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Production of pantropic retrovirus by ecotropic packaging cell line

Research Article

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Summary

The range of infectivity (tropism) of the packaged retrovirus is generally determined by env protein expressed in packaging cell line. Therefore, conversion of the env component of existing packaging cell line to another one can alter the tropism of packaged retrovirus. To achieve this, the VSV-G vector encoding the VSV-G env glycoprotein was cotransfected with the retroviral GFP expression vector into BOSC23 ecotropic packaging cell line which stably express the MMLV env protein. Unfortunately, the produced virus could only infect few human 293F cells, indicating the VSV-G can not totally replace the MMLV env as an env component of the packaged virus in BOSC23 cells. Therefore, RNAi technology was used to silence the MMLV *env* gene expression. The high-titer pantropic retrovirus were obtained by cotransfection of BOSC23 cells with MMLV *env* silencing vector, VSV-G vector, and retroviral GFP expression vector. When the pantropic retrovirus was applied to gene transfer of human umbilical cord blood $CD34^+$ cells, the enriched $CD34^+$ cells were successfully transduced. These results suggest that a method by which different tropic (pantropic) retrovirus can be packaged using an existing, tropism-fixed (ecotropic) packaging cell line has been successfully developed. This method is especially useful for researchers in developing countries who have one packaging cell line with tropism not compatible with the target cells and can not easily obtain the comercial packaging cell lines because there might not be enough suppliers to provide the commercial products of packaging cell lines in developing countries.

I. Introduction:

Packaging cell lines for retroviral gene transfer systems are important tool to produce infectious retroviral particles which can transfer gene of interest into target cells by infection. Packaging cells express necessary viral structural proteins including gag-pol and env (envelpoe) proteins which package the recombinant viral RNAs into infectious, replication-incompetent particles when retroviral expression constructs are transfected the packaging cells. The env protein packaged on the retroviral particles determines the host range (tropism) of the retrovirus. Therefore, the packaging cell lines can be classified into ecotropic, amphotropic, dualtropic, and pantropic packaging systems according to the expressed envelopes which bind to the receptors to enter host cells. Virus packaged in ecotropic packaging system can only infect mouse and rat cells because the virus recognizes a receptor mCAT1 found only on mouse and rat cells (Albritton et al., 1989). Similarly, virus packaged in amphotropic or dualtropic packaging system can infect mammalian cells expressing amphotropic receptor Ram-1 or mammalian cells expressing Ram-1 or GALV receptors (Weiss and Tailor, 1995), respectively. The pantropic retrovirus can infect both mammalian and non-mammalian cells because this virus is pseudotyped with the envelope glycoprotein from the vesicular stomatitis virus (VSV-G) which mediates viral entry of various cell types through LDL receptor (LDLR) (Finkelshtein et al., 2013).

Several kinds of packaging cell lines have been established and can be obtained commercially, e.g. NIH3T3-based packaging cells such as PT67, PA317 etc., HEK 293-based packaging cells such as Phoenix-E, Plat-E, GP2-293 etc. Nevertheless, if we can find a simple method by which different tropic retrovirus can be packaged using an existing, tropism-fixed packaging cell line at packaging stage, it will be useful because 1) various tropic packaging cell lines can be easily obtained in developed countries, but may not easily in developing countries because there might not be enough suppliers to provide the commercial products of packaging cell lines; 2) some researchers own a preferred packaging cell line which have been proved to be efficient for packaging retrovirus during long time of their bench experiments, however, the tropism of virus produced from the preferred packaging cell line might not be compatible with the target cells; 3) some of the amphotropic comercial packaging cell lines can not package high-titer retrovirus (Robbins et al., 1998; Friedmann and Yee, 1995, Miller, 1992).

How can different tropic retrovirus be packaged using an existing, tropism-fixed packaging cell line at packaging stage? This goal can be achieved by replacing the env protein in packaging cell line with a new env protein because the tropism of the retrovirus is determined by env protein. Based on this principle, we developed a strategy for conversion of the tropism of retrovirus produced by existing packaging cell line by substitution of VSV-G or other envelopes for the intrinsic envelope using RNAi technology. To prove the conversion is feasible, the BOSC23 cell line which is proved to be a good ecotropic packaging cell line was chosen to be converted to pantropic packaging cell line. Bosc23 is a retroviral packaging cell line stably expressing *gag-pol* and *env* of moloney murine leukemia virus (MMLV) (Pear et al., 1993). Our study demonstrates that BOSC23 can be successfully converted to pantropic packaging cell line which can produce high titer pantropic retrovirus.

II. Materials and Methods:

A. Vector-based gene silencing of *env* gene in BOSC23 using RNA interference

The human U6 Pol III promoter was amplified from pSilencer 2.1-U6 Hygro (Ambion, Austin, TX, USA) with pfu DNA polymerase (Tiangen, Beijing, China) and primer Set, 5'-ATAGTCGACGGGCAGGAAGAGGGCCTATTTC-3' and 5-AGTGGATCCCGCGTCCTTTCCA-3. The human H1 promoter was excised between EcoRI and BamHI from pSilencer 3.1-H1 Hygro (Ambion, Austin, TX, USA). The PCR fragment and the excised fragment were inserted into the corresponding restriction sites of pSP73 vector (Promega, Madison, WI, USA), one after the other. The resulting pSP73-H1/U6 vector contain opposing H1 and U6 promoters was used for vector-based gene silencing (**Figure 1A**).

The *env* gene in BOSC23 is derived from Moloney murine leukemia virus (MMLV) genome, therefore, we selected two sequences (AAGCCCTCCTCATCATGGGATT, AAGCAGTCGAGGGTCCTATTAT) that target MMLV *env* gene as the templates for producing the shRNAs (sense-loop-antisense). A DNA containing the two shRNA templates and poly T was designed, synthesized, and ligated into the BamHI site of the pSP73 H1/U6 vector to build the env silencing vector (**Figure 1A**). To verify the gene-silencing efficacy of the selected sequences that target MMLV *env* gene, we inserted a GFP expression cassette into the ecotropic *env* silencing vector (**Figure 1B**) so that the transfected cells (GFP positive cells) could be collected by FACS for quantitative polymerase chain reaction (q-PCR) analysis. A control siRNA vector which was constructed in our previous study (Wang et al., 2008b) was used as control. The control siRNA vector also contains the H1/U6 promoters which drive expression of siRNA that does not target the MMLV *env* gene. Results showed that the amount of mRNA of MMLV *env* gene in BOSC23 cells was silenced by 90%, suggesting the selected sequences that target MMLV *env* gene work well and can be used for the silencing of the ecotropic *env* gene (**Figure 1C**).

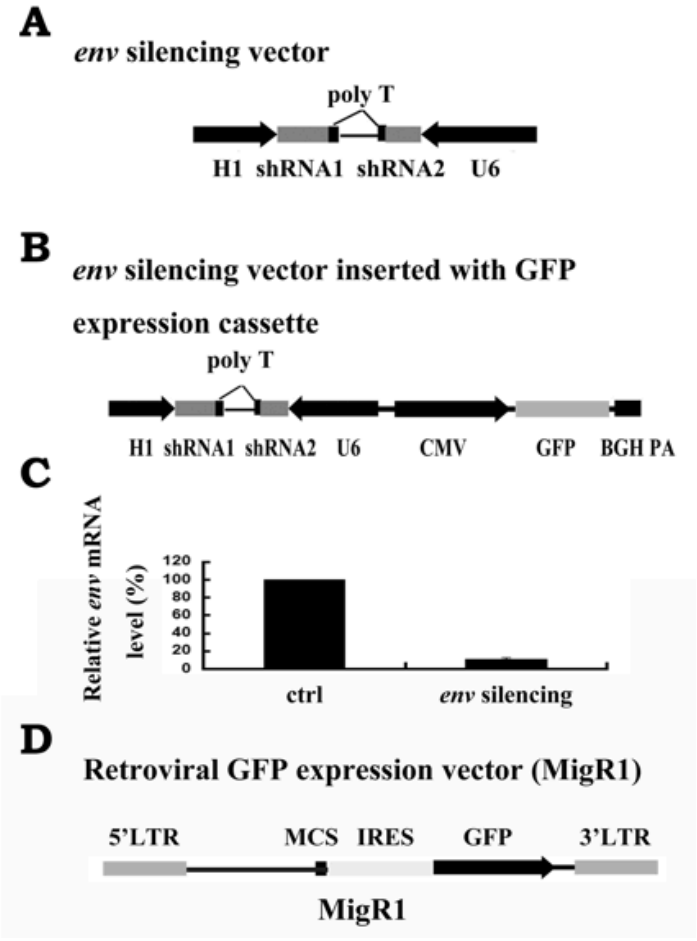

Figure 1. Schematic representation of the vectors and silencing efficiency for silencing vector. (A) Map of *env* silencing vector. (B) Map of the *env* silencing vector inserted with GFP expression cassette. (C) Silencing efficiency of the *env* silencing vector inserted with GFP expression cassette. BOSC23 cells were transfected with the *env* silencing vector inserted with GFP expression cassette and GFP^+ cells were sorted by FACS. A control siRNA vector was also transfected into BOSC23 cells and the GFP^+ cells were sorted. Total RNA was isolated from GFP^+ cells and silencing of MMLV *env* gene was analyzed by quantitative RT-PCR. Values are mean±SD for three independent experiments. (D) Map of retroviral GFP expression vector MigR1

B. Retroviral GFP expression vector and generation of retroviral supernatants

The retroviral vector MIGR1 was used as retroviral GFP expression vector which contains the murine stem cell virus (MSCV) promoter

and an internal ribosomal entry site element (IRES) followed by the green fluorescent protein (GFP) gene (**Figure 1D**). Retroviral supernatants were generated by transient transfection of BOSC23 cells with retroviral GFP expression vector and/or VSV-G vector and/or *env* silencing vector using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, 1.25×106 of BOSC23 cells per well were plated in six-well plate. Next day the subconfluent BOSC23 were transfected with 3μg of retroviral GFP expression vector and/or 1μg of VSV-G vector and/or 1μg of env silencing vector using lipofectamine 2000 according to the manual instruction. The retroviruses were collected 48 hours after transfection.

C. Titration on NIH 3T3 and 293F cells

Virus titers were determined by scoring GFP-positive target cells by FACS analysis (Ghani et al., 2007). Briefly, NIH-3T3 or 293F cells were inoculated at a density of 2×10^5 cells per well in six-well plates and cultured in 1.2 ml of medium (DMEM medium (Gibco, Grand Island, NY, USA), 10% fetal bovine serum (ES specific, Gibco, Auckland, NZ), 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 1% Pen/Strep (100x, Gibco, Grand Island, NY, USA)) overnight. The medium from each well was replaced with serial dilutions of virus supernatants in a 1.2 ml medium containing 8 μg/ml polybrene. After 48 h, cells were trypsinized and analyzed for GFP fluorescence by FACS. Infections resulting in 2–20% of GFP-positive cells were considered for titer calculation based on the linear range of the assay. The virus titer was calculated using following formula:

Virus titer=(total number of cells at the time of infection)×(proportion of GFP positive cells)×(dilution ratio)÷ (viral volume applied).

F. Real-time qPCR analysis

Total RNA was isolated 30 hours post-transfection using Trizol Reagent (Invitrogen). The cDNA was synthesized with MLV reverse transcriptase (TaKaRa Biotech, Dalian). Real-time PCR was performed on an ABI 7500 using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) following the manufacturer's protocol. The following primers were used: 5'-GAGAGATCCAACCGACACAAA-3' and 5'-AGCAGTAGTCCCTGTTCCTATT-3' for MMLV *env* gene and 5'-CTCTCTGCTCCTCCTGTTCGAC-3' and 5'-GCCCAATACGACCAAATCCG-3' for human GAPDH. Data were normalized to GADPH.

E. Isolation and transduction of CD34 Progenitor Cells

Umbilical cord blood samples from healthy donors were obtained from Hangzhou Zhengyin Bio-Tech Co., LTD. with approval from the local Ethical Committee. Mononuclear cells were separated by Ficoll–Hyaque density centrifugation. CD34+ cells were collected using miniMACS (Miltenyi Biotec, Gladbach, Germany) according to the instructions provided by the manufacturer. 1×105 Enriched CD34+ cells per well were cultured in 24-well plate in the medium [high glucose DMEM medium (Gibco, Grand Island, NY, USA), 15% fetal bovine serum (ES specific, Gibco, Auckland, NZ), 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 0.1 mM non-essential amino acid (Gibco, Grand Island, NY, USA), 1μM hydrocortisone, 0.1μM β-mercaptoethanol, 1% Pen/Strep (100x, Gibco, Grand Island, NY, USA)] supplemented with 100 ng/ml stem cell factor (rhSCF, from R&D Systems Inc.), 100 ng/mL Flt3 ligand (Flt3-L, from R&D Systems Inc.), 50 ng/mL thrombopoietin (Tpo, from R&D Systems Inc.). After 48 h, the cells were washed and resuspended in retroviral supernatant containing SCF, Flt3-L and Tpo, and transferred to a retronectin (Takara)-coated 24-well tissue culture plate. The suspension was centrifuged at 900g for 45 minutes at room temperature and then incubated for an additional 2 hours at 33°C. Retroviral supernatants were replaced with the fresh medium supplemented with SCF, Flt3-L, and Tpo. The retroviral infected cells were cultured for an additional 48 hours at 37°C (Chadwick et al., 2007; Berger et al., 2001; Barquinero et al., 2000). Cells were then detached from the plates using 0.05%

Trypsin-EDTA (Invitrogen) and GFP positive cells were detected by FACS (Wang et al., 2008a).

F. Statistical Analysis

Statistical analyses were performed using Student 2-tailed t test, and all data were expressed as mean or mean±S.D.

III. Results

A. Expression of ecotropic envelope in BOSC23 cells inhibited VSV-G pseudotyped retrovirus packaging

BOSC23 is an ecotropic HEK 293-based packaging cell line that stably expresses *gag-pol* and *env* of moloney murine leukemia virus (MMLV) and produces high-titer recombinant retrovirus 24 to 72 hours after transfection (Pear et al., 1993). It is easy to transfect and produce retrovirus with a tropism limited to mouse and rat cells which express receptor mCAT1 recognized by the env protein expressed by BOSC23. BOSC23 cells were transfected with a retroviral GFP expression vector (Migr1 vector) **(Figure 1D)** to produce retrovirus (the mean of titers of 3 lots of produced supernatants reached 3.43±0.31×106 cfu/ml determined by infection of mouse cell line NIH 3T3 with serial dilutions of retroviral supernatant). The virus produced could infect mouse cells (e.g. NIH-3T3, mouse ES cell line CCE, mouse bone marrow cells, etc.), but not human cells (e.g. K562 cells and 293F cells, etc.) (**Figure 2A and 2B**) as the human cells do not express the ecotropic receptor mCAT1. Because VSV-G is a pantropic envelope that mediates virus entering mammalian and non-mammalian cells, we cotransfected BOSC23 packaging cells with the retroviral GFP expression vector and the pantropic envelope vector (VSV-G vector) to produce retrovirus which we expected to be able to infect human cells. **Figure 2A and 2B** showed that the retrovirus could infect few 293F cells but could infect more NIH-3T3 cells, indicating BOSC23 cells produce few pantropic retrovirus particles and more ecotropic retrovirus particles. These results suggest the VSV-G can not totally replace the MMLV env as an env component of the virus.

B. BOSC23 cells with ecotropic envelope silenced can package pantropic retrovirus

We wondered whether silencing of the ecotropic envelope gene expression may facilitate generation of VSV-G pseudotyped retrovirus. To silence the expression of the ecotropic envelope gene, RNAi technology was applied. We constructed a ecotropic *env* silencing vector bearing two shRNA expression cassettes which included human H1 and U6 pol III promoter to drive expression of two shRNAs targeting the MMLV *env* gene (**Figure 1A**). To verify the gene-silencing efficacy of the selected sequences that target MMLV *env* gene, we inserted a GFP expression cassette into the ecotropic *env* silencing vector (**Figure 1B**) so that the transfected cells (GFP positive cells) could be collected by FACS for quantitative polymerase chain reaction (q-PCR) analysis. Analysis showed the amount of mRNA of MMLV *env* gene in BOSC23 cells was silenced by 90% (**Figure 1C**), indicating the selected sequences that target MMLV *env* gene work well and can be used for the silencing of the ecotropic *env* gene.

Next, the *env* silencing vector was cotransfected into BOSC23 cells with VSV-G vector and retroviral GFP expression vector, 48 hours later we collected the supernatant containing retrovirus particles (the mean of titers of 3 lots of supernatants was 3.1±0.28×106 cfu/ml determined by infection of 293F cells with serial dilutions of retroviral supernatant). 293F and NIH-3T3 cells were infected with the retroviral supernatants and GFP expression was detected by FACS to determine the transduction efficiency. The transduction efficiency of 293F cells using

retroviral supernatant produced from BOSC23 cells cotransfected with *env* silencing vector, VSV-G vector, and retroviral GFP expression vector was significantly increased compared to that using retroviral supernatant produced from BOSC23 cells cotransfected with both VSV-G vector and retroviral GFP expression vector (**Figure 2A**). The efficiencies of transduction of NIH3T3 using above 3 kinds of retroviruses were similar (**Figure 2B**). These results suggest that when the ecotropic envelope protein expression is silenced, the retroviral RNA can be effectively packaged into pantropic VSV-G pseudotyped retrovirus, which is consistent with the observations that the envelope component of one retrovirus can be substituted by the envelope of another virus to produce ''pseudotyped'' virus particles that exhibit a cell tropism specified by the new env component (Sharma et al., 1997; Yee et al., 1994; Dong et al., 1992).

C. Application of the pantropic retrovirus

To examine whether the pantropic retrovirus packaged by BOSC23 cells using methods above could be applied to infection of primary human cells, we infected human umbilical cord blood CD34+ cells which is an attractive target for gene therapy.

Enriched CD34+ cells were infected with the produced pantropic retroviral supernatant using retronectin-coated plate. The transduction efficiency was 44.94±8.7% (Value is mean±SD for three independent experiments; **Figure 2C**), suggesting that the pantropic retrovirus packaged by BOSC23 cells using methods above can efficiently transduce the human umbilical cord blood CD34+ cells.

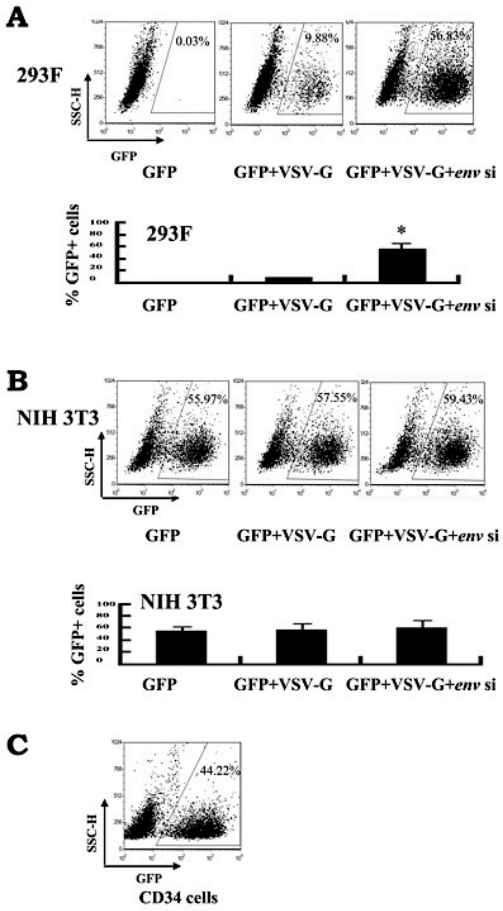

Figure 2. FACS analysis of retroviral transduction efficiency of 293F, NIH-3T3, or CD34$^+$ cells. (A) FACS analysis of transduction efficiency of 293F cells. 293F cells were transduced with retrovirus produced from BOSC23 cells transfected with only retroviral GFP expression vector (GFP), with retrovirus produced from BOSC23 cells cotransfected with both retroviral GFP expression vector and VSV-G vector (GFP+VSV-G), with retrovirus produced from BOSC23 cells cotransfected with retroviral GFP expression vector, VSV-G vector, and *env* silencing vector (GFP+VSV-G+*env* si). Representative dot plots (upper panel) together with mean±SD (*p<0.001 compared with GFP+VSV-G) for three replicates (lower panel) are shown. (B) FACS analysis of transduction efficiency of NIH-3T3 cells. NIH-3T3 cells were transduced with retrovirus produced from BOSC23 cells transfected with only retroviral GFP expression vector (GFP), with retrovirus produced from BOSC23 cells cotransfected with both retroviral GFP expression vector and VSV-G vector (GFP+VSV-G), with retrovirus produced from BOSC23 cells cotransfected with retroviral GFP expression vector, VSV-G vector, and *env* silencing vector (GFP+VSV-G+*env* si). Representative dot plots (upper panel) together with mean±SD for three replicates (lower panel) are shown. (C) FACS analysis of transduction efficiency of CD34$^+$ cells . The enriched and cytokine-stimulated CD34$^+$ cells were transduced with the pantropic retrovirus produced from BOSC23 cells cotransfected with retroviral GFP expression vector, VSV-G vector, and *env* silencing vector. One representative dot plot is shown.

IV. Discussion

In this study we have demonstrated that a simple and effective method for conversion of the env component of existing packaging cell line to another one is established. Using this method, pantropic retrovirus with high titer were simply produced from the ecotropic packaging cell line BOSC23 and this retrovirus could be successfully applied to transduction of human $CD34^+$ cells which is an attractive target for gene therapy. We are not aware of previous related methods that have the advantages that different tropic retrovirus can be packaged using an existing, tropism-fixed packaging cell line at packaging stage. To date, some efforts were exerted (Koch et al., 2006; Gollan and Green, 2002), such as transient expression of murine retrovirus receptor mCAT1 on human ES cells for transduction of human ES cells with ecotropic retrovirus (Koch et al., 2006), or insertion of short and nondisruptive peptide ligands into envelope to redirect retroviral tropism (Gollan and Green, 2002). However, these efforts obviously focus on either change of host cell tropism or creation of a new packaging cell line, which are different from ours.

The ecotropic packaging cell line BOSC23 have been proved to be efficient for packaging of high-titer ecotropic retrovirus in our lab, therefore, we wondered whether it could be used for packaging of high-titer pantropic retrovirus. When we just simply introduced the VSV-G vector and retroviral GFP expression vector into BOSC23 cells, the titer of resulting retrovirus determined by infection of 293F cells was very low, indicating the ecotropic envelope protein (MMLV env) interfers with the packaging of pantropic retrovirus. The interference of the ecotropic envelope protein in packaging of pantropic retrovirus was validated by the experimental evidence that silencing of the expression of the ecotropic envelope protein (MMLV env) in BOSC23 cells successfully facilitated production of high-titer pantropic retrovirus.

Although there are commercially and academically available packaging cell lines that just stably express the *gag* and *pol* genes (without *env* gene) and can produce pantropic retrovirus without the need to suppress existing env production (e.g. ANJOU65 and GP2-293 packaging cell lines), or co-transfection of three other plasmids, namely a gag/pol packaging construct, a VSV-G expression plasmid and a retroviral vector, into a parental cell line (e.g. HEK293 or NIH3T3 cells) would result in the generation of pantropic particles, our method is still useful for some researchers, , especially for researchers in developing countries. In developing countries, it may not be easy for researchers, who have one packaging cell line with tropism not compatilbe with the target cells, to comercially obtain the tropism-appropriate packaging cell lines because there might not be enough suppliers to provide the commercial products of packaging cell lines. It may also not be easy to obtain plasmids from other working groups in developing countries. Our method may also be useful for researchers who own a preferred, high-titer retrovirus-producing packaging cell line with tropism not compatible with the target cells. For these researchers, conversion of the env component of existing packaging cell line to another one which is compatible with the target cells may be their best option.

In our study, the produced pantropic retroviral supernatant should contain a small amount of ecotropic retroviral particles because the env protein expression could not be totally inhibited by RNAi. Therefore, this retroviral supernatant might not be suitable for clinical gene therapeutic applications. Nevertheless, it is suitable to be used for delivery of genes into human cells in lab research applications.

The method described here is for transient transfection because the VSV-G protein is toxic to the cells. However, it can still be used for generate stable retrovirus-producing cell lines if nontoxic amphotropic or GALV env expression vector is used to pseudotype amphotropic retrovirus.

In conclusion, we successfully developed a method by which different tropic (pantropic) retrovirus can be packaged using an existing, tropism-fixed (ecotropic) packaging cell line at packaging stage.

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Conflict Of Interest

The authors declare no conflict of interest.

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