Establishing a dual knock-out cell line by lentivirus based combined CRISPR/Cas9 and Loxp/Cre system

Ya Li · Weifeng Zhang · Junli Zhao · Sai Li · Linlin Shan · Jiuling Zhu ·
Yan Li · He Zhu · Qinwen Mao · Haibin Xia

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Abstract The clustered regulatory interspersed short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system has been widely used for gene knock-out. Lentiviral vectors have been commonly used as a delivery method for this system, however, prolonged Cas9/sgRNA expression due to lentiviral integration can lead to accumulating off-target mutations. To solve this issue in engineering a gene knock-out cell line, this study established a novel system, which was composed of two lentiviral vectors. One lentiviral vector carried simultaneously sgRNAs and CRISPR/Cas9 expression cassettes targeting single or multiple gene(s); the other lentiviral vector carried Cre that could remove excess sgRNAs and Cas9 expression cassettes in the genome after gene

targeting was achieved. To prove the principle, two candidate genes, extracellular matrix protein 1 (ECM1) and progranulin (PGRN), both highly expressed in MDA-MB-231 cells, were selected for testing the novel system. A dual knock-out of ECM1 and PGRN was successfully achieved in MDA-MB-231 cell line, with the sgRNAs and Cas9 expression cassettes being removed by Cre. This system should have great potential in applications for multiple genes knock-out in vitro.

Keywords CRISPR/Cas9 · Gene editing · Lentivirus · Loxp/Cre system · Off-target

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Y. Li · W. Zhang · J. Zhao · S. Li · L. Shan ·
J. Zhu · Y. Li · H. Zhu · H. Xia (✉)
Laboratory of Gene Therapy, Department of
Biochemistry, College of Life Sciences, Shaanxi Normal
University, 199 South Chang'an Road,
Xi'an 710062, Shaanxi, People's Republic of China
e-mail: hbxia2001@163.com

Q. Mao
Department of Pathology, Northwestern University
Feinberg School of Medicine, 303 E. Chicago Avenue,
Ward 3-140, Chicago, IL 60611, USA

Introduction

In recent years, a highly promising system, the clustered regulatory interspersed short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system, has been used as a versatile approach for editing the genome of eukaryotes. This system creates sequence specific dsDNA breaks that are repaired by the error-prone non-homologous end joining pathway (NHEJ), resulting in gene inactivation by creation of frame shift alleles (Cong et al. 2013; Dang et al. 2015; Jinek et al. 2013; Mali et al. 2013; Woong et al. 2013). The CRISPR/Cas9 system has been successfully used for gene knock-out in human cells (González et al.

2014; Kim et al. 2014; Li et al. 2015; Liao et al. 2015; Rahdara et al. 2015), embryos of mice (Fujii et al. 2013; Wang et al. 2013; Yang et al. 2013), zebrafish (Auer et al. 2013; Sung et al. 2014) and rabbit (Song et al. 2016).

Although CRISPR/Cas9 system enables highly efficient and specific cleavage activities, the off-target mutation is still a challenge for the application of CRISPR/Cas9 system (Fu et al. 2013; Lin et al. 2014b). This has been especially a significant concern when using this technique to generate a stable knock-out cell line. One of the main reasons is that the CRISPR/Cas9 system can tolerate 3–5 base mismatches between the target DNA and single guide RNA (sgRNA), especially when the mismatches occur on the 5' end of the sgRNA (Fu et al. 2013; Ran et al. 2013). To achieve genome editing with high specificity and low off-target effects by using the CRISPR/Cas9 system, the first critical step is the design of a highly specific and efficient guide RNA (gRNA) that will guide the Cas9 nuclease to the complementary DNA target sequence. The suggested gRNA length is 17- or 18-nt of complementarity. This length of gRNA has shown best specificity and lowest off-target effects (Fu et al. 2014). The second important step is to choose a suitable delivery method for Cas9/sgRNA expressing cassettes into target cells. For mammalian cells, plasmid transfection is the most common way to deliver the Cas9/sgRNA expressing cassette (Dang et al. 2015). However, transfection can cause overexpression of both Cas9 protein and sgRNA, and then induce off-target mutagenesis (Hsu et al. 2013). In addition, many cell types are hard-to-transfect cells, such as hematopoietic cells (Li et al. 2005; Ramezani et al. 2000; Walker et al. 2012), immune cells (Perez et al. 2005; Qin et al. 2003), stem cells (Chang et al. 2014; Han et al. 2015; Joseph et al. 2010), etc. Microinjection is an option (Wang et al. 2013; Yang et al. 2013) but with a low efficiency of only one cell per injection. Several groups used adenovirus to deliver Cas9/sgRNA expressing cassettes (Cheng et al. 2014), but the construction and amplification of recombinant adenovirus was laborious and time consuming. Shalem et al. designed a single lentiviral vector to simultaneously deliver Cas9 and sgRNA expressing cassettes into the target cells (Shalem et al. 2014). This system will enable genome editing in most cell types of interest. However, prolonged expression

of Cas9/sgRNA due to lentiviral integration aggravated off-target effects (Hsu et al. 2013).

To decrease the off-target mutagenesis of Cas9/sgRNA owing to lentiviral integration when generating a stable knock-out cell line, we developed a new system, which is composed of two lentiviral vectors. The first vector carried both CRISPR/Cas9 and sgRNAs expression cassettes to achieve the knock-out of single or multiple gene(s). The second vector carried Cre to remove the excess sgRNAs and Cas9 expression cassettes in the genome after the gene-editing was achieved. As a proof of concept, extracellular matrix protein 1 (ECM1) and progranulin (PGRN), which are highly expressed in MDA-MB-231 cells, were knocked out by using this system, with sgRNAs and Cas9 expression cassettes eventually removed by Cre.

Materials and methods

Cells and cell culture

HEK 293A, HEK 293T and MDA-MB-231 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin–streptomycin and 1% L-glutamate. The cell lines were cultured in a humidified incubator with 5% CO₂ at 37 °C.

Establishment of MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} cell line

To generate the lentiviral vector carrying simultaneously sgRNAs and Cas9 for targeting genes of interest (GOI) in MDA-MB-231, sgRNAs targeting GOI (gene of interest) were designed, then sgRNA oligonucleotides were formed by annealing at room temperature and then cloned into the pU6-sgRNA1.0 vector constructed by Xiao et al. (2016). The resultant plasmid was named pU6-GOI sgRNAs. The positive clones were confirmed by restriction enzyme digestion and sequencing. The Cas9 digested by *Cla* I and *Spe* I from pGEMT/Cas9 was subcloned into pCMV-MCS-SV40pA vector by restriction sites *Cla* I and *Spe* I. The resultant plasmid was named pCMV-Cas9-SV40pA.

To screen sgRNAs with highly biological activities, pCMV-Cas9-SV40pA and each pU6-GOI sgRNA were co-transfected into the HEK293A cell line and the genomic DNA was extracted after 72 h for T7E1 (Biolab, Ipswich, MA, USA) assay. pCMV-Cas9-SV40pA and pU6-sgRNA1.0 vector were co-transfected into the HEK293A cell line as a negative control. For the purpose of the T7E1, the genomic DNA above was used as a template for amplifying target sequence fragment (TSF) flanking the binding sites of sgRNA. The purified PCR products were denatured, reannealed, and digested with T7E1. Then the digested products were separated using DNA gel electrophoresis and photographed.

To construct the lentiviral vector containing two loxp sites oriented in the same direction, a *Sal* I-*loxp*-*Xho* I-*Sfu* I-*Xba* I linker formed by annealing of primers shown in Supplementary Table 1 was inserted into the C-terminus of the CMV through *Sal* I and *Xba* I enzyme sites, the obtained plasmid was called pCDH-CMV-*loxp*-MCS-EF1 α -Puro vector. Then, *Xba* I-*Kpn* I-*Nhe* I-*Not* I-*loxp*-*Not* I M linker obtained by annealing of primers shown in Supplementary Table 1 was inserted into the N-terminus of the EF1 α through *Xba* I and *Not* I enzyme sites to obtain pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-*Nhe* I-*Not* I-*loxp*-EF1 α -Puro vector. The fragment carrying multiple sgRNA expression cassettes from pU6-GOI sgRNAs vector digested with *Not* I and *Spe* I was cloned into pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-*Nhe* I-*Not* I-*loxp*-EF1 α -Puro vector digested by *Not* I and *Nhe* I. The resulting plasmid was named pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-GOI sgRNAs-*Not* I-*loxp*-EF1 α -Puro vector. Then Cas9 gene fragment from pCMV-Cas9-SV40pA digested by *Cla* I and *Spe* I was ligated with *Sfu* I and *Xba* I digested pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-*Nhe* IM-GOI sgRNAs-*Not* I-*loxp*-EF1 α -Puro vector. The final plasmid was named pCDH-CMV-*loxp*-Cas9-GOI sgRNAs-*loxp*-EF1 α -Puro vector. Recombinant lentivirus was produced by the co-transfection of pCDH-CMV-*loxp*-Cas9-GOI sgRNAs-*loxp*-EF1 α -Puro vector and packaging plasmids (Addgene, Cambridge, MA, USA) into HEK 293T cells. The recombinant lentivirus was named Lenti-*loxp*-Cas9-GOI sgRNAs-*loxp*-EF1 α -Puro.

To test the novel system, the target genes of ECM1 and PGRN highly expressed in MDA-MB-231 cell line were selected for the experiment. The primers for ECM1 and PGRN sgRNAs are listed in

Supplementary Table 1. sgRNA oligonucleotides were formed by annealing and then cloned into the pU6-sgRNA1.0 vector. The resultant plasmid was named pU6-ECM1 sgRNA1, pU6-ECM1 sgRNA2, pU6-ECM1 sgRNA3, pU6-ECM1 sgRNA4, pU6-PGRN sgRNA1, pU6-PGRN sgRNA2, pU6-PGRN sgRNA3 and pU6-PGRN sgRNA4, respectively. To screen sgRNAs with highly biological activities for ECM1 and PGRN, pCMV-Cas9-SV40pA and pU6-ECM1 sgRNA1, pU6-ECM1 sgRNA2, pU6-ECM1 sgRNA3, pU6-ECM1 sgRNA4, pU6-PGRN sgRNA1, pU6-PGRN sgRNA2, pU6-PGRN sgRNA3 or pU6-PGRN sgRNA4 were co-transfected into the HEK293A cell line and the genomic DNA was extracted after 72 h for T7E1 assay.

To obtain a vector containing multiple sgRNA expression cassettes, the pU6-ECM1 sgRNA X1 (where X1 stands for one of the best two ECM1 sgRNAs) vector linearized with *Kpn* I and *Spe* I was ligated with sgRNA X2 expression cassette from pU6-ECM1 sgRNA X2 vector (where X2 stands for another one of the best two ECM1 sgRNAs) digested with *Kpn* I and *Xba* I. The obtained vector was named pU6-ECM1 sgRNA X1&X2. By using a similar strategy, PGRN sgRNA Y1 and sgRNA Y2 (where Y1 and Y2 stand for one of the best two PGRN sgRNAs) expression cassettes were sequentially cloned into the pU6-ECM1 sgRNA X1&X2 vector. The resultant plasmid containing four independent sgRNA expression cassettes was named pU6-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2.

The fragment carrying multiple sgRNA expression cassettes from pU6-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2 vector digested with *Not* I and *Spe* I was cloned into pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-*Nhe* I-*Not* I-*loxp*-EF1 α -Puro vector digested by *Not* I and *Nhe* I. The resulting plasmid was named pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2-*Not* I-*loxp*-EF1 α -Puro vector. Then Cas9 gene fragment was ligated with pCDH-CMV-*loxp*-*Sfu* I-*Xba* I-*Nhe* IM-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2-*Not* I-*loxp*-EF1 α -Puro vector. The final plasmid was named pCDH-CMV-*loxp*-Cas9-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2-*loxp*-EF1 α -Puro vector. Recombinant lentivirus was produced by co-transfection of pCDH-CMV-*loxp*-Cas9-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2-*loxp*-EF1 α -Puro vector and packaging plasmids into HEK 293T cells. The recombinant

lentivirus was named Lenti-loxp-Cas9-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2-loxp-EF1 α -Puro.

To remove sgRNAs/Cas9 expression cassettes, the Cre gene fragment from pGEMT/Cre digested by *Xho* I and *Xba* I was cloned into lentiviral vector pCDH-CMV-*Xho* I-*Xba* I-EF1 α -Neo vector through restriction sites *Xho* I and *Xba* I. The obtained plasmid was named pCDH-CMV-Cre-EF1 α -Neo vector. Recombinant lentivirus was produced by the co-transfection of pCDH-CMV-Cre-EF1 α -Neo vector and packaging plasmids into HEK 293T cells. The recombinant lentivirus was named Lenti-Cre.

To build a dual genes knock-out MDA-MB-231 cell line, MDA-MB-231 cells were transduced with Lenti-loxp-Cas9-ECM1 sgRNA X1&X2/PGRN sgRNA Y1&Y2-loxp-EF1 α -Puro and then selected with medium containing puromycin for 3 ~ 4 days. The screened MDA-MB-231 cells were infected with Lenti-Cre and cultured in medium containing neomycin for 3 ~ 4 days. Finally, a single cell clone with dual genes knock-out and the removal of sgRNAs and Cas9 expression cassettes was obtained through limited dilution.

Southern blot analysis

The Cas9 DNA probe was amplified from pAd5-E1-CMV-*Cla* I-Cas9-*Spe* I-SV40pA with LA Taq DNA polymerase (Takara, Dalian, China) using primers shown in Supplementary Table 2, then labeled with biotin using the Biotin DecaLabel DNA Labeling Kit (Fermentas, Hanover, MD, USA). 100 μ g genomic DNA from MDA-MB-231 integrated the sgRNAs and CRISPR/Cas9 cassettes (Cre(–)) and MDA-MB-231 ECM1^{–/–} & PGRN^{–/–} C3 cells (Cre(+)) digested overnight with *Spe* I & *Nde* I at 37 °C was used as experimental group, and 15 ng of pCDH-CMV-loxp-Cas9-ECM1 sgRNA 2&4/PGRN sgRNA 2&3-loxp-EF1 α -Puro vector cut by *Spe* I & *Nde* I was used as positive control. Then Southern blot analysis was performed according to the previously published method (Xiao et al. 2016).

Analysis of Cre-mediated removal of loxp-flanked DNA sequence in the genome

The genomic DNA was extracted using the TIANamp Blood DNA Kit (TIANGEN BIOTECH, Beijing, China). The specific Cre-mediated gene excision was

detected by PCR analysis with primers (Supplementary Table 2) flanking the full length of Cas9. The PCR products were subjected to DNA gel electrophoresis and photographed.

Genotyping and off target analysis

The genomic DNA was extracted from cells using the TIANamp Blood DNA Kit (TIANGEN BIOTECH, Beijing, China). The fragments between the binding sites of two sgRNAs and the fragments covering potential off-target sites were amplified from the cells integrated with the sgRNAs and CRISPR/Cas9 cassettes or control group cells by PCR using the detection primers (Supplementary Table 2 and Supplementary Table 3). The PCR products from the region targeting by two sgRNAs were confirmed by sequencing, or denatured and reannealed, then purified using an AxyPrep DNA Gel Extraction Kit (Axygen, Hangzhou, China) followed by T7E1 assay. The PCR products from potential off-target regions of MDA-MB-231 cells integrated with the sgRNAs and CRISPR/Cas9 cassettes (Cre(–)) and MDA-MB-231 ECM1^{–/–} & PGRN^{–/–} C3 cells (Cre(+)) with the removal of the sgRNAs and CRISPR/Cas9 cassettes were analyzed by sequencing.

Real-time PCR

The total RNA of MDA-MB-231 ECM1^{–/–} & PGRN^{–/–} C3 cells (Cre(+)) and MDA-MB-231 wild type cells were extracted and cDNA was synthesized from 0.5 μ g of total RNA using PrimeScript RT reagent kit (Takara). The cDNA was amplified by SYBR Premix Ex Taq II (Takara) using the primers shown in Supplementary Table 4. The Real-time PCR was performed using Bio-Rad CFX ManagerTM software (Bio-Rad, Hercules, CA, USA).

Western blot

The total proteins of MDA-MB-231 ECM1^{–/–} & PGRN^{–/–} C3 cells (Cre(+)) and MDA-MB-231 wild type cells were extracted and applied to 10% SDS-PAGE, followed by electrotransfer onto methanol pretreated polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA, USA). For immunoblotting, the PVDF membranes were incubated with anti-ECM1 Mab 1C4 (Li et al. 2016), anti-

PGRN MAb 2A5 (Chai et al. 2011) or anti-GAPDH MAb (Proteintech, Chicago, IL, USA) for 1 h at room temperature. Membranes were washed and then detected by the HRP conjugated goat anti-mouse IgG antibody (1:10,000 dilution, Zhong Shan, Beijing, China) followed by enhanced chemiluminescence (ECL) Western blot detection reagents (Thermo, Rockford, IL, USA), according to the manufacturer's protocol.

Results

A novel lentiviral system for efficient gene editing and low off-target effects

We generated a novel system to achieve efficient genome editing with low off-target effects. The novel system is composed of two lentiviral vectors. The first lentiviral vector simultaneously contains a Cas9 expression cassette driven by CMV promoter, sgRNA expression cassette(s) under the control of U6 promoter for single or multiple gene targeting, and two loxp sites located in the C-terminal region of CMV and N-terminal region of EF1 α , respectively (Fig. 1a). The second lentiviral vector contains a CMV promoter-controlled Cre expression cassette for the removal of the excess Cas9/sgRNAs cassettes from the genome after the gene targeting is achieved (Fig. 1b).

Generation of MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} cell line by the novel system

ECM1 and PGRN, which are highly expressed in MDA-MB-231 cell line, were selected to test the novel system for dual sgRNA-directed genes knock-out with

low off-target effects. sgRNAs targeting ECM1 or PGRN genes were designed according to the genomic sequence from the National Center for Biotechnology Information (NCBI). Then, sgRNA activity was screened by T7E1 assay, and the results showed that ECM1 sgRNA2 ~ 4 and PGRN sgRNA1 ~ 4 possessed high targeting activities (Supplementary Fig. 1). ECM1 sgRNA2, ECM1 sgRNA4, PGRN sgRNA2 and PGRN sgRNA3 were selected for the assembly of the multiple sgRNA expression cassettes in the first lentiviral vector, named Lenti-loxp-Cas9-ECM1 sgRNA 2&4/PGRN sgRNA 2&3-loxp-EF1 α -Puro (Fig. 1a). After the lentivirus was produced, it was used to transduce MDA-MB-231 cells followed by puromycin selection. Then, the cell genome was subjected to PCR using a pair of primers located at the upstream of the 5' terminal sgRNA binding region, and the downstream of the 3' terminal sgRNA binding region individually. ECM1 PCR product from the mutant MDA-MB-231 cells revealed a fragment of 200 bp, a length smaller than that of the wild type ECM1, indicating a deletion between ECM1 sgRNA 2&4. As expected, a band with a size similar to that of wild-type ECM1 allele was also present (Fig. 2a). PGRN PCR product from the mutant MDA-MB-231 cells showed a fragment of about 500 bp in addition to the band amplified from the wild type PGRN, indicating a deletion between PGRN sgRNA 2&3 (Fig. 2a). The band from the mutant MDA-MB-231 cells with the size similar to that of the wild-type ECM1 or PGRN was further subjected to T7E1 assay, which revealed high gene-editing activity (Fig. 2b, c). Then, the MDA-MB-231 cells with dual ECM1/PGRN knock-out were infected by Lenti-Cre followed by the treatment of G418. The single cell clone was then obtained through limited dilution. A total of 3

Fig. 1 The novel lentiviral system for gene knock-out with low off-target effects. **a** Schematic of recombinant lentivirus Lenti-loxp-Cas9-GOI sgRNAs-loxp-EF1 α -Puro, which carries single

or multiple sgRNA expression cassette(s) for targeting single gene or multiple genes. GOI, the gene of interest. **b** Schematic of recombinant lentivirus Lenti-Cre

Fig. 2 RNA-guided mutagenesis of ECM1 and PGRN in MDA-MB-231 cells. **a** PCR products of targeted region of ECM1 and PGRN in MDA-MB-231 cells. Line 1, PCR product of ECM1 in wild type MDA-MB-231 cells; line 2, PCR product of ECM1 in mutant MDA-MB-231 cells; line 3, PCR products of PGRN in wild type MDA-MB-231 cells; line 4, PCR product of PGRN in mutant MDA-MB-231 cells. WT, wild type cells; MT, mutant cells. Arrows indicate expected positions of DNA bands. The numbers at the bottom indicate estimated indel frequency. **b** Cleavage activity in ECM1 site revealed by T7E1 assay on the “wild-type” PCR product of ECM1 in mutant MDA-MB-231 cells (MT). WT, wild-type MDA-MB-231. Red asterisks indicate expected positions of DNA bands cleaved by T7E1. The numbers at the bottom indicate estimated indel frequency. **c** Cleavage activity in PGRN site revealed by T7E1 assay on the “wild-type” PCR product of PGRN in mutant MDA-MB-231 cells (MT). WT, wild-type MDA-MB-231. Red asterisks indicate expected positions of DNA bands cleaved by T7E1. The numbers at the bottom indicate estimated indel frequency. **d** Sequences of the mutated ECM1 and PGRN alleles in MDA-MB-231 cells. The target sequences complementary to

ECM1 and PGRN sgRNAs are shown in red; base substitution mutations are shown in purple; deletion or insertion mutations are shown in blue or green; insertions (+) or deletions (–) are indicated at the right of each allele. **e** Schematic diagram of Lenti-loxP-Cas9-ECM1 sgRNA 2&4/PGRN sgRNA 2&3-loxP-EF1α-Puro, and the location of the probe for Southern blot. **f** Southern blot confirmed that the sgRNAs and CRISPR/Cas9 cassettes were removed by Lenti-Cre. Line 1, the genome of MDA-MB-231 integrated with the sgRNAs and CRISPR/Cas9 cassettes (Cre(–)) cut by *Spe* I & *Nde* I; line 2, the genome of MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 (Cre(+)) cut by *Spe* I & *Nde* I; line 3, vector of pCDH-CMV-loxP-Cas9-ECM1 sgRNA 2&4/PGRN sgRNA 2&3-loxP-EF1α-Puro cut by *Spe* I & *Nde* I. Arrow indicates expected positions of DNA bands. **g** Detection of the specific Cre-mediated gene excision in MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3. The Cas9 gene was amplified by PCR. Line 1, wild type of MDA-MB-231 cells; line 2, MDA-MB-231 cells integrated with the sgRNAs and CRISPR/Cas9 cassettes (Cre(–)); line 3, MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 (Cre(+)). Arrows indicated expected positions of Cas9. (Color figure online)

primary clones were isolated and examined by sequencing. All 3 clones had the insertion or deletion mutations in PGRN gene, and 2 clones had the insertion or deletion mutations in ECM1 gene. Clone 3 had insertion or deletion mutations in both PGRN and ECM1 (Fig. 2d). Then, the cell line derived from the clone 3, named MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 (Cre(+)), was analyzed by Southern blot and PCR analysis, which showed that the sgRNAs and CRISPR/Cas9 expression cassettes were successfully removed by Cre (Fig. 2e–g).

Characterization of MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} cell line

The protein and mRNA expression levels of ECM1 and PGRN in MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 (Cre(+)) were evaluated by Western blot analysis and real-time PCR. The results showed that the protein levels of ECM1 and PGRN in this cell line were below the limit of detection, while high levels of both proteins were detected in wild-type MDA-MB-231 cells (Fig. 3a). The relative mRNA expression levels

Fig. 3 The ECM1 and PGRN expression levels in MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 (Cre (+)) detected by Western blot **a** and Real-time PCR **b**. WT, wild-type MDA-MB-231

cells. C3, MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 (Cre (+)) cell line. ***P* < 0.01 by one-way ANOVA. Error bars represent standard deviation of triplicates

of ECM1 and PGRN were decreased by sixfold and 20-fold, respectively, in the mutant cells as compared with the wild-type cells (Fig. 3b).

CRISPR/Cas9 system can tolerate 3–5 mismatched bases between the target DNA and sgRNA, especially when the mismatched bases occur at the 5' end of the sgRNA (Fu et al. 2013; Ran et al. 2013). We next examined the off-target mutations in the genome of MDA-MB-231 cells integrated with the sgRNAs and CRISPR/Cas9 cassettes (Cre(-)) and MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 cells (Cre(+)) by sequencing. The potential off-target sites that contain 1- to 3-bp mismatches for the sgRNA target sequence were searched in the entire human genome, and a total of 20 sites were found. Eight potential off-target sites in the genome of MDA-MB-231 cells integrated with the sgRNAs and CRISPR/Cas9 cassettes (Cre(-)) and MDA-MB-231 ECM1^{-/-} & PGRN^{-/-} C3 cells (Cre(+)) were sequenced, and the results revealed that prolonged expression of Cas9/sgRNA induced off-target activities (off target 3), and no off-target activities were shown in any of these loci after the Cas9/sgRNA cassette was removed by Lenti-Cre (Fig. 4).

Discussion

In this study, a dual lentiviral system was established. One of the vector carried both sgRNAs and CRISPR/Cas9 expression cassettes for targeted knock-out of gene(s); the other vector carried Cre to remove the excess sgRNAs and Cas9 expression cassettes after genome editing was achieved. By using this system,

we successfully generated a MDA-MB-231 cell line with dual knock-out of ECM1 and PGRN genes, and sgRNAs and Cas9 cassettes being removed eventually.

The CRISPR/Cas9 system is a versatile tool for generating a gene knock-out cell line due to the highly efficient targeting of DNA sequences complementary to their RNA guide strands (Cong et al. 2013; Mali et al. 2013; Woong et al. 2013). But the applications of Cas9-mediated gene modification might be limited due to its potential off-target effects. It was reported that 3–5 base mismatches could be tolerated by CRISPR/Cas9 system, which potentially cuts at hundreds of sites across the genome, especially in large mammalian genomes having similar DNA sequences with the difference of only a few base pairs (Fu et al. 2013; Ran et al. 2013). However, off-target effects could be significantly reduced by controlling the expression of Cas9 (Lin et al. 2014a). CRISPR/Cas9 systems can be delivered by different methods that have varying efficiency and off-target effects. Plasmid transfection has been commonly used in various cell lines, however, overexpression of Cas9 and sgRNA by plasmid transfection has been shown to cause off-target mutagenesis. In addition, many cell types are hard-to-transfect (Chang et al. 2014; Han et al. 2015; Joseph et al. 2010; Li et al. 2005; Perez et al. 2005; Qin et al. 2003; Ramezani et al. 2000; Shalem et al. 2014; Walker et al. 2012). Microinjection is an approach but efficiency is low. Adenoviral vector can be useful, however, the construction of the viral vector, and the production of viruses are laborious and time-consuming. Lentiviral vectors have long been used for delivering various exogenous genes into the host

Fig. 4 Analysis of off-target activities of RGENs in the MDA-MB-231 cells integrated with sgRNAs and CRISPR/Cas9 cassettes (Cre(−)) and MDA-MB-231 ECM1^{−/−} & PGRN^{−/−} C3 cells (Cre(+)) by sequencing. Sequencing was conducted using genomic DNA samples from MDA-MB-231 cells integrated with sgRNAs and CRISPR/Cas9 cassettes (Cre(−)) and MDA-MB-231 ECM1^{−/−} & PGRN^{−/−} C3 cells (Cre(+)). The putative off-target sites bearing 2 to 3-bp mismatches were examined. Mismatched bases in red, and PAM (Protospacer adjacent motif, PAM) in blue. Arrows indicate the mutant site of the putative off-target site. (Color figure online)

cells including dividing and non-dividing cells (Kim et al. 1998; Semple-Rowland and Berry 2014), and have shown to be very useful for delivering Cas9 and sgRNAs. A single lentiviral vector carrying both Cas9 protein and sgRNA expressing cassettes successfully mediated targeted genome editing (Shalem et al. 2014). However, there was evidence showing that prolonged expression of Cas9/sgRNA due to lentiviral integration worsened the off-target effects (Hsu et al. 2013). The novel system established in this study includes two lentiviral vectors. One lentiviral vector simultaneously contains a Cas9 expression cassette driven by CMV promoter and sgRNA expression cassette(s) controlled by U6 promoter, which is similar to currently used single lentiviral system. The other lentiviral vector of the novel system carrying Cre expressing cassette could be used for removing sgRNA and Cas9 expression cassettes through two loxp sites after gene targeting is achieved. The issue that prolonged expression of Cas9/sgRNA due to lentiviral integration worsened the off-target effects was solved in this study by using loxp/Cre system, which successfully removed the sgRNAs and Cas9 after gene-targeting was achieved. The high efficiency of Cre mediated the removing of sgRNAs and CRISPR/Cas9 expression cassettes was verified by southern blot and PCR analysis (Fig. 2f, g). Furthermore, the sequencing results of potential off-target sites revealed that no off-target activities were shown in any of these loci after the Cas9/sgRNA cassette was removed by Lenti-Cre while prolonged expression of Cas9/sgRNA induced off-target activities (Fig. 4). This suggested that Cre caused excision of Cas9 would reduce long-term accumulation of off-target mutations. Alternatively, an inducible CRISPR/Cas9, which was shown to be able to decrease the off-target effects in vitro (González et al. 2014), has been introduced in the lentiviral system. Targeting the Cas9

with an additional sgRNA to prevent prolonged protein expression of the nuclease (Moore et al. 2014) can be another different approach. However, both methods would not produce a cell line as Cas9/sgRNA-free as Cre system.

Studies have shown the close relationship between ECM1 and PGRN in chondrogenesis (Kong et al. 2010; Xu et al. 2007) and cancer progression including proliferation (Cheung et al. 2004; Lee et al. 2014) and metastasis (Chen et al. 2011; Tangkeangsirisin and Serrero 2004; Wu et al. 2012, 2014). By using the novel lentiviral system, this study successfully generated a MDA-MB-231 cell line with dual knock-out of ECM1 and PGRN, with the Cas9 and sgRNA expression cassettes being removed. This will be a “clean” ECM1 and PGRN knock-out cell line without on-going off-target mutagenesis activity, which will be very useful for the functional studies of ECM1 and PGRN. We searched the genome of the dual knock-out cell line for potential off-target sites, none of which showed mismatches by sequencing. Interestingly, while protein levels of ECM1 and PGRN of the established cell line were both below the limit of detection, the relative mRNA levels were still detectable, and it was believed to be produced by the degradation mechanism of posttranscriptional mRNA processing.

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Compliance with ethical standards

Conflict of interest The authors declares that they have no conflicts of interest.

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