Available online at www.sciencedirect.com
SciVerse ScienceDirect
Journal of Genetics and Genomics 39 (2012) 275−280
JGG

ORIGINAL RESEARCH

Rapid and Cost-Effective Gene Targeting in Rat Embryonic Stem Cells by TALENs

Chang Tong, Guanyi Huang, Charles Ashton, Hongping Wu, Hexin Yan, Qi-Long Ying*

Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research at USC, Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA

Received 21 March 2012; revised 18 April 2012; accepted 23 April 2012
Available online 9 May 2012

ABSTRACT

The rat is the preferred animal model in many areas of biomedical research and drug development. Genetic manipulation in rats has lagged behind that in mice due to the lack of efficient gene targeting tools. Previously, we generated a knockout rat *via* conventional homologous recombination in rat embryonic stem (ES) cells. Here, we show that efficient gene targeting in rat ES cells can be achieved quickly through transcription activator-like effector nuclease (TALEN)-mediated DNA double-strand breaks. Using the Golden Gate cloning technique, we constructed a pair of TALEN targeting vectors for the gene of interest in 5 days. After gene transfection, the targeted rat ES cell colonies were isolated, screened, and confirmed by PCR without the need of drug selection. Our results suggest that TALEN-mediated gene targeting is a superior means of establishing genetically modified rat ES cell lines with high efficiency and short turnaround time.

KEYWORDS: TAL effector; *BMPR2*; Rat ES cells; Gene-targeting

1. INTRODUCTION

Embryonic stem (ES) cells were first isolated from mouse embryos three decades ago. ES cells can be maintained in culture indefinitely while retaining the capacity to generate nearly any type of cell in the body. The pluripotency of ES cells and the availability of gene-targeting technology have enabled the creation of mouse animal models with a variety of genetic modifications. These models have become important tools for understanding gene function and modeling human diseases (Austin et al., 2004). Although the rat is the preferred animal model in many human health-related research fields, mainly due to its bigger size, more-developed brain and other humanized features (Abbott, 2004), the unavailability of authentic rat ES cells had, until recently, prevented the application of gene-targeting technology in this species. Instead, several alternative methods had been developed to generate genetically modified rats without using ES cells. Those methods include *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (Zan et al., 2003), transposon-tagged mutagenesis (Kitada et al., 2007), and zinc finger nuclease (ZFN)-based pronuclear injection (Geurts et al., 2009). Unfortunately, all of these non-ES methods are expensive and/or difficult to apply.

By using small-molecule inhibitors to block mitogen activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3), we successfully derived ES cells from rat embryos in 2008 (Li et al., 2008). A gene knockout rat was generated 2 years later *via* conventional homologous recombination in rat ES cells (Tong et al., 2010). The application of conventional gene-targeting methodology in rat ES cells makes it possible for researchers to achieve any type of

* Corresponding author. Tel: +1 323 442 3308, fax: +1 323 442 4040.
E-mail address: qying@med.usc.edu (Q.-L. Ying).

doi:10.1016/j.jgg.2012.04.004

genetic modification in rats, as has been the case in mice for decades. However, the generation of knockout animals *via* the traditional method is a time-consuming and laborious process, and therefore, a more-efficient tool is preferred for generating knockout rats.

Recently, two independent groups reported that transcription activator-like (TAL) effector, a protein secreted by *Xanthomonas* bacteria, can bind to specific DNA sequences *via* repetitive amino acid residues in the central domain (Boch et al., 2009; Moscou and Bogdanove, 2009). It has been shown that the 12th and 13th amino acid residues in sequential repeats actually determine the DNA binding specificity and thereby the TAL effector's target site. The simple relationship between amino acids in the TAL effector and the DNA bases in its target provides the possibility of engineering TAL effector proteins with an affinity for a pre-determined DNA sequence. Fusion proteins carrying the DNA binding domain of the TAL effector and the DNA cleavage domain of restriction enzyme *Fok* I can create a double-strand break at a particular genomic site among a wide range of species, from yeast to humans (Li et al., 2011; Miller et al., 2011). These engineered TAL effector nucleases (TALENs) have been successfully applied to disrupt gene function in the rat through pronuclear injection (Tesson et al., 2011).

The assembly of the repeat variable di-residues (RVD) containing a highly conserved repetitive sequence in TALENs, however, is challenging for researchers using the regular cloning method, and chemical synthesis of the entire RVD region is relatively expensive. In 2009, a type IIs restriction enzyme-based DNA cloning method called Golden Gate Shuffling was reported (Engler et al., 2009). Golden Gate cloning allows a plasmid to be assembled from 10 separate input plasmids without the introduction of any mutation. This feature of the technique makes it possible to assemble more than 20 RVDs in just two rounds of cloning. The first successful assembly of pre-designed TALENs using the Golden Gate cloning method was recently performed to target a promoter sequence driving GFP expression in a transgenic plant (Weber et al., 2011). Here, we modified the Golden Gate cloning system and applied it to construct TALENs that can be used to generate gene-targeted rat ES cells with high efficiency.

2. MATERIALS AND METHODS

2.1. DNA cloning

Escherichia coli (*E. coli*) strains carrying each plasmid included in the Golden Gate cloning system for TALEN construction were kindly provided by Dr. Daniel F. Voytas. Plasmid DNA was extracted using a Mini-prep kit from Qiagen Inc. (USA). Concentrations of all plasmids were adjusted to 150 ng/mL with ddH_2O for Golden Gate cloning. All DNA cloning procedures were performed according to the protocols provided on the Addgene Website (http://www.addgene.org/TALeffector/goldengate/voytas/). To appropriately modify the backbone plasmid pTAL3 in the Golden Gate cloning system, pCAG-EGFP-ires-Pac was digested with *Bam*H I and *Eco*R I to remove the EGFP-ires-Pac cassette and was then ligated with the bGHpolyA fragment generated by PCR amplification to obtain plasmid pCAG-bGHpolyA. The fragment extending from restriction site *Bgl* II to *Afl* II in the pTAL3, including the selection marker gene LacZ and cleavage domain of *Fok* I, was subcloned into pCAG-bGHpolyA *via Afl* II and *Bam*H I to form pCAG-TAL3. Golden Gate TALNs assembly was carried out as previously described (Cermak et al., 2011).

2.2. Rat ES cell and hepatocellular carcinoma (HCC) cell culture

Rat ES cells were routinely cultured on γ-irradiated mouse embryonic fibroblasts (MEFs) in N2B27 medium supplemented with 3 μmol/L of CHIR99021 and 1 μmol/L of PD0325901 (2i medium). Rat ES cells were passaged every 2−3 days as described previously (Tong et al., 2011). The rat HCC cell line derived from diethylnitrosamine (DEN)-induced rat liver tumors was maintained in N2B27 medium supplemented with 10 ng/mL of EGF, 10 ng/mL of bFGF and 3 μmol/L of CHIR99021. Rat HCC cells were cultured on Matrigel-coated dishes and passaged every 3 days or until 70% confluent.

2.3. Amaxa nucleofection

Rat ES cells were treated with 0.025% trypsin for 5 min to obtain a single cell suspension. After neutralization of trypsin with 10% fetal bovine serum (FBS), 10^6 rat ES cells were washed with 10 mL of PBS twice. Nucleofection was performed according to the instructions in the Amaxa Mouse ES Cell Nucleofector Kit (Amaxa, VPH-1001, USA). Briefly, 90 μL of Nucleofector solution was mixed with 20 μL of supplement, and 1.5 μg each of the TALEN-expressing plasmid pair pCAG-TAL-F and pCAG-TAL-R was dissolved into 10 μL of the Nucleofector solution mixture. The cell pellet was resuspended with 100 μL of the mixture and then combined with the plasmid solution. Nucleofection program A-13 was applied to introduce pCAG-TAL-F and pCAG-TAL-R into the rat ES cells. After transfection, rat ES cells were seeded into four 100 mm dishes plated with MEF feeder cells and cultured in 10 mL of 2i medium. Single colonies emerged 5 days after nucleofection and were ready for picking up.

2.4. Genomic PCR screening in 96-well plates

Genomic DNA of the isolated rat ES cell colonies was prepared using ZR-96 quick gDNA kit (Zymo Research, USA, D3012) and eluted in 30 μL of ddH_2O. 5 μL of DNA solution was used to provide 50−200 ng of genomic DNA for the PCR template. Paq5000 DNA polymerase was used for amplifying the bone morphogenetic protein receptor 2 (*BMPR2*) target region with the primer pairs F: 5′-AGATGCCATACCCA GATGAGAC-3′ and R: 5′-AGGCTCTGCTGCATTGATTG-3′.