Master of Science

Establishment of an *in vitro* investigation model to discover molecular mechanisms of B cell lymphoma development using CRISPR-Cas9 system

B 세포 림프종 발달의 분자기전 규명을 위한
CRISPR-Cas9 시스템 기반 *in vitro* 연구 모델 확립

The Graduate School
Of the University of Ulsan
Department of Medicine
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Establishment of an *in vitro* investigation model to discover molecular mechanisms of B cell lymphoma development using CRISPR-Cas9 system

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Establishment of an *in vitro* investigation model to discover molecular mechanisms of B cell lymphoma development using CRISPR-Cas9 system

This certifies that the master's thesis of Bo-Ra Nam is approved.

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Abstract

Diffuse Large B-Cell Lymphoma (DLBCL) is the most common histologic subtype of non-Hodgkin lymphoma and a heterogeneous disease with a variety of molecular aberration and diverse clinical outcomes. Currently, therapeutic advances in DLBCL have been achieved, however; 40% of the patients fail to achieve durable remission and developed relapsed/refractory disease. To further improve and develop the therapeutic effects for patients of DLBCL, investigation on the gene functions of DLBCL is essential. However, up to now, many of the functions of genes in DLBCL was not completely identified due to the difficulty of gene manipulation in human B cells. Here, we established an *in vitro* investigation model to discover molecular mechanisms of B cell lymphoma development using measles virus glycoprotein displaying lentiviral transduction based CRISPR-Cas9 system. We transduced Cas9 into various DLBCLs, which are hard to transduce. The Cas9 expression was confirmed by western blot. Functionality of the transduced Cas9 was assessed by GFP knockout efficacy using GFP gRNA-GFP-BFP expression vector system. Transduction CD45-gRNA expressing vector knocked out by 70% of the cells, as well as transfection of CD45-sgRNA knock out by 20%. Thus, this gene functional analysis system is compatible with both cell lines for long-term culture and primary cells for short-term culture. Collectively, this system allowed us to manipulate genes in B cells with high efficiency and provide novel insight into therapeutic modalities for the DLBCL.

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is aggressive malignancy of mature B lymphocytes and the most common non-Hodgkin lymphoma worldwide.[1-3]. Therapeutic advances in DLBCL have been achieved with the addition of the anti-CD20 monoclonal antibody rituximab to cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP)[4,5]. Although a significant amount of DLBCL patients can be cured with current therapies, a substantial proportion of patients die because of progressive disease[6,7]. Therefore, it is necessary to improve therapeutic effects.

Particularly for the genetics identification, immune therapies continue to be the main therapeutic tools for treating disease[8,9]. Genome editing, the introduction of a desired change to the sequence of genomic DNA, is a revolution in the biomedical sciences and has the potential to provide future treatments for a lot of human diseases with a genetic component[10]. Many genome editing technologies have showed up in recent years, including zinc-finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs) and the RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 (Cas9) nuclease system[11]. The CRISPR-Cas9 system is a variable technology that provides the ability to add or delete DNA in the genome in a sequence-specific manner[12]. CRISPR-Cas9 is an RNA-guided, targeted genome-editing platform with great potential in both basic research and clinical applications[13]. The most broadly used *Streptococcus pyogenes* Cas9 (SpCas9) system requires a trans-activating CRISPR RNA (tracrRNA), which is fused with the crRNA to form a single-guide RNA (sgRNA)[13,14]. The tracrRNA sequences with the sgRNA change to a specific conformation, to form base pairs with DNA target sequences, can make Cas9 possible to introduce a site-specific double-strand break in the DNA[15]. This technology is markedly easier to design guide sequence, highly specific, efficient and can be applied high through-put and multiplexed gene editing for a variety of cell types and organisms[11]. Thus, this novel technology will be of great potential for application in molecular study, as well as clinical applications[16,17].

B lymphocytes are attractive targets of genetic diseases for gene therapies associated with B-cell

dysfunction and for immunotherapy[18]. Lentiviruses incorporating Edmonston measles virus (MV) glycoproteins hemagglutinin (H) and fusion protein (F) on their surface, named H/F-LVs, were able to transduce almost completely quiescent B cells in the absence of any exogenous stimulus[19]. This novel viral transduction system allows the introduction of foreign genes into the human B cells and/or DLBCLs with high efficiency[20,21]. By using this, we generated Cas9-expressing DLBCL cell lines, which are hard to transduce genes, and established the model system for the investigation on gene functions in DLBCLs through CRISPR-Cas9 system. This investigation model can manipulate easier to researcher for study the mechanism of molecular level. So, we expected that this model will be a breakthrough therapy for lymphoma.

MATERIALS AND METHODS

Cell culture and Reagents

All DLBCL cell lines were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and ATCC (American Type Culture Collection) (Table 1). All cell lines were maintained in a 5% CO_2 atmosphere at 37℃. U2932, Bjab, SU-DHL-2, OCI-LY3 were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific). OCI-Ly7 was cultured in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific) containing 10% FBS. U2932, BJAB ($5x10^5$ cells/ml), SU-DHL-2 ($3x10^5$ cells/ml), OCI-Ly7, OCI-Ly3 ($2x10^5$ cells/ml) cells were subcultured every 2-3 days.

Cell counting grid of hemocytometer

To determine the amount of cells growing in the flasks, the cell number were counted with trypan blue by using hemocytometer. Briefly, cells were resuspended by pipetting (RPMI 8226, GC-B cells, and *in vitro* generated PBs) or Trypsin/Ethylenediaminetetraacetic acid (EDTA) treatment (CD40L expressing L cells and HS-5 cells). Ten microliter of cell suspension was mixed with 10 μL of 0.4% Trypan Blue and the 10 μL cell suspension-Trypan blue mixture was loaded on the V-shaped groove of the hemocytometer. The cells in the 4 quadrant of each corners of the counting grid were counted under microscopy. Trypan blue stained dead cells (blue) were excluded and only live cells (white) cells were calculated.

To calculate the cell density (number of cells/mL), the average count number from each quadrant of the hemocytometer was multiplied by 10,000 then multiply by 2 (dilution number).

Number of cells/mL = Average number of cells × dilution number (2) × 10^4

Virus Plasmids, constructs and production

The cytoplasmic-tail mutated glycoproteins H and F from Edmonston expressing plasmids pCG-H△24 and pCG-F△30 were kindly provided by Dr. Els Verhoeyen[22], for production of measles gp displaying LV. Procedures of the virus production was performed as follows:

Day1 - PsPAX2 8.6 μg, Target vector 8.6 μg, H del-24 2.75 μg, F del-30 2.75 μg, CaCl2 55 μl, H2O up to 450 μl and add into 450 HBSS (Thermo Fisher Scientific) media drop by drop.

Day2 - Media change into 6 ml Opti-MEM in 100 π cell culture dish.

Day4 -Virus harvest, and centrifuge 1,800 rpm, 5 min, 4□. And harvest the soup and filtering 0.45 μm syringe. And PEG of 1/5 volume media and inverting 2 days in 4□.

Day7 - Centrifuge 2,300 rpm, 60 min, 4□ and harvest the pallet. Concentrated the virus in 500 ul PBS. Aliquots each solution into 50 ul in e-tube. Storage at -80□.

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Viral transduction

The cells were seeded on the culture dish with the half volume of original culture condition. The viruses were added into the culture at the concentration of 20 μl/ml to various DLBCLs with 8 μg/ml of polybrene. After 24 hours, the media was changed to the original volume of cell culture and added antibiotics (blasticidin) to select the transduced cells.

Institutional review board approval

This study was approved by the Asan Medical Center Institutional Review Board (IRB; Approval number 2013–0864). The need for informed consents was waived by the Institutional Review Board on the following bases: 1) there was no additional risk to the participants. Remainder tissue samples from routine tonsillectomy and pathologic exam were used for the *in vitro* experiment to study the general phenomena; 2) patient identities were anonymized and completely delinked from unique identifiers. GC-B cells were isolated from human tonsils obtained from remainder tissues of routine diagnostic pathologic examination after therapeutic tonsillectomy.

Tonsillar mononuclear cell extraction

Human tonsil tissue was obtained from the tissue remaining from tissues of routine tonsillectomy and was handled in accordance with IRB-approved protocol (2013–0864). Tonsillar mononuclear cells (MNCs) were extracted from human tonsils by mechanical disruption. To extract cells in the tonsils, the specimens were cut into 3 to 10 mm fragments and mashed in RPMI 1640 containing 10% bovine calf serum (BCS) using scissors and forceps. The extracted cells were collected and tissue debris were removed using disposable pipette. The cell suspension volume overlayed on Ficoll-Paque (GE Healthcare) and follow Ficoll density gradient centrifugation. After the centrifugation, lymphocyte layer (interface layer; containing MNCs) was collected.

GC-B cell isolation

GC-B cells were purified from tonsillar MNCs via magnetic-activated cell sorting (MACS; Miltenyi Biotec). In briefly, 3 × 107 of MNCs were incubated with mouse anti-IgD, mouse anti-CD3 (OKT3), and mouse anti-CD44 in PBS for 20 minutes under dark conditions on ice. After washing with RPMI 1640 containing 10% BCS, the cells were incubated with goat anti-mouse magnetic microbeads (Miltenyi Biotec), according to the manufacturer's instructions. The cells were washed and resuspended in RPMI 1640 containing 10% FBS. A LS magnetic separation column (Miltenyi Biotec) was placed in this magnet, and the cell suspension was applied at the top of this column and allowed to pass through; the effluent was collected as negative fraction. The purity was greater than 95%, as assessed by CD20 and CD38 expression.

Flow Cytometric Analysis

For each group, 2×10^5 cells were prepared and treated with 10 ng/mL antibody before incubation at 4℃ for 20 minutes. After the cells were resuspended by 100 μL of PBS. The fluorescence was measured using a FACS Calibur flow cytometer and analyzed using FlowJo software (Tree Star).

Measurement of cell surface protein expression using Flow cytometry

For measurement of surface protein expression, cells were spun down at 1,200g for 5 minutes and resuspended with 10 μL of Flow cytometry buffer. The cells were stained with various combination of immunofluorescence dye-conjugated antibodies in a dark condition on ice for 20 minutes. After incubation, 100 μL of Flow cytometry buffer was added, and acquired by flow cytometer.

All flow cytometric analyses were performed on an Accuri C6 flow cytometer (BD biosciences). Data were analyzed using FlowJo software (FlowJo, LLC). Viable cells within the cells alone dot plot were gated and used to directly compare and analyze the various Ab combinations. Histograms were drawn to observe any possible shifts in fluorescence, and to compare appropriate negative control with the primary Abs.

Electroporation

CD45 knockout with sgRNA transfection was performed using a The Neon® Transfection System (MPK5000, Invitrogen) System efficiently delivers into DLBCL cells with a high cell survival rate according to the manufacturer's instructions. For each electroporation, 1×10^6 per cells were harvested and washed twice in PBS. Then, the cells were resuspended in 100 μL resuspension buffer, transfected with 1 μM of CD45-targeting sgRNA or negative control sgRNA at 1200-40-1 (voltage-width-number). Cells were immediately transferred to a 6-well plate containing prewarmed media without antibiotics and incubated at 37°C. CD45-targeting sgRNA was designed as follows:

CD45 #1: 5'-GTCCTCCAGCTCCTATATGA-3' (targeted exon6 of CD45),

CD45 #2: 5'- GAGGATCCTCAGGCACCCCG-3' (targeted exon9 of CD45).

Negative control siRNA (non- targeting scrambled RNA [Scr]) was purchased from Genolution Pharmaceuticals.

Quantitative PCR

For quantitative RT-PCR, total cellular RNA was prepared from 5×10^5 cells using Nucleo-Spin RNA II (Macherey-Nagel). For reverse transcription, cDNA was synthesized from 1μg isolated total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The gene-specific primers were designed as follows:

Targeted exon6 (CD45 #1):

5'-CTTAGGGAGCATCTTATGATGC-3' (forward),

5' -CACTGAATACAGTCAGTCTACCCTCC-3' (reverse);

Targeted exon9 (CD45 #2):

5'-GTAGTACATGCAAGTCCTGCAC-3' (forward),

5'-CTACCTGCAGTGCACCACAATG-3' (reverse).

One microliter of the cDNA template and 1 μM of the specific primer were used in the Power SYBR-Green PCR kit (Applied Biosystems) with a quantitative PCR reaction mixture (20 μL). The following PCR conditions were used in the Step-One Real-Time PCR System (Applied Biosystems): 95°C for 10

minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Melting curve analysis was performed to control for the specificity of the PCR product fluorescence. Fold induction was calculated using the comparative Ct method (Livak & Schmittgen, 2001), and the expression of the ribosomal protein S18 was used as a reference. Data are presented as the means ± standard deviation (SD) of triplicate experiments.

Western blot

Fifty micrograms of protein were separated using 6% SDS-polyacrylamide gel and electrophoretically transferred to an Immune-Blot Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Laboratories). The membrane was blocked for 1 hour at RT by using TBS/0.1% Tween 20 that containing 5% BSA, and then incubated overnight with the primary Ab. The unbound primary Ab was removed by washing the membrane 3 times with TBS/0.1% Tween 20, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary Ab (diluted 1:3000 in TBS/0.1% Tween 20). The protein was then visualized using an enhanced chemiluminescence solution (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific) and the ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences). For quantitative analysis of the western blot images, Gel Analyzer software (http://www. gelanalyzer.com) was used. Gel Analyzer enables the measurement of peak height and volume of the band with the expected molecular weight. The background subtraction tools facilitate the subtraction of the defined background from the intensity values. Equal loading was confirmed by stripping the western blot and re-probing for b-actin. The values of the western blot represent the relative density of the bands normalized to b-actin.

Statistics

Data are presented as mean ± standard deviation. All comparisons were analyzed using Student's t-test.

RESULTS

1. Measles virus glycoprotein displaying lentiviral system dramatically increased transduction efficacy in B cells.

To investigate the function of genes in DLBCLs, we used H/F-LV system. This system is the mutant system of lentivirus incorporating measles virus glycoproteins (H) and (F) and makes transduction efficiency much higher in B cells (Figure 1A). We applied this H/F-LV system to our experiment. We tried to transduction into DLBCL cell lines using H/F-LV system and the transduction efficiency increased from 5% to 65% (Figure 1C).

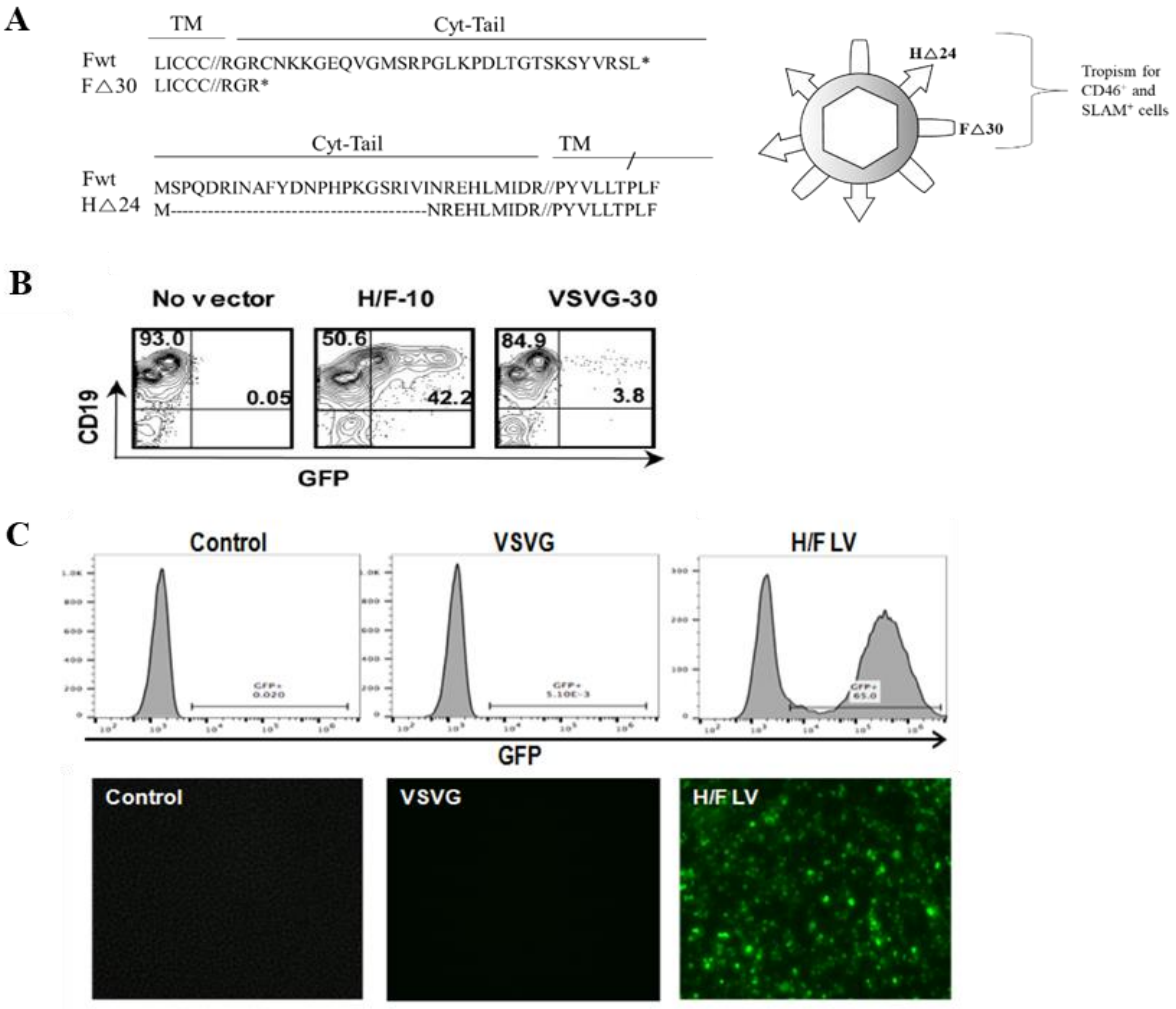


Figure 1. The H/F-displaying lentiviral vectors efficiently transduced B cells. (A)Schematic representation of the lentiviral vectors displaying hemagglutinin (H) and fusion (F) glycoproteins derived from Edmonston strain MV. The combination of the cytoplasmic-tail mutants of MV glycoproteins, HΔ24 and FΔ30, allowed efficient co-incorporation on the LV surface, resulting in high-titer HIV vectors (hereafter named H/F-LVs). These H/F-LVs conserved the parental tropism as confirmed by transduction of CD46+ and SLAM+ cell lines. The picture was adapted from Frecha C., et al (2009) Blood[20]. (B)Percentage of GFP measured by FACS at day 2 after transduction of SAC/IL-2 pre-stimulated B cells transduced with H/F-LVs and VSVG-LVs20. (C)Percentage of GFP measured by FACS and fluorescence microscope image after IM9 cell line transduced with H/F-LVs and VSVG-LVs.

2. General information of DLBCL lines used in this study.

To investigate the molecular mechanisms of DLBCL, We chose DLBCL cell lines for study our experiments and got information about DLBCL cell lines to use for the experiment. BJAB and OCI-Ly7 are GCB (Germinal center B-cell) - DLBCL cell type and OCI-Ly3, SU-DHL-2, U2932 are ABC (Activated B-cell, Non-GCB) - DLBCL cell type. Because Myc and Bcl-2 are related to lymphoma, these features were important for studying the DLBCL. OCI-Ly7 has Myc translocation and BJAB, OCI-Ly3, SU-DHL-2, U2932 have Myc amplification. And OCI-Ly7 has Bcl-2 deletion, OCI-Ly3, SU-DHL-2, U2932 have Bcl-2 amplification (Table 1).

Table 1. General information of DLBCL lines used in this study

ID	Cell type	Provider	Myc	Bcl-2
BJAB	GCB-DLBCL	DSMZ	Amplification	-
OCI-Ly7	GCB-DLBCL	DSMZ	Translocation	Deletion
OCI-Ly3	ABC-DLBCL	DSMZ	Amplification	Amplification
SU-DHL-2	ABC-DLBCL	ATCC	Amplification	Amplification
U2932	ABC-DLBCL	DSMZ	Amplification	Extensive amplification

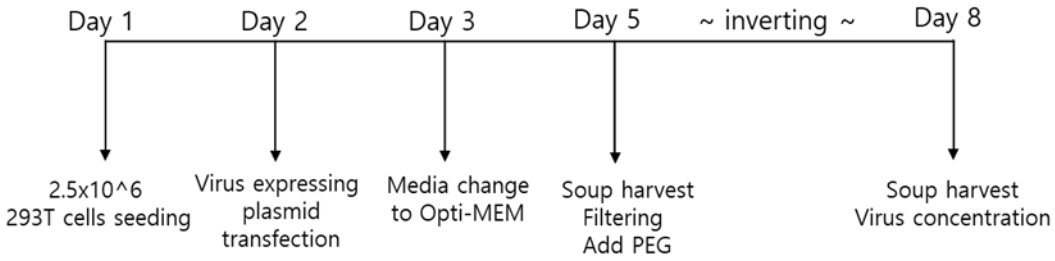
3. Generation of the virus for Cas9 transduction and selection of Cas9 transduced cells using the antibiotics.

To manipulate gene expression, we applied CRISPR-Cas9-mediated gene editing system. CRISPR-Cas9 system is a powerful tool to edit the genomes of human cells. To express Cas9 in various DLBCLs, we obtained LentiCas9-Blast from Addgene (LentiCas9-Blast, Plasmid #52962) (Figure 2A) and Cas9 was transduced with H/F-LV system. LentiCas9-Blast vector has a gene of resistance of blasticidin, therefore, we can select the introduced Cas9 cells. When the procedure of virus making was finished, we transduced the virus into targeted DLBCL cell lines (Figure 2B and C).

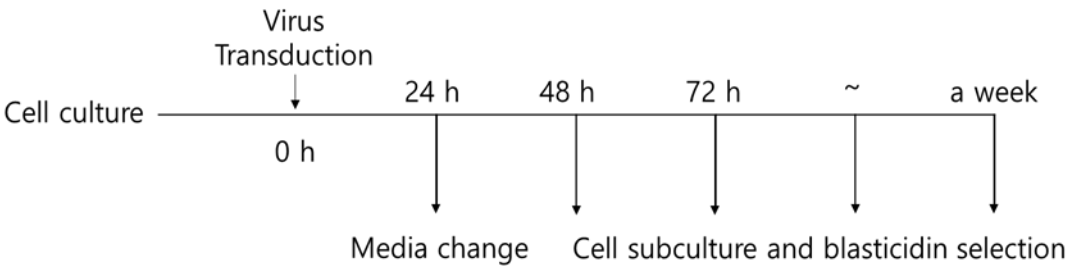
A


P2A
BSD
Cas9

B


Day 1
Day 2
Day 3
Day 5
~ inverting ~
Day 8
2.5x10^6
293T cells seeding
Virus expressing
plasmid
transfection
Media change
to Opti-MEM
Soup harvest
Filtering
Add PEG
Soup harvest
Virus concentration

C


Virus
Transduction
Cell culture
24 h
48 h
72 h
~
a week
0 h
Media change
Cell subculture and blasticidin selection

D

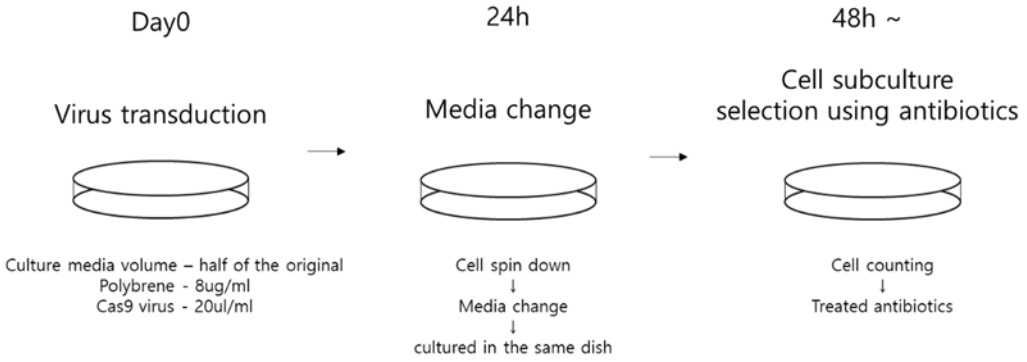

Day0
24h
48h ~
Virus transduction
Media change
Cell subculture
selection using antibiotics
Culture media volume – half of the original
Polybrene - 8ug/ml
Cas9 virus - 20ul/ml
Cell spin down
↓
Media change
↓
cultured in the same dish
Cell counting
↓
Treated antibiotics

Figure 2. Illustration of Cas9 expressing lentiviral vector and experimental scheme of the viral transduction. (A)Illustration of the LentiCas9-Blast. The vector has Cas9 and blasticidin resistance gene (blasticidin S deaminase, DSB). The size of the vector is 12,838 base pairs. (B)Experimental scheme of the virus production. Day1, 293 T cells were seeded into 100π dish by 2.5 x 10^6 cells. Day2, plasmids (psPAX2, H△24, F△30) were transfected into 293T cell. Day3, Media was changed to Opti-MEM. Day5, the supernatant containing the lentiviruses was collected, filtered through a 0.45μm filter, and saturated by polyethylene glycol precipitation. Day8, the precipitated virus was concentrated. (C)Procedure of virus transduction. Day1, Cas9 expressing H/F-LV virus transduction to DLBCL cells. Day2, Cell counting and media change. Day3, cell subculture and selected cell counting.

4. Measles virus glycoprotein displaying lentiviral system allowed to introduce the Cas9 gene in various B cells.

To transduce Cas9 into the DLBCL cell lines, we checked the average cell growth rate (Figure 3A). In addition, for antibiotic selection to enrich the Cas9-expressing cells, we titrated the blasticidin concentration that the cell died in a week (Figure 3B). Optimal concentration of blasticidin for BJAB and, OCI-Ly7 is 10 μg/ml, and for OCI-Ly3, SU-DHL-2, and U2932 is 3 μg/ml (Figure 3C). Blasticidin selection was commenced at the time of Cas9 transduction was accomplished. BJAB, U2932, and OCI-Ly3 had high transduction efficacy, because they almost little cells were died, but OCI-Ly7 showed 5%, SU-DHL-2 showed 20% transduction efficiency (Figure 3D). Then, we confirm the Cas9 induced state in protein levels. All DLBCL cell lines expressed the Cas9 that detected molecular size 154kda (Figure 3E).

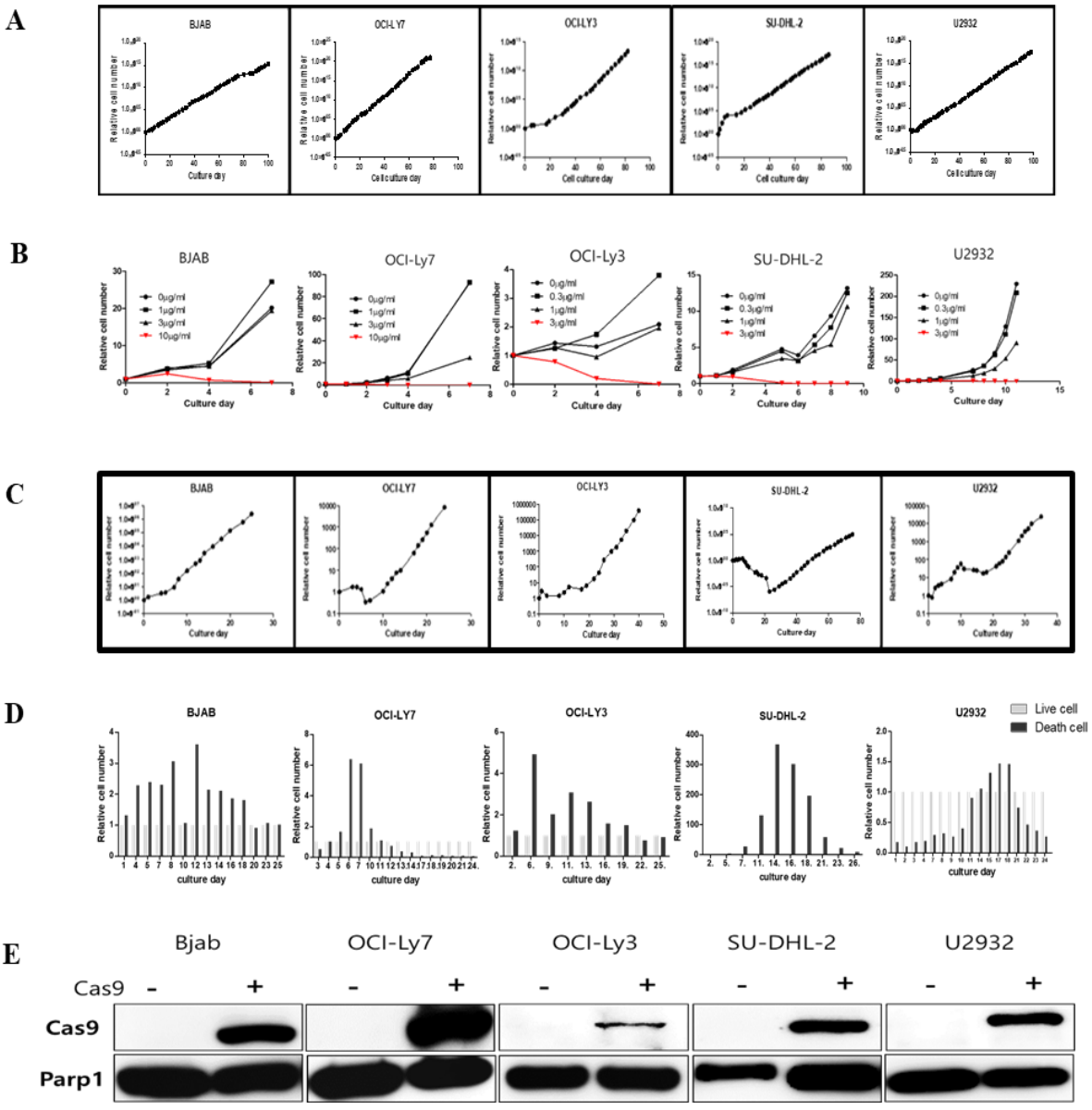

A
BJAB
OCI-LY7
OCI-LY3
SU-DHL-2
U2932
Relative cell number
Culture day
B
BJAB
OCI-Ly7
OCI-Ly3
SU-DHL-2
U2932
0μg/ml
1μg/ml
3μg/ml
10μg/ml
0.3μg/ml
Relative cell number
Culture day
C
BJAB
OCI-LY7
OCI-LY3
SU-DHL-2
U2932
Relative cell number
Culture day
D
BJAB
OCI-LY7
OCI-LY3
SU-DHL-2
U2932
Live cell
Death cell
Relative cell number
culture day
E
Bjab
OCI-Ly7
OCI-Ly3
SU-DHL-2
U2932
Cas9
Cas9
Parp1

Figure 3. Cas9 transduction by H/F-LV and the antibiotic selection successfully generated Cas9-expressing DLBCLs. (A)Growth rate of DLBCL cell lines. (B)Titration of the blasticidin concentration. Concentration of blasticidin for BJAB, OCI-Ly7 was 10μg/ml, concentration of blasticidin for OCI-Ly3, SU-DHL-2, U2932 was 3μg/ml. (C)Cas9 transduced DLBCL cell lines were selected in 2 weeks. (C and D) The ratio of the live cells vs death cells. (E)Confirmation of Cas9 expression using western blot and detection with LAS (ImageQuant LAS 4000).

5. Cas9 introduced B cells are functional.

To assess Cas9 functionality, we used CRISPR functional screening vector, which encodes gRNA for GFP knockout, BFP, and GFP. When Cas9 is expressed in the cells, sgRNA in this vector can knockout GFP, accordingly, they inhibited the GFP expression (Figure 4A). As a result, the cells introduced BFP-GFP expressing vector showed on BFP positive location in FACS, the cells introduced BFP-GFP expressing vector and Cas9 also worked well and showed on BFP positive, GFP negative location (Figure 4B). In this way, we tried this method to all DLBLC cell lines to confirm the Cas9 working. BJAB and OCI-Ly7's Cas9 working efficiency value were 95% and 94%, respectively, and the value was very high in GCB type DLBCL cell lines. OCI-Ly3, SU-DHL-2, U2932's Cas9 working efficiency value were 32%, 12% and 45%, respectively (Figure 4C).

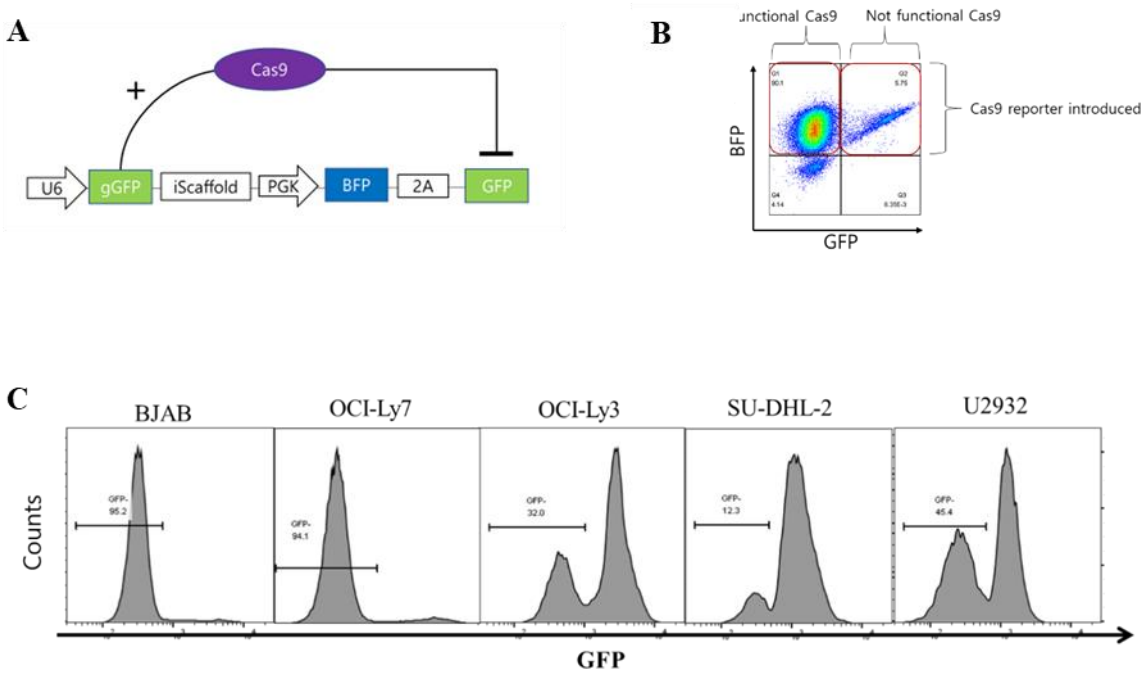


Figure 4. The transduced Cas9 effectively knocked out the GFP expression by gRNA targeting GFP transduction. (A) Schematic diagram of Cas9 functional reporter vector. (B)FACS analysis of GFP-BFP expressing vector introduced in OCI-Ly7. (C)Cas9 working efficiency using FACS. GFP negative percentage was BJAB; 95.2%, OCI-Ly7; 94.1%, OCI-Ly3; 32.0%, SU-DHL-2; 12.3%, U2932; 45.4%.

6. Cas9-expressing B cells successfully decreased the CD45 expression by H/F-LV transduction.

We tried to knockout the CD45 gene using H/F-LV virus. We designed a vector CD45 sgRNA with puromycin resistance gene. We transduced the vector into OCI-Ly7 cell and the next step proceeded as described in Figure 2C. After 7 days of transduction, CD45 knockout ratio of CD45 #1, CD45 #2 was 33% and 24%, respectively. After 7 days of transduction, we treated puromycin for select the guide-CD45 introduced cells. 10 days after puromycin treatment, CD45 knockout cell percentage of CD45 #1, CD45 #2 was by 50% and 70%, respectively. The knockout efficiency maintained 30 days after transduction (Figure 5A). The cell number decreased when the puromycin treated, but 10 days after, the cells recovered to the original growth rate (Figure 5B). We confirmed the CD45 gene knockout efficiency in the mRNA levels using real-time PCR. CD45 #1 decreased by 50% of the CD45 gene and CD45 #2 decreased by 70% of the CD45 gene (Figure 5C). This result was consistent with previous results of FACS data (Figure 5A and B).

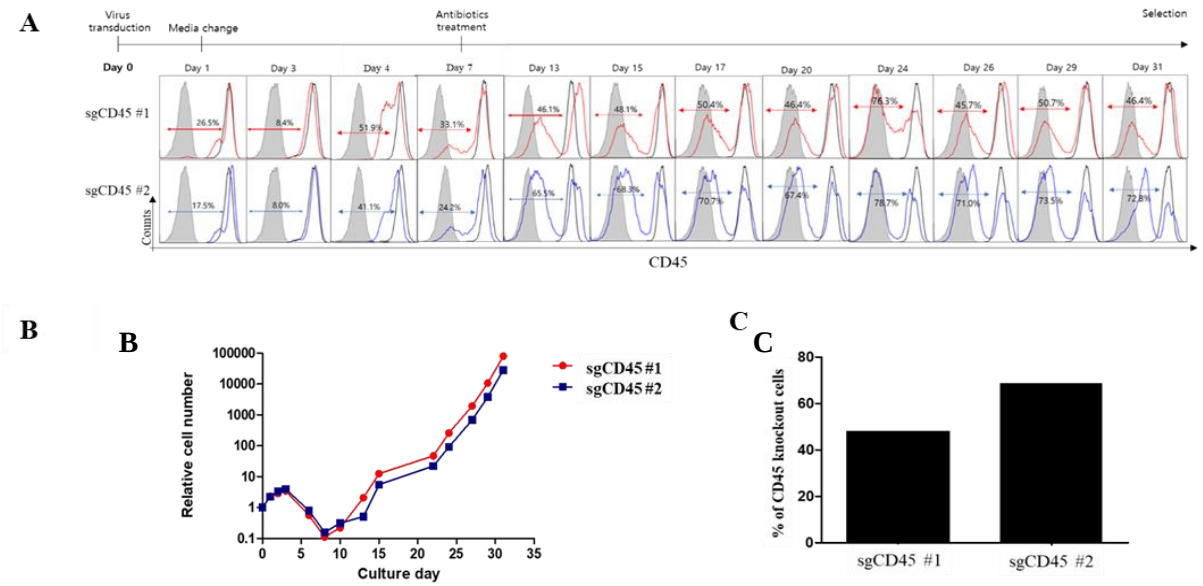


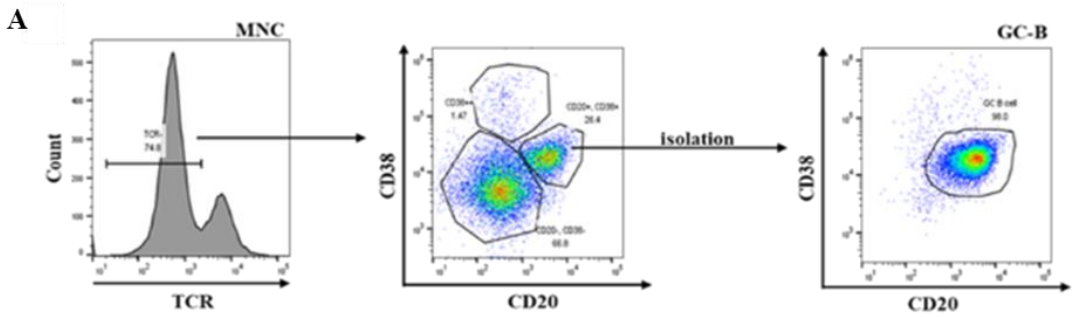
Figure 5. The Cas9-expressing DLBCL was successfully knocked out the CD45 expression by viral transduction. (A)CD45 transduced at day0, and the next day, cell media changed the new media. At 7 days after transduction, puromycin treated for selection. (B)Cell growth curve of CD45 transduced OCI-Ly7. (C)CD45 knockout efficiency of OCI-Ly7 in mRNA level using real-time PCR.

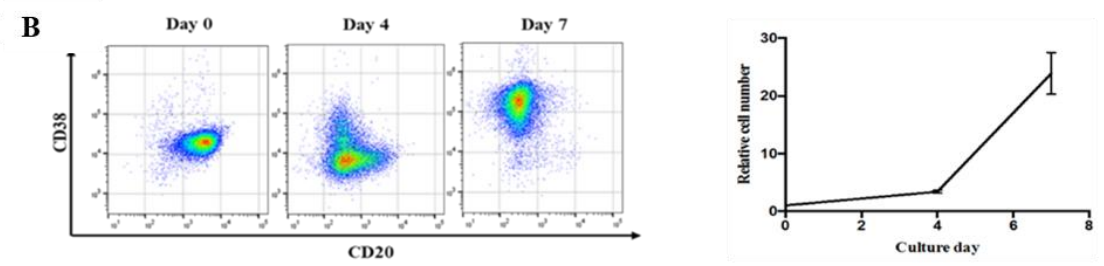
7. Primary germinal center B cell and the B cell line were differentiated into plasma cells.

To isolate GC-B cells from tonsillar mononuclear cells MNCs, we initially identified the MNC subtypes by multicolor flow cytometric analysis. Tonsillar T and B cells were identified based on the presence of CD3 and CD19. Among CD3-B cells, the subsets of tonsillar B lineage cells were further examined by gating these cells according to CD38 and IgD expression: IgD +CD38−, naïve B cells; IgD −CD38−, memory B (MB) cells; IgD − CD38+, GC-B cells; and IgD − CD38++, PCs. The GC-B cells were also identified with CD20 and CD38. The average proportion of GC-B cells was 40% on average (Table 2 and Figure 6A, B). When the GCB differentiation method was equally applied to OCI-Ly7, it showed the same result as GCB differentiation. Blimp1(differentiation marker) was high after the differentiation in both cells and this was confirmed using real-time PCR (Figure 6C).

Table 2. Summary of GC-B cell isolation and plasma cell differentiation

Tonsil (gender/age)	MNC subtypes proportion				GC-B cell isolation		Differentiation to plasmablasts						
							Cell number (10^5 cells/mL)				Surface CD38 (MFI)		
	TCR^- (%)	TCR^+ (%)	$CD38^-CD20^-$ (%)	$CD38^+CD20^+$ (%)	Yield (%)	Purity (%)	Day 0	Day 4	Day 7		Day 0	Day 4	Day 7
M/5	75.5	23.3	51.5	39.7	47	96	2	5	2.2		7296	3084	29133
M/4	76.2	23.2	47.2	42.6	54	95	2	7.7	2.3		7385	5869	46267
F/4	78.3	20.5	49.5	40.5	50	95	2	5.6	3		6957	2928	27570
M/5	82.6	16	38.4	51.9	45	96	2	7.5	1.2		7750	765	20127
M/4	80.1	17.7	49.1	38.4	65	95	2	8.2	3.2		5145	2504	13945
M/3	83.5	15.6	34.8	57	41	97	2	4.85	3.1		5646	2245	18110
F/4	77	22	52.1	37.7	57	97	2	9.4	1.2		5657	2594	16656
M/3	70.3	28.3	67.3	21.6	50	94	2	5.8	2.9		5222	2148	21868
Average	77.94	20.83	48.74	41.18	51.13	95.63	2	6.76	2.39		6382.25	2767.13	24209.5
SD	4.24	4.3	9.77	10.51	7.51	1.06	0	1.67	0.82		1068.15	1439.82	10308.36


A
MNC
Count
TCR
CD38
CD20
isolation
GC-B
CD38
CD20


B
Day 0
Day 4
Day 7
CD38
CD20
Relative cell number
Culture day

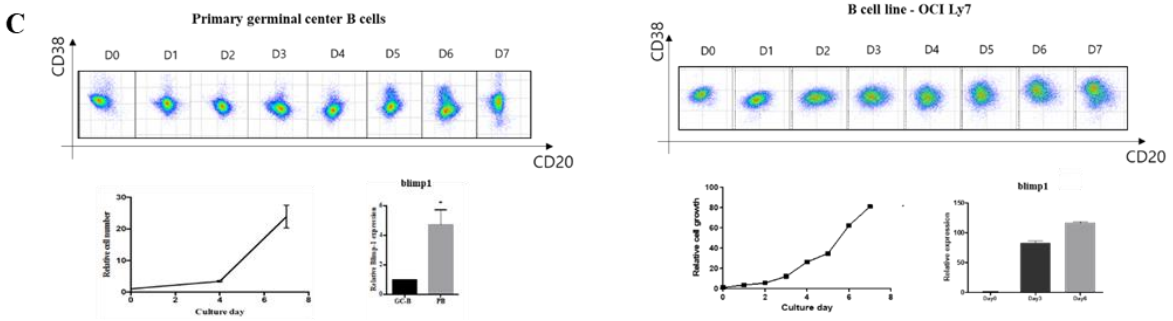

C
Primary germinal center B cells
CD38
D0 D1 D2 D3 D4 D5 D6 D7
CD20
Culture day
blimp1
B cell line - OCI Ly7
CD38
D0 D1 D2 D3 D4 D5 D6 D7
CD20
Culture day
blimp1

Figure 6. Primary germinal center B cell and OCI-Ly7 were differentiated. (A)To isolate GC-B cells, we used the MACS negative selection method. B cell isolation from MNCs using MACS. Representative flow cytometric analysis of GC-B cell isolation. (B)GCB cell differentiated into the plasma cells. GCB; CD20+ CD38-, PB; CD20-, CD38++. (C)FACS analysis of GCB, OCI-Ly7 differentiation. Blimp1 expression was confirmed using real-time PCR.

8. Cas9-expressing B cells reduced the CD45 expression by the transfection of CD45 sgRNA.

Non-immortalized primary cells have a limitation on culture period, they only can culture only short periods of time. (Figure 6C). For these short-lived cells, we tried to transfect sgRNA to OCI-Ly7. To test whether nucleotides can be delivered to the cells with high efficacy by electroporation, we transfected the GFP-expressing vector into OCI-Ly7. GFP was expressed from the first day after transfection, and highest at day 4 (Figure 7A). To knockout the CD45 gene, we transfected CD45 sgRNA to OCI-Ly7. These 2 transfection methods have no significant effect on cell growth. Four days after transfection, CD45 knockout efficiency showed by 5% and 20% in sgCD45 #1 and sgCD45 #2 target gene, respectively (Figure 7B).

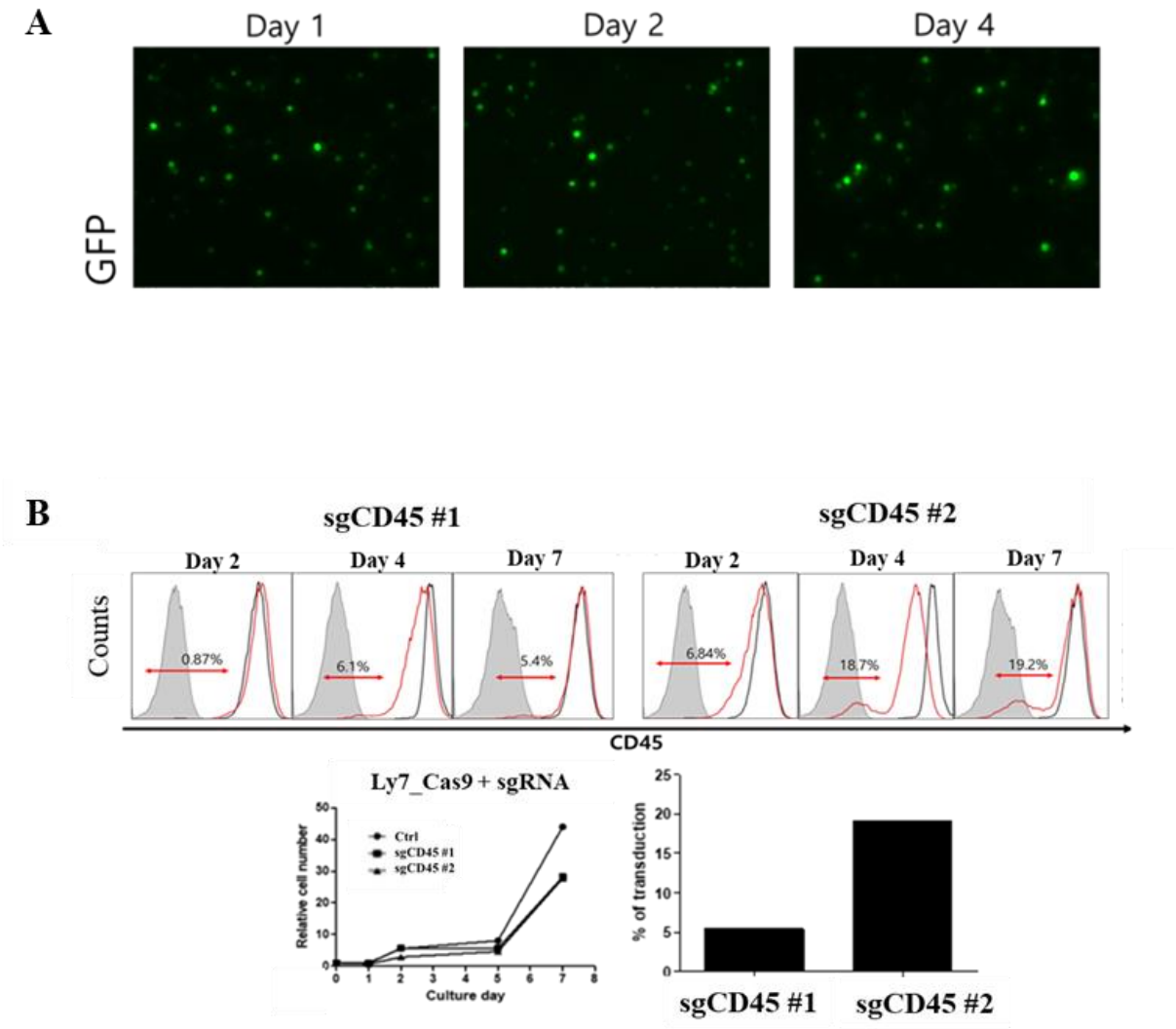


Figure 7. The Cas9-expressing DLBCL was successfully knocked out the CD45 expression by viral transduction. (A)GFP transfection using Neon electroporator in OCI-Ly7. (B)FACS analysis of CD45 gene knockout in OCI-Ly7. CD45 expression was confirmed by real-time PCR.

Discussion

Diffuse large B- cell lymphoma is the most common non- Hodgkin lymphoma (NHL), accounting for 30–40% of all lymphoma cases[23]. Although most patients are cured with 6–8 cycles of rituximab with cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) chemotherapy, about 10–15% have primary refractory disease and a further 20–30% relapse[24]. Therefore, there is a need for urgent molecular studies to treat for patients with DLBCL. However, B cell lymphoma is hard to study because they are difficult to manipulate gene expression. Thus, we used a new lentivirus-based tool that can effectively transduce B cells in the absence of any other stimulus[20,21]. Additionally, we introduced the CRISPR-Cas9 system that provides the ability to add or remove DNA in the genome in a sequence-specific manner to study molecular level [12].

Using this method, we established an investigation model to study the molecular mechanism of the DLBCL cell lines. We cultured five DLBCL cell lines (BJAB, OCI-Ly7, OCI-Ly3, SU-DHL-2, U2932) and we successfully transduced Cas9 to DLBCL cells. We detected the Cas9 expression using western blot. To confirm the Cas9 functionality, we used GFP gRNA-GFP-BFP expression vector system. GCB-DLBCL cell lines' (OCI-Ly7, BJAB) Cas9 activity was 95.2% and 94.1%, that activity value was high. However, ABC-DLBCL cell lines' (OCI-Ly3, SU-DHL-2, U2932) Cas9 activity was 32.0%, 12.0% and 45.4%, respectively. ABC-DLBCL seemed to be need single cell cloning to find cells that have high Cas9 activity.

To confirm a specific gene knockout, we examined with knockout of two gRNAs that targeted different site of CD45 gene sequences. To perform this experiment, we used the virus first. The CD45 gene knockout efficiency was 33.1% and 24% respectively, before the antibiotic selection. After the antibiotic selection, the percentage of the knocked out cells was significantly increased by 50.4% and 70.7% respectively. Furthermore, this ratio was lasted for a month. Secondary, we used electroporation to knockout CD45 gene in OCI-Ly7. The cells were successfully differentiated to plasma cells.

Therefore, they have the advantage of being able to replace the primary cells that difficult to manipulate. Electroporation was for short-lived cells, such as differentiating cells or primary cell, and the knockout efficiency was up to 5.4% and 19.2%, respectively. This suggests that the knockout is highly variable and may dependent on gRNA target sequences.

This study provided in patient basis an investigation model of DLBCL and established to study the B cell lymphoma in molecular levels. This system will make it easier to manipulate the B cell lymphoma to researchers related to B cell lymphoma study. Therefore, this is expected to contribute to the development of a treatment for DLBCL disease.

Conclusions

DLBCL is one of the most common lymphomas and difficult to achieve durable remission[25]. Because DLBCLs are hard to manipulate its gene expression, the investigation of the role of specific genes are extremely difficult. Consequently, the functions of many genes in DLBCLs (may have an important role in lymphomagenesis or disease progression) are poorly identified. To overcome these obstacles, we established the investigation model of B cell lymphoma. There are two method that we suggested for the study. One is viral transduction, the other is electroporation. Viral transduction method is compatible for stable cell lines to knockout genes-of-interest, and electroporation method is compatible for transient cells to knockout. Thus, this system allows us to manipulate genes in B cells with high efficiency and provide novel insight into therapeutic modalities for the DLBCL.

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Abstract in Korea

확산성 B 세포 림프종(DLBCL)은 비호지킨 림프종의 가장 흔한 조직 학적 아형이며, 다양한 분자적 수차와 다양한 임상 결과를 가진 이질성 질환이다. 현재 DLBCL의 치료적 진보가 이뤄지고 있음에도 불구하고, 아직 환자의 40%는 지속적인 완화에 실패하여 재발성/난치성 질환을 보인다. DLBCL 환자의 치료 효과를 더욱 향상시키고 발전시키기 위해서는, DLBCL의 유전자 기능 연구가 필수적이다. 그러나 지금까지 DLBCL의 많은 유전자 기능은 인간 B 세포에서 유전자 조작의 어려움으로 인해 완전히 밝혀지지 않았다. 이 연구에서, 우리는 CRISPR-Cas9 시스템을 기반으로 한 Measles virus glycoprotein displaying lentiviral 형질 도입방법을 이용하여, B 세포 림프종 발달의 분자적 기작을 밝히기 위한 *in vitro* 모델을 확립하였다. 우리는 Measles virus 시스템을 사용하여 조작하기 어려운 DLBCL에 Cas9를 형질 도입했다. 우리는 Western blot을 이용하여 Cas9발현을 확인하였고, 성공적으로 DLBCL로 형질 도입된 것을 확인하였다. Cas9의 활성은 GFP-BFP 리포터를 이용하여 확인하였다. Cas9의 활성은 GCB-DLBCL에서 90 % 이상, ABC-DLBCL에서 10-50 % 사이인 것을 확인하였다. 우리는 B 세포에서 안정적으로 발현되는 CD45 유전자 제거를 시험하였다. 이 방법에는 2가지가 있는데, 하나는 수명이 긴 세포주에 적합한 viral transduction 방법이고, 다른 하나는 primary 세포 또는 분화하는 세포와 같은 수명이 짧은 세포에 적합한 electroporation방법이다.

이러한 시스템을 통하여, 우리는 B 세포의 유전자를 고효율로 조작할 수 있었으며, 이는 DLBCL의 치료 방법에 대한 새로운 통찰력을 제공 해 줄 것이다.