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ORIGINAL ARTICLE

Quantitative assessment of timing, efficiency, specificity and genetic mosaicism of CRISPR/Cas9-mediated gene editing of hemoglobin beta gene in rhesus monkey embryos

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Abstract

Gene editing technologies offer new options for developing novel biomedical research models and for gene and stem cell based therapies. However, applications in many species demand high efficiencies, specificity, and a thorough understanding of likely editing outcomes. To date, overall efficiencies, rates of off-targeting and degree of genetic mosaicism have not been well-characterized for most species, limiting our ability to optimize methods. As a model gene for measuring these parameters of the CRISPR/Cas9 application in a primate species (rhesus monkey), we selected the β -hemoglobin gene (*HBB*), which also has high relevance to the potential application of gene editing and stem-cell technologies for treating human disease. Our data demonstrate an ability to achieve a high efficiency of gene editing in rhesus monkey zygotes, with no detected off-target effects at selected off-target loci. Considerable genetic mosaicism and variation in the fraction of embryonic cells bearing targeted alleles are observed, and the timing of editing events is revealed using a new model. The uses of Cas9-WT protein combined with optimized concentrations of sgRNAs are two likely areas for further refinement to enhance efficiency while limiting unfavorable outcomes that can be exceedingly costly for application of gene editing in primate species.

Introduction

New technologies allowing gene editing and stem cell manipulation offer new hope for addressing serious human genetic

diseases such as hemoglobin disorders. The development of these new approaches will be facilitated by the availability of animal models that allow physiologically relevant testing of novel therapeutic approaches. Nonhuman primates offer an

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outstanding model species for such applications because of their similar genetic makeup, and similar developmental, endocrinological, behavioral, metabolic and physiological characteristics. But the application of these technologies in primates, especially the most promising method using zygotic gene editing, must address challenges not encountered with other model species (rodent, pig, etc.), such as the long generation time, availability, husbandry cost, and other procedural costs. As a result, issues of efficiency and specificity of gene editing take on increased importance for implementation in primates. To date, the efficiency, specificity, timing, and genetic mosaicism features of zygotic gene editing have not been characterized in detail in any primate species. This study reports the first detailed, quantitative analysis of these parameters of gene editing in any primate species.

Previously, genetic engineering among mammalian species has been most successful in mice using zygote-based microinjection approaches for transgenesis, and gene knockout/knock-in approaches using embryonic stem cells. Genetic engineering in other mammals, for which embryonic stem cells have not been available and zygote microinjection has not proven efficient, has been achieved predominately through transgenic manipulation of somatic cells coupled with somatic cell nuclear transfer to generate progeny. More recently, germ cell manipulation and transplantation emerged as additional approaches for modifying animal germ lines. Techniques based on using the clustered regularly interspaced short palindromic repeats (CRISPR) associated bacterial Cas9 enzyme to engineer targeted DNA modifications in zygote stage embryos have opened up new possibilities for genetic engineering in virtually any animal where embryos or germ cells can be accessed. A high efficiency and a high degree of specificity were observed when CRISPR was applied in rodents (1–3). High efficiencies were also reported for nonhuman primates (4,5), and human embryos (6,7). However, protocols have not been optimized, and the incidence of undesirable outcomes has not been well characterized, nor has the timing of gene editing been thoroughly studied. In some studies results are available only for small tissue biopsies taken from progeny delivered at term, or tissues from arrested fetuses. Most studies experienced very high rates of developmental arrest (can be 90% or greater loss), due in part to arrested early development, and apparently in part to pregnancy loss associated with multiple gestation pregnancies following multiple embryo transfer (4,8–10). This severely limits insight into the genetic modifications that might have occurred in most embryos, and further raises concerns about non-genetic technical factors contributing to the low rates of survival. In studies where pre-implantation embryos were examined, the methods of analysis limited insight into the nature of gene targeting events, and overall efficiency. For example, the Surveyor method provides a rough estimate of targeting efficiency based on gel band intensity, but no insight into the nature of editing events, whereas methods employing cloning and sequencing of PCR products provide at least partial information on the nature of gene editing events, but limitations in sampling can compromise estimates of efficiencies and timing of these events, and may not detect lower abundance alleles that arise during later cleavage stages (4,5,8–10). Collectively, studies to date in primates demonstrate the promise and potential for genome editing but also highlight the need for more detailed understanding of CRISPR/Cas9 gene editing in these species, the reasons for high rates of mortality, and approaches to maximize efficiency and specificity without excess genetic mosaicism. Thus, acquiring data for improving the success of gene editing through methodological

variations in a primate species remains an important area for addressing major concerns related to practical implementation, including efficiency, genotoxicity caused by off-target editing, and genetic heterogeneity that can arise due to occurrence of multiple and temporally distinct DNA modification events during cleavage divisions.

As a model gene for measuring these parameters of the CRISPR/Cas9 application in a primate species (rhesus monkey), we selected the β -hemoglobin gene (*HBB*), due to its relevance to the potential application of gene editing and stem-cell technologies for treating human disease. Hemoglobin disorders, such as sickle-cell disease (SCD) and the thalassemias, are the most common monogenic diseases in the world, with an incidence of 300,000 to 400,000 severely affected infants born annually worldwide, and a prevalence of about 100,000 severely affected individuals in the United States alone (11). Current treatments are largely supportive, such as antibiotics, hydroxyurea, transfusions, and chelation. Allogeneic hematopoietic stem cell transplantation is limited by donor scarcity and immune incompatibility. The *HBB* gene is mutated in both SCD and β -thalassemia. Various mouse models have been produced for these disorders, but none completely mimics the human conditions. No large animal model for the hemoglobin disorders is available, which is a challenge for the development of novel therapies. Therapeutic gene editing strategies include correction of the *HBB* mutation and induction of fetal hemoglobin (HbF) (12). In particular, most vertebrates, including mice, lack HbF, but old-world primates share this potentially disease ameliorating hemoglobin with humans. Thus, the β -hemoglobin gene *HBB* was our target for studying the efficiency, timing, specificity, and genetic mosaicism effects of gene editing in a nonhuman primate species, with the future goal to develop improved methods for gene editing in primates.

Using a strategy designed to introduce a single-base change in the rhesus *HBB* gene, we compared the efficacy of spCas9 variants (wild-type and nickase), choice of Cas9 mRNA versus protein microinjection, and use of an individual guide RNA or dual guide RNAs designed to nick opposite DNA strands. We then used a sample processing and library preparation approach capable of fully quantifying the CRISPR/Cas9 induced editing for all the DNA copies in the embryo at the *HBB* target locus as well as off-target sites. This provided a detailed assessment of the efficiencies of different CRISPR/Cas9 treatments, specificity of editing, the timing of editing events, and the degree of genetic mosaicism resulting from zygotic microinjection of the CRISPR/Cas9 reagents. Our data demonstrate that efficiency of editing can be very high and that off-target events can be essentially avoided with proper selection of guide RNAs. Our data also provide a quantitative assessment of the nature, number, and frequencies of different alleles that contribute to genetic mosaicism in a given embryo, and when they arise during cleavage. These results provide valuable insight for further refining the gene editing methods, along with novel methods for quantitatively assessing efficiency and specificity of outcomes in support of further development of novel primate disease models and refinement of methods for possible therapeutic applications.

Results

Our overall goal was to assess the targeting efficiencies, specificities, timing, and genetic mosaicism outcomes of four different methodological variations, which collectively allowed us to compare outcomes of using wild type (WT) versus nickase Cas9,

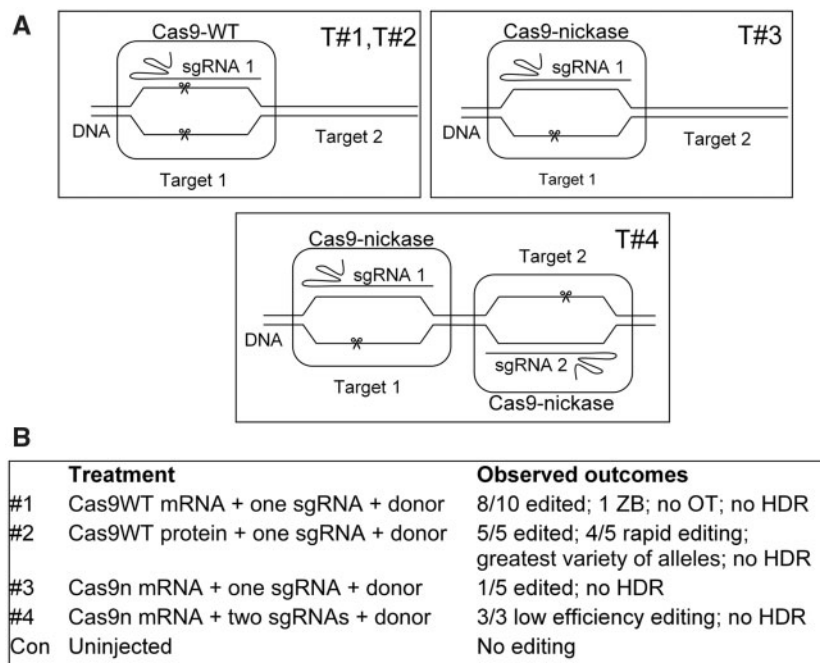


Figure 1. Summary of treatments, expected (A) and observed (B) outcomes. OT, off-targeting (only tested in Treatment #1); HDR, homology directed repair; ZB, biallelically disrupted zygote.

Cas9 mRNA versus protein, single or multiple sgRNAs, and inclusion of donor single-stranded DNA (ssDNA) template for homologous recombination. We used the *HBB* locus as a test locus, and selected sgRNAs to minimize the likelihood of off-target events. The tradeoff between maximizing sample sizes and addressing multiple aspects of the method that need to be optimized for application in NHPs was considered. The availability of rhesus zygotes for study is more limited than in other species, due primarily to cost. Thus, to maximize information gained concerning methodological variations, we elected to pursue four different Treatments (Fig. 1). Through the analysis of embryos subjected to these four treatments, it was possible to draw important conclusions about efficiency, specificity, timing, and genetic mosaicism, while also identifying procedural features that are beneficial to successful outcome.

The four mixtures of reagents (Treatments #1–4) were chosen to maximize the opportunity to observe the effects of using the individual reagents. Additional combinations were not attempted due to cost and limited number of rhesus monkey embryos available. In addition, we used two sgRNAs in a mixture with Cas9-nickase, because this could generate indels with paired single strand cuts (13). Gene targeting reagents were microinjected into rhesus monkey zygotes, which were then cultured for up to six additional days and lysed at stages ranging from eight-cell to blastocyst, when no further development was noted. Analysis of the number and frequencies of generated mutant alleles offered insight into the efficiency of targeting, type of genetic modification, extent of genetic mosaicism, and estimates of the cleavage stage(s) at which individual targeting events were likely to have arisen.

We examined the cleavage and blastocyst formation rates for the four treatment groups and the control uninjected embryos (Table 1). The rates of cleavage and the rates of cleaved embryos forming blastocysts were all within the range of values typically seen for unmanipulated embryos from different oocyte donors. Although the rates of cleavage were reduced for Treatments #1

and #3, the study did not incorporate a sufficient number of females to assign significance to this, as genetic factors could have contributed to the difference. There was no statistical difference in blastocyst formation rates for any of the treatment groups compared to the control group, indicating that there was no apparent compromise in preimplantation development assignable to the treatments.

A total of 41 embryos were processed for sequencing (Table 2). Across all injected embryos combined, we observed 18 indel alleles for which the observed editing frequency passed a threshold to exceed sequencing error rate, as described in Methods (Tables 2 and 3), and classified them as deletions (D1-12), insertions (I1-4) and complex indels (deletions + substitutions, C1-2). The remaining reads were reported as a group labeled "Other"; these consist of reads for indel alleles that didn't pass the threshold, and the reads that the automated classification method could not recognize as either WT, or as any of the 18 indel alleles.

The 18 uninjected embryos displayed little to no apparent gene editing above the threshold considered biologically relevant given the numbers of cells present in each embryo. One uninjected embryo had traces of an indel allele (0.3%). None of the other uninjected samples included any indels at a level above the relevant thresholds.

Treatment #1—*HBB* target-1 sgRNA, donor template and Cas9-WT mRNA

The objectives with this first treatment were to assess the incidence of off-targeting and to determine efficiency and mosaicism resulting from targeting at *HBB* using a single sgRNA (target-1 sgRNA). To acquire data for both the *HBB* target and two off-target sites, an initial multiplexed PCR amplification step-0 reaction was used.

A total of 10 samples representing embryos injected with Cas9-WT mRNA (embryos of various stages), *HBB* target 1

Table 1. Number of injected, cleaved, and embryos developed to blastocyst

Treatment	Number of oocytes	Number of cleaved embryos	% Cleaved Embryos	Blastocyst		
				n	% of total	% of cleaved
#1	17	12	70.6	5	29.4	41.7
#2	11	10	90.9	5	45.5	50.0
#3	9	5	55.6	2	22.2	40.0
#4	12	11	91.7	3	25.0	27.3
Control	39	39	100.0	21	53.8	53.8

sgRNA and donor template were analyzed. These embryos displayed varying levels of editing ranging from almost complete (samples 1.3 and 1.10) to mostly unaffected (samples 1.7 and 1.9) (Table 2). Two of the embryos appeared nearly unedited, with <0.5% targeted reads detected. Both alleles were targeted at the zygote stage in one of the ten embryos. Although this treatment group encompassed embryos lysed at a range of stages, the percentage of edited alleles was not correlated with embryo stage. Although a donor DNA template was included to provide an opportunity for gene mutation by homologous recombination, none of the treated embryos displayed substitutions expected to be guided by the ssDNA donor template with this treatment, or any of the other 3 treatments.

No reads indicated editing at the two tested off-target sites in these 10 embryos and three controls. Because these two loci were estimated most likely to show off-target editing, but failed to yield any evidence of off-target editing, we did not pursue off-target analysis for additional embryos.

Treatment #2—Cas9-WT protein and single guide RNA

All five blastocysts injected with Cas9-WT protein had some level of editing. Four of five embryos displayed evidence of rapid editing, most likely occurring during the one-cell (2 embryos) or two-cell stage (2 embryos). Thus, injecting Cas9 protein appears to provide for more rapid gene editing than Cas9-WT mRNA. Most embryos still displayed genetic mosaicism, indicating later events occurred as cleavage progressed.

Treatments #3 and #4—Cas9-nickase mRNA and single or dual guide RNAs

Editing was expected to be more efficient with the use of two sgRNAs (Treatment 4) than one (Treatment 3), making Treatment 3 essentially a negative control. In contrast to the high rate of success seen with Cas9-WT mRNA or protein, samples injected with Cas9-nickase mRNA and *HBB* target 1 sgRNA exhibited very low levels of editing; adding *HBB* target 2 sgRNA to Cas9-nickase mRNA and *HBB* target 1 sgRNA increased editing, but Cas9-nickase mRNA was still less efficient than Cas9-WT mRNA or Cas9 protein. There were fewer distinct editing effects caused by addition of target 2 sgRNA compared to the effects observed in embryos injected with only target 1 sgRNA. This may have been due to a low efficiency of Cas9-nickase function.

Comparison of editing efficiencies for treatments #1–4

Editing efficiencies obtained by four treatment methods (Table 2, column Editing Outcomes/Tot.) were compared with Wilcoxon rank-sum test. Regardless of delivery method Cas9-

WT is significantly more efficient than Cas9-nickase [$P=0.002$ for comparison of Cas9-WT mRNA (Treatment #1) and Cas9-nickase (Treatment #3); $P=0.008$ for comparison of Cas9-WT protein (Treatment #2) and Cas9-nickase (Treatment #3)]. Cas9-WT protein appeared, on average, to be more efficient than Cas9-WT mRNA, however both treatments produced a wide range of editing efficiencies and the difference was not statistically significant. Cas9-WT protein was more efficient than Cas9-WT mRNA in achieving events during the first two cell cycles (Fig. 2). Co-injecting two different sgRNAs with Cas9-nickase mRNA (Treatment #4) increased the average editing efficiency over injecting just one sgRNA with Cas9-nickase mRNA (Treatment #3). Statistically, the apparent difference was marginal and did not pass the threshold of significance ($P=0.071$); this may reflect the small numbers of samples being compared, so that a potential benefit of using two sgRNAs should not be entirely disregarded.

Timing of gene editing and emergence of genetic mosaicism

Knowing the time of editing in the context of different methodologies could help optimizing the methods further. We estimated times of editing events for the four Treatments employed by comparing the observed frequencies of edited alleles to the expected frequencies resulting from a single or two editing events (Tables 2 and 4). For example, the frequency of D1 in sample 1.2 (37.3%) is within the interval [34.2%, 40.5%] (Table 4, columns 1-2), i.e. the nearest expected frequency is 37.5% (Table 4, column 6), which corresponds to one editing event at two-cell stage (25%) and another editing event at four-cell stage (12.5%).

For combinations of two or three editing events, only those combinations that could not be explained by a simpler combination of editing events (or a single editing event) were considered. For example, for sample 1.3 (morula) we inferred that both alleles were edited at the one-cell stage, with a deletion (D1) in one allele and an insertion (I2) in the other allele. For the frequency of D1 (51.3%) there is a discrepancy of 1.3% compared to the expected frequency (50%), which can be explained either as error arising during PCR and sequencing or as an additional editing event at 32-cell stage (1.6%). We attribute the discrepancy of 1.3% to stochasticity of PCR and sequencing, and consider a single D1 editing event at one-cell stage to be the most parsimonious explanation for the observed frequency of 51.3%.

Other studies have reported on the percentage of biallelic or monoallelically targeted embryos. However, because of the variability in timing of editing events and the resulting diversity in genetically mosaic embryos, it is more appropriate to consider potential fraction of biallelically targeted cells in each embryo. This information is relevant to the likely manifestation of

Table 2. Summary of editing outcomes

Stage	Embryo Editing outcomes ^a (%)														WT	Other (%)								
	(Animal)	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	I1			I2	I3	I4	C1	C2	Tot.		
Treatment #1: HBB target-1 sgRNA, Cas9-WT mRNA, Donor template ssDNA																								
8-16 Cell	1.1 (a)	26.7(2,+)																			26.7	72.2	1.1	
	1.2 (a)	37.3(2,4)			13.0(4)									29.6(2,+)							79.8	19.9	0.3	
Morula	1.3 (b)	51.3(1)																			98.3	1.2	0.5	
32-64 Cell	1.4 (b)	16.0(4,+)	2.3(32,+)											7.4(8,+)	47.0(1)						26.9	72.1	1.0	
	1.5 (b)	20.0(4,8)																			20.0	79.1	0.9	
Ear. B.	1.6 (a)	12.8(4)	0.5							2.8(16)				0.3							16.5	83.2	0.4	
Blast.	1.7 (b)	0.4												12.0(4)							0.4	99.2	0.5	
Exp. Blast.	1.8 (a)																				12.0	87.6	0.4	
	1.9 (a)	0.3																			0.3	99.3	0.4	
	1.10 (a)	51.0(1)	10.5(8,+)							9.4(8,+)				21.9(4,+)							92.8	6.9	0.3	
Treatment #2: HBB target-1 sgRNA, Cas9-WT protein, Donor template ssDNA																								
Blast.	2.1 (c)	24.2(2)		1.5(32)						8.6(8,+)											34.3	65.0	0.7	
	2.2 (c)	27.6(2,+)	26.9(2,+)								1.6(32)										56.1	42.2	1.7	
	2.3 (c)	46.2(2,4,8)		34.8(2,4)													1.0(64)	0.4	4.0(16,+)		86.4	12.3	1.3	
	2.4 (c)	2.2(32,+)			2.9(16)					0.6(64)											5.8	93.3	1.0	
	2.5 (c)	54.4(1,+)			13.9(4,+)																68.3	31.3	0.4	
Treatment #3: HBB target-1 sgRNA, Cas9-nickase mRNA, Donor template ssDNA																								
8-16 Cell	3.1 (d)	4.3(16)																			4.3	92.7	3.1	
	3.2 (d)																				98.5	1.5		
	3.3 (d)																				99.6	0.4		
Exp. Blast.	3.4 (d)																				99.5	0.5		
	3.5 (d)																				99.6	0.4		
Treatment #4: HBB target-1 & target-2 sgRNA, Cas9-nickase mRNA, Donor template ssDNA																								
Exp. Blast.	4.1 (e)				0.5						1.7(32)		1.5(32)								3.7	93.6	2.7	
	4.2 (e)	0.5		0.2																	2.6(32,+)	18.9	79.7	1.5
	4.3 (e)				24.2(2)																24.2	74.1	1.7	
Uninjected																								
Stage (Num. of samples) Sample ID/Animal ID (WT %, Other %, editing outcomes)																								
8-16 Cell (4)					5.1/a (99.9,0.1,-); 5.2/a (99.7,0.3,-); 5.3/d (99.8,0.2,-); 5.4/d (99.9,0.1,-)																			
Early Blast. (1)					5.5/a (99.8,0.2,-)																			
Exp. Blast. (9)					5.6/a (99.2,0.4,D1:0.3); 5.7/a (99.9,0.1,-); 5.8/d (99.9,0.1,-); 5.9/c (99.8,0.2,-); 5.10/c (99.8,0.2,-); 5.11/f (99.9,0.1,-); 5.12/f (99.8,0.2,-); 5.13/f (99.9,0.1,-); 5.14/f (99.9,0.1,-)																			
Hatching Blast. (4)					5.15/c (99.9,0.1,-); 5.16/f (99.9,0.1,-); 5.17/d (99.9,0.1,-); 5.18/f (99.6,0.4,-)																			

^aDeletion (D1-D12), insertion (I1-I4) and complex indel (C1-2) allele sequence are listed in Table 3. Estimated editing times are shown in parentheses (e.g. 51.3(1) in means that 51.3% of reads were mapped to Deletion #1, which was estimated to occur at 1-cell stage), a '+' after number indicates that editing occurred twice (e.g. (8,+)) means that editing occurred at the 8-cell and at a later stage or stages).

Table 3. Editing outcomes

		Deletions ^a		
		<-Target-1	<- - Expected cut sites - ->	Target-2->
WT ^b	ACAGACACCATGGTGCATCTGACT <u>CCTGAGGAGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT</u>			
D1	ACAGACACCATGGTGCATCTGACTCCT—GAGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D2	ACAGACACCATGGTGCATCTGACTCCTGA—GAGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D3	ACAGACACCATGGTGCATCTGACTCCT—GAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D4	ACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGAATGCCGTCACCACCCTGT—GGTGAACGTGGATGAAGTTGGTGGT			
D5	ACAGA—CACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D6	ACAGACACCATGGTGCATCTGACTCCTG—AGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D7	ACAGACACCATGGTGCATCTGACT—CCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D8	ACAGACACCATGGTGCATCTGACTCCT—GAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D9	ACAGACACCATGGTGCATCTGACTCCTG—GAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D10	ACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGAATGCCGTCACCACCCTGT—GGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D11	ACAGACACCATGGTGCATCT—CCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
D12	ACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGAA—TGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT			
		Insertions ^c		
		<-Target-1	<- - Expected cut sites - ->	Target-2->
WT ^b	ACAGACACCATGGTGCATCTGACT <u>CCTGAGGAGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT</u>			
I1	ACAGACACCATGGTGCATCTGACTCCTGAGAgaaatgcccgtaccaccctgtgggGAGAAGAATG.....			
I2	ACAGACACCATGGTGCATCTGACTCCTGAGgGAGAAGAATG.....			
I3	ACAGACACCATGGTGCATCTGACTCCTGAGGAGAagaatgccgtcAGAATGCCGT.....			
I4	ACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAatgaAGTTG.			
		Complex Indels ^{a,d}		
		<-Target-1	<- - Expected cut sites - ->	Target-2->
WT ^b	ACAGACACCATGGTGCATCTGACT <u>CCTGAGGAGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT</u>			
C1	ACAGACACCATGGTGCATCTGACTCCTG—AGAAGAATGCCGTCACCACCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT tcttgaaccttgataccaacctgccaggcctcaccacca			
C2	ACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGAATGCCGTCACCACCCTGTGG—GGTGAACGTGGATGAAGTTGGTGGT tggg			

^aDeleted bases are shown as “-”.

^bProtospacer adjacent motifs (PAM) are underlined.

^cLowercase bold letters are used for inserted bases, “...” represents continuation of original sequence.

^dInserted bases are shown under the deleted segments.

phenotypic effects during development or postnatally. Further complicating consideration of editing outcomes, while the fraction of edited DNA copies within an embryo can be estimated from the sequencing data, it is impossible in many embryos to determine from the sequencing data whether a given edited allele was paired with another edited allele in a cell with biallelic editing, or with an unedited allele in a cell with monoallelic editing. For some embryos estimating the fractions of cells with biallelic and monoallelic editing is a trivial task, such as embryos with one or both alleles edited at the one-cell stage (Supplementary Material, Fig. S1A and B). For some embryos with multiple editing events, the final outcome is unique (in terms of number of cells with biallelic and monoallelic editing), such as when the first editing event occurs at the one-cell stage and is followed by a second editing event at a later stage (Supplementary Material, Fig. S1C and D), the second editing event will for certain affect the second DNA copy in a cell where one copy has already been edited. However, in many cases, particularly when the first editing event occurs after the one-cell stage, the status of the cell that is affected by the second and later editing events is ambiguous and the final outcome cannot be uniquely determined (Supplementary Material, Fig S1E and F). For such embryos we can simulate all possible outcomes and calculate the lowest and highest possible value for the fraction of biallelically or monoallelically affected cells. The estimated fractions (or possible ranges of fractions) of cells with monoallelic and biallelic editing for the embryos in this study are listed

in Table 5. Sample 1.3 had 100% of cells with biallelic editing and two more samples (1.10, 2.5) had 100% of cells with editing, part biallelic and part monoallelic. Several more samples (1.2, 2.2, 2.3) may have undergone editing in 100% of cells, but in those samples such outcomes were not certain because there are other possible outcomes where less than 100% of cells were affected.

Without accounting for number of gene copies present at each stage, the majority of editing events occurred at the eight-cell and later stages (Fig. 2). But when the number of available WT allele DNA copies is taken into account, editing was the most efficient at one-cell, two-cell and four-cell, after which the efficiency started to decline. Deletion D1 was the most frequent editing event, except for Treatment #4, where the addition of another sgRNA changed the set of outcomes. Treatment #2 with Cas9-WT protein produced the greatest variety of alleles.

Discussion

To our knowledge, this is the first study to quantify in detail the effects of CRISPR/Cas9 gene editing approaches on the whole population of cells in preimplantation stage primate embryos, providing a comprehensive assessment of timing, efficiency, and specificity of gene editing, thorough characterization of nature and abundance of alleles generated, along with overall levels of genetic mosaicism arising. Other studies have relied on genotyping various tissues of fetuses or progeny, or methods of

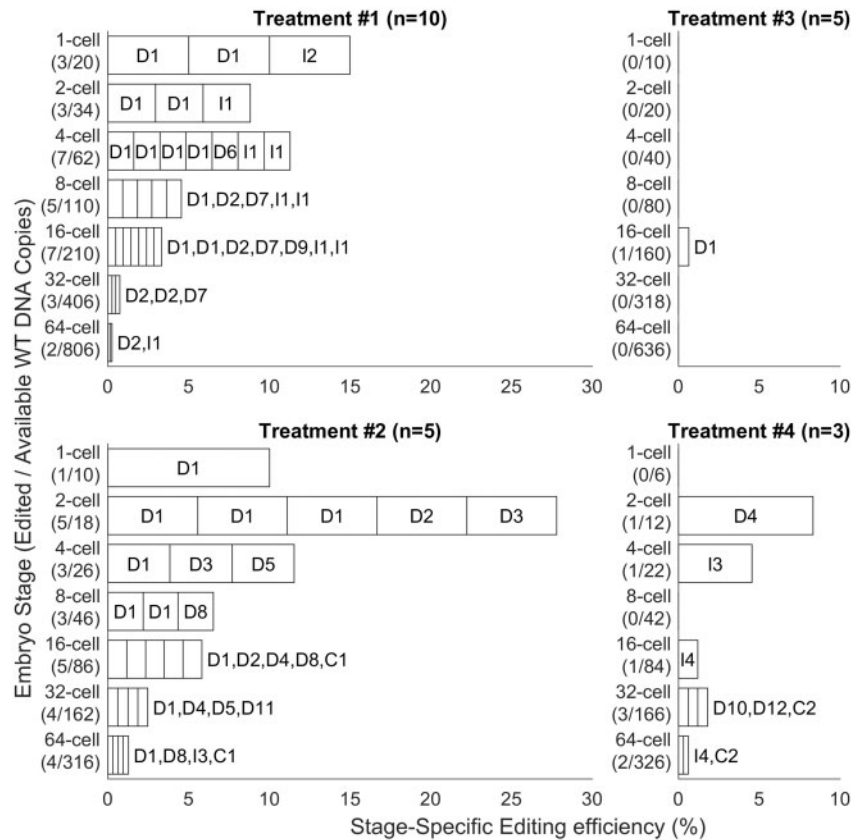


Figure 2. Stage-specific editing efficiency; efficiency at each stage is defined as the ratio of number of edited DNA copies and the number of WT DNA copies that were available for editing.

limited quantitative assessment of outcomes in preimplantation embryos, and thus may not have detected all relevant gene editing events. Furthermore, they may be subject to selection effects, particularly given the high rate of embryo loss to term. Our approach has enabled an estimate of the time frame of the first editing event and subsequent events. It has also revealed the extent and character of mosaicism that may have been missed by other sample collection and processing approaches in other studies.

The main results of our study are that 1) gene targeting can be very efficient, affecting 80–100% of embryos, 2) there is substantial genetic mosaicism arising as editing events occur in multiple cells during cleavage, with up to 5 distinct targeted alleles in some embryos, and first targeting events occurring from the zygote to 16 cell stage, 3) editing in both parental alleles (at the one-cell stage) can occur at a reasonably high efficiency (1 in 35 embryos overall), 4) off-targeting does not appear to be a frequent event with proper selection of sgRNAs, and 5) homologous recombination using a ssDNA donor template was not observed.

The most frequently observed indel in this study was a deletion of three bases, which causes an in-frame mutation that does not introduce a premature stop codon and gene knockout. The high propensity for this outcome appears to be due to microhomology mediated end joining based repair (14), associated with a repeat of three bases (GAG) on both sides of the cleavage site specified by the guide RNA. Such information can be taken into account when designing guide RNAs for gene knockout to maximize the likelihood of premature stop-codon inducing non-homologous end joining (NHEJ).

The use of a multiplexed PCR pre-amplification step allowed simultaneous amplification of several different loci (target and

off-target loci). This approach was more efficient than pre-amplification approaches that rely on whole genome amplification. It ensured that the small aliquots of the amplified material were all highly similar – in terms of the ratios of various alleles for the amplified loci – to the initial population of DNAs in the lysate. Therefore, it allowed simultaneous sequencing of both the target and the predicted off-target regions. We tested two off-target regions which were predicted most likely to be affected and observed no editing. This was not an unexpected result, given the relatively low similarity between the guide RNA and the off-target loci. One study reported no off-targets were detected by screening 84 off-target sites for 5 targets (9–21 off-target sites per target) in another nonhuman primate (4), while one study in human embryos reported off-target effects (6).

The efficiency and targeting specificity seen in our studies indicate that gene editing can be applied in primate embryos to good effect. This capability will enable gene editing to be applied in nonhuman primates to advance biomedical research through the production of novel research models, and indicates that gene editing in human cells could be practical for novel therapies, such as stem cell based therapies to address devastating genetic diseases. Some of the primate studies to date are laudable for their success in generating gene edited monkeys (4,8). However, it is also clear that those notable accomplishments were achieved at very high cost in terms of the numbers of embryos used. Some studies report limited viability to blastocyst stage, and very limited (~10%) viability of transferred embryos to term (10). This inefficiency is a major barrier to practical use of the technology in nonhuman primates. Embryos are costly to generate, embryo transfers are costly to

Table 4. Inferring timing of editing events from observed allele frequencies

Observed Frequency (%)		Assigned timing of editing events			Expected Frequency of Editing Events (%)
From ^a	To ^a	1 st	2 nd	3 rd	
93.5	100	1-cell	1-cell		100
81.0	93.5	1-cell	2-cell	4-cell	87.5
68.5	81.0	1-cell	2-cell		75.0
59.3	68.5	1-cell	4-cell		62.5
53.0	59.3	1-cell	8-cell		56.3
46.8	53.0	1-cell			50.0
40.5	46.8	2-cell	4-cell	8-cell	43.8
34.2	40.5	2-cell	4-cell		37.5
29.6	34.2	2-cell	8-cell		31.3
26.5	29.6	2-cell	16-cell		28.1
23.4	26.5	2-cell			25.0
20.3	23.4	4-cell	8-cell	16-cell	21.9
17.1	20.3	4-cell	8-cell		18.8
14.8	17.1	4-cell	16-cell		15.6
13.3	14.8	4-cell	32-cell		14.1
11.7	13.3	4-cell			12.5
10.1	11.7	8-cell	16-cell	32-cell	10.9
8.56	10.1	8-cell	16-cell		9.38
7.41	8.56	8-cell	32-cell		7.81
6.63	7.41	8-cell	64-cell		7.03
5.85	6.63	8-cell			6.25
5.06	5.85	16-cell	32-cell	64-cell	5.47
4.28	5.06	16-cell	32-cell		4.69
3.49	4.28	16-cell	64-cell		3.91
2.71	3.49	16-cell			3.13
1.91	2.71	32-cell	64-cell		2.34
1.10	1.91	32-cell			1.56
0.55	1.10	64-cell			0.78

^aAll interval boundaries, except for 100% and 0.55%, are geometric means of expected frequencies in pairs of consecutive rows, e.g. $93.5 = (100 \cdot 87.5)^{1/2}$.

perform, and animal husbandry is costly. Reasons for inefficiency might include biallelic targeting resulting in null lethal phenotypes, genetic mosaicism leading to overall less fitness through monoallelic deficiencies in a large fraction of cells, and non-genetic factors such as multiple implantations [selective embryo reduction may not solve this limitation (9)], stress of the embryo manipulations, or non-specific effects of the targeting reagents on the early embryo. Off-targeting does not appear to be a major limitation, at least for the *HBB* locus, and for some other loci tested (4,8,10), in contrast to one report for CRISPR/Cas9 application in tripronuclear human embryos (6). Off-targeting potential may largely depend on the specific sgRNA sequences utilized. Genetic mosaicism appears to be a significant limitation of existing technologies. Due to the longer life cycle of the monkey, use of breeding strategies to segregate individual alleles is problematic. Methods to minimize mosaicism would be advantageous.

Our high targeting efficiency (fraction of embryos bearing edited alleles) resembles that observed in progeny of other species such as pig [100% (15)], mouse [80% (3)], cynomolgus monkey [80% (4)], or in human embryos [52% (6)], but was higher than seen in at least one study (9). Gene knock-out through non-homologous end joining would be predicted to produce null alleles, which in the case of *HBB* could enable the development of large animal models of β -thalassemia. The level of editing (percentage of cells with edited alleles) was variable, with biallelic editing at one-cell stage occurring in only 1 embryo. Editing also occurred at the two-cell stage and later, although it started declining at or after the four-cell stage. There was a

wide variety of editing outcomes, and for the embryos in which editing occurred, results were not uniform across all edited alleles, with at least two (and frequently three or more) different indel alleles arising in many embryos.

Further resembling other studies is the difficulty in achieving homologous recombination with zygotic injection (4,6,15). Although the failure to accomplish single-base substitution through homologous recombination – neither with WT nor nickase variant of Cas9 – might have been due to inappropriately selected sgRNAs, the selected concentration of donor template or the lack of or lower activity of homology directed repair (HDR) mechanism, this barrier appears to span species and gene target. Methods to enhance homologous recombination with CRISPR/Cas9 for gene editing would be a clear advance for the field.

Some technical considerations to enhance outcomes emerge from our data. First, our data indicate that injecting Cas9 protein instead of mRNA likely improves speed and efficiency of editing, although the number of embryos tested was small. A similar finding was reported for mice (16). The greater speed and efficiency achieved with Cas9-WT protein was accompanied by a greater variety of alleles. Second, Cas9-nickase was less efficient for editing than Cas9-WT mRNA or protein. Third, a single sgRNA produced high efficiency editing with Cas9-WT. Using more than one sgRNA may be advantageous for generating larger indels. Finally, the concentration of Cas9 mRNA (50 ng/ μ l) was less than in some other studies where higher concentration appeared detrimental (e.g., 200 ng/ μ l in (8)), and our sgRNA concentration was higher (250 versus 5–10 ng/ μ l). Thus,

Table 5. Estimated fractions of edited cells per injected embryo

Sample ID	Estimated number of indel events per stage (...-cell)							% Reads with indels	% Cells with monoallelic editing	% Cells with biallelic editing	% Unedited cells
	1-	2-	4-	8-	16-	32-	64-				
1.1		1			1			26.7	43.8 – 56.3	0.0 – 6.3	43.8 – 50.0
1.2		2	2		1			79.8	6.3 – 43.8	56.3 – 75.0	0.0 – 18.8
1.3	2							98.3	0.0	100.0	0.0
1.4			1	1	1	2	2	26.9	0.0 – 53.1	0.0 – 26.6	46.9 – 73.4
1.5			1	1				20.0	12.5 – 37.5	0.0 – 12.5	62.5 – 75.0
1.6			1		1			16.5	18.8 – 31.3	0.0 – 6.3	68.8 – 75.0
1.7								0.4	0.0	0.0	100.0
1.8			1					12.0	25.0	0.0	75.0
1.9								0.3	0.0	0.0	100.0
1.10	1		1	3	3	1		92.8	15.6	84.4	0.0
2.1		1		1	1	1		34.3	28.1 – 71.9	0.0 – 21.9	28.1 – 50.0
2.2		2			2	1		56.1	3.1 – 84.4	15.6 – 56.3	0.0 – 40.6
2.3		2	2	1	1		2	86.4	3.1 – 28.1	71.9 – 84.4	0.0 – 12.5
2.4					1	1	2	5.8	0.0 – 12.5	0.0 – 6.3	87.5 – 93.8
2.5	1		1	1		1		68.3	59.4	40.6	0.0
3.1					1			4.3	6.3	0.0	93.8
3.2									0.0	0.0	100.0
3.3									0.0	0.0	100.0
3.4									0.0	0.0	100.0
3.5									0.0	0.0	100.0
4.1						2		3.7	0.0 – 6.3	0.0 – 3.1	93.8 – 96.9
4.2			1		1	1	2	18.9	12.5 – 37.5	0.0 – 12.5	62.5 – 75.0
4.3		1						24.2	50.0	0.0	50.0

the higher concentrations of Cas9-WT mRNA in other studies may generate a higher rate of mutated embryos, including biallelic mutants, but reduces embryo viability and could increase mosaicism rates. In our studies, Treatments 1 and 3 may have negatively impacted initial cleavage rate compared to uninjected controls, but effects on the subsequent development to blastocyst were minor. This indicates improvements to enhance initial embryo survival by minimizing trauma to the cell may improve the number of embryos available for establishing pregnancy.

We note that because the four experimental groups used embryos obtained from different females, one can speculate that genetic factors might have contributed to differences in outcomes or embryo viability. However, the uninjected controls used three of the same four females as those used in Treatments 1 and 2 (the main groups for inter-treatment comparison) and Treatment 3, allowing any maternal genetic effects to be shared between those treatment groups and controls. We also note that single cell genotyping would be required to fully characterize the fraction of monoallelic and biallelic cells generated. Because our data provide a foundation for further optimization studies, this was not deemed necessary. Rather, it will be of greater interest to focus future efforts on refinements that provide more rapid and efficient editing without increasing mosaicism.

Practical application of gene editing in nonhuman primates in biomedical research can be considered in the context of two general strategies. In one strategy, genes for editing may be selected for study with the expectation that their disruption will create developmental abnormalities that will be evident in fetal or early post-natal life. These studies will yield short-term insights, and will be particularly valuable for developmental features that are unique in primate biology. The second strategy is to establish genetically modified lines of primates for the study

of human disease, and novel approaches to treat or preventing disease. This will be particularly valuable for disease for which rodent or other animal models are not well suited, such as brain disease and behavioral disorders. The insights to be gained from these studies will be long-term and will require resources to be dedicated over many years to the support and expansion mutant lines of animals for study. This latter mission will benefit from limiting genetic mosaicism to minimize the number of generations needed to obtain well-defined mutants, and reducing early embryo demise. For both strategies, editing efficiency and specificity and embryo viability must be maximized whilst minimizing emergence of genetic mosaicism. Additionally, for both strategies, the ability to achieve biallelically modified cells (and fetuses or progeny), or to acquire multiple monoallelically modified animals in a single generation will accelerate success. Our analysis demonstrates potential success rates in these outcomes, and suggests ways to enhance success.

Overall, our data demonstrate an ability to achieve a high efficiency of gene mutation in rhesus monkey zygotes, with no detected off-target effects in the top two potential off-target loci. Considerable genetic mosaicism and variation in the fraction of embryonic cells bearing targeted alleles are observed. The use of optimized amounts of Cas9-WT protein, modified Cas-9 protein (17), optimized concentrations of sgRNAs, and chemically modified sgRNAs (18) are likely areas for further refinement to enhance efficiency while limiting unfavorable outcomes that can be exceedingly costly for application of gene editing in primate species. Other technical aspects can influence efficiencies and outcomes. These include biological variations between embryos, and variation in the microinjection process. The results described here should help to streamline the implementation of improvements in gene editing methods and achieve the promise of important advances in primate and human biomedical research.

Materials and Methods

Guide RNA and donor single-stranded DNA template design

Candidate CRISPR guides near the homologous sequence to the SCD point-mutation in rhesus macaque *HBB* exon 1 were evaluated using the methodology described on <http://crispr.mit.edu:8079/about>; date last accessed April 28, 2017 and in (19). Two guides on opposite strands were selected (coordinates given for Mmul8.0.1 assembly): GTGACGGCATTCTTCTCCTC (*HBB* target 1), chr14:61968455-61968474(-), and AAGGTG AACGTGGATGAAGT (*HBB* target 2), chr14: 61968488-61968507(+); a G was added at 5' to facilitate IVT. Production of the sgRNAs is described in detail in Supplemental Methods.

Donor template ssDNA was designed based on the 199-base sequence (chr14:61968356-61968554) around the sickle-cell point-mutation locus (chr14: 61968456), to guide the homology directed repair and induce a single-base substitution at the locus, as well as four silent (i.e. synonymous) mutations at both *HBB* target 1 and *HBB* target 2 in order to prevent further editing of edited DNAs.

Four treatments tested

We subjected embryos to microinjection with four different combinations of Cas9 mRNA or protein, DT ssDNA, and sgRNAs (Table 6). Treatment #1: embryos received *HBB* target 1 sgRNA and Cas9-WT mRNA (Trilink; L-6125-100). Treatment #2: embryos received *HBB* target 1 sgRNA and cas9-WT protein (PNA Bio; CP01-50). Treatment #3: embryos received *HBB* target 1 sgRNA and Cas9-nickase mRNA (Trilink; L-6116-100). Treatment #4: embryos received *HBB* target 1 and target 2 sgRNAs and Cas9-nickase mRNA. Other combinations were not tested due to limited availability of embryos and associated cost. Additional ($n = 18$) uninjected embryos served as controls. Two other control embryos received EGFP mRNA but were later found to contain a substantial fraction of edited alleles (2–11%, data not shown), indicating likely contamination, and were not considered in the final analysis.

Rhesus macaque embryo production, microinjection and culture

Adult female rhesus macaques (*Macaca mulatta*), housed at the California National Primate Research Center, were housed as described (20). All procedures for maintenance and handling of the animals were reviewed and approved in advance by the Institutional Animal Use and Care Administrative Advisory Committee at the University of California at Davis. The criteria for selection included age range from 6 to 12 years, history of successful pregnancy, and normal menstrual cycles. The control group consisted of seven females 8.5 ± 3.1 (mean \pm SD) years of age and weight 8.01 ± 1.78 kg. The treatment group consisted of nine females 8.2 ± 1.9 years of age and weight 8.08 ± 1.57 kg. Menstrual bleeding was monitored daily and body weights were recorded weekly for the duration of study.

Oocytes were obtained from six female rhesus monkeys by controlled ovarian stimulation with twice daily injections of human rFSH (37.5 IU) for 7 days and 1000 IU of hCG on Day 8. On Day 9, oocytes were obtained by ultrasound-guided needle aspiration as described in detail (21). Oocytes were inseminated and cultured in vitro up to the blastocyst stage as described (21). Due to limited numbers of monkeys available for each experiment,

limited numbers of oocytes obtained from each animal and cycle, and practicalities of the microinjection set up, each treatment employed embryos from different animals, and the control group incorporated two of these same animals.

At approximately 12 h after insemination, presumptive zygotes were transferred to 30 μ l drops of TL-Hepes-PVA medium (20) under oil and transferred to the 37.5°C environmental chamber of a Nikon Eclipse TE300 microscope equipped with Hoffman Modulation optics and Narishige microinjectors (Nikon Instruments Inc., Melville, NY). Holding pipettes were purchased from Origio Inc, Charlottesville, VA. Injections were performed using an Eppendorf FemtoJet Injector system and FemtoTips (Eppendorf North America, Hauppauge, NY). Following injection (approximately 10 pl per embryo), zygotes were returned to embryo culture medium and continued on standard culture protocol for up to 6 days until there was no further development or reaching the blastocyst stage. At the end of the culture period single embryos were transferred in less than 5 μ l to a tube containing 20 μ l QuickExtract buffer (Epicentre, Madison, WI).

PCR amplification of target and off-target loci and library preparation

Each embryo lysate was subjected in its entirety to PCR amplification of *HBB* target and two off-target loci for Target 1 sgRNA. Two off-target loci with the highest scores assigned by the sgRNA evaluation procedure (19) were selected, where higher score indicates higher similarity to the sgRNA target locus and therefore higher propensity for off-target editing. The scores for two loci were 5.1 (mismatches between target and off-target locus in positions 2 and 3), and 3.2 (mismatches in positions 1 and 6). All other potential off-target loci had scores < 2 , with higher number of mismatches and/or mismatches in positions closer to protospacer adjacent motif (PAM).

PCR pre-amplification (step-0) was performed (Supp. Methods, Table 7) via Platinum® Taq DNA polymerase kit (Life Technologies; 10966-018). When sequencing target plus off target loci, step-0 employed multiplexed PCR. Step-0 products were aliquoted and processed with the standard 2-step PCR approach using Herculase II Fusion Enzyme with dNTPs Combo kit (Agilent; 600677) (Supplementary Material, Supplementary Methods). Locus-specific primers (with 5' overhangs) were used in step-1 (Table 7). Nextera sequencing primers were used in step-2. When sequencing multiple loci, for each of the samples all step-1 products from various loci were assigned the same pair of barcodes, resulting in pooled libraries that were later split into locus-related parts in silico. Gel purification and size selection (Qiagen; 28704) of step-2 products was performed. Final products were quantified (Qubit DNA HS, Kappa qPCR), pooled to the same molar concentration, and sequenced on Illumina MiSeq to produce 150 base paired-end reads.

Data analysis

Sequencing data with single-locus libraries were analyzed with CRISPResso (22), while multiple-locus libraries were analyzed with CRISPRessoPooled, which includes a step to split reads into sets based on locus, followed by CRISPResso for each set. CRISPResso performed average quality based filtering ($-q 30$), clipping of adapter read through and low-quality 5' ends (*trimmomatic* options ILLUMINA_CLIP and -SLIDING_WINDOW:6:15), merging pairs of reads into extended fragments (*flash*), and

Table 6. Cas9 treatments

#	Cas9-WT mRNA	Cas9-nickase mRNA	Cas9-WT protein	HBB Target 1 sgRNA	HBB Target 2 sgRNA	Donor Template ssDNA
1	50ng/ μ l			250ng/ μ l		100ng/ μ l
2			1 μ g/ μ l	250ng/ μ l		100ng/ μ l
3		50ng/ μ l		250ng/ μ l		100ng/ μ l
4		50ng/ μ l		250ng/ μ l	250ng/ μ l	100ng/ μ l

Table 7. PCR primers

Locus	Step		
HBB target region	0	Forward	GGCTGAGGGTTTGAAGTCCA
		Reverse	CCTCTGGGTCCAAGGGTAGA
		Locus	chr14:61968204-61968689
	1	Product length	486
		Forward ^a	[FwdPref]-CTACAGTTGGCCAATCTACTCC
		Reverse ^b	[RevPref]-CCACATGCCAGCTTCTATT
HBB target 1, offtarget 1	0	Locus	chr14:61968301-61968585
		Product length ^c	285 (+33 + 34)
		Forward	CGCTCCAGAAGTCAAGTGT
	1	Reverse	CCTCTGGGTCACTGCTGTG
		Locus	chr4:117524460-117525190
		Product length	731
HBB target 1, offtarget 2	0	Forward ^a	[FwdPref]-AACCCAGGATGCAGAGGTTG
		Reverse ^b	[RevPref]-GTGAGGGCTTCTGTCTGCTT
		Locus	chr4:117524785-117524999
	1	Product length ^c	215 (+33 + 34)
		Forward	TGCTTTTCTCTGGGTGCCAA
		Reverse	GCTCCTGTGGCCTCATTCT
HBB target 1, offtarget 2	0	Locus	chr1:211187784-211188350
		Product length	567
		Forward ^a	[FwdPref]-GGAAACAGAGCCAGACCTC
	1	Reverse ^b	[RevPref]-CACGTTGTGCCAACATCTGG
		Locus	chr1:211188073-211188304
		Product length ^c	232 (+33 + 34)

^aForward prefix FwdPref: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG.

^bReverse prefix RevPref: GTCTCGTGGCTCGGAGATGTGTATAAGAGACAG.

^cStep 1 products consist of amplified sequence flanked by 33 base forward and 34 base prefixes.

aligning extended fragments to the amplicon sequence (*needle*) (23–25). Alignments produced by *needle* were processed with custom python scripts to quantify wild-type (WT) and indel alleles and nucleotide substitutions within 30 bases of intended cut sites; indels outside of this interval were ignored as sequencing artifacts. Indel alleles were ignored as biologically unfeasible (classified as “Other”, rather than wild-type) if they occurred with a frequency lower than the minimal expected frequency for embryo stage: 2.78% for 8–16 cell, 2.08% for morula, 0.69% for 32–64 cell, 0.22% for blastocyst. Formula for threshold $100\% / (2 \times \text{cell number} \times 1.5)$ was based on an estimated number of cells at each stage, multiplied by a factor of 1.5 to allow for DNA replication (G_2 phase) in a part of cells.

For each indel allele with frequency above the threshold, the timing of editing was inferred by finding a combination of up to three editing events that best approximate the observed editing frequency (Table 4). Table 4 was constructed by first including stages up to 64-cell for single editing events, followed by pairs of stages for combinations of two editing events, and finally triplets of stages for combinations of three editing events. At each step, any combination of editing events for which the

expected frequency was within $\pm 10\%$ error interval of expected frequency for some combination that was already in the table was ignored. For example, expected frequencies for pairs *one-cell* & *16-cell*, *one-cell* & *32-cell*, and *one-cell* & *64-cell* are all within the $50\% \pm 5\%$ interval, where 50% is the expected frequency for single editing event at one-cell stage, and $50 \pm 5\%$ is its 10% error interval, therefore they were excluded. Similarly, after including triplets of events occurring at three consecutive stages (such as *one-cell* & *two-cell* & *four-cell*), the expected frequencies for all other triplets were within the 10% error interval for at least one combinations already in the table.

For samples with one editing event, the percentage of affected cells was estimated based on the stage when editing occurred. For example, if one allele is edited in one cell of a two-cell embryo, approximately 50% of cells inherit one edited allele. For samples affected by multiple editing events, all editing events after the first event may affect either a previously unaffected cell or a cell with one edited allele; which of these two possible events has occurred cannot be determined from the sequencing data. This ambiguity means that in many cases there is no unique final outcome. Therefore, we used a strategy of

simulating all possible outcomes and calculating percentages of cells affected by monoallelic and biallelic editing.

Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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