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LETTER TO THE EDITOR

Gene Targeting Through Homologous Recombination in Monkey Embryonic Stem Cells Using CRISPR/Cas9 System

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EDITOR: Genome editing is a critical tool in both basic biomedical research and gene therapy. Compared with zinc finger nucleases and transcription activator-like effectors, the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system offers a more efficient and user-friendly design and utilizes DNA-RNA recognition instead of DNA-protein recognition, and it has been successfully applied in various species [1]. The nonhuman primate is ideal to model human diseases and evaluate the potential applications of embryonic stem cells (ESCs) in regenerative medicine. Therefore, it will be important to develop a technology to genetically manipulate monkey ESCs. A recent report has indicated that the CRISPR/Cas9 system has recently been used to generate the gene-modified cynomolgus monkey [2]. While this is an important proof-of-concept experiment, a genetically modified nonhuman primate is expensive to maintain and requires facility that is only available to a small number of laboratories. Instead, monkey ESCs can undergo unlimited self-renewal while maintaining the potential to give rise to all cell types and therefore an in vitro alternative of the monkey model. In this study, we demonstrated the feasibility to genetically modify the ESCs of rhesus monkey using the CRISPR/Cas9 system for cell tracing.

To test whether spCas9 can induce precise gene targeting by means of homologous recombination (HR) in rhesus monkey ESCs, we designed the experiment to employ spCas9 and a long DNA donor template to introduce the *Brainbow2.1* coding region into the *HPRT* locus, which is a housing keeping gene on X-chromosome [3] (Fig. 1A). We used the *Brainbow* gene that can be used for cell tracing [4]. In this context, the Cre-*loxP* recombination can be used to create a stochastic choice of XFP expression, resulting in the heritable marking of ESC-derived cells with multiple distinct colors. In the long DNA donor template (Fig. 1B), a *Brainbow2.1* expression cassette (CAG-*Brainbow2.1*-polyA) and a selection cassette (CAG-*neo*-IRES-*Puro*-polyA) were inserted into the two *HPRT* homologous arms around 1 kb (Fig. 1C). Four sgRNAs (Fig. 1D), which targeted the junction site of the two homologous arms, were designed according to the (N)20NGG rule (See Supplementary Data for sgRNA; Supplementary materials are available online at www.liebertpub.com/scd). It was expected to make double nicking of genomic DNA by paired sgRNAs (sgRNA3 and sgRNA4) and the mutant Cas9 (Cas9n) that confers a higher targeting specificity and is applicable in human ESCs and induced pluripotent stem cells [5,6]. After electroporation and puromycin selection, 80 monkey ESC clones survived by means of cotransfection of four single sgRNAs with wt Cas9 and paired sgRNAs (sgRNA3 and sgRNA4) with Cas9n. We used PCR screening to identify 22 positive clones (See Supplementary Data for Primer sequences) (Fig. 1E). To further confirm HR, we used Southern blotting with the *Avr*II digestion and hybridization to the upstream, downstream, and internal probes (See Supplementary Data for Probe sequences). As shown in Fig. 1F, 9 of the 22 clones had precise HR at both arms with no random integration. The knockin ESC lines (termed ormBB cells) exhibited typical ESC morphology (Fig. 1J-a). To determine the pluripotency of ormBB cells, we performed teratoma assays. When injected into severe combined immune deficiency (SCID) mice, both ormES22 (WT) and ormBB cells formed well-differentiated teratomas, and subsequent histological analysis identified representative cell types of all three germ layers (Fig. 1H). To test the expression of *Brainbow* fluorescent genes that can be used for single-cell labeling, the plasmid-encoding Cre recombinase was transiently electroporated into ormBB ESCs (electroporated cells were termed ormBB-Cre cells). Three days later, XFP signals were demonstrated by FACS and microscopy analyses (Fig. 1I, J-b).

To test whether ormBB cells could be applied to track cell lineage in vitro, ormBB-Cre cells were differentiated into the embryoid body followed by conditions for neural lineage differentiation [7]. XFP-expressing cells could be differentiated into typical neural rosette-like structures (Fig. 1J-c),

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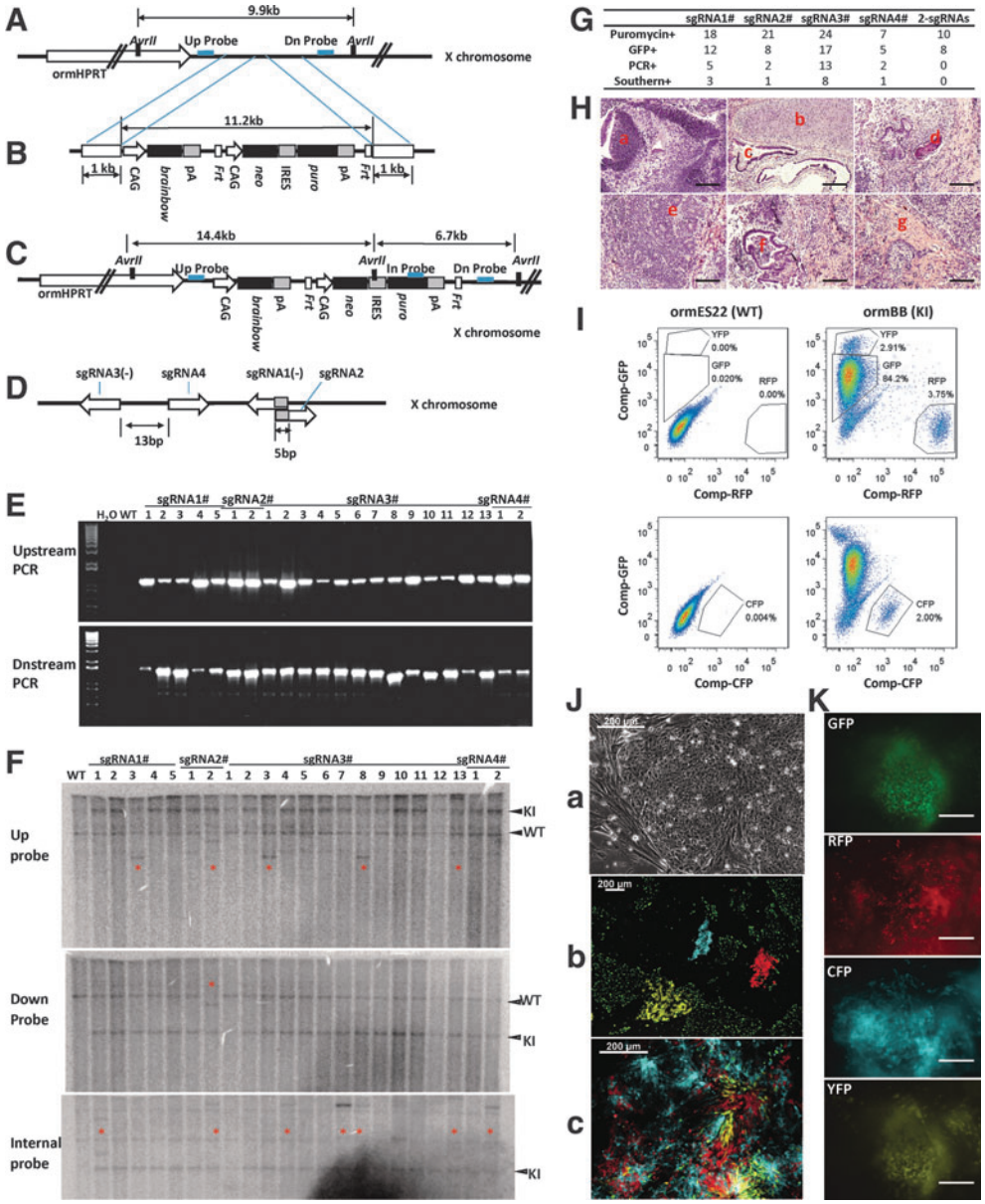

FIG. 1. *Brainbow* Knockin strategy in rhesus monkey embryonic stem cells through CRISPR/Cas9 and XFP expressions in ormBB-derived cells. **(A)** The endogenous rhesus monkey *HPRT* locus. The *arrow* indicates the *HPRT* gene. The locations of upstream and downstream probes for Southern blotting and the sizes of WT *Avr*II restriction fragments are shown. **(B)** The DNA donor template with *Brainbow* fragment and the *Frt* flanked selection cassette. The sizes and locations of homologous arms are indicated. **(C)** The configuration of the knockin allele. The location of internal probe for Southern blotting and the sizes of mutant *Avr*II restriction fragments are indicated. **(D)** The targeting sites of sgRNAs on X chromosome. The gap sizes between sgRNA3 and sgRNA4 or the overlapping sizes between sgRNA1 and sgRNA2 are shown. **(E)** Clones with recombined upstream arm or downstream arm are identified by PCR screening. Primers used to screen homologous recombination at both streams cover more than the primary homologous arm. **(F)** Southern blotting analysis of the PCR-positive clones. Genomic DNA was digested with *Avr*II and hybridized to the upstream, downstream, and internal probes sequentially with stripping between probes. The WT and KI bands are indicated. *, bands generated by random or partial integration. **(G)** Summary of *Brainbow* knockin clones. **(H)** *Brainbow* knockin ormESCs formed well-differentiated teratomas in severe combined immunodeficiency (SCID) mice. Cells of each of the three germ layers were identified in the teratomas by hematoxylin and eosin staining, including (a, e) ectoderm (neural rosette); (b, d, g) mesoderm (cartilage, bone, smooth muscle); (c, f) endoderm (GE, gut-like epithelium). Scale bar is 2,000 μm. **(I)** FACS analysis for XFP expression levels in *Brainbow* knockin ormESCs after Cre recombinase plasmid electroporation. Wild-type ormESCs were used as negative control. All XFP percentages are indicated. GFP/YFP share the same channel, but the MFI of YFP is much higher compared with GFP. **(J)** Cell morphology of ormBB cells and XFP expressions during differentiation. **(a)** ormBB cells, **(b)** ormBB-Cre cells, **(c)** typical neural rosettes differentiated from ormBB-Cre cells. Scale bars are indicated. **(K)** Microscopy analysis of XFP expression in the fresh teratomas derived from ormBB-Cre cells. All differentiated cells and fresh tissues were observed under the Olympus IX81S1-F3 microscope and analyzed by ImageJ. Scale bar is 200 μm.

indicating that ormBB-Cre ESCs could be used to individually labeled cells during differentiation. To test whether the cells derived from ormBB-Cre ESCs could be tracked in vivo, we took advantage of the capability of ESCs to form teratomas in SCID mice, which consisted of various cell types of the three germ layers. We harvested fresh teratomas formed by ormBB-Cre ESCs, which were cut into tiny pieces and examined under the immunofluorescence microscope. As shown in Fig. 1K, cells individually labeled by one of the four fluorescent colors could be found throughout the teratomas. Therefore, ormBB cells could be applied in individual cell tracing.

To our knowledge, our report represents the first description of the genomic editing of monkey ESCs by the CRISPR/Cas9 system. In the context of WT Cas9 system, the targeting efficiency is high (33% with sgRNA3, Fig. 1G) in rhesus monkey ESCs. However, in the context of the nickase Cas9n together with paired sgRNAs (sgRNA3 and sgRNA4), the targeting efficiency is very low in monkey ESCs, consistent with the notion that Cas9n confers a much lower targeting efficiency than WT Cas9. In addition, our findings also show a high frequency of random integrations, indicating that the CRISPR/Cas9 system could target nonspecific genomic sites.

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Author Disclosure Statement

No competing financial interests exist.

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