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Innovative Approaches to Genome Editing in Chickens

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Abstract—Obtaining productive animals, including chickens, with specified characteristics is a promising area of modern animal husbandry. The most relevant traits for chickens are increased meat and egg productivity, resistance to infectious diseases, products with reduced allergenicity, and the production of integrated transgenic proteins. The rapidly developing methods of molecular genetics, such as genome editing, allow for solving these problems. In birds, unlike mammals, access to the unicellular zygote—when editing the genome is most effective—is difficult due to the special structure of the reproductive system. As a result, innovative methods have been developed for genetic engineering of birds, the most common of which is the use of primordial germ cells (PGC), precursors of poultry reproductive cells. This review provides a brief description and discussion of modern methods of editing the chicken genome using endonucleases, such as transcription activator-like effector nucleases (TALEN) and the system of clustered short palindromic repeats CRISPR/Cas9. Particular attention is paid to methods of gene editing in birds using primordial germ cells (PGC). Various strategies for the delivery of guide RNA (gRNA) and Cas9 protein into poultry cells based on the use of plasmid vectors, an alternative method of delivery of genetic constructs using spermatozoa (STAGE), the RNP method, which consists in direct delivery of gRNA and Cas9 protein in the form of a complex ribonucleoproteins, and the RNP method based on the use of nanobubbles were considered.

Keywords: genome editing, TALEN, CRISPR/Cas9, NHEJ (nonhomologous end joining), HDR (homologous direct reduction), primary germ cells (PGC), chicken embryo

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INTRODUCTION

Poultry farming makes up a large part of the livestock industry worldwide, which makes poultry an important object in biology and agriculture and arouses increased interest in innovative genome-editing technology to improve productivity and other economically useful traits in various types of productive animals, including chickens. Technological breakthroughs and the rapid improvement of genetic engineering tools in the past 10 years have allowed scientists to make precise targeted modifications to the chicken genome and effectively solve the above problems. Along with the development and improvement of new genome-editing tools based on the use of endonucleases, such as transcription activator-like effector nucleases (TALEN) and the powerful short palindromic cluster repeat technology (CRISPR/Cas9), there has also been progress in technologies used to deliver editing tools to cells. In mammals, edit constructs are usually delivered at the time of fertilization or into fertilized unicellular embryos in vitro, which are subsequently cultured and then implanted into the recipient animal. In birds, due to the peculiarity of the reproductive system, it is impossible to use such an approach, and, therefore more complex technologies for the delivery of editing constructs into the chicken embryo have been developed. One of these approaches is the transformation and introduction into the embryo of cultured primordial germ cells (PGCs), which are precursors of spermatozoa and oocytes, which migrate through the embryonic circulatory system to the developing gonads and can be selected at this time for genetic modification. Advances in avian primordial cell (PGC) genome editing technology allowed for the creation of bird models with improved production and low allergenic and disease-resistant egg laying. The method based on the technology of clustered regularly interspaced short palindromic repeats (CRISPR) made gene editing relatively simple and highly efficient, which opens up wide opportunities for making targeted modifications to the genotype and phenotype of an animal in accordance with a specific scientific or industrial purpose. Gene knockout chickens created by genome editing are used as model objects for studying aspects of the functioning of target genes as well as for research in the field of developmental biology, immunology, physiology, and neurology.

The purposes of this article are to review modern methods of editing the chicken genome using endonucleases and the prospects for their application in poultry farming and to describe the technology for using primordial germ cells and current approaches to delivery of editing tools into avian embryos.

MODERN GENOMIC EDITING TOOLS

Recent advances in the targeted modification of complex eukaryotic genomes with endonucleases have ushered in a new era of genetic engineering. The use of genomic editing tools was first described almost 20 years ago using the homologous recombination technique, when the targeted integration of exogenous DNA into double-stranded breaks (DSBs) caused by rare cutting endonucleases in eukaryotic cells was demonstrated (Rouet et al., 1994). Eukaryotic cells repair DNA breaks in two ways: nonhomologous end joining (NHEJ) or, less commonly, homologous direct repair (HDR). NHEJ is the main DNA repair mechanism for double-strand breaks (DSBs) and involves the alignment of one to several additional bases for religation of the two ends, which can lead to small deletions, insertions (indel), or single nucleotide substitution at the break point. By introducing a double-stranded break in the target gene, as a result of indel mutations caused by nonhomologous end-linking, a complete knockout of the target gene can be performed by shifting the reading frame or introducing a STOP codon. The second DSB repair mechanism is the HDR pathway, which uses an allelic gene from a sister chromatid as a DNA template to restore the original sequence (Johnson and Jasin, 2000). The DNA template provides information to accurately repair the damaged chromosome region (Yeh et al., 2019). This repair system is highly specific and accurate, but its occurrence in eukaryotic cells is much lower due to the high prevalence of NHEJ (Riordan et al., 2015). HDR occurs during the G2 phase of the cell cycle (Zhao et al., 2017). However, the efficiency of homologous recombination is extremely low in most cell types, and another problem was the insertion of an exogenous DNA fragment into an off target. To improve the efficiency and accuracy of traditional gene targeting, a new method has been developed based on site-specific nucleases (SSNs), such as zinc finger nucleases (ZFN) (Porteus et al., 2005; Bibikova et al., 2002) and transcription activator-like effector nucleases (TALEN) (Li et al., 2011; Miller et al., 2011), as well as the powerful technology of clustered regularly interspaced short palindromic repeats or CRISPR/Cas9 (Jinek et al., 2012; Pennisi, 2013; Barrangou, 2014; Wu et al., 2014). The latter method revolutionized genome editing and allowed researchers to generate mutations and cut DNA in a very precise way, activating double-stranded breaks (DSB) and recognizing target sequences.

Zinc finger technology (ZFN) or chimeric nucleases were developed in 2001 (Bibikova et al., 2001) and are designed to target and accurately cut DNA sequences (Qomi et al., 2019). However, ZFN-mediated gene editing has not yet been reported in poultry. In the search for more efficient gene-editing tools, a new generation of effector nucleases similar to the transcription activator (TALLEN) emerged in 2009. Originally discovered in pathogenic bacteria of the genus Xanthomonas, effectors similar to transcriptional activators (TALE) are DNA-binding and FokI-nuclease domains (Gaj et al., 2013), as in the ZFN system, performing a double-strand break at the desired position. The difference between the two systems is that the DNA-binding domains in TALEN contain 33–35 amino acid repeating motifs, each of which binds only one nucleotide and not a triplet of nucleotides as in the ZFN system, which makes TALEN more site-specific and less likely causes cleavage outside the target (Khan, 2019). The use of TALEN for gene editing has been demonstrated in a number of animals, including chickens. Pak et al. performed a targeted knockout of the ovalbumin gene (OVA) using the TALEN method in domestic chicken. In this study, cultured PGCs were transfected with plasmids encoding OVA-TALEN. This resulted in 33% of the culture of PGCs containing deletions from 6 to 29 nucleotides in the OVA gene. PGCs containing OVA modifications were transplanted into recipient embryos and brought to puberty. These chimeric males gave birth to heterozygous OVA knockout chicks with an efficiency of 10% (Park et al., 2014). More recently, Tylor and others have used TALEN in combination with homology-directed repair (HDR) to produce sterile chickens. Park and others edited the DDX4 (vasa) locus by the TALEN method using a plasmid transfected primordial germ cell culture (PGC). This study also included an HDR matrix containing a reporter (topuromycin fusion with GFP-2A) to enable the selection of targeted PGCs. After 2 weeks of culture, 8.1% of the PGCs were found to express GFP, indicating a successful HDR. The resulting edited heterozygous male cells were transplanted into recipient embryos and grown to puberty. After crossing these founding males, modified offspring were obtained with an efficiency of 6% (Taylor et al. 2017).

Although the use of ZFN and TALEN have made significant improvements in gene manipulation, these methods require specially designed proteins, which makes the technology expensive and complex, because each new target site requires the development and creation of a new site-specific nuclease, which are used for precise targeting and gene knockout. Unusual short palindromic cluster repeats were first discovered by Ishino et al. in 1987 while studying Escherichia coli bacteria. Years later, in 2002, these repeats, which represent the natural defense system of bacteria against phage and plasmids, were named CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) (Mojica et al., 2000; Hsu et al., 2014).

The main components of the system are the operon, which encodes the Cas protein(s) responsible for endonuclease activity and two small RNAs, crRNA and tracrRNA, combined into one guide RNA to target a specific target sequence (Mojica et al., 2005). Optimization of a single guide RNA (gRNA), which is much easier to design and create, in contrast to the ZNF and TALEN editing systems, makes CRISPR/Cas the most popular genome-editing tool in eukaryotic cells (Cong et al., 2013). Critical disadvantages include off-target effects and the need for a short DNA sequence protospacer motif (PAM) contiguous, which is required for compatibility with a used Cas protein, such as NGG in the case of the most widely used Cas9 from Streptococcus pyogenes (Fagerlund et al., 2015; Komor et al., 2017). The main components of the system are the operon, which encodes the Cas protein(s) responsible for endonuclease activity and two small RNAs, crRNA and tracrRNA, combined into one guide RNA to target a specific target sequence (Mojica et al., 2005). Optimization of a single guide RNA (gRNA), which is much easier to design and create, in contrast to the ZNF and TALEN editing systems, makes CRISPR/Cas the most popular genome-editing tool in eukaryotic cells (Cong et al., 2013). Critical disadvantages include off-target effects and the need for protospacer adjacent motif (PAM) of a short DNA sequence, which is required for compatibility with a used Cas protein, such as NGG in the case of the most widely used Cas9 from Streptococcus pyogenes (Fagerlund et al., 2015; Komor et al., 2017). Numerous studies using early CRISPR/Cas agents have shown that more than 50% of RNA-dependent mutations caused by endonucleases had an off-target effect (Fu et al., 2013; Cho et al., 2014). gRNA recognizes a 20 bp target DNA sequence, which it binds and cleaves to "edit" the DNA sequence, but this process can tolerate up to several base pair mismatches, which means the potential to bind to thousands of possible binding sites and cause a number of experimental problems (Hsu et al., 2014).

Several engineering and screening techniques have been described to reduce off-target mutations throughout the genome, including nuclease mutation, sequence modification of a protospacer adjacent motif (PAM), gRNA truncation, and the discovery of new nucleases (Kadam et al., 2018). For example, Fu et al. reported in 2013 that the target specificity of nuclease increased up to 5000-fold when truncating gRNA from <20 bp up to 17 or 18 bp, and incidents of mismatch above three bases became very rare or did not occur at all (Fu et al., 2013). Therefore, CRISPR/Cas9 can be used to modify any genomic sequence, thereby providing a simple, easy, and cost-effective means of editing target genes of the entire genome (Jiang and Doudna, 2017). In 2013, the first editing of the mammalian genome using the CRISPR/Cas9 system was successfully performed (Cong et al., 2013). The first study using CRISPR in chickens was published in 2015 and involved electroporation of chick embryos with plasmids encoding Cas9 and guide RNAs targeting the transcription factor PAX7, resulting in an 80–90% reduction in PAX7 expression compared to control embryos (Veron et al., 2015). This result clearly demonstrated that the CRISPR/Cas9 method was able to effectively cope with the task of gene editing in chick embryos.

APPROACHES TO EDITING GENES IN BIRDS WITH CULTIVATION OF PRIMORDIAL GERM CELLS (PGC)

The use of avian genome-editing techniques poses special challenges related to the structure of the fertilized egg and early embryo. In mammals, editing genetic constructs are inserted directly into the zygote; as a result, animals carry mutations in one or both sister chromatids, but the same strategy does not apply to birds due to the peculiarities of the reproductive system. In chickens, the oocyte is transported and fertilized in the oviduct funnel within 15 min after ovulation, and the egg is laid within ~24 h (Sang, 2004). After fertilization, the 1-cell zygote divides rapidly and reaches a size of ~ 60000 cells for egg laying (Pokhrel et al., 2017). As a result of the rapid proliferation of the cells of the fertilized egg in the oviduct, access in vivo at the first stage of zygote development for genome editing is difficult. The classical method of microinjection of a genetic construct into an amniotic zygote is associated with problems in determining the accuracy of ovulation, oocyte opacity, a large amount of yolk in the egg, strong cytoplasm compaction near the pronucleus, difficulties in extracting, and manipulating and cultivating early embryos (Love et al., 1994; Shimada et al., 2014). As a result, methods in birds for editing the chicken genome, supplemented by the method of introducing exogenous cultured primordial germ cells (PGC) into the embryo, were developed.

Primordial germ cells (PGCs) are precursors of sperm and oocytes in adults and are well suited for in vitro culture and genetic modification (Macdonald et al., 2010). Unlike other species, embryos of birds and some reptiles have the unique ability to use blood circulation to transport PGC to the gonadal primordium at an early stage of development, which makes it possible to manipulate them by intravascular injection (Nakamura, 2017).

In chickens, PGCs are usually localized in the central area pellucida on the ventral surface of the epiblast of freshly laid eggs (stage X). PGCs are formed in the embryonic sickle and begin to accumulate inside the blood vessels at the HH10 or HH11 stage (Hamburger and Hamilton, 1951). Unlike mammals, chicken PGCs enter the blood vessels and circulate in the blood until the HH17 stage. Between HH10 and HH12, PGCs use the circulatory system to migrate along the dorsal mesentery and finally settle and accumulate on the genital crests (Nakamura et al., 2013).

Table 1. Main methods of delivery of the CRISPR/Cas9 system to chicken cells

Delivery means	Format of Cas9 delivery	Advantages	Disadantages
Physical delivery methods			
Microinjection	In vitro and ex vivo microinjection of plasmid DNA; mRNA(Cas9 + gRNA); RNP complex	Suitable for all CRISPR-Cas9 formats; high specificity and reproducibility, controlled delivery of a known amount of cargo; control over inappropriate exposure; good for PGC	Causes cell damage; high level of complexity of manual labor; low throughput; low delivery efficiency; expensive equipment (microinjector, microscope)
Electroporation, nucleofection	In vitro and ex vivo plasmid DNA mRNA(Cas9 + gRNA); RNP complex	Suitable for all CRISPR-Cas9 formats; suitable for any type of cells; high efficiency of delivery and transfection; ease of execution	Ability to cause significant cell death; nonspecific transfection; usually not suitable for in vivo use; high price
Nonviral delivery methods			
Lipofection Lipid nanoparticles/ liposomes	In vitro and ex vivo (Cas9 + gRNA); mRNA(Cas9 + gRNA); RNP complex	Suitable for all CRISPR-Cas9 formats; safe technique; low risk of cell damage; low cost; ease of execution	Low delivery efficiency, endosomal degradation of the cargo; work efficiency depends on the type of cells
Cationic polymer carriers (polyethyleneimine (PEI))	In vitro and ex vivo microinjection of plasmid DNA	Effective for transient transfection of plasmid DNA. The efficiency of PEI-based transfection is influenced by many factors	Polyethyleneimine (PEI) is toxic to cells if overused
Sperm delivery method (STAGE)	Ex vivo mRNA(Cas9 + gRNA)	Relevant for the creation of edited heterozygous and homozygous wild birds in the first generation	Low delivery efficiency
Virus-assisted deliver			
Retroviral-*mediated delivery*	In vitro, ex vivo in vivo, microinjection of plasmid DNA	High efficiency in vivo, integration of the target gene into the genome of the host cell; loading capacity up to 10 kb	High risk of off-target effect, insertional mutagenesis; activation of oncogenes
Delivery using lentiviruses	In vitro and ex vivo microinjection of plasmid DNA	High efficiency of integration of the target gene into the genome of the host cell, high throughput in vitro and in vivo; load capacity 8 kb; long-term gene expression	High risk of inappropriate off-target effect; prone to rearrangements of target genes, to silencing of transgenes
Adenovirus-mediated delivery	In vivo, in vitro, ex vivo microinjection of plasmid DNA	High in vivo efficiency; large storage capacity up to 35 kb; nonpathogenicity, immunoreactivity, difficult to produce on a large scale, limited load carrying capacity; high price	High risk of inappropriate off-target effect; prone to rearrangements of target genes, to silencing of transgenes
Delivery using gesicles	In vitro, ex vivo, in vivo RNP complex	Short-term exposure; universal format provides binding and fusion with membranes of a wide range of target cells; low cost; reduced the likelihood of off-target effects; finer control of Cas9 expression	Limited quantification method; small load capacity 1.8ii kb (significantly compensated by the TAMEL method

PGC in blood reaches its maximum at the HH13–15 stage, which is the most preferred time of their release (Nakamura et al., 2007; Jeong et al., 2002; Li et al., 2005; Mozdziak et al., 2005; Yamamoto et al., 2007; Jung et al., 2017). It has been reported that PGCs obtained at various stages of development (from 2.5 to 8.5 days) have a diameter of approximately 9 to 20 μm (Motono et al., 2008).

A study by Nakamura et al. (2007) showed that the number of PGCs in chick embryos from stage X (newly laid egg) to stage HH10 gradually increases from approximately 130 to 439 cells. However, according to Bernando et al. (2012), from HH5, when PGCs are present in the germinal sickle, to HH19 stages, when they reached the genital crests, the number of PGCs remained constant and ranged from 200 to 400. There is no direct calculation of the PGC number at HH14–16 due to cell migration during this period.

The first birds with genome editing were obtained using PGCs isolated from chick embryos at the HH11 stage (Vick et al., 1993). Nakamura et al. (2010) treated fertilized eggs with a busulfan emulsion to reduce the amount of endogenous PGCs in the blood at the HH14–16 stage and produced offspring of exclusively donor origin. Van de Lavoir et al. (2006) were the first to develop a long-term culture system for chicken PGCs isolated from whole blood at stages HH13–15 and obtained germ-line chimeras from them. In addition, they injected cultured chicken PGCs into xenogeneic embryos and produced offspring with a donor species phenotype using sperm from a xenogeneic donor (van de Lavoir et al., 2012). These studies demonstrate that PGCs represent a major breakthrough in the conservation of avian genetic resources. Since then, techniques for modifying PGCs and constructing plasmids for chicken transgenesis have been greatly improved.

However, PGCs are characterized by their low ability to induce efficient and persistent transfection. In addition, there are several obstacles to this process, since germ cells are relatively transcriptionally immobile and tend to turn off the expression of the transgene (Seydouxand and Braun, 2006); therefore, the development of effective methods for culturing chicken PGCs without loss of germline competence was the main task, the solution of which provided more opportunities for genetic modification and precise gene editing (Song et al., 2014; Naito, 2015; Whyte et al., 2015; Lee et al., 2016).

The method for creating targeted gene modifications in birds consists of three processes: isolation and cultivation of primordial germ cells (PGCs), modification of the PGCs genome in vitro, and injection of genetically modified PGCs into embryos. The ability of cultured PGCs to retain germline competence after they are introduced back into host embryos is unique to chickens. PGC lines can be grown in culture for more than 150 days without losing germ-line competence, thereby providing a virtually limitless cell resource (Schusser et al., 2013). The isolation and transplantation of cultured PGCs is possible only during the period of their circulation in the circulatory system of the embryo and is limited in time (Nakamura et al., 2017). After culturing of chicken PGCs and making site-specific changes using TALENs or CRISPR/Cas9 nucleases, the modified PGCs are screened by antibiotic selection or fluorescence-labeled cell sorting. Individual cells are then proliferated and sequenced to detect cells with an altered genome. Selected PGCs are injected into the dorsal aorta of recipient embryos so that exogenous edited PGCs can be deposited in the genital ridge along with endogenous PGCs to eventually produce genome-altered offspring. Moreover, exogenous PGCs from various chicken breeds can be introduced into host embryos to preserve and restore rare chicken breeds (Woodcock et al., 2019).

After creating a chimeric chicken using a potential germ line, the chimeras are mated with wild-type partners to produce Wt (+/+) wild-type chickens and Mut (+/–) heterozygous mutant chickens. The offspring of Mut +/– mate with Mut +/– producing chickens Wt (+/+), Mut (+/–) and Mut (–/–) (Fig. 1 according to Lee et al., 2020).

There are a number of studies on gene editing by primordial germ cell (PGC) culture in chicken. Thus, Dimitrov et al. used a combination of CRISPR and HDR to target the chicken immunoglobulin heavy chain locus in cultured PGCs. Using electroporation, the cultured PGCs were modified with two plasmids, one of which encoded gRNA and Cas9, and the other encoded a template for HDR. PGCs containing the desired modifications also had antibiotic resistance for subsequent selection of transduced cells. The modified PGCs were then introduced into recipient embryos to produce chimeric birds, which were raised to puberty and the offspring were evaluated for modification. In this study, it was found that the germline transmission rate of 13 chimeric males averaged 14.5%. The results showed the first successful modification of PGC in vitro using the CRISPR/Cas9 system and a gRNA donor vector for HDR, which opens up many potential applications for efficient genetic modification in birds (Dimitrov et al., 2016).

In the studies of Oishi et al. (2016), a successful work was carried out on the knockout of two genes of the egg protein ovalbumin (OVA) and ovomucoid (OVM) using a plasmid to introduce the CRISPR/Cas9 system into PGC, which allowed them to obtain eggs without ovomucoid proteins and ovalbumin, which significantly reduced egg allergenicity.

Cultured PGCs were transfected with plasmids carrying Cas9/gRNA and a gene encoding antibiotic resistance using lipofection. HDR was not used in this work. NHEJ was relied on to create mutations. Using this approach, the authors found deletions ranging in size from 1 to 21 bp in the *OVM* gene. Interestingly, no

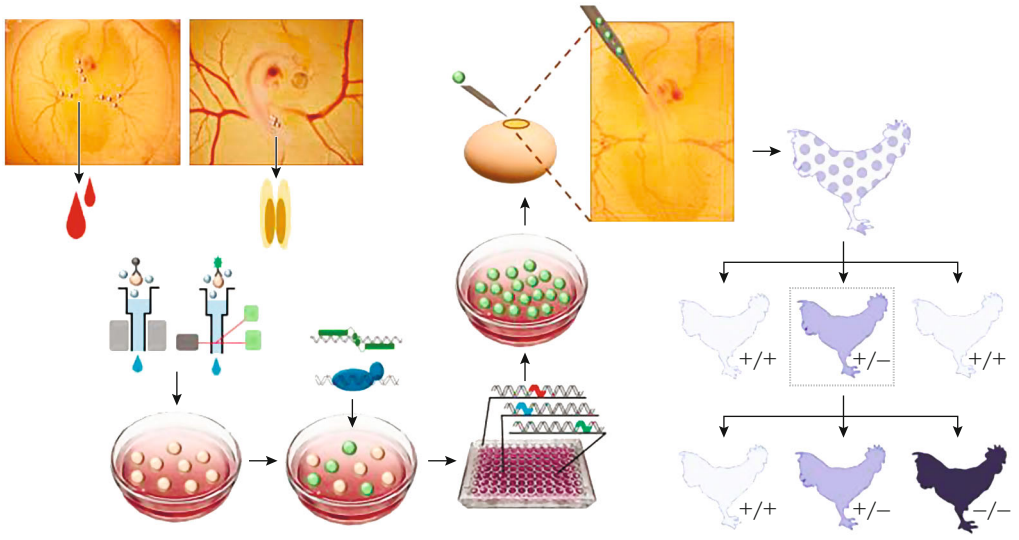

Fig. 1. Primordial germ cell-mediated chicken genome editing (PGC) method (Lee et al., 2020).

deletions were found in the 13 sequenced clones. As in the previously described studies, modified PGCs containing the desired mutations were selected using antibiotic selection prior to injection into recipient embryos. Genomic fragments, including gRNA target sites, were amplified using PCR and sequenced. This analysis showed that the frequency of the desired mutation ranged from 13 to 92%. The modified PGCs were injected into chick embryos that produced healthy offspring. Progeny analysis showed that two males had OVM mutations with 58% germline transmission. In this study, cultured PGCs were obtained from different strains of birds other than recipients permitted for color selection of donor chicks from chimeric males. It was found that 53% of the chickens of donor origin (average 73%) had mutations in the *OVM* gene. In this study, the authors also obtained *OVM* knockout homozygous birds that were healthy but not tested for reproductive ability. In subsequent studies, Oishi et al. (2018) showed that transgenic chickens could potentially serve as bioreactors for the commercial production of recombinant egg white proteins using the CRISPR/Cas9 method mediated by the PGC culture method. The integration of human beta interferon (hIFN-β) in the locus of chicken ovalbumin for the production of hIFN-β in egg white was carried out. Using the CRISPR/Cas9 system, the *hIFN*-β gene was inserted into chicken primordial germ cells, and then chimeric roosters were obtained by classical transplantation of modified PGCs into recipient embryos. Two zero-generation founding roosters produced offspring with an integrated *hIFN*-β gene, and all female offspring produced abundant amounts of hIFN-β egg white (~3.5 mg/mL). Although the female offspring of the first generation were infertile, the males of the same generation were fertile and produced the second generation of females in which the production of hIFN-β in the egg white was comparable to that of the first generation. These results showed that the insertion of an exogen into the locus of chicken ovalbumin leads to stable expression of the exogenous protein deposited in egg white and can be used for industrial applications.

Lee et al. (2019) also reported the production of genetically modified chickens as model organisms using CRISPR/Cas9 and PGCs by introducing a donor plasmid containing CRISPR/Cas9 recognition sites. Using the method of introducing modified PGCs into the recipient embryo, the authors obtained a bird carrying a cassette of the expressing green fluorescent protein (GFP) gene, specifically located on the Z chromosome, which allowed for identify gender during early embryogenesis: male offspring can be distinguished from female offspring by the expression of GFP with characteristic green glow. By allowing identification of male embryos, this model has the potential to prevent huge economic losses resulting from chick culling, and it can also be used to study the molecular mechanisms that govern sex determination. Avian leukemia virus subgroup J (ALV-J) is a serious problem in the poultry industry. Replication of ALV-J depends on a functional cell type one receptor regulating metabolism of Na+/H+ (chNHE1) in the cell. The tryptophan residue at position 38 of chNHE1 (W38) in the extracellular part of this molecule is a key amino acid for the penetration of the virus. Kozlova et al. performed a CRISPR/Cas9-mediated deletion at the locus encoding W38 in primordial chicken germ cells and successfully produced modified birds. Homozygous ΔW38 chicks showed resistance to ALV-J both in vitro and in vivo, in contrast to Δ W38 heterozygotes and wild-type birds, which were sensitive to ALV-J. Removal of W38 had no visible side effects. This study provides evidence for the principle that

editing a receptor gene recognized by a virus can generate resistance to the virus and related diseases. Highly efficient CRISPR/Cas9 gene editing in primary germ cells represents a significant addition to the development of chickens as an important food source and model for biological research.

APPROACHES TO EDITING GENES IN BIRDS WITHOUT CULTIVATION OF PGC

There are reports of obtaining genetically modified chickens by direct transfection of PGC in vivo without their preliminary cultivation.

In 2013, Tyack et al. reported successful in vivo direct transfection of chicken PGC. In this study, they used the miniTol transposon system, consisting of two plasmids: the first plasmid contained the EGFP transgene under the control of the CAGGS promoter and was flanked by the ITR Tol2 (pMiniTol-EGFP), while the second plasmid (pTrans) encoded Tol2 transposase under the control of an immediate early CMV promoter for expression of transposase and subsequent transposition of miniTol-EGFP from the plasmid into the genome of transfected cells. In this study, two plasmids were combined, which were introduced by liposome transfection into embryos at the 14HH stage (approximately 2.5 days of embryogenesis). Using this approach, they were able to create chimeric roosters capable of passing the transgene to the next generation. This study set the stage for the use of direct delivery by injection of a plasmid carrying gene-editing tools, such as TALENs and CRISPR, to generate genome-edited birds. In 2020, Challagulla et al. reported germline targeting to modify the endogenous chicken interferon alpha gene and subunit beta receptor 1 (IFNAR1) through in vivo transgenic expression of high-precision Cas9 (Cas9-HF1) and gRNA in chickens. The authors developed a Tol2 transposon vector carrying the transgenes Cas9-HF1, IFNAR1-gRNA (IF-gRNA), and green fluorescent protein (GFP) (pTgRCG) and tested it on the DF1 chicken fibroblast culture. The plasmid pTgRCG was then directly injected into the dorsal aorta of chick embryos at embryonic day 2.5, targeting circulating primordial germ cells (PGCs). The resulting chimera roosters generated fully transgenic first-generation chickens (G1) with constitutive expression of Cas9-HF1 and IF-gRNA (G1_Tol2-Cas9/IF-gRNA). The spectrum of induced indel mutations in loci targeting gRNA was revealed. In chicken G1_Tol2-Cas9/IF-gRNA, indel mutations were stably inherited by the G2 offspring. Selection of chickens G1_Tol2-Cas9/IF-gRNA resulted in up to 10% transgene-free heterozygous IFNAR1 mutants after zero segregation of the Tol2 insert.

The methods described here provide new possibilities for genome editing in chickens and other avian species in the absence of the possibility of cultivating PGC. The disadvantage of the direct approach to in vivo transfection is the impossibility of enriching the modified population of PGC, which may lead to a lower frequency of obtaining modified G1 offspring from gonadal chimeric males using this approach.

METHODS OF CRISPR/CAS9 SYSTEM DELIVERY INTO CELLS

The CRISPR/Cas9 system can be delivered to the cytoplasm and then to the cell nucleus in several different formats, each with its own advantages and disadvantages. The methods based on viral delivery systems make up a significant part of the delivery approaches for the CRISPR/Cas9 system, such that delivery formats can be described as "viral" or "nonviral." It is noteworthy that the plasmids for the Cas9 protein and gRNA can be included in viral vectors created by nature for the transfer of genetic material, with subsequent expression in the cell. The efficiency of component delivery is largely due to the inherent ability of viral vectors to introduce exogenous genetic material into the cell, which usually leads to high transfection efficiency (Luther et al., 2018). In the CRISPR/Cas9 gene-editing system, which uses viral-based delivery methods, Cas9 and gRNA are packed onto plasmid DNA, which is delivered via a lentiviral or retroviral vector to the target cell. These vectors provide highly efficient but nonspecific integration of genetic material into the host genome. Nonspecific integration has risks associated with inclusion in random regions of the host genome, including a tendency to insert mutagenesis in vital host genes (Luther et al., 2018). The use of adenovirus (AdV) in viral vector delivery systems minimizes the side effect, since AdV shows very minimal potential for integration into the target cell genome (Qin et al., 2019). Since the application of viral approaches faces certain barriers to safety and practicality (Luther et al., 2018), for these reasons, alternative systems, such as some plasmids, mRNA, ribonucleic complexes, and gesicles, have been studied as carriers for the introduction of the CRISPR/Cas9 system into the cell.

A popular approach to editing CRISPR/Cas9 is based on the use of a plasmid encoding the Cas9 protein and gRNA. The advantages of this strategy are simplicity, prevention of multiple off-target transfections, and increased stability. One of the methods of using enriched gRNA/Cas9 plasmids is the transfection of PGCs followed by the introduction of modified PGCs into the host embryos at the appropriate stage. However, this approach also has limitations, such as a large number of off-target effects and the need to deliver the plasmid to the nucleus, which requires the selection of the correct method. For this reason, a wide variety of methods have been developed to facilitate the introduction of plasmids carrying the Cas9 protein and gRNA sequence, mainly electroporation, lipofection, and the use of polyethyleneimine (PEI). Zhang et al. (2017) constructed three gRNAs that were used to knock out the STRA8 gene in DF-1 cells and

chicken embryo stem cells (ESC). The plasmid Cas9/gRNA was introduced into cells by lipofection. The knockout efficiency in DF-1 and ESC cells was 25 and 23%, respectively. The PEI (polyethyleneimine) method was used to introduce the Cas9/gRNA plasmid into chick embryos. Analysis using the T7EI assay showed that the STRA8 gene knockout efficiency in embryos was 12%. Abu-Bonsrah et al. (2016) introduced the Cas9/sgRNA plasmid into chick embryos to edit the DGCR8 gene in embryonic nerve cells using the electroporation method. As a result, HIRA, TYRP1, DICER, MBD3, EZH2, and six other knockouts were obtained in two cell lines (cell lines DF-1 and DT-40) with a similar efficiency (26–68%). In addition to the desired mutations, this direct procedure caused heart deformities in over 41% of individuals. In mutant mice, a decrease in DGCR8 gene expression also led to impaired development of the cardiovascular system (Chapnik et al., 2011). Similar studies were carried out by Zuo et al. (2016), where the authors constructed three gRNAs for knockout of the C2EIP gene and investigated the efficiency of gene knockout in chicken fibroblasts DF-1 and chicken embryo stem cells (ESC). To evaluate the effects of this knockout in cells, they used luciferase single-stranded annealing (SSA) recombination assay, TA clone sequencing, and T7 endonuclease I (T7EI). The results of this analysis showed that the knockout efficiency was 27%. The same gene was knocked out in chick embryos. The aPEI-encapsulated CRISPR/Cas9 vector was introduced to the recipients. Knockout of the C2EIP gene was induced in three of 20 embryos (efficiency 15%), which was confirmed by T7EI analysis and sequencing of TA clones (Zuo et al., 2016). Delivery based on mRNA encoding Cas9 is another widely used approach for introducing the CRISPR genome-editing system into the cell. CRISPR/Cas9 components can also be delivered directly as mRNA carrying Cas9 and gRNA to target cells. Genome editing in cells begins after the expression of the Cas9 protein and the formation of the Cas9/gRNA complex inside the cells. Lower cytotoxicity and transient Cas9 expression was demonstrated by Li et al. (2014) in avian cell lines and primordial germ cells. In addition, smaller off-target effects and easy injection into the cytoplasm to manifest their effects are the main advantages of using this strategy. Unfortunately, mRNA has low stability, which is a disadvantage of this approach.

However, Cooper et al. (2017) successfully demonstrated an alternative strategy for the delivery of Cas9 mRNA and guide RNA to male sperm using lipofection during artificial insemination of chickens. This STAGE method is successfully combined with the CRISPR/Cas9 gene-editing system and uses the ability of sperm to deliver nucleic acids. In the past, researchers have tried to use sperm as a delivery mechanism for transgenic constructs (Collares et al., 2011). Although sperm have proven to be very efficient in delivering DNA constructs, the integration of the transgene into the sperm genome remains a huge obstacle (Ball et al., 2008). This study showed that transfected spermatozoa are detected and fertilized with the transfer of an integrated transgene to the offspring. The targets used for knockout combined the GFP gene, the endogenous doublesex gene, and the mab-3-related transcription factor 1 (DMRT1). The STAGE validation work was carried out using liposome transfection to deliver CRISPR/Cas9 instruments to the cytosol of spermatozoa so that these instruments were active only in newly fertilized embryo and allowed for the modification of genes after syngamia and decondensation of genomic gametes. The maximum efficiency of this method was 26% (Cooper et al., 2017). STAGE is especially relevant for the creation of edited heterozygous and homozygous wild birds in the first generation since modern methods such as the method using primordial germ cells (PGC) require two generations. Editing primordial germ cells (PGCs) in culture with their subsequent transfer into developing embryos (Oishi et al., 2016) and direct in vivo transfection of circulating PGCs into embryos (Tyack et al., 2013) lead to gonadal mosaicism in birds. These birds must be raised to puberty and then mated to produce a bird with the desired genotype in all cells with a transmission rate from 0.5 to 40%. STAGE is designed to induce mutations in the early zygote, preferably in the unicellular zygote, in order to produce full gene knockout animals in the first generation; however, it can also induce gene mutations in the multicellular zygote, leading to mosaicism (Cooper et al., 2017). The STAGE method uses RNA-based components since avian oocytes and early embryos of most avian species are in a state of transcriptional dormancy (Malewska and Olszanska, 1999).

One of the nonviral delivery methods used for gene editing is the RNP format, which consists in direct delivery of sgRNA and Cas9 protein in the form of a Cas9/gRNA ribonucleoprotein complex. The characteristic features are high efficiency of gene editing, reduced off-target effects and less toxicity. In addition, promoter selection and codon optimization are not required. In the work of Lin et al. (2014), PNP Cas9/gRNA in combination with nucleofection (by the method of nonviral transfection of cell lines, using electrical impulses and subsequent introduction of genetic material into the cell) were used to edit the EMX1 gene in HEK 293 T cells, primary human neonatal fibroblasts and human embryonic stem cells. The efficiency of HDR-mediated genome editing was at 38%. A recently developed delivery technology is based on the combination of a complex of ribonucleoproteins (RNP) Cas9/gRNA with nanobubble derivatives called gesicle. Vesicles contain native Cas9 protein in complex with gRNA specific for the gene of interest and glycoproteins on their surface that allow binding and fusion with membranes of a wide range of

target cells. Gesicles contain native Cas9 protein in complex with gRNA specific for the gene of interest and glycoproteins on their surface that allow binding and fusion with membranes of a wide range of target cells. Delivery of the native Cas9 protein means that the target cells lack the gene encoding Cas9, which eliminates the problem of persistent and increased Cas9 expression. These features allow gesicles to provide targeted delivery of the genome-editing complex into cells with higher efficiency compared to plasmid-based delivery methods. Also, the use of this method allows one to control the dose and duration of exposure to the Cas9-gRNA complex in the cell, further reducing the likelihood of off-target effects.

Vesicles are produced by a specially modified 293 T cell line (Gesicle Producer Cell Line, Takara Bio, Shiga, Japan) as a result of cooverexpression of glycoprotein G of vesicular stomatitis virus. The resulting nanobubbles containing target-specific Cas9/gRNA RNP complexes are fused with target cells, after which the Cas9/gRNA complex is released and transferred to the nucleus to perform site-specific gene editing (Hsu et al., 2013). The low capacity of nanobubbles is compensated by the TAMEL technique, which 42-fold increases the active loading of mRNA (1.8 kb) into the gesicles (Hung et al., 2016). Comparison of nanobubble and plasmid technology as a system with two Cas9/gRNA delivery modes for editing the EMX1 gene in HEK 293 T cells was demonstrated by scientists from Takara (Chojnacka-Puchta and Sawicka, 2020). They treated HEK 293 T cells with gesicles transfected with plasmids containing the Cas9 gene sequence and gene-specific gRNA. Four potential off-target loci were selected. The presence of indel polymorphisms was detected using the resolvase digestion system. As expected, delivery by gesicles did not lead to the observed formation of indel polymorphisms outside the locus site in comparison with transfection with plasmids. This versatile tool for genome modification provides a direct and rapid method of delivery to target cells. The first preliminary study on the use of this system for the delivery of the Cas9/gRNA complex into hard-to-transfect and very sensitive primordial germ cells (PGCs) of chicken has been performed. The results confirmed that this system is suitable for PGCs and could be a powerful technique for genome manipulation in chicken PGCs. The new method can reduce adverse events and eliminate problems with constitutive expression of the Cas9 protein in target cells (Chojnacka-Puchta and Sawicka, 2020).

CONCLUSIONS

The development of the technology for editing the chicken genome will allow for the accelerated improvement of the productive qualities of all types of poultry. The new CRISPR/Cas9 method has the potential to address global food security concerns. Establishing meat and egg lines of birds using the latest advances in genome editing can have a significant impact on improving poultry-related performance, such as feed conversion, digestibility, increased egg production, growth, and overall performance improvements. Scientists all over the world are actively using advanced genome-editing technologies to solve applied problems. The use of the TALEN and CRISPR/Cas9 editing systems allowed for obtaining chickens laying eggs with reduced egg white allergenicity as well as to knock out the myostatin gene (MSTN), which can be used in poultry meat farming to increase poultry weight gain. It has been proven that the CRISPR/Cas9 system can be successfully used for the production of recombinant proteins, such as interferon beta (hIFN-β), in chicken egg white. Creation of human immune factors, hormones, etc. using the methods of editing the genome of bird bioreactors will provide an alternative therapeutic approach that will be relevant to the medicine of the future. Innovations in the field of editing the bird genome are promising for such areas of development of poultry farming as improving productivity, creating resistance to infectious diseases, and producing vaccines. This, in turn, will increase the safety of food and vaccine production using chicken eggs, which is an important element in the poultry industry and definitely affects the improvement of human safety. Future applications of CRISPR technology in poultry have promising and enormous opportunities in agriculture and industrial biotechnology that can benefit from the wide range of opportunities for both food and vaccine production and the treatment and prevention of poultry and human diseases.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interests. This article does not contain any studies involving animals or human participants performed by any of the authors.

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