Neurosci Bull June 1, 2014, 30(3): 381–386. http://www.neurosci.cn
DOI: 10.1007/s12264-014-1434-8

·Report·

Generation of a monkey with *MECP2* mutations by TALEN-based gene targeting

Zhen Liu[1,#], Xue Zhou[1,#], Ying Zhu[1,#], Zhi-Fang Chen[1], Bin Yu[1,2], Yan Wang[1], Chen-Chen Zhang[1], Yan-Hong Nie[1], Xiao Sang[1,2], Yi-Jun Cai[1], Yue-Fang Zhang[1], Chen Zhang[3], Wen-Hao Zhou[2], Qiang Sun[1], Zilong Qiu[1]

[1]*Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*

[2]*Department of Neonatology, Children's Hospital of Fudan University, Shanghai 201102, China*

[3]*State Key Laboratory of Biomembrane and Membrane Biotechnology, School of Life Sciences; PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing 100871, China*

[#]These authors contributed equally to this work.

Corresponding authors: Zilong Qiu and Qiang Sun. E-mail: zqiu@ion.ac.cn, qsun@ion.ac.cn

ABSTRACT

Gene editing in model organisms has provided critical insights into brain development and diseases. Here, we report the generation of a cynomolgus monkey (*Macaca fascicularis*) carrying *MECP2* mutations using transcription activator-like effector nucleases (TALENs)-mediated gene targeting. After injecting TALENs mRNA into monkey zygotes achieved by *in vitro* fertilization and embryo transplantation into surrogate monkeys, we obtained one male newborn monkey with an *MECP2* deletion caused by frame-shifting mutation in various tissues. The monkey carrying the *MECP2* mutation failed to survive after birth, due to either the toxicity of TALENs or the critical requirement of *MECP2* for neural development. The level of MeCP2 protein was essentially depleted in the monkey's brain. This study demonstrates the feasibility of introducing genetic mutations in non-human primates by site-specific gene-editing methods.

Keywords: monkey; TALEN; MeCP2; Rett syndrome

INTRODUCTION

Model organisms with genetic manipulations have proven to be valuable tools for understanding brain development and diseases. Gene editing in non-human primates would be particularly valuable for studying neuropsychiatric diseases with symptoms that are difficult to identify in rodents and other model organisms[1]. Recently, site-specific gene-editing methods, such as the use of transcription activator-like effector nucleases (TALENs) and the clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated protein (Cas9) system, have been developed for gene editing in various organisms, including worms, fish, and rodents[2-7]. Two recent studies have successfully generated monkeys with genetic mutations *via* the CRISPR/Cas9 or TALEN methods[8, 9]

The gene methyl-CpG binding protein 2 (*MECP2*) encodes a methyl-DNA-binding protein, and mutations of this gene in humans lead to Rett syndrome, a severe neurodevelopmental disorder[10, 11]. Not only does MeCP2 play a critical role in regulating gene expression transcriptionally, it also controls nuclear microRNA processing and regulates gene expression *via* microRNAs[12-14]. Mice with *mecp2* deletion appear to have severe health issues and mimic human patients with Rett syndrome[15, 16]. However, whether the loss of *MECP2* in non-human primates yields similar outcomes is yet to be determined.

In this study, we used TALEN-based methods to introduce mutations in the *MECP2* gene and acquired

a male monkey carrying these mutations, but it failed to survive after birth. We found that this monkey carried *MECP2* mutations in various tissues and the MeCP2 protein level in the brain was significantly decreased compared to that of wild-type monkeys. This result suggests that loss of *MECP2* in primates may still allow the full development of the brain and provides a useful path through which we can understand the role of MeCP2 in primate brain development.

MATERIALS AND METHODS

Animal Ethics Statement

The use and care of animals complied with the guideline of the Biomedical Research Ethics Committee at the Shanghai Institutes for Biological Science, Chinese Academy of Sciences, which approved the application entitled "Reproductive physiology of cynomolgus monkeys and establishment transgenic monkeys" (#ER-SIBS-221106P).

Design and Validation of TALENs

A total of 10 TALENs were designed against exon 3 of the monkey *MECP2* gene by the Beijing Biocytogen Co. Ltd., using the TALEN GoldenGate system (Figs. S1 and S2). TALENs were transfected into COS7 cells and the PCR products, amplified by primers (F: gcctcattgagcattcattc, R: cctatagaggcaggagttgctc), were sequenced. Overlapped peaks from sequencing results were found and indicated that the TALENs were active. TALEN #52 was chosen for injection into monkey zygotes.

Production of TALEN mRNA

The TALEN plasmid was digested by the AflII restriction enzyme. The digested product was gel-purified and used as the template for *in vitro* transcription using the mMESSAGE mMACHINE T7 kit (#AM1344; Life Technologies, Shanghai, China). Both TALEN-52L and TALEN-52R mRNAs were purified by phenol-chloroform extraction and isopropanol precipitation.

Collection of Oocytes, Gene Delivery, and Embryo Construction

Oocytes were collected at laparoscopy by aspiration from follicles 2–8 mm in diameter, 32–36 h after stimulation with human chorionic gonadotropin[17, 18]. The collected oocytes were cultured in pre-equilibrated maturation medium[17]. Monkey semen was collected by penile electro-ejaculation. Oocytes arrested in metaphase II were selected for intracytoplasmic sperm injection (ICSI). For ICSI, a single sperm was immobilized and aspirated tail-first. A single oocyte was fixed by a holding pipette, and the injection pipette was pushed through the zona pellucida and subsequently through the oolemma to release the spermatozoon[17]. After ICSI, zygotes were cultured in pre-equilibrated hamster embryo culture medium 9 at 37°C under 5% CO_2 for 6–8 h, and then selected for injection of TALEN mRNA at 25 or 12.5 ng/μL.

Selection of Surrogate Females and Embryo Transfer

The menstrual cycles of females were recorded daily. To synchronize the developmental stage of embryos with the recipient, monkeys were chosen for tubal embryo transfer at 0–3 days after ovulation, and a stigma or a new corpus luteum on the ovary was observed at laparoscopy. One to six embryos at pronuclear stage were selected for tubal transfer to each surrogate female[18].

Genotyping PCR

DNA was extracted from small samples of various tissues collected from newborn monkeys. Samples were digested with proteinase K overnight at 65°C and precipitated for DNA and PCR with specific primers against monkey *MECP2* as follows: MK-Mecp2-F: CTTGTCAGCCATTGAGCCCAGAG and MK-Mecp2-R: AGCTTCCGTGTCCAGCCTTCA. PCR products were sequenced for *MECP2* mutations.

Immunohistochemistry of Transgenic Monkey Tissues

Stillborn *MECP2* mutant and wild-type (WT) monkeys were deeply anaesthetized with ketamine hydrochloride (5–10 mg/kg) and perfused with 0.9% saline. The brain was dissected, cut into small blocks, fixed with 4% paraformaldehyde in phosphate buffer, and equilibrated in 30% sucrose. Fixed and equilibrated tissue blocks were cut at 40 μm on a Microm HM525 cryostat. Sections were washed for 5 min in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) and 0.3% TritonX-100, and incubated with primary antibodies (in PBS with 3% BSA and 0.3% TritonX-100) overnight at 4°C and

subsequently with the corresponding secondary antibodies (Alexa Fluor-conjugated at 1:1 000; Invitrogen). DAPI was used to label the nuclei and sections were mounted with 75% glycerol. The other antibodies used were anti-NeuN (#MAB377; Millipore, Billerica, MA) and anti-MeCP2 (#3456S; Cell Signaling Technology, Danvers, MA).

Western Blotting

The brain tissues were homogenized in RIPA buffer (containing 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, protease inhibitor cocktail, and phosphatase inhibitor cocktail) on ice and then centrifuged at 1 000 g for 10 min at 4°C. The supernatant was stored at –80°C until use. Protein concentration was measured by the BCA method. Protein from each sample (30 μg) was loaded on 10% SDS-PAGE and run at 120 V. A current of 0.36 mA was used for transblotting. Blots were probed with primary antibodies (1:1 000–5 000) overnight at 4°C. After three washes, blots were incubated with goat anti-rabbit secondary antibody (1:5 000) at room temperature for 2 h. Chemiluminescence was used to visualize the protein bands. The antibodies used were anti-MeCP2 (#3456S; Cell Signaling) and anti-GAPDH (8245; Abcam, Cambridge, UK).

RESULTS AND DISCUSSION

To delete the *MECP2* gene from the monkey genome, we decided to target exon 3, which is one of two major coding exons in this gene (Fig. 1A). We synthesized 10 TALEN pairs and tested their efficiency by transfecting them into COS7 monkey cells (Fig. S1A). After sequencing the *MECP2* gene in the COS7 cells expressing various TALENs, we found that TALEN #52 successfully introduced genomic deletions (Fig. S1B). The complete targeting module of TALEN #52 is shown in Fig. S2 (see also Fig. 1A, B).

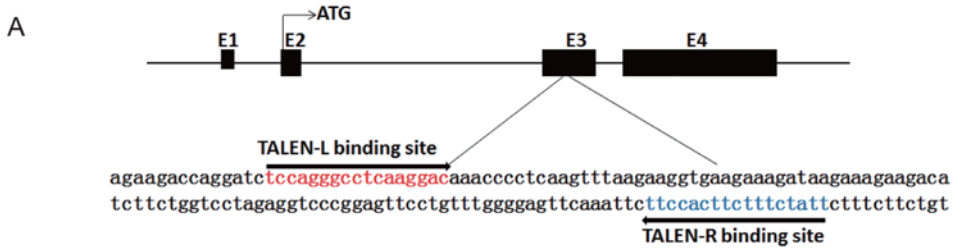
We then injected the mRNA of TALEN #52 (12.5 ng/μL) into 52 cynomolgus monkey zygotes obtained by intracytoplasmic sperm injection (Fig. 1C), and transferred 51 embryos into 9 surrogate female monkeys. Three surrogates became pregnant and gave birth to 5 offspring (A, B1-3, and C; Table 1); all but one were stillborn. The low pregnancy and survival rates may reflect the toxicity of TALEN mRNA.

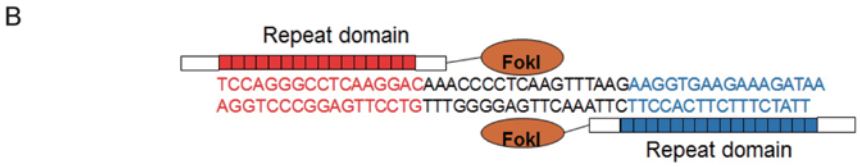
Further sequencing of the TALEN targeting site showed mutations in the *MECP2* gene in one male monkey (A, out of 5 monkeys): a 38-bp deletion together with a 4-bp insertion that resulted in frame-shifting of the *MECP2* gene (Fig. 1D). Monkey A (with the *MECP2* deletion) failed to survive after birth, due to either the toxicity of TALENs or the critical requirement of *MECP2* for embryonic development, as suggested by the lethality of male human fetuses with *MECP*2 mutations[10]. Mutations were found in all tissues of monkey A examined: heart, kidney, muscle, cerebral cortex, cerebellum, and liver (Fig. S3A–F). Furthermore, these mutations in the *MECP2* gene appeared to be mosaic within each tissue, showing overlapped signals of wild-type and mutated sequences (Fig. S3A–F). Therefore, we examined the mutation rate of the *MECP2* gene by sequencing genomic DNA from various tissues and found that the highest mutation rates, >30%, were in the cerebrum and liver (Fig. 1E). This mosaicism may be due to the delayed activity of TALENs in monkey zygotes, similar to that found in TALEN-mediated gene targeting in mouse and rat[2, 4].

To determine whether the level of MeCP2 protein was altered by the genomic deletion caused by TALENs, we performed Western blotting analysis using cortical tissues from monkeys A (with the *MECP2* deletion), B2, and B3 and found that the level was largely depleted only in the lysate from monkey A (Fig. 1F). Further immunohistochemical study of the MeCP2 expression in brain sections showed that MeCP2 staining was detectable in monkey B1 but not in monkey A (Fig. 1G). Thus, the level of MeCP2 protein was essentially depleted in the monkey with the *MECP2* mutation.

In summary, we have shown that the TALEN method can be used to generate monkeys with a specific gene deletion. The mosaicism we found in the monkey with *MECP2* deletion may reflect delayed targeting of TALENs during early embryonic development. Whether other methods such as CRISPR/Cas9 may yield higher efficiency of site-specific gene targeting for the *MECP2* gene in non-human primates remains to be determined.

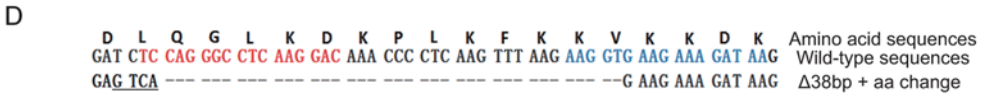
A recent study reported that investigators obtained a female cynomolgus monkey with *MECP2* mutations using the TALEN-based gene-editing method[8]. But male monkeys carrying *MECP2* mutations in the study failed to





C

Dose of TALEN-*MeCP2* mRNA (ng/μl)	Injected zygotes	Transferred embryos (Percentage of injected)	Surrogates	Pregnancy	Newborns (Percentage of transferred)	Mutant (Percentage of newborns)	Mutant monkey number
12.5	52	51 (98.1%)	9	3	5 (9.8%)	1 (20%)	#A



E

Tissues	Cerebrum	Cerebellum	Kidney	Liver	Heart	Umbilical cord
Mutation rate (%)	33.3	20	22.2	33.3	16.6	20
Sequencing clones (positive/total)	5/15	4/20	2/9	6/18	1/6	1/5

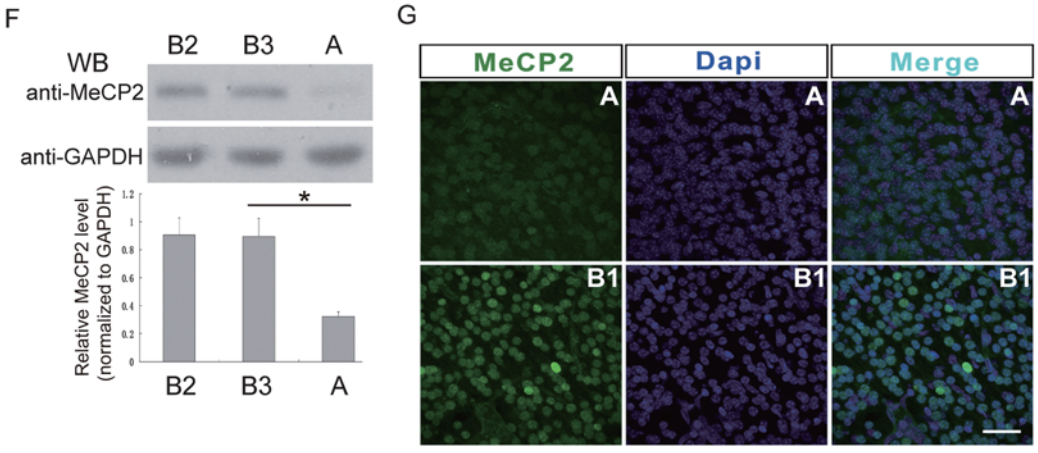


Fig. 1. Generation of cynomolgus monkey with *MECP2* deletion by TALENs. A: The TALEN-targeting region in the monkey *MECP2* genomic locus. B: TALEN-binding sequences (in red and blue) and the space region. C: TALEN-based *MECP2* gene-targeting in cynomolgus monkeys. D: Genomic DNA sequences of the *MECP2* locus from the wild-type monkey *versus* monkey A with *MECP2* deletion. Underlined sequence indicates new nucleotide substitution. E: Mutation analysis by sequencing the targeted genomic *MECP2* locus from various tissues of monkey A. F: Protein levels of MeCP2 from monkeys with (A) and without (B2 and B3) *MECP2* deletion. Lysates from postmortem cortical tissues were homogenized and analyzed by SDS-PAGE, using the indicated antibodies. The histograms below show quantitation of immunoblots. Error bars indicate SEM; **P* <0.05, *t* test. G: Immunostaining for MeCP2 and the nuclear marker DAPI in sections of brains from monkeys with (A) and without (B1) *MECP2* deletion. Scale bar, 30 μm.

Table 1. *MECP2* mutations in newborn monkeys

Surrogate monkeys #	Newborn	Gender	Conditions	*MECP2* mutations
189	A	Male	Stillbirth	Positive
12 (Triplets)	B1,2,3	B1:Female	Stillbirth	B1 Negative
		B2:Male		B2 Negative
		B3:Female		B3 Negative
42	C	Female	Deceased after birth due to mother-inflicted head trauma	Negative

reach term due to severe miscarriages. The results from our work suggest that male primates with significant loss of MeCP2 protein still form a grossly normal brain and survive until natural delivery. The in-depth analysis of monkey tissues lacking MeCP2 protein would yield valuable insights about its role in the neural development of primates.

SUPPLEMENTARY DATA

Supplemental data include three figures and can be found online at http://www.neurosci.cn/epData.asp?id=182.

ACKNOWLEDGEMENTS

We thank Mu-Ming Poo for comments on this manuscript. This work was supported by the National Basic Research Development Program of China (2011CBA00400 and 2011CB809102), the CAS Strategic Priority Research Program of China (XDB02050400), the National Key Technology R&D Program of China (2014BAI03B00), the CAS Hundreds of Talents Program of China (to Z.Q. and Q.S.), and the National Science Foundation of China (91232712).

Received date: 2014-03-16; Accepted date: 2014-03-20

REFERENCES

[1] Nestler EJ, Hyman SE. Animal models of neuropsychiatric disorders. Nat Neurosci 2010, 13: 1161–1169.

[2] Tesson L, Usal C, Menoret S, Leung E, Niles BJ, Remy S*, et al.* Knockout rats generated by embryo microinjection of TALENs. Nat Biotechnol 2011, 29: 695–696.

[3] Huang P, Xiao A, Zhou MG, Zhu ZY, Lin S, Zhang B. Heritable gene targeting in zebrafish using customized TALENs. Nat Biotechnol 2011, 29: 699–700.

[4] Sung YH, Baek IJ, Kim DH, Jeon J, Lee J, Lee K*, et al.* Knockout mice created by TALEN-mediated gene targeting. Nat Biotechnol 2013, 31: 23–24.

[5] Yang H, Wang HY, Shivalila CS, Cheng AW, Shi LY, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 2013, 154: 1370–1379.

[6] Li DL, Qiu ZW, Shao YJ, Chen YT, Guan YT, Liu MZ*, et al.* Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat Biotechnol 2013, 31: 681–683.

[7] Wang HY, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F*, et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 2013, 153: 910–918.

[8] Liu H, Chen Y, Niu Y, Zhang K, Kang Y, Ge W*, et al.* TALEN-mediated gene mutagenesis in rhesus and cynomolgus monkeys. Cell Stem Cell 2014, 14: 323–328.

[9] Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L*, et al*. Generation of gene-modified cynomolgus monkey via Cas9/RNA-Mediated gene targeting in one-cell embryos. Cell 2014, 156: 836–843.

[10] Nan XS, Campoy FJ, Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell 1997, 88: 471–481.

[11] Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nature Genetics 1999, 23: 185–188.

[12] Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC*, et al.* Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 2003, 302: 885–889.

[13] Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y*, et al.* DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science 2003, 302: 890–893.

[14] Cheng TL, Wang Z, Liao Q, Zhu Y, Zhou WH, Xu W*, et al.* MeCP2 suppresses nuclear microRNA processing and

dendritic growth by regulating the DGCR8/Drosha complex. Dev Cell 2014, 28: 547–560.

[15] Chen RZ, Akbarian S, Tudor M, Jaenisch R. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat Genetics 2001, 27: 327–331.

[16] Guy J, Hendrich B, Holmes M, Martin JE, Bird A. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genetics 2001, 27: 322–326.

[17] Chan AWS, Yang SH. Generation of transgenic monkeys with human inherited genetic disease. Methods 2009, 49: 78–84.

[18] Sun Q, Dong J, Yang WT, Jin YJ, Yang MY, Wang Y*, et al.* Efficient reproduction of cynomolgus monkey using pronuclear embryo transfer technique. Proc Natl Acad Sci U S A 2008, 105: 12956–12960.