Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9

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SUMMARY

Genome editing via CRISPR/Cas9 has rapidly become the tool of choice by virtue of its efficacy and ease of use. However, CRISPR/Cas9 mediated genome editing in clinically relevant human somatic cells remains untested. Here, we report CRISPR/Cas9 targeting of two clinically relevant genes, *B2M* and *CCR5*, in primary human CD4^{+} T cells and CD34^{+} hematopoietic stem and progenitor cells (HSPCs). Use of single RNA guides led to highly efficient mutagenesis in HSPCs but not in T cells. A dual guide approach improved gene deletion efficacy in both cell types. HSPCs that had undergone genome editing with CRISPR/Cas9 retained multi-lineage potential. We examined predicted on- and off-target mutations via target capture sequencing in HSPCs and observed low levels of off-target mutagenesis at only one site. These results demonstrate that CRISPR/Cas9 can efficiently ablate genes in HSPCs with minimal off-target mutagenesis, which could have broad applicability for hematopoietic cell-based therapy.

INTRODUCTION

The hematopoietic system is at the forefront of cell-based gene therapies due to the fact that the cells can be readily obtained, manipulated, and reintroduced into patients. The development of genome editing methodologies such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENS) (Urnov et al., 2010);(Joung and Sander, 2013; Scharenberg et al., 2013) have enabled site-specific gene repair or ablation and raised the possibility of treating a broad range of diseases at the genetic level (Pan et al., 2013). Despite much promise, limitations associated with these technologies, including low targeting efficacy and *de novo* engineering of proteins for each target have precluded wide-spread adoption of these technologies for therapeutic use (Silva et al., 2011). The recent emergence of the clustered, regularly interspaced, palindromic repeats (CRISPR) system for gene editing has the potential to overcome these limitations (Jinek et al., 2012). The CRISPR technology utilizes a fixed nuclease, often the CRISPR-associated protein 9 (Cas9) from *Streptococcus pyogenes,* in combination with a short guide RNA (gRNA) to target the nuclease to a specific DNA sequence (Cong et al., 2013; Jinek et al., 2012; Jinek et al., 2013; Mali et al., 2013). CRISPR/Cas9 relies on simple base-pairing rules between the target DNA and the engineered gRNA rather than protein-DNA interactions required by ZFNs and TALENS (Gaj et al., 2013; Wei et al., 2013). As a result, the CRISPR/Cas9 system has proven extremely simple and flexible. Perhaps most important, this system has achieved highly efficacious alteration of the genome in a number of cell types and organisms (Ding et al., 2013; Hwang et al., 2013; Niu et al., 2014; Wang et al., 2013; Wei et al., 2013).

Given the importance of the hematopoietic system in cell-based gene therapies, we tested the CRISPR/Cas9 system in primary human CD4^{+} T cells and CD34^{+} hematopoietic stem and progenitor cells (HSPCs) targeting two clinically relevant genes, beta-2 microglobulin (*B2M*) and chemokine receptor 5 (*CCR5*). *B2M* encodes the accessory chain of major histocompatibility complex (MHC) class I molecules and is required for their surface expression (Bjorkman et al., 1987; Zijlstra et al., 1990). Deletion of *B2M* is a well-

established strategy to ablate MHC class I surface expression (Riolobos et al., 2013), and could be used to generate hypoimmunogenic cells for transplantation and adoptive immunotherapy. *CCR5* is the main co-receptor used by CCR5-tropic strains of HIV-1 (Trkola et al., 1996) and a validated target for gene ablation, as mutations resulting in loss of protein expression or haploinsufficiency protect against HIV infection (Catano et al., 2011; Hutter et al., 2009; Martinson et al., 1997; Samson et al., 1996). Moreover, transplantation of CCR5 homozygous mutant HSPCs provides long-term protection against HIV rebound even after discontinuation of antiretroviral therapy (Allers et al., 2011; Hutter et al., 2009). Several attempts have been made to target *CCR5* in T cells (Perez et al., 2008; Tebas et al., 2014) and HSPCs (Holt et al., 2010; Schleifman et al., 2011) though the efficiency of gene targeting was not sufficient to protect against viral recrudescence (Tebas et al., 2014). Recently, *CCR5* has been targeted using CRISPR/Cas9 in cell lines (Cho et al., 2013) and iPS cells (Ye et al., 2014). However, CRISPR/Cas9 gene editing in primary human hematopoietic cells remains untested. Here we report that use of CRISPR/Cas9 with single gRNAs led to highly efficient *CCR5* ablation in CD34^{+} HSPCs but not *B2M* in CD4^{+} T cells. Employing a dual gRNA approach improved gene deletion efficacy in both cell types with biallelic inactivation frequencies reaching 34% for *B2M* in CD4^{+} T cells, and 42% for *CCR5* in CD34^{+} HSPCs. Importantly, CRISPR/Cas9 *CCR5*-edited CD34^{+} HSPCs retained multi-lineage potential *in vitro* and *in vivo* upon xenotransplantation. Deep target capture sequencing of predicted on- and off-target sites in CD34^{+} HSPCs revealed highly efficacious on-target mutagenesis, and exceedingly low off-target mutagenesis.

RESULTS

We designed gRNAs to target Cas9 to the *B2M* gene (Figure 1A). Each guide was first tested for the ability to direct site-specific mutations in HEK293T cells. Using flow cytometry we measured the efficiency of each gRNA to direct Cas9-mediated ablation of B2M surface expression 72 hours post-transfection (Figure 1B). We observed that B2M was abrogated in ~7% (±1.02 SEM, n=3) to 48% (±1.80 SEM, n=3) of HEK293T cells depending upon the gRNA utilized (Figure 1C and S1A). Similar results were observed using the Surveyor assay, with gRNA-specific mutation frequencies of 0–26% in HEK293T cells (Figure S1B). We also designed gRNAs to target Cas9 to the *CCR5* gene (Figure 1D). Upon introducing these into K562 cells, we measured targeting efficacy using the Surveyor assay and observed mutation frequencies ranging from 22–40% (Figure 1E). Variation in the efficiency with which a specific gRNA directed Cas9-mediated ablation was observed, even between gRNAs targeting the same exon or nearly overlapping sites (Figure 1A–E) indicating that on-target efficiency of site directed mutation is highly gRNA dependent as previously noted (Hsu et al., 2013).

Next, we tested selected single gRNAs in CD4^{+} T cells and CD34^{+} HSPCs. Surprisingly, gRNAs that were highly efficacious at targeting *B2M* in HEK293T cells exhibited lower targeting efficiencies in primary CD4^{+} T cells ranging from 1.4% (±0.2 SEM, n=6) to 4.7% (±0.9 SEM, n=6) ablation of *B2M* expression (Figure 1F and S1C-D) or 3–11% using the Surveyor assay (Figure S1B, E). For instance, crB2M_13 exhibited more than 10-fold reduced efficacy in CD4^{+} T cells (4.7%±0.9) as compared to HEK293T cells (48.0%±1.8) (Figure 1F and S1C). Interestingly, single gRNAs targeting *CCR5* showed comparably

mutation frequencies in CD34+ HSPCs as observed in K562 cells (Figure 1E,G). To explore this further we performed direct Sanger sequencing of several hundred colonies derived from HSPC clones targeted with crCCR5_A or crCCR5_B from two donors and observed very high mutation frequencies in all cases (Figure 1H). As only cells expressing Cas9 were analyzed, it is unlikely that differences in on-target mutation efficiency were due to differential transfection efficiencies, although we cannot rule out differential transfection of individual guides, but rather may reflect intrinsic properties of certain primary hematopoietic cell types.

We reasoned that using two gRNAs directed against the same locus might generate predictable mutations (deletions) more frequently than that achieved by error-prone non-homologous end joining (NHEJ), which represents the predominant DNA double strand break (DSB) repair pathway in HSPCs (Beerman et al., 2014). Indeed, this approach has previously been utilized for ZFNs, TALENs and the CRISPR/Cas9 system to achieve predictable deletions (Bauer et al., 2013; Canver et al., 2014; Gupta et al., 2013; Lee et al., 2010; Wang et al., 2014; Zhou et al., 2014). Six dual gRNA combinations targeting *B2M* with DNA sequence lengths between their predicted Cas9 cleavage sites ranging from 81 to 2261 nucleotides were introduced in CD4+ T cells together with Cas9 (Figure 2A). We observed a trend of improved targeting efficacy for most of the tested gRNA pairs and greatly improved efficacy for one gRNA pair (crB2M_13+8), which resulted in 18.0% (±8.35 SEM, n=3) ablation of B2M surface expression (Figure 2B-C and S2A). B2M ablation led to a concomitant reduction of MHC class I cell surface expression (Figure S2B). We further interrogated mutation frequency at a clonal level via single-cell quantitative PCR (qPCR), which revealed 28.2% (n=301 cells analyzed) of CD4+ T cells were homozygous null for *B2M* (Figure S2C). Upon Sanger sequencing across the predicted Cas9 cutting sites we observed deletion of the intervening sequence (Figure S2D).

We next applied the dual guide strategy to primary CD34+ HSPCs by introducing three gRNA pairs along with Cas9 (Figure 2D). Sorted CD34+ HSPCs expressing Cas9 were plated into methylcellulose and emergent clonal colonies were picked two weeks post-plating for analysis. Individual colonies were analyzed by PCR to quantify the deletion efficacy of *CCR5* (Figure 2D, E). Remarkably, although variation in *CCR5* ablation was noted among different donors and gRNA pairs, we consistently observed high monoallelic and biallelic inactivation of *CCR5* in all cases (Figure 2E and S2E). For example, one dual gRNA combination (crCCR5_D+Q) generated biallelic *CCR5* deletion in CD34+ HSPCs at a rate of 26.8% (±7.1 SEM) across 4 donors (Figure 2E and S2E). It should be noted however that the mutation rates determined by this PCR strategy underestimate actual mutation frequency since small insertions or deletions (InDels) are not detected by this approach. A similar dual gRNA approach targeting *CCR5* (crCCR5_A+B) in CD4+ T cells resulted in a biallelic inactivation rate of 8.8% at the single cell level (n=363) (Figure S2F). Again, after Sanger sequencing we noted excision of the DNA between the Cas9 cleavage sites (Figure S2G). Taken together, these data demonstrate that highly efficacious ablation of clinically relevant genes can be achieved in primary hematopoietic CD4+ T cells and CD34+ HSPCs using a dual gRNA strategy.

In order to determine whether CD34^{+} HSPCs that had undergone genome editing with CRISPR/Cas9 retained their potential to differentiate into effector cells, we performed *in vitro* and *in vivo* differentiation assays. Towards this *CCR5*-edited CD34^{+} HSPCs were plated in methylcellulose and clonal colonies that emerged two weeks post-plating were counted and scored for contribution to granulocyte, macrophage, erythrocyte and megakaryocyte lineages. Comparable colony numbers and colony types were observed regardless of whether single, dual or no gRNAs were used demonstrating that CD34^{+} HSPC colony forming potential was not impacted by CRISPR/Cas9 (Figure 3A) despite the high *CCR5* mutation frequencies observed in these experiments (Figure 1H, 2E).

We next tested the *in vivo* reconstitution potential of HSPCs following CRISPR/Cas9 targeting of *CCR5* by xenotransplantation of control (Cas9-only), and *CCR5*-edited (Cas9 + crCCR5 D+Q) CD34^{+} HSPCs into NOD-*Prkdc^{Scid}-IL2rγ^{null}* (NSG) recipients. Recipients were sacrificed at 12 weeks post-transplantation and human hematopoietic cell engraftment (hCD45^{+}) was examined in the bone marrow revealing CD19^{+} lymphoid cells and CD11b^{+} myeloid cells (Figure 3B). Human CD45^{+} hematopoietic cells were also found in the spleens of transplanted mice (Figure 3C). PCR analysis on DNA isolated from sorted human CD45^{+} hematopoietic cells from reconstituted mice demonstrated that *CCR5* edited cells (ΔCCR5) robustly contributed to human hematopoietic cell chimerism (Figure 3D). Taken together these results demonstrate that CRISPR/Cas9 *CCR5*-edited CD34^{+} HSPCs retained multi-lineage potential *in vitro* and *in vivo*.

CRISPR/Cas9 has been shown to generate off-target mutations depending upon experimental setting and cell type (Cho et al., 2014; Cradick et al., 2013; Fu et al., 2013; Fu et al., 2014; Hruscha et al., 2013; Lin et al., 2014). To examine this we performed target capture sequencing of CD34^{+} HSPCs subjected to CRISPR/Cas9 *CCR5*-editing. These experiments captured each gRNA target site (n=5) and predicted off-target sites (n=126) with expanded capture intervals of 500 base pairs flanking each site to ensure accurate detection of any genetic lesion occurring at or near the selected sites (Figure 4A, Table S1). We have previously shown this approach can identify structural variations, such as translocations and inversions, in proximity to the capture site (Talkowski et al., 2011). Sorted CD34^{+} HSPCs treated with Cas9 alone or in combination with multiple single or dual gRNA combinations were sequenced to a mean target coverage of 3,390X across each 23 bp gRNA sequence and PAM (Figure 4B). Analysis of these data revealed highly efficacious on-target mutagenesis with a diverse array of mutated sequence variants observed in both single and dual gRNA treatments (Figure 4C). As expected, we detected small InDels of up to 10bp in addition to single nucleotide substitutions at the predicted target sites in single gRNA conditions. Strikingly, in each dual gRNA experiment, no fewer than 15 alternate mutant alleles were observed at either one of the gRNA sites (Tables S2–S4). Notably, the sequencing depth of our analysis permitted estimation of mutation frequency for each particular variant, including mutations that were observed in only a few hundredths of a percent of the sample sequenced (Table S5). Predicted deletions (i.e. deletions between the two Cas9 target sites) were the most common mutations observed, while small InDels were also frequent (Figure 4C). Interestingly, for two combinations, crCCR5_A+B and crCCR5_D+Q, we also observed inversions between the predicted Cas9 cleavage sites. The

most efficacious combination crCCR5_D+Q led to mutations in approximately 48% of the captured sequence reads.

We next examined the capture sequence reads at predicted off-target sites in the genome (Table S1). An N-fold enrichment analysis was performed, wherein we compared the total number of non-reference sequencing reads at each predicted off-target site in gRNA treated and control (Cas9 only) samples. This analysis generated a ratio where 1.0 indicates an equivalent number of non-reference sequence reads in both treated and control samples, values less than 1.0 indicate fewer non-reference reads in treated samples, and values greater than 1.0 indicate a greater number of non-reference reads in treated samples (Figure 4D). This analysis found that the mean enrichment of mutations at off-target sites in all the gRNA-treated samples compared to control closely conformed to the null hypothesis (i.e., 0.99-fold enrichment compared to controls) indicating that off-target mutation events were extremely rare. Indeed, statistical evaluation of all captured off-target sites yielded a single site (1/126; 0.6%, Figure 4D) in the sample treated with crCCR5_B alone that passed multiple test correction for a statistically significant enrichment for off-target InDels versus controls (p 7.6×10^{-11}) (Table S5). When we scrutinized the sequencing reads from this site, which was located in the highly homologous *CCR2* gene (Figure S3A), we found that all sequence variants (36 out of 5,963 total reads) were one or two base InDels, (Figure S3B). Of note, the other sample in which crCCR5_B was used (in combination with crCCR5_A) only 13 out of 5,339 reads supported mutation, however these events did not meet statistical significance above controls (Figure S3B, Table S5). Thus, off-target mutagenesis was exceedingly rare and moreover, the use of two gRNAs in combination did not increase the very low incidence of off-target mutagenesis. We also performed analyses for structural variation at all sites and though we could readily detect on-target inversions in crCCR5_A+B and crCCR5_D+Q treatments, there was no evidence for inversion or translocation at any off-target sites. These data indicate that on-target mutagenesis efficiency was very high, and further that off-target mutagenesis was extremely infrequent for both single and dual gRNA treatments.

DISCUSSION

In this study we utilized the CRISPR/Cas9 system in human primary CD4^{+} T cells and CD34^{+} HSPCs to target two clinically relevant genes *B2M* and *CCR5*. Surprisingly, the activity of the CRISPR/Cas9 system was remarkably variable in different human cell types, with the same gRNA exhibiting highly efficacious on target mutagenic activity in HEK293T cells but little activity in CD4^{+} T cells. In contrast, the targeting efficacy in K562 cells and CD34^{+} HSPCs was comparable. Moreover, consistent with previous reports (Hsu et al., 2013) we observed that the efficiency of the CRISPR/Cas9 system was gRNA specific, as even gRNAs with partially overlapping sequences displayed significantly different targeting efficiencies. Further, a dual gRNA approach yielded increased gene ablation efficacy in both CD4^{+} T cells and CD34^{+} HSPCs leading to predicted deletions at the targeted loci.

The lack of CRISPR/Cas9 activity observed in T cells especially with single gRNAs may be due to a number of factors including, inefficient plasmid DNA delivery, the innate immune response of T cells to foreign nucleic acid (Monroe et al., 2014), and/or active DNA repair

machinery. Given the efficacy of the CRISPR/Cas9 system in a wide variety of cell types and species both *in vitro* and *in vivo* (Sander and Joung, 2014), the lack of activity observed in T cells is likely the exception and not the rule. Nonetheless, our results highlight that CRISPR/Cas9 targeting efficacy can differ between cell lines and primary cells. Ultimately, further studies will be necessary to determine how variable the activity of CRISPR/Cas9 is in different primary human cell types.

Our mutational analysis revealed highly efficacious mutagenesis of on-target sites in CD34^{+} HSPCs. Single gRNAs generated a range of mutations with the vast majority comprised of small InDels. In contrast, dual gRNA combinations largely led to predicted deletions though a diverse array of mutations including InDels and even inversions were detected. Importantly, we only identified one statistically significant off-target site in the highly homologous *CCR2* gene, which occurred in one out of 6 experimental settings (gRNA crCCR5_B alone). Sequence analysis of crCCR5_B in comparison to the identified off-target site in *CCR2* indicated that it perfectly matched in the seed region and contained 3 sequence mismatches at the 5′ end of the gRNA sequence. This data is consistent with studies showing that mismatches in the 5′ end of the gRNA are tolerated by Cas9 (Lin et al., 2014; Wu et al., 2014). Our data support the idea that judicious guide design is critical for minimizing off-target mutations. Of note, our very deep sequencing analysis enabled detection of the lone off-target event, whereas analysis performed at lower sequencing depth — such as 50X coverage used in previous studies (Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014) — would have been unable to detect this event. Overall, our analysis of CRISPR/Cas9 mutational activity in CD34^{+} HSPCs revealed very high on-target mutation rates and extremely low incidence of off-target mutagenesis.

The ability to direct efficient and predictable deletions using dual gRNAs opens the possibility of using this strategy to target non-coding regions in the genome such as enhancers and silencers that control expression of disease relevant genes. For example, recent studies have identified regulatory regions that control expression of fetal hemoglobin (Bauer et al., 2013), which if deleted increase fetal globin expression in cells otherwise restricted to expressing adult β-globin (Bauer et al., 2013; Xu et al., 2011). Targeted deletion of such regions in CD34^{+} HSPCs followed by transplantation into patients may provide a durable therapy for the treatment of β-hemoglobinopathies such as sickle cell anemia and β-thalassemia (Xu et al., 2011). Overall, our data demonstrate that CRISPR/Cas9 can be used to ablate genes of clinical significance in CD4^{+} T cells and CD34^{+} HSPCs with an efficiency that is therapeutically meaningful for a number of clinical settings, such as the treatment of HIV. Our demonstration that CRISPR/Cas9 targeted CD34^{+} HSPCs retain multi-lineage potential *in vitro* and *in vivo*, combined with very high on-target and minimal off target mutation rates suggests that CRISPR/Cas9 could have broad applicability enabling gene and cell-based therapies of the blood.

EXPERIMENTAL PROCEDURES

Molecular Biology

All guides were designed using the online optimized design tool at http://crispr.mit.edu. gRNA and primer sequences are enlisted in supplemental data.

Transfection of Cells

Human primary CD4+ T cells and CD34+ HSPCs were transfected with Cas9-2A-GFP and gRNA encoding plasmids using respective Amaxa Nucelofection kits using cell-specific Nucleofection program with Nucleofector II device.

Surveyor assay

Amplicons spanning the different targeted regions were PCR amplified using the Phusion polymerase and HF Buffer (New England Biolabs) and CEL assay was carried out using the Surveyor Mutation detection kit (Transgenomic) as per manufacturer's instructions.

In vivo transplantation of CD34+ HSPCs

75,000-sorted CD34+ HSPCs expressing Cas9 alone (control group, n=2) or Cas9 with crCCR5_D+Q gRNAs (experimental group, n=5) were transplanted in to NSG recipient mice. At 12 weeks post-transplantation, all mice were euthanized and blood, bone marrow, and spleen samples were taken for characterization of human hematopoietic cell chimerism. Human CD45+ cells were sorted for DNA isolation and analysis of *CCR5* deletion.

Off-Target Prediction and Capture Sequencing

Each guide RNA target site (n=5) and predicted off-target site (n=126) was selected for capture sequencing (Table S1) using the Agilent SureSelectXT Target Enrichment System. Capture Sequencing was performed as described earlier (Talkowski et al., 2011).

For details on experimental procedures, see supplemental data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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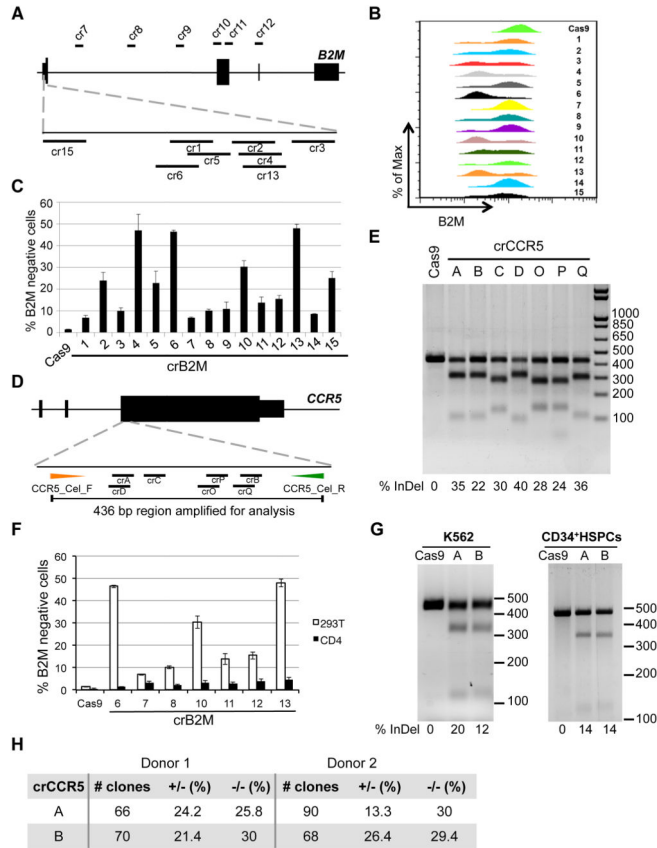
Figure 1. Targeting clinically relevant loci in human cells using CRISPR/Cas9

A) Schematic of gRNAs targeting *B2M*. B) Histogram of B2M surface expression in HEK293T cells. C) B2M deletion efficiency with various gRNAs in HEK293T cells; n=3 (mean±SEM). D) Schematic of gRNAs targeting *CCR5*. Orange and green arrows represent primer pairs used to amplify the region for analysis. E) Surveyor assay of each gRNA targeting *CCR5* in K562 cells. % InDels is indicated under each guide. F) B2M deletion efficiency of selected gRNAs in primary CD4+ T cells in comparison to 293T cells; n=6 (mean±SEM). G) Surveyor assay of crCCR5_A and crCCR5_B targeting *CCR5* in K562 cells and HSPCs. H) Clonal deletion efficiency of crCCR5_A and crCCR5_B targeting of *CCR5* in HSPCs (n=2) as determined by Sanger sequencing. (Note: crB2M_14 is not depicted in panel A schematic, as it is located 20 Kb downstream of coding sequence.). See also Figure S1.
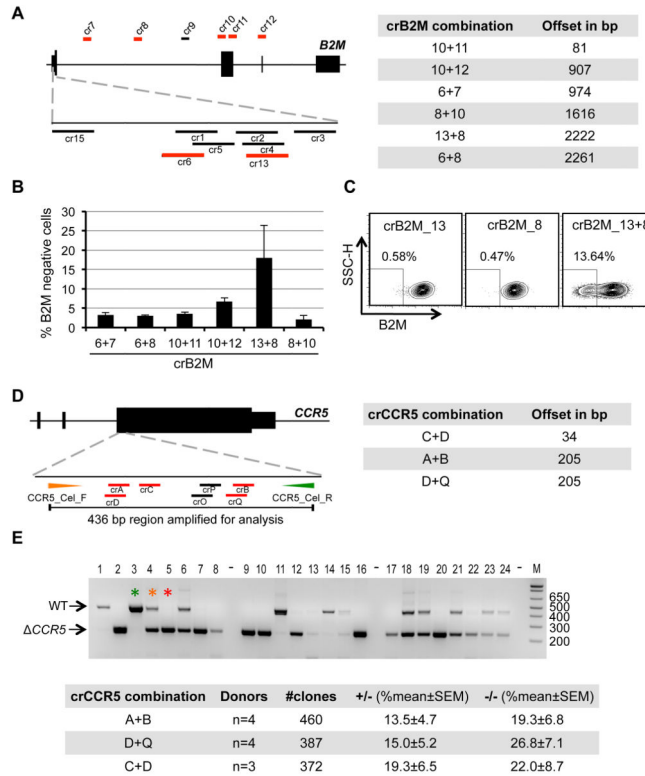
Figure 2. A dual gRNA approach for CRISPR/Cas9 genome editing in primary human hematopoietic stem and effector cells

A) Schematic of dual gRNA approach for targeting the *B2M* locus. gRNA pairs are in red. The offset in base pairs between Cas9 sites for each gRNA combination (right panel). B) B2M deletion efficiency in CD4$^+$ T cells for 6 dual gRNA combinations (n=3; mean±SEM). C) FACS plots showing loss of B2M expression of either crB2M_13 or crB2M_8 alone or in combination in primary CD4$^+$ T cells. D) Schematic of dual gRNA approach for targeting *CCR5*. gRNA pairs are shown in red. Orange and green arrowheads represent primer pairs used to amplify the region. The offset between the Cas9 sites of each gRNA pair (right panel). E) Gel electrophoresis image of CD34$^+$ HSPCs derived clones targeted with crCCR5_D+Q analyzed by PCR. Note the deletion of the 205 bp region between the two gRNA cutting sites (top panel; WT: wild type; *CCR5*: deleted; green * = WT; orange * = heterozygote; and red * = null clone). Clonal deletion efficiency for three dual gRNA combinations targeting *CCR5* in CD34$^+$ HSPCs (n=4; % mean±SEM; bottom panel). See also Figure S2.
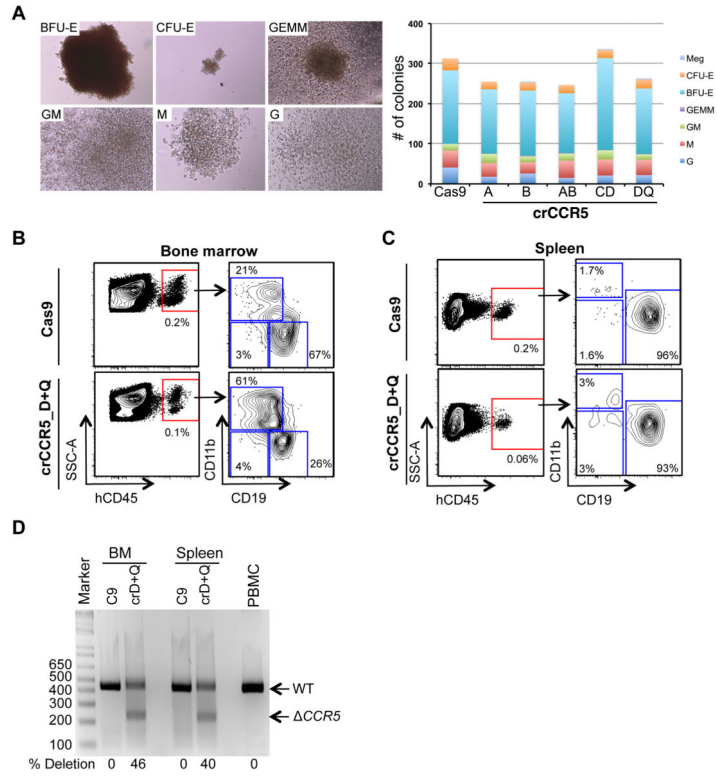
Figure 3. *CCR5*-edited CD34^{+} HSPCs retain multi-lineage potential
A) Representative pictures of colonies formed in methylcellulose CFC assay (left panel) with quantified data on colony number and types are presented (right panel). Representative FACS plot showing human hematopoietic cell (hCD45^{+}) engraftment and multi-lineage reconstitution at 12 weeks post-transplantation in the bone marrow (B) and spleen (C) of NSG recipient mice. D) PCR results confirmed predicted deletion of targeted region at *CCR5* locus in human hematopoietic cells sorted from NSG mice transplanted with CRISPR/Cas9-treated HSPCs. PBMC (human peripheral blood mononuclear cells) from healthy donor taken as control. (WT: wild type, Δ*CCR5*: deleted)
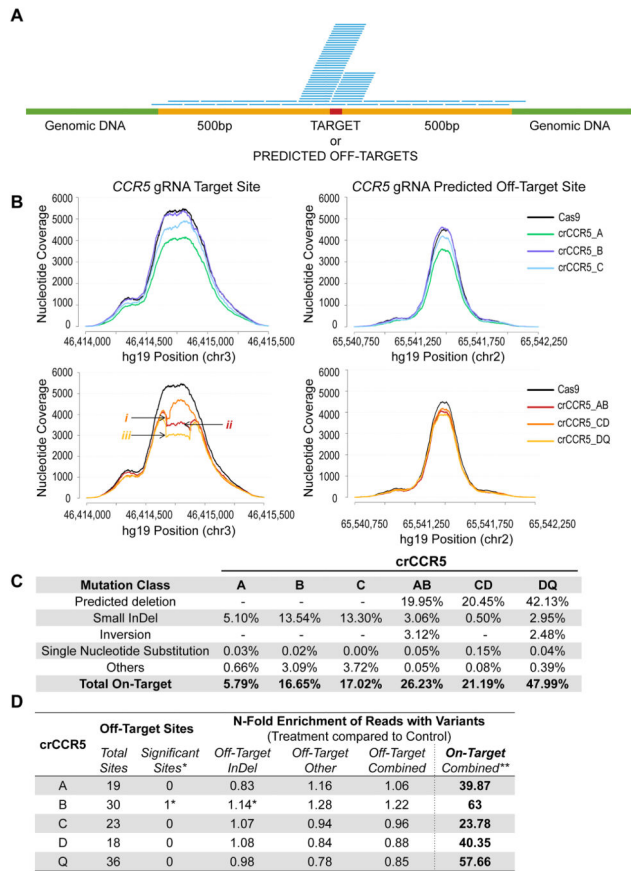
Figure 4. Targeted capture and extremely deep sequencing of on-target and predicted off-target sites in CD34^{+} HSPCs

A) Schematic of targeted capture deep sequencing of on-target and predicted off-target sites (red bar); probe sets are indicated in blue. A 500 bp region flanking the site (in yellow) was included for detection of structural rearrangements (i.e. translocations). B) Plots showing sequencing depth coverage at both on-target (left panel) and off-target (right panel) sites, achieving a coverage exceeding 3,000x for all on-target sites. Decrease in sequencing depth at the on-target sites in dual-gRNA libraries is marked by arrow, supporting predicted deletions (bottom left; i=35 bp, ii=205 bp, iii=205 bp). C) Precise estimation of on-target mutation allele frequencies by capture sequencing. Notably, the rate of mutation exceeds previous estimates by PCR of predictable deletions, as smaller InDels and inversions also occur at appreciable frequencies. D) Estimation of mutation frequencies at predicted off-target sites (*One off-target site was statistically different from controls following correction for multiple comparisons; p 7.6×10^{-11}). N-fold enrichment is determined based on the ratio of non-reference reads in treated libraries compared to untreated library and represents the average of all off-target sites for a given experiment. Enrichment of 1 is equivalent to untreated control. **For reference to on-target enrichments, on-target combined represents the proportion of non-reference reads (including single and dual-gRNA treatments using a given gRNA) to total reads at on-target sites in treatment compared to control. See also Tables S1–S5.