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Retroviral vectors for homologous recombination provide efficient cloning and expression in mammalian cells

Eiji Kobayashi, Hiroyuki Kishi *, Tatsuhiko Ozawa, Masae Horii [1], Hiroshi Hamana, Terumi Nagai, Atsushi Muraguchi

Department of Immunology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

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ABSTRACT

Homologous recombination technologies enable high-throughput cloning and the seamless insertion of any DNA fragment into expression vectors. Additionally, retroviral vectors offer a fast and efficient method for transducing and expressing genes in mammalian cells, including lymphocytes. However, homologous recombination cannot be used to insert DNA fragments into retroviral vectors; retroviral vectors contain two homologous regions, the 5′- and 3′-long terminal repeats, between which homologous recombination occurs preferentially. In this study, we have modified a retroviral vector to enable the cloning of DNA fragments through homologous recombination. To this end, we inserted a bacterial selection marker in a region adjacent to the gene insertion site. We used the modified retroviral vector and homologous recombination to clone T-cell receptors (TCRs) from single Epstein Barr virus-specific human T cells in a high-throughput and comprehensive manner and to efficiently evaluate their function by transducing the TCRs into a murine T-cell line through retroviral infection. In conclusion, the modified retroviral vectors, in combination with the homologous recombination method, are powerful tools for the high-throughput cloning of cDNAs and their efficient functional analysis.

1. Introduction

A variety of gene cloning strategies have been established and utilized for the cloning of cDNAs to analyze the biological functions of their protein products. These cloning strategies can be divided into two major categories: the viral and non-viral methods [1,2]. In the viral methods of transducing cDNAs into mammalian cells, retroviral, adenoviral, adeno-associated viral and herpes simplex viral vectors are often used [3]. Retroviral vectors have an advantage in the transduction of cDNAs over the non-viral conventional methods, as they efficiently and stably integrate the cDNAs into the genome of the cells. The integrated cDNA is stably expressed, and the functions of its protein products can then be analyzed. A large repertoire of well-characterized-retroviral vectors has been developed during the last decade and used for the treatment of human diseases [4,5].

Ligation-dependent cloning is most commonly employed to introduce DNA fragments into vectors [6,7]. However, ligation-dependent cloning often requires multiple rounds of enzyme treatments and purification of both the inserts and vectors. Furthermore, the limited number of appropriate restriction enzyme sites for the insertion of the DNA into the vector DNA limits the flexibility of the vectors in constructing recombinant molecules. These processes hamper high-throughput cloning. In contrast, homologous recombination technologies enable the seamless insertion of any DNA fragment into any desired position [8,9]. Although the homologous recombination method offers several advantages for high-throughput cloning, it cannot be applied to vectors that contain homologous sequences within them, as homologous recombination preferentially occurs between those sequences, and the DNA of interest cannot be inserted [10]. Therefore, the homologous recombination method cannot be applied to retroviral vectors because they contain two homologous long terminal repeats (LTR).

In this study, we designed a modified retroviral vector into which PCR-amplified DNA fragments can be selectively and efficiently inserted using homologous recombination. We cloned TCR cDNA prepared from a large number of human single T cells into the retroviral vector using homologous recombination and analyzed the functions of these cDNAs in a high-throughput manner.

* Corresponding author. Fax: +81 76 434 5019.
E-mail address: immkishi@med.u-toyama.ac.jp (H. Kishi).
[1] Present address: Department of Environmental and Preventive Medicine, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan.

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2. Materials and methods

2.1. Vector construction

The pMX-CH vector was constructed from the pMX vector (kindly provided by Dr. Kitamura, University of Tokyo) [2] by inserting the human IgG Cγ1 gene and the *Bacillus subtilis sacB* gene encoding levansucrase. In *Escherichia coli*, the expression of *sacB* in the presence of sucrose is lethal [11]. Briefly, the pCMVtag1 vector (Agilent technology) was modified at the multiple cloning site (MCS), and the *sacB* gene was inserted into the modified MCS (pCMVtag1-SacB) [12]. Constant region of human IgG cDNA was then inserted into the pCMVtag1-SacB vector. The DNA fragment containing the *sacB* and *Cγ* genes was removed from the pCMVtag1-CH vector by digestion with BamHI and NotI and was inserted into the pMX vector to construct the pMX-CH vector. To prepare the pMX-KmAmpR-TCR-Cα or Cβ vector, the ampicillin resistance gene (*AmpR*) from the pMX vector was first substituted with the kanamycin resistance gene (*KmR*) from the pCMVtag1 vector (Promega) (pMX-Km). The *AmpR* gene was amplified from pBR322 (Promega) by PCR and was then inserted into the BstXI site of the pMX-Km vector (pMX-KmAmpR). To prepare the DNA fragment containing the *sacB* gene and the constant region of TCRα (Cα) or TCRβ (Cβ), the Cα and Cβ cDNAs were amplified from human T cells by RT-PCR and were inserted into the NruI and NotI sites (pCMVtag1-Cα and pCMVtag1-Cβ, respectively) [13]. DNA fragments containing the *sacB-Cα* and *sacB-Cβ* genes were prepared from the pCMVtag1-Cα and pCMVtag1-Cβ plasmids and were inserted into the BamHI and NotI sites of the pMX-KmAmpR vector to construct the pMX-KmAmpR-TCR-Cα and Cβ vectors, respectively. Expression vectors for the human CD3γ, CD3δ, CD3ε, ζ and CD8 cDNAs were constructed by linking these cDNAs with the 2A sequence from the foot-and-mouth disease virus, as previously described (2A-hCD3-hCD8 vector) [14]. All human CD3γ, CD3δ, CD3ε, ζ and CD8 cDNAs were purchased from Origene.

2.2. Preparation of human peripheral blood lymphocytes and HLA typing

Human experiments were performed with the approval of the Ethical Committee at the University of Toyama. Informed consent was obtained from all subjects. Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood samples by density gradient centrifugation using Ficoll-Hypaque (Immuno-Biological Laboratories). Screening for HLA-A24 haplotype positivity was performed by staining the PBLs with FITC-conjugated anti-HLA-A24 (MBL). The cells were then analyzed by flow cytometry.

2.3. Cell lines

RPMI 1640 and DMEM media (Wako Pure Chemical) were supplemented with 10% fetal bovine serum (Biowest), 100 μg/ml streptomycin and 100 U/ml penicillin. Human CD8 (hCD8)-expressing TG40 cells [15] were kindly provided by Dr. Ueno (Kumamoto University) with permission from Dr. Saito (Riken) and were maintained in RPMI 1640 medium. The retroviral packaging cell lines, PLAT-E and Phoenix-A, were kindly provided by Dr. Kitamura (University of Tokyo) and by Dr. G. Nolan (Stanford University), respectively, and were maintained in DMEM medium. The 2A-hCD3-hCD8-293T cells were established by transducing the 2A-hCD3-hCD8 vector into HEK293T cells (purchased from ATCC).

2.4. Antibody and MHC tetramer staining

EBV-specific T cells were stained with PE-conjugated HLA-A*2402/peptide tetramers. The amino acid sequences of the HLA-A*2402-restricted EBV peptides are as follows: TYPVLEEMF (BRLF-1 198-206), DYNFVKQLF (BMLF-1 320-328), IYVLVMLVL (LMP2 222-230), RYSIFFDYM (EBNA3A 246-254) and TYSAGIVQI (EBNA3B 217-225). All tetramers were purchased from MBL. The FITC-conjugated anti-human CD8 antibody (MBL), APC-conjugated anti-human CD3ε antibody (TONBO Biosciences), biotin-conjugated anti-murine CD3ε antibody (eBioscience) and APC-conjugated streptavidin (eBioscience) were used for flow cytometry.

2.5. Single-cell RT-PCR

Single-cell RT-PCR of the *TCR* gene from human T cells was performed using the single-cell 5′-RACE method as previously described [16]. The PCR products were analyzed by either direct sequencing or sequencing after subcloning into an expression vector. The TCR repertoire was analyzed using the IMGT/V-Quest tool (http://www.imgt.org/) [17].

2.6. Homologous recombination reaction in the competent cells

Homologous recombination in the competent cells was performed according to the instructions of the manufacturer (GENE BRIDGES). Briefly, competent cells harboring the pRedET expression plasmid were mixed with the pMX-KmAmpR-TCR-Cα or Cβ vector that was linearized by NruI digestion to remove *sacB* gene and the amplified cDNAs encoding TCR-Vα or Vβ, respectively. After transformation of the competent cells with the mixtures, the competent cells were streaked onto LB agar plates containing 4% sucrose and 100 μg/ml ampicillin. After incubation at 37 °C overnight, ampicillin-resistant *E. coli* cells were expanded. Plasmid DNAs were purified, digested with BamHI and NotI, and separated by agarose gel electrophoresis.

2.7. Retroviral transfection

The cDNAs encoding the TCR α or β chain were independently inserted into the pMX-KmAmpR-TCR-Cα or pMX-KmAmpR-TCR-Cβ plasmids, which were then transfected into the retroviral packaging cell line PLAT-E using FuGENE 6 (Roche). The culture supernatant from the transfected PLAT-E cells was collected 72 h after transfection and was added to hCD8-TG40 cells along with polybrene (Sigma–Aldrich). The transfection was monitored by the cell surface expression of murine CD3ε. For the retroviral transduction of the TCR cDNAs into 2A-hCD3-hCD8 293T cells, the recombinant retroviruses were produced using the packaging cell line Phoenix-A and infected with 2A-hCD3-hCD8 293T cells as described above.

3. Results

3.1. Undesirable homologous recombination between the 5′- and 3′-LTRs in the pMX retroviral vector

To insert cDNA fragments into the retroviral vector using homologous recombination, we first constructed a retroviral vector with the human IgG constant region (Cγ) and the *sacB* gene (Fig. 1A). For examining the cDNA insertion into the vector using homologous recombination, we amplified the V_H genes encoding human IgG V_H from single human B cells using the 5′-RACE method. The amplified V_H genes were mixed with the linearized vectors and introduced into the competent cells containing the pRED-ET plasmid [9], which induces homologous recombination in these

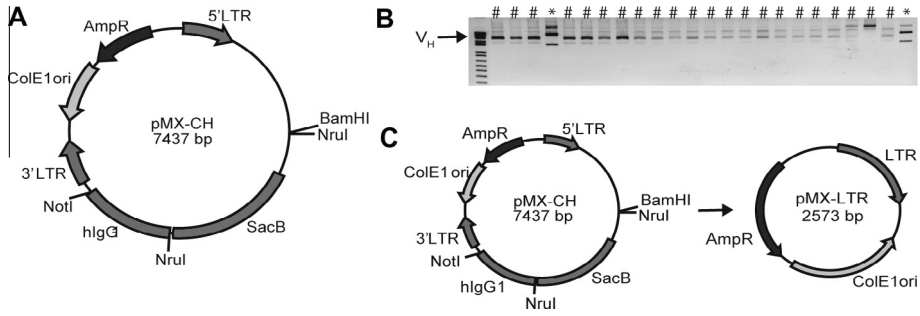

Fig. 1. Undesirable homologous recombination in the conventional retroviral pMX vector. (A) Map of the pMX-CH vector. The pMX-CH vector contains the *sacB* gene and the gene encoding the constant region of human IgG (B) Analysis of the homologous recombination products generated by the pMX-CH vectors. The PCR-amplified variable region of the antibody heavy chain (V_H) from human single B cells was inserted into the pMX-CH vector using homologous recombination. Plasmid DNA was prepared from the transformed colonies, digested with the BamHI and NotI restriction enzymes, and analyzed by agarose gel electrophoresis. The black arrow shows the target V_H gene. The asterisks indicate the pMX-CH vectors with the properly inserted V_H gene. "#" indicates vectors resulting from unsuccessful homologous recombination. (C) Scheme of unsuccessful homologous recombination in the pMX-CH vector. See the sequence analysis in Supplementary Fig. 1. The pMX-LTR results in the pMX- CH vector following unsuccessful homologous recombination.

cells [12]. The transformed cells were cultured on LB plates including ampicillin. Plasmid DNA from the ampicillin-resistant colonies was prepared, and the insertion of the V_H gene was examined by the restriction enzyme digestion. As shown in Fig. 1B, only 8% of the ampicillin-resistant cells contained pMX-Cγ vectors into which the V_H genes were properly inserted, whereas the rest of the colonies did not. Sequence analysis of the plasmid DNA that did not contain the V_H gene revealed that it only contained a single *LTR*, and the sequence between the 5′-LTR and 3′-LTR was deleted (Supplementary Fig. 1). These results demonstrated that homologous recombination preferentially occurred between the 5′- and 3′-LTRs of the pMX-C_H vectors, as illustrated in Fig. 1C.

3.2. Modification of the pMX retroviral vector for the insertion of DNA fragments using homologous recombination

In conventional retroviral vectors, *E. coli* selection markers, such as AmpR or KmR genes, are located adjacent to the *Col-E1* replication origin (*ori*). Consequently, homologous recombination between the 5′- and 3′-LTRs in these retroviral vectors resulted in the production of the vector shown in Supplementary Fig. 1. We assumed that the frequency of homologous recombination between the 5′- and 3′-LTRs was much higher than that of homologous recombination between the retroviral vectors and the target DNA fragments when the recombination reaction was conducted between linearized retroviral vectors and the target DNA fragments because the distance between the 5′- and 3′-LTRs is stochastically much shorter than the distance between the vector and the target DNA fragment.

To specifically select the *E. coli* cells that contained the retroviral vector into which the target DNA was properly inserted, we constructed a retroviral vector in which the *E. coli* selection marker gene was located adjacent to the insertion site of the target DNA fragment by homologous recombination (Fig. 2A). When we inserted the target DNA into the modified retroviral vector using homologous recombination, three possible products may be produced. When homologous recombination occurs between the 5′-LTR and 3′-LTR, the products (i) and (ii) in Fig. 2A will be produced. When homologous recombination properly occurs between the vector and the target DNA fragment, then product (iii) will be produced. Product (i) contains the *ori* but lacks the *AmpR* gene, and the *E. coli* harboring this product fail to grow in medium containing ampicillin. Product (ii) contains the *AmpR* gene but lacks the *ori*, and the *E. coli* harboring this product fail to grow in the medium because the plasmid DNA cannot be replicated. Only the *E. coli* harboring product (iii) can grow in the medium containing ampicillin, as this product contains both the *ori* and the *AmpR* gene. Therefore, we may obtain only the proper product (iii) through homologous recombination. According to this hypothesis, we inserted the *AmpR* gene into the pMX-Km vector, in which the ampicillin resistance gene was replaced with the kanamycin resistance gene at a site between the 3′-LTR and the multiple cloning site (pMX-KmAmpR) (Fig. 2B).

3.3. High-throughput cloning using the modified retroviral vectors with homologous recombination

To confirm the applicability of the vector for cloning by homologous recombination, we inserted the *sacB* gene and the constant region of either human TCRα or TCRβ into the vector (pMX-KmAmpR-TCR α/β) (Fig. 3A). We amplified the V region cDNA of either the TCRα or TCRβ gene from single human $CD8^+$ T cells using the 5′-RACE method (Fig. 3B) and inserted them into the *Nru*I-linearized pMX-KmAmpR-TCR α/β vector using homologous recombination, as described in Section 2. We picked the resultant ampicillin resistant colonies, grew them in medium containing ampicillin and prepared the plasmid DNA. We then digested the plasmid DNA with restriction enzymes to examine the proper insertion of the TCR Vα (TRAV) or Vβ (TRBV) cDNA. Of the ampicillin resistant cells, 100% and 81.3% of the competent cells contained the vectors in which the TRAV- and TRBV-cDNAs were properly inserted, respectively (Fig. 3C). These results showed that the pMX-KmAmpR-TCR α/β vectors enabled the efficient cloning of the TCR gene using homologous recombination.

3.4. Usefulness of the pMX-KmAmpR-TCR α/β vectors for the functional evaluation of the cloned TCR cDNAs in a high-throughput and comprehensive manner

To evaluate the usefulness of the vector for high-throughput cloning, we cloned the TCR genes from Epstein-Barr virus (EBV)-specific $CD8^+$ T cells derived from HLA-$A24^+$ latent healthy donors. We detected the EBV-specific $CD8^+$ T cells using a HLA-A∗2402-restricted tetramer mixture of the five EB virus epitopes (BRLF-1, BMLF-1, LMP2, EBNA3A and EBNA3B) and then single-cell sorted the tetramer-positive cells from 10 donors whose frequencies of EBV tetramer-positive cells were more than 0.06% of the $CD8^+$ T-cell population. We amplified 444 pairs of TRAV and TRBV cDNAs from the sorted single cells using the 5′-RACE method and cloned the TCR α and β cDNAs into the pMX-KmAmpR-Cα or pMX-KmAmpR-Cβ vectors, respectively. Next, to determine the antigen-specificity of the cloned TCRs, we retrovirally transferred the

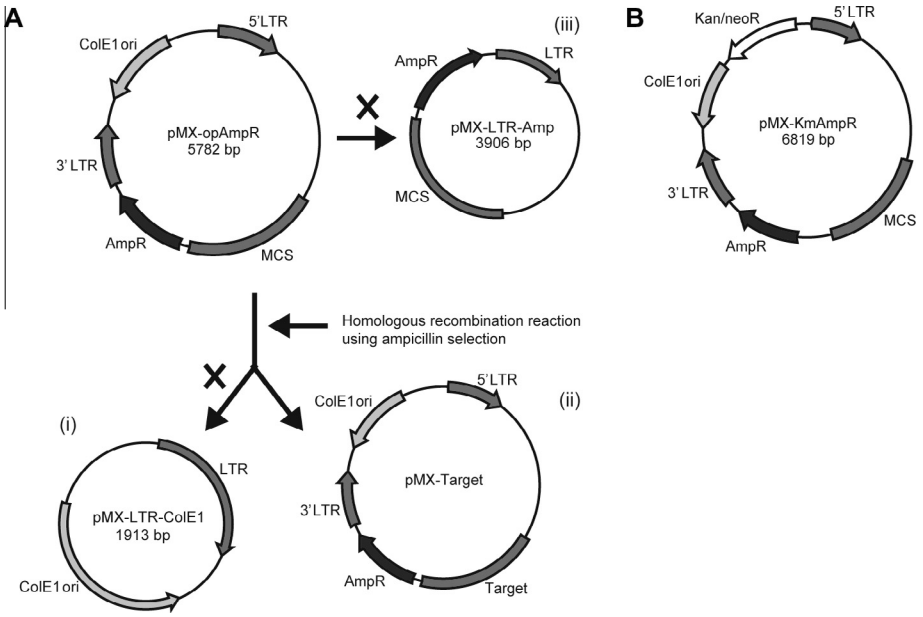

Fig. 2. Schematic illustration of the modified retroviral pMX vector. (A) Schematic illustration of the homologous recombination using the pMX-opAmp vector. (i) and (ii) Products of homologous recombination between the 5′- and 3′-LTRs. (iii) Product of homologous recombination between the target gene and the vector. (B) The pMX-Km vector contains the *AmpR* gene in the opposite position of the Col-E1 ori relative to two LTRs (pMX-KmAmpR).

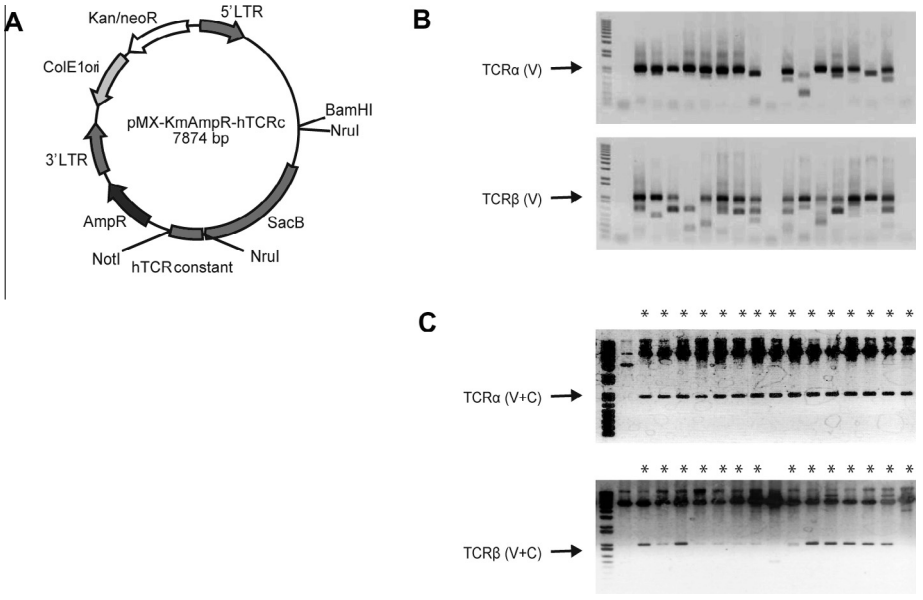

Fig. 3. Cloning of PCR-amplified cDNAs into the modified retroviral pMX vector through homologous recombination. (A) Schematic illustration of the modified pMX-KmAmpR vector for TCR cloning. (B) Amplified products of the variable regions of the TCRα or β genes from single human T cells. The amplified products were analyzed by agarose gel electrophoresis. Black arrow shows the target TCR-Vα (upper) or Vβ (lower). (C) Cloning of the PCR-amplified TCR-Vα or Vβ genes into the pMX-KmAmpR-TCR-Cα/Cβ using homologous recombination. The PCR-amplified TCR-Vα or Vβ were cloned into the pMX-KmAmpR-TCR-Cα or Cβ vectors using homologous recombination, respectively. Plasmid DNA was prepared from ampicillin-resistant colonies, digested with BamHI and NotI and separated by agarose gel electrophoresis. The black arrows show TCRα (upper) or TCRβ (lower), respectively. Asterisks indicate the plasmid that underwent successful homologous recombination.

TCR cDNAs into the TG40 cell line, which does not express endogenous TCR, and stained them using the EBVpep/HLA-A∗2402 tetramer mixture. Ninety-five percent of the TCRs that were expressed on TG40 cells bound the tetramer (data not shown). The analysis of the cloned TCRs showed that the repertoire of the EBV-specific TCRs was highly restricted; in particular, Vβ5 was frequently used with TRBV (Fig. 4A). Furthermore, the number of T cell clones obtained from each donor was inversely correlated with the percentage of tetramer^{+} CD8^{+} T cells (Fig. 4B), suggesting that the specific clones were expanded in each donor to regulate EBV latency. During flow cytometric analysis, we found that some TCRs were hardly expressed on TG40 cells. The TG40 cells expressed only mouse CD3 molecules. We speculated that some human TCRs were difficult to associate with mouse CD3 molecules. To examine this possibility, we transduced two groups of human TCRs into the HEK293T-hCD3 cells that expressed human CD3 molecules: Ones that could be expressed on a large proportion of TG40 cells and ones that were hardly expressed on TG40 cells. Flow cytometric analysis (Fig. 4C) revealed that similar percentages of HEK 293T-hCD3 cells that were transduced with TCRs of each group

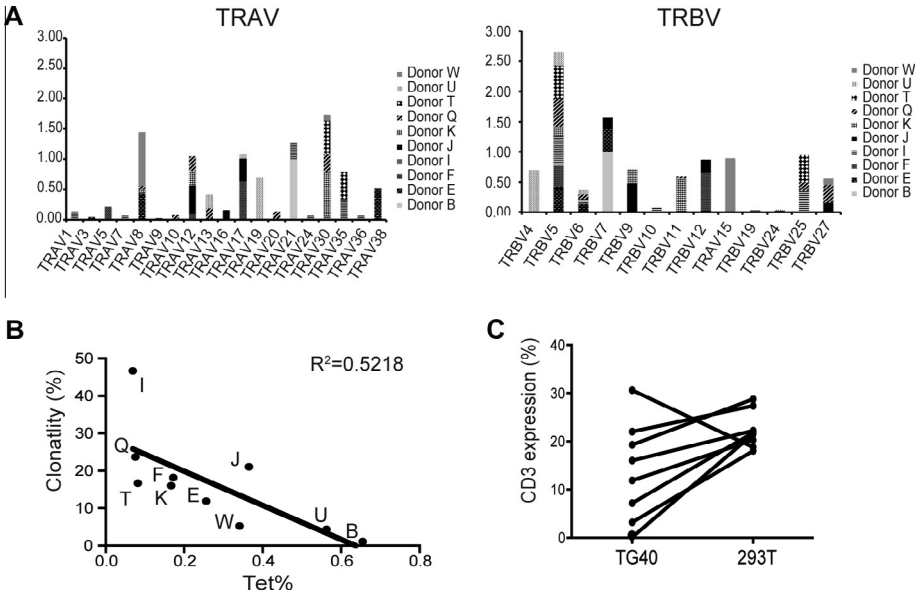

Fig. 4. Functional analysis of the cloned TCRs using the retroviral transduction method. (A) Comprehensive repertoire analysis of the EBV-specific TCRs from 10 latent healthy donors. The V regions of TCRα/β were obtained from single $CD8^+$ T cells that were stained with EBVpep/HLA-A*2402 tetramer mixture and were cloned into the pMX-KmAmpR-TCR-Cα/Cβ vectors using homologous recombination. The EBV-specificity of the cloned TCRα/β pairs was analyzed as described in Section 2. The frequency of the TCR repertoire (%) was calculated using the following formula: Frequency of TCR repertoire (%) = (the number of cloned TCR)/(the number of analyzed T cell clones) × 100. (B) Relationship between the number of cloned TCRs and the percentage of EBV-specific tetramer-positive cells in the $CD8^+$ T cells of 10 latent healthy donors. Clonality (%) was calculated using following formula: Clonality (%) = (the repertoire number)/(the number of analyzed T-cell clones) × 100. "R^2" shows the index correlation. The index correlation was calculated using the GraphPad Prism6 software. (C) Comparison of the cell surface expression of retrovirally-transduced TCRs. Representative TCRs of two groups: Ones that could be expressed on a large proportion of TG40 cells and ones that were hardly expressed on TG40 cells were transduced into 2A-hCD3-hCD8-293T cells and the cell surface expression of the TCRs was analyzed.

expressed human CD3 on the cell surface. The results showed that the expression of some human TCR molecules on the cell surface was affected by the species of the CD3 molecules.

4. Discussion

This study was performed to construct a retroviral vector in which the gene of interest can be inserted by homologous recombination and that is suitable for high-throughput cloning. Retroviral vectors contain 5′- and 3′-LTRs of more than 600 base pairs that exhibit sequence homology of more than 99%. Consequently, it is extremely difficult to insert a target gene into a retroviral vector because homologous recombination preferentially occurs between the LTRs rather than between the target gene and the vector [9,10]. To overcome this difficulty, we integrated the DNA replication origin *Col E1 ori* and the *E. coli* selection marker at opposite positions relative to the 5′- and 3′-LTRs. Because both elements are necessary for *E. coli* to grow, only *E. coli* that harbor a retroviral vector containing the properly inserted target DNA fragment can grow in the selection medium.

Using our modified retroviral vectors, we cloned 444 pairs of TRAV and TRBV cDNAs from EBV-specific $CD8^+$ T cells and analyzed their antigen-specificity and function [16]. Numerous studies on the TCR repertoire of antigen-specific T cells have been performed using conventional analysis methods, such as a FACS-based method with a panel of mAbs specific to each TCR β (TRB) V gene family product [18] or PCR-based methods with a panel of TRBV-specific primers [19,20]. Additionally, our group and others have reported a single cell RT-PCR protocol that allows for the simultaneous identification of the CDR3 α and CDR3 β transcripts of TCRs in human [13] and mice [21]. However, these protocols could not retrieve the TCR α/β pairs and analyze their functions, including antigen-specificity. In contrast, the retroviral vector reported here enabled the high-throughput and comprehensive cloning of TCRs using homologous recombination and efficient confirmation of the antigen-specificity of the cloned TCRs through retroviral transduction.

We used TG40 cells that expressed murine CD3 to analyze the human TCRs. It is worth noting that some human TCRs are difficult to express on the cell surface in association with the murine CD3 molecules, whereas they can be efficiently expressed on the cell surface in association with human CD3 molecules. The TCRα/β heterodimer is expressed in association with the CD3γ, CD3δ, CD3ε and ζ molecules [22,23]. A remarkable feature of the transmembrane domains of these receptor components is the presence of nine basic/acidic residues. Mutations of some of these polar residues resulted in a loss of receptor expression at the cell surface, demonstrating that these polar residues are essential for the expression of TCR-CD3 at the cell surface [24,25]. In contrast, our data showed that the V regions of the TCRα/β heterodimer also affected TCR-CD3 expression at the cell surface, at least in the case of the human TCR-murine CD3 complex.

In conclusion, target cDNA can be cloned into our modified retroviral vector using homologous recombination, making this vector a powerful tool for high-throughput cloning and the functional analysis of cDNA products.

5. Conflict of interest

E.K., H.K., T.O., M.H., and A.M., have a patent regarding the modified retroviral vector described in this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.049.

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