Citation: Molecular Therapy — Methods & Clinical Development (2014) **1,** 14050; doi:10.1038/mtm.2014.50

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ARTICLE

Genetic rearrangements of variable di-residue (RVD)-containing repeat arrays in a baculoviral TALEN system

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Virus-derived gene transfer vectors have been successfully employed to express the transcription activator-like effector nucleases (TALENs) in mammalian cells. Since the DNA-binding domains of TALENs consist of the variable di-residue (RVD)-containing tandem repeat modules and virus genome with repeated sequences is susceptible to genetic recombination, we investigated several factors that might affect TALEN cleavage efficiency of baculoviral vectors. Using a TALEN system designed to target the *AAVS1* locus, we observed increased sequence instability of the TALE repeat arrays when a higher multiplicity of infection (MOI) of recombinant viruses was used to produce the baculoviral vectors. We also detected more deleterious mutations in the TALE DNA-binding domains when both left and right TALEN arms were placed into a single expression cassette as compared to the viruses containing one arm only. The DNA sequence changes in the domains included deletion, addition, substitution, and DNA strand exchange between the left and right TALEN arms. Based on these observations, we have developed a protocol using a low MOI to produce baculoviral vectors expressing TALEN left and right arms separately. Cotransduction of the viruses produced by this optimal protocol provided an improved TALEN cleavage efficiency and enabled effective site-specific transgene integration in human cells.

Molecular Therapy — Methods & Clinical Development (2014) **1**, 14050; doi:10.1038/mtm.2014.50; published online 29 October 2014

INTRODUCTION

Recent advances in site-specific genome editing using zinc finger nucleases (ZFNs),[1,2] transcription activator-like effector nucleases (TALENs),[3,4] and RNA guided clustered regulatory interspaced short palindromic repeats (CRISPR)-associated nuclease Cas9 systems[5,6] promise to have profound impacts on biological research and could lead to new clinical applications of gene and cell therapies. These programmable chimeric nucleases enable robust and precise genetic modifications by inducing targeted DNA double-strand breaks that stimulate non-homologous end joining (NHEJ)- and/or homology-directed repair (HDR)-based cellular DNA repair machinery. When a sequence-specific nuclease is delivered together with a homologous donor DNA construct, HDR will incorporate the homologous strand as a repair template into the targeted site. The application of ZFN technology for gene therapy is currently undergoing early-phase clinical trials (ClinicalTrials.gov identifiers: NCT00842634, NCT01252641, and NCT01044654). RNA guided CRISPR-Cas9 systems is highly attractive due to the multiplexable genome engineering potential.[6] Nevertheless, TALEN remains one of the most promising tools for targeted genome editing because its off-target effects are less observed.

Given the great potential of viral vectors in gene and cell therapy, a number of studies have employed these vectors to deliver functional ZFNs into human cells for targeted genome editing. The adenoviral,[7,8] adeno-associated viral,[9] baculoviral,[10–12] and integrase-defective lentiviral vectors[13,14] permitted efficient delivery and transient expression of functional ZFNs. Recently, lentiviral and retroviral delivery vehicles have been used to express both a codon-optimized Cas9 and its synthetic guide RNA for site-specific genome editing.[15] Furthermore, a genome-scale lentiviral RNA guided CRISPR-Cas9 knockout library was adopted for genome-wide targeted mutations or loss-of-function genetic screening in mouse embryonic stem cells,[16] human cancer and pluripotent stem cells.[17,18] Despite large advances in TALEN-based genome editing, many technical challenges remain regarding the use of viral vectors to deliver TALENs.

A functional TALEN is generated by fusing the TALE DNA binding domain to the DNA cleavage domain FokI.[19] The modular tandem repeats of the DNA binding domain self-associate to form a right-handed superhelix that wraps around the DNA major groove.[20] Each repeat typically comprises a highly conserved 34 amino acid sequence with the exception of a repeat-variable di-residues (RVDs) at 12th and 13th amino acids.[21] The 12th residue stabilizes the local conformation of the RVD loop, whereas the 13th residue makes a base-specific contact to the DNA sense strand which confers DNA specificity.[20,21] Since a single unit of repeat bearing an RVD-containing loop forms a left-handed, two-helix bundle to recognize each DNA base in the target sequence, TALENs generated with the tandem array of repeats can recognize target sequences predicated by this simple DNA recognition code.[20,21]

Similar to the hurdles associated with cloning repetitive DNA sequences, the repetitive sequences of an assembled TALE repeat arrays may promote unwanted homologous recombination, impairing the DNA targeting specificity of the TALEN. Since segments of

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Received 15 August 2014; accepted 17 August 2014

virus genome containing repeated sequences are unstable and prone to genetic rearrangements, the use of a viral vector to package and deliver functional *TALEN* genes could be even more challenging.[22] To date, only a few studies attempted to use viral vectors carrying the *TALEN* genes for genomic modification in human cells. The first study used the adenovirus-based vector to accommodate and deliver intact *TALEN* genes into various human cells for efficient genome editing.[23] A set of protocols detailing the rescue, propagation and purification of adenoviruses expressing TALENs was subsequently described.[24] Through Gateway LR recombination, the assembled *TALEN* genes can be easily transferred into an adenoviral vector system, which facilitates delivery of functional TALENs into mammalian cells.[25] Although lentiviral vector was initially shown to have failed to transfer intact TALEN genes,[23] a more recent study successfully used recoded TALEs to eliminate the repetitive TALE RVD array sequences and to generate functional lentivirus carrying TALENs with high targeted genome editing efficiency in human induced pluripotent stem cells (iPSCs).[26] Taking advantage that the single-stranded DNA-based genome of adeno-associated viruses (AAV) is less susceptible to recombination, light-inducible transcriptional effectors containing a customizable TALE DNA-binding domain have been packaged into an AAV vector and used for targeting the mouse genome in primary neurons and the cortex for optical control of endogenous transcription and epigenetic states.[27]

We have recently developed a baculoviral TALEN system for targeted genomic manipulation in iPSCs.[28] Unlike other viral delivery vehicles, baculoviral vectors offer several advantages such as high transduction efficiency in a broad range of cell types including human pluripotent stem cells, large cloning capacity with an ability to package long DNA inserts, replication incompetent in mammalian cells, and low cytopathic effect on transduced cells.[10–12,28–33] While a high targeted integration efficiency in human iPSCs was achieved by using our BV-TALEN system, the structural integrity and functional stability of the TALE DNA-binding domain in the baculoviral delivery vectors are yet to be assessed. Herein, we report the development of a method to detect genetic rearrangements of TALE repeat arrays in baculoviral vectors carrying the *TALEN* gene and describe a number of advances in the production of the baculoviral vectors that enable to minimize these rearrangements, thus providing an improved efficiency for site-specific genome modification.

RESULTS

Development of a new method to detect genetic rearrangements of TALE repeat arrays

To characterize rearrangement events in TALE repeat arrays in a viral vector, we first developed a TA cloning- and RE digestion-based method to examine size change of the arrays (Figure 1a). We used a plasmid vector constructed previously to produce BV-TALEN(L-R), a baculoviral vector with a long repetitive sequence after inserting both left and right TALEN arms into a single expression cassette, to test the method.[28] Our initial effort using Platinum Taq DNA Polymerase High Fidelity to amplify the TALE repeat arrays of the plasmid led to a ladder of PCR products differing from the targeted 1.76 kb DNA fragment by multiples of the repeated unit (Figure 1b, left). Generation of such shadow bands is commonly observed when tandem repeat regions are PCR amplified using primers complementary to unique sequences that flank the repeat region and probably due to slipped-strand misalignment by Taq DNA polymerase.[34,35] Since Elongase provides a high processivity by editing nascent strands to allow subsequent polymerization by Taq DNA Polymerase, this enzyme mixture was used together with Platinum Taq DNA Polymerase High Fidelity. We also increased the PCR annealing temperature to 72 °C and included 5–8% DMSO as a PCR additive to improve amplification specificity and reduce the slipped-strand extension by Taq DNA polymerase.[36]

The optimized condition generated PCR products with one pronounced band around 1.76 kb (Figure 1b, right), indicating that the full-length of the TALE DNA-binding domains of the plasmid was successfully amplified. Although the plasmid template tested contains two TALEN arms and the primers used may bind to both, we used a shorter PCR denaturation and extension times to preferentially amplify single arm fragments. This is possible given the facts that a complete primer extension is greater for the shorter templates and longer PCR products may be less efficient denatured than shorter products.[34] Importantly, amplification of single TALEN arm fragments only facilitates characterization of TALE repeat arrays performed below. The PCR products were then cloned into a linearized TA-vector and individual E. coli clones with the inserts were picked for plasmid DNA extraction and RE digestion with EcoRI. Detection of DNA inserts of 1.76 kb indicates no significant deletion or addition event in the DNA-binding domains of the TALENs (Figure 1c). DNA sequencing analysis further confirmed the correct TALE RVD repeat arrays (Figures 1d). These results demonstrate that our TA cloning/RE digestion approach provides a reliable and quick way to characterize the rearrangement events in TALE repeat arrays.

Characterization of genetic rearrangements of TALE repeat arrays using TA cloning and RE digestion

Using the above method, we then performed a series of experiments to systematically investigate the rearrangement events in TALE repeat arrays in DNA samples used to generate working baculoviral vectors. These samples include the donor plasmids containing TALEN genes designed to target the *AAVS1* locus, the composite bacmids that are generated with the donor plasmids and can be used as baculovirus shuttle vectors for virus production after transfection into host insect cells, passage 1 (P1) virus stocks, P2 virus stocks, and P3 working virus stocks. According to the protocol for Bac-to-Bac Baculovirus Expression System, the recommended MOI of the initial P1 viral stock used to infect insect cells to generate the P2 viral stock is 0.05 pfu per cell. For further amplification to produce high titer P3 viruses typically used for protein expression, an MOI of 0.10 of P2 viral stock is often used. To determine effects of MOI used to generate P3 working viruses on the structural integrity of the TALE repeat arrays, we used various amounts of P2 viral stocks to infect insect cells. We were also interested to find out whether there is any difference in susceptibility to genetic rearrangement between BV-TALEN(L-R) that contains a long repetitive sequence and baculoviral vectors containing a short repetitive sequence with one TALEN arm only, BV-TALEN(L) and BV-TALEN(R). In total, we analyzed 613 DNA samples (Table 1).

In PCR products amplified using the donor plasmids and composite bacmids as templates, we did not observe any obvious size change after RE digestion (Figure 2a). However, in PCR products amplified from baculoviral genomic DNA, the fragments smaller than 1.76 kb, indicating large genomic deletion in TALE repeat array, were often observed (Figure 2a,b). Out of 534 individual TA clones analyzed, 184 clones displayed PCR insert size change (34.5%) and the frequency of the change increased with serial passage of baculoviruses, from 23.4% in P1 (n = 64), 25.3% in P2 (n = 83), to 38.2% in P3 (n = 387) viral stocks (Figure 2, Table 1). Taken these findings together, we can infer that genetic rearrangement events in the TALE repeat arrays occurred mainly during baculovirus propagation

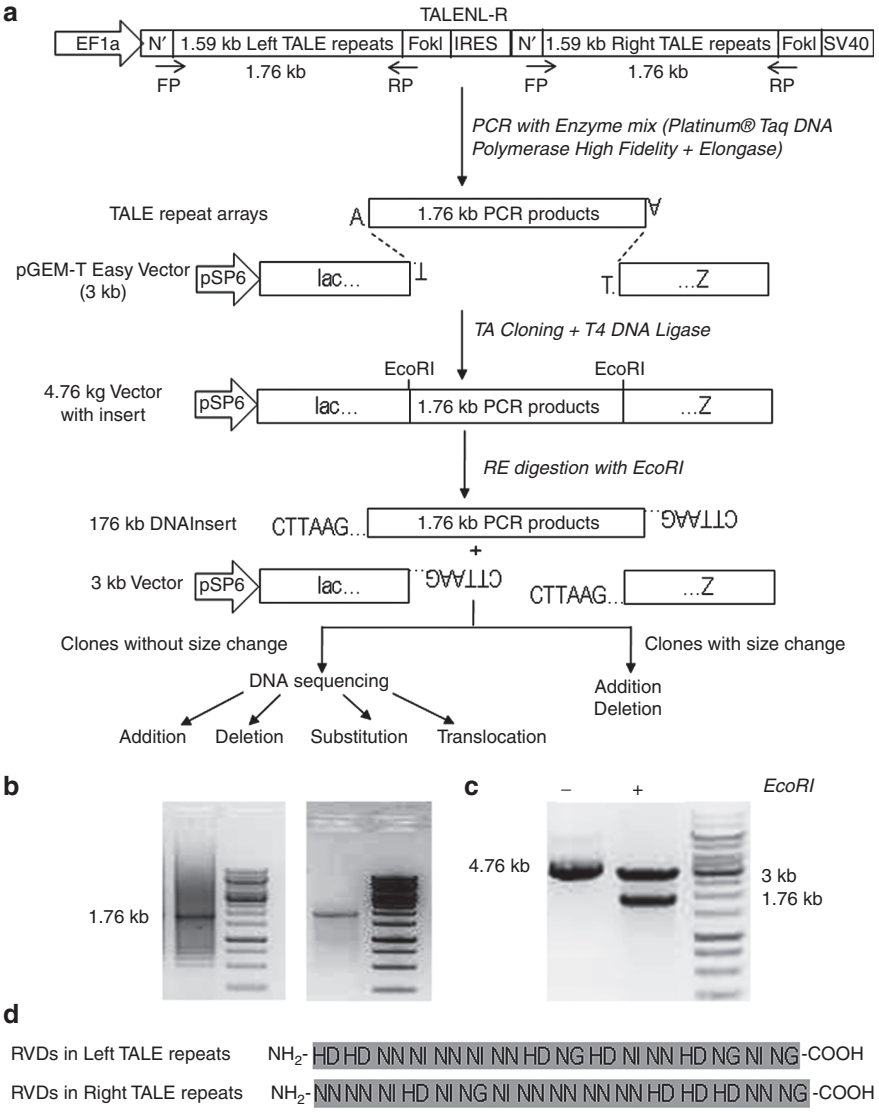

Figure 1 Development of a TA cloning- and RE digestion-based method to examine genetic rearrangements of TALE repeat arrays. (**a**) Work flow chart. A fusion expression cassette bearing both left and right TALEN arms (TALEN L-R) is amplified with forward and reverse PCR primers (FP and RP) designed to amplify the full-length of tandem repeat arrays. The PCR products are inserted into a TA cloning vector. EcoRI digestion of individual clones followed by agarose gel electrophoresis is used to detect the size change of the TALE repeat arrays. The detection of a 1.76 kb DNA fragment indicates that the number of the tandem repeat units remains correct in the TALEN expression cassette. To further classify the genetic rearrangement events, the clones with no obvious size changes are subjected to DNA sequencing. (**b**) The use of an enzyme mixture allows PCR amplification of single band. Left: Platinum Taq DNA Polymerase High Fidelity. Right: Platinum Taq DNA Polymerase High Fidelity mixed with Elongase (1:2). (**c**) TA cloning and EcoRI digestion detect no change in the size of the TALE repeat arrays in plasmid vector samples. The band at 3 kb after EcoRI digestion is the linearized cloning vector, while the DNA band at 1.76 kb is the DNA insert bearing a TALE repeat array. "–" and "+": Without and with EcoRI. One kb DNA ladder was used as a molecular weight standard. (**d**) DNA sequencing analysis confirms no change in DNA recognition specificity by RVDs in left *(top panel)* and right *(bottom panel)* TALEN arms in the above plasmid samples. Amino acid sequence alignment was performed after translating DNA sequences into amino acids. The pair letters in the sequence represent a RVD (HD, NI, NG, and NN) for each TALE repeat unit in a TALEN arm.

in insect cells. We further detected a significant increase in number of the clones displaying size change when the MOI of P2 viral stocks used to generate P3 viruses was increased from 0.005 to 0.2. Using an MOI of 0.2, most of the generated viruses (54 out of 75 examined clones) displayed DNA insert size smaller than 1.76 kb (Table 1). Overall, the use of a low MOI up to 0.05 appeared to result in less rearrangement events in the tested viruses (Table 1). Between long and short repetitive sequences in viral genome, we observed a pronounced increase in large genomic deletion/addition in the baculoviral vectors with a long repetitive sequence, regardless of whether P1, P2, or P3 viral stocks were examined (Figure 2, Table 1). The above findings suggest that to minimize large genomic deletion/addition of TALE repeat arrays in baculoviral vectors, left and right TALEN arms should be cloned separately into two different vectors and working viruses should be produced using a low MOI to infect insect cells.

Characterization of genetic mutations of TALE repeat arrays using DNA sequencing method

Genetic rearrangement in TALE DNA-binding domain may not lead to obvious size alterations but causes changes in DNA sequence. We then performed DNA sequencing of the TALE DNA-binding domains in all clones that carried a 1.76 kb PCR insert as revealed by the RE digestion method above. Totally 429 clones

Table 1 Summary of using RE digestion and DNA sequencing methods to detect rearrangements of TALE repeat arrays in plasmid vectors, recombinant bacmid, and BV[a]

			(A) RE digestion			(B) DNA sequencing			Total (A+B)		
DNA Template				Clones with rearrangements			Clones with rearrangements			Clones with rearrangements	
Sample	TALEN construct[b]	MOI used[c]	No. clones analyzed	No.	%	No. clones analyzed	No.	%	No. clones analyzed	No.	%
Plasmid	L	N/A	10	0	0	10	0	0	10	0	0
	R	N/A	10	0	0	10	0	0	10	0	0
	L-R	N/A	20	0	0	20	0	0	20	0	0
Bacmid	L	N/A	10	0	0	10	0	0	10	0	0
	R	N/A	10	0	0	10	0	0	10	0	0
	L-R	N/A	19	0	0	19	0	0	19	0	0
	Subtotal		79	0	0	79	0	0	79	0	0
BV-P1	L	N/A	17	3	17.7	14	1	7.1	17	4	23.5
	R	N/A	19	2	10.5	17	3	17.7	19	5	26.3
	L-R	N/A	28	10	35.7	18	5	27.8	28	15	53.6
BV-P2	L	0.05	33	6	18.2	27	2	7.4	33	8	24.2
	R	0.05	25	3	12.0	22	6	27.3	25	9	36.0
	L-R	0.05	25	12	48.0	13	6	46.2	25	18	72.0
	Subtotal		147	36	24.5	111	23	20.7	147	59	40.1
BV-P3	L	0.005	25	3	12.0	22	4	18.2	25	7	28.0
		0.025	24	5	20.8	19	4	21.1	24	9	37.5
		0.05	25	4	16.0	21	4	19.1	25	8	32.0
		0.10	25	11	44.0	14	4	28.6	25	15	60.0
		0.20	26	18	69.2	8	3	37.5	26	21	80.8
	R	0.005	22	3	13.6	19	4	21.1	22	7	31.8
		0.025	27	7	25.9	20	2	10.0	27	9	33.3
		0.05	30	5	16.7	25	6	24.0	30	11	36.7
		0.10	26	11	42.3	15	5	33.3	26	16	61.5
		0.20	24	15	62.5	9	4	44.4	24	19	79.2
	L-R	0.005	31	10	32.3	21	7	33.3	31	17	54.8
		0.025	24	6	25.0	18	9	50.0	24	15	62.5
		0.05	28	12	42.9	16	5	31.3	28	17	60.7
		0.10	25	17	68.0	8	3	37.5	25	20	80.0
		0.20	25	21	84.0	4	2	50.0	25	23	92.0
	Subtotal		387	148	38.2	239	66	27.6	387	214	55.3

BV, baculoviral vectors; RE, restriction enzyme.
[a]TA cloning followed by RE digestion was first used for the examination. Clones with a 1.76 kb DNA fragment as revealed by RE digestion, indicating no size change in TALE repeat arrays, were subject to DNA sequencing examination. [b]Left (L) and right (R) TALEN constructs contain 15 RVDs, respectively, with different RVD compositions for *AAVS1* recognition specificity. A fusion TALEN construct (L-R) is composed of both left and right TALEN arms with 30 repeat units in total. [c]Multiplicity of infections (MOIs) of the initial BV stock used to infect insect cells for the generation of the passage 2 (P2) or 3 (P3) viruses.

were subjected to DNA extraction and sequencing analysis and 89 of them were found to display genetic mutations (Table 1). Once again, we did not observe any changes in PCR products amplified using the donor plasmids and composite bacmids as templates, and genetic mutations were detected only in PCR products amplified from baculoviral genomic DNA.

The changes in DNA sequences of the TALE repeat arrays of these samples included deletion, addition, substitution, and translocation. Deletion and addition caused changes in the number of RVD-containing TALE repeat units, while substitution mutation resulted in codon change. DNA strand exchange between left and right TALEN arms led to translocation of a portion of a TALE DNA binding

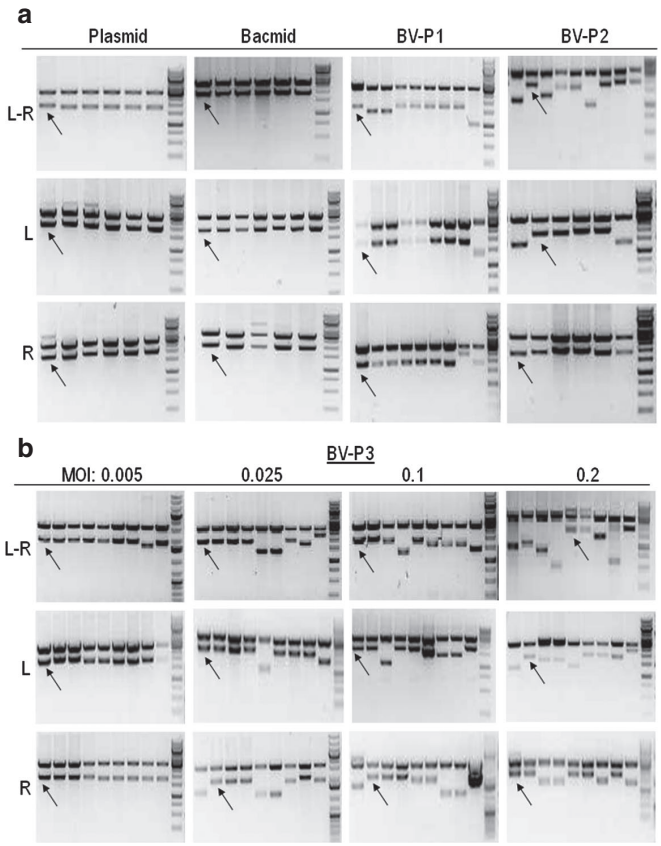

Figure 2 Deletion or addition events in TALE repeat arrays detected by the TA cloning and RE digestion method. (**a**) Testing using donor plasmids, composite bacmids and baculoviral genomic DNA isolated from P1 and P2 virus stocks. (**b**) Testing using baculoviral genomic DNA isolated from P3 working viruses generated with various MOIs of initial P2 virus stocks. DNA with a fusion expression cassette bearing both left and right TALEN arms (L+R), left (L), or right (R) TALEN arm was analyzed in **a** and **b**. Upper bands in each panel: 3 kb linearized cloning vector. Lower bands in each panel: PCR inserts. Arrows indicate a 1.76 kb PCR product. Bands with a size smaller or larger than 1.76 kb indicate significant deletion or addition events, respectively.

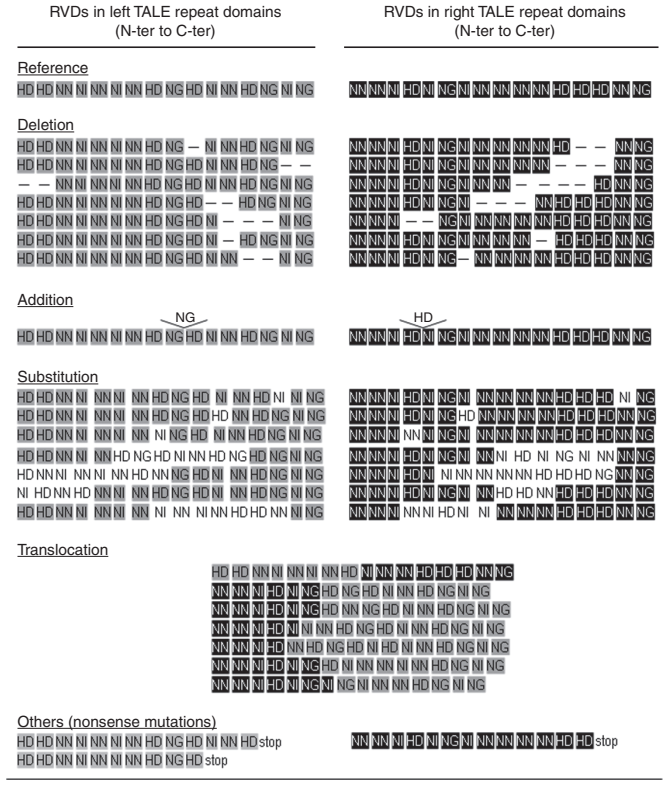

Figure 3 Types of mutations in TALE repeat arrays identified by DNA sequence analysis. Clones with a 1.76 kb DNA fragment of PCR insert as revealed by the RE digestion method were subjected to DNA sequencing examination. The samples of P3 baculovirus genome (*n* = 239) were analyzed. The correct TALE repeat arrays in left and right TALEN arms are listed on the top.

domain. The results obtained from genomic DNA isolated from P3 viruses including BV-TALEN(L), BV-TALEN(R) and BV-TALEN(L-R) are shown in Figure 3. In these samples, more than 60% of genetic mutations were attributed to deletion, which could involve 1 to 4 RVD-containing repeat units (Supplementary Table S2). As expected, translocation was observed in BV-TALEN(L-R) with a reduction in the frequency of intra-array recombination within the left or right TALE DNA binding domain (Supplementary Table S2). Apart from missense mutations, other nucleotide sequence changes related point mutation, including frameshift and nonsense mutations, were detectable but at a negligible frequency. DNA sequence alignment revealed no evidence of silent mutation in the RVD-containing repeat arrays. When looking at effects of MOIs of viral stocks used to generate baculoviruses, we noticed that a higher MOI could increase the incidence of genetic mutation, although it did not cause obvious change in mutation type (Supplementary Table S3).

Characterization of genetic rearrangements of TALE repeat arrays using Southern blot analysis

We further performed Southern blot analysis to examine the structural integrity of the TALE repeat arrays in the donor plasmids, composite bacmids, and baculoviral genome. Plasmid, bacmid, and viral genomic DNA were digested with NsiI and XbaI before agarose gel electrophoresis. Instead of trying to differentiate the extent of mutations between left and right TALEN arms, we used these two REs to determine the overall structural integrity of the two TALEN arms in the expression cassette L-R. The Southern blot probe was designed to bind to the FokI region, which is located downstream of a TALE DNA binding domain (Figure 4a). The detection of a single 2.4 kb DNA fragment bearing the FokI and TALE DNA binding domains in the donor plasmids and composite bacmids demonstrated the TALE repeat arrays remain unchanged in these samples (Figure 4b). Upon transfection of insect cells with the composite bacmids to generate infectious recombinant baculovirus, "shadow" bands were observed in Southern blots for the early passage (P1) baculoviruses, indicating possible genetic rearrangements in the TALE repeat arrays. These events appeared to increase from P1 to P3, possibly due to the accumulation of genetic rearrangements over virus passaging. We then examined the genomic DNA of late passage (P3) baculoviruses generated with various MOIs of P2 viral stocks, from 0.005, 0.025, 0.05, 0.10 to 0.20 pfu per cell, and observed a 'ladder' of bands starting at ~600 bp and every 100 bp up to ~2,300 bp (Figure 4c). With an increase in MOI of P2 viral stock, P3 BV-TALEN(L-R) bands with intact DNA size (2.4 kb) became less noticeable, indicating the disappearance of functional TALENs. Thus, our Southern blot analysis, although unable to provide quantitative evaluation, shows again that low multiplicity of infection to produce baculoviral vectors with one TALEN arm only will be favorable to generation of functional viral TALEN vectors.

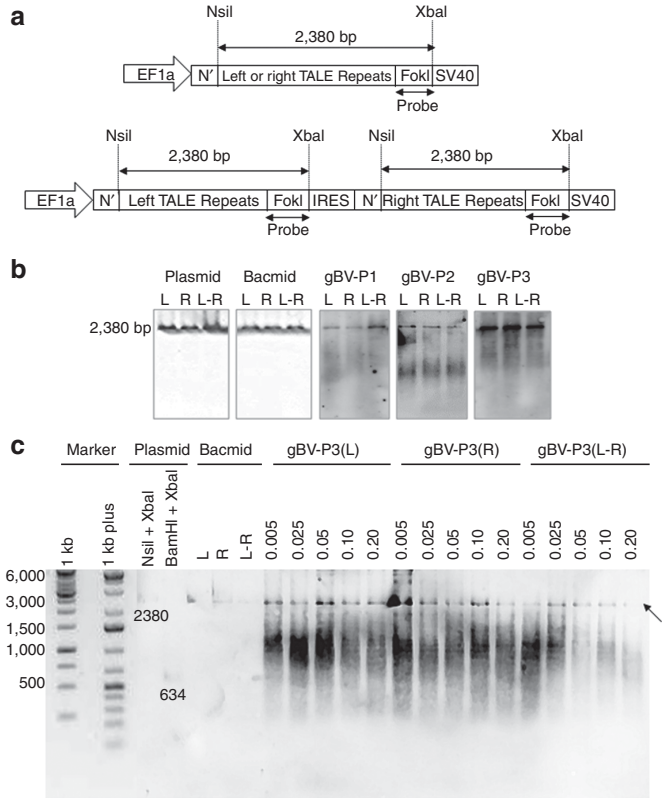

Figure 4 Southern blot analysis to examine the structural integrity of TALE repeat arrays. (**a**) The probe binding sites used for Southern blot analysis. The probe used was amplified from the FokI cleavage domain in the donor plasmid carrying a fusion expression cassette (TALEN L-R). DNA bearing the TALEN expression cassette was digested with NsiI and XbaI to generate 2.4 kb DNA fragments containing the central TALE repeat array and FokI cleavage domain. (**b**) The structural integrity of TALE repeat arrays in the donor plasmids, composite bacmids and baculoviral genome. Genomic DNA of recombinant baculovirus (gBV) was extracted after serial passages (P1, P2, and P3). (**c**) The influence of MOI used to infect insect cells and the length of tandem repeats in TALEN constructs on structural integrity. As a positive control, a TALEN expression cassette in a donor plasmid vector was digested with BamHI and XbaI to generate a DNA fragment consisting of the FokI cleavage domain only (634 bp).

Evaluation of the optimal BV-TALEN system

To evaluate the DNA disruption efficiency by BV-TALEN vectors, we constructed a luciferase reporter vector bearing the targeted AAVS1 site and performed a single strand annealing assay (Figure 5a) in human U87 cells. When P3 viruses generated with an initial MOI from 0.025 to 0.2 pfu per insect cell were used, their DNA disruption efficiency decreased with increase in MOI used for virus generation, regardless of whether the cells were transduced with BV-TALEN(L-R) or cotransduced with BV-TALEN(L) and BV-TALEN(R) (Figure 5b). These results are consistent with the above findings that the viruses generated with a higher MOI bear more incorrect DNA binding sequences, thus being less effective in cleavage of a target site. Likewise, cotransduction of U87 cells with BV-TALEN(L) and BV-TALEN(R) provided a more robust TALEN activity than transduction with BV-TALEN(L-R) generated at the same MOI (Figure 5b). Being consistent with the observations that P2 viruses carry less functionally defective RVD-containing repeat units than P3 viruses (Table 1), the highest TALEN activity was achieved when the cells were cotransduced with two P2 viruses, BV-TALEN(L) and BV-TALEN(R), exhibiting an activity to 156% of that provided by two P3 viruses generated with the same MOI. These results support the notion that placing two TALEN arms into two viral vectors, generating viruses with a low MOI, and using a low passage number of working viruses could be an optimal protocol to reduce the sequence instability of a TALE repeat array in a baculoviral vector.

To examine the efficiency of the *AAVS1*-directed homologous recombination driven by the optimal BV-TALEN system, a donor baculoviral vector containing the eGFP reporter and neomycin resistant genes was used together with P2 BV-TALEN(L) and P2 BV-TALEN (R) for cotransduction in human U87 cells (Figure 6a). After G418 selection for 20 days, most of the survived cells became eGFP-positive (Figure 6b). These cells exhibited persistent eGFP expression for at least 3 months (Figure 6c). PCR genotyping of the genomic DNA of the transgenic cells was performed. The amplification of a 2.9 kb fragment confirmed the successful site-specific integration of the donor cassette into the *AAVS1* locus driven by the TALEN-mediated homologous recombination (Figure 6d).

DISCUSSION

Highly repetitive DNA sequences in TALE repeat arrays are unstable and prone to genetic recombination. Even in circular plasmid vectors, superhelical stress can generate secondary structures that are susceptible to deleterious mutations in host *E. coli* cells, particularly in regions that contain numerous tandem or palindromic repeats.[37] However, we did not detect any genetic rearrangements in TALE repeat arrays in DNA samples isolated from the donor plasmid vectors used to generate the composite bacmids, including the donor plasmid constructed for generating BV-TALEN(L-R) that contains a long repetitive sequence, indicating that TALE repeat array sequences alone are not enough to promote genetic rearrangement in *E. coli*.

Baculovirus shuttle vector bacmids are produced by Tn7-mediated site-specific transposition of an expression cassette in a donor plasmid into a baculovirus genome region in *E. coli* cells. Baculovirus genome is also a supercoiled circular double-stranded DNA. Similar to what happens in circular plasmid, torsional strain inherent to baculovirus genome may induce localized melting to generate cruciform and other secondary structures, which may bring two DNA fragments physically closer together, favoring genetic recombination or deletion of repetitive DNA sequences.[38] Pausing of DNA replication at these secondary structures could promote template switching between direct repeats. However, once again, we did not detect any genetic rearrangements in TALE repeat arrays in DNA samples isolated from the composite bacmids. Bacmids contain a mini-F replicon and thus can replicate in *E. coli* at a low copy number.[39] Furthermore, *E. coli* cells used for the composite bacmid generation were DH10Bac competent cells, which is a recombination-deficient RecA mutant strain. These two features provided by bacmids and DH10Bac could play important roles in maintaining genetic stability of the TALE repeat arrays in the composite bacmids.

On the other hand, we detected genetic rearrangement in P1 viruses generated in insect cells and observed an increased mutation rate over serial passage of baculoviruses and when a higher MOI of P2 virus stocks was used to produce P3 viruses. Taken together with above observations, these results lead us to speculate that the observed genetic rearrangements in the tested TALE repeat arrays are possibly associated with the expression of virus genes in host insect cells. Homologous recombination (HR) between baculoviruses is often observed during virus DNA replication in host insect cells, stressing the concurrency of these two mechanisms.[40–42] Detected baculovirus recombinants are cumulatively generated during several DNA replication cycles in a single

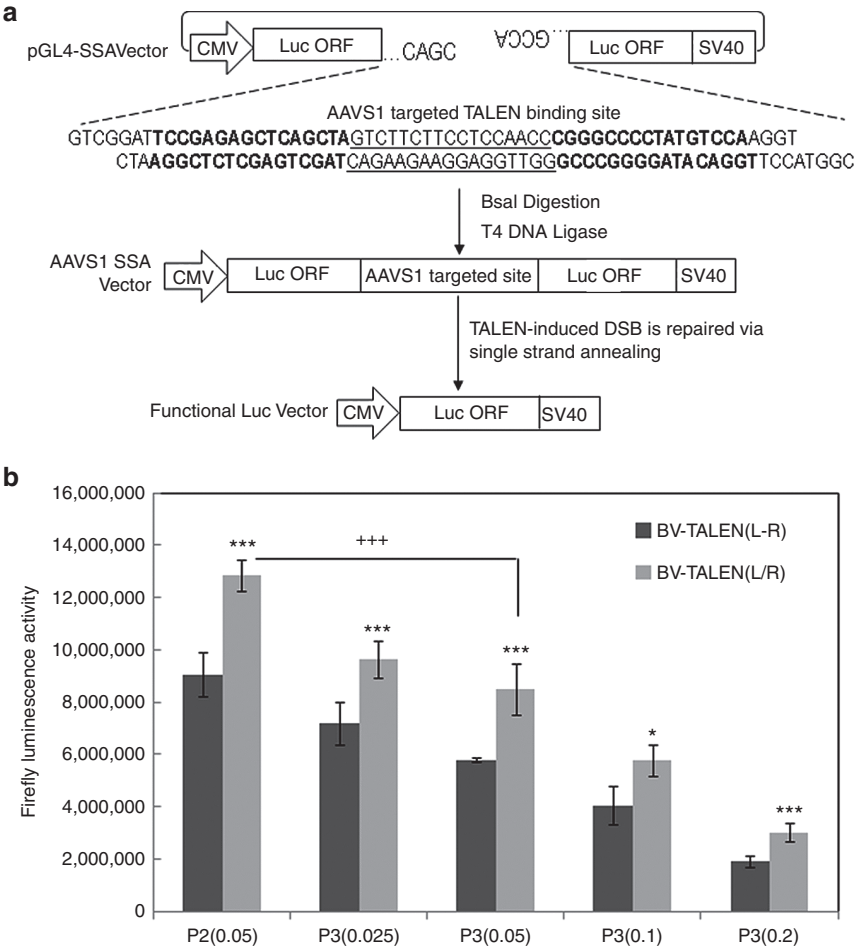

Figure 5 Quantification of the TALEN cleavage efficiency using single strand annealing (SSA) assay. (**a**) Schematic representation of SSA assay designed to quantify the TALEN cleavage efficiency at the targeted *AAVS1* site. An AAVS1 SSA assay testing vector is constructed by cloning annealed oligonucleotides bearing the targeted *AAVS1* site into BsaI-treated pGL4-SSA vector through their sticky ends. TALEN-induced double strand break (DSB) at the *AAVS1* site of the testing vector is repaired via SSA to generate a functional luciferase reporter vector. (**b**) Comparisons of TALEN cleavage efficiency of TALEN baculoviral vectors prepared with different ways. U87 cells were transfected with the AAVS1 SSA vector, followed by transduction of BV-TALENs at an MOI of 50. BV-TALEN (L-R): Transduction with viruses containing an expression cassette with both left and right TALEN arms. BV-TALEN (L/R): Cotransduction with two types of viruses carrying left and right TALEN arms, respectively. P2 or P3 viruses used for cell transduction and the initial MOI used to generate these BV-TALENs (in brackets) are indicated. Luciferase activity assay was performed 48 hours after transduction, which is directly proportional to the cleavage efficiency mediated by the TALENs. Values present mean ± SD (n = 5). *, ***: $p < 0.05$ and $p < 0.001$ versus BV-TALEN(L-R) by Student's *t*-test. +++: $p < 0.001$ between P2 BV- TALEN(L/R) and P3 BV-TALEN(L/R) by Student's t-test. The background readings for the AAVS1 SSA vector were 291,027 ± 121,415.

passage.[43] In *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), the viral genes that are necessary for efficient HR include *ie-1*, *ie-2*, *lef-7*, and *p35*, with *ie-1* and *ie-2* being sufficient to promote HR in the absence of other viral genes. Furthermore, in the absence of the AcMNPV genes, the cellular machinery is unable to promote high levels of HR.[42] It can be envisioned that after the TALE repeat arrays analyzed in this study are inserted into bacmids and transduced into insect cells the expression of the above AcMNPV genes from the composite bacmids and newly generated baculoviruses will promote HR between these structures as they do for HR between two baculovirus genomes, causing genetic rearrangement. Also, during DNA replication of tandem repeats, the slipped misalignment between the nascent strand and another homology template strand can lead to deletions,[44] which may contribute to the deletions observed in this study. Naturally, the number of viral replication cycles that a virus undergoes prior to harvesting will play a great role in increasing the sequence instability of the TALEN expression cassettes in the virus genome.

To minimize deleterious mutations in the TALE DNA-binding domains in baculovirus vectors, using a low MOI for virus production is recommended, which could be helpful in preserving the genetic integrity of viruses and preventing the buildup of defective interfering particles (DIPs).[45,46] DIPs are virus-like byproducts of infection with partial genomes packaged by complementation from intact genomes coinfected in the same cell. DIPs tend to emerge and outgrowth during high MOI infection, where multiple copies of the viral particles infect each host cell. Genome deletions in DIPs could occur between TALE tandem repeat arrays and/or distant homologous repeat regions in baculoviral genome. DIPs are shorter in genome size that makes their replication faster than that of intact viruses. At a low MOI, the probability that both an intact virus and a DIP will infect the same cell is low. Moreover, virus yield declines more rapidly for passages performed at a higher MOI, as predicted with a mathematical model based on single-passage behavior of cells coinfected with virus and DIPs.[46] Hence, a low MOI used during the initial infection in insect cell culture would lead to a purifying selection against baculovirus deletion mutants.

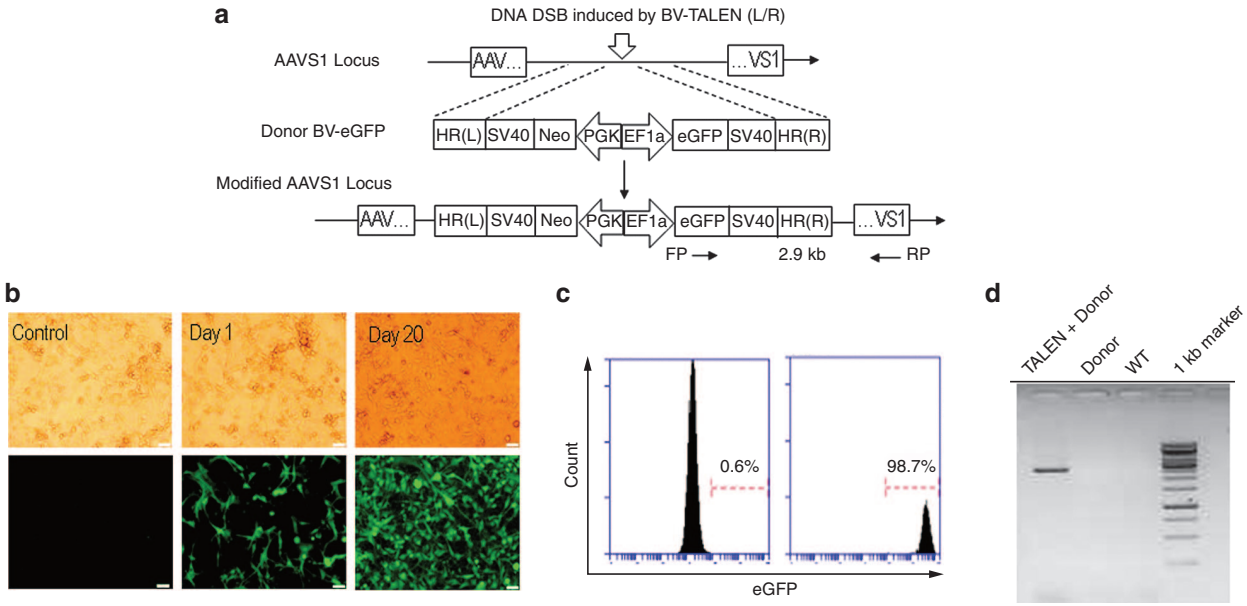

Figure 6 Stable eGFP expression in human U87 cells after HR mediated by BV-TALEN (L/R). (**a**) Schematic diagram showing *AAVS1*-locus integration of the *Neo-eGFP* expression cassette in the DNA donor BV-eGFP following DNA double-strand break (DSB) triggered by a pair of BV-TALENs. HR(L) & HR(R): Left and right arms for homologous recombination. FP & RP: Binding sites for PCR forward and reverse primers. (**b**) eGFP expression in U87 cells. After G418 selection for 20 days, most of the cells are eGFP-positive. Representative phase and fluorescence images, top and bottom panels respectively, are shown. (**c**) Flow cytometry analysis to determine the percentage of eGFP-positive cells 3 months after BV transduction. The cells were maintained without G418. (**d**) AAVS1-specific transgene integration. Genomic DNA was extracted from original, wild-type U87 cells (WT), U87 cells transduced with BV-eGFP alone (Donor) and U87 cells cotransduced with BV-TALEN and BV-eGFP (TALEN+Donor). PCR analysis demonstrates the cotransduction-mediated, site-specific integration of the 2.9 kb eGFP cassette at the *AAVS1* locus.

We noticed an increased genetic rearrangement in TALE repeat arrays in baculoviral vectors with both left and right TALEN arms in a single expression cassette as compared to the viruses containing one arm only. Due to supercoiling stress in a circular baculovirus genome, a long tandem repeat array may increase DNA instability and induce cruciform or hairpin secondary structures to cause rearrangement. As shown in bacteriophage T7, the frequency of deletion of DNA inserts with longer direct repeats increases exponentially as the length of the repeats increased.[38] As expected, two baculoviral vectors with a short repetitive sequence, BV-TALEN(L) and BV-TALEN(R), display a similar trend in genetic rearrangement, demonstrating that the composition or combination of RVDs, which contributes only minor variation to the overall sequence of a repetitive array in a TALE, may have little effect in determining the frequency of rearrangement events. In fact, the genetic rearrangement of TALE repeat arrays was mainly attributed to highly conserved arrays of modular repeat units, regardless of the nucleotide sequences encoding two variable amino acids of each RVD. Despite the significant impairment of the structural integrity in the TALE repeat arrays in baculoviral vectors, a high targeting efficiency could still be achieved with the remaining intact TALENs as demonstrated in our previous study.[28] Thus, the baculoviral vectors with the mutated TALE repeat arrays are most likely to be functionally defective in targeted genome editing. Crystal structure analysis has demonstrated that a single mismatch between a RVD and a target base will result in a longer distance from the RVD to the base.[20] Functionally, a previous study has also revealed that TALEs with fewer repeats are largely inactive because a minimal number of TALE monomers is needed to recognize the target DNA box and to function efficiently.[47]

To improve the efficiency of a baculoviral TALEN system, we have optimized the virus generation protocol in the current study to minimize genetic rearrangements in the TALE DNA-binding domains by cloning left and right TALEN arms separately into two different vectors and using P2 viruses produced by a low MOI. Since baculoviruses are potent in transduction of various types of human cells including human pluripotent stem cells,[32] our simple method should prove a useful tool to facilitate targeted genome editing in these cells. Alternatively, sophisticated ways, such as the construction of hybrid DNA binding domain architectures with minimal tandem repeats, could be used to circumvent the problem of sequences instability caused by long repetitive arrays of a TALE repeat array and for correctly packaging them into viral vectors. For instance, the use of a pair of hybrid nucleases combining the two distinct DNA binding domains based on ZFN and TALEN platforms respectively could reduce the number of TALE repeat units by half.[48] A single-chain nuclease architecture designated megaTAL has been developed recently, which is generated by fusing the DNA binding domain of a TALE with a meganuclease cleavage domain and provides high efficiency with perfect on target genomic specificity.[49] As only one single TALE is used, we would expect a low frequency of genetic rearrangement when megaTALs are packaged into baculoviral vectors. Looking ahead, advances in the production of viral vectors for the delivery of *TALEN* genes will be valuable for efficient targeted genome editing in various types of human cells.

Derived from insect viruses, baculoviral vectors hold intrinsic immunostimulatory capacities in human cells and can mediate significant innate immune responses.[32,50,51] While the innate immune responses may attenuate transgene expression in mammalian cells,[52] this ability make baculoviral vectors promising adjuvant candidates for vaccination. Baculoviral vectors can also induce adaptive immune responses in mice, which can impact transgene expression *in vivo*.[53] However, since baculoviruses are not infectious to human cells, preexisting baculovirus-specific antibodies and T cells are not detectable in humans,[53] which circumvents the problem of preexisting immunity encountered with vectors derived from human

viruses, like adenovirus and adeno-associated viruses. Also, baculoviral transduction in certain types of cells, for example mesenchymal stem cells, dose not provoke systemic induction of immune cells.[54] Nevertheless, it is worthy of further studies on the design and development of baculoviral vectors with minimized immunostimulatory activity.

MATERIALS AND METHODS

Recombinant baculoviral vectors

Left and right TALEN expression plasmids were constructed using a custom TALEN service provided by Cellectis Bioresearch (Paris, France). TALEN-coding sequences were subcloned into pFastBac1 donor plasmid (Invitrogen, Carlsbad, CA) to produce pFB-TALEN(L-R) that contains both left and right TALEN arms fused by internal ribosome entry site (IRES) element as described previously.[28] Two pFastBac1 vectors contain left and right TALEN arms respectively were also constructed (Supplementary Figure S1 and Sequence Data S1–S3). Recombinant baculoviruses, including BV-TALEN(L), BV-TALEN(R), BV-TALEN(L-R), and BV-eGFP, were generated using pFB-TALEN(L), pFB-TALEN(R), pFB-TALEN(L-R), and pFB-eGFP, respectively, and propagated in *Sf9* insect cells according to the protocol of the Bac-to-Bac Baculovirus Expression System from Invitrogen. Recombinant DNA research in this study followed the National Institutes of Health guidelines.

PCR amplification, TA cloning, restriction enzyme digestion, and DNA sequencing

Virion DNA was isolated using High Pure Viral Nucleic Acid kit (Roche Applied Science). The purified viral genome was used as DNA templates for PCR amplification. An enzyme mixture containing Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and Elongase (Invitrogen) was used to amplify the full length of TALE DNA binding domains. An optimized reaction buffer consisted of 1 µl of 200 ng/µl DNA template, 20 µl Platinum Taq DNA Polymerase High Fidelity master mix, 1 µl Elongase enzyme mix, 0.5 µl for 10 µmol/l of each forward and reverse primer, and 2 µl DMSO. The optimal thermal cycler program for two-step PCR was set as follows: an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles at 94 °C for 20 seconds and 72 °C for 120 seconds with a final extension step at 72 °C for 10 minutes. Amplified products were analyzed on a 1% agarose gel. Primers used to amplify the full-length of TALE repeat arrays are listed in Supplementary Table S1.

The PCR products bearing a TALE repeat array were then cloned into pGEM-T Easy Vector (Promega, Madison, WI). White E. coli colonies containing the PCR products of interest were selected for plasmid extraction (GeneJET Plasmid Miniprep Kit, Fermentas) and confirmed with restriction enzyme (RE) EcoRI digestion. Since the EcoRI sites border both ends of the cloned fragments, EcoRI digestion would release the cloned fragment containing a 1.76 kb TALEN repeat array and a 3 kb pGEM-T Easy Vector. After the RE digestion, the size of DNA fragments was analyzed on a 1% agarose gel. Those clones with the correct size of DNA insert were selected for DNA sequencing using the forward and reverse M13 universal primers.

Southern blot analysis

For Southern blot analysis, plasmid, bacmid and viral genomic DNA (1 µg) was digested overnight with NsiI and XbaI. The digested DNA was loaded on a 2% agarose gel and electrophoresis was performed for 8 hours at 25 V. Using the iBlot Dry Blotting System (Invitrogen), the DNA was then transferred to the iBlot DNA Transfer Stack (Invitrogen) containing a positively charged nylon membrane. The membrane was incubated in 1.5 mol/l NaCl/0.5 mol/l NaOH denaturing solution for 10 minutes immediately after transfer and air-dried. After UV cross-linking at 130 mJ/cm^2, the membrane was hybridized overnight with DIG Easy Hyb (Roche, Indianapolis, IN). DIG-labeled probes targeting the FokI region of the TALEN expression cassette were synthesized using the PCR DIG Probe Synthesis Kit (Roche). Following hybridization, the membrane was stringently washed, blocked, and then incubated with an anti-digoxigenin-AP conjugate (DIG DNA Labeling and Detection Kit, Roche) that was detected by CDP-Star, ready-to-use (Roche). The membrane bearing DNA was exposed to Chemiluminescent Image Analyzer (ImageQuant LAS 4000 mini, GE Healthcare Biosciences, Pittsburgh, PA) for 15 minutes. Primers used for synthesizing DIG-labeled probes are listed in Supplementary Table S1.

Single strand annealing assay

A SSA assay testing vector was constructed by inserting annealed oligonucleotides bearing an AAVS1 TALEN target sequence into BsaI-treated pGL4-SSA firefly luciferase reporter vector (Addgene). The design of custom oligonucleotides was previously described.[55] Briefly, U87 cells were seeded into a 96-well plate at a density of 2,000 cells/well on a day before plasmid transfection. The constructed single strand annealing (SSA) assay testing vector was then transfected using Lipofectamine 2000 (Invitrogen) into U87 cells cultured in Dulbecco's modified Eagle's medium (DMEM) (high glucose) medium with 10% fetal bovine serum (Invitrogen). Four hours after the transfection, the cells were transduced with TALEN-expressing baculoviruses at multiplicity of infection (MOI) of 50 plaque forming units (pfu) per cell. The ONE-Glo Luciferase Assay System (Promega) was used 48 hours later to quantify the produced luminescent signals.

TALEN-mediated homologous recombination

For targeted transgene integration of U87 cells, 2×10^4 cells seeded on a six-well plate were cotransduced with BV-TALEN(L), BV-TALEN(R), and BV-eGFP (as a donor template) at an MOI of 100 in serum-free DMEM medium for 4 hours. The viruses were purified and resuspend in PBS for cell transduction. Briefly, the insect cell medium containing viruses was transferred to sterile tubes. The tubes were centrifuge at 1,000*g* for 5 minutes to remove cells and large debris. The virus supernatant was then filtered through a 0.2 µm, low protein binding filter. A day before the cell transduction, the filtered working virus stock was centrifuged at 28,000*g* (4 °C) for 1 hour 30 minutes. The supernatant was discarded after the high-speed centrifugation and the virus particle pellet was resuspend with PBS. G418 selection at 400 mg/ml was started at day 3 after transduction, and the medium was changed every 2 days for 20 days. For PCR genotyping to verify AAVS1-site specific integration driven by the BV-TALEN system, genomic DNA of U87 cells was isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). PCR amplification was performed using the following parameters: an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles at 94 °C for 15 seconds, 65 °C for 45 seconds, and 72 °C for 150 seconds with a final extension step at 72 °C for 10 minutes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Singapore Ministry of Health's National Medical Research Council (NMRC/CIRG/1367/2013), (NMRC/1284/2011), the Singapore Ministry of Education (MOE2011-T2-1–056), and Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore). Funding for open access charge: (NMRC/CIRG/1367/2013).

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