CHAPTER 16

# Transgenic Animals

## SUMMARY

Similarly to crop plants, selective breeding of animals over a long period of time has produced desirable traits, such as woollier sheep and smarter dogs. Animals can also be created through genetic engineering to contain transgenes, which encode desirable traits. Once a transgene has been created, it is typically delivered into the animal cell using the process of nuclear microinjection of the transgene into the pronucleus of a sperm at fertilization. The pronuclei from the egg and sperm eventually fuse, and an embryo forms. The embryo is implanted into a surrogate mother and eventually develops into a baby animal. The progeny are screened for the presence of the transgene using various biotechnology techniques. Progeny receiving two copies of the transgene are homozygous and particularly important for future breeding to produce more homozygous animals.

There are alternate approaches to creating transgenic animals. Retroviruses can be used to introduce foreign DNA molecules into the chromosomes of animal cells, including embryonic stems cells, which have the ability to develop into any tissue. There are a few drawbacks to using retroviruses to generate transgenic animals. Mainly, it is impossible to create fully transgenic animals in this way because the retrovirus does not always insert in the appropriate location on the genome. It is possible, however, to generate chimeric animals having some tissue that is transgenic.

Transgenic mice have been produced that have a gene encoding a rat growth hormone (somatotropin) inserted into their genomes. The mice grew larger and more robust, although not as large as a rat. Proper expression of the transgene relies on the presence of appropriate host promoters upstream of the gene of interest. Recombinant human somatotropin has been successfully used to treat a specific type of dwarfism in humans. Recombinant proteins from livestock have been produced in bacteria, such as recombinant bovine somatotropin (rBST). This growth hormone is used to increase production of milk in cattle. Other recombinant proteins from livestock have also been produced for experimental purposes. In transgenic goats, recombinant tissue plasminogen activator (rTPA) is produced in the mammary glands. This protein helps dissolve blood clots.

Mice have not only been made transgenic for the production of a protein, but also can be engineered to knock out a particular gene. The effects of that knockout are then monitored to determine the role of the gene's protein product. It must be understood that some genes are required for normal functions in the mice. Generating knockout mice that are homozygous for the defect might be impossible because of deleterious effects due to the absence of the protein, thus leading to increased mortality.

Several alternative approaches to nuclear injection can be used to generate transgenic animals. Retroviruses can infect and introduce a single copy of a transgene into the genome of the host cell. A disadvantage of using retroviruses to create transgenic animals is that the animals are almost always chimeric. Embryonic stem cells can also be used to generate transgenic animals. The cells are cultured and new DNA is introduced during the culturing process. These engineered cells can then be inserted into the central cavity of a blastocyst. The result is a genetic chimera.

Expressing the transgene within the animal is dependent upon the location of the inserted foreign DNA. Nearby regulatory elements, insertion within heterochromatin, histone acetylation, and DNA methylation can all affect the level of expression. To help alleviate this issue, scientists can target the foreign DNA to a specific location on the chromosome by adding sequences to the ends of the transgene that are homologous to the sequences at a desired location on the chromosome.

It is possible to construct transgenes that can be induced to produce protein. Linking the transgenes to an inducible promoter allows the expression to be controlled. However, ill effects have been observed using inducible promoters. The effects either come from the

inducing molecule or the event itself, or from some other unidentified source. These harmful effects can largely be avoided by constructing recombinant promoters instead. Recombinant systems have included the use of bacterial repressors that are modified to work in animal cells. The *lac* and *tet* systems are of bacterial origin and work well in eukaryotes. Steroid receptors also work quite well in animal systems and are activated to bind DNA and regulate gene expression in the presence of the steroid. The main problem associated with this system is preventing induction of genes that normally are associated with the steroid. This can be accomplished by using steroid receptor/steroid combinations that do not naturally occur in the host, such as an insect system in a mammalian host or vice versa.

Control of the transgene can also be accomplished by site-specific recombination using Cre recombinase from bacteriophage P1 or Flp recombinase (flippase) from yeast 2-micron plasmid. The Cre/loxP system, as well as the Flp/FRT system, can be used to delete reporter genes or selectable markers and activate the transgene by removing a blocking sequence, engineering chromosomes *in vivo* through large-scale deletions or rearrangements, and creating conditional knockout mutants. Bacterial recombinases, such as phiC31 integrase, can also be used to rearrange segments of DNA or moderate gene expression.

Several nonmammalian animals have been genetically modified to contain foreign genes. Transgenic fruit flies (*Drosophila*) have been generated using transposable elements, known as P elements, which can be inserted into germline cells. Mosquitoes have also been genetically modified by the introduction of transgenes through a similar process to that of *Drosophila*. The *piggyBac* transposon from the cabbage looper and the *Minos* transposon from a *Drosophila* species are widely used transposons for the insertion of DNA into the genomes of mosquitoes. The goal is to help eliminate the spread of mosquito-borne diseases such as malaria, yellow fever, and dengue fever, by adding genes that could help eliminate the pathogens in the arthropod host before they are transmitted to humans or prevent colonization of the pathogen in the vector. Strategies include increasing expression of a mosquito immune system protein, defensin A, and also producing transgenic mosquitoes that express phospholipase from bees. Another strategy involves producing a transgenic mosquito that expresses a human antibody against the pathogen within the mosquito's salivary glands. The presence of a symbiotic bacterium called *Wolbachia* also has the potential to stop the transmission of diseases in arthropod vectors.

In addition to genetically engineered mosquitoes and the fight against disease spread, there are other numerous practical applications for transgenic animals. Transgenic animals can help fight disease. Influenza easily spreads between humans, birds, and swine. A transgenic chicken that encodes an RNA hairpin structure has been shown to decrease the spread of H5N1 influenza when cohabiting with uninfected chickens. Cattle and goats have been genetically engineered to produce lysozyme in their milk. The lysozyme targets peptidoglycan in bacterial cells, such as *E. coli*, and can thus limit diarrheal disease. Transgenic goats produce some pharmaceuticals, such as antithrombin, in their milk. Transgenic fish have been produced to grow larger. Transgenic pigs and sheep produce more essential fatty acids. Some animals can even be modified to remove allergens. Enviropig™ is a transgenic pig that can utilize grains more effectively and cuts back on the phosphate waste generated by the pig, which is normally hazardous to the environment.

Perhaps one of the more innovative outcomes of transgenic animals is the production of fluorescent silk used in a wedding dress. The transgenic silkworm produced a fluorescent silk by introducing the genes for green fluorescent protein from jellyfish or red fluorescent protein from coral under the control of a silkworm promoter. The wedding dress was made out of this silk and fluoresced under ultraviolet light.

Antisense transgenes can be introduced into organisms by inverting DNA sequences. Once transcribed, the antisense RNA will bind to mRNA and silence expression of the protein product through RNA interference. Ribozymes can also be inserted into transgenic organisms.

Finally, it is possible to acquire genes horizontally from other organisms, such as plants or animals, simply by consuming them. Enzymes in the digestive tract of animals degrade most of the ingested DNA. However, recent data suggest that some DNA does survive and is capable of crossing into the blood stream, where it might be picked up by a cell and incorporated. For this to have any impact on the progeny, the DNA would need to be incorporated into germline cells. It is unclear whether or not this actually happens.

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

**Yuyu Niu et al. (2014). *Cell* 156, 836–843.**

Human diseases are modeled effectively using monkeys. The ability to genetically manipulate these models is paramount for investigating disease progression and generating effective therapeutics. Transgenic monkeys have previously been created using retroviral or lentiviral vectors. However, the authors desired a more precise targeting strategy. The CRISPR/Cas9 system has been used to successfully edit genes in rats and mice.

With the success of the CRISPR/Cas9 system in rats and mice, the authors expanded the targets of this system to early-cleavage-stage embryos of monkeys. In their approach, founder animals were obtained harboring two modifications.

**What is the CRISPR/Cas9 system?**

CRISPR stands for clustered regularly interspaced short palindromic repeats. Cas9 is a nuclease. Together, the CRISPR/Cas9 system uses a small guide RNA for targeted editing of the monkey genome in this research article's example.

**The CRISPR/Cas9 system has been previously shown to target loci effectively in mouse and rat models, and also mammalian cell lines. How did the authors determine that the CRISPR/Cas9 system worked in primates?**

The authors selected three target genes: *Nr0b1* (nuclear receptor subfamily 0 Group B member 1), *Ppar-$\gamma$* (peroxisome proliferator-activated receptor gamma), and *Rag1* (recombination activating gene 1). Small guide RNAs (sgRNAs) were designed for each of these genes, which were then cotransfected with Cas9 into an African green monkey kidney cell line. Upon isolation of DNA from these cells followed by PCR, the authors detected various mutation sizes and determined that the selected sgRNAs worked with Cas9 in the monkey cell line.

**The authors determined that the CRISPR/Cas9 system worked in monkey cell lines. How did they determine that this system was effective in monkey embryos?**

Cas9 mRNA was mixed with equal parts of each of the five sgRNAs from the target genes and then microinjected into the fertilized eggs of cynomolgus monkeys. The eggs were cultured, and a total of 15 eggs developed normally into blastocyst stages. Through various experiments, the authors determined that modifications were present at all three targeted genes, indicating that this system functions in embryos. Furthermore, some embryos had combinations of targeted modifications, indicating that the system was quite efficient.

**The CRISPR/Cas9 system was successfully used to edit the genomes of monkey kidney cell lines and also cynomolgus monkey embryos. Were the authors able to produce genetically modified cynomolgus monkeys? If so, how?**

Yes. The same procedure that was used to generate genetically modified embryos was employed to produce genetically modified monkeys.

**How successful were the authors in generating genetically modified cynomolgus monkeys? Were any offspring actually genetically modified?**

Out of 198 collected oocytes, 186 were injected zygotes. Out of these zygotes, 83 were transferred into 29 surrogate females, in which only 10 pregnancies occurred. One pregnancy was miscarried. At the time of press, a set of twin female babies has been delivered, and 8 other pregnancies were still ongoing. Tissues from the infant twins were positive for genomic modifications in both *Ppar-$\gamma$* and *Rag1*, but not for *Nr0b1*.

**Were any of the genetic modifications ubiquitous? How did the authors determine any ubiquitous modifications?**

Ear punch tissue from each infant twin was analyzed and found to contain the same experimental results from the analysis as observed for the previous tissues. Additionally, wild-type *Rag1* was not detected in the ear punch tissue of one of the infant twins.

**Primates are diploid, meaning each somatic cell contains two copies of each gene. Did the authors consider any allelic effects of targeting? If so, how was this investigated, and what was the outcome?**

Yes. The authors considered allelic effects of targeting. Single-nucleotide polymorphisms (SNPs) of parent monkeys were tagged. Different combinations of these tags were detected into the founder twins. Upon further investigation, the results showed that both alleles for *Rag1* in one of the twins were targeted in the CRISPR/Cas9 system.

The authors were able to successfully utilize the CRISPR/Cas9 system for genetically modifying monkey cell lines, embryonic stem cells, and producing founder monkeys. This system is specific for the targets due to the use of sgRNAs derived from those targeted sequences. Additionally, multiple specific genetic mutations occur, indicating the efficiency of the system. On the other hand, little detectable off-target mutagenesis occurs. This further supports the specificity and efficiency of using the CRISPR/Cas9 system in the targeted genome editing of primates.

# 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 (*Ppar-γ* 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., 2013a). 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., 2013b; Wang et al., 2013) 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; Shen et al., 2013; Ma et al., 2014). 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., 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% $CO_2$. 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 (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.

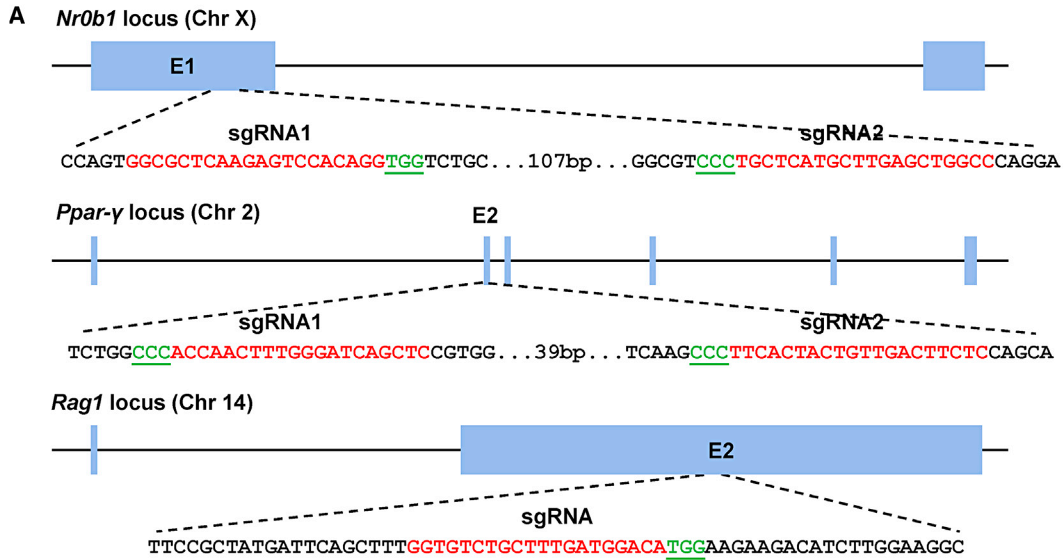
### Cas9/RNA Enables One-Step Multiple Gene Modifications in Monkeys

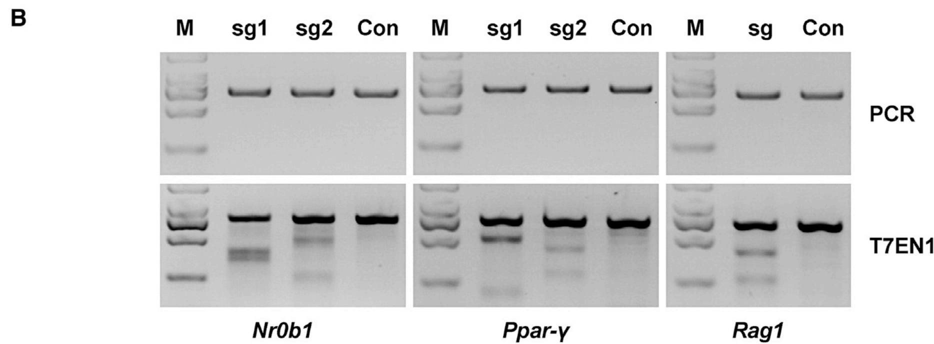
With these successes, we set out to generate genetic modified cynomolgus monkeys. A total of 198 MII oocytes were collected. After fertilization by intracytoplasmic sperm injection (ICSI), Cas9 mRNA and sgRNA mixtures of five sgRNAs were injected as described above. A total 83 out of 186 injected zygotes 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 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).




**C**

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Nr0b1   TCTGGTGCGCTGCGGCCAGTGGCGCTCAAGAGTCCACAGGTGGTCTGCGAGGCAGCCTCAGCT (WT)
 sg1    TCTGGTGCGCTGCGGCCAGTGGCGCTCAAGAGTCCACA:GTGGTCTGCGAGGCAGCCTCAGCT (-1,1/18)
        TCTGGTGCGCTGCGGCCAGTGGCGCT::::::::::CAGGTGGTCTGCGAGGCAGCCTCAGCT (-10,1/18)
        TCTGGTGCGCTGCGGCCAGTGGC:::::::::::::::::::::::::::::::::::::::: (-43,1/18)
        ::::::::::::::::::::::::cctggtgggacacc::::::::::::::::::::::::: (-336,+14,1/18)

Nr0b1   GGTGCGCAACTGCTGGGCGTCCCTGCTCATGCTTGAGCTGGCCCAGGACCGCTTGCAGTTCGA (WT)
 sg2    GGTGCGCAACTGCTGGGCGTCCCTGCT::TGCTTGAGCTGGCCCAGGACCGCTTGCAGTTCGA (-2,1/15)
        GGTGCGCAACTGCTGGGCGTCCCTGCTCATGCTTGAGCTGGCCCAGGACCGCTTGCAGTTCGA (+1,1/15)
                                   ^
                                   c
        :::::::::::::tcctaaattgccc::::CTTGAGCTGGCCCAGGACCGCTTGCAGTTCGA (-49,1/15)

Ppar-γ  ACACAGAGATGCCATTCTGGCCCACCAACTTTGGGATCAGCTCCGTGGATCTCTCCGTAATGG (WT)
 sg1    ACACAGAGATGCCATTCTGGC:tc:::ACTTTGGGATCAGCTCCGTGGATCTCTCCGTAATGG (-6,+2,1/20)
        ACACAGAGATGCCATTCTGGCCCACC:::::GGGATCAGCTCCGTGGATCTCTCCGTAATGG (-6,1/20)

Ppar-γ  CCCACTCCTTTGACATCAAGCCCTTCACTACTGTTGACTTCTCCAGCATTTCTGCTCCACATT (WT)
 sg2    CCCACTCCTTTGACATCAAGCCCTTCACTACTGTTGACTTCTCCAGCATTTCTGCTCCACATT (+1,5/20)
                                   ^
                                   a

Rag1    TTCCGCTATGATTCAGCTTTGGTGTCTGCTTTGATGGACATGGAAGAAGACATCTTGGAAGGC (WT)
 sg     TTCCGCTATGATTCAGCTTTGGTGTCTGCTTTGATG:ACATGGAAGAAGACATCTTGGAAGGC (-1,3/21)
        TTCCGCTATGATTCAGCTTTGGTGTCTGCTTTGAT::ACATGGAAGAAGACATCTTGGAAGGC (-2,1/21)
        TTCCGCTATGATTCAGCTTTGGTGTCTG::::::::GACATGGAAGAAGACATCTTGGAAGGC (-8,1/21)
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(legend on next page)

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 notifying 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 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 fascicularis*), 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. MII (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 embryo culture medium-10 (HECM-10) containing 10% fetal calf serum (Hyclone Laboratories, SH30088.02) at 37°C in 5% $CO_2$. The cleaved embryos with high quality at two-cell to blastocyst stage were transferred into the oviduct of the matched recipient monkeys. Twenty-nine monkeys were used as surrogate recipient, and typically, three embryos were transferred into each female. The earliest pregnancy diagnosis was performed by ultrasonography about 20–30 days after the embryo transfer. Both clinical pregnancy and number of fetuses were confirmed by fetal cardiac activity and presence of a yolk sac as detected by ultrasonography (Chen et al., 2012).

### DNA Constructs

Codon optimized Cas9 expression construct, Cas9-N-NLS-flag-linker (Addgene No. 44758), was synthesized and inserted into pST1374 vector as described before (Shen et al., 2013). The pUC57-sgRNA expression vector used for in vitro transcription of sgRNAs was described as before (Zhou et al., 2014). pGL3-U6-sgRNA-PGK-Puro vector, containing the U6-PGK-Puro fragment amplified from pLKO.1 (Addgene No. 8453), sgRNA scaffold amplified from pUC57-sgRNA, and pGL3-Basic plasmid backbone (Promega,

**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.

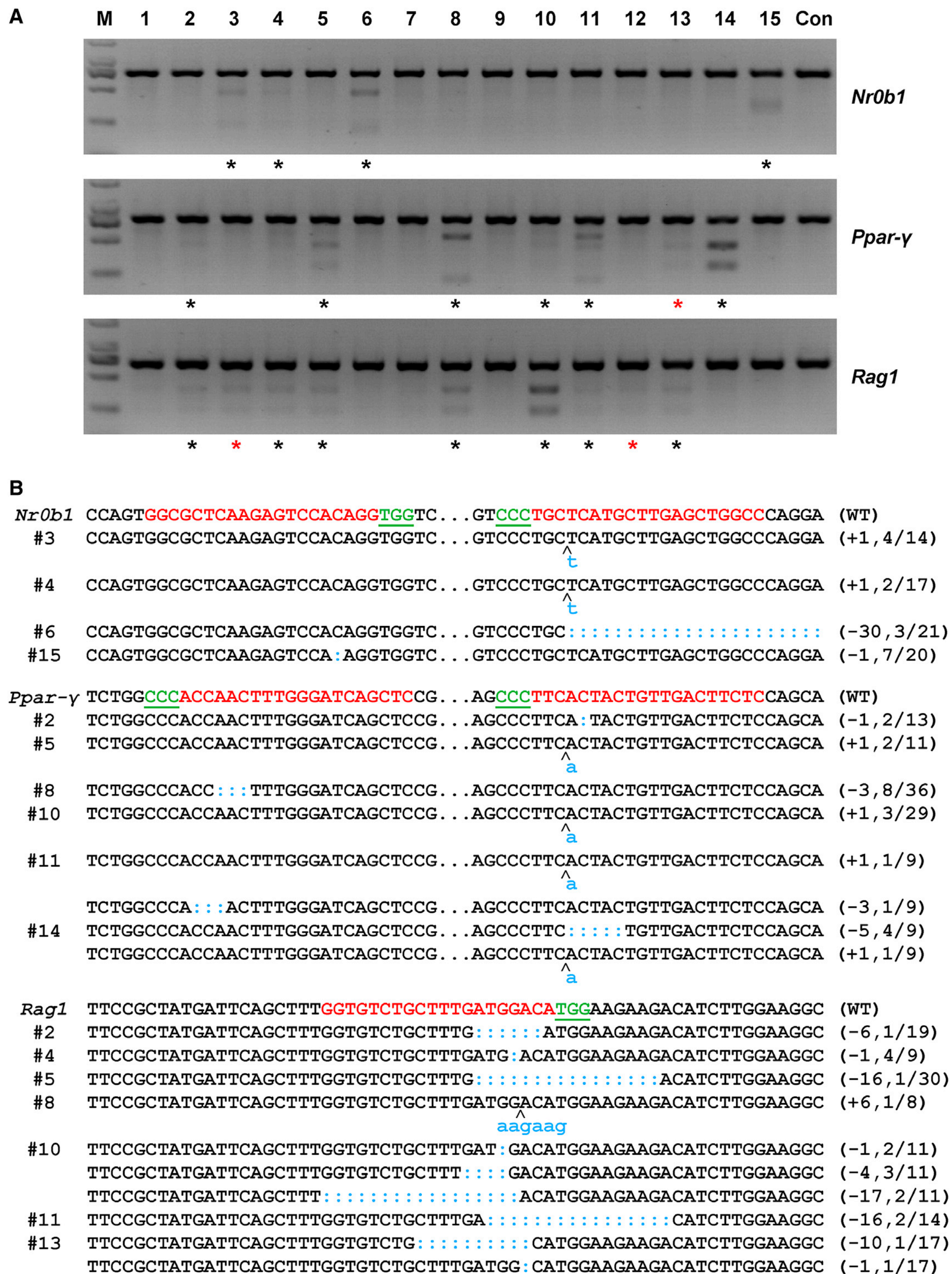


**Figure 2. sgRNA:Cas9-Mediated Modifications of *Nr0b1*, *Ppar-γ*, and *Rag1* in Cultured Embryos**

(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.

**Table 1. Summary of Embryo Microinjection of Cas9 mRNA and sgRNAs**

| MII Oocyte | Injected Embryos | Embryos for ET | Pregnancies /Surrogates | Single Pregnancy | Multiple Pregnancy | Fetuses |
|---|---|---|---|---|---|---|
| 198 | 186 | 83 | 34.5% (10/29) | 4[a] | 3 twins, 3 triplets | 19 |

[a]One miscarried 36 days after embryo transfer.

E1751) was used for expression of sgRNAs in cells. Oligos for the generation of sgRNA expression plasmids (Table S4) were annealed and cloned into the BsaI sites of pUC57-sgRNA or pGL3-U6-sgRNA-PGK-Puro. pGL3-U6-sgRNA-PGK-Puro was deposited in Addgene (Addgene NO. 51133).

### Cell Culture and Electroporation

COS-7 cells (ATCC, CRL-1651) were cultured in DMEM/high glucose (HyClone) with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml); $2 \times 10^6$ cells were electroporated (BioRad Gene Pulser XL) with four micrograms of Cas9 expression plasmids and two micrograms of pGL3-U6-sgRNA-PGK-Puro. Empty pGL3-U6-sgRNA-PGK-Puro plasmid was used as control. Cells were collected 72 hr postelectroporation.

### 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 by alcohol precipitation. Genomic DNA from cultured embryos was amplified by REPL1-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 T7EN1 (NEB, M0302L) for 30 min and separated by 2.5% agarose gel. To detect T7EN cleavage products of *Nr0b1* (localized on chromosome X) in

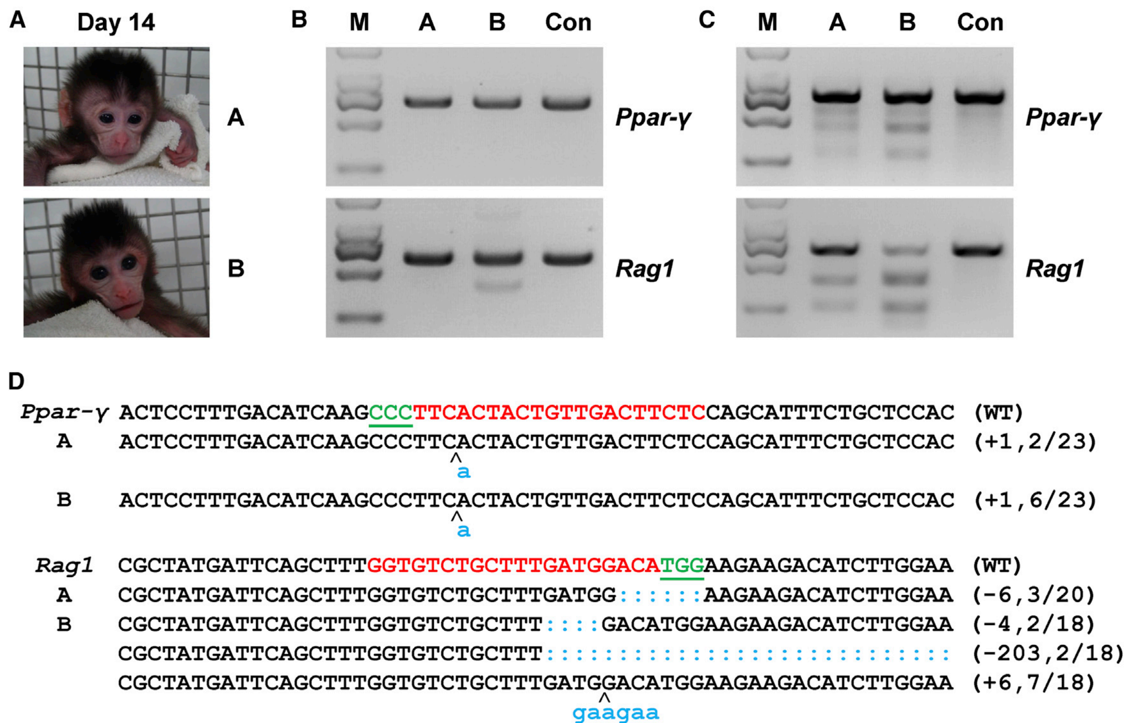


**Figure 3. sgRNA:Cas9-Mediated Modifications of *Ppar-γ* and *Rag1* in Founder Cynomolgus Monkeys**

(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 umbilial 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.

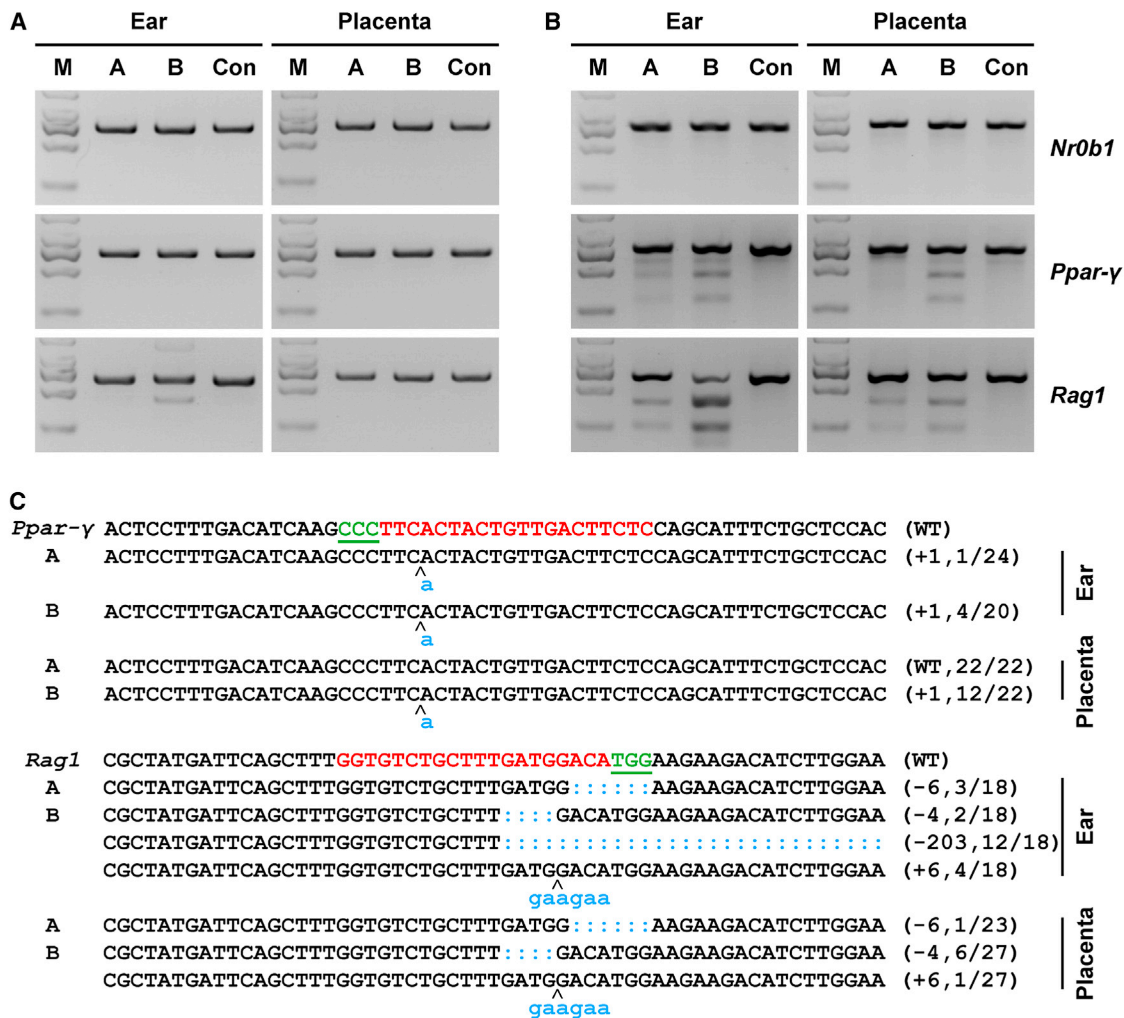


**Figure 4. sgRNA:Cas9-Mediated Modifications of *Nr0b1*, *Ppar-γ*, and *Rag1* in Ear and Placenta of Founders**

(A) PCR products of the targeted region of *Nr0b1*, *Ppar-γ*, and *Rag1* in founders. Target regions of *Nr0b1*, *Ppar-γ*, and *Rag1* loci were PCR amplified from the ear and placenta genomic DNA of A and B founders. M, DNA marker; Con, wild-type control.

(B) Detection of sgRNA1:Cas9-mediated on-target cleavage of *Nr0b1*, *Ppar-γ*, and *Rag1* by T7EN1 cleavage assay.

(C) DNA sequences of *Nr0b1*, *Ppar-γ*, and *Rag1* loci. The PCR products were analyzed by DNA sequencing. The PAM sequence 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 S3 and S4.

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*, *Pparg*, 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 rhesus genome (BGI CR_1.0/rheMac3), allowing for ungapped 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 at http://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 modification analysis, off-target assay, etc. Y.K., X.Z., W.S., W.L., A.P.X., C.S., H.W., T.L., T.T., X.P., F.W., and S.J. assisted in monkey work. J.W, L.W., J.Z., X.G., Y.B., B.H., G.D., and Z.Z. assisted in genome manipulation and analysis.

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