Efficient Retrovirus-Mediated Gene Transduction Into Murine Hematopoietic Stem Cells and Long-Lasting Expression Using a Transwell Coculture System

By Wilfred T.V. Germeraad, Norio Asarni, Shinji Fujimoto, Osam Mazda, and Yoshimoto Katsura

The neomycin phosphotransferase (neo) gene was transduced into murine hematopoietic stem cells by culturing a recombinant retrovirus-producing cell line in a Transwell (Costar, Cambridge, MA) (bottomed with a porous membrane) hung into a Dexter-type long-term bone marrow (BM) culture. Gene transduction into stem cells retaining long-term reconstitution ability was successfully performed by using protocols of total 15 to 18 days of culture including establishment of the Dexter culture, transduction, and G418 selection. In the irradiated recipients of these cells, a large majority of the BM, thymus, and spleen cells as well as peripheral blood (PB) leukocytes were of donor origin and the neo gene was present in these organs up to 21 weeks after cell transfer. One third to two thirds of the in vitro colony-forming cells in the BM of the recipient mice were resistant to cultivation with G418. It was further found that the hematopoietic system of secondary recipients given BM cells from a primary recipient mouse was predominated by original donor-type cells. The transduced neo gene was detected in the PB, BM, thymus, and spleen cells of these secondary recipients. These results indicate that our procedure of retroviral vector-mediated gene transfer is highly effective in safely introducing a gene into pluripotent hematopoietic stem cells.

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Use of Replication-defective recombinant retroviruses is currently the most successful method to introduce a gene into somatic cells. The advantages of retrovirus-mediated gene transduction are no direct damage of the target cells, a high efficiency of transfer and a high expression of the transduced gene seen especially in vitro. In earlier experiments, the neomycin phosphotransferase (neo) gene transduced with a recombinant retroviral vector into hematopoietic stem cells was used as a lineage marker, and contributed to elucidate the developmental capacity of hematopoietic stem cells. Along with the improvement in the construction of vectors and safe packaging cell lines, the applicability of the retrovirus-mediated gene transfer method is broadening to be used in studies of molecular mechanisms of organogenesis as well as gene therapy.

Basic studies for gene therapy are being undertaken by introducing a variety of genes in model animals to become able to overcome congenital diseases. At present, clinical trials have begun in patients with an inherited immunodeficiency caused by dysfunction of the adenosine deaminase gene and in patients with certain forms of cancer. Important improvements necessary for a gene transfer system to be effective in gene therapy concern the efficiency of gene transduction and the level of expression. Although much effort has been paid on improving the construction of vectors and cultivation methods of BM cells, more effective therapy of inherited as well as acquired diseases will be possible after further advances in gene transduction technology. In the present work, we inserted a modified N2 vector into plasmid p574 containing a mutant polyoma enhancer (PyF101) in the long terminal repeat (LTR) and the constructed new vector was termed W5. An ecotropic virus-producing cell line containing the W5 provirus (M3-W5) with a titer of 3 × 10⁷ colony-forming units (CFU)/mL was established. By coculturing M3-W5 cells in a Transwell that physically separates the producer cells from the BM cells maintained in a Dexter-type long-term bone marrow culture (LTBMC), we have succeeded in introducing the neo gene into pluripotent stem cells.

Materials and Methods

Mice. Adult C57BL/6 (B6) mice (Ly5.2) were purchased from the Japan SLC Co Ltd (Shizuoka, Japan), and Ly5 congenic C57Bl/Ly5.1 (B6Ly5.1) mice were kindly donated by Dr H. Nakashima (Institute of Physical and Chemical Research, Tsukuba, Japan). All animals were bred and maintained in a laminar flow rack in our animal facility. All mice were 12 to 16 weeks old when used in experiments. Recipient animals were given acidified water starting several days before the irradiation.

Retroviral vector construction. The retroviral vector W5 (Fig 1A) was constructed by ligating the 3.0-kb NheI fragment from the retroviral vector W8, which contains the 5'LTR and the neo gene, into the NheI site of p574 (a kind gift of Dr D. Valerio, TNO Rijswijk, The Netherlands). Plasmid p574 contains a hybrid 3'LTR in which the enhancer of the Moloney murine leukemia virus (MoMuLV) was replaced by the enhancer (PyF101) of a mutant polyoma virus.

Generation of a high-titer producer cell line. Ecotropic (GP + E86) and amphotropic (GPenvAml2) packaging cell lines were kindly supplied by Dr A. Bank (Columbia University, New York, NY) and maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY), containing 10% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH), 15 µg/mL hygromycin (Sigma Chemical Co, St Louis, MO), 250 µg/mL xanthine (Sigma) and 25 µg/mL mycophenolic acid (Sigma). In the case of the amphotropic producer line, 200 µg/mL hygromycin B (Boehringer Mannheim, Mannheim, Germany) was included in the medium. These cells were maintained in humidified air containing 7.5% CO₂ at 37°C. Plasmid W5 (20 µg) was twice purified with a CsCl/ EtBr gradient and was transfected into GP + E86 cells using a modified calcium-phosphate technique. Fourteen days later, several...
G418 ( Gibco, final concentration 800 µg/mL)-resistant clones were isolated and virus-containing supernatant from the ecotropic clone with the highest titer was used to infect GPenvAml2 cells. Two days later, supernatant with amphotropic virus was added to a monolayer of GP + E86 cells. This infection cycle was repeated several times. After G418 selection, 12 ecotropic clones were titered on NIH/3T3 and the clone (M3-W5) with the highest titer (3 x 10^7 CFU/mL) was used in all experiments for gene transduction into hematopoietic stem cells. No replication-competent virus was detected in the supernatant of W5-infected NIH/3T3 cells.

Transduction of the retroviral vector into BM cells and transplantation of the cells into irradiated recipients. A Dexter LTBMC was initiated with the BM of four femurs from B6Ly5.1 mice per 6-well plate (Costar, Cambridge, MA) using a-minimal essential medium (a-MEM; Gibco) supplemented with 20% horse serum (Cell Culture Laboratories) and 10^-7 M hydrocortisone (Sigma) (see Fig IB). These cultures were maintained in humidified air containing 6% CO2 at 33°C. Four to 7 days later, a porous, collagen-coated Transwell (Costar, pore size 0.45 µm) was hung into each well, and virus-producing cells (5 x 10^4) were inoculated into the Transwell. Polybrene (Sigma, final concentration of 8 µg/mL) was added to the LTBMC. Upon reaching confluence after 4 days, a new Transwell with new producer cells was hung into each well. At the same time, half of the medium was replaced with fresh medium containing polybrene. This transduction cycle was continued for 5 to 19 days, after which G418 was added to the medium (final concentration of 600 µg/mL). The cells were collected 4 to 7 days later, and viable hematopoietic cells were counted by dye exclusion. The cells were thoroughly washed with modified Eagle’s medium (MEM) (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) and were transferred intravenously into irradiated B6 mice via a lateral tail vein. The recipients used for assaying splenic colony-forming cells (CFU-S) were irradiated with 9.7 GY of γ-rays from a 60Co source and 10^5 cells were injected. Mice used in long-term repopulating assays received a radiation dose of 9.2 GY and 2 x 10^6 cells. In one experiment, 1 to 5 x 10^6 BM cells from a primary recipient were injected into 9.2 GY-irradiated secondary recipients.

Southern blot analysis. High molecular-weight DNA was prepared from CFU-S colonies, spleen, thymus, lung, peripheral blood lymphocytes (PBL), and BM from reconstituted mice according to standard procedures. DNA was digested by Sac I, which cuts once in each proviral LTR, or BamHI, which recognizes an unique site in W5. Ten micrograms of digested DNA was electrophoresed through a 0.8% agarose gel and blotted onto a nylon membrane (Gene Screen plus, New England Nuclear Research Products, Boston, MA) using the alkaline-transfer method, followed by overnight hybridization in a solution containing 50% formamide at 42°C with a 32P-labeled 920-bp PstI fragment of the neo gene as a probe. After washing, the membranes were analyzed by an imaging analyzer (Fuji BAS2000, Fuji Photo Film Co, Ltd, Tokyo, Japan).

Polymerase chain reaction analysis. DNA from 6 µL PB was prepared with Instagenic matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s recommendations. Twenty microliters of each sample prepared this way or 1 µg of digested, genomic DNA obtained as described above was used as template in a PCR that permitted the amplification of a 450-bp sequence specific
for the W5 provirus with the following primers: sense, 5'-TGTAGGAG-
TTACCCCTACCTTAGG-3' (P5) and antisense, 5'-GCCACCT-
TGATATCCGTGCTTAAC-3' (M1). Control primers were: sense, 5'-
ACTGGGACATTAGATGCCTGG-3' and antisense, 5'-
AGCAGGATTCTCCACAGAG-3' that produce a 210-bp fragment in the mouse skeletal α-actin gene. Each 50-μL PCR reaction mixture consisted of the appropriate amount of DNA, 50 nmol/ 
L KCl, 10 mMol/L TRIS-HCl (pH 8.8), 1.5 mMol/L MgCl2, 1% Triton X-100, 1 μmol/L of each primer, 0.2 mMol/L of each dNTP and 1 U of Taq DNA polymerase. Routinely, 30 cycles of 1-minute denaturing at 94°C, 1-minute annealing at 56.5°C and 2-minute extension at 72°C were performed. Five microliters of each sample was electrophoresed through a 5% polyacrylamide gel and visualized by EtBr staining.

Antibodies and fluorescence-activated cell sorting (FACS) analysis. Supernatant of hybridoma clones RA3-82B (anti-B220), M1/70 (anti-Mac-1) and 8C5 (anti- Gr-1) were used for indirect staining with phycoerythrin (PE)-conjugated antirat IgG (Biomeda, Foster City, CA) as secondary antibody. Biotin-conjugated anti-Ly5.1 and fluorescein isothiocyanate (FITC)-conjugated anti-Ly5.2 were kindly provided by Drs H. Nakauchi and G. Spangrude (National Institutes of Health, Hamilton, MO), respectively. PE-conjugated anti-Thy-
1.2 was purchased from Caltag Laboratories (San Francisco, CA).

Depending upon the other staining reagents, FITC-conjugated streptavidin (Becton Dickinson, Mountain View, CA) or PE-conjugated streptavidin (Biomed) were used as secondary reagents. The basic methods for staining and analysis have been described elsewhere.77 PB (about 100 μL) was taken from the ocular plexus, and after pelleting red blood cells were lysed in 1 mL of 0.17 mol/L ammonium chloride to obtain PBLs. Cell suspensions of the spleen and thymus were made by disecting the organs with scissors and gentle mincing over stainless steel mesh. BM was obtained by flushing the femurs and a cell suspension was made by gentle pipetting.77

Analysis of neo gene expression in vitro colony-forming cells (CFU-C). Five months after transplantation, BM cells from irradiated recipient mice reconstituted with cells from a neo gene-transduced donor were obtained by flushing the femurs and a cell suspension was made by gentle pipetting.77

In all experiments 5 × 10^5 BM cells from B6Ly5.1 mice and the producer cell line M3-M5 were used. In systems 1, 2, and 3, a-MEM supplemented with 10% FCS was used. The culture conditions of system 4 are detailed in Material and Methods.

* In system 1, BM cells were transduced with 4 mL supernatant for 6 hours, 2 days before the addition of G418. IL-1α (50 U/mL), IL-3 (50 U/ 

| Table 1. Comparison of Various Culture and Transduction Systems for Their Effectiveness in Introducing the Neo Gene Into BM Cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Transduction System* | Viability (%) Without G418 (dl†) | Viability (%) With G418 (dl†) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1. Supernatant | 0 2 4 7 10 | 0 2 4 7 10 |
| 2. Coculture | 45 22 0 ND ND | 45 22 0 ND ND |
| 3. PA6neo Feeder + Transwell | ND ND ND ND ND | ND ND ND ND ND |
| 4. LTBM + Transwell Coculture | 99 98 98 98 98 |

† Viability was determined on the indicated days after the end of the transduction by trypan blue dye exclusion.

‡ Viability was determined on the indicated days after the end of the transduction when G418 was added to the transduction cultures. Untransduced BM cells had died within 4 days under all culture conditions.

§ Aggregates of PA6neo stroma cells and BM cells were repeatedly found during the transduction (usually 6 days after the initiation of the culture), making it impossible to determine the cell number. For this reason, cells were not subjected to G418 selection.

RESULTS

Comparison of various culture and transduction systems. We first compared various methods for gene transduction into BM cells, which included culturing BM cells with supernatant of the virus-producing cell line M3-M5, coculture of BM cells on irradiated M3-M5 cells, and coculture of BM cells with nonirradiated M3-M5 cells separated by a porous membrane (Transwell). The virus can pass unobstructedly through the membrane of the Transwell into the lower compartment (data not shown). The proportion of viable cells in these cultures with and without G418 selection is summarized in Table 1. With the supernatant or by cocultivation with irradiated M3-M5, it was hard to transduce the neo gene into BM cells. This may be because of the inability of these culture conditions to maintain BM cells for a period long enough to guarantee gene transduction. The use of a BM-derived stromal cell line PA6neo (a neo gene transduced clone of PA6 that kindly donated by Dr S.-I. Nishikawa, Kyoto University) as a feeder layer improved the cell viability dramatically. Moreover, the presence of proviral DNA could repeatedly be detected by PCR analysis of cells harvested on day 5 (data not shown). However, this culture system could not conveniently be used for the present purpose because, around 6 days after addition of BM cells, the PA6neo monolayer detached and formed a large aggregate with the BM cells to such an extent that it became hard to harvest the cells and subject them to G418 selection.

The last culture system we investigated was the coculture of virus-producing cells maintained in a Transwell hung into 35-mm culture dishes in Iscove's modified Dulbecco's medium (GIBCO), containing 10 mMol/L 2-ME, 10% fetal calf serum (FCS), 1% bovine serum albumin (fraction V), 10% pokeweed mitogen-stimulated lymphocyte-conditioned medium, and 0.8% methylcellulose. After 7 days of incubation, colonies consisting of more than 50 cells were scored on an inverted microscope.

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Fig 2. Photomicrographs of LTBMC after G418 selection (A) and PCR analysis of individual day-12 splenic colonies (B). In A, an LTBMC was established, and 4 days later, virus-producing cells (M3-W5) were added to a Transwell and transduction continued for 18 days followed by G418 (600 μg/mL) selection. Photographs were taken 5 days after the addition of G418. Hematopoiesis continues in the transduced culture (left panel), whereas in the untransduced culture (right panel), all cells have died. Original magnification × 20. In B, mice were transplanted with 10^7 cells obtained 7 days after G418 addition to the same LTBMC as in A. Splenic colonies were enumerated on day 12, and genomic DNA from eight randomly chosen splenic colonies from six recipient mice was analyzed. Correct amplification results in a 450-bp fragment that was detected in every sample. Molecular-weight markers are indicated on the left side of the figure. -, negative control (splenic DNA isolated from a control mouse); +, positive control (DNA isolated from the W5-transduced and G418-selected human T-cell line CEM containing 1 copy/cell).

an LTBMC. An LTBMC initiated 4 days earlier was cultured together with M3-W5 cells for 18 days. The cells were then selected with G418 (600 μg/mL). The cells transduced according to this protocol were completely resistant to G418 and hematopoiesis continued for several weeks in the presence of G418 (Table 1 and Fig 2A), suggesting that the neo gene was successfully introduced and expressed in virtually all the cells including hematopoietic progenitors present in the LTBMC. From these results, we considered it most advantageous to use the Dexter LTBMC and the Transwell coculture system for transducing a gene into BM cells.

Transduction of the neo gene into CFU-S. We next examined whether hematopoietic cell progenitors capable of forming spleen colonies were maintained in the transduced BM cells and contained the neo gene. Cells were harvested from several wells of the same LTBMC (4 days establishment, 18 days transduction, and 7 days G418 selection), and 10^5 hematopoietic cells were injected into irradiated recipient mice. Spleens were removed 12 days later and the colonies enumerated. The average colony number was 8 per spleen, which is about 20% of that seen in recipients given the same number of fresh BM cells, indicating that a substantial proportion of day-12 CFU-S was still present in the LTBMC 29 days after initiation of the culture. DNA was extracted from eight randomly chosen, individually excised colonies from six different spleens. The PCR assay showed the presence of the proviral DNA in every sample (Fig 2B). These results provided distinct evidence that the Dexter LTBMC and Transwell coculture system is highly effective in introducing a gene into early progenitors.

Successful transduction of the neo gene into stem cells with long-term reconstitution ability. Although the gene transduction protocol described in the preceding section was highly effective in introducing the neo gene into progenitors, it was impossible to reconstitute irradiated mice with such transduced cells (data not shown). It seemed a prerequisite to shorten the total culture period to warrant the presence of pluripotent stem cells in vitro. On the other hand, the transduction period should still be long enough to successfully introduce the gene into the stem cells. We tried four different transduction protocols, varying from a total of 5 to 18 days, and the transduced cells were transferred into irradiated recipients for investigating their reconstituting ability and gene expression.

All mice that received 2 × 10^6 transduced LTBMC cells that had been maintained in vitro for no longer than 15 days...
markers (Fig 3B) suggested that all myeloid and B cells and that most of the cells in these organs were donor derived. thymic T cells were replaced by donor-derived cells, whereas face antigen expression. The Ly5 expression profile showed all organs contained proviral DNA with the organs, PBL and lung, when analyzed by Southern blotting showed a complete reconstitution of the B lineage, whereas around 50% of the T cells were host derived (data not shown). However, in mice that had received LTBMC cells transduced only for 5 days (experiments 3 and 4), proviral DNA became undetectable in their PBLs 14 weeks after reconstitution.

Two primary recipient mice P1.1 and P2.2 (experiments 1 and 2 in Table 2) were killed 21 weeks after reconstitution and the BM, thymus, and spleen cells were assayed for surface antigen expression. The Ly5 expression profile showed that most of the cells in these organs were donor derived (Fig 3A), and detailed analysis of these cells with lineage markers (Fig 3B) suggested that all myeloid and B cells and thymic T cells were replaced by donor-derived cells, whereas a small proportion of host-type T cells remained in the spleen. DNA from the same organs and PBLs were analyzed by Southern blotting, and the results showed that proviral DNA was present in all hematopoietic organs of both mice (summarized in Table 3). Together these data indicate that a culture period of 15 to 18 days is necessary to effectively transduce a gene into stem cells that maintain their long-term repopulating ability.

Expression of the neo gene in progeny of transduced stem cells confers complete resistance to G418 selection. To test if the transduced neo gene is expressed in progenitors and their progeny, BM cells obtained from two mice (P1.1 and P2.2 in Table 3) that were killed 5 months after reconstitution were injected into 9.2 Gy-irradiated B6 recipients.

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Table 2. Protocols for Successful Transduction of the Neo Gene Into Stem Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Est (d)</th>
<th>Tr (d)</th>
<th>Sel (d)</th>
<th>No. of Recipients</th>
<th>Day of Analysis†</th>
<th>Flow Cytometry % Ly5.1†</th>
<th>PCR*/Analysed</th>
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<tr>
<td>1</td>
<td>4</td>
<td>10</td>
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<td>21</td>
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<td>63</td>
<td>81.99</td>
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<td>2</td>
<td>4</td>
<td>49</td>
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<td>0</td>
<td>5</td>
<td>42</td>
<td>79-82</td>
<td>5/5</td>
</tr>
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</table>

Abbreviations: Est, establishment; Tr, transduction; Sel, G418 selection (800 μg/mL).

* BM from B6Ly5.1 mice were transduced according to the indicated protocol and 2 × 10⁶ cells from the LTBMC were injected into 9.2 Gy-irradiated B6 recipients.

† PBL were taken on the indicated days from the ocular plexus.

‡ In experiment 1, two mice had died after 42 days leaving two mice available for analysis, whereas in the other experiments all mice survived.

§ The numbers indicate the range in which donor cells were detected in each group.

Two mice were killed for use in other experiments.

We also found that normal hematopoiesis occurred when the BM of recipient mouse P2.1, which was sacrificed 13 weeks after reconstitution, was cultured on a monolayer of the stromal cell line PA6neo in a Dexter LTBMC containing G418 (data not shown). RT-PCR and Northern blot assays confirmed the presence of a neo gene-specific message in the PB of these mice (data not shown). From these results it was concluded that the neo gene is functionally expressed in hematopoietic cells and their progenitors derived from transduced stem cells.

Generation of donor-derived cells in secondary recipients transplanted with BM cells from a primary recipient. We next investigated whether the stem cells transduced with the neo gene retain self-renewal capacity. In this experiment, a primary recipient mouse, P2.1, sacrificed 13 weeks after transplantation was used as the donor of BM cells. The FACS profiles of cells from lymphoid organs of this mouse indicated that virtually all cells in the BM and spleen were Ly5.1⁺. Thymocytes of Ly5.1⁺, though, in this mouse, the proportion of Ly5.1⁺ thymocytes was very small (Table 3). Although the reason why the thymic reconstitution in this mouse is exceptionally low is unclear, this low level can not be attributed to the inability of transduced stem cells to generate T cells, as the expression of the neo gene in progeny of transduced stem cells confers complete resistance to G418 selection.

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GENE TRANSDUCTION BY TRANSWELL COCULTURE

Fig 3. FACS analysis of cells in BM, spleen, and thymus obtained from recipient mouse P2.2. This mouse was killed 5 months after transplantation of 2 x 10^6 cells from a retrovirus-transduced LTBMC. Cells in the BM and thymus were almost completely donor derived, whereas host cells were still present in the spleen (A). Detailed analysis with lineage markers is shown in (B). Cells of the myeloid and B lineages as well as thymic T cells were completely donor derived, whereas about 50% of the T cells in the spleen were remaining host cells.

Hemopoietic cells including myeloid, B, and T cells in the secondary recipient mice are derived from the stem cells in the original donor mouse (Ly5.1). It was also found that hemopoietic cells in three of six recipients were highly positive for the transduced gene, whereas in the remaining three mice, the level was much lower.

To determine the number of transduced stem cells that contributed to the reconstitution of irradiated recipients, we investigated the site-specific integration pattern of DNA obtained from BM and spleens from two primary recipients (mouse P1.1 and P2.1) and a secondary recipient (mouse S2.5) that had received BM cells from mouse P2.1. The DNA was digested with BamHI, which cuts the provirus only once, followed by Southern blot analysis. As shown in Fig 4B, in the two primary recipients, three bands of different length, each representing one integration site, were detected in both BM and spleen. The sizes of these bands were different between the two primary recipients. These results strongly suggest that primary recipient P1.1 and P2.1 were reconstituted with at least three different stem cells each. An alternative interpretation is that both P1.1 and P2.1 were reconstituted by a single stem cell containing three proviruses each. However, this seems unlikely because the relative intensities vary between bands within one lane.

There is no difference in the integration pattern between mouse P2.1 and its secondary recipient S2.5 in both the BM and spleen, meaning that the three stem cells present in P2.1, which were derived from the transduced LTBMC underwent
self renewal. The data further showed that stem cells with a
minor contribution (lane 4, lowest band) to the hematopoiesis
in the primary recipient had retained enough capacity to take
part in reconstituting the secondary recipient.

**DISCUSSION**

In the present work, we have established an efficient and
safe protocol for introducing a gene into murine hematopoietic
stem cells using a recombinant retroviral vector contain-
ing the neo gene. Lots of efforts are being paid by re-
searchers in this field to optimize the method for safely
and efficiently introducing a gene into pluripotent stem cells
able of generating hematopoietic cells for a lifetime. These
include: (1) construction of a vector that can perma-
nently express the introduced gene in the stem cells as well
as in their progeny; (2) isolation of recombinant virus-pro-
ducing cells with high titers; (3) insurance of safety from erro-
neous production of replication-competent virus and from
contamination of virus-producing cells in the cell population
to be transplanted; (4) maintenance of stem cells in vitro as
well as stimulation of these stem cells to enter the cell cycle
to ensure the transduction of the gene.

As for the first point, we used the retroviral vector W5,
which is an N2-based vector,18 carrying the mutant polyoma
e enhancer PyF101 instead of the MoMuLV enhancer.20 Our
data confirmed that this enhancer is effective in transcribing
the gene in the progeny of transduced stem cells.14,21 A high
titer of the recombinant virus is one of the most crucial
factors required for a highly efficient transduction. It has
been shown that by coculturing ecotropic and amphotropic
producer cell lines to allow heterologous infection, the titer
can be raised by more than one order.20,31 We adopted the
protocol to repeatedly infect ecotropic and amphotropic
producer cell lines to allow heterologous infection, the titer
can be raised by more than one order.20,31 One of the ecotropic
clones (M3-W5) obtained this way produced the virus at a
very high titer (3 x 10^{7} CFU/mL), without the generation
of replication competent, wild-type virus.

One of the dangers in performing retroviral-mediated gene
transfer is the contamination of the transduced BM cells by
virus-producing cells. This might cause a serious problem
in recipients given BM cells that were cocultured on the
virus-producing packaging cells while keeping the advan-
tages of the coculture system.

Because retroviruses predominantly infect dividing cells,
stem cells have to be stimulated in the culture to enter the
cell cycle. In preliminary experiments, we had tried various
conditions for cultivation of BM cells to efficiently introduce
a gene into stem cells with the recombinant retrovirus. Be-
cause various cytokines have been shown to enhance the
transduction efficiency,15,32,33 we added various combinations
of cytokines including interleukin-1α (IL-1α), IL-3, IL-6,
and stem cell factor (SCF) to the BM cultures. However, in
our hands, the addition of cytokines was less effective in

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**Table 3. Analysis for Surface Phenotypes and Presence of the Transduced Gene in Various Organs of Recipient Mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day of Analysis</th>
<th>BM</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Southern Blot Analysis</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>M/G*</td>
<td>Total</td>
<td>M/G*</td>
<td>B*</td>
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<td>Secondary recipients</td>
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<td>S2.11</td>
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<td>16</td>
<td>ND</td>
<td>80</td>
<td>ND</td>
</tr>
<tr>
<td>S2.2</td>
<td>28</td>
<td>16</td>
<td>ND</td>
<td>80</td>
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<td>S2.6</td>
<td>50</td>
<td>35</td>
<td>30</td>
<td>70</td>
<td>78</td>
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</table>

Abbreviations: S, spleen; Th, thymus; M/G, macrophages and granulocytes; B, B lymphocytes; T, T lymphocytes; ND, not determined.

* Percentages indicated refer to percent donor (Ly5.1) cells that also express the indicated lineage marker.
† Naming: P and S indicate primary and secondary recipient mice, respectively, followed by the number of the experimental group as indicated in Table 2 and an individual number.
§ Mouse S2.1 to S2.4 were injected with 10^{6} BM cells, whereas mouse S2.5 to S2.6 received 5 x 10^{6} BM cells.

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**Table 4. Expression of the Neo Gene in CFU-C Derived From Transduced Stem Cells**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Colony No. -G418</th>
<th>Colony No. +G418</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>17.0 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>P1.11</td>
<td>36.0 ± 5</td>
<td>12.3 ± 2</td>
</tr>
<tr>
<td>P2.21</td>
<td>32.6 ± 3</td>
<td>20.6 ± 3</td>
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</tbody>
</table>

Data depicted are indicated as the mean ± SE from triplicate cultures.

* 5 x 10^{6} BM cells were plated per dish.
† 2 x 10^{6} BM cells were plated per dish.
GENE TRANSDUCTION BY TRANSWELL COCULTURE

transducing the gene into BM cells than the Dexter LTBMC (Table 1). The use of the BM-derived stromal cell line PA6neo as the feeder cells for BM culture improved the maintenance of BM cells, but we were unable to recover the hematopoietic cells because of the influence of the growth factors on the PA6neo cells. We found that the gene transduction occurred most efficiently when the Transwell coculture system was combined with a Dexter LTBMC, which does not require any addition of exogenous growth factors. This has been attributed to the production of physiologic amounts of various growth factors by the stromal layer present in the culture.34

We had previously noticed that the number of remaining stem cells 2 and 4 weeks after the initiation of the LTBMC was 10% and 1%, respectively, of that in fresh BM cells (Uozumi and Katsura, unpublished data, 1987). Similar results were also obtained by Weilbaecher et al.,35 who have shown that after 28 days in LTBMC, about 0.5% of the cells were still Thy-1+, Lin- , and Sca-1+. Thus we presumed that a period of 4 weeks was the upper limit to maintain the BM in vitro for gene transduction into stem cells. BM cells that were transduced for less than a week in LTBMC were able to reconstitute all recipients completely, but the neo gene-positive hematopoietic cells were observed transiently, indicating that 5 days of transduction is not enough to introduce a gene into stem cells. On the other hand, we succeeded in introducing a gene into the pluripotent stem cells by protocols of a total of 15 to 18 days of culture (Tables 2 and 3), whereas longer periods resulted in the loss of stem cells. These results suggest that the optimal gene transduction into the pluripotent stem cells occurred mainly in the second week of LTBMC.

Several other groups have also tried gene transduction in a Dexter LTBMC16,17 by inoculation of culture supernatant containing recombinant retrovirus. The disadvantage of culture supernatant is that the virus titer declines rapidly. As Nolta et al.17 reported, it is possible to introduce a gene into LTBMC cells with this method, but a large proportion of the cells were killed by G418 selection. This is in marked contrast with our experiments in that virtually no cell death was observed (Fig 2A). Thus, a coculture using a Transwell in which virus-producing cells are continuously proliferating is much more efficient in transducing a gene into BM cells than infection with virus-containing supernatant.

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The authors would like to thank Drs Y. Hirai (Otsuka Pharmaceutical Co, Ltd, Tokushima), T. Sudo (Toray Industries Inc, Kanagawa), and T. Hirano (Osaka University, Osaka) for supplying us with IL-1α, IL-3, and IL-6, respectively; Dr S.-I. Nishikawa (Kyoto University, Kyoto) for SCF and the PA6neo stromal cell line; Drs Jun-ichiro Gyotoku, Tatsuo Kina, Keisuke Teshigawara, and Kazuhiro Iwai for helpful discussions; Daiki Okude for excellent technical assistance; Yuko Takaoki for the preparation of this manuscript; and Ryuichi Komatsu for irradiation of mice.

REFERENCES

7. Lemischka IR, Rauler DH, Mulligan RC: Developmental po-

Fig 4. Southern blot analysis of DNA obtained from various organs of primary and secondary recipients. In A, one animal (P2.1) was killed 13 weeks after transplantation of $2 \times 10^6$ hematopoietic cells (transduction protocol as in experiment 2 of Table 1) into irradiated recipient mice. DNA was prepared from the BM (B), PBL (P), spleen (S), thymus (T), and lung (L), digested with Sac I and analyzed with a $32^P$-labeled neo probe. In all organs, proviral DNA was detected, the amount being correlated with their engraftment. In B, DNA was obtained from the spleen (lanes 1, 3, and 5) and BM (lanes 2, 4, and 6) of primary recipient mice P2.1 (lanes 3 and 4) and P1.1 (lanes 5 and 6), as well as secondary recipient mouse S2.5 (lanes 1 and 2) that received $5 \times 10^6$ transduced BM cells from the primary recipient P2.1. Digestion was performed with Bam HI to analyze differential proviral integration. Hybridization was done with the same probe as in A. All animals were reconstituted with at least three different stem cells.
tential and dynamic behavior of hematopoietic stem cells. Cell 45:917, 1986
Efficient retrovirus-mediated gene transduction into murine hematopoietic stem cells and long-lasting expression using a Transwell coculture system

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