Original Article

Efficient of Toll-Like Receptor 4 Knockout in Mouse Zygotes by CRISPER/Cas9

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Abstract

Background: Transgenic animals are genetically modified animals to create a specific trait that imitates an indication of pathogenesis in humans. Toll-like receptors (TLRs) are implicated in immune regulation of the female reproductive tract and, subsequently, infertility rate. This study produced Toll-like receptor 4 (Tlr4) knockout blastocysts with single-guide RNA targeting for Tlr4 by CRISPER/Cas9 technique.

Materials and Methods: Web CRISPER design tools designed single-guide RNAs (sgRNAs) targeting *Tlr4* gene were designed by web CRISPER design tools. Then, two strands of sgRNAs were cloned into a linearized vector for producing a gRNA-expressing eCAS9-GFP vector. The vector was then injected into the male pronucleus in the fertilized oocytes in vitro fertilization (IVF) and do polymerase chain reaction (PCR) and sequencing.

Results: Gene deletion with acceptable efficiency (38%, $p<0.05$) successfully was confirmed by sequencing and PCR analysis.

Conclusion: Our result showed that the CRISPER/Cas9 technique is an effective knockout method in mouse zygotes, potentially producing disease animal models.

Keywords: Transgenic, sgRNAs, CRISPER/Cas9, Toll-like receptors, Knockout

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Introduction

To understand the role and function of each gene, its interaction with other genes, and determine how impaired expression or lack of that gene affects a particular disease, the production of modified alleles in laboratory animals such as rodents is a strong tool[1, 2]. First time more than thirty years ago, created knock-in and knockout allele mice were appointed[3]. The production of transgenic animals and their importance and application in research centers has increased the attention of transgenic animals in medical, pharmaceutical, biotechnological, and immunological centers[4]. The Transgenesis process is created by the insertion of a particular human DNA into cells[5,6,7]. Many methods have been utilized for genome targeting technology, but CRISPER/Cas9 has rapidly grown because it is a simple, cheap, and efficient tool for gene editing. Crisper applies a protein called Cas9; Cas9 is an endonuclease that binds to guide RNA and finds the DNA sequence in host̕ s genome that is complementary with the guide RNA and then cleaves it[8, 9]. Adjusting the immune system of the female reproductive tract due to the unique requirements has made it responsive to the entry of invading pathogens or normal flora and semi-allogeneic fetus[10, 11]. The innate immune system that can detect self-molecules from non-self is the body´s first line of immune defense.

One of the most basic innate immune receptors that detect pathogen-associated molecular patterns (PAMPs) is Toll-like receptors. TLRs are classified according to their cell location and the type of PAMPs ligands (TLRs)[12, 13]. TLRs are implicated in immune regulation of the female reproductive tract and, subsequently, infertility rate[14]. Especially, TLR4 distinguishes bacterial lipopolysaccharides (LPS), short-strand RNA, and un-methylated cytosine-phosphate-guanine dinucleotide (CpG) DNA and then recruits MyD88 and TRIF dependent pathways. Activation of these pathways results in inflammatory cytokines such as TNF, IL-1, IL-6, MCP-1, IL-8, and type 1 interferon[15-18]. Studies were demonstrated that TLR4 is expressed in trimester trophoblast cells in placenta tissues and its activation in these cells promotes the production of cytokines. Although the *Tlr4* expression in the embryonic membrane (such as chorionic and amnion) is not as placenta, its expression level increases during parturition and chorioamnionitis. *Tlr4* expression has been reported to increase in pregnant myometrium and especially during parturition, and its function can be suppressed by progesterone[14]. Studies have shown that *Tlr4* expression increased in response to LPS and PG and stimulation of decidual cells *Tlr4* expression has higher than trophoblasts, suggesting decidual cells are the primary targets for bacterial infection[15]. Activating MyD88 and TRIF dependent pathways by TLR4 in cumulus cells of ovulated cumulus oocyte complexes (COCs), which produce chemokine and cytokine, is involved in sperm capacitation and subsequent fertilization rate[19, 20]. In addition, hyaluronidase fragments can stimulate TLR4. Hyaluronidase fragments can impact immune cell responses through NF-kB pathway activation in cumulus cells and then cause inducing sperm capacitation during IVF procedures[19]. We can identify the important role of different genes, pathological mechanisms of disease and discovery of new therapeutic approaches for human complications through gene disruption using CRISPR-Cas9 technique[8, 9]. Here, we use CRISPER/Cas9-mediated genome editing, creating *Tlr4* deficient blastocysts to suppress TLR4 activity.

Methods

All the materials used in this research were purchased from Sigma Company, except for those mentioned separately. B6D2F1 (C57BL/6×DBA/2) female and male mice were obtained from Royan Institute (Tehran, Iran). The code of ethics approved for this research by the Research and Ethics Committee of the Shahid Beheshti University of Medical Sciences is 5540.

Designing and ordering guide RNA for CRISPER/Cas9 genome editing and cloning of sgRNA into eCAS9-GFP vector

Web CRISPER design tools designed single-guide RNAs (sgRNAs) targeting Tlr4 gene was designed by web CRISPER design tools (Table 1). To increase the efficiency of RNA polymerase for transcription, add guanine nucleotide to 5́ end of them if the first nucleotide was no guanine. The linear eCAS9-GFP vector was purified using the gel extraction kit (Qiagen, Hilden, Germany). A sense and antisense strands of each designed sgRNA were annealed and then cloned into linearized vector for producing gRNA-expressing eCAS9-GFP vector. Next, the CaCl2 transformation method was used to transform the recombinant eCAS9-GFP vector into competent cells.

In vitro fertilization (IVF) procedure

Sperm and oocyte preparation

Preparation of spermatozoa for IVF was started by isolation of sperm from caudal epididymis in male mice

Table 1: Designed sgRNAs for Tlr4 gene.

gRNA	sequence	length
gRNA1	F 5' CACCGTAATATTACCTACCAATGCA	25n
	R 5' AAACTGCATTGGTAGGTAATATTAC	
gRNA2	F 5' CACCGATGCATTGGTAGGTAATATT	25n
	R 5' AAACAATATTACCTACCAATGCATC	
gRNA3	F 5' CACCGTTTCTGATCCATGCATTGGT	25n
	R 5' AAACACCAATGCATGGATCAGAAAC	

Table 2: Comparison of the developmental competence rate in the control and pronuclear injection (PNI) (test group) groups.

Groups	Oocyte NO	Fertilization% (mean±SD)	Four cells% (mean±SD)	Compact% (mean±SD)	Blastocyst% (mean±SD)	GFP Positive% (mean±SD)	Tlr4-K0% (Knock-out) (mean±SD)
control	10	90±1.98	89±0.78	85±1.3	81±0.67	___	___
PNI	37	76±1.43	67±0.84	54±0.96	49±2.34	45±1.34	38±0.45

KO: Knock-out. *Significantly difference $P<0.05$

(1-0-12 weeks old), and sperm suspension was then placed in drops containing human tubal fluid medium (HTF) supplemented with 4 mg/mL bovine serum albumin (BSA). The prepared sperm were capacitated in the incubator (5% CO_2, 37℃) for 45 min. In order to isolate a large number of oocytes from female mice (6-8 weeks old), we have superovulated them with 10 IU pregnant mare serum gonadotropin (PMSG) using intraperitoneal (IP) injection and 48 h later with 10 IU human chorionic gonadotropin (HCG) injection. After 14 of HCG injections in superovulated mice, cumulus-oocyte complexes (COCs) were derived from the oviduct ampulla and transferred into HTF medium (contained 4% BSA)[21].

IVF

COCs were inseminated with approximately 10^6 sperm/mL in drops of HTF medium and the mentioned above condition were co-incubated for six h. Next, fertilized oocytes (two pronuclear zygotes) were incorporated in a potassium simplex optimized medium (KSOM) that contained amino acids and 4% BSA. Embryonic development after fertilization until blastocyst formation was followed[21].

Mouse zygotes transformation by pronuclear

Table 3: PCR primer for all guide RNAs.

Primer	Sequence	Tm	Length
F- Seq-TLR4:	GGGAACACACGGTTGGAAAC	59℃	20n
R- Seq-TLR4:	GCCCATCCAACTGAAACCAG	59℃	20n

microinjection

About six h after insemination, both pronucleus is quite clear in the fertilized oocytes. The male pronucleus is usually applied for microinjection because it is both larger and better positioned than the female pronucleus. Each pronucleus, approximately 12 pI of DNA solution (with a concentration of 2 ng DNA µl-1) was injected during the microinjection. 96 h after microinjection, formed blastocysts were collected, and PCR was done using specific primers against the target sites and sent to the sequencing for final approval[22].

Statistical analysis

All statistical analyses were done using SPSS version 20 software (IMB, Chicago, IL, USA). The data have been presented as means ± SD with a significance of P<0.05.

Results

Establishment of TLR4 embryo mice by CRISPER/Cas9 microinjection

To create a TLR4-embryo mice model, we designed three gRNAs targetings in exons (exons 1, 2, and 3) of the *Tlr4* gene. Cloning of gRNA targeting was confirmed by colony PCR with specific primer for gRNA and PCR product of approximately 250bp confirmed the successful cloning of gRNA targeting in the eCAS9-GFP vector (Fig. 1). (F: ACTTCATTCAAGACCAAGCCTTTC; R: GATACACCTGCCAGAGACATTGC)

Nuclear transfer to create TLR4-modified embryo mice model

gRNA-expressing eCAS9-GFP vectors were delivered to fertilized oocytes by microinjection. Table 2 demonstrates the developmental competence rate in the control and test groups in the IVF, which the blastocyst rate in the control group was 81%, whereas it was 49% for the test group (injected group) (P<0.05). Of these blastocysts in the test group, 45% and 38% were GFP-positive and knockout in the *Tlr4* gene, respectively (Fig. 2). *Tlr4* gene mutation in zygotes mice were screened by PCR with the specific primer for the three exons (Table 3) and results were presented in the Figure 3 and approved treated samples using PCR. Data of confirmation by sequencing was not shown. Our results were shown that bands approximately 400bp in control groups and non-band in test groups confirmed that *Tlr4* gene have knockout in the injected blastocysts (Fig. 3).

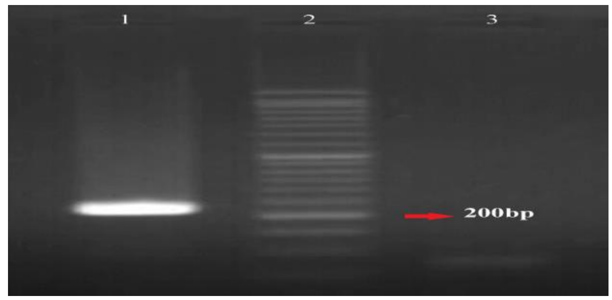

Figure 1. Confirmation of gRNA cloning into the eCAS9-GFP vector. (A) Colony polymerase chain reaction (PCR): Lane 1 is positive clone with approximately 250bp, a lane 2 is the 50bp DNA ladder and Lane3 is negative clone.

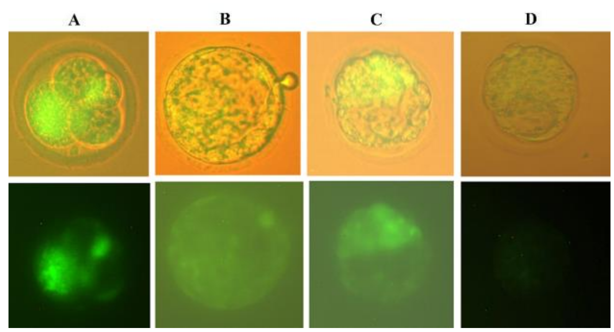

Figure 2. Microscopy image of zygotes in control and pronuclear injection (PNI) groups. A) Fluorescence microscopy image of the compact cell (up), light microscopy image of the compact (down) in the PNI group. B and C) Fluorescence microscopy images of the blastocyst (up), light microscopy images of the blastocyst (down) in the PNI group. D) Fluorescence microscopy image of the blastocyst (up), light microscopy image of the blastocyst (down) in the control group.

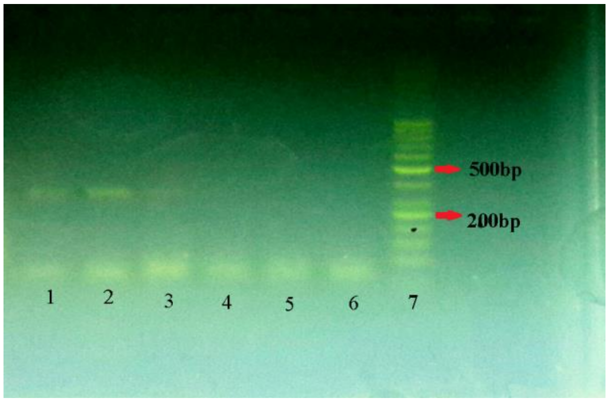

Figure 3. PCR confirmation of knockout *Tlr4* gene in the GFP positive blastocysts. Lanes 1, 2 and 3 are control groups with different DNA concentration (respectively 1, 0.5 and 0.01µg/µl DNA) with fragment size of 400bp (Note that the band in row 3 is too weak). Lanes 4, 5 and 6 are blastocysts containing gRNAs 1, 2 and 3, respectively that knockout the *Tlr4* gene. Lane 7 is 50bp DNA ladder.

Discussion

The CREISPER/Cas9 technique has been used as one of the powerful genomes editing tools for generating knockout and knock-in mice by zygote microinjecting[8]. Researches have shown that the expression of *TLR4* changes during mensuration under the influence of sex hormones such as progesterone and estrogen. It states that the expression level of *TLR4* is affected by hormonal changes and the immune system challenges in pregnancy[16,20]. We designed three gRNAs targeting's *TLR4* gene, and these gRNAs demonstrated have a good mutation efficiency in the mice zygotes (38%). The use of the Cas9/gRNA targeting for the genes in goat primary fibroblasts demonstrated a high efficiency mutation[23]. Researchers were applied CRISPER/Cas9 system for generated Plac8 deficient models in mice[24]. In the three different CRISPER/Cas9 genome editing strategies in zebra fish have been demonstrated, that efficiency of gene modification and editing are ~24-60%[25], ~35%[26], and 75-90%[27], respectively. Gene knockout in zygotes by CRISPER/Cas9 and microinjection has some advantages over conditional mutagenesis by embryonic stem cells (ES), in which the most important advantage is easier and less time-consuming of this method. Producing transgenic animals using a direct genetic modification of the zygote genome is important because it prevents causing mosaic or hypomorphic mutation in the transgenic animals and requires more birth to obtain the desired animals (homozygous)[28, 29].

Conclusion

Our finding indicates that gene knockout using the CRISPER/Cas9 system is helpful for gene editing and distinguishing the different functional roles of genes.

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