Review

Gene targeting using the human Nalm-6 pre-B cell line

Noritaka Adachi[1,*], Hitoshi Nishijima[2,3], Kei-ichi Shibahara[2,3,*]

[1] *International Graduate School of Arts and Sciences, Yokohama City University, Yokohama, Kanagawa, Japan;*
[2] *Department of Integrated Genetics, National Institute of Genetics, Mishima, Shizuoka, Japan;*
[3] *The Graduate University for Advanced Studies, Mishima, Shizuoka, Japan.*

Summary **Gene targeting by homologous recombination is a powerful tool to precisely manipulate the genome in order to study the function of a gene of interest (*GOI*). Indeed, it has become a routine methodology in yeasts, murine embryonic stem cells, and a chicken DT40 cell line. However, gene targeting has not been used often in human somatic cells to date since the relative efficiency of gene targeting (the ratio of homologous integrations to random integrations) is remarkably low. In this review, we introduce a fundamental strategy and a protocol to generate a null allele and/or 'tetracycline-inducible conditional gene knockout' for the *GOI* by gene targeting in the human Nalm-6 pre-B cell line. The Nalm-6 is a rare cell line in which gene targeting by homologous recombination takes place efficiently, and it carries a stable near-diploid karyotype with a doubling time of around 20 h. In addition, the tetracycline-regulated gene depletion (Tet-Off) system is steadily applicable to this cell line. Therefore, gene targeting systems using the Nalm-6 cell are used increasingly and offer promise in the study of human gene functions. This review should prove useful to researchers in a wide rage of fields.**

Keywords: Gene targeting, Gene knockout, Nalm-6

1. Introduction

Although RNA interference is now used routinely, gene targeting by homologous recombination is a powerful approach to studying gene function. However, homologous recombination in human somatic cells occurs infrequently except for in a limited number of cell lines including the HCT116 colon cancer cell line (*1*) and the Nalm-6 pre-B cell line (*2*) (Figure 1A). The Nalm-6 cell line, originally established from the peripheral blood of a patient with acute lymphoblastic leukemia (*3*), is highly efficient for gene targeting by homologous recombination (*2*,*4*,*5*). It also carries a stable near-diploid karyotype (Figure 1B) (*6*), unlike the HCT116 cell line. In addition, a tetracycline-regulated gene depletion (Tet-Off) system (*7*), a highly convenient inducible gene expression system, can be efficiently applied to this cell line with some technical improvements (Ono *et al*., submitted). A 'tetracycline-inducible conditional gene knockout' can thus be generated for a human gene of interest (*GOI*) in this cell line, in which the expression of the *GOI* can be depleted in a tetracycline-dependent manner

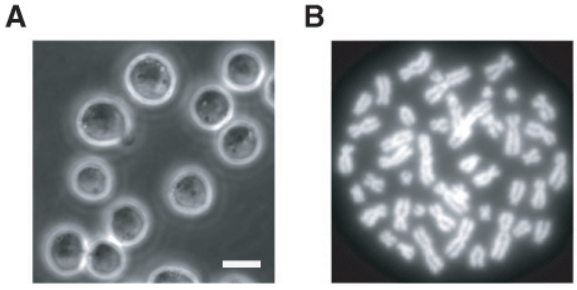

Figure 1. (A) Phase contrast images of the living Nalm-6 human pre-B cell line. Scale bar = 10 μm. (B) Staining metaphase chromosomes of the Nalm-6 cell with 4',6-diamidino-2-phenylindole (DAPI), showing the chromosome number of 2N = 46.

**Correspondence to:* Dr. Kei-ichi Shibahara, Department of Integrated Genetics, National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan; e-mail: kshibaha@lab.nig.ac.jp; Dr. Noritaka Adachi, International Graduate School of Arts and Sciences, Yokohama City University, Seto 22-2, Kanazawa-ku, Yokohama 236-0027, Japan; e-mail: nadachi@yokohama-cu.ac.jp

on a knockout background. This method is useful and potentially applicable to any human genes, and thus gene-targeting systems using the Nalm-6 cell line will be used popularly in the near future in the study of human gene function.

2. General strategy

The general strategy described in this review uses a replacement-type vector as a targeting vector. The standard features of replacement-type vectors are a plasmid backbone containing a positive selection cassette placed between two regions of chromosomal homology (these regions are termed the 'left arm' and 'right arm' hereafter, they are also called the '5'-arm' and '3'-arm' for their position, or the 'long arm' and 'short arm' for their length) and a negative selection cassette adjacent to one of the homologous arms (*8*). With those negative and positive selections, the efficiency of homologous recombination between the targeting vector and the endogenous locus in the Nalm-6 cell line increases to a tractable range (the ratio between homologous recombinants and random integrants is around 1-5%). To design the targeting vector, a gene locus and the exon-intron structure of the *GOI* need to be defined. The University of California at Santa Cruz's Genome Browser web site is an extremely convenient way to obtain almost all of the information required for these purposes.

Unless the *GOI* is an essential gene, the first desired mutation to generate is a null allele. If protein-coding exons span a relatively small genomic distance (< 10 kb), almost all of the protein coding exons can be deleted. Using this strategy, the generation of a functional null allele is almost guaranteed because it is almost impossible to generate a protein product from the target locus. However, if the protein-coding exons of the *GOI* span a relatively large distance (greater than > 10 kb), more thought is required to determine which regions of the *GOI* should be deleted because conventional gene targeting strategies are less efficient at generating very large deletions (data not published). The first strategy suggested is to generate up to a 5-kb deletion (hopefully less than 3 kb) to remove as many of the protein coding exons as possible, including the exon containing the translation initiation codon (Figure 2A). Although this does not always guarantee the generation of a null allele, it increases its likelihood. Additionally, since neighboring genes may lie very close to the *GOI*, care must be taken not to delete portions beyond the known sequence of the gene. The second strategy suggested is to delete a coding region that encodes crucial functions of the proteins (Figure 2B). Again, the size of the deletion is limited up to 5 kb, and an in-frame stop codon can be placed in the downstream primer of the left arm when the left arm resides in the exon (Figure 3).

When the *GOI* is an essential gene or compensation experiments are required, a 'tetracycline-inducible conditional gene knockout' needs to be generated in which expression of the *GOI* can be depleted in a tetracycline-dependent manner on a knockout background. To generate a tetracycline-inducible conditional null allele, the tetracycline-inducible expression cassette of the *GOI* needs to be introduced into the cells before or after the targeting of the first allele, as outlined in Figure 5. A full-length protein or a tag-conjugated full-length protein can be expressed as the expression cassette of the *GOI* depending on plans for subsequent experimentation. Often expressed are FLAG-HA-tagged fusion proteins for the subsequent immuno-affinity purification of the functional protein complex or green fluorescence protein (GFP) conjugates to examine their cellular distribution. Expressing tagged-fusion proteins, instead of full-length proteins, is also beneficial because it facilitates confirmation of the tetracycline-regulated expression of the transgenes by Western blot analysis, as described in section 6.4. However, the most critical point here is that tagged-fusion proteins must be functionally intact to rescue the knockout phenotype.

3. Design of the targeting vector

3.1. *Finding the target locus to be deleted*

This is the most critical step to design the targeting vector as described above.

3.2. *The size and location of the target arms*

One of the most important parameters of the gene-targeting vector that influences targeting efficiency at a given genetic locus is generally assumed to be the length and the sequence identity of the homologous DNA regions on the vector (*9*). Shorter homologous arms might reduce the targeting efficiency and longer homologous arms hamper the construction of the targeting vector. In Nalm-6 cells, gene targeting is practicable with relatively shorter arms. The shorter arm should be 1.0~2.5 kb and the longer arm should be 2.5~6.0 kb in the Nalm-6 system. Genomic PCR is usually performed as the first screening to select the targeted clones, and thus one of the primer for the PCR is preset inside the drug resistance cassette (Figure 2). Given the efficacy of PCR amplification, the length of the DNA fragment amplified in a mass screening of PCR should be less than 3 kb (see section 6.3.2). Homologous arms generally include intron sequences or other untranslated segments of the endogenous gene. Untranslated regions of the genes often contain stretches of interspersed repetitive DNA that might influence targeting efficiency. Those repetitive sequences can be sought using the Human Genome Browser Gateway (*http://genome.ucsc.edu/cgi-bin/hgGateway*). Merrihew *et al*. (*10*) proposed an idea that random integration into the

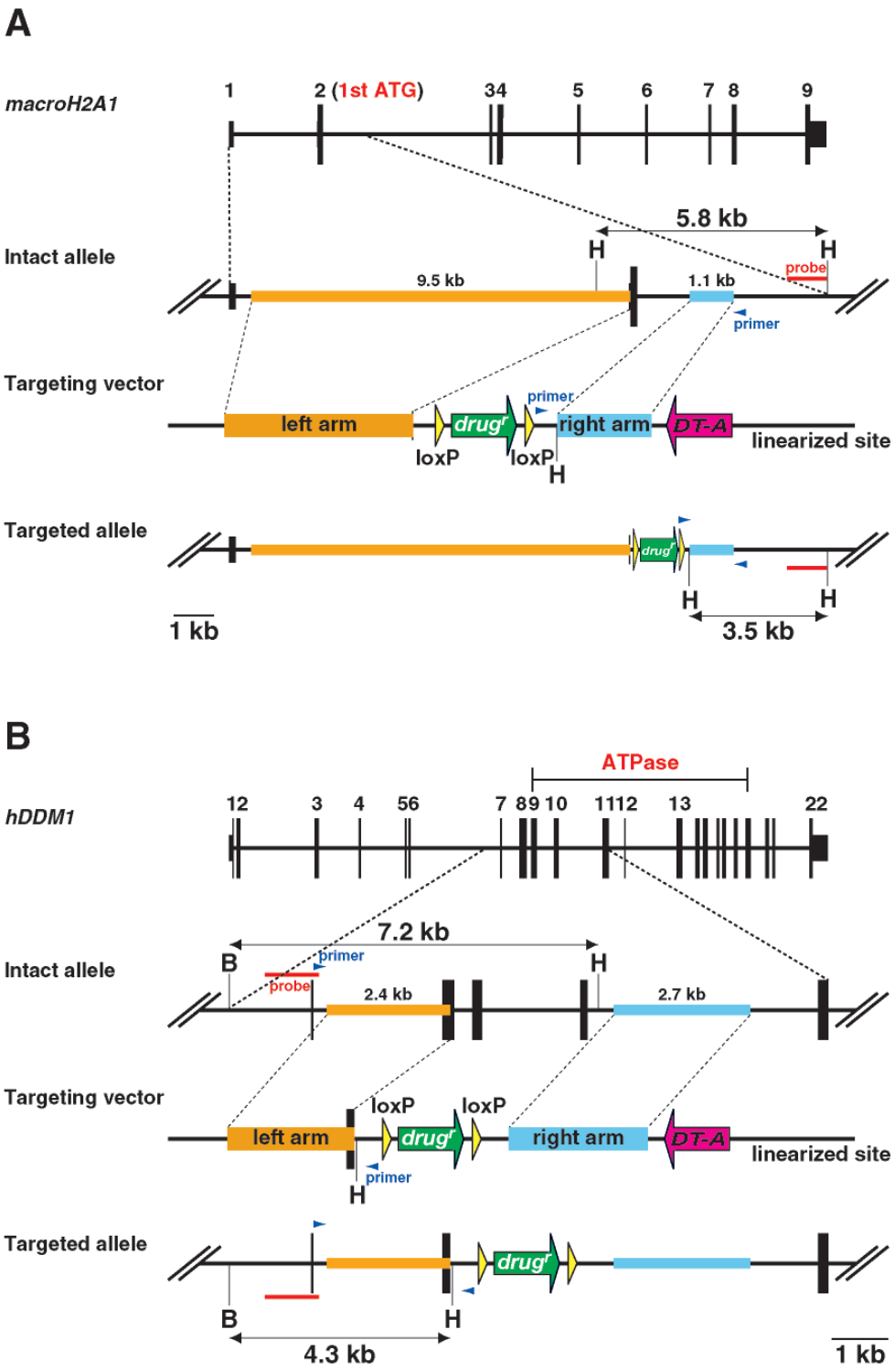

Figure 2. The strategy of knockout of the *GOI* in a Nalm-6 cell line. (A) An example of deletion of the exon containing the translation initiation codon. Diagrams to show the disruption of the *macroH2A1* locus. The *macroH2A1* gene consists of 9 exons with a histone fold (exon 2-4) and macro domain (exon 6-9). Codons coding methionine exist in exon 2 and exon 9. Therefore, gene-targeting vectors were constructed to delete the first ATG in exon 2. Black thin lines and closed boxes represent genomic DNA and exons, respectively. Orange and cyan box represent the left arm and the right arm of the targeting vector, respectively. The *drug*^{r} (green arrow box) and *DT-A* (magenta arrow box) cassettes are used for positive and negative selection, respectively. Through homologous recombination, exon 2 (including the 1st ATG) and introns are replaced with drug-resistant genes (*drug*^{r}): the histidinol resistance (*his*^{r}) gene for the first targeting, and the hygromycin resistance (*hyg*^{r}) gene for the second targeting. Yellow triangles represent *lox*P sequences, so *drug*^{r} could be removed from the targeted allele, if needed. In Southern blot analysis, the probe and expected bands are shown by a thick red line above the wild-type allele and double pointed arrows with their sizes and restriction sites: H for *Hin*dIII. In genomic PCR, the locations of the primers are indicated as blue arrowheads. (B) An example of the deletion of functional important coding exons. Diagrams to show the disruption of the human homolog of a decrease in DNA methylation 1 (*hDDM1*) locus. The *hDDM1* gene consists of 22 exons with a conserved ATPase domain extending from exon 9 to exon 19. Therefore, gene-targeting vectors were constructed to delete part of exon 8 and all of exons 9/10, in which the left arm harbored the stop codon in the frame and other frames (Figure 3); translation of *hDDM1* mRNA derived from targeted allele should be stopped. Through homologous recombination, a part of exon 8 and entire exons 9/10 including introns are replaced by *drug*^{r}. The symbols and terms used are the same as in A except where noted. The *puro*^{r} gene is used for the second targeting. The restriction sites B for *Bam*HI and H for *Hin*dIII are used for Southern blot analysis. These figures are drawn to scale.

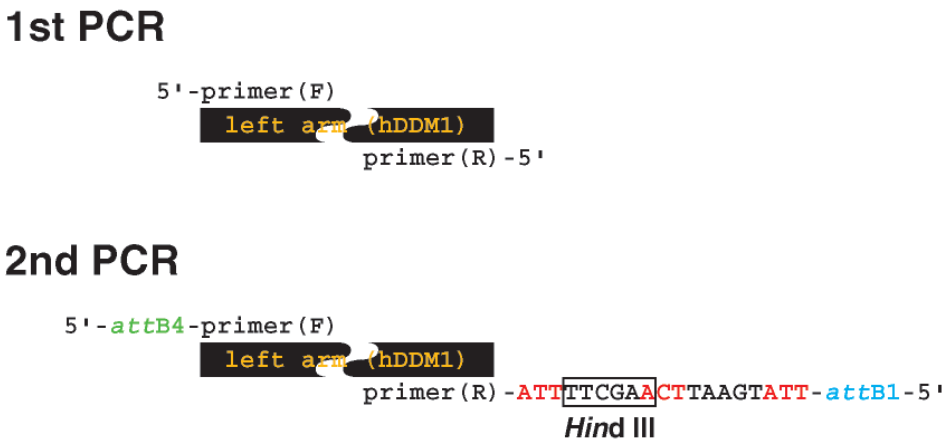

Figure 3. Primer design to amplify the DNA fragment with additional appended sequences using two-step PCR for the arm of the targeting vector. In the first step, the target genomic region is amplified using the primers simply corresponding to genome sequences. In the second step, the arm fragment is re-amplified using the primers with additional sequences: *att*B site for the MultiSite Gateway system (Figure 4), stop codon for the translation stop, and restriction enzyme site for Southern blot analysis. In this case, three frame stop codons (red characters) and a restriction enzyme site (*Hin*dIII, boxed AAGCTT) are appended at the 5'-end of the reverse primer for the left arm. The *att*B sequences differ from one another: *att*B4 sequence (green character) for the forward primer of the left arm, *att*B1 sequence (cyan character) for the reverse primer of the left arm, *att*B2 sequence for the forward primer of the right arm, and *att*B3 sequence for the reverse primer of the right arm.

genome was stimulated by short homological sequences. It is suggested that highly repetitive sequences should be excluded from the homologous arms as much as possible. However, the impact of repetitive sequences on gene targeting is a difficult issue, since it was reported that a flanking repetitive sequences enhanced the homologous recombination (*11*). Gene targeting of the *GOI* with pseudogenes or highly homologous genes often causes complications, Therefore, special care is needed in those cases. A BLAST search of the cDNA of the *GOI* or the arm sequences should always be run against the human genome assembly (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) before finally deciding the arms. The sequence of the arms should be as identical as possible to the corresponding genomic regions, but for convenience the arms can, using the genomic DNA prepared from Nalm-6 cells as a template, be amplified by genomic PCR using proof-reading taq polymerases. Even with these proof-reading polymerases, amplification of the genomic regions often results in sequence errors, and thus the best clone with the sequence matching the corresponding genomic regions is chosen from the database.

3.3. *Positive selection cassette*

There are several positive selection marker genes available, including neomycin, hygromycin, puromycin, and histidinol resistance genes. The promoter for the marker expression used in the Nalm-6 system is mouse phosphoglycerate kinase (PGK) promoter or chicken β-actin promoter. Virus-derived promoters such as SV40 (Simian virus 40)-derived promoters should be avoided because some virus-derived promoters do not ensure stable expression in Nalm-6 cells (unpublished data). The cassette can be placed in either forward or reverse orientation relative to the direction of the *GOI*. The presence of a selectable marker cassette with its exogenous promoter may influence gene expression at the targeted locus or its surrounding genes. To avoid these unexpected and undesirable possibilities, the marker gene cassette should be flanked by loxP sequences or FRT sequences to provide the option for the subsequent removal of the marker gene cassette with Cre recombinase (*12*) or Flp recombinase (*13*). In addition, this option provides the opportunity to remove the selection marker cassette from the targeted locus for the subsequent targeting of an additional gene(s) to generate double or triple gene knockout cell lines. A point of note is that a "promoterless" drug resistance cassette is often used efficiently; the exons proximal to a promoter region are replaced and an endogenous promoter for the *GOI* is borrowed for the expression of the drug resistance gene (*4*).

3.4. *Negative selection cassette*

There are primarily two different ways of negative selection commonly used in gene targeting; these are used to preferentially kill the cells in which the targeting construct is incorporated in a random fashion. The first is a herpes simplex thymidine kinase gene expression cassette with gancyclovir or FIAU (1-2-deoxy-2-fluoro-1-β-D-arabinofuranosyl-5-iodouracil). An alternative strategy is to express the diphtheria toxin A-chain (DT-A) gene (*14*). Use of DT-A is preferred by the current authors because no drug need be added to the culture medium. The negative selection cassette can be placed in either forward or reverse orientation relative to the direction of *GOI* transcription. However, an important point is that even after positive/negative selection many of the resulting colonies are nontargeted clones (random integration or ectopic homologous recombinants; see 7. Troubleshooting).

3.5. *Linearization of the targeting vector*

The targeting vector must be linearized outside of the regions of homology before introduction into the Nalm-6 cells, and thus adequate restriction sites should be prepared in advance. The current authors usually place the restriction sites outside the negative selection cassette, as outlined in Figure 2 (see section 6.2.3).

3.6. *Southern blot analysis*

In the course of design of the targeting vector, genomic regions that can serve as Southern blot probes to recognize targeting events must also be determined. Southern analysis is performed with probes that are external to the regions of homologous arms in the targeting vector (Figure 2). Therefore, an important aspect to consider is restriction enzyme sites that can be used to discriminate between targeted and nontargeted alleles prior to construction of the targeting vector. Not all enzymes provide complete DNA restriction, and thus a pilot reaction with the enzyme of choice should be performed before screening is started. For convenience, ideal restriction enzyme sites are introduced in the downstream 3' primer to amplify the left arm or the upstream 5' primer to amplify the right arm, as shown in Figure 2. These restriction enzyme sites should be located outside the genomic region of the homologous arm and also at the genomic region to be disrupted (Figure 2). In this way, diagnostic digestion will yield a smaller DNA fragment for the mutant allele to serve as the recognized external probe. For the clear resolution of the DNA in agarose gels, the agreeable length of DNA fragment would be less than 15 kb. The length of the probe is also important for the efficiency of random oligo labeling (see section 6.2.4), and thus a 300~1,000 bp fragment is usually generated for the probe. Another step to take is avoiding the inclusion of highly-repetitive sequences in the probe and checking the probe sequence against the human genome assembly to exclude pseudogene loci and genetic regions with highly identical sequences. The probe is obtained by PCR amplification and its sequence confirmed before use.

3.7. *Primer design*

Genomic regions are amplified in two steps. In the first step, the genomic regions are simply amplified using the primers with 20-25 nucleotide sequences corresponding to the targeting locus. Sequences of primer with 55~68°C of the calculated Tm value and with G or C at the 3′ end are selected. Genetic analysis tools are also used to avoid primer sequences that tend to form a primer-primer dimer or hairpin-like structures as much as possible. The amplified DNA fragments are always confirmed by sequencing and/or restriction mapping analysis. In the second step, the confirmed DNA fragment is re-amplified with primers harboring additional sequences required for subsequent analysis. For instance, an in-frame stop codon and restriction sites for Southern blot analysis are added at the 5′ ends of the reverse primer for the left arm and the forward primer for the right arm (Figure 3). In addition, *attB* sequences are added to all of the primers at their 5′ ends when the MultiSite Gateway System is used to construct the targeting vector (see section 6.2.3).

4. Conditional gene targeting

Conditional gene targeting, in which gene disruption or gene inactivation occurs in a conditional manner, is especially valuable when null mutations are lethal or compensation experiments are required. The two main strategies for conditional targeting are using an inducible gene expression system such as a tetracycline-inducible gene expression system (Tet-Off system) or an inducible recombination such as *loxP* sequences and Cre site-specific recombinase (Cre/*loxP* system), CreMer, which carries a mutated hormone-binding domain of estrogen receptor that binds to the antagonist 4-hydroxytamoxifen (*15*). Both systems have advantages and disadvantages. For instance, the Tet-Off system is more tractable and suppression of gene expression can be achieved in a more synchronous manner. In addition, depleted gene product can be rapidly recovered by removing tetracycline. On the other hand, the Cre/*loxP* system can generate null alleles in principle and allows the use of endogenous promoters. However, Cre-mediated deletion does not occur synchronously and thus may not occur in all of the tamoxifen-treated cells (*16*). The Tet-Off system can be used efficiently in the Nalm-6 cell line with technical modifications (Ono *et al*., submitted). Use of the Tet-Off system is therefore recommended in Nalm-6 cell lines as the first choice to render cells conditionally null for the *GOI* because depletion is more synchronous and the system is more tractable.

5. Materials

5.1. *Web resources*

1. Human Genome Browser Gateway: *http://genome.ucsc.edu/cgi-bin/hgGateway*
2. NCBI BLAST Home: *http://blast.ncbi.nlm.nih.gov/Blast.cgi*

5.2. *Cell cultures*

1. Medium: 500 mL ES medium (Nissui, Tokyo, Japan) supplemented with 50 mL (10%) fetal bovine serum (FBS; Kohjin-Bio, Saitama, Saitama, Japan; *Note*: tetracycline-negative!), 5 mL (1%) penicillin-streptomycin solution (Sigma-Aldrich,

St Louis, MO, USA), 5 mL (2 mM) glutamine (Sigma-Aldrich), and 50 μM 2-mercaptoethanol (2-ME). *Note*: Adding too much 2-ME causes poor growth of cells!
2. Petri dish for suspension culture (10 cm × 2 cm; Greiner Japan, Tokyo, Japan).
3. Cell banker for frozen stock of cells (Nihon Zenyaku, Fukushima, Japan).

5.3. *Construction of the targeting vector*

1. GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich)
2. Proteinase K solution (Sigma-Aldrich)
3. RNase A solution (Sigma-Aldrich)
4. GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA)
5. PrimeSTAR HS DNA polymerase (Takara Bio, Ohtsu, Japan)
6. Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA)
7. PCR-Script Amp Cloning Kit (Stratagene, La Jolla, CA, USA)
8. Chemically competent DH5α or XL10-Gold cells
9. TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA
10. QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan)
11. MultiSite Gateway System (Invitrogen, Carlsbad, CA, USA)

5.4. *Gene targeting screening*

1. Transfection: Nucleofector I (Amaxa Inc., Gaithersburg, MD, USA), Nucleofector Kit T (Amaxa Inc.).
2. Drug screening: 1.0 mg/mL of L-histidinol (Sigma-Aldrich), 0.2 μg/mL of puromycin (BD Biosciences, San Jose, CA, USA), 1.0 mg/mL of G418 (GIBCO, Grand Island, NY, USA), or 0.35 μg/mL of hygromycin B (Clontech, Mountain View, CA, USA). *Note*: Optimal concentrations for drug screening should be checked beforehand.
3. 96-well, 24-well, and 6-well flat-bottom plates for suspension culture (Sumitomo Bakelite Co. LTD, Tokyo, Japan).
4. 96-well V-bottom plate (Nunc, Roskilde, Denmark)
5. 8-channel and 12-channel pipettes (Thermo Scientific Inc., Rockford, IL, USA)
6. Lysis buffer for genomic DNA preparation: 100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 μg/mL RNase A, and 100 μg/mL Proteinase K.
7. Plate seal 1 (Nunc)
8. Aluminum plate seal 2 (Simport, Beloeil, QC, Canada)
9. Optical 96-well reaction plate and MicroAmp 8-cap strip (ABI)
10. Takara Ex Taq (Takara Bio)
11. Takara LA Taq (Takara Bio)
12. Gel-loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in water
13. Biodyne B membrane (Pall, Pensacola, FL, USA)
14. Denaturation solution (for Southern): 1.5 M NaCl, 0.5 M NaOH
15. Neutralization buffer (for Southern): 1.5 M Tris-HCl (pH7.4), 1.5 M NaCl
16. Megaprime DNA labelling systems (GE Healthcare, Piscataway, NJ, USA)
17. Illustra ProbeQuant G-50 micro columns (GE Healthcare)
18. Hybridization oven HI-80M (Kurabo, Osaka, Japan)
19. Hybridization solution: 6× SSC, 5× Denhardt's reagent, 0.5% SDS, 50% formamide, 100 μg/mL salmon sperm DNA
20. [α-^{32}P] dCTP

6. Protocols

6.1. *Culture of Nalm-6 cells*

The Nalm-6 cell line is maintained in the medium described in section 5.2. The doubling time of the cells is around ~20 h and thus the cells 1:10 were diluted in three days. The cells grow to a maximum of 1.5 × 10^6 cells/mL in a logarithmic manner, but cells were kept under a concentration of 0.1~0.8 × 10^6 cells/mL. Cloning efficiency of the cells is quite good and the cells can be diluted when needed.

6.2. *Construction of the targeting vector and preparation of Southern probe*

6.2.1. *Preparation of genomic DNA*

The genomic DNA was prepared manually or by using the GenElute Mammalian Genomic DNA Miniprep kit. Starting cells are usually 5 × 10^6~1 × 10^7. Other kits or any manual protocols can also be used to prepare genomic DNA.

6.2.2. *Amplification of arms*

For the amplification of the genomic fragments, proofreading polymerase (with a 3′-5′ exonuclease activity) should be used. Here, PrimeSTAR HS DNA polymerase was used. A typical reaction is as follows:

1. Prepare a 50 μL reaction mixture as follows:

5× Polymerase buffer	10.0 μL
Genomic DNA (section 6.2.1)	2.0 μL (50 ng)
Forward primer (fin. 0.2 μM)	0.5 μL
Reverse primer (fin. 0.2 μM)	0.5 μL
dNTP (fin. 0.2 mM each)	4.0 μL
PrimeSTAR HS DNA polymerase	0.5 μL (1.25 U)
MilliQ water	32.5 μL

2. PCR program:

98°C	3 min	
98°C	10 sec	] 30 cycles
55°C	5 sec	
72°C	1 min/kb	
4°C	∞	

3. After checking and confirming the amplification of the DNA fragment through agarose gel electrophoresis, an amplified DNA band with an expected size is cut and purified by Wizard SV Gel and PCR Clean-Up System. *Note*: A recommended final elution volume is 50 μL according to the manufacturer's protocol, and ethanol precipitation is often performed here to concentrate DNA for subsequent cloning.
4. The purified DNA fragment is cloned into pPCR-Script Amp SK(+) cloning vector, as described in the manufacturer's protocol. For transformation, chemically competent DH5α or XL10-Gold cells were used. *Note*: Confirm the sequence of the cloned DNA because sequence matches influence targeting efficiency.

6.2.3. *Construction of the targeting vectors with the MultiSite Gateway System*

The MultiSite Gateway System, as mentioned previously (Figure 4; *ref. 17*), is quite useful at constructing targeting vectors, and the following is an example of constructing targeting vectors with this system. In this system, four DNA fragments of the left arm, the drug resistance expression cassette, the right arm, and the negative selection cassette/the plasmid backbone were conjugated efficiently in a one-tube reaction (Figure 4). In addition, two targeting vectors each containing different drug resistance gene cassettes are usually required to target the 1st and 2nd allele. Actually, three different targeting vectors can be generated simultaneously with the same arms but different drug resistant cassette vectors, as shown in Figure 4.

Step I:

1. Re-amplify the arm fragment with the primers containing attB sequences to fit the Gateway recombination system (Figure 4).
2. Prepare 10 μL reaction mixtures as follows:

	Left Arm	Right Arm
pDONR vector	50 fmoles (P4-P1R)	50 fmoles (P2R-P3)
Re-amplified PCR product	50 fmoles	50 fmoles
TE buffer	to 8.0 μL	to 8.0 μL

3. Add 2.0 μL BP Clonase II enzyme to the reaction mixture and mix well.
4. Incubate at 25°C for 1 h.
5. Add 1.0 μL Proteinase K and incubate at 37°C for 10 min to stop reaction.
6. Transformation of DH5α competent cells with 1 μL reaction mixture. *Note*: Transformants should be spread on pre-warmed LB plates containing 50 μg/mL kanamycin.
7. Prepare the plasmid DNA using the QIAprep Spin Miniprep Kit and select appropriate recombinant plasmid DNA by restriction enzyme digestion.
8. Confirm the sequence of the prepared recombinant plasmid DNA in this step and the resulting plasmids are termed as 5'-Entry clone and 3'-Entry clone.

Step II:

1. Prepare 20 μL reaction mixtures as follows:

Destination vector (pDEST DTA-MLS)	25 fmoles
Entry vector (pENTR lox-hisD)	25 fmoles
or (pENTR lox-puro)	25 fmoles
5'-Entry clone (L4-left arm-R1)	25 fmoles
3'-Entry clone (R2-right arm-L3)	25 fmoles
5× LR Clonase Plus Reaction Buffer	4.0 μL
TE buffer	to 16.0 μL

2. Add 4 μL LR Clonase Plus enzyme mix to the reaction mixture and mix well.
3. Incubate at 25°C for 16 h.
4. Add 2 μL Proteinase K solution and incubate for 10 min at 37°C to stop reaction.
5. Transformation of XL10-Gold competent cells with 1 μL reaction mixture. *Note*: Transformants should be spread on pre-warmed LB plates containing 100 μg/mL ampicillin.
6. Prepare the plasmid DNA using the QIAprep Spin Miniprep Kit and select appropriate recombinant plasmid DNA by restriction enzyme digestion.

6.2.4. *Preparation of southern probe*

For the amplification of the Southern probe, PrimeSTAR HS DNA polymerase should be also used. Basically subclone the amplified DNA fragment into the pPCR-Script Amp SK(+) cloning vector and confirm the sequence before use. The subcloned DNA fragment derived from pPCR-Script using the appropriate restriction enzyme was purified through agarose gel electrophoresis and stored.

6.3. *Targeting of the 1st allele of the GOI*

6.3.1 *Transfection of Nalm-6 cells and screening for drug-resistant colonies*

Day 0:

1. Linearize the targeting vector prepared in section

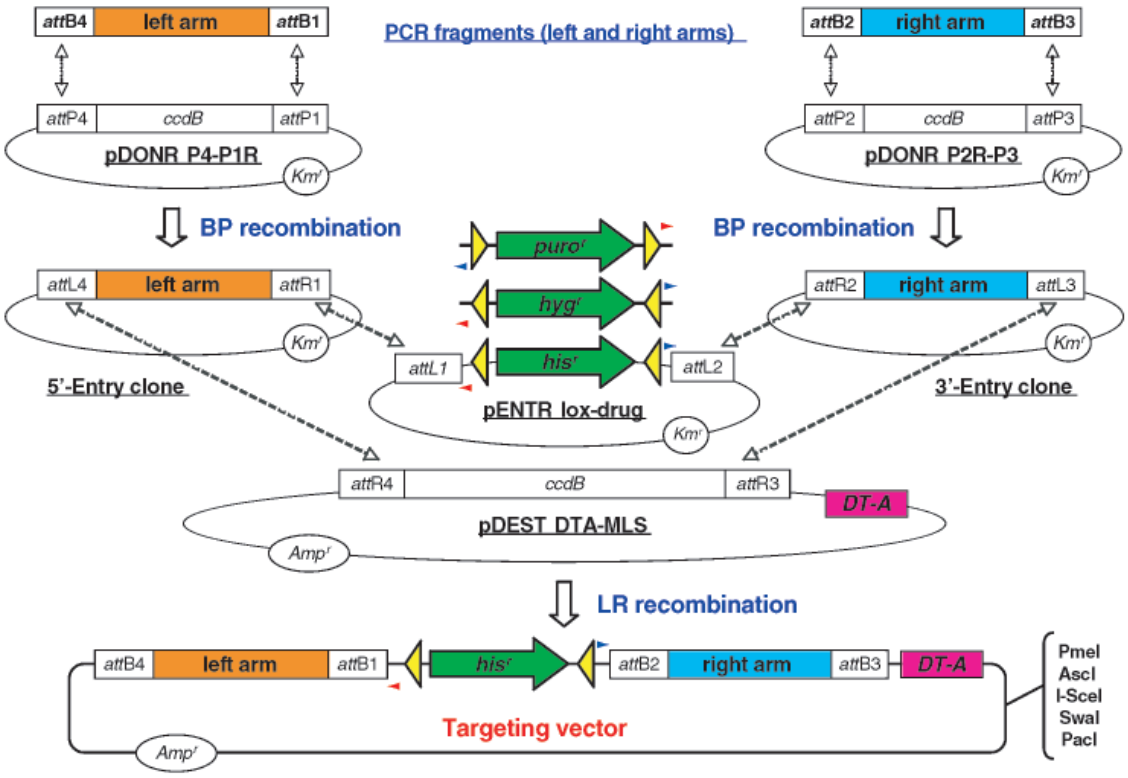

Figure 4. Diagram of the method of constructing a targeting vector based on the MultiSite Gateway system. This system consists of three steps: (1) PCR amplification with attB-containing primers, (2) BP recombination between each arm fragment and donor vector, and (3) LR recombination to construct the targeting vector using four DNA fragments. BP recombination is carried out between the left arm and pDONR P4-P1R donor vector and the right arm and pDONR P2R-P3 donor vectors, resulting in a 5'-Entry clone and 3'-Entry clone, respectively. In LR recombination, two targeting vectors for two rounds of targeting are generated containing different drug resistance gene cassettes; this was done using the same Entry clone but different drug resistant cassette vectors. Arrowheads indicate the two universal primers outside the *lox*P sequence. Yellow triangles represent *lox*P sequences for removal of the selection marker cassette from the targeted locus. *his*^{r}, a histidinol resistance gene; *hyg*^{r}, a hygromycin resistance gene; *puro*^{r}, a puromycin resistance gene; *DT-A*, a gene that codes for a diphtheria toxin A fragment; *Km*^{r}, a kanamycin resistance gene; *Amp*^{r}, an ampicillin resistance gene.

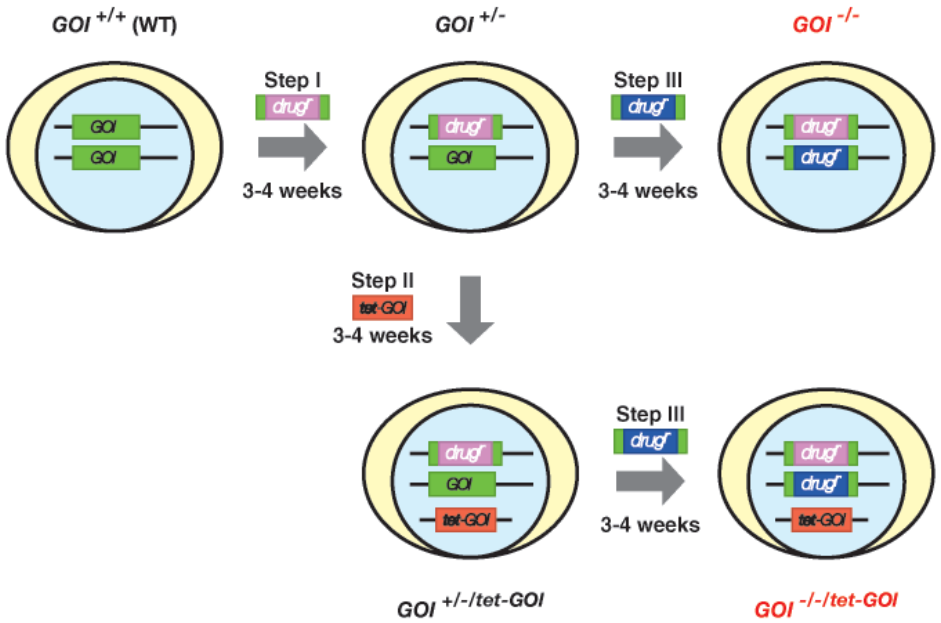

Figure 5. A flow chart for the generation of gene knockout human Nalm-6 cell clones. If the *GOI* is on the autosome, the cell harbors two alleles of the *GOI* (green box). Step I represents the first targeting of one *GOI* using the targeting vectors containing one drug resistant cassette (magenta box) to produce heterozygous *GOI*$^{+/-}$ cell clones, which takes 3-4 weeks. Step II is a process to obtain *GOI*$^{+/-/tet\text{-}GOI}$ cell clones expressing the exogenous *GOI* (orange box) in a tetracycline-regulated manner, which takes 3-4 weeks. This step is required only when tetracycline-inducible conditional gene-targeted cell clones are generated. Step III represents the second targeting of the other *GOI*, using the targeting vectors containing another drug resistant cassette (blue box), to generate homozygous *GOI*$^{-/-}$ or *GOI*$^{-/-/tet\text{-}GOI}$ cell clones, which also takes 3-4 weeks.

6.2, using a single cutter.
2. Digest overnight.

Day 1:

3. Perform phenol-chloroform extraction and ethanol precipitation and then rinse with ice-cold 70% ethanol.
4. Dry the DNA on a clean bench and suspend the DNA pellet in sterile MilliQ water.
5. Check the concentration of the DNA before transfection.
6. Pre-warm the complete culture medium (0.5 mL per one sample) in a 50 mL tube at 37°C.
7. Pre-warm the Nucleofector Solution T at room temperature (RT).
8. Perform a cell viability check for Nalm-6 cells. *Note*: Cells for transfection should be healthy and 2 × 10^6 cells per each transfection are required.
9. Harvest the cells by centrifugation at 90 g for 10 min at 22°C and completely aspirate the supernatant (Do not lose cells because cell pellets are less-tightly packed in this step!).
10. Suspend the cells with 100 μL of Nucleofector Solution T.
11. Add the linearized plasmid DNA (up to 2 μg) to the suspended cells and transfer the cell/DNA mixture into an Amaxa certified cuvette.
12. Perform transfection with Nucleofector I using the C-05 program of the machine.
13. Quickly add 500 μL pre-warmed culture medium into the cuvette and mix it well but slowly.
14. Transfer the cell/DNA mixture to a 6-well plate with a pipet.
15. Culture the cells in a humidified 5% CO_2 incubator at 37°C for 24 h.

Day 2:

16. Prepare the screening medium containing a selection drug at the appropriate concentration. *Note*: The optimal concentration of the drug should be determined whenever new lots of the drug are used!
17. Count the cells to check the viability of the transfected Nalm-6 cells. *Note*: Around 80% cell viability is preferred.
18. Seed the cells at 1,000-2,000 viable cells per well in the screening medium on 96-well flat-bottom plate.

Days 14-21:

19. After 2-3 weeks of culturing, drug-resistant colonies were selected and transferred to new 96-well flat-bottom plates.
20. These cell colonies were grown for the preparation of genomic DNA.

6.3.2. *Preparation of genomic DNA*

1. Transfer 150 μL cell (around 5 × 10^4 cells) suspensions from the 96-well flat-bottom plate to a 96-well V-bottom plate. *Note*: Wells on a flat-bottom plate should be filled with new medium and cells should then be cultured.
2. Centrifuge at 1,000 rpm (190 g) for 5 min at 4°C, and discard the supernatant by turning the plate over on a paper towel.
3. Add 50 μL lysis buffer per well, and seal the plate with a plate seal 1.
4. Mix the cells completely but gently using a plate mixer.
5. Incubate the mixtures at 55°C for more than 60 min.
6. After incubation, add 16 μL of 5 M NaCl per well, and mix them completely but gently using the plate mixer (Low speed, 1/3 max, 5 sec).
7. Add 70 μL 2-isopropanol per well, seal the plate with an aluminum plate seal 2, and mix vigorously using the plate mixer.
8. Centrifuge at 3,500 rpm (2,380 g) for 15 min at 4°C and discard supernatants by turning the plate over on a paper towel.
9. Resuspend the DNA pellet with 100 μL of ice-cold 70% ethanol per well, centrifuge at 3,500 rpm (2,380 g) for 5 min at 4°C, and discard supernatants by turning the plate over on a paper towel.
10. Dry for 10 min at RT.
11. Add 100 μL of TE buffer per well, seal the plate with a plate seal 1, and mix for more than 30 min using the plate mixer to completely dissolve the DNA pellet.
12. Store at 4°C for subsequent analysis.

6.3.3.*Target screening (Genomic PCR)*

1. Prepare a reaction pre-mixture as follows:

10× Ex Taq buffer	2.0 μL (×sample number)
Forward primer (100 μM)	0.1 μL (×sample number)
Reverse primer (100 μM)	0.1 μL (×sample number)
dNTP mixture (2.5 mM each)	1.6 μL (×sample number)
Ex Taq polymerase (5 units/μL)	0.1 μL (×sample number)
MilliQ water	15.1 μL (×sample number)
	19.0 μL (×sample number)

2. Add 1 μL of the genomic DNA prepared in section 6.3.2. to a MicroAmp Optical 96-well reaction plate.
3. Add 19 μL of well-mixed reaction mixture to the 96-well plate and mix it gently by pipetting, using the 8-channel multipipette.
4. Seal the plate tightly with MicroAmp 8-cap strip.
5. Perform PCR with the GeneAmp PCR System 9700 as follows:

98°C	5 min	
98°C	5 sec	] 40 cycles
65°C	30 sec	
68°C	1 min/kb	
72°C	5 min	
4°C	∞	

6. Check the PCR screen through agarose gel electrophoresis.
7. Select appropriate clones and transfer to a 6-well flat-bottom plate and then to a 10-cm plate for subsequent preparation of genomic DNA for Southern blotting. *Note*: If the target DNA fragment was not amplified, a different DNA polymerase could be used, such as LA taq, or parameters for PCR could be changed. If possible, several primers should be prepared.

6.3.4. *Target screening (Genomic Southern blotting)*

Day 0: Preparation of genomic DNA

The genomic DNA for Southern blotting is prepared from cell clones grown in a 24-well flat-bottom plate ~ 10-cm plate, as described in section 6.2.1.

1. Digest 10-20 μg genomic DNA with one or two restriction enzymes.

Day 1: Blotting

2. If necessary, concentrate the DNA fragments by ethanol precipitation and dissolve in ~15 μL of TE.
3. Add 0.15 vol of gel-loading buffer and separate the fragments of DNA by electrophoresis through an agarose gel (for most genomic DNAs, a 0.7% gel cast in 1× TBE may be used). Maintain a low voltage through the gel (about < 1 V/cm) to allow the DNA to migrate DNA slowly. *Note*: If the digested DNA has been stored at 4°C, it should be pre-heated to 56°C for 2-3 min! This heating disrupts any base pairing.
4. After electrophoresis is complete, stain the gel with ethidium bromide and photograph the gels. *Note*: Place a ruler alongside the gel in order to estimate the length of migrated DNA fragments.
5. Then soak the gel for 45 min at RT in a sufficient volume of denaturation solution with constant gentle agitation on a horizontal shaker.
6. Rinse the gel briefly in MilliQ water and then neutralize it by soaking for 30 min at RT in a sufficient volume of neutralization buffer with constant gentle agitation. Change the neutralization buffer and continue soaking for 15 min.
7. Use of the Biodyne B nylon membrane is suggested. Prepare the membrane as described (Molecular Cloning, 3rd edition).
8. Assemble the transfer apparatus and transfer the DNA to the prepared membrane with 0.4 M NaOH overnight, as described (Molecular Cloning, 3rd edition).

Day 2: Probe labeling

9. Labeling reaction: Prepare the following 50.0 μL reaction mixture.

probe	25 ng
random primers	5.0 μL
MilliQ water	to 33.0 μL

10. Incubate at 100°C for 5 min and transfer it to RT.
11. Add the following solutions to the mixture,

labeling buffer	10.0 μL
[α-^{32}P] dCTP	5.0 μL
Enzyme (Klenow fragment)	2.0 μL

12. Incubate at 37°C for 10 min.
13. Purify the labeled probe using the G-50 micro column (see Section 5.4).
14. Incubate the eluted DNA at 100°C for 5 min and rapidly cool it on ice for at least 2 min.

Day 2: Hybridization

15. Soak the membrane by floating it with a sufficient volume of neutralizing buffer in a tray for 15 min at RT.
16. Soak the membrane with a sufficient volume of 6× SSC in a tray for 5 min at RT.
17. Pre-hybridization: Place the membrane in a hybridization bottle containing 5 mL of hybridization solution, and incubate in a hybridization oven at 42°C for 2 h.
18. Pour off the hybridization solution from the hybridization bottle.
19. Hybridization: Add a 5 mL fresh hybridization solution containing a labeled probe, and incubate in a hybridization oven at 42°C over night.
20. Washing: wash the membrane as follows. *Note*: During washing, monitor the amount of radioactivity on the membrane using a hand-held Geiger counter!

In 2× SSC, 0.5% SDS	at RT for 5 min
In 2× SSC, 0.1% SDS	at RT for 5 min
In 0.1× SSC, 0.1% SDS	at 65°C for 30 min
In 0.1× SSC, 0.1% SDS	at 65°C for 1 h

21. Place the damp membrane on a sheet of plastic wrap, and expose the membrane to X-ray film for 36 h at –80°C with an intensifying screen.

6.4. *Isolation of stable cell clones expressing a tetracycline-inducible GOI*

As described in the section above, the most critical point here is to determine whether a full-length protein

or a tag-conjugated full-length protein is expressed to rescue the knockout phenotype. When a tagged-fusion protein is expressed, tetracycline-regulated expression of the transgenes is much easier to confirm with Western blot analysis, although tag-fusion may sometimes cause functional disability. cDNA is amplified by reverse-transcription PCR (RT-PCR) or can be purchased from Invitrogen (full-length cDNA of human genes is commercially available as Image clones). Detailed protocols for the applications of the improved Tet-Off system to the Nalm-6 cell line will be discussed in another opportunity (Nishijima *et al.*, in preparation).

6.5. *Targeting of the 2nd allele of the GOI*

To generate the homozygous null ($GOI^{-/-}$) or tetracycline-inducible conditional homozygous null ($GOI^{-/-/tet\text{-}GOI}$), the heterozygote for the *GOI* ($GOI^{+/-}$ or $GOI^{+/-/tet\text{-}GOI}$) is transfected with the 2nd targeting vector, as described in section 6.3.1. To screen for the $GOI^{-/-}$ or $GOI^{-/-/tet\text{-}GOI}$, genomic PCR (see sections 6.3.2 and 6.3.3), genomic Southern blot (see sections 6.2.1 and 6.3.4), or Western blot analysis can be used. Here, genomic PCR is usually performed for the first screening. For PCR screening, a specific primer outside of a homologous arm region is used with either of the two universal primers outside of the *loxP* sequences (Figure 4). If targeting vectors harboring a histidinol resistance cassette for the 1st targeting and a puromycin resistance cassette for the 2nd targeting are used, a universal primer with corresponding red arrowhead for the 1st screening and blue arrowhead for the 2nd screening can be used with a specific primer upstream of the left arm as was previously prepared. Additionally, PCR is performed using a primer set that corresponds to the region to be replaced by homologous recombination.

7. Troubleshooting

In addition to random integration, undesirable 'ectopic gene targeting' has often been observed in the Nalm-6 cell line as well as in mouse ES cells and a chicken DT40 cell line (unpublished data and personal communications). It is believed that ectopic gene targeting is an aborted event of homologous recombination in which a recombinant DNA molecule produced accidentally through homologous recombination between the introduced targeting vector and the target locus is randomly integrated into another genomic locus without completing targeting. Differentiating between this 'ectopic gene targeting' and true homologous recombination during screening for the heterozygotic allele is critical; otherwise, homozygotic alleles ($GOI^{-/-}$ or $GOI^{-/-/tet\text{-}GOI}$) can never be isolated. Thus, the signal strength of the bands derived from a wild-type allele and a targeted allele is always carefully compared in Southern blot analysis, as shown in Figure 6. The band intensity of the target allele is weaker than that of a wild-type allele in the cell clones in which 'ectopic gene targeting' takes place. In addition, a Southern probe is usually prepared at both the 5′ and 3′ ends to make sure.

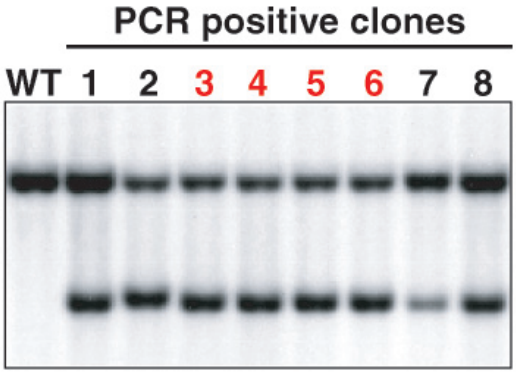

Figure 6. Southern blot analysis for 1st targeting. Genomic DNA was digested by restriction enzyme and hybridized with the [α-^{32}P] dCTP-labeled probe. Ten micrograms of DNA was loaded on each lane. The upper and lower bands are derived from a wild-type allele and a targeted allele, respectively. The signal strength of upper bands is no more than that of lower bands in clones 3, 4, 5 and 6, in which target gene disruption is performed through homologous recombination in all likelihood. The signal strength of upper bands in these clones is obviously weaker than that in the wild-type (WT). This does not apply, however, to clones 1, 7, and 8, in which 'ectopic gene targeting' takes place. These cell lines were not selected as a heterozygotic clone as they would never yield a knockout cell. Clone 2, in which the lower band has a different than expected size, was also not selected.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research in Priority Areas and by the City Area Program of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and grants from the Transdisciplinary Research Integration Center, Research Organization of Information and Systems, the Takeda Science Foundation and the Naito Foundation, Japan. This work was also supported in part by Yokohama City University (Strategic Research Project). H.N. acknowledges the Seed Of Excellence Foundation in Shizuoka Prefecture.

References

1. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998; 282:1497-1501.
2. Grawunder U, Zimmer D, Fugmann S, Schwarz K, Lieber MR. DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. Mol Cell 1998; 2:477-484.
3. Hurwitz R, Hozier J, LeBien T, Minowada J, Gajl-Peczalska K, Kubonishi I, Kersey J. Characterization of a leukemic cell line of the pre-B phenotype. Int J Cancer

1979; 23:174-180.
4. Adachi N, So S, Iiizumi S, Nomura Y, Murai K, Yamakawa C, Miyagawa K, Koyama H. The human pre-B cell line Nalm-6 is highly proficient in gene targeting by homologous recombination. DNA Cell Biol 2006; 25:19-24.
5. So S, Adachi N, Lieber MR, Koyama H. Genetic interactions between BLM and DNA ligase IV in human cells. J Biol Chem 2004; 279:55433-55442.
6. Wlodarska I, Aventin A, Ingles-Esteve J, Falzetti D, Criel A, Cassiman JJ, Mecucci C, Van den Berghe H, Marynen P. A new subtype of pre-B acute lymphoblastic leukemia with t(5;12)(q31q33;p12), molecularly and cytogenetically distinct from t(5;12) in chronic myelomonocytic leukemia. Blood 1997; 89:1716-1722.
7. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 1992; 89:5547-5551.
8. Mortensen RM., Zubiaur M, Neer EJ, Seidman JG. Embryonic stem cells lacking a functional inhibitory G-protein subunit (alpha i2) produced by gene targeting of both alleles. Proc Natl Acad Sci U S A 1991; 88:7036-7040.
9. Hasty P, Rivera-Perez J, Bradley A. The length of homology required for gene targeting in embryonic stem cells. Mol Cell Biol 1991; 11:5586-5591.
10. Merrihew RV, Marburger K, Pennington SL, Roth DB, Wilson JH. High-frequency illegitimate integration of transfected DNA at preintegrated target sites in a mammalian genome. Mol Cell Biol 1996; 16:10-18.
11. Kang YK, Park JS, Lee CS, Yeom YI, Chung AS, Lee KK. Efficient integration of short interspersed element-flanked foreign DNA *via* homologous recombination. J Biol Chem 1999; 274:36585-36591.
12. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A 1988; 85:5166-5170.
13. Babineau D, Vetter D, Andrews BJ, Gronostajski RM, Proteau GA, Beatty LG, Sadowski PD. The FLP protein of the 2-micron plasmid of yeast. Purification of the protein from Escherichia coli cells expressing the cloned FLP gene. J Biol Chem 1985; 260:12313-12319.
14. McCarrick JW 3rd, Parnes JR, Seong RH, Solter D, Knowles BB. Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. Transgenic Res 1993; 2:183-190.
15. Zhang Y, Riesterer C, Ayrall AM, Sablitzky F, Littlewood TD, Reth M. Inducible site-directed recombination in mouse embryonic stem cells. Nucleic Acids Res 1996; 24:543-548.
16. Fujimori A, Tachiiri S, Sonoda E, Thompson LH, Dhar PK, Hiraoka M, Takeda S, Zhang Y, Reth M, Takata M. Rad52 partially substitutes for the Rad51 paralog XRCC3 in maintaining chromosomal integrity in vertebrate cells. EMBO J 2001; 20:5513-5520.
17. Iiizumi S, Nomura Y, So S, Uegaki K, Aoki K, Shibahara K, Adachi N, Koyama H. Simple one-week method to construct gene-targeting vectors: application to production of human knockout cell lines. Biotechniques 2006; 41:311-316.

(*Received September 25, 2008; Revised October 15, 2008; Accepted October 16, 2008*)