GENETICS

Lentivirus Transduction of Bone Marrow Hemopoietic Precursor Cells with Lin⁻c-kit⁺ Phenotype *Ex Vivo* Using a Genetic Construct Containing Green Fluorescent Protein Gene

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We developed a technology of labeling bone marrow precursor cells with the Lin⁻c-kit⁺ phenotype in culture by green fluorescent protein gene using a lentivirus vector. The proposed system provides effective transduction of bone marrow precursor cells and high transgene expression level *in vitro* (27%). The integration of the transgene into the transduced cell genome *in vivo* was verified by the method of splenic colonies.

Key Words: *bone marrow precursor cells; lentivirus vectors; transduction; splenic colonies*

Recent progress in methodology of gene transfer into mammalian cells is due to the construction of vectors delivering genes to appropriate cells with research or therapeutic purposes [2-4]. Lentivirus vectors allowing gene introduction into non-dividing cells, *e.g.* stem cells, attract special attention [8]. The maximum efficiency of transduction of bone marrow hemopoietic cells with lentivirus constructs *in vitro* does not surpass 22% [7].

We created an effective method for gene transfer into undifferentiated bone marrow cells by lentivirus transduction *ex vivo*. Green fluorescent protein (GFP) gene serves as the reporter gene for labeling of bone marrow cells *in vitro*. Integration of a genetic construct containing GFP gene into bone marrow hemopoietic precursors (BMHP) *in vitro* was verified by the method of splenic colonies by the presence of vector sequence WPRE (woodchuck post-transcriptional regulatory element, a vector component) in genomic DNA of descendants from these cells.

MATERIALS AND METHODS

Male and female (CBA×C57Bl/6)F_1 mice (18-20 g) were used in the study.

Subpopulations of early BMHP with Lin⁻c-kit⁺ phenotype were obtained by two-staged magnetic separation of cells using MACS MicroBeads magnetic microcarriers from colloid iron in accordance with manufacturer's protocol (Miltenyi Biotec). During stage 1, cells not carrying linear markers on their surfaces were separated from the total bone

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marrow cell population. To this end, mouse bone marrow cell suspension was stained with a mixture of monoclonal antibodies to various differentiation markers (CD5, CD45R, CD11b, Ly6G, and Ter 119) conjugated to microcarriers. The suspension was passed through a LS Column (Miltenyi Biotec) packed with ferromagnetic material and exposed to magnetic field. During stage 2, cells carrying surface receptor for stem cell factor c-kit were isolated. To this end, cells isolated during stage 1 were stained with antibodies to c-kit conjugated with microcarriers and passed through the column. The obtained population of BMHP was analyzed on a FACSCalibur flow cytofluorometer (BD Immunocytometry Systems) after staining with specific antibodies to marker proteins of stem and low-differentiated cells c-kit, Sca-1, and CD34 (BD Biosciences).

Two variants of genetic constructs created on the basis of feline and human immunodeficiency viruses (FIV and HIV, respectively), were used for BMHP transduction. Both constructs contained *GFP* gene as the reporter gene under human cytomegalovirus promotor. In order to provide highly effective transduction of BMHP we compared the capacity FIV- and HIV-based genetic constructs to integrate into genome of these cells *in vitro*.

For obtaining HIV-based pseudoviral particles, two packing plasmids pVSV-G and pHIVpack (System Biosciences) were used in addition to the pLA-CG-H1 transporter plasmid (System Biosciences).

FIV particles were packed using pCopGXL-H1 transport plasmid (System Biosciences) and pVSV-G-C34N packing plasmid obtained by fusion of two plasmids (pVSV-G and pC34N; System Biosciences).

Pseudoviral particles were obtained using HEK293-T cells, which were co-transfected with plasmid preparations using lipofectamine. Pseudoviral particles were concentrated using polyethylene glycol. The titer of particles evaluated on H1299 cells varied from 1.5×10^6 to 1×10^7 infective particles/ml.

For transduction, the fresh-isolated cells with $Lin^-c\text{-}kit^+$ phenotype were seeded into 24-well plate in a growth medium containing 10% heat-inactivated serum; cytokines IL-3 and IL-6 and stem cell factor were added. After addition of pseudoviral particles and polybrene (final concentration 5 μg/ml), the cells were incubated at 37°C and 5% CO_2 for 24 h, washed, fresh medium was added, and culturing was continued for 1 week. The percent of cells expressing GFP was evaluated on a flow cytofluorometer.

The transduction efficiency *in vitro* was evaluated in BMHP culture with the $Lin^-c\text{-}kit^+$ phenotype. After transduction with pseudoviral particles with different titers, the cells were cultured for 1 week and analyzed in a flow cytofluorometer for evaluating the percentage of GFP-expressing cells. The cytotoxic effect of pseudoviral particles was visually evaluated during culturing.

In order to obtain splenic colonies, (CBA×C57Bl/6)F_1 females were irradiated in two equal doses (total dose 10 Gy) with a 3-h interval on an IPK ^{137}Cs apparatus. After irradiation recipient females were intravenously injected with bone marrow cell suspension from males of the same strain. The animals were then divided into 3 groups (1 experimental and two control) and donor cells were injected in quantities needed for obtaining discrete splenic colonies (Table 1). Individual colonies were isolated from the spleens on day 10 after irradiation. Genome DNA was isolated from cells of these colonies, and two marker fragments in DNA were detected by PCR. The presence of Y chromosome DNA fragment confirmed donor origin of the colonies, while detection of vector WPRE sequence fragment confirmed integration of the lentivirus construct into DNA of cells in the colonies.

The donor origin of colonies was confirmed using primers specific for male Y region: direct AAG TTG GCC CAG CAG AAT and reverse CTC CGA TGA GCC TGA TAT [5]. The WPRE vector sequence fragment was detected using commercial primers (System Biosciences). PCR products obtained by DNA amplification were separated by electrophoresis in agarose gel.

Some spleens with colonies were fixed in Bouin's fluid, dehydrated, and embedded in paraffin; paraffin sections were stained with hematological stain after Giemsa (Sigma), and examined under a microscope.

TABLE 1. Characteristics of Animal Groups

Group	Male donor cells	Number of injected cells	Incubation with pseudoviral particles (transduction)
1 (experimental; *n*=8)	$Lin^-c\text{-}kit^+$	2×10^3	+
2 (control; *n*=4)	$Lin^-c\text{-}kit^+$	2×10^3	—
3 (control; *n*=4)	Nonfractionated bone marrow cells	3×10^4	—

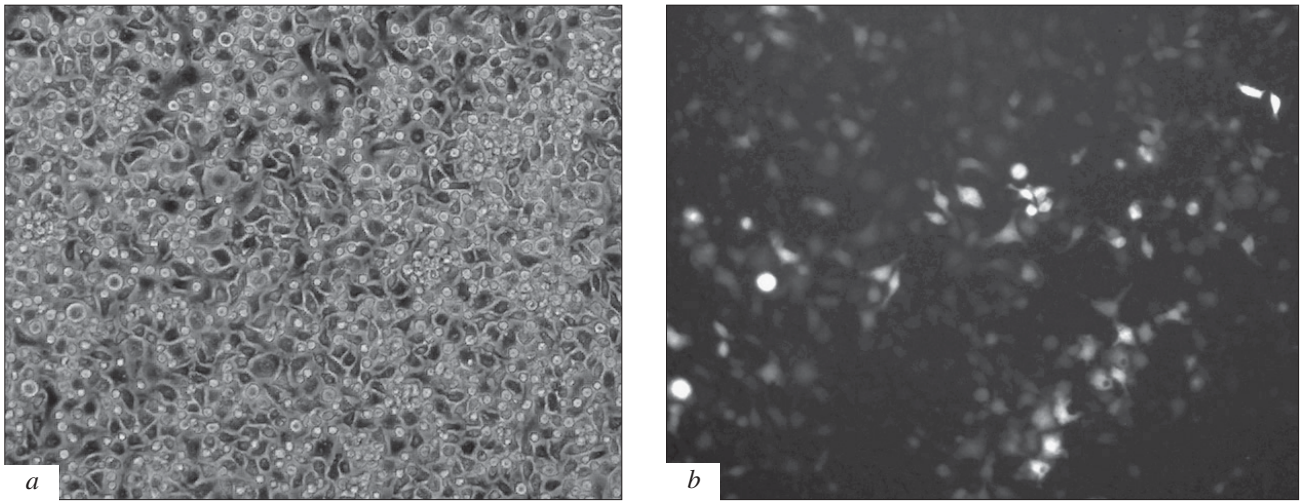

Fig. 1. Expression of GFP in culture formed by bone marrow cells with Lin⁻c-kit⁺ phenotype transduced with pseudoviral HIV particles on day 7 of culturing. *a*) phase contract, ×150; *b*) fluorescent microscopy, ×150.

RESULTS

After isolation of BMHP subpopulation with Lin⁻ c-kit⁺ phenotype by magnetic separation its cell composition was analyzed on a flow cytofluorometer. Polypotent BMHP with Lin⁻c-kit⁺Sca-1⁺ CD34⁺ phenotype (0.4-1.0%) were detected in this population; according to published data, these cells can differentiate in all hemopoietic directions [6].

After transduction of BMHP with pseudoviral FIV particles, the percentage of GFP-expressing cells was 8-27%. Virtually similar results were obtained by using HIV-based genetic construct; the percentage of cells expressing the transgene after its transduction was 2.5-27%.

Higher efficiency of BMHP transduction by FIV particles resulted in the appearance of abundant cell detritus, while conglomerations of maturing hemopoietic cells were rarely seen. Pseudoviral particles created on the basis of HIV virus with a titer of 1×10^7 infective particles/ml also produced a significant toxic effect on cells. HIV particles with a titer of 2×10^6 infective particles/ml were characterized by high transduction efficiency (27%) and produced minimum cytotoxic effect *in vitro*. The phase contrast and fluorescent microscopic picture of BMHP culture transduced with these particles almost did not differ from intact culture (Fig. 1, *a*, *b*).

After comparative analysis of the efficiency of two transduction systems, the preparation of HIV-based pseudoviral particles with a titer of 2×10^6/ml was chosen for further *in vivo* experiments.

Histological study of splenic sections from group 3 mice (Table 1) injected with nonfractionated bone marrow cells after irradiation showed all characteristic types of splenic colonies: erythroid (Fig. 2, *a*), myeloid, megakaryocytic, and mixed, which agreed with published data [1]. In groups 1 and 2, undifferentiated colonies (Fig. 2, *b*) consisting of morphologically unidentifiable lymphocyte-like and blast elements predominated among splenic colonies (Table 1). This result indicated that the subpopulation of BMHP with the Lin⁻c-kit⁺ phenotype was appreciably enriched with early hemopoietic precursors during its isolation.

Donor origin of splenic colonies was verified by the analysis of DNA isolated from cells of individual colonies for the presence of the male Y chromosome fragment; cell DNA from colonies in group 1 was also analyzed for the presence of WPRE sequence in the genome.

According to PCR analysis, DNA of each isolated colony in all groups of animals contained Y chromosome fragment (Fig. 3, *a*), that is, all studied splenic colonies in the recipient females originated from male donor BMHP.

Cell elements in 30% splenic colonies from recipients injected with transduced cells contained a fragment of WPRE vector DNA (Fig. 3, *b*) and hence, the genetic construct used in the study was integrated into the genome of descendants from donor BMHP.

Hence, the genetic construct created on the basis of HIV lentivirus is capable of stable and effective (27%) transduction of hemopoietic precursors with the Lin⁻c-kit⁺ phenotype *in vitro*. Experiments on *ex vivo* transgenesis showed that donor BMHP containing the transgene contained CFU capable of forming splenic colonies, including undifferentiated colonies, in lethally irradiated mice.

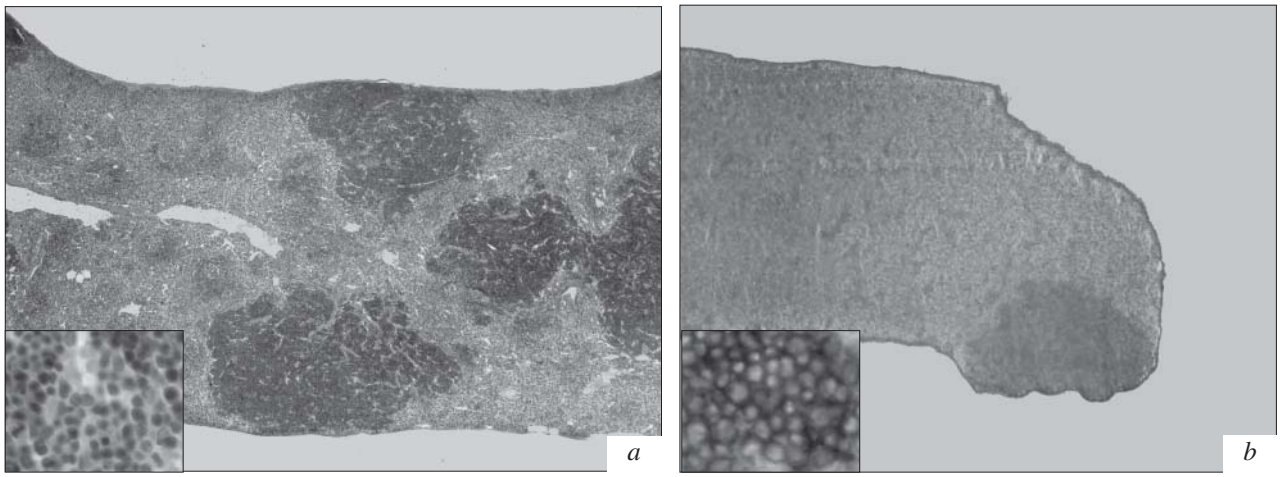

Fig. 2. Sections of spleens from recipient females 10 days after transplantation of donor bone marrow cells from males. Hematological staining after Giemsa. *a*) erythroid colonies in the spleen of a mouse from group 3 (nonfractionated bone marrow cells injected after irradiation; ×60). Insert: fragment of erythroid colony, ×600; *b*) undifferentiated colony in the spleen of a mouse from group 1 (bone marrow cells with $Lin^{-}c\text{-}kit^{+}$ phenotype injected after irradiation; ×60). Insert: fragment of a colony, ×600.

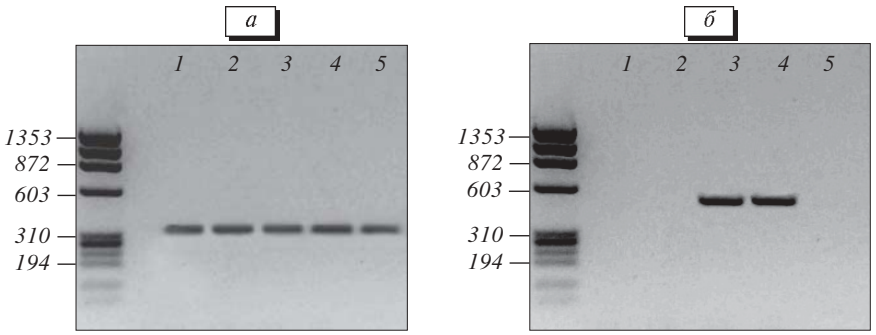

Fig. 3. Analysis of DNA from splenic colonies of group 1 mice for Y chromosome marker (*a*) and WPRE vector sequence (*b*). Electrophoresis of DNA amplification products in agarose gel. *1-5*: numbers of colonies.

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