Generation of Gene-Modified Cynomolgus Monkey via Cas9/RNA-Mediated Gene Targeting in One-Cell Embryos

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SUMMARY

Monkeys serve as important model species for studying human diseases and developing therapeutic strategies, yet the application of monkeys in biomedical researches has been significantly hindered by the difficulties in producing animals genetically modified at the desired target sites. Here, we first applied the CRISPR/Cas9 system, a versatile tool for editing the genes of different organisms, to target monkey genomes. By coinjection of Cas9 mRNA and sgRNAs into one-cell-stage embryos, we successfully achieve precise gene targeting in cynomolgus monkeys. We also show that this system enables simultaneous disruption of two target genes (Pparγ and Rag1) in one step, and no off-target mutagenesis was detected by comprehensive analysis. Thus, coinjection of one-cell-stage embryos with Cas9 mRNA and sgRNAs is an efficient and reliable approach for gene-modified cynomolgus monkey generation.

INTRODUCTION

Monkeys have served as one of the most valuable models for modeling human diseases and developing therapeutic strategies due to their close similarities to humans in terms of genetic and physiological features (Chan, 2013). The genetic modification is invaluable for generation of monkey models. Although several transgenic monkeys have been successfully generated using retroviral or lentiviral vectors (Chan et al., 2001; Niu et al., 2010; Sasaki et al., 2009; Yang et al., 2008), precise genomic targeting in monkeys is the most desired for generating human disease models and has not been achieved so far (Chan, 2013; Shen, 2013). The recently described clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 system confers targeted gene editing by small RNAs that guide the Cas9 nuclease to the target site through base pairing (Jinek et al., 2012). The CRISPR/Cas9 system has been demonstrated as an easy-handle, highly specific, efficient, and multiplexable approach for engineering eukaryotic genomes (Mali et al., 2013). By now, this system has been successfully used to target genomic loci in the mammalian cell lines (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013a; Ma et al., 2014) and several species, including mice and rat (Li et al., 2013a; Li et al., 2013b; Ma et al., 2014; Shen et al., 2013; Wang et al., 2013). But whether it's feasible in primates is still unclear.

By taking the advantages of CRISPR/Cas9, we achieved efficient gene targeting in mice and rats by coinjection of one-cell-stage embryos with Cas9 mRNA and sgRNAs (Li et al., 2013b; Wang et al., 2013). Encouraged by our successes in CRISPR/Cas9-mediated gene targeting, as well as gene manipulation in early-cleavage-stage embryos of monkeys (Niu et al., 2010), here, we have extended the application of the CRISPR/Cas9 system to multiplex genetic engineering in one-cell-stage embryos of monkeys and successfully obtained founder animals harboring two gene modifications.

RESULTS AND DISCUSSION

Cas9/RNA Effectively Mediates Gene Disruptions in Monkey Cell Line

We selected cynomolgus monkey (Macaca fascicularis) as the model animal because of its body size, availability, similar
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menstrual cycle to human, and efficient reproduction ability (Sun et al., 2008). Three genes, namely Nr0b1 (Nuclear Receptor Subfamily 0 Group B Member 1), Ppar-γ (Peroxisome Proliferator-Activated Receptor Gamma), and Rag1 (Recombination Activating Gene 1), were selected as the target genes. Two sgRNAs separated by 117 bp for Nr0b1, 2 sgRNAs separated by 49 bp for Ppar-γ, and 1 sgRNA targeting Rag1 (Figure 1A), were designed as described (Mali et al., 2013a, 2013b). The efficiency of all sgRNAs was first tested by cotransfection with Cas9 into the COS-7 cell line derived from African green monkey kidney. Genomic DNA was isolated from cells harvested 72 hr after transient transfection and screened for the presence of site-specific gene modification by PCR amplification of regions surrounding the target sites as well as T7EN1 cleavage assay (Figure 1B). The cleavage bands were visible in all target genes. Further characterization of the cleavage by sequencing showed, different indels were detected at all five target sites with various mutation sizes (−336 ∼ +1 bp) at the efficiency of 22.2% for Nr0b1-sgRNA1, 20% for Nr0b1-sgRNA2, 10% for Ppar-γ-sgRNA1, 25% for Ppar-γ-sgRNA2, and 23.8% for Rag1-sgRNA (Figure 1C). These data demonstrated that the selected sgRNAs worked effectively with Cas9 on monkey genomes.

**Cas9/RNA Induces Efficient Genomic Targeting in Monkey Embryos**

Although microinjection of ZFN or TALEN mRNA into embryo has been successfully used for creation of gene target animals, but they have not been feasible in monkeys so far (Chan, 2013). To test whether the CRISPR/Cas9 system works in monkey embryos, the Cas9 (Addgene No. 44758) and sgRNAs were transcribed by T7 RNA polymerase in vitro as described (Shen et al., 2013). Twenty nanogram/μl Cas9 mRNA and 25 nanogram/μl of mixtures containing equal amount of each 5 sgRNAs were pooled and microinjected into 22 one-cell fertilized eggs of cynomolgus monkeys. The eggs were further cultured at 37°C in 5% CO2. A total of 15 embryos with normal development to morula or blastocyst stages were collected and examined for the presence of site-specific genome modification analysis by PCR, T7EN1 cleavage assay, and sequencing as described above. The results showed (Figures 2 and S1 available online), different sgRNAs function by different efficiency. Targeted modification with a range of sizes (−30 ∼ +6 bp) in monkey embryos occurred at all three target genes with efficiency of 4/15 for Nr0b1, 7/15 for Ppar-γ, and 9/15 for Rag1. Intriguingly, 6 of 15 embryos (2, 5, 8, 10, 11, and 13) harbored simultaneously mutations in both Ppar-γ and Rag1; whereas 2 of 15 embryos (embryos 3 and 4) harbored simultaneously mutations in both Nr0b1 and Rag1, demonstrating that the CRISPR/Cas9 system functions well in monkey embryos.

**Cas9/RNA Enables One-Step Multiple Gene Modifications in Monkeys**

With these successes, we set out to generate genetic modified cynomolgus monkeys. A total of 198 MII oocytes were collected. After fertilization by intracytoplasmic sperm injection (ICSI), Cas9 mRNA and sgRNA mixtures of five sgRNAs were injected as described above. A total 83 out of 186 injected zygotes were transferred into 29 surrogate females. Of the recipient mothers, ten pregnancies were established (34.5%; 10 out of 29), one of which was miscarried 36 days after embryo transfer. Among the pregnancies, three were twins, three were triplets, and the remaining four were single pregnancies (Table 1). So far, a set of twin female babies (A and B) were successfully delivered at full term (155 days) by caesarean section (Figure 3A). The other eight surrogate females are still in the gestation period. The noninvasively available tissues of the two infant monkeys, including placenta, umbilical cord, and ear punch tissues, were collected. Cas9/RNA-mediated genome modifications were first screened using genomic DNA from umbilical cord as described above. An additional band with smaller molecular size was observed by PCR amplification of the target region of Rag1 in infant B (Figure 3B), suggesting that the genomic modification occurred in this founder animal. Next, all the PCR products were subjected to the T7EN1 cleavage assay (Figure 3C). Cleavage products were detected in both infants in Rag1 and around the second sgRNA target site of Ppar-γ, indicating the presence of multiple genomic modifications in the founder monkeys. As expected, different kinds of indels (one for Ppar-γ, four for Rag1) were detected by sequencing of the PCR products (Figure 3D), further confirming the occurrence of multiple genomic modifications in the founder monkeys. Of note, no cleavage band was detected at Nr0b1 (Figure S2), which may be due to the lowest mutation efficiency of this gene in the embryonic test described above.

The presence of gene modification was further analyzed using genomic DNA from ear punch tissues and placenta. The same PCR bands, cleavage bands, and modifications were detected in Rag1 and Ppar-γ genes in both monkeys (Figure 4), further demonstrating the targeting success and confirming that CRISPR/Cas9 induces global genome modification in monkey embryos. Very impressively, no wild-type Rag1 sequence was detected in the ear punch of founder B (Figure 4C), demonstrating that the target modification has been ubiquitously and efficiently integrated into different tissues, most likely including the germline.

We also further substantiated the allelic targeting effects by tagging single-nucleotide polymorphisms (SNPs) of parent monkeys. A 3.8 kb fragment harboring Rag1-sgRNA target site was PCR amplified from ear genomic DNA of the parents and sequenced. Two different combinations of 4 SNPs tagging the

**Figure 1. sgRNA:Cas9-Mediated Modifications of Nr0b1, Ppar-γ, and Rag1 in COS-7 Cells**

(A) Schematic diagram of sgRNAs targeting at Nr0b1, Ppar-γ, and Rag1 loci. PAM sequences are underlined and highlighted in green. sgRNA targeting sites are highlighted in red.

(B) Detection of sgRNA1-Cas9-mediated cleavage of Nr0b1, Ppar-γ, and Rag1 by PCR and T7EN1 cleavage assay. M, DNA marker; sg1, sgRNA1; sg2, sgRNA2; Con, control.

(C) Sequences of modified Nr0b1, Ppar-γ, and Rag1 loci detected in COS-7 cells. At least 15 TA clones of the PCR products were analyzed by DNA sequencing. The PAM sequences are underlined and highlighted in green; the targeting sequences in red; the mutations in blue, lower case; deletions (−), insertions (+). N/N indicates positive colonies out of total sequenced.
Figure 2. sgRNA:Cas9-Mediated Modifications of Nr0b1, Ppar-γ, and Rag1 in Cultured Embryos

(A) Detection of sgRNA1:Cas9-mediated on-target cleavage of Nr0b1, Ppar-γ, and Rag1 by T7EN1 cleavage assay. PCR products were amplified and subjected to T7EN1 cleavage assay. Samples with cleavage bands were marked with an asterisk ‘*’.

(B) DNA sequences of marked samples. TA clones from the PCR products were analyzed by DNA sequencing. Mutations in three PCR products (labeled with red asterisk) indentified by T7EN1 cleavage assay were not detected by TA sequencing because of limited amount of colonies. The PAM sequences are underlined and highlighted in green; the targeting sequences in red; the mutations in blue, lower case; deletions (−), and insertions (+). N/N indicates positive colonies out of total sequenced. See also Figure S1.
parents derivation were detected downstream of the target site of *Rag1*-sgRNA (Figure S3A, Tables S1 and S5). The tagging SNP combinations of the parents and the founder twins were further determined by TA cloning and sequencing (Figures S3B and S3C). The results showed that two tagging SNP combinations segregate in accordance with Mendel’s laws. The *Rag1*-sgRNA target site in the ear of founder B showed high target efficiency was further sequenced. The results (Figure S3D) showed that both alleles identified by tagging SNPs harbored target modifications, indicating two alleles from both parents could be modified by Cas9/RNA-mediated targeting in monkeys.

Surprisingly, only one genotype with a single-nucleotide insertion for *Ppar-*γ at different tissues of both founder animals was detected (Figures 3D and 4C). To exclude the possibility that this single-nucleotide insertion was a SNP rather than a real mutation, the target sites of the parents and surrogate mother were amplified to perform T7EN1 cleavage assay and sequencing (Figure S4). The results excluded the presence of the same single nucleotide, confirming that the insertion was indeed caused by CRISPR/Cas9 modification to the *Ppar-*γ gene. Taken together, we have successfully achieved Cas9/RNA-mediated site-specific modifications in monkey genome by one-cell embryo microinjection.

**Mosaicism**

It is worth noticing that the sequence data of both cultured embryos and founder animals showed multiple genotypes (Figures 2B, 3D, and 4C), suggesting the CRISPR/Cas9-mediated cleavage had occurred multiple times at different stages of monkey embryogenesis and resulted in mosaicism of the modification, as have been observed in other species (Sung et al., 2013; Tesson et al., 2011). Currently, the founder babies are housed in dedicated facilities and developing normally. Due to the limited access of tissues from the founder infants, more thorough characterization of the genomic modifications as well as phenotype

| Table 1. Summary of Embryo Microinjection of Cas9 mRNA and sgRNAs |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| MII Oocyte Injected Embryos | Embryos for ET Pregnancies | Single Pregnancy | Multiple Pregnancy | Fetuses |
| 198                        | 186                        | 83                  | 34.5% (10/29)    | 4^a | 3 twins, 3 triplets | 19 |

^aOne miscarried 36 days after embryo transfer.

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**Figure 3. sgRNA:Cas9-Mediated Modifications of Ppar-*γ* and *Rag1* in Founder Cynomolgus Monkeys**

(A) Photographs of 14-day-old founder infants A and B.

(B) PCR products of the target region of *Ppar-*γ and *Rag1* in founders. Targeted region of *Ppar-*γ and *Rag1* loci were PCR amplified from the umbilical cord genomic DNA of A and B founders. M, DNA marker; Con, control umbilical cord from wild-type cynomolgus monkey, which was born 9 days after birth of A and B.

(C) Detection of sgRNA:Cas9-mediated on-target cleavage of *Ppar-*γ and *Rag1* by T7EN1 cleavage assay. PCR products from (B) were subjected to T7EN1 cleavage assay.

(D) Sequences of modified *Ppar-*γ and *Rag1* loci detected in founders. At least 18 TA clones of the PCR products were analyzed by DNA sequencing. The PAM sequences are underlined and highlighted in green; the targeting sequences in red; the mutations in blue, lower case; deletions (−), and insertions (+). N/N indicates positive colonies out of total sequenced. See also Figure S2 and S4.
remains to be performed. This has to be awaited until the founder monkeys have developed into adulthood. In addition, more full-term founders will be born and provide more samples for further assessment of CRISPR/Cas9-mediated genome modification in monkeys.

**Off-Target Analysis**

The off-target effect is of a major concern for the CRISPR/Cas9 system (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). We observed CRISPR/Cas9 induced heritable off-target mutation in mice (B.S., W. Zhang, J. Zhang, J. Zhou, J.W., L. Chen, L. Wang, A. Hodgkins, V. Iyer, X.H., and W.C. Skarnes, unpublished data). To test whether off target occurred in these genetic modified monkeys, we screened the monkey genome and predicted a total of 84 potential off-target sites (OTS), including 9 for site 1 of Nr0b1, 20 for site 2 of Nr0b1, 14 for site 1 of Ppar-γ, 20 for site 2 of Ppar-γ, and 21 for Rag1, respectively (Table S2). The off-target effects were comprehensively assessed as on-target effect analysis using genomic DNA from umbilical cord. The fragments around all the potential off-target loci were PCR amplified, then subjected to T7EN1 cleavage assay. Seventeen PCR products yielded cleavage bands were precisely examined by TA sequencing. Surprisingly, all the cleavage were caused by SNP or repeat sequences, and no authentic mutation was detected (Table S3). These results demonstrated that Cas9/RNA does not induce detectable off-target mutation in our study. Considering that the off-target effect is site-dependent, and more specific strategies using mutated Cas9 have already been established (Ran et al., 2013), the off-target mutagenesis can be minimized by optimizing the procedure, suggesting...
CRISPR/Cas9 could be a reliable genome target tool for monkeys.

In summary, our current studies demonstrate that site-specific gene modification can be effectively achieved in monkeys by coinjection of Cas9 mRNA and sgRNAs into the one-cell fertilized eggs. We also demonstrate that the multiple genetic mutations can be established at once without detectable off-target effects, providing the success of creating genome engineered primates and confirming the CRISPR/Cas9 system is applicable for monkey genome targeting.

EXPERIMENTAL PROCEDURES

Animals
Healthy female cynomolgus monkeys (Macaca fuscata), ranging in age from 5 to 8 years and having body weights of 3.62 to 5.90 kg, were selected for use in this study. All animals were housed at the Kunming Biomed International (KBI). The KBI is an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. All animal protocols are approved in advance by the Institutional Animal Care and Use Committee of Kunming Biomed International.

Embryo Collection
Embryo collection and transfer were performed as previously described (Niu et al., 2010). In brief, 11 healthy female cynomolgus monkeys aged 5–8 years with regular menstrual cycles were selected as oocyte donors for superovulation, which were performed by intramuscular injection with rhFSH (recombinant human follitropin alfa, GONAL-F, Merck Serono) for 8 days, then rHCG (recombinant human chorionic gonadotropin alfa, OVIDREL, Merck Serono) on day 9. Oocytes were collected by laparoscopic follicular aspiration 32–35 hr after rHCG administration. All (first polar body present) oocytes were used to perform intracytoplasmic sperm injection (ICSI) and the fertilization was confirmed by the presence of two pronuclei.

Cas9/sgRNA Injection of One-Cell Embryos
The zygotes were injected with a mixture of Cas9 mRNA (20 ng/μl) and five sgRNAs (5 ng/μl each). Microinjections were performed in the cytoplasm of zygotes using a Nikon microinjection system under standard conditions. The zygotes then were cultured in the chemically defined, protein-free hamster zygotes using a Nikon microinjection system under standard conditions. The described before (Shen et al., 2013). The pUC57-sgRNA expression vector pGL3-U6-sgRNA-PGK-Puro vector, containing the U6-PGK-NLS-Flag gene modification can be effectively achieved in monkeys by coinjection of Cas9 mRNA and sgRNAs into the one-cell fertilized eggs. We also demonstrate that the multiple genetic mutations can be established at once without detectable off-target effects, providing the success of creating genome engineered primates and confirming the CRISPR/Cas9 system is applicable for monkey genome targeting.

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In Vitro Transcription
In vitro transcription was performed as described (Zhou et al., 2014). Briefly, the pST1374-Cas9-N-NLS-flag-linker vector was linearized by Age1 enzyme and in vitro transcribed using T7 Ultra Kit (Ambion, AM1345). Cas9-N-NLS-flag-linker mRNA was purified by RNeasy Mini Kit (Qiagen, 74104). sgRNA oligos were annealed into pUC57-sgRNA expression vector with T7 promoter. Then expression vectors were linearized by Dra I and transcribed by MEGashortscript Kit (Ambion, AM1354) in vitro. The sgRNAs were purified by MEGAClear Kit (Ambion, AM1908) and recovered by alcohol precipitation.

T7EN1 Cleavage Assay and Sequencing
Different samples, including cells, placenta, umbilical cord, and ear punch tissues, were collected and digested in lysis buffer (10 μM Tris-HCl, 0.4 M NaCl, 2 μM EDTA, 1% SDS, and 100 μg/ml Proteinase K). The genomic DNA was extracted from lysate by phenol-chloroform recovered after alcohol precipitation. Genomic DNA from cultured embryos was amplified by REPLI-g Single Cell Kit (Qiagen, 150343) according to the manufacturer’s instructions. T7EN1 cleavage assay was performed as described (Shen et al., 2013). In brief, targeted fragments were amplified by PrimerSTAR HS DNA polymerase (Takara, DR010A) from extracted DNA, and purified with PCR cleanup kit (Axygen, AP-PCR-50). Purified PCR product was denatured and reannealed in NEBuffer 2 (NEB) using a thermocycler. Hybridized PCR products were digested with T7EI (NEB, M0302L) for 30 min and separated by 2.5% agarose gel. To detect T7EI cleavage products of Nr0b1 (localized on chromosome X) in cultured embryos, 50 ng of PCR fragment from wild-type control embryos was mixed with 150 ng of PCR fragments from embryos injected with Cas9 mRNA and sgRNAs. PCR products with mutations detected by T7EN1 cleavage assay were sub-cloned into T vector (Takara, D103A). For each sample, colonies were picked up randomly and sequenced by M13-47 primer. Primers for amplifying Nr0b1, Ppar delta, and Rag1 targeted fragments are listed in Table S5.

Off-Target Assay
All potential off-target sites with homology to the 23 bp sequence (sgRNA+PAM) were retrieved by a base-by-base scan of the whole genome (BGI CR_1.0/neMac3), allowing for untargeted alignments with up to four mismatches in the sgRNA target sequence. In the output of the scan, potential off-target sites with less than three mismatches in the seed sequence (1 to 7 base) were selected to PCR amplification using umbilical cord genomic DNA as templates. The PCR products were first subject to T7EN1 cleavage assay. The potential off-target sites yielding typical cleavage bands were considered as candidates, then the PCR products of the candidates were cloned and sequenced to confirm the off-target effects. The primers for amplifying the off-target sites are listed in Table S6.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and six tables and can be found with this article online athttp://dx.doi.org/10.1016/j.cell.2014.01.027.

AUTHOR CONTRIBUTIONS
J.S., W.J., X.H., and Q.Z. initiated the project, designed the experiments, and wrote the manuscript. J.S. organized and supervised the whole project. W.J. organized and supervised all monkey work; X.H. organized and supervised all genome manipulation and analysis; Q.Z. organized the teams and provided guidance on the whole project. Y.N. and Y. Chen performed monkey work, including superovulation, microinjection, embryo transfer, animal care, etc. B.S. and Y. Cui performed genome manipulation and analysis, including Cas9 and sgRNA design and construct, in vitro transcription, genome

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