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# Reversible Immortalization of Human Primary Cells by Lentivector-Mediated Transfer of Specific Genes

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**We exploited the ability of lentiviral vectors to govern the stable transduction of cells irrespective of their cycling status to induce the reversible immortalization of human primary cells. First, bicistronic HIV-derived lentiviral vectors expressing GFP– and the HSV1 thymidine kinase and containing the LoxP sequence in their LTR (HLox) were used to transduce HeLa cells. Cre expression led to efficient proviral deletion, and unexcised cells could be eliminated by ganciclovir treatment. A human liver biopsy was then exposed to a combination of HLox vectors that harbored either the SV40 large T (TAg) or the human telomerase (hTERT) DNAs in place of GFP. This led to the isolation of liver sinusoidal endothelial cell (LSEC) clones that exhibited an immortalized phenotype while retaining most of the features of primary hLSEC. Complete growth arrest of these cells was observed in 2 days of Cre expression, and the resulting stationary culture could be kept for at least 2 weeks. Transduction of human adult pancreatic islets with HLox vectors coding for Tag and Bmi-1 also induced the proliferation of insulin-positive cells. These results indicate that lentivectors can be used to mediate the reversible immortalization of primary nondividing cells and should allow for the production of large supplies of a wide variety of human cells for both therapeutic and research purposes.**

*Key Words:* **HIV vectors; lentiviral vectors; conditional immortalization; reversible immortalization; gene therapy; gene transfer; LoxP-Cre; SV40 large T; Bmi-1; telomerase; pancreatic beta cell; insulin.**

## INTRODUCTION

Many fundamental studies on primary human tissues are hampered by the paucity of obtainable substrates. Also, even though cell-based therapy holds great promises for the treatment of a number of hereditary as well as acquired disorders, it will mostly remain a theoretical prospect unless the requirements for providing a high number of therapeutic cells can be met. The controllable expansion of primary cells, be they early progenitors or differentiated effectors, represents an attractive means of producing large quantities of cells for either experimental or therapeutic purposes. In a prototypic approach, a cell harvested *in vivo* is induced to grow indefinitely *in vitro,* a process referred to as immortalization. Furthermore, the primary phenotype of the cell, including its nondividing status if relevant, can be restored at will. Cell lines thereby obtained can be expanded, cloned, extensively characterized both in their immortalized and "de-immortalized" (i.e., after removal/silencing of the immortalizing genes) states, and used for analyses that range from basic physiology to proteomics, for the production of specific proteins and, in selected cases, for transplantation.

Murine primary cells can be readily immortalized through the expression of oncogenes (1, 2), although they often lose in the process part of their phenotypic characteristics. The simian virus 40 (SV40) gene encoding the large tumor (T) antigen (TAg) has thus been widely used for obtaining continuously growing cell lines from transgenic mice (3, 4). Unlike most other oncogene products, and owing to its multiple effects on the cell cycle, TAg alone can immortalize cells in the absence of other oncoproteins (5). In one typical example, a murine pancreatic islet cell line was established through the transgenic, repressible expression of TAg. The viral protein was produced under the control of the *tet* repressor-VP16 system, allowing the generation of a conditionally

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immortalized beta cell line, the proliferation of which could be stopped by tetracycline (6). Once arrested, these cells responded to glucose.

Although by analogy it should be possible to generate reversibly immortalized human cell lines, human tissues are generally more refractory to this manipulation than their mouse counterparts. Indeed, whereas continuously growing murine cell lines are rather easy to obtain through the expression of a single oncogene (7), this so-called single-hit immortalization usually fails in human cells (8, 9). The introduction of cell cycle inducers such as SV40 TAg (9), or the elimination of negative regulators such as p21 (10), do not cause the immortalization but rather the *in vitro* life span extension of human cells. After a certain number of cell divisions a crisis occurs, which results in the rapid senescence and death of the cells in culture (9). Cumulated evidence indicates that this event reflects a critical attrition of telomere length, and can be overcome by expressing the catalytic component of the enzyme telomerase (hTERT) (11). However, the ectopic expression of hTERT alone does not induce changes associated with transformation (12, 13). Instead, it was recently demonstrated that the tumorigenic conversion of human cells is a stepwise process that requires the combined expression of a series of growth-promoting and anti-senescence genes (14). It should therefore be possible to induce the controlled proliferation of human cells through proper genetic manipulation.

To be reversible, an immortalization procedure requires that the production or function of the proteins responsible for triggering cell division be repressible. This can be achieved through externally controllable gene expression systems, such as those derived from the *tet* repressor (6, 15) or from the dexamethasone-responsive mouse mammary tumor virus (MMTV) promoter (16). Alternatively, one can utilize a conditionally active growth promoting factor, for instance a temperature-sensitive mutant of SV40 large T (17). Finally, transgenes can be introduced in a configuration that permits their subsequent excision by DNA recombinases such as Cre from bacteriophage P1 or FLP from *Saccharomyces cerevisiae* (18, 19). The latter approach, obviously the most irrevocable, was exploited to obtain the temporary expression of genes transferred by oncoretroviral vectors (20–22). In the integrated provirus derived from these vectors, the transferred cDNAs were flanked by LoxP or FRT sites, allowing for their excision via the Cre or FLP recombinases, respectively (22). The Cre-sensitive retroviral vector-mediated expression of SV40 TAg was used to induce a limited life span extension of bovine smooth muscle cells, rabbit kidney cells, human foreskin cells (22) and human myogenic cells (23). In these cases, however, although the cells exhibited increased growth rates and plating efficiencies, they were not properly immortalized and ultimately underwent senescence (22, 23). More recently, TAg was successfully used to immortalize human hepatocytes (24). Whether it indicates that this tissue constitutively expresses hTERT or the selected clones contained a fortuitous mutation remains to be determined.

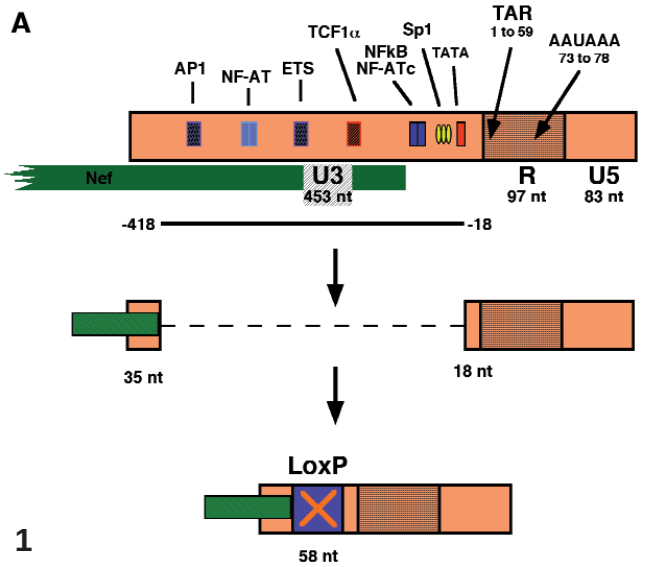
One major hurdle to the stable genetic manipulation of many primary cells, be it for immortalization or for other purposes, is their failure to divide readily in culture. This particularity decreases considerably their susceptibility to conventional gene delivery systems, including oncoretroviral vectors. The recent development of vectors derived from lentiviruses has changed this perspective, since lentiviral vectors are capable of governing the efficient delivery, integration and long term expression of transgenes into nondividing cells both *in vitro* and *in vivo* (25–28). This property provides an invaluable advantage for targets such as neurons (25), pancreatic islet cells (29, 30), hematopoietic progenitors (31), dendritic cells, hepatocytes, or smooth muscle cells (32).

In the present work, we demonstrate that the ability of lentiviral vectors to transduce cells irrespective of their proliferating status can be exploited to obtain the conditional immortalization of human primary nondividing cells. In one example provided here, human liver sinusoidal endothelial cells (hLSEC) were reversibly immor-

**FIG. 1.** HLox vector design and predicted life cycle. (A) Schematic diagram of the HLox 3'LTR. The sizes of the LTR segments (U3, R, U5) and positions of major transcriptional elements are indicated. After removal of the –418 to –18 segment of the HIV-1 LTR, only 53 nt are left upstream of R, comprising 35 nt of the proximal region of U3 and 18 nt of the distal end of U3. The LoxP sequence (58 nt) is inserted within the SIN deletion. (B) Schematic diagram of the HLox vector life cycle. The basic elements of HIV-based vectors, i.e., LTRs, SD, SA (splice donor and acceptor, respectively), Ψ (packaging signal), Ga (fragment of *gag*), RRE (Rev-responsive element), have been described (25). CMV: human cytomegalovirus immediate early promoter; EGFP: enhanced green fluorescent protein; IRES: internal ribosomal entry site of the encephalomyocarditis virus; HSV1-TK: thymidine kinase of herpes simplex virus type 1. In related constructs, TAg Bmi-1 and hTERT DNAs were inserted in place of EGFP.

**FIG. 3.** Morphology and phenotypic analysis of the immortalized hLSEC line. (A) Western Blot analysis of TAg expression in four clones (E4, F10, G9, G10) of hLSECs. The same filter was probed with a tubulin-specific antibody as internal control. Large T positive (+) and negative (–) cells served as references. Molecular masses are indicated in kDa. (B) Phase-contrast microscopy analysis of hLSECs (magnification 200×). (C) Staining of hLSECs with anti-von Willebrand factor monoclonal antibody. (D) Uptake of acetylated-LDL by hLSECs.

**FIG. 4.** Immortalized hLSECs strictly depend on oncogene for division. Time-course fluorescence analysis of Cre-transduced vs control hLSECs. hLSECs stably expressing farnesylated GFP were stained with PKH26 vital dye, replated and incubated overnight with Cre-expressing adenoviral vector at a m.o.i. of 100. On the day prior to the introduction of Cre and on day 1, 2, and 4 postexcision, cells were analyzed by FACS for GFP and PKH26. Results of the FACS analysis are represented as dot plots of GFP fluorescence (*x*-axis, 4-decade log scale) versus PKH26 fluorescence (*y*-axis, 4-decade log scale). Results of FACS analysis on day 1, 2, and 4 postexcision are represented as merged dot plots of two independent conditions: green dots represent control cells (–Cre) and red dots represent Cre-treated cells (+Cre). Similar results were obtained with two independent clones and in two separate experiments.

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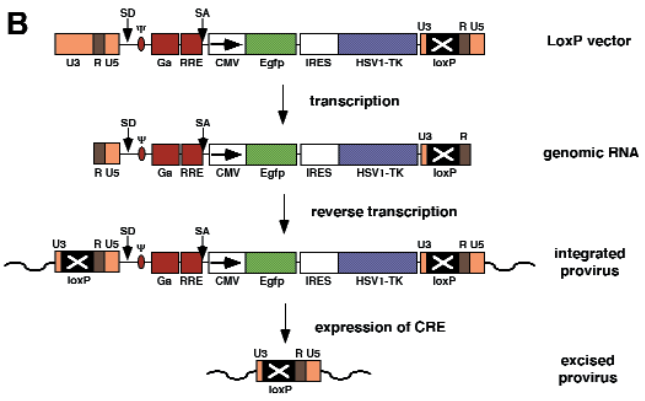


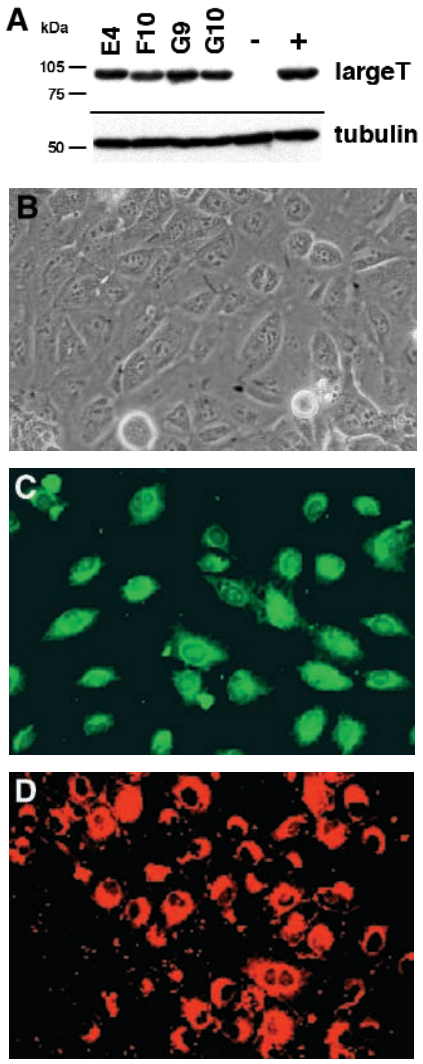


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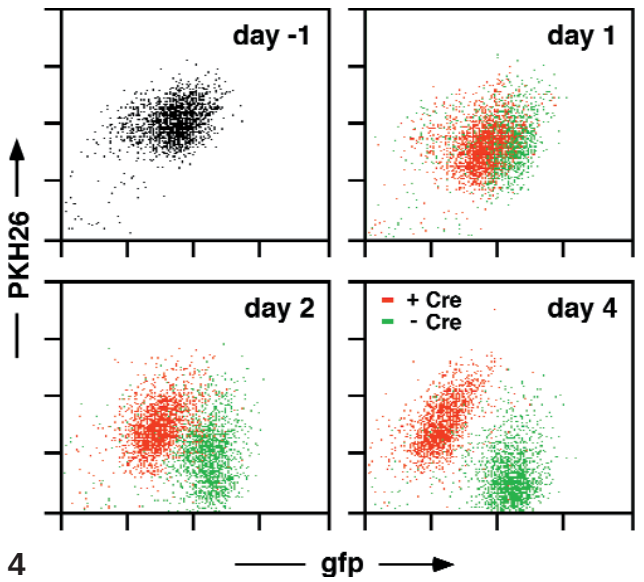


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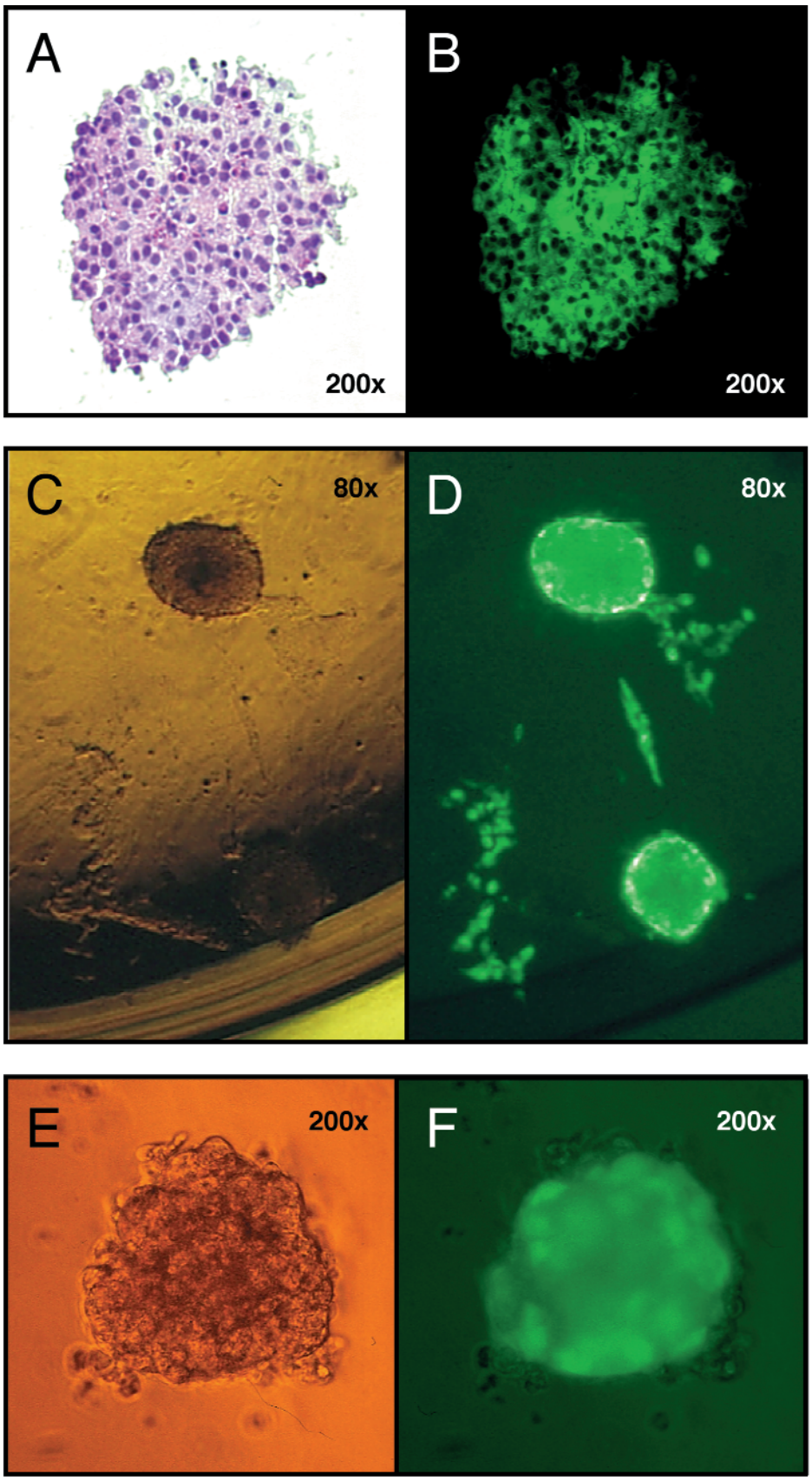


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talized by the sequential transduction of material derived from a liver biopsy with two LoxP-containing excisable lentiviral vectors, one expressing TAg and the other expressing hTERT. The resulting cell line has been growing robustly in minimal medium for more than a year and one hundred passages, and can therefore be considered as genuinely immortalized. It further exhibits numerous phenotypic markers of sinusoidal endothelial cells. Upon Cre expression, complete growth arrest is observed within 2 days, and the nondividing cells survive for up to 2 weeks. In another example, the expansion of insulin-producing cells was obtained following the lentivector-mediated transduction of nondividing adult pancreatic islet cells with TAg and Bmi-1, with or without hTERT.

## MATERIALS AND METHODS

*Vectors and plasmids*. Production of HIV-derived vectors pseudotyped with the VSV G envelope protein was achieved by transient cotransfection of three plasmids into 293T epithelial cell line as previously described (25). The HIV-derived packaging construct was pCMVΔR8.91, which encodes the HIV-1 Gag and Pol precursors, as well as the regulatory proteins Tat and Rev (28). VSV G was expressed from pMD.G. The HIV-LOX vector plasmids (pLOX) were derivatives of the original pHR′ backbone (25), with the following modifications. As depicted in Fig. 1, the LoxP sequence was inserted in the 3′ LTR U3 region of the previously described SIN-18 vector (33). The resulting LTR is described in GenBank No. AF237862. An internal cassette composed of two cistrons was introduced in this background, under the transcriptional control of the immediate-early promoter of the human cytomegalovirus. The first cistron comprised (i) the gene coding for the enhanced green fluorescent protein (EGFP) (resulting vector: pLOX-GFP) or the farnesylated form of EGFP (Clontech Laboratories), as the reporter gene, or (ii) the gene of interest, i.e., SV40 large T DNA (GenBank No. J02400, nt 2691 to 5163), the human hTERT cDNA (GenBank No. AF018167), or the murine Bmi-1 cDNA (50). The second cistron was the thymidine kinase of Herpes simplex virus type 1 (HSV1-TK), downstream of the internal ribosomal entry site (IRES) of encephalomyocarditis virus. In the experiments shown in Fig. 5, the CMV promoter from the pLOX-GFP vector was replaced by the promoter of the rat insulin II gene promoter. This sequence was obtained by PCR amplification of nt 252 to 946 (numbered after GenBank No. J00748) of the rat insulin II promoter (from the –660Ins2Luc plasmid kindly provided by J. Philippe). PCR primers contained *Cla*I and *Bam*HI sites to clone into *Cla*I and *Bam*HI sites of pLOX vector plasmid, in place of the CMV promoter. After transient transfection of the three plasmids in 293T cells by calcium phosphate precipitation, the supernatant was harvested, concentrated by ultracentrifugation, and resuspended in serum-free Cellgro medium (Boehringer Ingelheim Bioproducts, Heidelberg, Germany). Viral stocks were stored at –70ºC and titers determined by enzymatic assay of reverse transcriptase or by transduction followed by flow cytometry analysis of GFP expression in HeLa cells as previously described (28). Purified adenoviral vectors expressing the Cre recombinase under the control of the CMV promoter (Ad1.0-CMVCre, kindly provided by M. Perricaudet, IGR, Villejuif, France) were stored at –80ºC at a titer of $10^6$ transducing units/ml. The Cre-expression plasmid (pOG231, a gift of S. O'Gorman, The Salk Institute for Biological Studies, La Jolla, CA) comprised a CMV promoter, a synthetic intron and the Cre sequence coupled to a NLS signal. The CD8 vector expressed murine CD8.1 gene in the pSG5 plasmid background. Details on pLOX constructs are available at http://www.medecine.unige.ch/~salmon and http://www.tronolab.unige.ch.

*Cells, transfection, and FACS analysis*. Human liver endothelial cells were isolated from liver biopsies of patients undergoing segmental hepatectomies, according to institution guidelines. HeLa and 293T cells were grown in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 1% penicillin–streptomycin, and 10 mM Hepes (GibcoBRL, Life Technologies). hLSECs were grown in DMEM or CMRL with the same supplements. For cloning of the hLSECs by limiting dilution, initial cultures were carried out in 20% FCS. Acyclovir (ACV, Zovirax, Wellcome) and Ganciclovir (GCV, Cymevene, Roche) were prepared from injectable solutions. HeLa and 293T cells were transiently cotransfected with 35 μg of Cre-expression plasmid and 5 μg of mCD8-expression plasmid, using the calcium phosphate precipitation method. FACS analysis after cotransfection was performed by labeling the cells with PE-conjugated anti-mCD8 monoclonal antibody (Mab) (Ly-2, Caltag Laboratories, South San Francisco, CA). For cell division analysis, cells were incubated with PKH26 (Sigma) following the manufacturer guidelines. Briefly, $3 \times 10^6$ hLSECs were stained with PKH26 at $4 \times 10^{-6}$ M in 400 μl for 5 min. The cells were then washed and distributed in 5 wells of multiwell-6 dish. One aliquot (day –1) was kept for GFP and PKH26 fluorescence analysis in the FL1 and FL2 channels of a FACScan (Becton–Dickinson). Live cells were gated on the basis of their forward versus side scatter. The following day (day 0), cells from 3 wells were incubated overnight with adenoCre vectors (m.o.i. = 100). On day 1, 2, and 4 cells were detached and analyzed by FACS for GFP and PKH26 fluorescence. Human islets were isolated as previously described (51).

*Western blot analyses*. Cells were harvested in lysis buffer containing PBS, 1% Triton X-100, 1 mM aprotinin, and PMSF. Nuclei were pelleted by centrifugation (14,000*g* for 10 min), and the supernatants were evaluated by the BCA protein assay (Pierce). Aliquots containing 10 μg of protein were subjected to electrophoresis on 8% polyacrylamide gels containing SDS and electroblotted onto PVDF transfer membranes (Polyscreen; NEN Life Science). Membranes were blocked overnight in TBS-T with 5% nonfat powdered milk, incubated with mouse monoclonal antibody against SV40 large T antigen (Santa Cruz), and washed extensively with TBS-T. Incubation was then carried out with 1:1500 diluted anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham), and membranes were washed again before development using chemiluminescent detection (ECL, Amersham Corp.). Confirmation of equivalent protein loading was obtained using a mouse monoclonal anti-β-tubulin antibody (Boehringer Mannheim). TBS-T is composed of Tris–Cl (50 mM; pH 8), NaCl (137 mM), and 0.1% Tween 20 containing 5% dried milk.

*Microscopic analyses*. Immunocytochemistry analyses (von Willebrand factor expression and uptake of acetylated LDL) were performed as previously described (52). Briefly, LSEC were plated in a chamber slide and grown to subconfluence. Rabbit antiserum to human von Willebrand factor (Sigma) with a corresponding FITC-conjugated goat anti-rabbit IgG antibody (Sigma) was used for immunofluorescence staining. Human umbilical vein endothelial cells (HUVEC) and human fibroblasts were used as positive and negative controls, respectively. Uptake of acetylated LDL was demonstrated as follows. Briefly, after incubation of LSEC with 10 μg/ml Dil-Ac-LDL (Paesel-Lorei, Frankfurt, Germany) during 4 h at 37°C, the medium was removed and the cells fixed by 10% buffered formalin. HUVEC and human smooth muscle cells (gift from G.

**FIG. 5.** Transduction and induction of proliferation of human pancreatic islet cells**.** A-B. Human islets were transduced with an HIV-based vector encoding for GFP under the control of the CMV promoter as described in materials and methods. After five days, islets were fixed and stained with hematoxylin–eosin (A) or directly observed for GFP fluorescence under UV microscope (B). (C, D) Islets were transduced with a cocktail of three different HIV-based vectors encoding for TAg or Bmi-1 under the control of the CMV promoter, or for GFP under the control of the rat insulin promoter (see Materials and Methods). After 3 weeks, live islets and cells were observed under an inverted fluorescence microscope using phase contrast (C) or UV light and GFP filter (D). (E, F) Same as C and D but with no TAg or Bmi-1. Results shown are representative of three independent experiments, two of which were done with the further addition of a hTERT-encoding vector, without noticeable difference.

Gabianni, Geneva Medical School) were taken as positive and negative control, respectively.

*Tumorigenicity assay.* Immunodeficient NUDE mice were anaesthetized with Enflurane and $10^6$ HeLa cells or LSECs (clone E4 or G9) were injected subcutaneously. Mice were examined every week for a period of 11 weeks. Mice were euthanized either when bearing tumors larger than 1 cm (HeLa cells controls, after 4 weeks), or at the end of the observation period (LSEC clones, 11 weeks).

## RESULTS

### *Design of Excisable Lentiviral Vectors*

A 58-nucleotide sequence containing the LoxP site of bacteriophage P1 (18) was inserted in the 3′ long-terminal repeat (LTR) of a so-called self-inactivating human immunodeficiency virus type 1 (HIV-1)-derived vector (33), near the junction between the U3 and R sequences (Fig. 1A). An internal promoter derived from human cytomegalovirus (CMV) was placed in the vector upstream of a bicistronic coding cassette, in which the green fluorescent protein (GFP) and the Herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) genes were linked by the sequence encoding the internal ribosome entry site of encephalomyocarditis virus. During reverse transcription, the U3 region of the 3′LTR is duplicated, so that LoxP sites end up flanking the genome of the integrated provirus. Upon expression of Cre, the DNA sequence located between the recombinase target sites is excised, leaving in the chromosome a solitary LTR remnant that is devoid of all transcriptional activity (Fig. 1B).

### *Excision of the Transgene and Conditional Ablation of Unexcised Cells*

To test the functionality of the excisable lentiviral vector system, HeLa cells were transduced with the above vector, called HLox.CMV.GFP.IRES.TK, and clones stably expressing GFP were selected. Following cotransfection with a CD8- and a Cre-expressing plasmid, cells that acquired CD8 on their surface became GFP-negative, while untransfected, CD8-negative cells remained GFP-positive (Fig. 2A, upper panel). Transfection of the CD8 vector alone did not affect the GFP status of the cells (Fig. 2A, middle panel), nor did introduction of Cre in HeLa cells previously transduced with a control lentiviral vector expressing GFP but devoid of LoxP sites (Fig. 2A, lower panel). To improve the efficiency of Cre delivery, a Cre-expressing adenoviral vector was used to trigger the excision of the provirus. The rate of GFP removal was directly proportional to the dose of Cre adenoviral vector applied to the cells, ranging from 4% at a multiplicity of infection (m.o.i.) of 0.1 to 95% at a m.o.i. of 100 (Fig. 2B). Finally, the function of the built-in conditional ablation system was assessed. Cells producing HSV TK gene are sensitive to nucleoside analogs such as acyclovir (ACV) and ganciclovir (GCV), which are converted by the viral enzyme into nucleotides that are toxic upon incorporation into DNA. A Cre plasmid was electroporated in a population of Hela cells previously transduced with the HLox.CMV.GFP.IRES.TK vector. The low efficiency of the Cre delivery procedure resulted in a high proportion of the cells conserving GFP expression (Fig. 2C). Addition of ganciclovir at a concentration 1 μM for 10 days resulted in an almost complete elimination of the GFP-positive cells (0.8% remaining) without affecting the growth rate of the GFP-negative cells (Fig. 2C). Similar results were obtained using 293T human embryonic kidney cells as targets (not illustrated).

### *Immortalization and Characterization of Human Liver Sinusoidal Endothelial Cells*

To immortalize primary human cells two additional Lox-containing lentiviral vectors were constructed using HLox.CMV.GFP.IRES.TK as background. GFP was replaced in one of these vectors by the TAg gene, and in the other vector by the hTERT gene. A preparation of dissociated cells from a human liver biopsy was first transduced with the HLox TAg vector. After 2 weeks, a bulk culture of growing endothelial-like cells was harvested, transduced with the telomerase vector and subjected to cloning by limiting dilution. Clones arising from this procedure were extensively characterized and results from a few representative clones are shown. These cells have now been cultured for more than a year, corresponding to 100 passages, and can thus be considered immortalized. Cells that did not receive the hTERT vector stopped dividing around passage 12 and died shortly thereafter. Expression of TAg in the continuously proliferating clones was verified by Western blot (Fig. 3A). Furthermore, a PCR-based enzymatic assay revealed levels of telomerase activity that were more than two orders of magnitude higher than in extracts from control primary cells (not illustrated).

In light microscopy, the immortalized cells exhibit a cobblestone-like morphology characteristic of endothelial cells (Fig. 3B). Furthermore, they express von Willebrand factor (Fig. 3C) as well as CD31 and thrombomodulin (data not shown), and take up high levels of acetylated low-density lipoproteins (Fig. 3D). Other morphological and functional characteristics, such as fenestration, presence of Weibel–Palade bodies and a high susceptibility to hypoxia/reoxygenation-induced injury (not illustrated), together with their origin, qualify these cells as human liver sinusoidal endothelial cells (hLSEC).

Importantly, the subcutaneous injection of $10^6$ cells from two independent clones of LSECs did not lead to tumor development in NUDE mice after 11 weeks. This suggests that the LSEC lines, although immortalized, are devoid of tumorigenicity.

### *Cre-Induced Growth Arrest of the hLSEC Line*

One crucial issue was to verify that the hLSEC line was still dependent on the presence of the immortalizing gene cocktail for proliferation. To facilitate the monitoring of Cre-mediated provirus excision, the cells were first

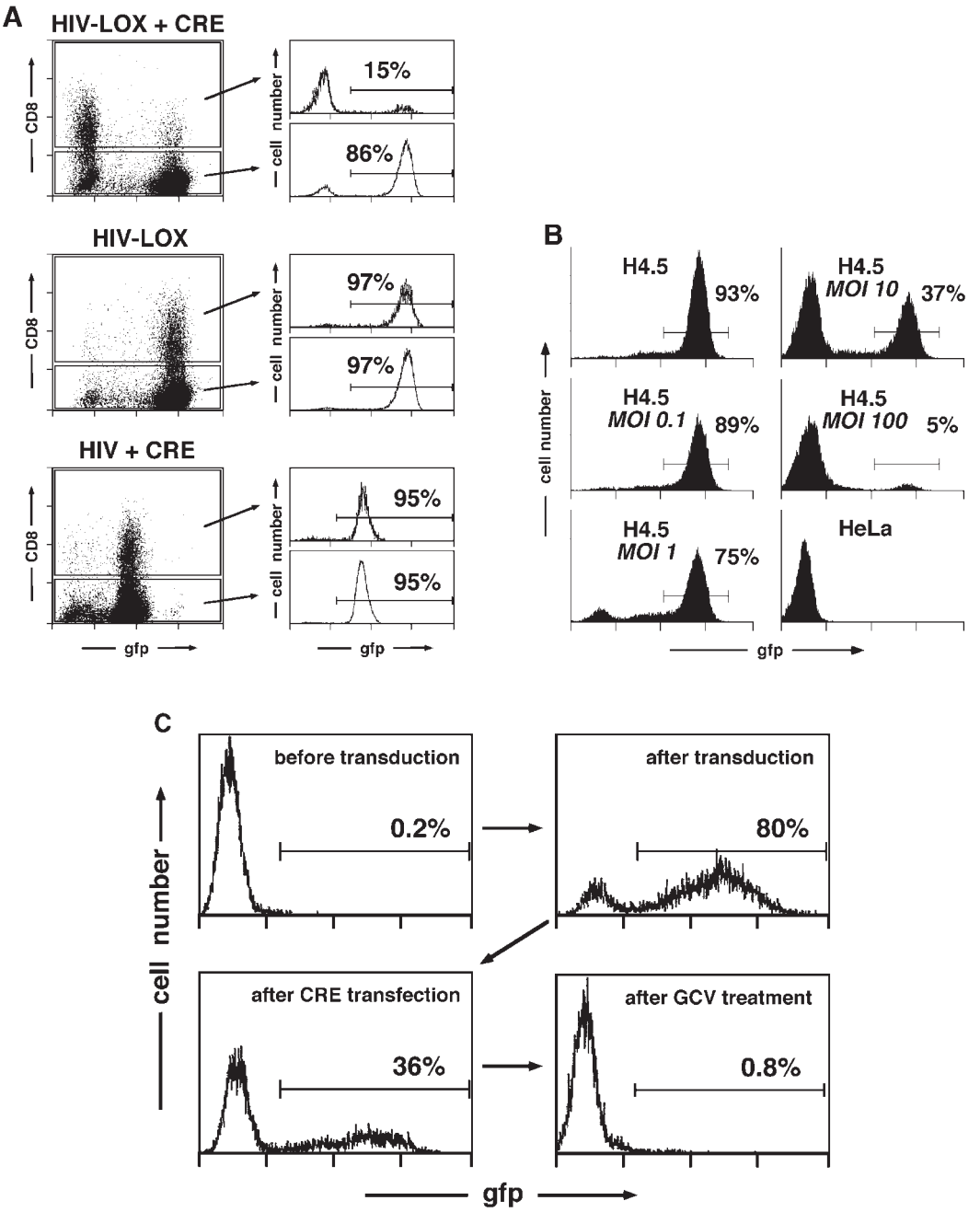


**FIG. 2.** Functional testing of the HLox system. (A) Excision after transfection of Cre. Stable clones of HeLa cells containing the HLox.CMV.GFP.IRES.TK provirus or a nonexcisable HR′.CMV.GFP vector were transiently cotransfected with a plasmid expressing murine CD8 and a control or a Cre-expressing construct. Three days later, cells were stained with PE-conjugated anti-mCD8 antibodies and analyzed by FACS for GFP (*x*-axis, 4-decade log scale) versus mCD8 (*y*-axis, 4-decade log scale). Gated $CD8^+$ (transfected) or $CD8^-$ (untransfected) cells were then displayed as histograms of GFP (*x*-axis, 4 decade log scale) versus cell number (*y*-axis, linear scale) and percentage of GFP-positive cells was determined (number above plots). Similar results were obtained with other clones of HeLa cells, clones of 293T cells, as well as in bulk cultures of HeLa or 293T transduced with HLox vectors. (B) Efficient excision after introduction of Cre via an adenoviral vector. A stable clone of HeLa cells (H4.5) containing the HLox.CMV.GFP.IRES.TK vector was incubated with various doses of adenoviral vectors expressing Cre (m.o.i. ranging from 0.1 to 100). After 7 days, the percentage of GFP-positive cells was determined by FACS, using untreated H4.5 and parental HeLa cells as positive and negative controls, respectively. All results are displayed as histograms of GFP (*x*-axis, 4 decade log scale) versus cell number (*y*-axis, linear scale); the percentage of GFP-positive cells is indicated above each plot. Similar results were obtained with another clone of HeLa cells. (C) Residual marker-positive HeLa cells are efficiently eliminated after treatment with ganciclovir. HeLa cells (upper left panel) were transduced with HLox.CMV.GFP.IRES.TK. After 6 days, a bulk culture of these cells was analyzed by FACS (upper right panel) and transfected with a Cre-expression plasmid. After 4 days, cells were analyzed by FACS (lower left panel) and subjected to treatment with 1 μM ganciclovir for 10 days. After treatment, cells were analyzed by FACS for residual GFP fluorescence. All results are displayed as histograms of GFP (*x*-axis, 4 decade log scale) versus cell number (*y*-axis, linear scale). Numbers above plots represent percentage of $GFP^+$ cells. Similar results were obtained with 293T cells.

transduced with the Lox sites-containing GFP expressing lentiviral vector HLox-CMV.GFP, and two hLSEC clones expressing high levels of GFP were selected for further analysis. These cells were stained with PKH26, a red dye that stably incorporates into the cell membrane lipid bilayer (34), and exposed to a Cre-expressing adenoviral vector. The excision of all Lox-flanked lentiviral integrants was predicted to have three consequences: first, the cells would lose GFP expression; second, provided that their growth was dependent on the presence of large T and/or telomerase, they would stop dividing; third, and as a consequence of this second point, if they became arrested they would keep high levels of PKH26, since the dye normally gets diluted together with membrane lipids as cells divide. At daily intervals after mock- or Cre-treatment, the cells were monitored by FACS analysis for the presence of red dye and GFP (Fig. 4). At day 1, no significant difference in PKH26-specific signal was observed between Cre-treated (red) and control (green) cells. However, a decrease of GFP fluorescence in the excised cells was already detected. At day 2, Cre-treated cells continued to lose GFP expression compared to untreated cells, but conserved levels of PKH26-specific signal that were unchanged compared to day 1. Conversely, the control cells retained their GFP fluorescence but showed a significant decrease in PKH26-derived signal. The FACS profile of cells analyzed at day 4 confirmed this trend, with a clear dissociation between excised and unexcised cells in terms of both GFP- and PKH26-specific signals. Of note, at that point very few of the Cre-treated cells exhibited a GFP-high and PKH26-low profile, attesting of the high efficiency of the gene excision process. The morphology of these cells was not fundamentally altered when they stopped dividing (not illustrated). Once arrested, they survived in culture for more than 2 weeks in minimal medium. The efficiency of gene excision was confirmed by the observation that significant cell growth could be detected by microscopic examination only after 2 weeks of culture of the Cre-treated population (data not shown). In that case, the foci that arose over the background of arrested cells were invariably GFP-positive, indicating that only unexcised cells were proliferating, hence that mutants capable of bypassing the requirement for the exogenous transgenes had not emerged. Taken together, these data demonstrate the strict dependence of the hLSEC line on the presence of the immortalizing genes for cell division.

### *Transduction and Induction of Proliferation of Human Pancreatic Islets Cells*

Primary LSEC are difficult to maintain in culture and grow little if at all *ex vivo* (35). As such, they are quite refractory to genetic modification by conventional gene delivery systems including oncoretroviral vectors. Still, to ascertain that lentiviral vectors carrying cell cycle promoting genes can induce the proliferation of truly nondividing primary cells, we used human adult pancreatic islet cells as a paradigm. As shown in Figs. 5A and 5B and as previously described (30), human islets are readily transducible with HIV-based vectors encoding for GFP, even in their innermost layers. Freshly purified adult islets were therefore exposed to a mixture of HIV vectors coding for TAg and Bmi-1, with or without hTERT, together with a lentivector coding for GFP under the control of the rat insulin II gene promoter (HLox.RiP.GFP). Preliminary experiments showed that GFP expression from this latter vector is restricted to insulin-positive cells (not illustrated). Introduction of the putatively immortalizing lentivector cocktail induced the proliferation and spreading of cells out of islets structures (Figs. 5C and 5D), while no such phenomenon was observed in islets transduced with the HIV-RIP-GFP vector only (Figs. 5E and 5F). Among the proliferating cells, a number of GFP-positive cells could be detected (Fig. 5D). Proliferation of such RIP-GFP positive cells could be observed as early as 2 weeks and could be followed for up to 6 weeks after transduction. However, the RIP–GFP positive cells were eventually lost over time, whether or not hTERT was one of the transgenes. Although this could be the consequence of their arrest of proliferation or of the transcriptional silencing of the RIP.GFP cassette, cumulated evidence indicates that, under our experimental conditions, the overgrowth of bystander cells plays an important role. Taken together, these data nevertheless show that HIV-based vectors coding for genes whose products promote the cell cycle are capable of inducing the proliferation of nondividing primary human cells without the aid of any additional specific growth factor.

## DISCUSSION

This work describes the reversible immortalization of human primary cells after transfer of specific genes using HIV-based vectors. Conditional immortalization of human liver sinusoidal endothelial cells was achieved through lentivector-mediated gene transfer of SV40 large T and telomerase. The immortalized cells divide continuously in the absence of specific growth factor yet retain many phenotypic and morphological characteristics of primary sinusoidal endothelial cells. Also, these cells do not induce tumors in NUDE mice, indicating that the cocktail of genes used to immortalize them does not lead to tumorigenic transformation. Most importantly, the design of the lentiviral vectors used to introduce the immortalizing genes allowed for the Cre-mediated excision of the proviral integrants. This resulted in a complete growth arrest of the cells within less than 2 days, indicating that the cells remain strictly dependent on the presence of the oncogene for cell cycling. These data demonstrate that Lox-containing lentiviral vectors represent a powerful tool to obtain reversibly immortalized cell lines of human origin through the expression of specific genes. The same type of lentiviral vectors was also

used to transduce nondividing human pancreatic islets with SV40 large T and Bmi-1, with or without hTERT. This resulted in the growth factor-independent proliferation of beta cells, another indication that HIV-based vectors coding for genes involved in cell proliferation can induce cell cycling in naturally resting human primary cells.

Lentiviral vectors have an unprecedented ability to mediate the stable integration and long term expression of transgenes in a wide variety of human primary cells, irrespective of their proliferative status. This will certainly represent an invaluable advantage when attempting to immortalize cells such as neurons because they are essentially nondividing, or hematopoietic stem cells because they are mostly quiescent and induction of proliferation results in loss of their pluripotentiality (36). The successful immortalization of liver sinusoidal endothelial cells and the induction of growth factor-independent proliferation of pancreatic beta cells by lentivector-mediated gene transfer therefore constitutes an exciting premise. LSECs are not truly resting, hence could theoretically be transduced with classical retroviral vectors. The efficiency of this process, however, is extremely low, a limitation that would most likely preclude the concomitant introduction of the several components of an immortalizing cocktail in a same cell. Lentivectors overcome this difficulty in this and many other similar situations. The case of the human pancreatic islet cells is even more representative, as these cells cannot be transduced with classical retroviral vectors (37). Durable proliferation of pancreatic cells after oncoretroviral-mediated gene transfer was obtained only after transduction of fetal islets, a structure in which cells still divide (38). The evidence we provide here that adult beta cells can be induced to proliferate after transduction with HIV vectors coding for proliferation inducing genes is thus very promising. Efforts aimed at obtaining the long-term expansion of these cells in culture, without overgrowth by non-beta cells, are in progress.

The terminology describing the different events that ultimately lead to cell transformation is still evolving. One emerging tendency is to use a specific term for each step of this process. The genes that trigger entry into the cell cycle can thus be called growth promoters, or cell cycle inducers. Prototypes of this category are the SV40 large T antigen, which acts through binding of tumor suppressors Rb and p53 (39), and Bmi-1, which downregulates tumor suppressor genes encoded by the *ink4a* locus (40). The label antisenescence gene can be reserved to telomerase, which prevents the progressive shortening of telomeres that occurs as the number of cell divisions augments (14, 41). Lastly, the qualifier transforming can be restricted to genes that provide the cell with strong pleiotropic signals, generally associated with loss of contact inhibition, growth in low serum, establishment and development of tumors in NUDE mice, as well as loss of tissue-specific properties, or dedifferentiation. The prototype of this category is the activated form of the Ras gene, H-Ras$^{V12G}$ (41). Here we use the term "immortalization" to describe a process in which primary cells have gained the capacity to grow indefinitely yet have conserved several properties of their primary parent, such as tissue-specific markers and functions and contact inhibition, and are not tumorigenic in NUDE mice. Of note, the absence of *in vivo* oncogenicity of the LSEC lines is in accordance with a recent report indicating that introduction of SV40 large T antigen and hTERT genes is not sufficient to induce the tumorigenic transformation of human cells (14).

The genuine immortalization of primary human cells is difficult to achieve with cell cycle inducers alone (42), but this hurdle can be overcome by coexpressing the hTERT gene (43–46). In rare occurrences, continuously growing human cell lines were obtained without the addition of the enzyme (8, 24, 42, 47, 48). It could be that, in these cases, a fortuitous mutation led to the spontaneous activation of telomerase, or that the parental tissue was one in which the enzyme is constitutively produced (9, 42).

The reversibility of the immortalization process lessens the importance of the phenotypic characteristics of the proliferating cell. Indeed, it is possible that in some cases, depending on the tissue and on the immortalization cocktail, specific traits of the parental cell might be lost during the growing phase and restored only after "deimmortalization." Our results with human muscle cells support this contention, by showing that the fusion score of immortalized myoblasts increases dramatically after excision of the growth-promoting genes (unpublished).

The induction of proliferation of human adult beta cells by lentivector-mediated gene transfer is encouraging, even though a number of obstacles will need to be overcome before cells obtained by such an approach can be envisioned for the treatment of type I diabetes. The hLSEC line, on the other hand, will be useful to study the mechanisms of angiogenesis and to examine the interactions that take place between hepatocytes and sinusoidal endothelial cells. It could also serve as a substrate to analyze the migration of cells or microorganisms through endothelial fenestrations, for instance to determine how malarial Plasmodia pass from the blood flow to the hepatocyte. Finally, the hLSEC line might help to understand the immunological tolerance to the resident intestinal flora as well as the innate and acquired immune responses against enteric pathogens, two phenomena in which liver sinusoidal endothelial cells apparently play a role (49).

More generally, reversibly immortalized cell lines will facilitate a very broad range of studies aimed at examining the physiology and the pathologies of human primary cells. Because lentiviral vectors can transduce targets that are nondividing, including at least some stem cells, the protocol described here will also permit to ask general questions about differentiation and oncogenesis. For instance, if a hematopoietic stem cell can be reversibly immortalized, does it conserve or ultimately recover its

pluripotentiality?

From a therapeutic standpoint, although the reversible immortalization of primary cells opens exciting perspectives for the field of cell transplantation, the clinical use of cells obtained by this technique poses important biosafety issues. Indeed, even if safeguards are multiplied and appropriate controls performed, it might be difficult to ensure the complete absence, in a given population, of a cell that is one step too close to a transformed state. Nevertheless, at least two situations come to mind in which this concern would not stand in the way of clinical applications. On the one hand, a conditionally immortalized cell could serve to produce a therapeutic factor that can be readily purified from its supernatant. A plasmocyte secreting a monoclonal antibody or an endocrine cell producing a hormone would be good examples. On the other hand, some cells can exert curative effects even if enclosed in semi-permeable containers preventing their dissemination. Current efforts aimed at treating diabetes via the implantation of encapsulated beta cells illustrate this general concept.

In conclusion, this work provides the proof-of-principle that the lentiviral vector-mediated transfer of specific genes can reversibly immortalize nondividing human primary cells. In view of the formidable ability of lentiviral vectors to deliver genes stably into a very large number of primary cells, this result suggests that the hereby described approach will allow the production of unlimited supplies of a broad variety of human cells for both therapeutic and research purposes.

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