Cell Stem Cell
Previews

Gene-ectomy: Gene Ablation with CRISPR/Cas9 in Human Hematopoietic Cells

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http://dx.doi.org/10.1016/j.stem.2014.10.014

CRISPR/Cas9 has recently been introduced as a gene editing tool and shows considerable promise. In this issue of *Cell Stem Cell,* Mandal et al. (2014) show efficient CRISPR/Cas9-mediated ablation of the CCR5 and B2M genes in primary human hematopoietic cells, two editing strategies that are potentially translatable into clinical application.

Experimental medicine through the use of cell and stem cell therapies has the potential to transform the way we treat specific diseases. In particular, the manipulation of the human hematopoietic system has clinically led the gene and cell therapy field. Gene-modified autologous stem and mature T cell products have now shown considerable clinical efficacy in the treatment of a number of monogenic diseases and acquired conditions. To date, gene delivery has relied on the use of integrating vectors that cannot be directed to a specific gene or locus and thus have the potential for semirandom genomic disruption.

The last 5 years have seen the emergence of gene editing strategies, including zinc-finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs) (e.g., Genovese et al., 2014). The principles of gene editing are those of DNA repair: we achieve site-specific DNA modification by damaging the genome and then taking advantage of the cell's DNA repair mechanisms. In general terms, the DNA repair machinery can opt for either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) if a DNA template is made available. Simultaneous generation of two or more double-strand breaks (DSBs) can also lead to genomic rearrangements such as deletions, inversions, and translocations.

The latest contender in the gene editing field is the CRISPR/Cas9 system, which consists of a nuclease protein (Cas9) that induces DSBs in target DNA after being directed by a short guide RNA molecule (gRNA). The advantages of CRISPR/Cas9 include the versatility of targeting and the ease of design, which allows for high-throughput gene editing (Zhou et al., 2014b). The design of the gRNA requires special attention, as this appears to determine the efficiency as well as the specificity of the approach.

In this issue of *Cell Stem Cell*, Mandal et al. report the ablation of the *CCR5* and *B2M* genes using CRISPR/Cas9 in human CD4$^+$ T cells and hematopoietic CD34$^+$ stem cells, respectively. Whereas CRISPR/Cas9 gene targeting has been previously successfully demonstrated in cell lines, the significant advance in this report lies in the proof of principle for successful gene ablation in primary human hematopoietic cells.

The *B2M* gene encodes for beta-2 microglobulin, a protein associated with the major histocompatibility complex (MHC) class I, or human leukocyte antigen (HLA) class I in humans. With the disruption of B2M, MHC class I molecules are not expressed on the cell surface and CD8$^+$ T cell immunogenicity and alloreactivity can be avoided (Riolobos et al., 2013), which could potentially generate a "universal" cell for allotransplantation. If successful, this approach could result in generic, cost-effective, off-the-shelf T cells that can be engineered for antiviral and anticancer immunotherapy (Torikai et al., 2013).

In the current report, the efficacy of multiple gRNAs targeting *B2M* was initially studied in HEK293T cells where a disruption efficiency of 6.9%–48% was achieved. However, a drop in the rate of NHEJ was found when these same gRNAs were moved to human primary CD4$^+$ T cells (1.4%–4.7%). The authors hypothesized that introducing two different gRNAs targeting a specific locus could result in a frequency of defined deletions, as described previously in other contexts. The results support the validity of the dual gRNA strategy: the predicted deletion was generally observed at rates similar to the NHEJ seen in the gRNAs when they were transfected separately. One of the pairs (crB2M_13+8) resulted in 18% of surface B2M ablation, implying that a synergic interaction between the two guides took place. This augmented effect in the dual gRNA CRISPR/Cas9 context has also been described in mouse embryos (Zhou et al., 2014a).

The *CCR5* gene encodes for chemokine receptor 5, the main coreceptor for the human immunodeficiency virus (HIV) in human T cells. It also became a major target for HIV treatment after the Berlin patient was reported as "cured" from the virus after allogeneic hematopoietic transplant from a *CCR5* deficient donor (Hütter et al., 2009). Research has tried to replicate this with gene therapy strategies to disrupt the *CCR5* gene with ZFNs (Holt et al., 2010) and such efforts were recently reported with the CRISPR/Cas9 system in hiPSCs (Ye et al., 2014).

Mandal et al. also looked to disrupt CCR5 expression in human CD34$^+$ hematopoietic stem cells. Similar to the CD4$^+$ T cell approach, several guides were first analyzed in the K562 cell line and then applied to CD34$^+$ cells. This time, however, NHEJ rates in primary CD34$^+$ cells (about 14%) were more similar to those observed in the K562s and comparable to the 17% NHEJ rate described in the same cells with ZFNs (Holt et al., 2010). Interestingly, when the dual gRNA approach was applied to primary CD34$^+$ cells, authors observed an increased deletion efficiency of 20%–42%, values that could have a bigger impact when applied into functional and preclinical models. It is important to note that the modified CD34$^+$ cells showed normal maintenance of stemness and normal differentiation function when tested in in vitro differentiation and in vivo murine reconstitution assays.

Ideally, gene editing should result in controlled, site-specific modifications. However, off-target effects have been described in all gene editing systems and we might expect even more off-targeting events if two (instead of one) gRNAs are introduced into cells. In the work of Mandal et al., the on-target ablation pattern and off-targeting safety was analyzed by next generation sequencing (NGS). While NHEJ is the main event in single gRNA transfections (5.1%–13.3%), they found that deletion takes the lead in the dual gRNA context (20.0% to 42.1%), with NHEJ and inversions set aside as minor events (2.5%–3.1%). Nevertheless, these disruptions still led to the silencing of CCR5. In terms of off-target disruption, such events were exceedingly rare. They used capture sequence reads at predicted off-target sites in the genome and found that only one site in the homologous *CCR2* gene was statistically significantly mutated above control levels. Structural variations such as inversions or translocations were also not evident at off-target sites.

Comparing the decreased efficiency of editing in T cells to that of editing in CD34$^+$ cells, it is tempting to speculate that some cell types are naturally more refractory to CRISPR/Cas9 gene editing than others. However, as pointed out by the authors, the differences seen could be due to multiple experimental factors: a real resistance of the T cell DNA repair machinery to edition, gene delivery issues, T cell defense against foreign DNA, or just the differences between gRNA sequences.

In summary, the work of Mandal et al. (2014) is one of the first steps toward successful gene editing of primary human T and CD34$^+$ cells with the CRISPR/Cas9 system. The potential applicability of targeted gene disruption is significant and the authors highlight its possible use in highly prevalent conditions such as β-thalasseamia and sickle cell anemia where disruption/deletion of *BCL11A* could increase fetal hemoglobin expression. The use of highly specific gene editing is also the first step to correcting genes by homology directed repair, which, if efficient, would overcome any concerns surrounding current gene augmentation strategies. Given the current momentum of CRISPR/Cas9 research and the rapid advances in gene modification and gene delivery technologies, the move to human clinical application is unlikely to be too far away.

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