THE EFFICACY OF CRISPR-CAS9-MEDIATED INDUCTION OF THE CCR5DELTA32 MUTATION IN THE HUMAN EMBRYO

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The editing of the CCR5 gene in the CD4+ T cell genome is an effective way of preventing HIV-1 proliferation. Very similar strategies can be used to protect the fetus of an HIV-infected female showing a weak response to antiretroviral therapy. Inducing the "natural" CCR5delta32 mutation in a zygote may guard the fetus against HIV infection both in utero and at birth. In this study, we optimize the CRISPR-Cas9 system to induce a homozygous 32-nt deletion similar to the naturally occurring CCR5delta32 allele in the human zygote at the S-phase. Edits were done in the abnormal tripronuclear zygotes unsuitable for IVF. Sixteen tripronuclear zygotes in the S-phase obtained from WT CCR5 donors were injected with an original CRISPR-Cas9 system designed by the authors. Upon injection, the zygotes were transferred into the Blastocyst (COOK) embryo culture medium and cultured for 5 days in a CO_2 incubator until blastocysts were formed (approximately 250 cells). Eight zygotes that successfully developed into blastocysts were PCR-genotyped to analyze the efficacy of genome editing. Of 16 zygotes injected with CRISPR-Cas9, only 8 reached the blastocyst stage. PCR genotyping revealed the absence of the initial WT CCR5 variant in 5 of 8 blastocysts (100% CCR5delta32 homozygous). Two had about 3% and one about 20% of WT CCR5 mosaicism. This leads us to conclude that the efficacy of the proposed CRISPR-Cas9 system for the induction of the CCR5delta32 mutation in human embryos is very high producing more than 50% of completely modified embryos.

Keywords: CRISPR-Cas9, genome editing, human embryo, CCR5, CCR5delta32, HIV resistance

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ЭФФЕКТИВНОСТЬ СОЗДАНИЯ ДЕЛЕЦИИ CCR5DELTA32 МЕТОДОМ CRISPR-CAS9 В ЭМБРИОНАХ ЧЕЛОВЕКА

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Изменение гена CCR5 путем редактирования генома CD4+-Т-клеток является одним из способов предотвращения распространения ВИЧ-1-инфекции. Однако похожая стратегия защиты от ВИЧ может быть использована и для защиты плода ВИЧ-инфицированных женщин со слабым ответом на антиретровирусную терапию. Создание «естественного» аллеля CCR5delta32 на стадии зиготы может защитить плод от ВИЧ-инфекции во время внутриутробного развития и родов. Целью данного исследования была оптимизация системы CRISPR-Cas9 под создание гомозиготной 32-нуклеотидной делеции (аналогичной природному варианту CCR5delta32) в S-фазе зиготы человека. Для редактирования генома были использованы зиготы с аномальным числом пронуклеусов (более двух), непригодные для ЭКО. 16 аномальных зигот от доноров с WT CCR5 были инъецированы разработанной системой CRISPR-Cas9 в S-фазе. После инъекции зиготы помещали в культуральную среду Blastocyst (COOK) и культивировали в течение 5 дней в CO_2-инкубаторе до стадии бластоцисты (приблизительно 250 клеток). Для анализа эффективности редактирования генома 8 успешно развивавшихся эмбрионов были генотипированы методом полимеразной цепной реакции (ПЦР). Из 16 зигот, инъецированных системой CRISPR-Cas9, лишь 8 достигли стадии бластоцисты. ПЦР-генотипирование показало отсутствие исходного варианта WT CCR5 в 5 из 8 бластоцист (100% гомозиготы по CCR5delta32). Два эмбриона продемонстрировали около 3% и один — около 20% мозаицизма по WT CCR5. Таким образом, эффективность разработанной CRISPR-Cas9 системы для создания аллеля CCR5delta32 в эмбрионах человека довольно высока: более половины эмбрионов оказываются полностью модифицированными.

Ключевые слова: CRISPR-Cas9, редактирование генома, эмбрион человека, CCR5, CCR5delta32, устойчивость к ВИЧ

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In the past few years the rapid evolution of CRISPR-based technologies has expanded their application scope and paved their way to preclinical trails. The successful editing of the CD4+ T cell genome by either knocking out or modifying the gene encoding the chemokine receptor CCR5 has raised new hope for the true functional cure of HIV-1 infection [1–5].

Apart from the edits in the CCR5-encoding gene of T cells that block the development of AIDS in HIV-infected patients, genome editing techniques can be used to induce the CCR5delta32 mutation in the egg as part of *in vitro* fertilization (IVF) procedures to protect the fetus of an HIV-infected female showing a weak response to antiretroviral therapy [6, 7].

Injecting CRISPR-Cas9 components into the zygote will entail genome modifications in almost all cells of the organism, which has already been demonstrated for a few deleterious hereditary mutations [8–12]. Importantly, the edited genome will be passed on to subsequent generations.

A modification identical to the naturally occurring mutant allele CCR5delta32 can be expected to protect the fetus from HIV infection in utero and at childbirth. Another beneficial effect of this edit is a potential lifelong immunity to HIV.

In this study we optimize the CRISPR-Cas9 system to induce a homozygous 32-nt deletion similar to the mutant CCR5delta32 allele in the human zygote during the S-phase. Editing was performed on the abnormal tripronuclear zygotes unsuitable for IVF.

METHODS

Ethical approval and consent to participate

The study protocol was reviewed and approved by the Ethics Committee of Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (Approval Reference: No.2017/45). The study complied with the international guidelines for human embryo research. Written informed consent was obtained from each couple before they could donate tripronuclear zygotes. Only homozygous wild-type CCR5 pairs were included in the study.

Zygote collection procedures

Tripronuclear zygotes were donated by patients undergoing IVF treatment from September 2017 through April 2018 at Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (Moscow, Russia). In total, 21 tripronuclear zygotes were obtained from 11 couples, of which 16 were injected with CRISPR-Cas9 and 5 were used as a control.

Design, synthesis and *in vitro* activity of gRNAs

Guide RNAs (gRNAs) were designed to match the target locus of wild-type (WT) CCR5 and CCR5delta32 alleles from the National Center for Biotechnology Information database (USA) were used to design guide RNAs (gRNAs). A 200 bp-long DNA sequence was picked for further editing in which the sites for base pairing between gRNA and target DNA were selected adjacent to the PAM sequence (Fig. 1). In total, 9 gRNAs were designed to target the sites convenient for the subsequent homologous repair of double-stranded breaks (Table 1).

The transcription template was generated by pairwise annealing of primers (Evrogen; Russia) and PCR-amplified by Taq polymerase (Evrogen; Russia). Guide RNA was synthesized from the template using T7 RNA polymerase (SybEnzyme; Russia).

The activity of the resulting gRNAs was studied using a test plasmid coding for the wild-type CCR5 sequence. *In vitro*

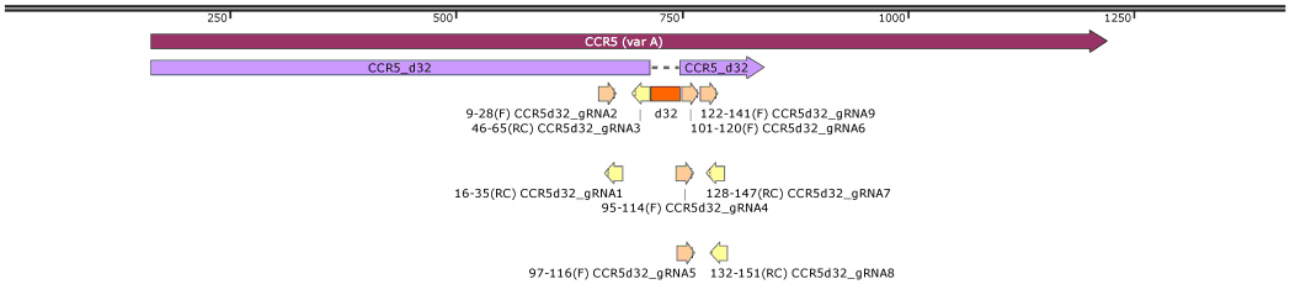

Fig. 1. gRNAs layout inside the human CCR5 gene

Table 1. Oligos for gRNA and DNA patches

CCR5_BamHI_F	GGATCCTAGGTACCTGGCTGTCGTCCATG
CCR5_XbaI_R	TCTAGAATGCAGCAGTGCGTCATCC
CCR5d32_gRNA1	TAATACGACTCACTATAGGAAGACCTTCTTTTTGAGATCGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA2	TAATACGACTCACTATAGGTTTACCAGATCTCAAAAAGAGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA3	TAATACGACTCACTATAGGGTATGGAAAATGAGAGCTGCGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA4	TAATACGACTCACTATAGGGACATTAAAGATAGTCATCTGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA5	TAATACGACTCACTATAGGACATTAAAGATAGTCATCTTGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA6	TAATACGACTCACTATAGGAAAGATAGTCATCTTGGGGCGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA7	TAATACGACTCACTATAGGTGACCATGACAAGCAGCGGCGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA8	TAATACGACTCACTATAGGCAGATGACCATGACAAGCAGGTTTTAGAGCTAGAAATAGCAAG
CCR5d32_gRNA9	TAATACGACTCACTATAGGGGTCCTGCCGCTGCTTGTCAGTTTTAGAGCTAGAAATAGCAAG
CCR5_big_leftpart_F	CAACTCTTGACAGGGCTCTATTTTATAGGC
CCR5_big_leftpart_R	GGACCAGCCCCAAGATGACTATCTTTAATGTATGGAAAATGAGAGCTGCAGGTGTAA
CCR5_big_rightpart_R	GCATAGCTTGGTCCAACCTGTTAG
CCR5_gib_rightpart_F	TTAAAGATAGTCATCTTGGGGCTGGTCC
CCR5_small_F	GTGATCACTTGGGTGGTGGC
CCR5_small_R	TTAGGATTCCCGAGTAGCAGATGAC
CCR5_small_hairpin_R	GCTAAGCGGGTGGGACTTCCTAGTCCCACCCGCTTAGGATTCCCGAGTAGCAGATGAC

DNA cleavage by the complex formed by RNA and EnGen® Cas9 NLS (New England Biolabs; USA) was performed as recommended by the manufacturer of the enzyme. The best results were shown by gRNAs #1 and #5, which were subsequently used for *in vivo* experiments mixed at a 1:1 ratio.

DNA patch

A standard overlap extension PCR technique was used to obtain a DNA patch. After the construct was assembled, a shorter single-stranded DNA product (perfect for promoting recombination over non-homologous end joining NHEJ) was amplified by asymmetric PCR with one of the primers (index F) used in excess. The resulting fragment reads as follows: GTGATCACTTGGGTGGTGGCTGTGTTTGCGTCTCT CCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTC ATTACACCTGCAGCTCTCATTTTCCATACATTAAAGATAGTCA TCTTGGGGCTGGTCCTGCCGCTGCTTGTCATGGTCATCTG CTACTCGGGAATCCTAA

Preparation of RNP complexes

The following components were used to prepare RNP complexes: Cas9 (20 μM), a mix of gRNA#1 and gRNA#5 at a ratio of 1:1 (30 ng/μl), ssDNA (100 ng/μl), a dilution buffer (0.25 mM EDTA/10 mM TrisHCl, pH 7.4).

The injectable solution was prepared by mixing 0.5 μl of Cas9 (20 μM) with 4.5 μl of the dilution buffer. Then, 1.56 μl of Cas9 (2 μM), 0.6 μl of the gRNA mix (30 ng/μl) and 2.5 μl ssDNA (100 ng/μl) were combined with 5.34 μl of the dilution buffer. The mix was incubated at 37 °C for 10 min and immediately used for injecting.

Injection of CRISPR-Cas9

The CRISPR-Cas9 complex was injected into tripronuclear zygotes in the S-phase according to the standard Intracytoplasmic Sperm Injection (ICSI) protocol [13]. The injection volume was 1 nl. After the injection, the zygotes were washed twice in the Sydney IVF Cleavage Medium (COOK Medical LLC; USA), then moved into the Sydney IVF Blastocyst Medium (COOK Medical LLC; USA) and incubated in the EmbryoPlan CO_2 Incubator (West trade LLC; Russia) under standard conditions for 5 days until blastocysts were formed (about 250 cells). Upon incubation each blastocyst was transferred into 12 μl of the dilution buffer and immediately analyzed by PCR.

PCR genotyping and data analysis

Genotyping by double-tube PCR was performed in the DTprime Real-Time PCR Cycler (DNA-Technology, Russia) as described in [14], but to exclude the gRNA region, another universal primer CCR5_check2_R was used with the following sequence: TCATTTCGACACCGAAGCAGA. PCR data were analyzed in the DTprime Real-Time PCR Cycler Software v.7.7 (DNA-Technology; Russia).

RESULTS

Of 16 zygotes injected with the CRISPR-Cas9 complex only 8 reached the blastocyst stage, whereas of 5 control zygotes injected with the dilution buffer 3 developed into a blastocyst. This is the standard rate of blastocyst formation for abnormal zygotes, meaning that the injection did not increase the risk of aborted development. PCR genotyping revealed the absence of the initial WT CCR5 variant in 5 of 8 blastocysts, so those embryos were 100% CCR5delta32- homozygous. Two embryos demonstrated about 3% and one about 20% of WT CCR5 mosaicism (Fig. 2). Cp values for each embryo are presented in Table 2. For each negative control sample (dilution buffer) each PCR was performed in two replicates. The reaction yielded no PCR products.

DISCUSSION

CRISPR-Cas9-mediated editing of the human zygote is an effective method for intracellular DNA modification capable of eliminating nearly 100% of the initial sequence in more than half of embryos

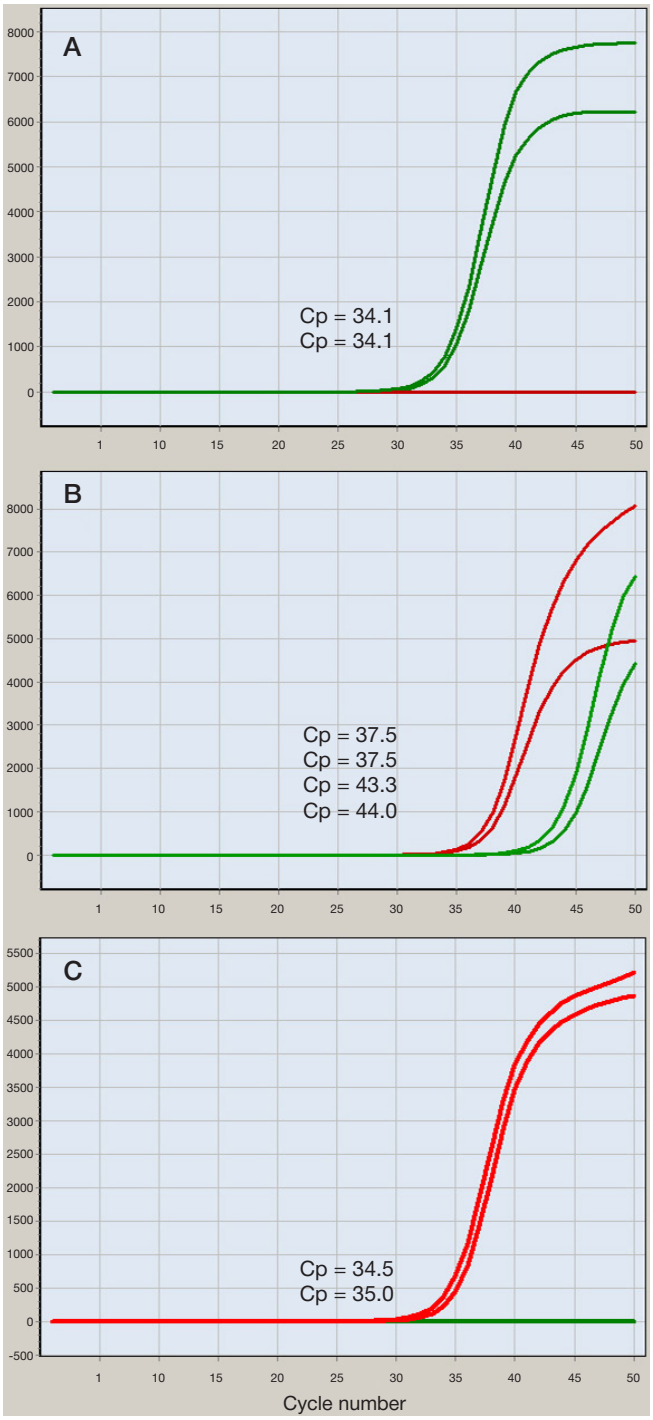

Fig. 2. Examples of real-time PCR curves for different genotypes: **A** — two control embryos, **B** — two mosaic embryos with about 3% of WT CCR5, **C** — two CCR5delta32 homozygous embryos

Table 2. Real-time PCR Cp values for each embryo

Embryo №	Cp WT	Cp del
Control 1	34.1	–
Control 2	34.1	–
Control 3	36.0	–
Exp 1	–	38.1
Exp 2	40.8	38.1
Exp 3		37.8
Exp 4	44.0	37.5
Exp 5	–	37.3
Exp 6	43.3	37.5
Exp 7	–	34.5
Exp 8	–	35.0

Note: – no PCR products.

[9, 10, 12, 15]. Our results are well correlated with those yielded by other GE-system models demonstrating very high efficacy.

In the past few years we have witnessed the rapid evolution of GE-systems. However, the off-target activity of such GE-systems still remains a challenge. Genome editing techniques can be introduced into clinical practice only if they have been proved to be safe for the patient.

CONCLUSIONS

This is the first study demonstrating the efficacy of CRISPR-Cas9-mediated induction of the CCR5delta32 mutation in the human embryo. Its efficacy is very high producing more than 50% of completely modified embryos.

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