β -Galactosidase marker genes to tag and track human hematopoietic cells

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Analysis of the behavior and fate of hematopoietic cells *in vivo* is an effective method of improving our understanding of normal hematopoiesis, which relies on a complex interactive network including cytokines, chemokines, physical contact, and undefined interaction kinetics, and of establishing the use of hematopoietic cells as therapeutic vehicles in gene therapy.¹

Analysis of animal models or patients reinfused with hematopoietic cells is considered important in addressing these issues. In an autologous context, the most relevant context for this purpose, the tracking of reimplanted cells is impossible to achieve without markers that are able to discriminate between reinfused cells and host cells; this emphasizes the need to develop safe, efficient, and easily performed marking strategies. The injection of nontoxic chemical markers has been widely used to study the embryonic development of animal models; however, fluorescein-conjugated dodecapeptides, rhodamine-conjugated dextran, or horseradish peroxidase molecules are diluted during successive divisions of marked cells, thus making long-term studies difficult.²⁻⁵ Differences in heterochromatin density in the quail/chicken chimera model have provided informative results but cannot be applied to other animal models.⁶ Defective retroviruses have been found to be very interesting cell markers in a number of situations, including preclinical and clinical models, because retroviruses are natural vectors that are able to stably integrate into the genome of the transduced cell; this allows detection of the provirus in the progeny of genetically modified cells, but *in situ* detection of a few transduced cells remains difficult.^{7,8}

A good experimental cell marker must exhibit the following properties: the expression of the marker must be (a) absent from the cells to be tagged, (b) genetically

transmissible, and (c) easily detectable *in situ*; in addition, the marker gene or its expression product should not be horizontally transmissible. To achieve these goals with the aim of tagging human hematopoietic cells, we decided to use the retroviral transfer of the bacterial β -galactosidase (β -gal) activity encoded by the *LacZ* gene as a cell-marking activity.

THE *LacZ* GENE

LacZ and neomycin-resistance (*neoR*) genes

Thus far, only the *neoR* gene that induces resistance to G418, a neomycin analog, has been used as a genetic marker in clinical trials dealing with hematopoietic cells. Several studies have been completed that have provided interesting information on hematopoietic recovery after stem cell transplantation, or on the origin of relapse in patients undergoing autologous transplantation. More than 20 gene-marking clinical trials using *neoR* have been performed or are under investigation and have demonstrated gene transfer and resistance to G418 in a very small percentage of the reinfused cells.⁹⁻¹² However, the use of *neoR* is hampered by several problems. Polymerase chain reaction analysis provides statistical data regarding only integration of the proviral sequence in the studied cell population. This assay does not necessarily demonstrate transgene expression, nor does it give information about individual transduced cells. An analysis of gene expression with the antibiotic resistance assay is limited to clonogenic cells that exhibit different sensitivities to G418 depending upon their nature (i.e., burst-forming units-E, granulocyte-macrophage colony-forming units (CFU), etc.).¹³ Finally, some reports suggest that the neophosphotransferase encoded by the *neoR* gene induces modifications of cellular metabolism or may act as a transcriptional silencer in eukaryotic cells.¹⁴⁻¹⁶ Therefore, the search for transgenes encoding an activity that is both innocuous and easily recognizable *in situ* has led to the use of marker genes such as the *LacZ* gene.

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The LacZ gene

General information. The bacterial *LacZ* gene which was isolated from the *z* cistron of the *Escherichia coli* lactose operon encodes β -gal, a glycoside hydrolase. The 3081-base pair coding sequence encodes a nonglycosylated protein of 1027 amino acids with a molecular mass of ~ 11 kDa.¹⁷ The active enzyme is a tetramer that is made up of four identical monomers encoded by the *LacZ* sequence.

Optimal pH and endogenous activity. Mammalian cells possess endogenous β -gal involved in lactose metabolism and glycolipid and mucosubstance catabolism. These enzymes are found mainly in lysosomes. Bacterial β -gal works optimally at a neutral pH, whereas mammalian β -gal requires an acidic pH (pH 4–5).¹⁸ Thus, by selecting appropriate reaction buffers, it is possible to preferentially observe the enzymatic activity attributable to the exogenous bacterial β -gal.¹⁹ In addition, chloroquine treatment of the cells can increase the internal pH of lysosomes and consequently prevent detection of the endogenous β -gal activity.^{20,21}

Subcellular localization. In mammalian cells, the *LacZ* gene product is found mainly in the cytoplasm.²² When chromogenic substrates are used for *in situ* detection, cells expressing *LacZ* can be visualized by their cytoplasmic staining surrounding a pale lighter area (the unstained nucleus). In CV-1 cells, it has been shown that β -gal has no membrane component and cannot be found exposed at the cell surface.²³ Moreover, β -gal is not able to cross intracellular or synaptic junctions; consequently, marking activity does not leak from *LacZ*-expressing cells to neighbor cells that do not express the *LacZ* gene.²⁴

Alternatively, the development of a fusion protein composed of the nuclear localization sequence of the simian virus 40 T antigen (Ag) at the 5' end of the *LacZ* coding sequence results in a β -gal enzyme that is located primarily in the nucleus of eukaryotic cells.^{22,25} Expression of the fusion protein nls-*LacZ* during mitosis suggests that the location of the enzyme is mostly perinuclear. Using photon microscopy, β -gal staining is not diffuse throughout the nucleus, but rather appears as a dark rim on the external face of the nuclear membrane. Galileo et al reported that the nuclear localization of nls- β -gal is not totally stable, with a cytoplasmic staining in 1% of postmitotic nls- β -gal⁺ embryonic cells *in vitro*.²⁶

Detection of expression of the LacZ gene. Expression of the *LacZ* gene or its derivatives can be detected using various substrates.²⁷ Cellular extracts can be analyzed using *O*-nitrophenol- β -D-galactoside or 4-methyl-umbelliferyl- β -D-galactoside, but these assays do not provide information for individual and identifiable cells. Only two substrates are used for the detection of single cells expressing a β -gal activity within a population of nonexpressing cells. Staining with 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal), which involves the degradation of X-Gal into galactose and a blue-indigo chromogene

product, is the most widely used assay for the detection of transduced cells.²⁸ It is worth noting that cells have to be fixed before addition of the substrate. X-Gal degradation can be inhibited by 4- β -D-galacto-D-glucose.⁷ A recent report suggests that the use of this detection strategy *in vivo* may underestimate the extent of gene expression compared with the immunostaining performed with monoclonal or polyclonal antibodies.²⁹ The use of fluorescein digalactose (FDG) combined with fluorescence-activated cell-sorting (FACS) analysis offers an interesting alternative. FDG is a galactose complex that traps and inactivates a fluorescein molecule which can be released as an active form by β -gal activity. The FDG-FACS assay is the most sensitive β -gal detection assay, because 1–10 enzyme molecules per cell are enough to detect a β -gal activity; in contrast, 100–1000 molecules per cell are necessary to achieve a positive X-Gal staining. In addition, the staining of cells with FDG does not require the fixation of cells and allows the sorting and subsequent culture of viable cells.^{27,30–33} However, because FDG is a large molecule, the staining procedure requires a gentle permeation of cell membranes using a mild osmotic shock, which makes this practical approach rather difficult when assaying primary hematopoietic cells. Lipophilic alternatives to FDG have been developed, but the interest of these molecules still remains to be demonstrated.³³

LacZ and cell behavior. Most of the results published thus far concern nonhematopoietic cell or tissue types; several examples are reported below. Sanes et al⁷ have shown that the growth of murine NIH-3T3 fibroblasts infected with a retrovirus coding for the *LacZ* gene is identical with that of noninfected NIH-3T3 cells. Furthermore, the same experiments have also shown that the percentage of *LacZ*-expressing cells remains stable over 50 cell divisions. The *LacZ* gene was also used as a reporter gene to analyze the transcription of specific or ubiquitous promoters in different cell and tissue types. *LacZ* was transferred to the cotton rat or to nonhuman primate airway epithelium using adenoviral vectors in preparation for a clinical trial of cystic fibrosis.^{34–36} The gene encoding β -gal was also transferred to neuronal^{37–40} and retinal cells,^{41–43} keratinocytes,^{44,45} bladder⁴⁶ and gallbladder cells,⁴⁷ mesangial and tubular cells,^{48,49} endothelial and other vascular cells,^{50–54} skeletal and heart muscle cells,^{55–59} hepatocytes,^{60–63} salivary gland cells,⁶⁴ and even thyroid follicular cells⁶⁵ using different, mostly adenoviral vectors in different species. In these experiments, limited side effects were observed and were believed to be related to the vector itself,^{34,51,57,65,66} rather than to the *LacZ* gene or its product. Moreover, in animal models, transgenic mice for *LacZ* did not exhibit toxicity,⁶⁷ and transfer of the *LacZ* gene into fertilized eggs⁶⁸ did not affect the embryonic and larval development of the African catfish.

To our knowledge, no report exists stating that β -gal can be toxic to human hematopoietic cells, which strongly express the *LacZ* gene. It has been assumed that the product of the nls-*LacZ* gene may be toxic in certain

conditions.^{69,70} However, our results demonstrate that expression of the *nls-LacZ* gene is not deleterious to human primary hematopoietic cells such as CD34⁺ progenitors and their progeny.⁷¹

β-gal and immunogenicity. An immune reaction against cells expressing a foreign Ag has already been shown for nonself proteins expressed in transduced cells reinfused into patients.^{72,73} As shown in animal models,⁷⁴ bacterial *β-gal* by itself exhibits immunogenic features, but it is worth noting that the long-term expression of a transduced *LacZ* gene into immunocompetent animals has already been reported in several instances.^{75,76} Whether or not the intrinsic immunogenic properties of a retrovirally transduced bacterial *β-gal* are sufficient to elicit an immune reaction which is able to eradicate transduced hematopoietic cells in humans remains unknown.⁶⁶ It should be expected that the combination of several parameters, including the vector strategy used, the reinfusion route, the level of expression, and the genotype of the recipient, will be involved in triggering off such an immune-mediated rejection of cells expressing *β-gal*.

Conclusion. Altogether, these results suggest that the use of native or modified *LacZ* genes does not affect cell behavior, and that *β-gal* can be envisaged as a safe marker for both *in vitro* and *in vivo* experiments.

In parallel to the *β-gal* activity encoded by the *LacZ* gene, alternative easily detectable markers should also be carefully considered; these markers include alkaline phosphatase and alcohol dehydrogenase⁷⁷ or proteins that can be directly detected by fluorescence, such as the green fluorescent protein from jellyfish and its derivatives.^{78–82}

CHOICE OF VECTORS AND PRODUCING CELL LINES

The efficiency and safety of nonviral transduction procedures, such as lipofection or wave-shock transfection, have to be clearly established before they can be used to genetically modify hematopoietic cells for clinical trials. Currently, transduction procedures using viral vectors seem to provide the most efficient approach.⁸³ Because early hematopoietic progenitors will actively proliferate to give rise to their differentiated and multilineage progeny, integration of the transgene in the genome is an absolute prerequisite to obtain long-term expression of the transgene. Therefore, the use of nonintegrating vectors such as adenoviruses is inappropriate in this situation and is restricted to therapies in which only short-term expression of the transgene is required.⁸⁴ Conversely, retroviral vectors and adeno-associated viral vectors can integrate the genome, and the transgene remains present in the progeny of the infected cells.

Retroviral vectors

Retroviruses are natural vectors that can be engineered to transfer genes.⁸³ For safety considerations and insert-

size limitations, *trans*-active viral sequences are excised and replaced with exogenous sequences to be transferred. Because these modified retroviruses are replication-defective, the production of viral particles is performed using transcomplementing packaging cell lines. During viral infection, viruses bind to target cells through specific receptors.^{85,86} After entry of the virus, reverse transcriptase transcribes viral RNA into double-stranded DNA that randomly integrates into the genome of the eukaryotic target cell. The new genetic information remains stable in the host cell and will be transmitted to its progeny during mitosis.⁸

Because the *LacZ* is only 3 kilobases long,¹⁷ it can be transferred using any available retroviral vector alone or in combination with another transgene.^{7,25,26,32,87–92} Most retroviral vectors derive from the Moloney murine leukemia virus; only a few of these defective vectors, such as GN1a and LNL6⁹³ or MFG derivatives,⁹⁴ have been used for gene-marking clinical trials. Vectors derived from murine retroviral backbones different from Moloney murine leukemia virus⁹⁵ are being tested for their potential to lead to a better expression of the *LacZ* gene or its *nls-LacZ* derivative in hematopoietic cells. The main limitation associated with the use of murine retroviral vectors is that integration in the genome requires cell division⁹⁶ and quiescent cells, such as very early hematopoietic progenitors, are difficult to infect. Promising strategies based on use of lentiviral-derived vectors able to transduce quiescent cells are now under development.⁹⁷

The choice of the packaging cell line to transduce human hematopoietic cells remains difficult, because no clear comparative data have been reported regarding the relative ability of Psi-Crip,⁹⁴ GP+envAm12,⁹⁸ PA317,⁹³ PG13,⁹⁹ 293 cell-derived BING,¹⁰⁰ or HT-1080-derived¹⁰¹ producing cell lines to transduce hematopoietic cells. Data obtained from different laboratories led to the hypothesis that the efficiency of transduction is not strictly related to viral titer and can differ from one producing clone to another.^{71,102–107} This hypothesis strongly argues for screening of the producing cell on the basis of its ability to infect the hematopoietic cell to be transduced rather than on the basis of titration assays performed on accessory cells such as murine fibroblasts.¹⁰⁷ In addition, prospects for *in vivo* gene therapy led to the conclusion that new packaging cell lines have to be designed that are able to produce viral particles not sensitive to the inhibitors present in human sera or able to produce targeted viral particles.^{101,108} In fact, all retroviral vector packaging cell lines currently developed can be used to produce *LacZ*-containing retroviral vectors.

Adeno-associated viruses (AAV)

Efficient gene transfer into CD34⁺ cells using AAV has been reported.^{109,110} The mechanisms of integration, efficiency of gene transfer (including efficient packaging cell lines), integration, and expression in dividing and nondividing hematopoietic cells remain to be clearly

defined for AAV.^{111,112} It will be especially interesting to learn whether the relative site-specific integration of AAV into the AAVS1 site on chromosome 19q13.3-qter will provide any advantage for gene transfer and gene therapy purposes.¹⁰⁹ The safety of viral supernatants (absence of replication-competent viruses) must also be improved for clinical purposes. Transfer of the *LacZ* gene with AAV-derived vectors into hematopoietic cells has already been reported, but long-term expression of this gene in transduced hematopoietic cells remains undocumented.^{69,70,110}

Combination of viral vectors

Very interesting investigations involve the combined use of nonintegrative vectors, such as adenoviral vectors, for the *in situ* delivery of retroviral vector sequences and transcomplementing genes, thus taking advantage of two different gene transfer strategies. Such strategies provide interesting approaches in transducing hematopoietic cells *in vivo* or in transducing target cells from transient packaging accessory cells *in vitro*, such as stromal cells.¹¹³

EX VIVO RETROVIRAL TRANSFER AND EXPRESSION OF *LacZ* ACTIVITY IN HUMAN HEMATOPOIETIC CELLS

LacZ activity in hematopoietic progenitors

As observed *in vivo* in humans and animal models, it is now established that the cell populations that participate in hematological reconstitution express the CD34 surface Ag and combine the expression of Thy-1 (CD90) or the absence of CD38 associated with the lack of expression of lineage-differentiated markers (Lin⁻). Thus, the CD34⁺ cell population originating from either bone marrow, mobilized peripheral blood, or cord blood is the most relevant cell compartment to target with the purpose of long-term maintenance of therapeutic information during the lifespan of a patient.¹¹⁴

Transfer of the *LacZ* gene into mononucleated bone marrow cells and the subsequent expression of cytoplasmic β -gal were first reported by Strair et al.³² The expression of β -gal was sufficient to allow cell sorting and the enrichment of X-Gal⁺ clonogenic cells. In our experience, expression of the *LacZ* gene in primary CD34⁺ cells has been difficult to detect with X-Gal staining, whereas detection of *nls-LacZ* was easier and more reproducible.⁷¹ In addition, discrimination between endogenous and exogenous β -gal activity is easier using *nls-LacZ*-mediated nuclear localization of the staining; the high nucleoplasmic ratio of immature cells favors the use of nuclear membrane staining.

As a model, we used TF-1, which is a growth factor-dependent cell line derived from an erythroleukemic patient.¹¹⁵ This cell line still expresses CD34 as well as CD33 and a variety of other Ags. Its growth is strictly dependent upon exogenous growth factors, including interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), or erythropoietin. By

cocultivating TF-1 cells with an MFG-*nls-LacZ* vector-producing cell line, $\leq 80\%$ of the TF-1 cells were transduced. The percentage of cells expressing the nuclear form of β -gal in the TF-1 cell population remained stable over a 2-month period. Subsequently, the percentage of *nls-LacZ*-expressing cells slowly decreased, reaching 1% after 12 months of culture, because of an inhibition of the long terminal repeat-mediated transcription of the transgene. Presence of the provirus is detectable in the unstained cell population using a polymerase chain reaction assay. Treatment with a demethylating agent such as 5'-azacytidine increases the percentage of X-Gal⁺ cells. This observation and others²⁸ suggest that DNA methylation may partly explain the decrease in β -gal expression in transduced cells. No morphological (Wright-Giemsa staining) or proliferative (growth curve kinetics, tritiated thymidine analysis, and the percentage of cells in different phases of the cell cycle) differences were observed between *nls-LacZ*-expressing and nonexpressing cells. The expression of differentiation markers, growth inhibition by transforming growth factor- β and interferon- α , and differentiation in response to IL-4 and erythropoietin were also unchanged in the transduced cell population.⁷¹

Using mobilized peripheral blood CD34⁺ cells at 2 days after the transduction step and cocultivating target cells and producers for 3 days or cultivating CD34⁺ cell populations with viral particles containing cell culture supernatants, 10–50% of the cells were determined to be transduced as assessed by X-Gal staining. Methylcellulose assays demonstrated the presence of transduced clonogenic cells with X-Gal⁺ blast-forming units-E, granulocyte CFU, macrophage CFU, and granulocyte-erythrocyte-megakaryocyte-monocyte-CFU. The same results were reported using cord blood CD34⁺ cells as targets.¹¹⁶ Expression of the *nls-LacZ* gene was also observed in colonies derived from more immature cells, such as high proliferative potential colony-forming cell colonies derived from transduced fetal liver CD34⁺CD38⁻Lin⁻¹¹⁷ or transduced peripheral blood CD34⁺Thy1⁺ cell populations.¹¹⁸

As indicated previously, transfer of the *LacZ* gene into immunoselected CD34⁺ human progenitor cells using AAV-derived vectors has already been achieved.^{69,70} Up to 70% of the transduced CD34⁺ cell population was found to express the transgene as assessed by X-Gal staining; however, detection of chromogenic activity of the bacterial β -gal in differentiated colonies was not reported. Transduction of human erythroleukemia cells using the same vector has also been reported, but with lesser efficiency ($\leq 3\%$).⁶⁹ It should be noted that transfer of the *LacZ* gene using adenoviral vectors⁸⁴ or human cytomegaloviral vectors¹¹⁹ has already been tested.

LacZ activity in differentiated cells

T lymphocytes. Among lymphoid cells, T lymphocytes are interesting targets for gene therapy.¹²⁰ T cells are affected in several genetic diseases or infected with HIV

in AIDS. In addition, they are (or could be) effector cells in cancer, recognizing tumor cells and contributing to their elimination through cellular- and humoral-mediated killing. Conversely, T cells are only alternative targets for gene therapy of genetic diseases, because they are not able to self-renew when reinfused into the patient, thus requiring a repeat patient treatment every 5–6 months. The *in vitro* generation of T cells from precursors using liquid or organotypic cultures still remains a technical challenge;¹²¹ however, in contrast with stem cells, T cells are neither rare nor difficult to identify and can be derived from hematopoietic or nonhematopoietic tissues.

Marking T cells with *LacZ* would provide information on survival, homing, the long-term proliferation of autologous, genetically modified T cells, and the long-term expression of transduced activity. Various coding sequences including neoR,¹²² herpes simplex virus thymidine kinase,^{123,124} human CD4,¹²⁵ and a truncated version of the human low-affinity nerve growth factor receptor,^{73,124} were retrovirally transduced into T cells derived from peripheral blood^{124,126} or resected tumors.¹²⁷ The culture and transduction of cells require stimulation, which can be performed with a combination of IL-2 and phytohemagglutinin^{123–125,128,129} or with a combination of antibodies directed against CD3, CD2, or CD28.¹²⁶ Retroviral transduction of the β -gal activity encoded by various genetic forms of the *LacZ* gene, including the regular *LacZ*, the *nls-LacZ* gene, and the *Sh-ble::LacZ* fusion gene has been successfully tested in prestimulated human T lymphocytes derived from peripheral blood cultivated in the presence or absence of feeder cells.^{126,129–131} When using a cocultivation procedure to transduce cells and a selected producing cell line that exhibits a good ability to transduce human hematopoietic cells, we found that $\leq 80\%$ of T cells were transduced, as assessed with X-Gal staining, without any deleterious effect on cell behavior.¹²⁶ Unfortunately, this good transduction efficiency was only obtained with a selected producing cell line. In our hands, the transduction of T lymphocytes usually ranges from 5 to 30% of X-Gal⁺ cells as reported by several laboratories. An analysis of CD4⁺ and CD8⁺ cells indicated that the two populations were equally transduced and expressed the *LacZ* gene.^{126,132} The proliferation and expression of various cell surface markers, including CD3, CD25, HLA-DR, CD49b (very late Ag-4), CD4, and CD8 were found unchanged after the transduction and expression of the marker.¹²⁶ It has also been shown that the *LacZ* product does not inhibit the T-cell differentiation of progenitor cells in fetal thymic organ cultures.¹³³

Dendritic cells (DCs). DCs are professional Ag-presenting cells that are involved in the activation of T lymphocytes during an immune reaction. DCs are considered as key targets to be manipulated to trigger an immune reaction against tumor cells. Transducing DCs with retroviral vectors has been approached both at the level of mature cells derived from the peripheral blood¹³⁴ and at the level of immature dendritic progenitors^{135,136}

present in the CD34⁺ fraction. In fact, an expression of bacterial β -gal activity was either reported in retrovirally transduced DCs originating from a peripheral blood mononuclear cell culture with GM-CSF and IL-4¹³⁴ or detected in CD1a⁺CD14[−] cells derived from transduced CD34⁺-mobilized peripheral blood cells cultured in the presence of GM-CSF and tumor necrosis factor- α (Celia Chishportich, C. B., and P. M., unpublished data).

Macrophages. It is possible to obtain macrophages either from a long-term culture of CD34⁺ cells or directly from peripheral blood or other nonhematopoietic tissues. The expression of *LacZ* activity into macrophage cells has been successfully assessed by X-Gal staining of macrophages generated from retrovirally transduced bone marrow, mobilized peripheral blood, or cord blood CD34⁺ cells.⁷¹ As of yet, no report has depicted the retroviral transfer of the *LacZ* gene or its derivative into fully differentiated human macrophages; however, the possibility of expressing this marker gene into cultured primary monocytes has already been shown using transfection procedures.¹³⁷ It is worth noting that macrophages usually exhibit a high level of endogenous β -gal activity, which makes the use of a nuclear localized version of this enzyme in the marking of these cells more interesting.

Natural killer (NK) cells. The expression of the *nls-LacZ* gene into NK cells by the generation of X-Gal⁺ NK cells from retrovirally transduced cord blood CD34⁺ cells cultivated in presence of IL-15 is under investigation. Experiments performed in fetal thymic organ cultures showed that NK cell differentiation of CD34⁺ progenitors was not modified by the expression of a retrovirally transduced *LacZ* gene.¹³³

FROM PRECLINICAL ANIMAL MODELS TO PATIENTS

Preclinical models

Most animal experiments were designed using the neoR gene rather than the *LacZ* gene to tag and track hematopoietic cells. For example, the use of retroviruses to transfer the neoR gene into hematopoietic progenitors has been widely used to study the contribution of different cell populations in hematopoietic recovery in mice,^{138–143} dogs,^{144–146} or nonhuman primates.^{103,147,148} Interesting data were reported by Champseix et al¹⁴⁹ using the expression of a cell surface marker coding sequence in a retrovirally transduced cord blood immature hematopoietic cell population reimplanted into immunoincompetent nonobese diabetic severe combined immunodeficient mice.

Retroviral transfer of the *LacZ* gene into avian leukemic cell lines or avian preosteoclastic cells, reinfusion of the cells into living embryos, and tracking of the cells in the hatched chicken using X-Gal staining have been reported.^{150,151}

In the murine model, Savatier et al⁶⁷ showed expression of a retrovirally transduced *LacZ* gene in the red

blood cells of transgenic mice. An infusion of retrovirally transduced CD34⁺CD38⁻Lin⁻ cells derived from the fetal liver into immunoincompetent severe combined immunodeficient-Hu mice led to a sustained expression of the *nls-LacZ* gene in primitive and committed clonogenic progenitors, mature myeloids, and B and T lineage cells for ≤ 4 months after injection.¹¹⁷

Clinical trials

Currently, neoR is the only coding sequence that has been used as a marker gene in clinical trials aimed at genetically tagging hematopoietic stem cells. Gene-marking clinical trials have focused on the contribution of infected progenitors to hematopoietic recovery in cancer patients receiving high-dose chemotherapy conditioning regimens⁹⁻¹¹ or on the role of the residual tumor cells present in the graft in the occurrence of relapse in a variety of malignancies.¹⁵² Previously, neoR was used by several groups to track tumor-infiltrating lymphocytes;¹²⁷ data have been published⁷³ that take advantage of a mutated form of the nerve growth factor receptor to detect transduced T lymphocytes in cancer patients.

CONCLUSION AND PERSPECTIVES

Using *LacZ* as a marker is an effective approach to (a) assess the efficacy of new transduction protocols or vectors and (b) tag and track hematopoietic cells *in vivo* in animal models or in clinical trials.

New procedures to genetically modify hematopoietic cells could be tested with the β -gal activity encoded by the *LacZ* gene. *Ex vivo* manipulation of hematopoietic cells still needs to be improved. The frequency of transgene integration could be assessed using X-Gal staining or FDG-FACS when testing new cytokine associations,^{153,154} the coculture of target cells and stromal cell lines,^{146,155,156} the use of extracellular matrix protein^{157,158} and antagonists or agonists of cycle protein regulators. In parallel, the improvement of a gene transfer tool to benefit both *ex vivo* gene therapy and future prospects for *in vivo* gene therapy can be approached using a marking strategy. Control of transgene expression must be improved, both in terms of sustained expression of a heterologous coding sequence and in terms of time and tissue specificity. Specificity of expression has already been envisaged using either specific promoters¹⁵⁹⁻¹⁶¹ that can direct expression of the transgene in specific cells or inducible promoters, such as those containing a Tat activating region element, whose expression is induced by the HIV Tat protein.^{162,163} Long-term expression of the transgene has to be addressed through the analysis of factors involved in the decrease of transgene expression,¹⁶⁴⁻¹⁶⁶ such as the hypermethylation of DNA,^{167,168} or interferences between cotransferred promoters.¹⁶⁹ New vector designs have already resulted from these studies but remain to be fully tested with primary hematopoietic cells.⁹⁵ Vectors coexpressing two different coding sequences sepa-

rated by an internal ribosomal entry site sequence¹⁷⁰ or coding for artificially engineered bifunctional protein^{171,172} could also improve expression in hematopoietic cells and/or the sorting of transduced cells. Interesting prospects can be anticipated with the construction of lentivirus-derived vectors that are able to integrate into quiescent cells.⁹⁷ In addition, the combination of different gene transfer tools has already been proven efficient, but still requires improvement.¹¹⁰ *In vivo* gene therapy will play a key role in gene therapy approaches in the near future, and the interest of using marker genes in animal models or preliminary marking clinical trials will be greatly emphasized in the development of this approach. In every aspect of gene transfer strategies, the use of a marker gene such as *nls-LacZ* will provide very informative results that accurately estimate transduction efficiency, gene expression, and the overall quality of the technical process tested. Identification and full characterization of new efficient marker genes in the marking of hematopoietic cells remains a challenge, and several approaches, including green fluorescent protein activities, cell surface proteins, and markers with low immunogenic potential are fully investigated by many laboratories, including our own.

Beyond *in vitro* experiments and animal models, study of the fate and behavior of hematopoietic cells in humans will remain the ultimate and most relevant strategy for improving our understanding of normal hematopoiesis and the use of hematopoietic cells in gene therapy. We currently propose the use of the retroviral transfer of *nls-LacZ* as a tool to follow the contribution of transduced autologous CD34⁺ peripheral blood progenitors in the reconstitution of lymphohematopoietic lineages in cancer patients undergoing treatment intensification. The short- and long-term expression of the transgene, immunogenicity of retrovirally transduced cells, and behavior and fate of autologous progenitors are important clues that will be addressed by this protocol precluding therapeutic gene therapy protocols.

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