vector delivery; Ly-6c was expressed in 3.8 ± 1.6% of splenocytes of noninjected SCID mice and was strongly up-regu-

mice; this represents an increase of ~100-fold, compared with findings obtained after injection of the retroviral vector (0.05 ± 0.07%; Table 1; $P < 0.005$). To investigate whether other ovarian cancer cell lines may be targeted by the lentiviral vector *in vivo*, we injected 3 SCID mice with OVCAR-3 cells, followed by administration of the lentiviral vector according to the same schedule used above, and found 2.9 ± 1.5% EGFP$^+$ tumor cells by FACS analysis. This finding shows that gene delivery to ovarian cancer cells is not restricted to a specific cell line.

The Lentiviral Vector Is Less Prone to Transcriptional Silencing *in Vivo* Than the Retroviral Vector. *In vivo* gene transfer experiments indicated a dramatic difference in the relationship between infection and transgene expression by the two vectors. In fact, in the case of the lentiviral vector, there was a good correlation between the number of cells carrying the transgene, quantified by real-time PCR assay, and transgene expression, quantified by FACS analysis of the EGFP$^+$ fraction of the tumors (Table 1). In marked contrast, in the case of tumors injected with the retroviral vector, we observed an apparent 10-fold difference between the number of cells carrying the transgene and those expressing it (Table 1). We hypoth-
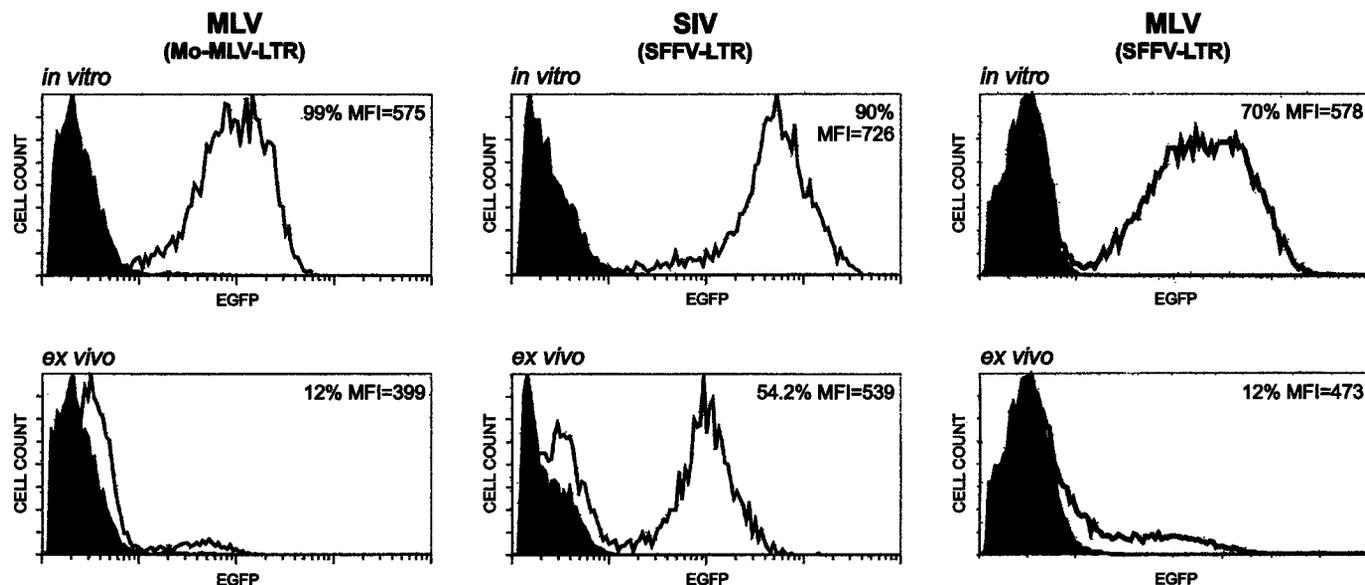
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Fig. 6. Quantitative analysis of EGFP and HLA class I expression by flow cytometry in retroviral (*MLV*) and lentiviral (*SIV*) vector-transduced IGROV-1 cells *in vitro* (*top panels*) and in the tumors formed after their i.p. injection into SCID mice (*bottom panels*). The promoter/enhancer used in each vector to drive EGFP expression is indicated in the *parentheses*. A sharp reduction in EGFP expression levels is observed in tumors formed by the retroviral vector-transduced cells carrying the Mo-MLV-LTR promoter. In contrast, lentiviral vector-transduced cells retain most EGFP expression *in vivo*. Transgene expression is also reduced in tumors formed by IGROV-1 cells transduced by a retroviral vector carrying the same promoter/enhancer as the lentiviral vector (*SFFV-LTR*).

lated in mice receiving the IFN-α_1-encoding lentiviral vector, where it was expressed at high levels by $16.0 \pm 6.5\%$ of the splenocytes, 6 weeks after vector injection. In terms of Ly-6c expression, SCID mice that had received the EGFP-encoding lentiviral vector disclosed figures similar to the untreated mice, thus indicating that lentiviral vector injection *per se* did not up-regulate endogenous IFN-α_1 in this system. No side effects were observed in tumor-bearing mice injected with either lentiviral vector. In our trial, all control animals died by day 27, with no significant differences between saline-injected and control vector-injected animals (Fig. 7). In contrast, animals injected with four doses of murine IFN-α_1-lentiviral vector had a 100% survival until day 52 and then started progressively to succumb to ovarian cancer (Fig. 7). Median survival was 68 days and was significantly prolonged compared with controls ($P < 0.005$), with 1 long-term survivor. These studies demonstrate that multiple doses of the IFN-α_1-lentiviral vector are remarkably effective in treating ovarian carcinoma as an early treatment strategy.

Table 2 *Estimation of EGFP copy number per infected cell by real-time PCR assay*[a]

| | Vector based on | | | |
| | MLV | | SIV | |
	Exp. 1[b]	Exp. 2[b]	Exp. 1[b]	Exp. 2[b]
In vitro	1.18	1.59	1.40	1.03
Ex vivo				
SCID #1	1.12	1.46	1.13	1.03
SCID #2	1.15	1.38	1.26	1.06
SCID #3	ND	1.42	1.14	1.01
Mean $\pm$ SD	1.13 ± 0.02	1.42 ± 0.04	1.17 ± 0.07	1.03 ± 0.02

[a] IGROV-1 cells (8×10^4) were plated in 6-well plates and infected with the retroviral (MLV) or lentiviral (SIV) vector using a MOI of 2. EGFP$^+$ cells were then sorted by flow cytometry; genomic DNA was extracted and analyzed by the real-time PCR assay to determine the *EGFP* copy number per infected cells, which is reported in the *in vitro* row of the table. Sorted cells were then injected i.p. in SCID mice (1×10^6 cells/mouse; 2–3 mice/group) and allowed to form tumors. Genomic DNA was extracted from tumors and underwent real-time PCR analysis to quantify transgene presence. The results of this analysis on *Ex vivo* samples are reported in the table. ND, not determined. The differences in the values of the *EGFP* copy number/cell between *In vitro* and *Ex vivo* samples were statistically not significant, according to the Student *t* test.

[b] EGFP copy/cell. Exp., experiment.

DISCUSSION

Gene delivery to ovarian cancer cells may allow new therapeutic approaches to this life-threatening disease. Thus far, this goal has been successfully achieved with adenoviral vectors that have been widely used both in preclinical models (27) and in clinical trials to transfer HSV-TK in ovarian cancer patients (11). Using adenoviral vectors, transduction efficiency is generally high, yet their clinical use might be limited by some vector-related toxicity, as well as short-term expression of the transgene, because of the non-integration of the therapeutic gene in the tumor cell DNA and to the clearance of adenoviral vector-carrying cells by the immune response. In view of these features, their exploitation has mainly been restricted to transfer of genes encoding prodrug-activating enzymes, such as HSV-TK (8, 11, 28) or the anti-erbB-2 intracellular single-chain antibody (10), the expression of which should last only 1–2 weeks from injection of the vector. On the other hand, some gene therapy strategies foresee delivery of genes whose product should be expressed long term; cytokines and other molecules that can activate the immune system against the tumor, as well as antiangiogenic molecules, such as angiostatin and endostatin, fall into this category.

The aim of this study was to identify a vector system that might be suitable for long-term expression of therapeutic genes in ovarian cancer cells. We compared the efficiency of gene transfer by lentiviral vectors and retroviral vectors, both of which stably integrate the proviral DNA in the target cell. Our findings demonstrate that both types of vectors transduce proliferating tumor cells *in vitro* with comparable efficiency. Interestingly, the lentiviral vector greatly exceeded the retroviral vector in its capability of transducing aphidicolin-treated, growth-arrested cells. This was an expected finding, because it has already been shown that lentiviral vectors in general (29, 30) and this SIV-based lentiviral vector in particular (17) can infect nonproliferating cells, although not previously demonstrated using growth-arrested ovarian cancer cells.

Several factors can contribute to the dramatic differences in gene transfer efficiency observed *in vivo*, including vector stability, requirement for dividing target cells, and readiness of transgene expres-
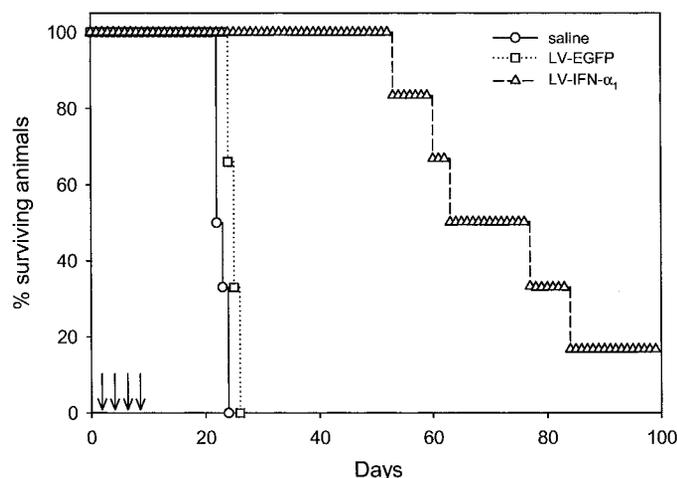
Fig. 7. Effects on survival after treatment with a murine IFN-α_1-encoding lentiviral vector in an ovarian cancer tumor model. Two days after the i.p. injection of IGROV-1 cells (5×10^6 cells/mouse), four doses of 0.4 μg of p24 equivalents of a murine IFN-α_1-encoding lentiviral vector ($\triangle$; *arrows*) were administered to SCID mice by the same route. The same dose of the lentiviral vector encoding EGFP ($\square$) was administered to another group of SCID mice. Control animals were injected with 500 μl of saline i.p. ($\bigcirc$). Animals ($n = 6$/group) were then followed for survival. Significant increases in survival ($P < 0.005$) were seen in IFN-α_1-treated animals compared with all of the other groups.

sion. Although we have not directly investigated vector stability *in vivo*, an *in vitro* comparison indicated that the half-life of the retroviral and lentiviral vectors at 37°C was comparable. Another major feature of the retroviral vector that might limit its efficacy *in vivo* is that it only infects dividing cells. In many solid tumors, a large fraction of the cancer cells is not actively dividing, and this can strongly reduce the chances for these cells to be transduced by the retroviral vector. We stained *in vitro* growing and *ex vivo* recovered IGROV-1 cells with propidium iodide and analyzed them by flow cytometry and observed that injection of tumor cells i.p. in SCID mice translates into a progressive shift of the cell cycle profile from the S-G$_2$ to the G$_0$-G$_1$ phases (data not shown); the reduction in the number of actively proliferating cells may thus partially explain the drop in gene transfer efficiency of the Mo-MLV vector *in vivo*, compared with *in vitro* findings obtained with the same vector.

The distribution of the i.p. administered virus was also investigated in our study. Clearly, the lentiviral vector used in this study was not specific for ovarian cancer cells, because it exploited the VSV-G envelope to interact with target cells, which has a very broad tropism for human and murine cells (16). In our system, FACS analysis of fresh tumor samples suggested that the large majority of EGFP$^+$ cells also expressed the HLA I marker and were not, therefore, host-derived cells. However, a PCR analysis of genomic DNA extracted from several organs of the injected mice revealed significant amounts of proviral DNA in the liver and the spleen of the injected mice as well, thus indicating that the transgene could be detected both in the peritoneal cavity and in some distant organs. These findings are not in contrast with the conclusions of a recent study involving i.v. injection of lentiviral vectors carrying the VSV-G envelope, which investigated the biodistribution of the transgene and found it predominantly in the spleen, liver, and bone marrow of the injected mice (31).

We observed that a significant fraction of tumor cells infected by the retroviral vector *in vivo* apparently did not express the transgene (Table 1). This finding may underlie a different propensity of the vectors to transcriptional silencing or be related to differences in the average vector copy number in cells infected by the retroviral vector compared with the lentiviral vector. However, *in vitro* infected cells harbored comparable amounts of proviral DNA (Table 2), indicating

that the retroviral and lentiviral vectors did not integrate different copy numbers of the transgene in the target cells at the MOI used in this study. Silencing *in vivo* has been observed after gene transfer by retroviral vectors (25); however, much less is known about the susceptibility of lentiviral vectors to this phenomenon. We addressed this issue by injecting tumor cells in SCID mice, which were *ex vivo* transduced by the two vectors, and expressed EGFP at comparable levels. Our experiments indicated a reduced susceptibility to transcriptional silencing of the lentiviral vector compared with the retroviral vector, which was not apparently attributable to differences in vector design. Future studies will be needed to investigate the duration of transgene expression in normal cells after gene delivery by the two types of vectors, as sustained transgene expression by non-tumor cells may suffice for many therapeutic applications, including angiogenesis inhibition.

In our study, we report on the therapeutic efficacy of this approach by delivering murine IFN-α_1 to ovarian cancer-bearing mice. Lentiviral vector-mediated gene transfer was followed by evidence of systemic expression *in vivo* and significantly prolonged the survival of the animals. Although the mechanism of the antitumor effect has not been directly addressed, IFN-α_1 therapy had efficacy in immunodeficient mice, thus ruling out a contribution of tumor-specific immunity, and may act through an activation of the natural immunity against tumors or angiogenesis inhibition. In this respect, we found recently that retroviral vector-mediated IFN-α_1 gene transfer is able to delay the growth of breast cancer cells and is associated with marked antivascular effects (20).

Overall, these findings indicate that lentiviral vectors might retain some advantages compared with the Mo-MLV vectors in terms of gene transfer efficiency and long-term expression of the transgene. Because lentiviral vector-mediated gene transfer is stable in nature, because of the integration of the transgene in the target cell DNA, we advance that lentiviral vectors could be convenient to transfer genes involved in the control of tumor-associated angiogenesis.

ACKNOWLEDGMENTS

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Gene Transfer in Ovarian Cancer Cells: A Comparison between Retroviral and Lentiviral Vectors

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