Cell Research (2015) 25:258-261.

www.nature.com/cr

LETTER TO THE EDITOR

# One-step generation of *p53* gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system

*Cell Research* (2015) **25**:258-261. doi:10.1038/cr.2014.158; published online 28 November 2014

**Dear Editor,**

Non-human primates (NHPs) are genetically, physiologically and psychologically more similar to humans than other model organisms, providing an important model for human development and diseases [1, 2]. Precise genome modification in non-human primates (NHPs) holds great promise for biomedical researches. However, owing to the long sexual maturation time and low reproduction rate of NHPs, it is very challenging to generate biallelic mutant NHPs for loss-of-function studies through breeding. Recently, the site-specific nucleases, namely TALENs (transcription activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) system, make it feasible to generate NHPs with precise genome modifications, yet the resulting transgenic animals exhibited mosaic mutations accompanied by the presence of wild type allele in different tissues [3, 4]. Thus, the feasibility of obtaining biallelic mutations in monkeys remains an open question. Here we report that by systematically optimizing the mutagenesis efficiency of the CRISPR/Cas-based method in monkeys, we have successfully obtained the first live *p53* biallelic mutant monkey, and achieved homology directed repair (HDR)-driven gene editing with nucleotide-level precision in monkey embryos.

We chose *p53*, a well-known tumor suppressor gene [5], as the target to produce biallelic mutant monkeys in one-step via zygote injection using a CRISPR/Cas system. Since previous studies have shown that both the targeting loci of sgRNAs and the injection concentrations of Cas9 mRNA/sgRNA had substantial effects on the mutation-generation efficiencies [6, 7], we first screened the targeting efficiency of sgRNAs by cell transfection (Supplementary information, Figure S1A). We designed a total of 6 sgRNAs targeting different sites of the monkey *p53* gene (sgRNA-*p53*-1~6). T7 endonuclease I (T7EI) assay showed that three sgRNAs (sgRNA-*p53*-3, sgRNA-*p53*-5, sgRNA-*p53*-6) could induce insertions or deletions (indels) with indel rates ranging from 17% to 34% at the target site, whereas the others were not functional (Supplementary information, Figure S1B). We further determined the mutation ratio (number of mutated sequencing reads versus number of total sequencing reads) of each sgRNA by Sanger sequencing in a more quantitative way. Consistent with the T7EI assay, sgRNA-*p53*-6 showed the highest mutation ratio (22/25, 88%) (Supplementary information, Figure S1C), indicating the sgRNA-*p53*-6 could efficiently target the *p53* gene. To screen for the most effective amount of injected RNAs, Cas9 mRNA/sgRNA of three concentrations (20:5 ng/μl; 100:10 ng/μl; 200:10 ng/μl) were injected separately into monkey zygotes constructed by intracytoplasmic sperm injection (ICSI). Only 42% of embryos injected with 200:10 ng/μl Cas9 mRNA/sgRNA developed to the morula/blastocyst stage, suggesting that high concentration of injected Cas9 mRNA/sgRNA might be toxic to monkey embryonic development (Figure 1A). Sanger sequencing of injected embryos showed that embryos of the two groups injected with 100:10 ng/μl and 200:10 ng/μl Cas9 mRNA/sgRNA were all biallelic mutants, whereas only about 63% of embryos from the lowest concentration group (20:5 ng/μl Cas9 mRNA/sgRNA) were biallelic mutants (Figure 1A and Supplementary information, Figure S1D). Taken together, our results demonstrated that using the optimized sgRNA sequence and Cas9 mRNA/sgRNA concentration could produce biallelic mutations in monkey embryos with relatively high efficiency via zygote injection in one-step.

Next, we used 100:10 ng/μl Cas9 mRNA/sgRNA-*p53*-6 for injection to produce live monkeys with biallelic *p53* mutations. Of 108 injected monkey zygotes, 62 embryos with good morphology were transferred into 13 surrogate mothers, and 4 surrogates were successfully pregnant. Two surrogate mothers completed the pregnancy cycle (~165 days) and delivered 3 infant monkeys (#1, #2, #3), while the other 2 surrogates miscarried at late gestation stage and delivered 2 miscarried fetuses (#m1 and #m2) (Figure 1B and 1C). T7EI assay and Sanger sequencing showed that two healthy infants (#1 and #3) and two miscarried fetuses (#m1 and #m2) carried *p53* gene mu-

**A**

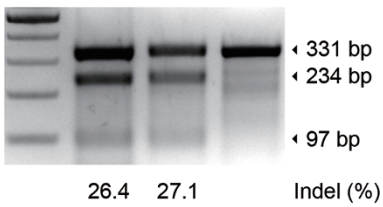
| Injection groups (ng/μl) | MII oocytes | 2-cell embryos | 4-8 cell embryos (% of 2-cell) | Morula/blastocyst (% of 2-cell) | Embryos sequenced | Biallelic mutation |
|---|---|---|---|---|---|---|
| 20 : 5 | 12 | 11 | 10 (90.9) | 8 (72.7) | 8 | 5 (62.5) |
| 100 : 10 | 15 | 13 | 12 (92.3) | 10 (76.9) | 9 | 9 (100) |
| 200 : 10 | 16 | 12 | 11 (91.7) | 5 (41.7) | 9 | 9 (100) |
| ICSI | 16 | 16 | 15 (93.8) | 13 (81.3) | - | - |

**B**

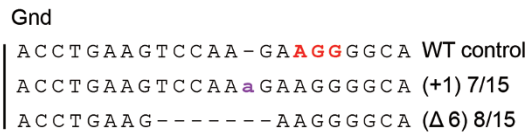
**C**

| Embryos transferred | Pregnancies in surrogates | Pregnancy | No. of offspring | Genotypes |
|---|---|---|---|---|
| 62 | 30.8% (4/13) | Triplets | # 1 | Monoallelic mutation |
| | | | # 2 | Wild-type |
| | | Singleton | # 3 | Homozygous mutation |
| | | Twins | # m1 | Biallelic mutation |
| | | Twins | # m2 | Monoallelic mutation |

**D**




**E**

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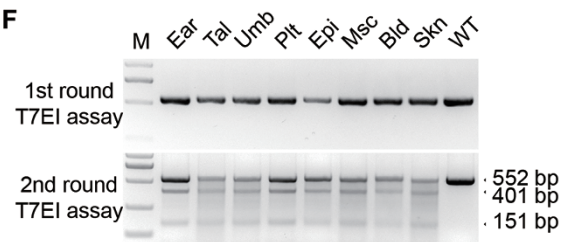
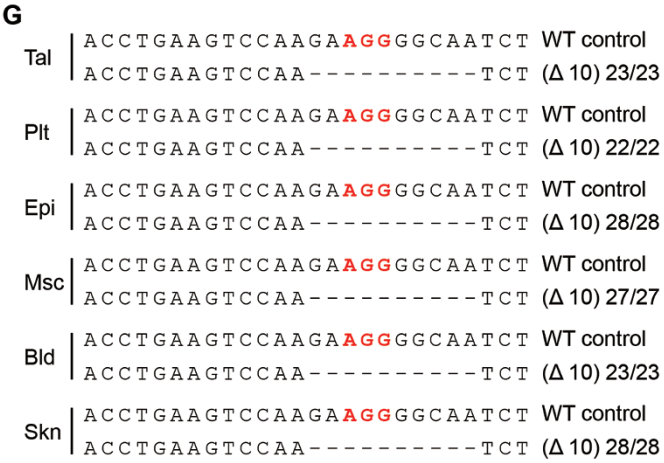
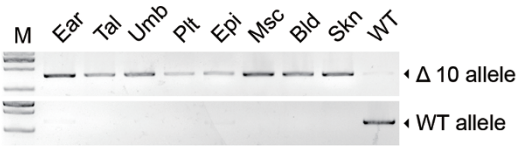


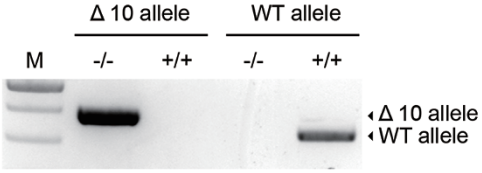

**G**



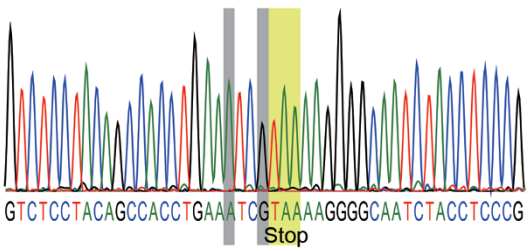

**H**




**I**




**J**




**K**

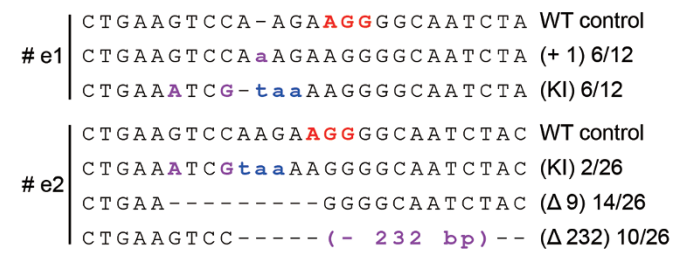


**Figure 1** Generation of *p53* gene biallelic mutant monkeys via CRISPR/Cas system. **(A)** Effects of injection concentrations of Cas9 mRNA/sgRNA on mutagenesis efficiency in monkey embryos. **(B)** Photograph of monkeys carrying biallelic mutation (#3, left panel) and monoallelic mutation (#1, right panel). **(C)** Summary of manipulated monkeys embryos and *p53* gene mutant monkeys. **(D)** T7EI assay of the PCR products encompassing the *p53* targeting site amplified from the genomic DNA prepared from gonad and umbilical cord of miscarried fetus #m1. Umb, Umbilical cord; Gnd, Gonad. **(E)** Sanger sequencing of PCR products encompassing the targeting site amplified from the gonad of miscarried fetus #m1. **(F)** Two rounds of T7EI assay of all the eight tissue samples dissected from live monkey #3. In the first round of T7EI assay, PCR products were re-annealed by themselves, then digested by T7EI, and no indel was detected. In the 2nd round of T7EI assay, the PCR products were annealed with PCR products from wild-type monkeys and then digested. M, Marker; Tal, Tail; Umb, Umbilical cord; Plt, Placenta; Epi, Oral epthelium; Msc, Muscle; Bld, Blood; Skn, Skin; WT, Wild-type. **(G)** Representative results of Sanger sequencing of PCR products from dissected tissues of #3. The fractions indicate the mutant reads number (numerator) out of total reads number (denominator). **(H)** Allele-specific PCR reveals that no wild-type allele can be detected in the eight dissected tissues of monkey #3. **(I)** Allele-specific RT-PCR reveals that no wild-type *p53* mRNA can be detected in monkey #3. **(J)** Sequences from HDR-modified embryos showing correct integration of the TAA stop codon into *p53* locus. **(K)** Sanger sequencing of PCR products encompassing the HDR-editing site from precisely modified embryos. The PAM sequence is shown in red bold uppercase. Targeted integration (KI) and the sizes of insertion (+), deletion (Δ) are presented on the right of each allele.

tations in the ear and umbilical cord tissues, whereas the other healthy infant (#2) exhibited the wild-type genotype, suggesting the failure of mutagenesis in this monkey. Notably, all the sequencing reads recovered from offspring #3 and #m1 contained mutations, suggesting that they are biallelic mutants (Figure 1E, 1G and Supplementary information, Figure S1E, S1F and Table S1). Since the CRISPR/Cas system-based mutagenesis could result in multiple and mosaic genotypes among different tissues, we further dissected more tissues for genotyping to examine the mosaicism. We dissected a total of 11 types of tissues from miscarried offspring #m1, 14 types of tissues from #m2, and 8 types of peripheral tissues from the healthy mutant monkey #3 (Supplementary information, Table S1). Both the T7EI assay and Sanger sequencing showed high rate of mosaicism of *p53* mutation in the monoallelic mutant offpring #m2, with mutation ratios ranging from 0% to 100% among the 14 examined tissues (Supplementary information, Figure S1G, S1H and Table S1). Sequences obtained from the 10 dissected somatic tissues of the miscarried offspring #m1 all contained mutations in the form of 3 different mutant genotypes without the presence of wild-type sequences, indicating that #m1 was biallelically mutated (Supplementary information, Figure S1I, S1J and Table S1). Notably, 100% mutation rate was also observed in the gonad tissue of #m1, which contained the same mutant genotypes as those observed in somatic tissues, suggesting that the Cas9-mediated mutations had been efficiently transmitted into germline (Figure 1E). For the live monkey #3, the T7EI assay detected the presence of hetero-duplexes annealed between PCR products of #3 and wild-type monkey, but not the homo-duplexes formed by the self-annealing of PCR products of #3 alone, as no PCR products could be cleaved by T7 endonuclease I. These results demonstrate that all tissues of monkey #3 contained only the mutated *p53* sequence. Consistent with the T7EI assay, the sequencing reads of all 8 tissues showed the same mutant genotype, deletion of 10 nucleotides (Δ10), further demonstrating that #3 was a homozygous mutant (Figure 1F, 1G and Supplementary information, Table S1). To confirm the biallelic mutation of *p53* gene in a more sensitive way, we designed a primer pair that could only amplify the wild-type but not the mutant allele, and used the sensitive PCR method to detect whether trace amount of wide-type *p53* allele still existed. No wild-type band was observed after 40 amplification cycles from any examined tissues of mutant monkey #3 (Figure 1H). We also used the same strategy to detect the presence of wild-type *p53* mRNA by RT-PCR. Consistently, we did not observe any wild-type band (Figure 1I). These results demonstrate that the live monkey #3 is a homozygous mutant in the *p53* gene. Sequencing the sgRNA-*p53*-6 targeting site in parents of all mutant offspring showed that the parental monkey genomes contained only the wild-type *p53* allele without any nucleotide variations, confirming that mutations in the offspring were generated by Cas9-mediated mutagenesis, but not inherited from the parents (Supplementary information, Figure S1K and S1L). Taken together, we demonstrate that live biallelic mutant monkey could be efficiently generated in one-step by zygote injection of an optimized CRISPR/Cas system.

It has been shown that homology directed repair (HDR)-driven gene editing for precise nucleotide modification can be achieved by the CRISPR/Cas system with the addition of desired template DNA [7]. To test the feasibility of HDR-driven genome editing in monkeys, we designed single-stranded DNA oligonucleotides (oligos) with homology to the targeting sequences, which would introduce a TAA stop codon and two synonymous nucleotide point mutations to the monkey *p53* gene (Supplementary information, Figure S1M). The DNA oligos were injected with 100:10 ng/μl Cas9 mRNA/

sgRNA-*p53*-6 into monkey zygotes. Sanger sequencing results revealed the presence of targeted integration of the TAA stop codon and two nucleotide variations in 2 of 9 assayed embryos (22%), demonstrating the feasibility of creating precise nucleotide-substitution mutations in monkey embryos (Figure 1J and 1K).

Since the CRISPR/Cas system may cause off-target mutagenesis [8], we examined potential off-target mutations in the mutant offspring #m1 and #3. A total of 18 potential off-target sites of sgRNA-*p53*-6 were identified by screening the monkey genome according to the homology of the sgRNA guide sequence to the genomic sequence (>12 base-pair identity to 3′ end of the sgRNA spacer), and all of them were located in intergenic regions or introns. Though T7EI assay showed that 4 sites of monkey #m1 and 7 sites of monkey #3 had cleavage, Sanger sequencing revealed that all cleaved PCR products had no mutation around the targeting PAM sites, but had sequence variations distant to the targeting sites (Supplementary information, Figure S1N-S1Q). Since the Cas9/sgRNA complex recognizes the protospacer adjacent motif (PAM) sequence NGG and cleaves the DNA around the PAM site [9], such sequence variations are unlikely to be induced by the off-target effect of the CRISPR/Cas system, but may arise from the high genomic polymorphism of intergenic and intron regions in monkeys [10, 11]. Taken together, we conclude that no off-target mutations were found in the mutant monkey offspring.

In summary, we have shown that biallelic gene mutation can be efficiently generated in monkeys by zygote injection with an optimized sgRNA Cas9/sgRNA combination in one-step. The long sex maturation time and low reproduction rate pose a huge challenge to generating biallelic monkeys through breeding. Therefore, generating biallelic mutant monkeys following an optimized procedure in a timely and efficient one-step method will bring genetically modified monkey models closer to practical application. Notably, we have produced the first live *p53* homozygous mutant monkey. Given the functional importance of p53 in tumorigenesis and other human diseases, our results not only demonstrate the feasibility of generating biallelic mutant monkeys in one-step through the CRISPR/Cas system, but also hold great values for biomedical researches. Moreover, we have also demonstrated the feasibility of HDR-driven precise gene editing in monkey embryos, which could be used to produce monkey models that more faithfully mimic human genetic defects.

## Acknowledgments

We thank all members of Group of Reproductive Engineering for discussion and help. This study was supported by grants from the China National Basic Research Program (2012CBA01300 to QZ), the "Strategic Priority Research Program" of the Chinese Academy of Sciences XDA01020100 (to QZ), the National Natural Science Foundation of China (91319308 to QZ), and the National Natural Science Foundation Project - Outstanding Young Scholars (31322036 to XYZ).

Haifeng Wan[1, *], Chunjing Feng[1, 2, *], Fei Teng[1, 2, *], Shihua Yang[3, 4, *], Baoyang Hu[1], Yuyu Niu[5], Andy Peng Xiang[6] , Weizhen Fang[7], Weizhi Ji[5], Wei Li[1], Xiaoyang Zhao[1], Qi Zhou[1]

*[1]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China; [2]University of Chinese Academy of Sciences, Beijing 100049, China; [3]College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong 510642, China; [4]Key Laboratory of Comprehensive Prevention and Control for Severe Clinical Animal Diseases of Guangdong Province, Guangzhou, Guangdong 510642, China; [5]Yunnan Key Laboratory of Primate Biomedical Research, Kunming, Yunnan 650500, China; [6]Center for Stem Cell Biology and Tissue Engineering, Key Laboratory for Stem Cells and Tissue Engineering, Sun Yat-Sen University, Guangzhou, Guangdong 510080, China; [7]Beijing Xieerxin Institute of Biological Resources, Beijing, China*
*These four authors contributed equally to this work.
Correspondence: Qi Zhou[a], Xiaoyang Zhao[b], Wei Li[c]
[a]E-mail: qzhou@ioz.ac.cn
[b]E-mail: xyzhao@ioz.ac.cn
[c]E-mail: liwei@ioz.ac.cn

## References

1 Bontrop RE. *Immunol Rev* 2001; **183**:5-9.
2 Capitanio JP, Emborg ME. *Lancet* 2008; **371**:1126-1135.
3 Liu H, Chen Y, Niu Y, *et al. Cell Stem Cell* 2014; **14**:323-328.
4 Niu Y, Shen B, Cui Y, *et al. Cell* 2014; **156**:836-843.
5 Ko LJ, Prives C. *Genes Dev* 1996; **10**:1054-1072.
6 Li W, Teng F, Li T, *et al. Nat Biotechnol* 2013; **31**:684-686.
7 Yang H, Wang H, Shivalila CS, *et al. Cell* 2013; **154**:1370-1379.
8 Hsu PD, Lander ES, Zhang F. *Cell* 2014; **157**:1262-1278.
9 Sternberg SH, Redding S, Jinek M, *et al. Nature* 2014; **507**:62-67.
10 Goswami DB, Ogawa LM, Ward JM, *et al. BMC Genomics* 2013; **14**:703.
11 Higashino A, Sakate R, Kameoka Y, *et al. Genome Biol* 2012; **13**:R58.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)