

# Transgenesis by lentiviral vectors: Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos

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**The introduction of foreign genes into early mouse embryos and embryonic stem (ES) cells is invaluable for the analysis of gene function and regulation in the living animal. The use of vectors derived from retroviruses as gene transfer vehicles in this setting has had limited success because of silencing of transgene expression. Here, we show that vectors derived from lentiviruses, which are complex retroviruses, can efficiently deliver genes to murine ES cells and that transgene expression is stable during proliferation of undifferentiated ES cells. The transgene is expressed during differentiation of ES cells *in vitro* (embryoid bodies) and *in vivo* (teratomas). Transfer of lentivector-transduced ES cells into blastocysts resulted in chimeric animals that expressed the transgene in multiple tissues. Embryos derived from crossings of chimeric mice expressed the transgene, indicating successful germ-line transmission. Infection of murine preimplantation embryos at morula stage with lentiviral vectors resulted in stable transduction and expression of the transgene in mouse embryos and in newborn mice. Finally, human ES cells were transduced by lentiviral vectors and expressed the transgene over several passages. Thus, lentiviral vectors represent a significant improvement over oncoretroviral vectors used previously for gene transfer into murine ES cells and preimplantation embryos. Ability to transfer foreign genes into human ES cells has potential relevance for the development of gene and cell-based therapies.**

**E**mbryonic stem (ES) cells are derived from early mammalian embryos and display characteristics of totipotency, i.e., after transfer to a suitable *in vivo* environment they contribute to the primary germ layers (ectoderm, endoderm, and mesoderm) and populate the germline of mice (1, 2). ES cells can be propagated in an undifferentiated state and genetically manipulated *in vitro*. Thus, transgenic animals can be generated by introducing foreign genes into ES cells, followed by transplantation of the ES cells into embryos and germ-line transmission.

The first reports of genetic manipulation of ES cells demonstrated that vectors derived from retroviruses can infect ES cells and that the integrated virus (provirus) is transmitted through the germline (3, 4). Furthermore, it was shown that retroviral vectors are able to infect preimplantation embryos, giving rise to transgenic animals that transmit the proviral DNA to offspring (5–8). However, further analysis revealed that both infected ES cells and preimplantation embryos lack significant provirus transcription. Two major mechanisms have been identified for retrovirus silencing (see references in ref. 9): trans-acting factors that bind to the viral promoters in the long terminal repeats (LTRs) and methylation of the integrated retroviral genome and flanking host DNA sequences. According to the organization of their genome (for review see ref. 10), one can distinguish simple retroviruses, such as the prototypic murine leukemia virus, from complex retroviruses like the lentiviruses. HIV type 1 (HIV-1) is one of the best-studied complex retroviruses and has the ability to infect nondividing cells presumably by import of the viral DNA through the nuclear pore and subsequent integration into the host genome (references in ref. 11). Vectors derived from lentiviruses can transduce a broad spectrum of terminally dif-

ferentiated, nondividing cells, as well as, hematopoietic stem cells of multiple mammalian species (references in ref. 11).

In this communication, we show that (i) unlike traditional oncoretroviral vectors, expression of transgenes introduced by lentiviral vectors into murine or human ES cells is not silenced. (ii) Transgene expression is not “shut off” during differentiation, and the transgene is expressed in multiple tissues of chimeric animals generated by transfer of lentivector-transduced ES cells in blastocysts. (iii) Germ-line transmission of transgenes introduced into ES cells by lentiviral vectors and (iv) preimplantation embryos at morula stage can be successfully transduced with lentiviral vectors, and the resulting progeny express the transgene. We therefore conclude that lentiviral vectors will be excellent tools for generating transgenic animals.

## Materials and Methods

**Virus Production.** LV-green fluorescent protein (GFP) was constructed by cloning the CAG promoter into the *Cl*I and *B*am*HI* sites of the vector LV-pGFP (12), thereby replacing the phosphoglycerate kinase (PGK) promoter. LV-Lac was cloned by introducing the LacZ-woodchuck hepatitis virus fragment into the *N*he*I* and *K*pn*I* sites of LV-GFP, thereby replacing the enhanced GFP (eGFP) cassette with LacZ. All viruses were produced as described (13). In brief, 293T cells were transfected with the vector, packaging plasmids, and a plasmid coding for the G protein of the vesicular stomatitis virus by the calcium-phosphate method. Virus was harvested over the following 3–4 days and concentrated by ultracentrifugation ( $68,000 \times g$ ). The titers of the virus preparations were determined by measuring the amount of HIV-1 p24 gag antigen by ELISA (Alliance; NEN). The multiplicity of infection (moi) was determined by infecting 293T cells, followed by flow cytometric quantification of eGFP-positive cells.

**ES Cells.** ES cells were grown on a feeder layer of irradiated embryonic fibroblasts in the presence of lymphocyte inhibitory factor as described (14). Single cell suspensions ( $1 \times 10^5$  ES cells) were infected overnight in a volume of 500  $\mu$ l with the lentiviral vectors. For flow cytometry analysis, ES cells were cultured on gelatinized plates. The number of eGFP-positive cells and the mean fluorescence were quantified by FACScan (Becton Dickinson).

**In Vitro and in Vivo Differentiation of ES Cells.** Embryoid bodies (EBs) were generated by cultivating ES cells in hanging drops (2

Abbreviations: ES, embryonic stem; LTR, long terminal repeat; HIV-1, HIV type 1; GFP, green fluorescent protein; eGFP, enhanced GFP; moi, multiplicity of infection; EB, embryoid body; PGK, phosphoglycerate kinase.

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days) and then in suspension for 6 more days. EBs were dissociated by using trypsin and then were plated on culture tissue culture slides (Falcon). Immunofluorescence analysis was carried out after fixation of EB-derived cells in 4% paraformaldehyde at 4°C. Fixed cells were incubated (1 h at room temperature) with primary Abs directed against myosin (Sigma), desmin (Chemicon), or neurofilament NF200 (Sigma). After washing, the cells were incubated for 1 h with anti-mouse or anti-rabbit IgG Cy5 (The Jackson Laboratory). Immunofluorescence and cellular green fluorescence were analyzed by using a confocal microscope (PASCAL, Zeiss).

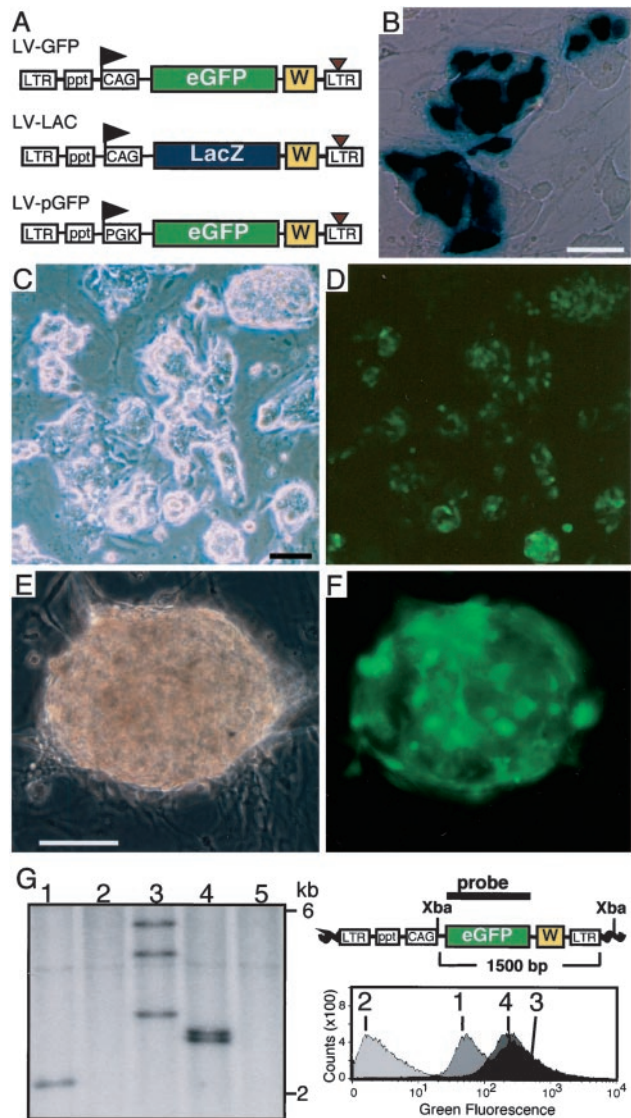
Teratomas were induced by injecting a single cell suspension of  $2 \times 10^6$  ES cells in a volume of 500  $\mu$ l of Hanks' buffer (GIBCO) s.c. (G25 needle) in nude mice (nu/nu, The Jackson Laboratory). Four weeks after injection, the mice were killed and the tumors were removed. Fragments of the teratomas were fixed in 4% paraformaldehyde before freezing in OCT freezing media (Tissue Tek, Sakura Finetek, Torrance, CA) and then sectioned on a cryostat.

**Fluorescence Imaging.** *In vivo* imaging of green fluorescence was carried out with a stereomicroscope (Zeiss SV-11) with an attached epifluorescence unit consisting of a mercury lamp and an eGFP filter. Images were recorded with a cooled charge coupled device camera either attached to an AxioVision imaging system (Zeiss) or to a video recorder.

**Infection of Preimplantation Embryos.** Six-week-old CB6F1 (C57BL/6  $\times$  BALB/c) females were superovulated with 5 units of pregnant mare serum gonadotropin (Sigma), followed 48 h later by injection of 5 units of human gonadotropin (Sigma), and mated with CB6F1 males. Morulae (8–16-cell embryos) were isolated by flushing the oviduct 2.5 days postcoitus with M2 medium (Sigma). Removal of the zona pellucida was achieved by acidic tyrode (15) treatment as described. Morulae were infected overnight with 20 ng of P24/ml in a volume of 5  $\mu$ l, covered with light paraffin oil (Fisher). Thirty hours after infection, blastocysts were transferred into the uteri of pseudopregnant CB6F1 mice. The presence of the lentiviral vector DNA was detected by PCR, using primers (5'-tgacctacggcgtcagtcg-3' and 5'-tcacctgatgcgcttcttct-3') that amplify a 300-bp fragment of the eGFP gene.

## Results and Discussion

To study lentiviral gene transfer into ES cells and preimplantation embryos, we used vectors derived from HIV-1 (16), which were further modified to contain the following changes (Fig. 1A): (i) The essential promoter/enhancer sequences of the LTR were deleted (SIN vectors), resulting in transcriptional inactivation of the integrated virus (provirus) (17, 18). (ii) The posttranscriptional regulatory element of the woodchuck hepatitis virus (19) and the central polypurine tract of the *pol* gene of HIV-1 were included, because these elements have been shown to enhance lentiviral gene expression in several cell lines, including hematopoietic stem cells (12, 20–22). (iii) To achieve transgene expression not only in ES cells but also in differentiated derivatives and tissues of adult animals, we chose the CAG promoter (23), which contains a modified chicken  $\beta$ -actin promoter and enhancer sequences from the cytomegalovirus, and the promoter of the PGK. Both promoters have been shown to be ubiquitously active in the mouse (24, 25). Three lentiviral vectors were designed (Fig. 1A): LV-GFP, which carries the expression cassette for eGFP under the control of the CAG promoter; LV-Lac, which contains the *LacZ* cDNA driven by the CAG promoter; and LV-pGFP, which carries eGFP under the control of the PGK promoter. After infection of D3 ES cells (26) with LV-Lac at an moi of 1,  $69.5\% \pm 4.7\%$  ( $n = 323$  colonies) of the ES cell colonies contained  $\beta$ -galactosidase-positive cells (Fig.



**Fig. 1.** Transduction of mammalian ES cells by using lentiviral vectors. (A) Schematic representation of the lentiviral vectors. (Top and Middle) Lentiviral vectors LV-GFP and LV-LAC contain the compound chicken  $\beta$ -actin/cytomegalovirus enhancer (CAG) promoter, which drives expression of the eGFP or the cDNA for  $\beta$ -galactosidase (*LacZ*), respectively. (Bottom) LV-pGFP contains the promoter of the PGK. All vectors carry a central polypurine tract of HIV-1 (ppt), a posttranscriptional regulatory element of the woodchuck hepatitis virus (W), and self-inactivating mutations (brown triangle) in the LTR. (B) Expression of *LacZ* in D3 ES cells transduced with LV-LAC. ES cell colonies were stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal). [Bar = 50  $\mu$ m.] (C and D) Representative bright field (C) and fluorescence microscopy (D) images of D3 ES cells 2 weeks after infection with LV-GFP (moi 50). [Bar = 200  $\mu$ m.] (E and F) Transduction of human ES cells with LV-GFP. Bright field (E) and fluorescence (F) images of one representative ES cell colony are shown. [Bar = 100  $\mu$ m.] (G) Southern blot and flow cytometer analyses of murine ES cell clones. (Left) Representative Southern blot of four ES cell clones (lanes 1–4) derived from D3 ES cells infected with LV-GFP (moi 5) and uninfected ES cells (lane 5). (Right Top) Schematic structure of the integrated lentivirus; wavy lines, mouse chromosome. Genomic ES cell DNA was digested with *Xba*I, which cleaves the provirus only once, blotted, and hybridized to the indicated probe (black bar) to assess provirus copy number. (Right Bottom) Flow cytometry analysis of the same ES cell clones (1–4). DNA from ES cell clone in lane 2 is negative for eGFP.

1B). To quantify the number of lentivirus-transduced ES cells, we infected D3 ES cells with LV-GFP (Fig. 1 C and D) and performed fluorescence-activated cell sorting (FACS) experi-



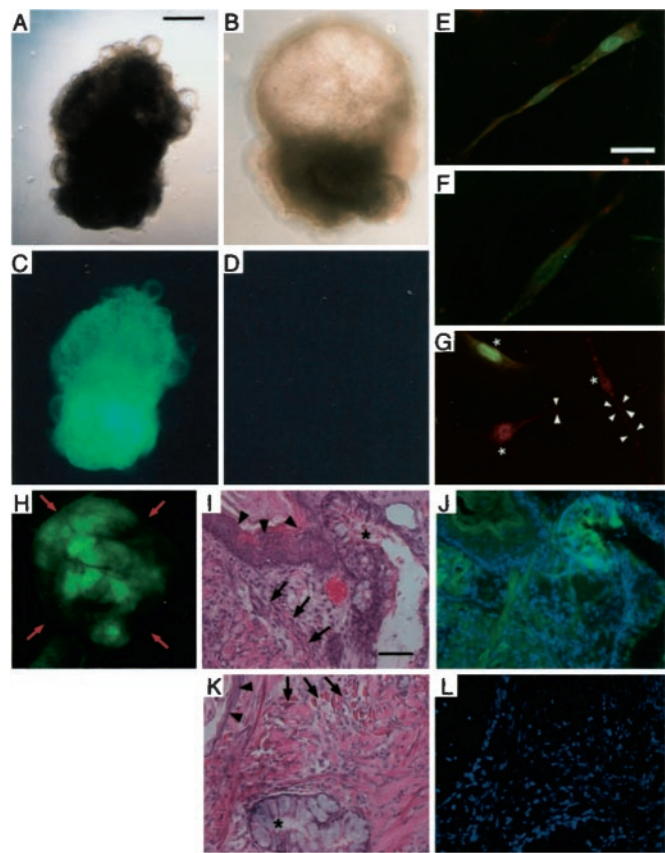
ments 4 and 14 days after infection. During the incubation period, the ES cells were passaged 6 times, but no significant reduction in the number of eGFP-positive cells was observed (data not shown). Fourteen days after infection with LV-GFP at an moi of 5 or 50, we observed eGFP expression in 52.5% and 95.5% of the cells, respectively (see Fig. 5, which is published as supporting information on the PNAS web site, www.pnas.org). Similar transduction efficacy was achieved by using LV-GFP in another ES cell line [R1 ES cells (27)] (see Fig. 5). These data demonstrate that lentiviral vectors carrying transgenes under the control of the CAG or the PGK promoter can efficiently transduce different ES cell lines, and that lentiviral transgene expression is sustained during undifferentiated proliferation of ES cells. Next, we tested whether lentiviral vectors can be used for gene transfer into ES cells derived from human embryos. Infection of human ES cells [cell line H1 (28)] with LV-GFP at an moi of 50 resulted in the transduction of almost 100% of the cells, which expressed eGFP over several passages (Fig. 1 E and F).

Southern blot analyses on genomic DNA isolated from transduced D3 ES cell clones revealed the number of proviral DNA integrations (ranging from 1 to 3 at an moi of 5; Fig. 1G). Interestingly, the amount of eGFP parallels the number of proviral DNA copies, suggesting the lack of transcriptional silencing (Fig. 1G).

To analyze lentiviral gene expression during *in vitro* differentiation of ES cells, we cultured murine ES cells in aggregates that form EBs (for review see ref. 29). All EBs derived from D3 ES cells, which were transduced with LV-GFP at an moi of 50, displayed eGFP expression (Fig. 2 A and C). No green fluorescence was detected in EBs derived from control ES cells (Fig. 2 B and D). After 8 days in suspension culture, several of the eGFP-positive EBs displayed contractile activity (see Fig. 5). To assess the function of the CAG promoter in the derivatives of the LV-GFP-infected ES cells, EBs were dissociated and analyzed by immunocytochemistry. Immunofluorescence staining with markers specific for muscle (myosin and desmin) and neuronal cells (neurofilament NF200) followed by confocal microscopy analysis revealed a more intense green fluorescence in myosin- and desmin-positive cells (Fig. 2 E and F) as compared with neuronal cells (Fig. 2G). Transgenic mice generated by pronuclear injection of CAG-GFP revealed a similar bias for muscle cells as compared with neuronal cells (24). We conclude that lentiviral transgene expression is sustained during *in vitro* differentiation of ES cells.

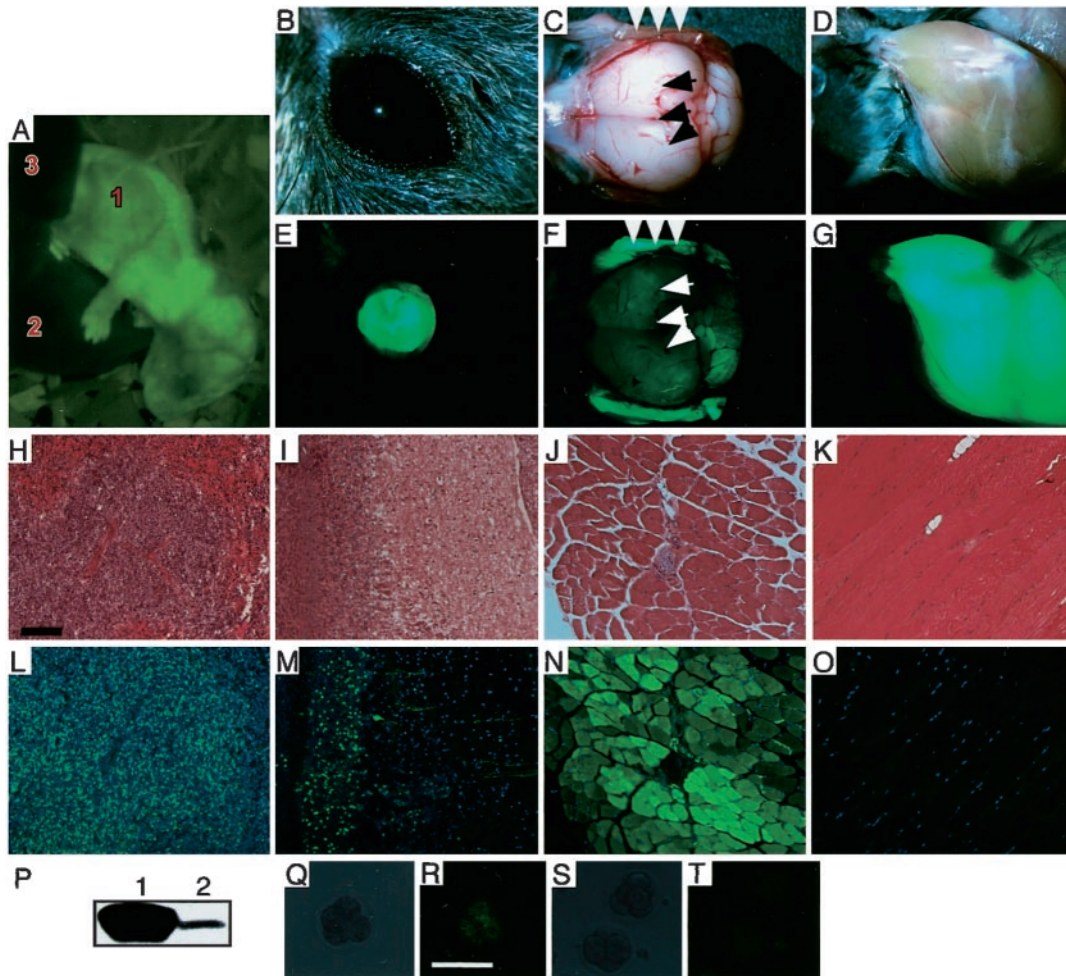
To determine whether lentiviral expression of transgenes is sustained during and after differentiation of ES cells, we analyzed the expression of eGFP during teratoma formation (30). Subcutaneous injection of LV-GFP-transduced ES cells (ES-LV-GFP) (moi 50) into nude mice produced teratomas in all mice ( $n = 4$ ). All ES-LV-GFP-derived teratomas expressed eGFP at such a high level that their fluorescence was observed externally, through the skin of the living animals by using *in vivo* fluorescence microscopy (Fig. 2H). Histological analysis revealed that the eGFP-positive teratoma included bone and muscle (mesoderm), gut epithelium (ectoderm), and stratified squamous epithelium (ectoderm) (Fig. 2I and J). No fluorescence was detected in the teratomas derived from control D3 ES cells (Fig. 2K and L). Thus, transduced ES cells participate in the formation of three primary germ layers, and the transgene introduced by lentiviral vectors is expressed during differentiation.

We next analyzed whether lentiviral transgene expression is sustained during embryo- and morphogenesis by injecting ES-LV-GFP into blastocysts from C57BL/6 mice. Bright green fluorescence was readily detectable in newborn chimeras by using *in vivo* fluorescence microscopy (Fig. 3A). Further analysis revealed eGFP expression in multiple organs of adult chimeras including the eye (Fig. 3B and E), brain (Fig. 3C and F), and muscle (Fig. 3D and G). Interestingly, immunoblot analysis of



**Fig. 2.** Expression of the lentiviral transgene during *in vitro* and *in vivo* differentiation of ES cells. (A–D) EBs derived from ES cells transduced with LV-GFP (A and C) vs. EBs derived from uninfected ES cells (B and D). Bright field (A and B) and fluorescent microscopy (C and D) images of the same EB are shown (see Fig. 5: contractile activity of the EB shown in A and C). [Bar = 200  $\mu$ m.] (E–G) Analysis of myosin (E), desmin (F), and neurofilament (NF200) (G) expression in EB-derived cells. Single-channel confocal microscopy analysis was performed (red channel, immunofluorescence stain; green channel, eGFP), and a merged-color image is shown. [Bar = 20  $\mu$ m.] The asterisks indicate the nucleus, and the arrowheads mark axon-like structures of two NF200-positive cells. (H–J) Transgene expression in teratomas derived from lentivirus-transduced ES cells. (H) *In vivo* imaging analysis of eGFP expression in a mouse injected with LV-GFP-transduced D3 ES cells (arrows). (I–L) Histological analysis of teratomas derived from LV-GFP-transduced ES cells (I and J) and control ES cells (K and L). The hematoxylin/eosin stain (I and K) and the fluorescence microscopy (J and L) images of adjacent sections (thickness, 5  $\mu$ m) are shown. The arrowheads indicate stratified squamous epithelium, whereas the arrows mark muscle. The asterisks indicate epithelial gut-like structures. [Bar = 50  $\mu$ m.] Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI), blue.

extracts from chimeric mice (Fig. 3P) revealed similar differences in the eGFP expression levels between skeletal muscle and brain as in those seen in CAG-GFP transgenic mice (24). Flow cytometry analysis revealed eGFP-positive cells in the spleen and bone marrow (see Fig. 5). Histological analysis demonstrated eGFP-positive cells in the germinal centers of the spleen (Fig. 3H and L), in the molecular layer of the cerebellum, in Purkinje cells (Fig. 3I and M), and in striated skeletal muscle (Fig. 3J and N). Interestingly, the amount of coat color chimerism (i.e.,  $\approx 40\%$  in the mice used for these experiments) correlated with the extent of green fluorescence observed in different tissues. To confirm that germ-line transmission is achieved, the male chimeric mice were bred with B6D2F1 superovulated female mice. After successful mating, the fertilized embryos were explanted and grown *in vitro* for 48 h. Results



**Fig. 3.** Lentiviral vector-transduced ES cells give rise to chimeras that express the transgene in different tissues. (A) *In vivo* imaging of green fluorescence in a 3-day-old chimera (no. 1) derived from LV-GFP-transduced ES cells and two nonchimeric animals (nos. 2 and 3). (B–G) eGFP expression in the eye (B and E), brain (C and F), and muscle (D and G) of an adult chimeric mouse with  $\approx 40\%$  coat chimerism. Shown are the bright field (B–D) and epifluorescence images (E–G); the arrowheads and arrows in C and F indicate eGFP-positive skeletal muscle and cerebrum, respectively. (H–O) Histological analysis of eGFP expression in the spleen (H and L), cerebellum (I and M), and skeletal muscle (J and N) of a chimeric mouse and skeletal muscle of a control mouse (K and O). The hematoxylin/eosin stain (H–K) and the fluorescence microscopy (L–O) images of adjacent cryosections are shown. [Bar = 100  $\mu\text{m}$ .] (P) Immunoblot analysis using polyclonal anti-GFP Abs on extracts of muscle (lane 1) and cerebrum (lane 2) of the same chimeric mouse shown in B–D. (Q–T) Germ-line transmission of the transgene. Fertilized eggs were isolated from superovulated females, and eGFP expression was analyzed by using a confocal microscope. Representative example of an eGFP-positive embryo (4-cell stage) derived from matings of male chimeric mice (Q and R) and eGFP-negative embryos derived from matings of control mice (S and T). [Bar = 100  $\mu\text{m}$ .]

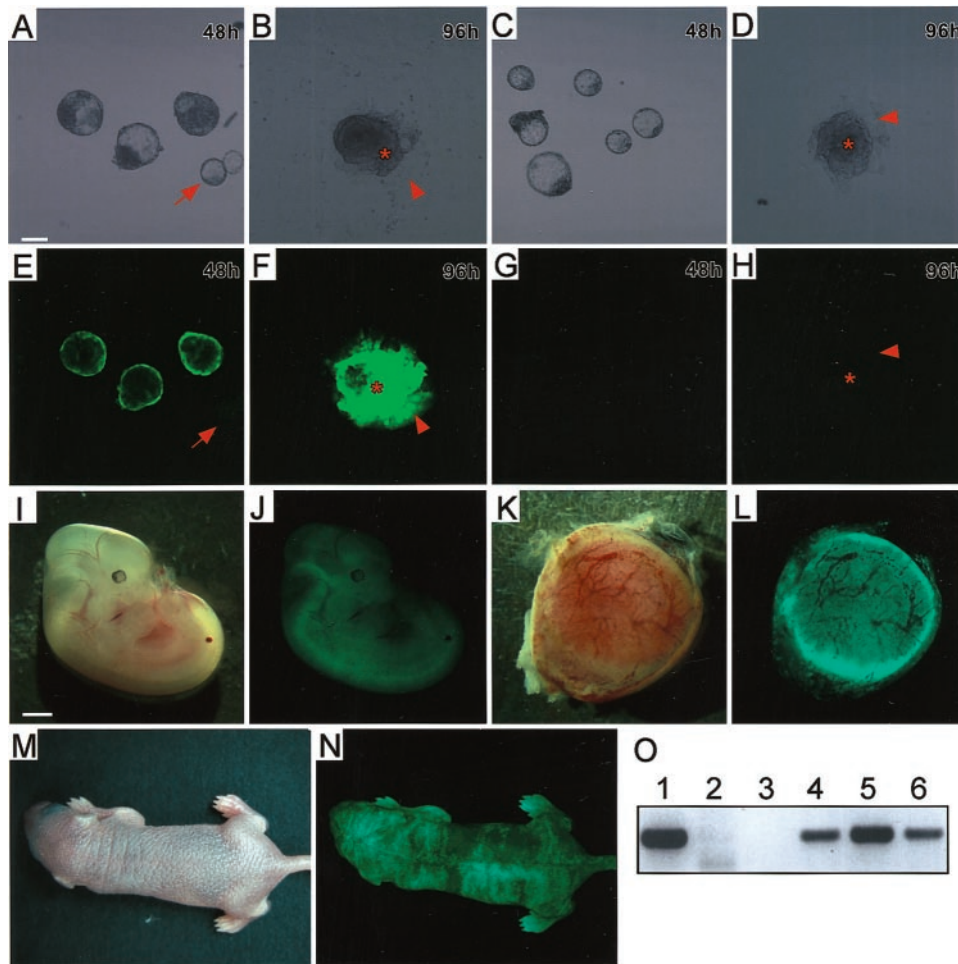
in Fig. 3 Q and R show expression of eGFP in 4-cell-stage embryos. No expression of eGFP was observed in control embryos (Fig. 3 S and T). We therefore conclude that transduction of ES cells by lentiviral vectors can lead to germ-line transmission.

Another way to generate a transgenic animal is to directly infect the embryo at an early stage. Four to eight cell-stage embryos (morulae) were isolated from CB6F1 mice, transduced with LV-GFP or LV-pGFP, and analyzed over the next 96 h for eGFP expression. Preimplantation embryos are surrounded by the zona pellucida, a layer of extracellular matrix synthesized by the growing oocyte (see references in ref. 15). Removal of the zona pellucida proved to be crucial for transduction of morulae by lentiviral vectors, because morulae surrounded by the zona pellucida were not transduced by lentiviral vectors, whereas all those embryos lacking the zona pellucida were eGFP-positive within 48 h after infection (Fig. 4 A and E). Within 96 h, the inner cell mass was distinguishable from trophoblast-like cells (Fig. 4 B and F). Both were positive for eGFP in the LV-GFP-

(E and F) and LV-pGFP-transduced (data not shown) embryos. The uninfected control embryos did not exhibit any detectable green fluorescence (Fig. 4 C and G and D and H). To analyze gene expression during embryogenesis *in vivo*, we transferred lentiviral vector-transduced embryos (29 blastocysts) into pseudopregnant females, which gave rise to three living embryos and mice. Analysis of eGFP expression at day 12 after fertilization revealed expression of eGFP throughout the whole body of the embryo and in the placenta derived from morulae transduced with LV-GFP (Fig. 4 I and J), demonstrating that lentiviral genes are expressed even during *in vivo* development of the mouse embryo. The placenta was also eGFP-positive (Fig. 4 K and L). Furthermore, we observed transgene expression in newborn mice by using *in vivo* fluorescence imaging (Fig. 4 M and N). PCR analysis revealed the presence of the provirus in all eGFP-positive embryos and mice (Fig. 4O).

The efficiency of gene transfer into ES cells and preimplantation embryos described here for a member of the retrovirus family is unprecedented. Vectors derived from murine leukemia and other





**Fig. 4.** Transduction of murine preimplantation embryos with lentiviral vectors. (A–H) eGFP expression in cultured blastocysts derived from morulae infected with LV-GFP. At 96 h after infection (B and F), eGFP is expressed in inner cell mass-derived components (asterisk) and trophoblast-like cells (arrow). Blastocysts derived from morulae that are surrounded by a zona pellucida (arrow in A and E) or from uninfected morulae (C and G) are eGFP-negative. [Bar = 100  $\mu\text{m}$ .] (I–L) Transgene expression in an embryo (day 12 postcoitus) derived from lentivirus-infected morulae. Bright field (I and K) and fluorescence (J and L) images of one representative embryo (I and J) and its placenta (K and L) are shown. [Bar = 1 mm.] (M and N) eGFP expression in a 3-day-old mosaic animal derived from an LV-GFP-infected morula; original magnification ( $\times 0.2$ ). The bright field (M) and the green fluorescence (N) images are shown. (O) PCR detection of lentiviral vector DNA. PCR analysis of genomic DNA isolated from a transgenic CAG-GFP mouse (1), control mouse (2), empty lane (3), embryo shown in I and J (4), placenta shown in K and L (5), and newborn mosaic mouse shown in M and N (6).

simple retroviruses are effectively silenced in murine embryos. It was postulated that provirus silencing is based on defensive mechanisms that have evolved during murine evolution to protect the embryo from viral infection (31). This behavior might explain why vectors derived from a virus, which is normally not infectious for murine cells (such as HIV-1), escape these defensive mechanisms and express transgenes during embryogenesis. Furthermore, the design of the lentiviral vectors used in this study incorporates several advantages over previously used vectors derived from simple retroviruses. Deletion of the essential enhancer/promoter regions from the LTRs of the lentiviral vectors counteracts provirus silencing by cellular transcriptional repressors, which bind to the LTRs and affect the initial expression of murine leukemia virus-based vectors (32, 33). That we observed transgene expression during murine ES cell differentiation and embryonic development demonstrates that long-term DNA methylation-dependent silencing (31, 34, 35)—the second principal silencing mechanism—has no major effect on transgene expression from lentiviral vectors. By using a low moi for the transduction of ES cells, we observed the insertion of single proviral copies into the genome. The use of lentiviral vectors will avoid the problem of concatamerization often

observed with pronuclear injections, making these vectors particularly useful for the introduction of regulatable transgenes and transgenes that contain the *loxP* recognition sites for the Cre recombinase. One immediate use of lentiviral vectors will be transgenesis in other species, especially nonhuman primates, to generate relevant disease model system.

The recent isolation of stem cells from human embryos brings new relevance to ES cell gene transfer. Efficient gene transfer into human ES cells is necessary to dissect the genetic pathways involved in human stem cell proliferation and differentiation and will help to elucidate developmental and differentiation mechanisms (e.g., the genetic requirements for lineage commitment). Furthermore, transfer of genes that regulate differentiation will improve our ability to generate specific cell types from undifferentiated ES cells for the treatment of many metabolic and degenerative diseases. We believe that lentiviral vectors offer excellent opportunity to transfer genes into a wide variety of cells, tissues, and organs to study growth, differentiation, and development.

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