Production of mutant alleles in mouse macrophage and microglial cell lines by CRISPR/Cas9-mediated genome editing

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ABSTRACT

CRISPR-Cas9 technology represents a significant improvement of traditional genome editing tools, reaching a new level of targeting, efficiency, and easy usability, thereby relieving many steps needed for generating mouse knockout alleles via traditional mouse ES cell technology. But with the restrictions of animal use regulations and housing costs, reduction or replacement of animal research is in great need, and, therefore, designing fast and inexpensive protocols to produce desired genomic modifications directly in the specific cell lines are becoming highly desirable. In this study we performed genomic modifications of mouse RAW264.7 and IMG cell lines using synthetic sgRNAs and oligo DNA, as well as targeting vectors. We assessed the efficiency of using this method to generate homozygous knockouts and point mutations. We were successful in producing homozygous knockout mutations. However, we couldn't achieve the correct point mutations when we used sgRNAs and oligo DNAs in either cell lines. When targeting vectors carrying selection markers were electroporated into the cells, only heterozygous mutations were obtained. Functional assays confirmed that the knockout cell lines do not express the target RNA, and can be successfully used in downstream functional applications. These cell models are great tools to validate in vivo results, define cell-special function, and carryout in depth mechanistic studies.

METHODS

sgRNA , DNA donor and vector design. Positive control Rosa26 sgRNA and primers were obtained from Synthego: Rosa26 sgRNA: 5'ACTCCAGTCTTTCTAGAAGA3'; mouse Rosa26 positive control forward PCR primer: 5'GAGGCGGATCACAAGCAATA3' and reverse PCR primer: 5'GGGAGGGGAGTGTTGCAATA3'. Editing efficiency was analyzed by Synthego's Inference of CRISPR Edits (ICE).

The following guide RNAs to knock out mouse Ghsr were designed using Synthego's algorithm and synthesized as modified sgRNA: gRNA1: GUGGAACGCGACGCCCAGCG (targeting Ghsr aa7-8) and gRNA2: CGGCACUCGUUGGUGUCCCG (targeting Ghsr aa192-193)(Figure 2).

The sgRNA for Ghsr Ala203Glu was designed using proprietary algorithm by CRISPR Core Partnership Program by Sigma Millipore (Merck KGaA, Darmstadt, Germany). The matching single stranded DNA oligo donor (ssDNA) was also produced by Sigma Millipore:

5'CTTCTGCAGCGCGGGGCCCATCTTCGTGCTGGTGGGCGTGGAGCACGAGAACGGCACAGATCCCCGGGACACCAACGAGTGCCGCGCCACTGAGT*TCGAG*GTGCGCTCTGGGCTGCTCACCGTGATGGTATGGGTGTCGAGCGTCTTCTTCTTCCTGCCGGTCTTCTGCCTCACTGTGCT 3'

Design features: GCT>GAG codon conversion (Ala203Glu) and ACC>ACT synonymous substitution to avoid re-cutting after editing; CRISPR gRNA target sequence; Left Homology Right Homology

The targeting vector to create the same Ala203Glu mutation was designed and produced by VectorBuilder. A different guide RNA targeting intron region was designed for electroporation of the vector: gRNA3 GAGGCACCAUUAACAAUUAG (Figure 7).

Cas9 protein was obtained from Synthego or IDT.

Electroporations. Electroporation of RAW264.7 and IMG cell lines with ribonucleoproteins (RNPs) was performed using protocols from Synthego [2] and BTX [4] and optimized using BioRad Gene Pulser Xcell, BTXpress buffer (# 45-0802) and either exponential or square-wave conditions, respectively. Concentrations were as follows: Cas9 1-5 uM per site, sgRNA 1.25-6.25 uM per site, 5uM carrier oligo (IDT #10007805 or extra sgRNA), 5-20uM ssDNA oligo, and 20ng/uL linearized vector. Square-wave conditions (420V, 8 pulses, 0.1ms pulse length, 0.5s interval) were eventually used for electroporating vector into both cell lines.

Isolation of mutant clones. Limiting dilution, where cells from each edited, pooled population are diluted to 0.5-1 cells per 100 µl and plated on 96-well plates, was used to isolate single cells for expansion. Both cell lines electroporated with donor plasmid and corresponding sgRNA went through the same limiting dilution clonal but with addition of G418 at 0.2 or 0.8 mg/ml as described [3].

Genotyping. Primers were designed to amplify the regions encompassing the edited region: Ghsr-F 5'CTCCTCAGGGGACCAGATTT3' and Ghsr-R 5'GAGCACAGTGAGGCAGAAGA3', expected bands were 740 bp (wt) or 150-700 bp (CRISPR del) (Figure 2). PCR was performed using LongAmp™ Taq Master Mix (New England Biolabs) under standard PCR conditions. The PCR products were then purified with a PCR Clean-Up System kit according to the manufacturer's instructions. Sanger sequencing was performed in Texas A&M Sequencing Core facility. The entire regions encompassing the guide cleavage sites were amplified to assess for genetic modifications.

A specific pair of primers was used to detect Ala203Glu mutation: Rm 5'CAGAGCGCACCTCGAACTCA3' and F3 5'GCCAAGGTGGTGGTCACCAA3' in both types of electroporations (ssDNA or plasmid) (Figure 7). Correct targeting was also confirmed by long distance PCR using genomic primers outside arms of homology.

RT-PCR and Western blotting Expression of Ghsr was induced in both WT and mutant RAW264.7 cell lines by addition of Lipopolysaccharide (LPS). Cell seeded at 1-1.5 million cells/ wells of 6-well plate and incubated in presence of LPS (1 or10 ng/mL) for 4 hrs after which cells were harvested. RT-PCR and Western blotting were done following standard procedures. Two-step SYBR real-time RT-PCR was performed with 80 ng of RNA for both target gene and endogenous control using ABI StepOnePlus™ (Applied Biosystems, CA) instrument. ABI High-capacity cDNA RT kit and Power SYBR Green PCR Master Mix were used. CT (cycle threshold) values were analyzed using the comparative CT(ΔΔCT) method as described by the manufacturer (Applied Biosystems, CA). The amount of target(2−ΔΔCT) was obtained by normalization to an endogenous reference standard (Cyclophilin) and relative to a wild-type cell sample. The following primers were used: Cyclophilin F 5'CTAAAGCATACAGGTCCTGGCATCTTG3' and -R 5'TGCCATCCAGCCATTCAGTCTTG3'; Ghsr1-F1 5'ACAGATCCCCGGGACACCAA3' and -R1 5'GCTCGACACCCATACCATCA3' (Figure 2). Western blotting was performed using the following antibodies:P62 antibody (Cell Signaling cat no. 5114S), B-actin antibody (Cell Signaling cat # 4976) and Phospho-IκBα antibody (Cell Signaling cat # 2859S)

RESULTS

Figure 1: ICE analysis on Rosa26 pooled samples A) RAW264.7 cells B) IMG cells C) Chromatogram sample showing editing

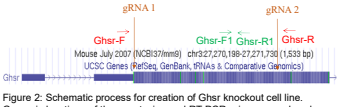

Figure 2: Schematic process for creation of Ghsr knockout cell line. Genomic locations of the genotyping and RT-PCR primers are also shown.

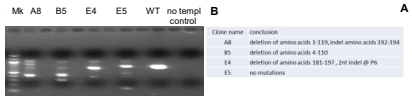

Figure 3: RAW264.7, genotyping confirmation A) gel B) conclusion based on the sequencing results

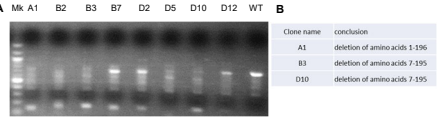

Figure 4: IMG, genotyping confirmation A) gel B) conclusion based on the sequencing results

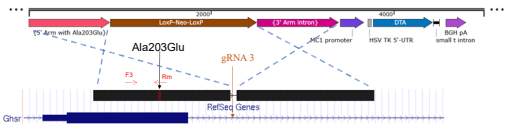

Figure 7: Schematic process for creation of Ghsr Ala203Glu cell Line using targeting vector

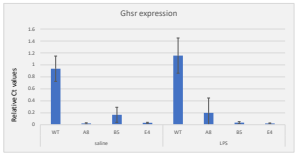

Figure 5: Ghsr gene expression in RAW264.7 clones by Real-time PCR. Ct values were normalized to Cyclo signal and are shown relative to wild-type sample. mRNA levels were analyzed in two sets of samples, with LPS treatment or without (saline).

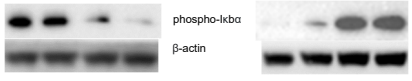

Figure 6: RAW264.7 Cell Line LPS experiments, Western blot analysis of expression of affected genes in relevant pathways in different KO clones

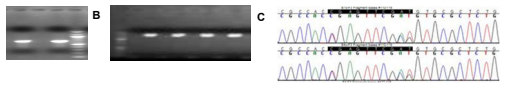

Figure 8: Ala203Glu mutation , genotyping confirmation A) RAW264.7 B) IMG C) Sequence confirmation of the correct mutation

To find optimal electroporation conditions we used Synthego's positive control sgRNA which is confirmed to make an indel mutation in Rosa26, and primers. ICE analysis showed that electroporation at 90V was the optimal condition for RAW264.7 and 280V for IMG cells respectively (Figure 1). We used these conditions to perform electroporation of the Ghsr RNP complexes as described [1]. After electroporations and subsequent dilutional clonals, several hundreds of clones were obtained for each cell line. 96 RAW264.7 clones were screened by PCR and 4 were identified (Figure 3), expanded and confirmed to carry homozygous mutations (4.2% homozygous targeting rate). 192 IMG clones were screened and 15 were identified as possibly carrying mutations (7.8% targeting rate); 8 of them were expanded and 3 confirmed to carry homozygous mutations (1.6% homozygous targeting rate) (Figure 4). All three RAW264.7 mutant clones were analyzed for expression of the target gene using RT-PCR. The results show that all clones exhibited significant reduction of the Ghsr mRNA (81-98%) (Figure 5). Downstream applications showed that RAW264.7 mutant clones exhibit up- or downregulation of gene expression in relevant autophagy and NFkB inflammatory pathway (Figure (6).

After production of the mutant clones was optimized for RAW264.7 and IMG cell lines, the protocol was tried to introduce point mutation Ala203Glu. Several initial electroporations were screened for the presence of the mutation in pooled samples first and one of the samples showed positive result (data not shown). A total of 200 clones were expanded from that electroporation and screened, but none carried the correct mutation. More electroporation rounds were performed using the same or similar conditions; both pooled samples and individual clones were screened, but no positive mutations were found.

The same Ala203Glu mutation designed for DNA oligo above was incorporated into 5' arm of the targeting vector (Figure 7) that also contained Neo and DTA to enrich cell populations for the correct targeting events. After the RAW264.7 Cell Line was electroporated, we found that individual colonies were hard to pick so we performed the same dilutional cloning under selection. Performing cell culture, we also determined that concentration of G418 at 0.2 mg/ml was optimal for selection. A total of 406 colonies were screened and further checked, 22 colonies were identified positive (5.4% targeting rate). Correct mutation was confirmed by sequencing, but no homozygous mutation was found (Figure 8). The same electroporation was performed with the IMG cell line, and a total of 152 colonies were screened of which 30 were positive (20% targeting rate) (Figure 8). Correct mutation was confirmed by sequencing, but as with RAW264.7 no homozygous mutation was found.

CONCLUSIONS

In this study we performed genomic modifications of mouse RAW264.7 and IMG cell lines using synthetic sgRNAs and evaluated the efficiency of using this method to generate homozygous knockouts and point mutations. We were successful in producing homozygous knockout mutations; at the same time, we couldn't obtain the correct point mutations when we used sgRNAs and oligo DNAs in either cell lines. Even though we obtained promising results with one pooled sample during electroporation optimization, we could not isolate a single clone with the correct mutation. The HDR-based mutations using oligo DNA and sgRNAs are very hard to achieve due to low efficiency as was confirmed by Synthego's technical team. When targeting vectors carrying selection markers were electroporated into the cells, only heterozygous mutations were obtained.

REFERENCES

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