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CREATION OF GENOME EDITING SYSTEMS BASED ON CRISPR-CAS9 FOR KNOCKOUT IN *FGF20* AND *HR* GENES OF EMBRYONIC AND GENERATIVE CELLS FROM CHICKEN AND QUAILS

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Abstract

Genome editing technologies using site-specific nucleases (ZNF, TALEN, CRISPR/Cas9) are used more and more in animal husbandry, including poultry farming. With the use of these technologies, scientists hope not only to speed up the process of creating breeds with improved economically useful traits, high resistance to infectious diseases, but also to create individuals carrying phenotypes, the introduction of which into animal and bird populations by traditional breeding methods is impossible or difficult. The creation of individuals devoid of plumage in order to improve the commercial qualities of poultry product is of interest for industrial poultry farming. For this, we selected the *FGF20* and *HR* genes associated with the development and growth of hair in mammals (F. Benavides et al., 2009) and feathers in birds (K.L. Wells et al., 2012). The aim of the study was to create a system for knocking out the *FGF20* and *HR* genes in chickens and *FGF20* in quails by genome editing techniques. We inactivated *FGF20* and *HR* genes in the region of the third exons based on the analysis of their structure. The optimal cutting regions of these genes and guide RNAs and primers for amplifying the *FGF20* and *HR* DNA fragments were selected bioinformatically and using internet resources (https://zlab.bio/guide-design-resources, https://www.ncbi.nlm.nih.gov/). To create genetic constructs for cutting in the regions encoding *FGF20* and *HR*, the vector pX458 was selected (F.A. Ran et al., 2013). The hybridized oligonucleotides 5′-CACCGAAAGATGGTACTCCCAGAGA-3′ and 3′-CTTTCTACCATGAGGGTCTCTCAAA-5′ (for *FGF20* gene in chicken), 5′-CACCGTCCATGTTTGTACACGTTGG-3′ and 3′-CAGGTACAAACATGTGCAACCCAAA-5′ (for *FGF20* gene in chicken and in quails); 5′-CACCGACGTGGCTGACGCGGCACT-3′ and 3′-CTGCACCGACTGCGCCGTGACAAA-5′ (for gene *HR*) were used for ligation. The effectiveness of cloning constructs was confirmed by sequencing. The plasmids that were obtained were used for edit the genome of embryonic (fibroblasts) and generative (primordial germ cells — PGCs, spermatogonia) chicken and quail cells in in vitro experiments. Target cells were transfected by electroporation. Efficiency of electroporation was evaluated on a high-performance fluorescent cell sorter BD FacsAria III («BD Biosciences», USA) by expression of the *eGFP* marker gene. The proportion of in vitro transfected embryonic fibroblasts, PGCs and spermatogonia from chickens with a knockout of the *FGF20* gene reached 5.7, 0.9, and 1.2 %, with a knockout of the *HR* gene — 7.4, 0.8, and 1.0 %, respectively. The percentage of embryonic fibroblasts, PGCs and spermatogonia from quails with a knockout of the *FGF20* gene was 6.3,

0.9, and 1.1 %, respectively. Genomic DNA was isolated from transformed chicken and quail cells and used for amplification and sequencing of the regions of the *FGF20* and *HR* genes in which deletions were introduced. The presence of multiple mutations in the amplified DNA regions was shown. The data obtained indicate the success of the knockout system creation for *FGF20* and *HR* genes in chickens and for *FGF20* gene in quails using genetic constructs based on the pX458 vector.

Keywords: genome editing, CRISPR/Cas9, chicken, quail, primordial germ cells (PGCs), spermatogonia cells, *FGF20*, *HR*

The development of genomic editing technologies using site-specific endonucleases (ZNF, TALEN, CRISPR/Cas9) has opened up new possibilities for introducing targeted genetic changes into animal and bird embryonic lines [1, 2]. These technologies are increasingly attracting attention due to their high efficiency and specificity [3]. In animal husbandry, including poultry, the researchers rely on genome editing to accelerate breeding for improved economically useful traits [4, 5], increased resistance to infectious diseases [6, 7] and also the creation of individuals with phenotypes which are diffecult or impossible to introduce into populations of animals and birds by traditional breeding methods [8, 9]. Obtaining lines of poultry that transmit the introduced genetic changes by inheritance requires manipulations with generative cells. However, the peculiarities of the embryonic development and reproductive physiology of birds do not allow application of methods used in mammals for genetic engineering manipulations, for example, microinjections and somatic cloning [10, 11]. Note that a significant part of the embryonic period in birds passes outside the body of females, which facilitates access to the embryo in the early stages of development. To date, a wide range of developed methods and approaches allow the introduction of genetic constructs into avian embryonic cells [12]. A number of research works confirm the successful production of genetically modified chickens and quails expressing the reporter genes *LacZ* [13] and *GFP* [14], the bacterial β-lactamase gene [15], human interferon α2b [16], human β-interferon [17], human granulocyte colony stimulating factor [18], monoclonal antibodies [19], β-interleukin receptor antagonist [20] and human growth hormone [21]. However, the technologies used to modify the genome of animals and birds until 2012 were non-specific, with the exception of model scientific systems. Since 2012, CRISPR/Cas9 technology has been consistently replacing other methods of industrial transgenesis. There are a number of works on the successful use of CRISPR/Cas9 for the modification of mammalian cells with the subsequent production of individuals with desired properties [22, 23]. In birds, until recently, the transformation of blastodermal cells at stage X using lentiviral and retroviral vectors was considered the most effective way to introduce hereditary changes into generative chicken cells [24]. In recent years, with the development of new methods for editing the genome, in particular TALEN and CRISPR, there has been an increasing interest in using generative cells, e.g., primordial germ cells (PGCs) and spermatogonia, as target cells for the introduction of genetic constructs. PGCs are embryonic cells characterized by pluripotency, that is, the ability to differentiate into both male and female germ cells. Spermatogonia refers to the stem cells of the testes of males. Spermatogonia are a small population of spermatogenic cells located on the basement membrane of the seminiferous tubules. They have the ability to self-renewal and differentiation with the formation of sperm, the male highly specialized mature germ cells.

The peculiarities of PGCs and spermatogonia open up wide opportunities for realizing their potential as targets for genome editing in poultry in order to create individuals with desired traits. The ability to manipulate cells of this type in vitro allows precise integration of expression constructs into a specific locus under a preselected endogenous promoter-enhancer system. Moreover, these technologies allow preservation of the endogenous gene expression. The use of PGCs as a

vector involves their isolation from donor embryos, transformation in in vitro culture, and introduction into recipient embryos. When working with spermatogonia, donor spermatogonia are subjected to transformation in vitro, selection of the transformed cells and their transplantation into the testes of sterile male recipients followed by the production of sperm for insemination of females to obtain genetically modified offspring.

TALEN and CRISPR technologies are applicable for genetic modification of chicken cells in vitro [25, 26]. A number of works have been published on the successful production of chickens and quails with desired properties using various genomic editing systems [27, 28]. One of the possible directions of genome editing, which is of practical interest for improving the commercial qualities of poultry products, is the creation of individuals devoid of plumage. The genes (*FGF20* and *HR*) required for the development and growth of hair in mammals [29] and feathers in birds [30] have been identified.

This report submits the results of studies on the creation of an editing system based on CRISPR/Cas9 for knockout of the *FGF20* and *HR* genes that control the development of plumage in birds. Here, on embryonic fibroblasts and generative cells (PCG and spermatogonia), we revealed the effectiveness of using the created constructs for introducing deletions in the *FGF20* and *HR* genes of chickens and quails under in vitro conditions.

Our goal was to develop a genome editing system for knockout of the *FGF20* and *HR* genes in chickens and quails, suitable for obtaining genetically modified poultry.

Materials and methods. Embryonic fibroblasts were isolated from 5-day-old chicken embryos (*Gallus gallus domesticus,* Russian White breed) or 4-day-old quail embryos (*Coturnix coturnix*, Japanese quail breed). Embryos were removed under aseptic conditions. For disaggregation, the embryos were first mechanically crushed with scissors, then subjected to enzymatic treatment by incubating tissue pieces in 0.15% trypsin solution (Gibco, Thermo Scientific, USA) for 15 min at 37 °C. Primordial germ cells were isolated from 6-day-old chicken embryos and 4-day-old quail embryos. Dissociation was carried out by successive mechanical and enzymatic treatments, as described above, but at a trypsin concentration of 0.05%. Spermatogenic cells were isolated by sequential mechanical and enzymatic treatment of the testis tissue of 1-week-old males. For enzymatic treatment, a 0.25% trypsin solution was used, incubated for 30 min at 37 °C.

The proportion of viable cells in the resulting cell suspension after mechanical and enzymatic treatment of embryos and testicular tissue was assessed by staining with 0.4% trypan blue for 10 min at 37 °C. Stained cells were counted in a Countess cell counter (Thermo Fisher Scientific, USA).

Suspensions of embryonic fibroblast cells and PGCs obtained after enzymatic treatment of embryos were transferred to Petri dishes and cultured in DMEM HG growth medium (Gibco, Thermo Scientific, USA) with a high glucose content (4.5 g/l), 10% fetal bovine serum (FBS, HyClone, USA), glutamine (2 mM), 2-mercaptoethanol (10^{-6} mM), and gentamicin (50 μg/ml). For the primary culture of testis cells, DMEM HG medium with a glucose content of 4.5 g/l supplemented with 20% FBS, alpha-glutamine (2 mM), MEM (100×), antimycotic antibiotic (100×) and ITS (100×) was used as a growth medium). Spermatogonia were cultured in DMEM HG supplemented with 5% FBS, 2 mM alpha-glutamine, MEM (10 μl/ml), antibiotic antimycotic (100×), ITS (10 μl/ml), mercaptoethanol (5×10^{-5} M), albumin (5 mg/ml), DL-lactic acid (1 μl/ml), EGF (20 ng/ml), bFGF (10 ng/ml), LIF (2 ng/ml).

Growth additives, amino acids, and antibiotics used in the culture media

are produced by Gibco (Thermo Scientific, USA) and Sigma (USA).

Chicken and quail cells were cultured at 37 °C and 5% CO_2. For passage and molecular genetic studies, the cells were removed from the substrate with a 0.25% trypsin solution.

Microscopy of the obtained cultures of embryonic fibroblasts, PGCs, and spermatogonia of chickens and quails was performed (an inverted microscope Nikon Eclipse TS100, Nikon, Japan).

Guide RNA sequences were designed using https://zlab.bio/guide-design-resources, https://www.ncbi.nlm.nih.gov/ tools, the GalGal5 genome assembly variant and gene names FGF20 (Gene ID: 428779) and HR (Gene ID: 107049623) for chicken and Coturnix japonica 2.0 and the gene name FGF20 (Gene ID: 107313688) for quail. Optimal cutting sites for the *FGF20* gene of chickens and the *HR* gene of quail were selected. To create genetic constructs for cutting selected genome regions, pairs of the following oligonucleotides were hybridized: 5'-CACCGAAAGAT-GTACTCCCAGAGA-3' and 3'-CTTTCTACCA-TGAGGGTCTCTCAA-5' (for the *FGF20* gene in chickens), 5'-CACCGTCCA-TGTTTGTACACGTTGG-3' and 3'- CAGGTACAAACATGTGCAACCCAAA-5′ (for the *FGF20* gene in chickens and quails); 5'-CA-CCGACGTGGCTGAC-GCGGCACT-3' and 3'-CTGCACCGACTGCGCCA-5' (for the *HR* gene of chicken). Hybridized oligonucleotides were ligated with plasmid pX458 (Addgene #48138) linearized with BbsI restriction endonuclease (ER1011, Thermo Scientific, USA) as described [31]. After ligation and transformation of *Escherichia coli* cells, the grown colonies were subcultured in liquid LB medium with ampicillin and used to isolate plasmids. Analysis of the results of cloning was performed by sequencing. Successfully cloned constructs were used to transfect cells.

The commercial QuickExtract™ DNA Extraction Solution kit (Lucigen Corporation, USA) was used as per the manufacturer's recommendations to isolate genomic DNA when creating constructs and evaluating the efficiency of cutting the target genome region. Amplification was carried out in a PCR mixture PCR MM based on Taq DNA polymerase (K0171, Thermo Scientific, USA) in a 25-μl mix volume at 60 °C for hybridization and at 72 °C for 1 min for elongation. Amplification of the *FGF20* gene region was performed with primers F20_CHK2F 5'-TGTTCCTTTGTGCAGGAGAAACT-2' and F20_CHK2R 5'-TCCCTCTCT-CCTCAGCTGTATC-3'.

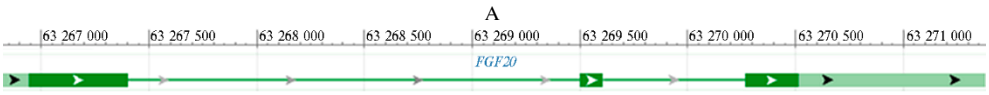
Editing systems were introduced into embryonic fibroblasts, PGCs, and spermatogonia by electroporation (a Neon™ Transfection System, Invitrogen, USA). Transfected cells were selected using a high-throughput BD FACSAria III cell sorter (BD Biosciences, USA).

Efficiency of cutting the target genome region was evaluated by amplification and sequencing of the corresponding genomic DNA fragments with the designed primers in the above modes (the sequences of the amplified fragments are shown in Figures 1 and 2). Sequencing (performed by OOO Sintol, Moscow) was performed by Sanger method with a direct primer for amplification.

Results. Development of the CRISPR/Cas9 genomic editing system for knockout of the *FGF20* and *HR* genes started with a bioinformatic search for the *HR* gene homologue in birds and guide RNA sequences to inactivate the *FGF20* and *HR* genes.

The *FGF20* gene of the chicken *G. gallus domesticus* is located on chromosome 4 (Fig. 1, A). To inactivate this gene, we chose exon III. The figure shows cut sites in the *FGF20* gene of chickens and quails, which are optimal for introducing target mutations into the genome with the CRISPR/Cas9 system, hybridization sites for two guide RNAs and primers for amplification of the selected gene

FGF20 regions. (see Fig. 1, B). One of the guide RNAs was universal for chicken and quail, the second one was complementary only for chicken. The primers for amplification of target regions of the *FGF20* gene corresponded to the genome of both chicken and quail.



B

Quail (*Coturnix japonica*)

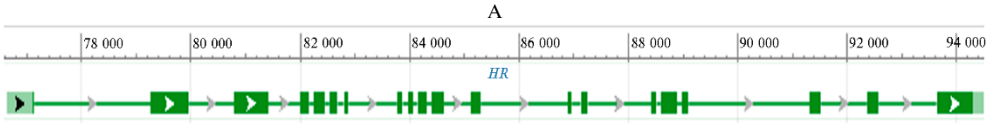
TGAGTGCATCTTCAGGGAACAGTTTGAGGAAAACTGGTACAACACTTACTCCTCCAA**CGTG**TACAAACATGGAGAT
TCTGGGCGGCGATACTTCGTAGCACTTAACAAGGACGGTACTCCC**AGAG**ATGGTGCAAGGTCCAAAAGACACCAGA
AATTCACACATTTCCTGCCCAGACCTGTGGATCCTGAAAGAGTTCCAGACT

Rooster (*Gallus gallus domesticus*)

GAGTGCATCTTCAGGGAACAGTTTGAGGAAAACTGGTACAACACTTACTCCTCCAA**CGTG**TACAAACATGGAGATT
CTGGGCGGCGATACTTCGTAGCACTTAACAAAGATGGTACTCCC**AGAG**ATGGAGCAAGGTCCAAAAGACACCAGAA
GTTCACACATTTCCTGCCCAGACCTGTGGATCCTGAAAGAGTTCCAGA

Fig. 1. Scheme of the *FGF20* gene (A) **and regions of hybridization of guide RNAs** (B) **in *Gallus gallus domesticus* and *Coturnix japonica*.** The genomic coordinate of chromosome 4 of *G. gallus domesticus* is shown with the location of exon (thick lines) and intron (thin lines) regions of the gene. Non-coding regions are marked in light green, coding regions in dark green. The arrows show the direction of transcription. Alignment of genome regions of quail *C. japonica* and chicken *G. gallus domesticus* with hybridization regions of the designed primers (marked in green) and guide RNA (marked in turquoise) is presented. PAM (protospacer adjacent motive) sites are shown in purple.

The *HR* gene is located on chicken chromosome 22 (Fig. 2, A). For its inactivation, exon III was chosen, encoding the amino acids of the active center of the HR enzyme, which is a lysine demethylase. Figure 2, B shows a fragment of the chicken *G. gallus domesticus* genome with selected regions for hybridization of primers and guide RNA.



B

GTCGGACGGTGAGAGCCCGGCGGGGGCGCACGGGGGCGTCCAGAAGCTGAAGAAGACGTGGCTGACGCGGCACTCGGA
GCAGTCGGGGCCGCGCTGTAGGGCGGCGCGGAGGGACGGAGCCGAAGAGGGCTTCAAACGCGCG

Fig. 1. Scheme of the gene *HR* (A) **and regions of hybridization of guide RNAs** (B) **in *Gallus gallus domesticus*.** The genomic coordinate of chromosome 22 of *G. gallus domesticus* is shown with the location of exon (thick lines) and intron (thin lines) regions of the gene *HR*. Non-coding regions are marked in light green, coding regions in dark green. The arrows show the direction of transcription. A genome region of chicken *G. gallus domesticus* with hybridization regions of the designed primers (marked in green) and guide RNA (marked in turquoise) is presented. PAM (protospacer adjacent motive) sites are shown in purple.

Oligonucleotides for the guide RNAs were synthesized and used to make genetic constructs for the inactivation of the *FGF20* and HR *genes*. Plasmid pX458 was treated with restriction endonuclease BbsI and ligated at the cleavage site with hybridized oligonucleotides F20C, F20U, and HR (Fig. 3).

F20C
5′-CACCGAAAGATGGTACTCCCAGAGA
CTTTCTACCATGAGGGTCTCTCAAA-5′

F20U
5′-CACCGTCCATGTTTGTACACGTTGG
CAGGTACAAACATGTGCAACCCAAA-5′

HR
5′-CACCGACGTGGCTGACGCGGCACT
CTGCACCGACTGCGCCGTGACAAA-5′

Fig. 3. Oligonucleotides providing specificity of guide RNAs and used to create plasmids for the *FGF20* and *HR* inactivation by the CRISPR/Cas9 technoogy.

After ligation and transformation of competent *E. coli* JM109 cells [32], and the clones were used for plasmid isolation. Sequencing of the obtained plasmids confirmed the success of cloning constructs which were further

used for transfection of chicken and quail cells.

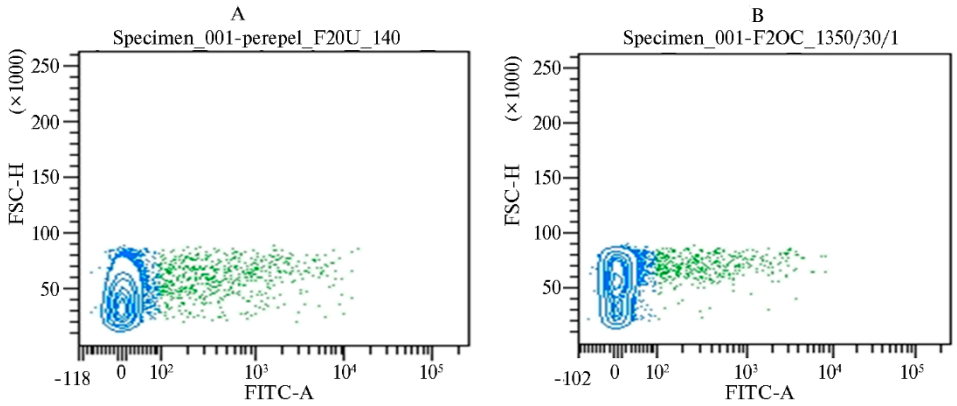


Fig. 4. An example of sorting a population of quail (A) **and chicken** (B) **embryonic fibroblast cells after transfection with pX458 plasmid-based constructs.** The X-axis shows the intensity of fluorescence in the green range, the Y-axis shows light scattering. Green indicates cells with green fluorescence.

The effectiveness of editing systems designed to knock out the *FGF20* and *HR* genes was first evaluated on embryonic fibroblasts due to the simplicity and availability of their production. Chicken and quail embryonic fibroblasts with a plasmid encoding the components of the CRISPR/Cas9 genomic editing system were separated from non-transfected cells using a cell sorter (Fig. 4). pX458 contains regions corresponding to the *Cas9* and *GFP* genes the coding regions of which are separated from each other by the sequence encoding the P2A peptide. Thereof, cells which synthesize Cas9 also contain GFP and can be separated from non-transfected cells due to fluorescence (see Fig. 4). The proportion of successfully transfected chicken embryonic cells (sample size of 10,000 cells) using systems for knocking out the *FGF20* and *HR* genes reached 5.7 and 7.4%, respectively. According to the distribution of cells by fluorescence intensity, the efficiency of transfection of quail embryonic cells using the editing system for the *FGF20* gene knockout was 6.3%.

Sorted transfected chicken and quail embryonic fibroblasts were used to isolate genomic DNA in order to evaluate the efficiency of editing the *FGF20* and *HR* genes. The isolated DNA was used for PCR amplification of loci containing regions of complementarity with guide RNA (see Fig. 1, 2). The isolated PCR products were analyzed by Sanger sequencing. The analysis of transfected chicken (Fig. 5, 6) and quail embryonic fibroblasts detected multiple microdeletions.

The data obtained (see Fig. 5, 6) allow us to conclude that our constructs are highly efficient to introduce microdeletions into the *FGF20* and *HR* genes. Of the two constructs targeting the *FGF20* gene, the F20U proved to be more effective and was used together with the HR construct.

When generating genetically modified birds, a directed modification of gonadal cells with the aim to further obtain offspring with a modified genome is of greatest interest. For these purposes, both mature germ cells and their precursors, PGCs and spermatogonia are suitable.

With disaggregation of chicken and quail embryos by mechanical and enzymatic treatment, we obtained a suspension of dissociated cells (the proportion of non-viable cells did not exceed 5%). The suspension contained different types of embryonic cells the separation of which by adhesion [33] maximally enriched population of embryonic cells with PGCs. The proportion of PGCs from the total number of other cell types in the culture of chicken and quail embryonic cells reached 88 and 81%, respectively. A small population of fibroblasts remaining

after cell separation served as a feeder layer on which PGCs were attached and cultured, forming colonies (Fig. 7).

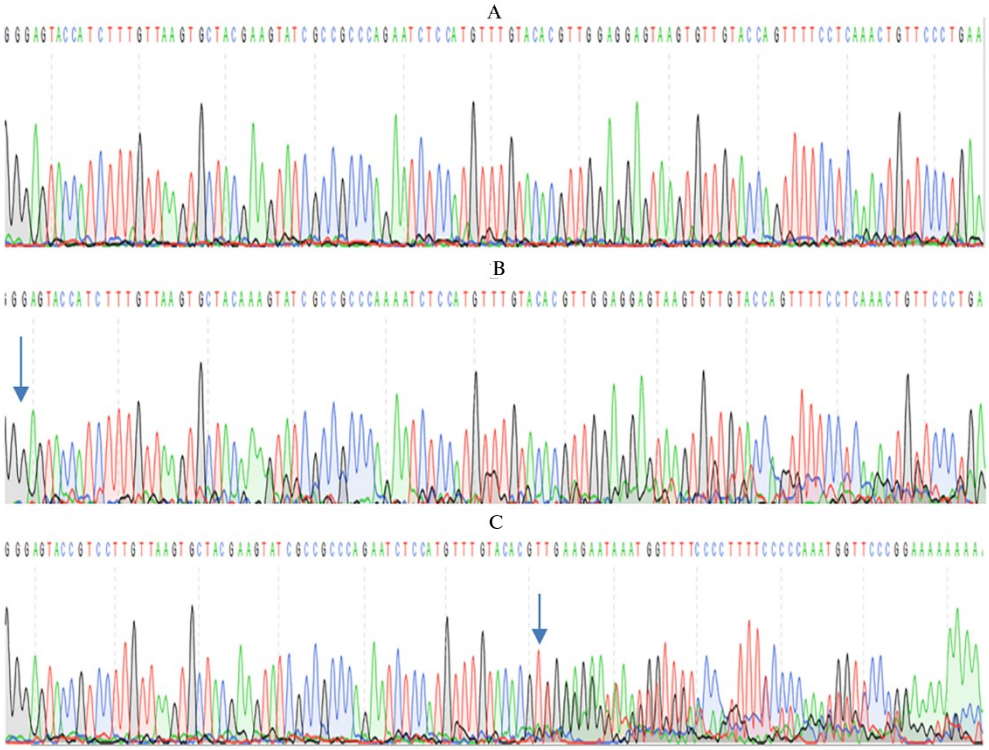


Fig. 5. Analysis of the CRISPR/Cas9 editing system efficiency for the *FGF20* gene by Sanger sequencing-based assay: A — unedited chicken *FGF20* gene; B — chicken *FGF20* gene after editing with Cas9 and F20C guide RNA; C — chicken *FGF20* gene after editing with Cas9 and F20U guide RNA. The arrows show the sites of gene cutting. You can see the overlap of the results of sequencing the products of multiple microdeletions. When sequencing heterogeneous microdeletion products, in contrast to a homogeneous wild-type amplicon, there is an overlap of peaks corresponding to different nucleotides (green for A, black for G, blue for C, and red for T).

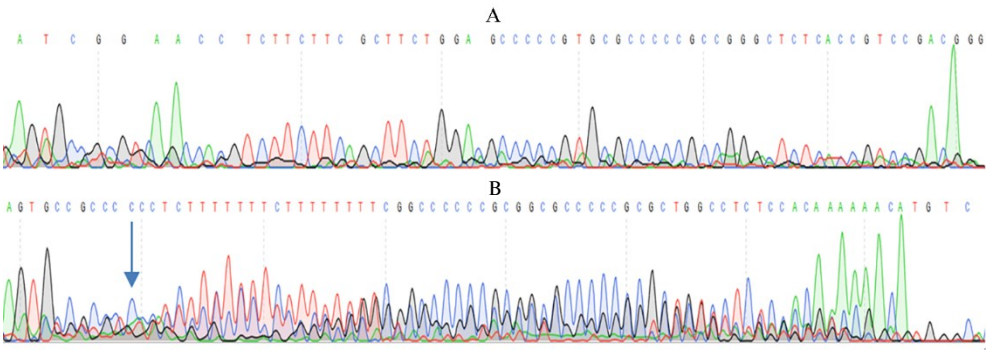


Fig. 6. Analysis of the CRISPR/Cas9 editing system efficiency for the *HR* gene by Sanger sequencing-based assay: A — unedited chicken *HR* gene; B — chicken *HR* gene after editing with Cas9 and HR guide RNA. The arrows show the sites of gene cutting. You can see the overlap of the results of sequencing the products of multiple microdeletions. When sequencing heterogeneous microdeletion products, in contrast to a homogeneous wild-type amplicon, there is an overlap of peaks corresponding to different nucleotides (green for A, black for G, blue for C, and red for T).

Disaggregation of the testis tissue of roosters and quails by trypsin created suspensions consisting mainly of Sertoli cells and spermatogonia. In culturiing, Sertoli cells spread flat on the surface of Petri dishes. Spermatogonia attached to Sertoli cells, forming colonies on days 7-8 of culture (Fig. 8).

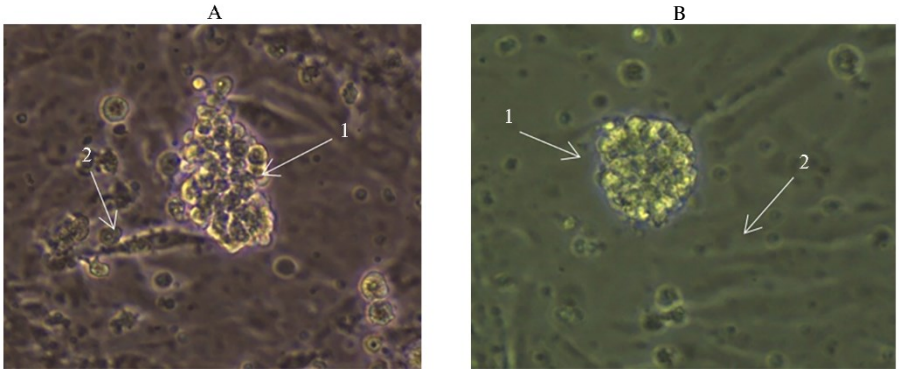


Fig. 7. Colonies of primordial germ cells (PGCs) **of chickens** (A) **and quails** (B) **used for transfection with constructs for knockout of the *FGF20* and *HR* genes:** 1 — PGCs, 2 — fibroblasts (feeder layer). Native preparation, light microscopy (Nikon Eclipse TS100, Nikon Co., Japan; magnification ×400).

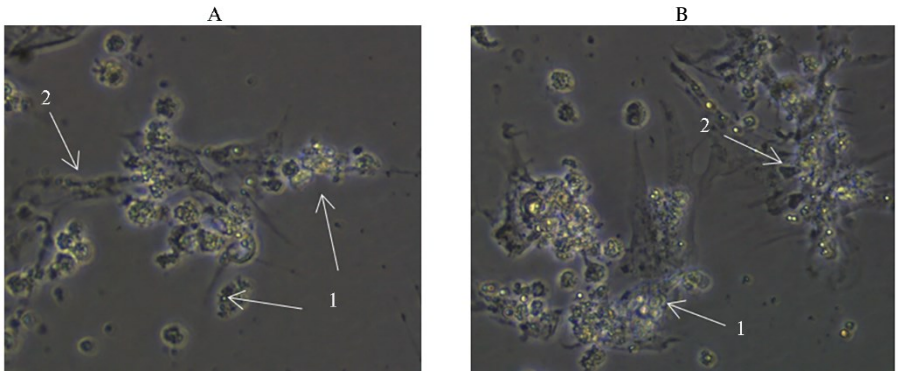


Fig. 7. Cultures of spermatogenic cells from testis of chickens (A) **and quails (B) used for transfection with constructs for knockout of the *FGF20* and *HR* genes:** 1 — spermatogonia, 2 — Sertoli cells. Native preparation, light microscopy (Nikon Eclipse TS100, Nikon Co., Japan; magnification ×400).

The resulting cultures of PZK and spermatogonia of chickens and quails were transfected with the created constructs for knockout of the *FGF20* and *HR* genes. The share of in vitro transfected PZK and spermatogonia of chickens with *FGF20* gene knockout reached 0.9 and 1.2%, respectively, with *HR* gene knockout 0.8 and 1.0%. The proportion of PGCs and quail spermatogonia with *FGF20* gene knockout was 0.9 and 1.1%, respectively. Note that the efficiency of transfection of these target cells was relatively low. However, we obtained a pure population of transfected cells by sorting and multiplied them in vitro to the required abundance.

Several research groups have studied the effectiveness of PGCs as target cells for gene editing to obtain birds with knockout of various genes, in particular, chickens with CRISPR/Cas9-mediated [5, 6, 8, 34] and TALEN-mediated [35] knockout of genes for myostatin [5], immunoglobulin heavy chain [34], DDX4 [35], ovomucin [8], and NHE1 [6]. In these works, two methods were used for PGC transfection, the electroporation [6, 34, 35] and lipofection [5, 8]. With electroporation, in most cases, the transfected cells were selected in growth media with a selective antibiotic. When the authors selected transfected PGCs by sorting without preliminary culture in a selective medium, the transfection efficiency was low (1%), which is consistent with our data.

We did not find information on the use of spermatogonia as targets for the introduction of gene editing systems in the available sources of information.

Thus, we developed gene editing systems for knockout of the *FGF20* and *HR* genes in chickens and the *FGF20* gene in quails. To find optimal cutting sites of these genes and to design the sequences of guide RNAs and primers for amplification of selected target DNA segments, we used bioinformatics tools. To introduce deletions into the regions encoding FGF20 and HR, gene constructs based on the pX458 vector were created. Our research results confirm the effectiveness of introducing microdeletions into these genes of chickens and quails with the constructs created. The transfection frequency is 5.7 and 6.3% for chicken and quail embryonic fibroblasts, respectively, 0.9 and 0.9% for primordial germ cells, 1.2 and 1.1% for spermatogonia. These findings indicate the successful creation of gene editing systems in poultry using gene constructs based on the pX458 vector.

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