TALEN-mediated genetic tailoring as a tool to analyze the function of acquired mutations in multiple myeloma cells

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Multiple myeloma (MM) is a clonal plasma cell malignancy that is initiated by a number of mutations and the process of disease progression is characterized by further acquisition of mutations. The identification and functional characterization of these myelomagenic mutations is necessary to better understand the underlying pathogenic mechanisms in this disease. Recent advancements in next-generation sequencing have made the identification of most of these mutations a reality. However, the functional characterization of these mutations has been hampered by the lack of proper and efficient tools to dissect these mutations. Here we explored the possible utility of transcription activator-like effector nuclease (TALEN) genome engineering technology to tailoring the genome of MM cells. To test this possibility, we targeted the HPRT1 gene and found that TALENs are a very robust and efficient genome-editing tool in MM cells. Using cotransfected green fluorescent protein as an enrichment marker, single-cell subclones with desirable TALEN modifications in the HPRT1 gene were obtained in as little as 3–4 weeks of time. We believe that TALENs will greatly facilitate the functional study of somatic mutations in MM as well as other cancers.

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INTRODUCTION
Cancer arises from a single normal cell that undergoes further progression through sequential acquisition of various pathogenic mutations (depicted in Figure 1a). As next-generation sequencing technology becomes more affordable, the number of mutations identified in various cancers continues to increase. In fact, the most frequent mutations in all major cancers have now been mapped and cataloged.1,2 Some of these mutated genes, for example, TP53, KRAS, BRAF, are well-known tumor suppressor genes or oncogenes, whereas the significance of mutations in other genes remains largely unknown. Although cancer is categorically clonal, tremendous subclonal heterogeneity is the unifying characteristic of all cancers due to prevalent mutation-driven clonal evolution.3,4 In this post-genome sequencing era, the biggest question we are facing is how these mutations individually and in combination drive the initiation and progression of cancer.

Theoretically, there are two ways to study cancer mutations in vivo. The first approach is to reconstruct cancer mutations in mice through homology-directed gene targeting, often referred to as gene knockouts or gene knockins.5 This approach replaces the endogenous locus of interest with a foreign DNA fragment that contains sequence homology on each end. This targeting approach has been successfully used in the past 20 years in mouse embryonic stem cells to generate mouse models. The major limitation of this gene targeting approach in mice is that only a limited number of mutated genes can be constructed in the mouse models through labor-intensive, time-consuming and expensive breeding strategies that intercross specific genetically manipulated mouse strains. Most importantly, the majority of mouse models do not faithfully phenocopy human diseases (depicted in Figure 1b).

The second way to study mutations in cancer is to deconstruct cancer mutations housed in human cancer cells from patients in reverse order by restoring the normality of these genes or by recreating the mutations (Figure 1c). However, the very same gene targeting approach used in somatic cells (or somatic knockout) is also notoriously inefficient and time consuming.6,7 There are only a handful somatic knockout cell lines in existence to this day. To that end, we believe a more efficient and flexible genome-editing method is urgently needed to revitalize the use of naturally occurring human cancer models, that is, human cancer cell lines, for functional studies of specific cancer mutations.

Recently, transcription activator-like effector nucleases (TALENs) have emerged as a highly effective tool for genome editing in various systems.8–11 A pair of designed TALENs specifically recognize nearby DNA sequences in the gene of interest on opposite strands. The targeting results in the dimerization of the FokI nuclease cleavage domain in the TALEN modules, which then cleave the DNA sequence between the targeting sites, leading to double-stranded DNA breaks in the target gene. The double-stranded DNA break lesion is then repaired primarily by the error-prone non-homologous end-joining DNA repair system. Typically, after TALEN editing and DNA repair, the target gene is efficiently disrupted through shifting of the reading frame. In addition to gene disruption, the same approach can be used to remove or modify pre-existing mutations by providing exogenous DNA with mutation-corrected DNA sequences. Because of its flexible modular design, TALEN technology can precisely target almost any gene of interest, making this approach highly suitable for editing cancer cell lines, thereby enabling functional analysis of complex cancer mutations.

To prove the principle that TALEN technology can be used to tailor the genome of human cancer cell lines, we used multiple

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myeloma (MM) as a representative cancer. MM is an incurable and fatal malignancy that originates from post-germinal center plasma cells. MM is also an excellent cancer model to study because it is preceded by an asymptomatic premalignant stage called monoclonal gammapathy of undetermined significance and the development of MM involves sequential acquisition of various gene mutations (Figure 1a). Of note, the precise roles that various mutations acquired during the transition from benign to malignant disease remain unknown in many cases. Thus, if TALENs can be used in MM cells, this technology would be a powerful tool to study mutated gene function in this disease. The goal of this study, therefore, was to determine whether TALEN technology could be used to modify the genome of human myeloma cell lines (HMCLs). We chose to first target the HPRT1 gene and show here that TALENs are indeed robust genome-editing tools for HMCLs.

**MATERIALS AND METHODS**

**TALEN design and construction**

The 15-15-15 TALEN design strategy was employed to generate TALENs for this project. Using Mohojand (http://taledesign.org), two 15-mer repeat variable di-residue (RVD) TALENs with a 15-bp spacer were designed to target exon 3 of the human HPRT1 locus (Figure 2a). The 15-bp spacer contained an XhoI site that could be used to screen for TALEN cutting efficacy by restriction fragment length polymorphism analysis. A frameshift mutation at this location is expected to disrupt the phosphoribosyltransferase-type I domain that is essential to HPRT1 function.

All TALEN constructs were synthesized with the Golden Gate method using the pc-GoldyTALEN scaffold (Addgene, Cambridge, MA, USA) described in Carlson et al. The repeat RVDs NI, HD, NG and NN (recognizing A, C, T and G bases, respectively) were used to construct TALENs targeting the HPRT1 locus. In the first Golden Gate reaction, RVDs 1-4 were synthesized individually. The library of 256 pFUS_B4 plasmids is available (represented by the dashed line). Our proposed TALEN-mediated HPRT1 disruption

**Detection of TALEN-mediated HPRT1 disruption**

To detect TALEN-mediated disruption of the HPRT1 gene, primers flanking the TALEN target site were designed using Primer3Plus (www.primer3plus.com). The primer sequences were HPRT1_F1 5'-CACAGCTTACTCTGGCAG-3' and HPRT1_R1 5'-ACAAGGATTGCTTCATGTA-3' (schematic depicted in Figure 2a). Genomic DNA was extracted from single-cell HMCL clones, amplified with the HPRT1 primers and the resulting products were subcloned into the pcRII vector using the TOPO TA cloning kit (Life Technologies) and sequenced with the M13 forward primer.

**Sensitivity of HPRT1 disrupted cells to 6-thioguanine killing**

Two independent TALEN-induced HPRT1 mutant subclones (S1 and S4) of ALMC-2 possessing a 192-nucleotide (nt) insertion and 2-nt deletion, respectively, along with the parental ALMC-2 cells were treated with increasing amounts (0, 1.0, 2.5, 5.0 and 10 μM) of 6-thioguanine (6-TG) in IMDM medium supplemented with 10% fetal calf serum, and 1 ng/ml recombinant IL-6. Cells were seeded at the density of 0.5×10^5/ml in a 24-well plate in quadruplicate. After 9 days of culturing, cell viability in each well was assessed using a Vi-Cell XR (Beckman-Coulter, Brea, CA, USA) cell counter.

**Figure 1.** Cartoon depiction of the genesis of tumors. (a) Generation of tumors such as MM driven by sequential acquisition of various pathogenic mutations in patients. (b) Reconstruction of cancer mutations in mice by gene targeting approach is laborious and often nonproductive due to adverse phenotypes or not feasible due to the limited number of gene mutations that can be imposed (represented by the dashed line). (c) Our proposed TALEN-mediated deconstruction of cancer gene mutations in cancer cells such as HMCLs. Due to the method’s flexibility, the TALEN approach can create or remove mutations as indicated by the multiple arrows.

**Figure 2.** TALEN structure and timeline for TALEN-mediated modification. (a) Sequence representation of TALEN-mediated HPRT1 gene targeting. PF and PR are PCR primers. (b) Timeline of the entire procedure shows its unmatched efficiency.
RESULTS

To test whether TALENs are effective in HMCls, we engineered a TALEN pair to target the HPRT1 gene as depicted in Figure 2a. The HPRT1 gene is located on the X-chromosome and encodes the protein hypoxanthine phosphoribosyltransferase that is necessary for the generation of purine nucleotides through the purine salvage pathway. Both male and female somatic cells have only a single functional copy of the HPRT1 gene because the second copy of the gene in female cells is transcriptionally silenced. The inactivation of the functional copy of the HPRT1 gene renders cells resistant to 6-TG, making it an ideal selection marker for cells in culture. To enrich for transfected cells, we cotransfected cells with an enhanced GFP (EGFP) expression plasmid that would allow us to use FACS to enrich for highly transfected cells that would have likely received the HPRT1 TALEN expression constructs as well. Thus, 48h after transfection of ALMC-2 and KAS-6/1 HMCls with the TALEN pair and pEgfp-N1 plasmid DNAs, GFP-expressing cells were isolated, allowed to rest for 2–4 days and then plated as single cells. After 2 weeks of expansion in culture, we were successful in obtaining 13 ALMC-2 and 10 KAS-6/1 healthy growing subclones.

We then examined whether the genomic sequence at the HPRT1 locus in these single-cell subclones was altered by the TALENs. Amplification of the unmodified HPRT1 gene using the primers described above should yield a 408-bp amplicon. As shown in Figure 3a, all 13 ALMC-2-derived subclones yielded a single sharp DNA band with the size of either ~400 or 600 bp on an agarose gel, whereas most of the KAS-6/1-derived subclones (no. 3–9) produced two major products with sizes of ~700 and 400 bp. Both cell lines were derived from female patients suggesting each cell line should have two copies of the X-chromosome, hence two TALEN targetable copies of the HPRT1 gene. This apparent difference in HPRT1 gene targeting by TALENs between ALMC-2 and KAS-6/1 MM cells prompted us to investigate the copy number of the HPRT1 gene given the fact that MM cells typically display abnormal ploidy. Indeed, prior array comparative genomic hybridization analysis of both cell lines had revealed that the ALMC-2 cell line had lost a copy of the X-chromosome, whereas the KAS-6/1 cell line retained both intact X-chromosomes (unpublished data). Therefore, the two amplified bands observed in the KAS-6/1 cell line may suggest that some of the KAS-6/1 subclones were modified at one allele while the others had identical HPRT1 mutations, or both alleles were differentially repaired resulting in distinct modifications in a single cell (Figure 3a).

We next examined the DNA nucleotide sequences of these PCR products. Because TALEN technology can disrupt gene function via the insertion or deletion of nucleotides by error-prone non-homologous end-joining, we subcloned the DNA fragments into the TOPO TA pCR-II vector, followed by sequencing of 6–8 DNA clones for each single-cell subclone. In all, 78 bacterial colonies derived from the TOPO-cloned HPRT1 PCR products from the 13 distinct ALMC-2 subclones showed evidence of TALEN-induced modifications. Further, sequence alignment analysis showed that only two types of sequence modifications occurred. The lower band with the size of 400 bp showed a 2-nt deletion at the TALEN target site, whereas the larger band of ~600 bp in size had a 192-nt insertion. We further searched the origin of the inserted 192-nt sequence and found it to be a fragment of the rDNA repeat origin sequence present in most plasmids including pC-GoldyTALEN and pEGFP-N1 used in our transfection. We also subcloned the DNA bands from TALEN-modified KAS-6/1 subclones 2, 3, 5 and 9. Although there were multiple amplification products in single-cell subclones 3 and 9, only the 400 bp band matched the HPRT1 sequence. Several bacterial colonies from each KAS-6/1 subclone were picked and those containing a 400 bp insert were sequenced. In total, 52 of these colonies were sequenced and no wild-type HPRT1 sequences were found. Furthermore, both HPRT1 alleles in each of the four cell subclones examined had different mutations (Figure 3b), suggesting that the modification of each allele is autonomous. These data clearly demonstrate that TALENs are a very robust genome-editing tool for HMCls.

After further analysis of TALEN-modified sequences from ALMC-2 subclones S1 and S4, we found both types of HPRT1 disruption resulted in frameshifting of the HPRT1 gene open reading frame (Figure 4a). This suggests that both types of mutation would render the subclones resistant to 6-TG selection. We therefore treated the S1 subclone with the 192-nt insertion and the S4 subclone with the 2-nt deletion along with unmodified parental ALMC-2 cells with increasing concentrations of 6-TG for 9 days. As shown in Figure 4b, although parental ALMC-2 cells were readily killed by 10 μM of 6-TG, both subclones were completely resistant to the drug. Our results therefore clearly suggest that TALEN technology is indeed a very robust system for editing the MM genome. Our data also demonstrate that GFP cotransfection coupled with FACS sorting is a very efficient way to select TALEN-modified cells in a very short period of time (Figure 2b).

DISCUSSION

The newly emerged TALEN technology has shown enormous flexibility and power in editing genomes in various model
systems including Caenorhabditis elegans, zebrafish and embryonic stem cells with great success. Chromosomal translocations and complex gene rearrangements are common characteristics of human cancer, and have not been successfully recreated in vivo due to the lack of specific targeting tools. However, Piganeau et al. successfully used specific TALENs to create t(11;22)(q24;q12) and t(2;5)(p23;q35) translocations found in Ewing sarcoma in human mesenchymal precursor cells, whereas Nyquist et al. used TALENs to engineer prostate-specific androgen receptor gene rearrangements. Here we show that TALENs are also efficient in targeting genes in HMCLs, providing a powerful tool to dissect existing somatic mutations in MM cells. We believe the method is also broadly applicable to other cancer cells, thereby providing a great tool for the functional study of cancer mutations in general.

Among the sequences analyzed, we found that several ALMC-2 cell-derived subclones carry a 192-nt DNA insert of plasmid DNA origin. However, it has previously been reported that exogenous DNA fragments have the tendency to be integrated into the double-stranded DNA break sites of host genomes. To determine whether the insert DNA was generated by TALEN off-target effects in the plasmid DNA, we examined the DNA plasmid sequences flanking the insert. However, we did not find any significant sequence homology that would qualify them as off-target sites for the HPRT1-specific TALENs. Within the exogenous insert and flanking genomic sequence in HPRT1, we found 6-base and 3-base microhomology that may have been exploited for microhomology-mediated end joining. Our observations are consistent with the findings of Lin and Waldman who found that only minimal 2 or 3 nt homologies at the junctions is needed to allow exogenous DNA insertion into host DNA at their ends. Such an efficient ‘absorption’ of foreign DNA into double-stranded DNA break sites actually achieves efficient disruption of the targeting gene, but may also be undesirable for other applications such as gene correction. In vitro-transcribed TALEN mRNA has been used with considerable success and could be used instead of plasmid DNA to avoid unwanted insertions and episomal effects.

Figure 4. Functional disruption of the HPRT1 gene in ALMC-2 subclones S1 and S4. (a) Protein-coding sequence alignment shows frameshifts caused by TALENs. (b) Comparison of 6-TG sensitivity of parental ALMC-2 cells with two subclones with HPRT1 gene disruption by TALEN technology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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