

## Precise Correction of the Dystrophin Gene in Duchenne Muscular Dystrophy Patient Induced Pluripotent Stem Cells by TALEN and CRISPR-Cas9

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### SUMMARY

Duchenne muscular dystrophy (DMD) is a severe muscle-degenerative disease caused by a mutation in the dystrophin gene. Genetic correction of patient-derived induced pluripotent stem cells (iPSCs) by TALENs or CRISPR-Cas9 holds promise for DMD gene therapy; however, the safety of such nuclease treatment must be determined. Using a unique *k*-mer database, we systematically identified a unique target region that reduces off-target sites. To restore the dystrophin protein, we performed three correction methods (exon skipping, frameshifting, and exon knockin) in DMD-patient-derived iPSCs, and found that exon knockin was the most effective approach. We further investigated the genomic integrity by karyotyping, copy number variation array, and exome sequencing to identify clones with a minimal mutation load. Finally, we differentiated the corrected iPSCs toward skeletal muscle cells and successfully detected the expression of full-length dystrophin protein. These results provide an important framework for developing iPSC-based gene therapy for genetic disorders using programmable nucleases.

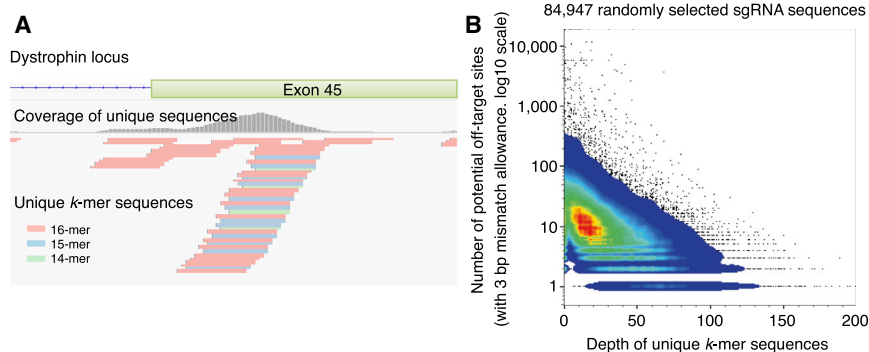
### INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe muscular degenerative disease caused by loss-of-function mutations in the dystrophin gene located on the X chromosome. The dystrophin gene consists of 79 exons, and disruption of the protein reading frame by small deletions, exon duplications, or loss of exons leads to DMD (Pichavant et al., 2011). The large size of the dystrophin gene hampers the delivery of therapeutic cDNA for gene augmentation. Therefore, the delivery of truncated microdystrophin or microdystrophin by an adeno-associated viral (AAV) vector (Okada and Takeda, 2013), lentiviral vector (Pichavant et al., 2011), or *Sleeping Beauty* transposon (Filareto et al., 2013) has been investigated for DMD gene therapy. However, restoration of the full-length dystrophin protein remains challenging. An exon-skipping approach that modulates mRNA splicing patterns using antisense oligonucleotides (Aartsma-Rus et al., 2009) has shown promising results in preclinical studies, but the effects are only transient. Genomic correction using programmable nucleases is an ideal approach that can correct the mutated dystrophin gene.

The development of programmable nucleases has provided a powerful tool for modifying target genome sequences. In particular, the transcription activator-like effector nuclease (TALEN) (Hockemeyer et al., 2011) and

the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR associated 9 (Cas9) endonuclease systems (Cong et al., 2013; Mali et al., 2013) provide greater flexibility than meganucleases or zinc-finger nucleases (ZFNs) with regard to selecting the target regions of interest (Li et al., 2014). Several studies have demonstrated the effectiveness of TALENs (Hockemeyer et al., 2011; Ding et al., 2013a) and CRISPR (Ding et al., 2013b; Mali et al., 2013) in human induced pluripotent stem cells (iPSCs) for reporter knockin, gene knockout, and gene correction. In fact, corrections of disease mutation by nucleases in iPSCs have been reported for several diseases, including  $\alpha$ 1-antitrypsin deficiency (Choi et al., 2013), epidermolysis bullosa (Osborn et al., 2013),  $\beta$ -thalassemia (Ma et al., 2013), AIDS (Ye et al., 2014), and Niemann-Pick Type C (Maetzel et al., 2014).

Before the TALEN and CRISPR systems can reach clinical application, however, target specificity must be improved, as high off-target mutagenesis rates in human cells have been reported (Fu et al., 2013; Hsu et al., 2013; Lin et al., 2014), although some reports have shown otherwise (Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014). Since target specificity depends on the design of the target site, the properties of the DNA-binding domain, and the epigenetic status of the targeting site, the risk of off-target mutagenesis should be examined with respect to each targeting nuclease in a therapeutic setting.



**Figure 1. Visualization of the Unique Sequences in the Human Genome**

(A) Example of the mapped unique  $k$ -mer sequences at the exon 45 region of the dystrophin gene. The mapped  $k$ -mers are indicated in the bottom panel and the coverage of the  $k$ -mers in each base position is indicated by the gray histogram. Within this region, unique sequences of 14- to 16-mer were identified.

(B) Pseudocolor dot plot for the depth of unique  $k$ -mers and the number of potential off-target sites. CRISPR-sgRNA targeting sequences (23 bp with “NGG” PAM,  $n = 84,947$ )

were randomly selected from the human genome and the depth of the unique  $k$ -mers and number of potential off-target sites (with up to 3 bp mismatches allowance) for each sgRNA sequence were calculated. Note that higher depth correlated with fewer potential off-target sites. See also [Table S1](#).

Immortalized myoblasts have been used for restoration of the dystrophin protein mediated by meganucleases (Rousseau et al., 2011; Popplewell et al., 2013), ZFNs (Rousseau et al., 2011), or TALENs (Ousterout et al., 2013). However, although primary myoblasts can be derived from patients, their clonal expansion requires transformation by oncogenes such as hTERT. In contrast, iPSCs (Takahashi et al., 2007) can be isolated from patients directly and still maintain pluripotency and an unlimited self-renewal capacity. Accordingly, when conjugated with made-to-order genetic correction technologies, human iPSCs derived from a patient with a genetic disorder (Park et al., 2008; Hotta et al., 2009) might be applicable to autologous transplantation as ex vivo gene therapy (Sebastiano et al., 2011; Soldner et al., 2011; Zou et al., 2011a, 2011b).

In this study, as a proof-of-concept of such gene therapy for DMD, we performed and demonstrated genetic correction of the dystrophin gene in patient-derived iPSCs by using three different methods: (1) disruption of the splicing acceptor to skip exon 45, (2) introduction of small indels to modulate the protein reading frame, and (3) knockin of the missing exon 44 to restore the full protein coding region. We then performed comprehensive genome-wide mutation analyses to assess the risk of off-target mutagenesis in 14 iPSC clones treated according to the TALEN or CRISPR approach. Our results demonstrate that genetic correction by these approaches in patient-derived iPSCs considerably lowers the risk of off-target mutagenesis and thus holds promise for DMD gene therapy.

## RESULTS

### Targeting a Unique Region in the Human Genome

The risk of off-target mutagenesis by programmable nucleases is associated with the specificity of the target sequence. For example, in the 23 bp of the single-guide RNA (sgRNA)

targeting region, up to 5 bp mismatches may be tolerated, which may lead to off-target mutagenesis (Fu et al., 2013; Hsu et al., 2013). To avoid this, the target sequence must be uniquely defined in the genome. Moreover, the uniqueness should be preserved when considering fragments of the sequence (i.e., 15 bp in length). Therefore, to systematically identify short unique sequences in the genome, we computationally generated all possible combinations of short  $k$ -mer sequences ( $k \leq 16$ ) and searched the human genome to determine how many identical sequences are found for each  $k$ -mer sequence. We then extracted the unique  $k$ -mer sequences only when they matched a single location (i.e., with no match to other regions; see [Table S1](#) available online). We stacked the mapped  $k$ -mer sequences as a histogram to visualize their uniqueness in the sequence depth of coverage (Koehler et al., 2011; [Figure 1A](#)). We confirmed that the higher the depth of the unique  $k$ -mer, the lower was the number of off-target sites, with up to 3 bp mismatches allowed ([Figure 1B](#)). Therefore, the genome regions with a higher depth of unique  $k$ -mers were considered good candidates for targeting by programmable nucleases, and regions with no peak were not considered. The benefit of this method is that it allows one to visually identify the targetable site quickly. Based on the histogram of the unique  $k$ -mers, we identified the 5' region of exon 45 in the dystrophin gene as a target site for designing TALENs and CRISPR-sgRNAs ([Figures 2B](#) and [S2A](#)).

### Generation of Integration-free DMD iPSCs

A DMD patient was diagnosed with a deletion of dystrophin exon 44 by a multiplex ligation-dependent probe amplification method. We performed a primer walking method to sequence the deleted region and identified that the deleted size was 75,484 bp (chrX: 32,215,020-32,290,503 [hg19]), including exon 44 ([Figure S1A](#)). We generated iPSC lines from fibroblasts obtained from this DMD patient using

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