AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1080-1094
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.5:57.08:577.21

doi: 10.15389/agrobiology.2019.6.1080eng
doi: 10.15389/agrobiology.2019.6.1080rus

TRANSGENIC POULTRY: DERIVATION AND AREAS OF APPLICATION
(review)

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The authors declare no conflict of interests
Received October 7, 2019
doi: 10.15389/agrobiology.2019.6.1080eng

Abstract

Transgenic poultry is a powerful instrument for the biotechnologic research in agriculture and medicine as well as a useful biological model (H. Sang, 2004). The technologies of transgenesis can be also aimed at the improvement of qualitative and quantitative characteristics of poultry products (L.G. Korshunova, 2011); development of poultry crosses genetically resistible to infectious diseases (L.G. Korshunova et al., 2014); derivation of poultry that can produce recombinant proteins of different usage areas within the eggs (D. Cao et al., 2015). The most popular way to induce transgenicity is the microinjection of foreign DNA into the ovicell in the proper moment when the organism consists of a single cell (zygote). Certain peculiarities of avian reproduction, however, constrain the induction of the transgenesis. A hen produces daily a single fertilized ovicell which is large in size and extremely sensitive to any manipulation like those to be performed on the mammal ovicells at the injection of foreign DNA. Furthermore, normal embryonic development in avian eggs requires the integrity of tertiary coats — albumen, inner shell membrane, and eggshell itself. The cleavage of chicken ovicell starts as early as in the magnum while freshly laid egg contains ca. 50,000-60,000 cells. As a consequence, first transgenic bird was produced via retroviral vectors. Retroviruses were the first contenders for the role of vectors in the gene transfer since they normally can enter the genomic DNA of the host with subsequent replication. At present the induction of retroviral (D.W. Salter et al., 1986; D.W. et al. Salter, 1987; D.W. et al. Salter, 1989; R.A. Bosselman et al., 1989; L.B. Crittenden, 1991; L.B. Crittenden et al., 1992) and lentiviral (H.A. Kaleri et al., 2011; A.H. Seidl et al., 2013; N.A. Volkova et al., 2015) transgenesis in chicken and quails was reported. The technologies of genomic modification in chicken and quails are continuing their development: e.g. the methods with the use of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) (T.S. Park et al., 2014), CRISPR/Cas9 (I. Oishi et al., 2016; Q. Zuo et al., 2016) were developed. The CRISPR/Cas9 technology allows for the further progress in the genetic manipulations to produce genome-edited lines of poultry (N. Veron et al., 2015). Avian embryos, primarily chicken (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*), served as a model for the embryologic studies in vertebrates for more than a century. Modern targeted genetic manipulations in chicken embryo as an in vivo model became possible via the CRISPR/Cas9 editing system (V. Morin et al., 2017). The alternative non-viral methods of the induction of transgenesis in avian species can be used; one of these methods involves the transfer of foreign embryonic cells (as foreign DNA vector) into the recipient embryo resulting in the chimeric birds (J.N. Petitte et al., 1990; J.Y. Han et al., 2017; N.A. Volkova et al., 2017). Another attractive technology for the transfer of foreign genetic material into avian embryos involves the use of spermatozoa as the vectors since artificial insemination is the traditional and common technique for poultry (E. Harel-Markowitz et al., 2009; A.V. Samoylov et al., 2013). The combination of spermatozoa vectors and CRISPR/Cas9 technology can result in transgenesis in the first generation and hence allows for the substantial savings in time and resources (C.A. Cooper et al., 2017). The microinjection of DNA into the zygote is still a classic technology of non-viral transgenesis. The method involves direct injection of gene construct into the cytoplasm of freshly fertilized ovicell and subsequent incubation of injected eggs. The ovicell for injection should be taken immediately after its fertilization which means that its movement down the oviduct should be interrupted; further development of the injected embryo require special cultivation system (C. Mather, 1994). Another microinjection technique for avian ovicells involves natural formation of tertiary coats in the oviduct. The method is based on the surgical operation to get the access to the ovicell, its microinjection with foreign DNA, and implantation of injected ovicell back

to the maternal oviduct for the formation of normal egg suitable for incubation (R.V. Karapetyan, 1995). The populations of transgenic chicken and quails with different foreign gene constructs were produced with the use of this method (R.V. Karapetyan, 1996; L.G. Korshunova et al., 2013).

Keywords: transgenesis, poultry, retroviruses, microinjection, primordial cells, sperm cells, genome editing

In the age of the developed poultry industry, the number of poultry species used in production has decreased significantly. Genotypes selected for certain conditions have become the most profitable but when market requirements change, the economic necessity for species and lines with new properties appears.

Genetic engineering provides powerful tools for changing living organisms. Since there is no species barrier for the integration of foreign genes, transgenic individuals of a specified type that cannot be obtained by classical breeding methods can be created. Transgenic organisms differ from natural ones in that they are obtained by genetic engineering methods and contain foreign genetic engineering material in their genome. They are capable of reproducing and transferring artificially altered genetic material to offspring. Transgenesis technologies in poultry farming can be aimed at improving the qualitative and quantitative characteristics of poultry products; developing poultry crosses genetically resistible to infectious diseases; deriving poultry that can produce recombinant proteins of different usage areas within the eggs. For practical use, a transgenic bird must have a phenotype that exceeds the level already achieved in poultry farming. Such signs may include an increase in growth rate, improved feed conversion, increased egg production, reduced body fat, increased disease resistance, etc. In comparison with traditional breeding methods, when the transfer of the desired gene from one bird line to another requires subsequent multiple backcrosses to remove unnecessary genes inherited during natural sexual hybridization, transgenesis gives a long-term advantage in time. In contrast to traditional breeding, transgenesis provides a unique opportunity to obtain a bird that produces and accumulates proteins useful for medical and industrial purposes in the egg, which can also have a wide practical application [1-4].

The purpose of this review is to summarize data on bioengineering methods for the creation and possible applications of transgenic poultry.

Research to develop practical methods of transgenesis in poultry has been ongoing since the creation of a transgenic mouse in the 1980s. The first manipulations with the chicken zygote initiated the development of more successful technologies [5]. Currently, there are several methods of genetic modification of the body. Most often, the microinjection of foreign DNA into the ovicells is used. At the same time, it is important to ensure that the transgene is contained in all cells of the body and obligatorily in the germ cells (for transfer to offspring). Genes are transferred at the earliest stages of the body's development, including the zygote stage. However, the peculiarities of birds breeding create serious problems here. A hen produces daily a single fertilized ovicell which is large in size and extremely sensitive to any manipulations. Normal embryonic development in avian eggs requires tertiary coats – albumen, inner shell membrane, and eggshell. The cleavage of chicken ovicell starts as early as in the magnum while freshly laid egg contains ca. 50,000-60,000 cells.

The first transgenic bird was produced via retroviral vectors. Retroviruses have become the first contenders for the role of vectors in the gene transfer since they normally can enter the genomic DNA of the host with subsequent replication. Many researchers have tried to introduce foreign genes into the germ line, infecting embryos with both replication-capable and non-replicating retroviral vectors [6, 7]. The frequency of successful embedding of the transgene in these exper-

iments ranged from 0.8 to 5%. A positive result on the transfer of genetic material to the germ line was obtained by introducing a recombinant vector of avian leukemia into the blastoderm of incubated chicken embryos [8, 9]. Complete transgenes were created with the help of the recombinant virus [10-12].

In the work of Lee *et al.* [3], the possibility of using chickens as a bioreactor to produce a human urokinase-type plasminogen activator (*huPA*) was studied. The recombinant *huPA* gene under the control of the Rous sarcoma virus promoting agent was injected into freshly laid eggs at stage X using retroviral vectors based on the mouse leukemia virus. In general, 38 chickens from 573 eggs injected with the virus hatched and contained the *huPA* gene in different parts of the body. The mRNA transcript of the *huPA* gene was present in various organs, including blood and ovicell, and was a germ line passed on to the next generation. The content of the active *huPA* protein in the blood of transgenic poultry was 16 times higher than that of non-transgenic poultry ($p < 0.05$). *HuPA* protein expression in eggs increased from 7.82 IU/egg in generation G_0 to 17.02 IU/egg in generation G_1. However, embryos expressing *huPA* had reduced survival and hatchability on Days 18 and 21 of incubation. In transgenic chickens, blood clotted much more slowly than in non-transgenic counterparts ($p < 0.05$). In addition, adult transgenic roosters had reduced fertility ($p < 0.05$): ejaculate volume, spermatozoa concentration and viability decreased. As a whole, the obtained data suggest that transgenic *huPA* chickens can be successfully obtained using a retroviral vector system. Such chickens can be used as a bioreactor in the production of the pharmacological *huPA* drug for the treatment of vascular diseases, as well as in the study of *HuPA*-induced bleeding and other disorders. Urokinase-type plasminogen activator (uPA; Swiss-Prot: P00749) – inducible serine protease (EC 3.4.21.73) found in blood and extracellular matrix plays an important role in fibrinolysis, extracellular proteolysis, and tissue remodeling.

In chicken transgenesis, the advantages of using a viral vector that carries its integrase were identified, which can be applied to embryos in newly laid eggs. This formed the basis for a more effective method using lentivirus vectors [13-16]. Byun *et al.* [4] used lentiviral vectors to create transgenic chickens that express the human extracellular superoxide dismutase (hEC-SOD) gene. Recombinant lentiviruses were injected into the sub-germ cavity of freshly laid eggs. The embryos were then incubated before hatching using phases II and III of the ex vivo surrogate shell culture system. Sixteen chickens (G_0) out of 158 such embryos hatched. In one of them, the polymerase chain reaction (PCR) method was used to identify a transgene in the germ line cells that form germ cells. This transgenic bird (G_0) was crossed with a normal one and, as a result, two transgenic chickens (G_1) were obtained. In G_2 transgenic chickens, the hEC-SOD protein was contained in egg white and showed antioxidant activity.

The ability to manipulate gene expression when creating transgenic chicken embryos using viral vectors has proven useful for analyzing gene functions in tissues. Modern transgenesis technologies using lentiviral vectors have significant potential for genetic research on embryos and adult birds. One of the main problems in vertebrate embryogenesis is to understand how differentiation and complete anatomical development are coordinated in organs consisting of many cell types, and how differences in gene expression affect the phenotype. These and many other issues can be solved by using transgenic bird lines [2, 17].

Currently, retroviral and lentiviral transgenesis is used in poultry. Lentiviral vectors have been successfully used to create several transgenic lines of chickens and quails [5, 18-21]. The development of this technology continues [22, 23]. However, despite the effectiveness of viral transduction, there are many

obstacles to its application due to the relatively low and variable frequencies of transgene transfer along the germ line, as well as safety problems when using viral vectors. Therefore, obtaining transgenic poultry using non-viral technologies remains a necessary condition for applying transgenesis in poultry farming practice [24, 25].

Effective genome modification technologies are currently available and continue to be developed: zinc finger nucleases method (ZFNs – "zinc fingers"), TALENs (transcription activator-like effector nucleases) [26], CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats – short palindromic repeats arranged in groups regularly) [27, 28]. All techniques of genomic engineering are available for poultry.

The possibilities and consequences of using CRISPR/Cas9 are being studied actively [29]. CRISPR/Cas9 is a revolutionary genome-editing system that provides a powerful tool for studying the molecular mechanisms that regulate development [30-32]. Recent achievements using programmable nucleases have increased the efficiency of making precise changes to the genomes of eukaryotic cells significantly. Genome editing technologies allow expanding knowledge about genetic diseases and create more accurate models of pathological processes [33]. The prospect of direct correction of genetic mutations in affected tissues and cells for the treatment of diseases that do not respond to traditional therapy is particularly tempting [34]. CRISPR/Cas9 can be used to accelerate progress in the genetic manipulation of poultry and create lines with the edited genome [35].

Let us note that due to the similarity in general ontogenesis and gene expression patterns, the bird can serve as an excellent model for sequestering genetic, molecular, and biochemical processes in mammals, including humans. For more than a century, bird embryos, mainly chicken (*Gallus gallus domesticus*) and quail (*Coturnix japonica*) have been the basis for studying vertebrate embryology, cell movement in morphogenesis, mechanisms of induction and differentiation, the pathogenesis of embryonic diseases, and toxicology. Transgenic birds remain one of the most powerful research tools in biotechnology.

Targeted genetic manipulations on a chicken embryo as an in vivo model were made possible by the CRISPR/Cas9 system [36-39]. Gandhi *et al.* [40] optimized CRISPR/Cas9 using a three-step strategy applied to early embryos. First, the authors used Cas9 with two signal sequences to improve nuclear localization. Second, due to the modified RNA (gRNA), there was no premature termination of transcription, and Cas9-gRNA interactions were stable. Third, the involved chicken-specific U6 promoting agent provided 4 times higher gRNA expression than human U6. For rapid in vivo gRNAs screening, a cell line of chicken fibroblasts expressing Cas9 was also created. To prove the principle, the authors conducted electroporation studies of loss of function in an early chicken embryo during protein knockout of Pax7 and Sox10, key transcription factors with a known role in the development of the neural crest. CRISPR/Cas9-mediated deletion has been shown to cause loss of the corresponding proteins and transcripts. The results confirmed the usefulness of the optimized CRISPR/Cas9 method for targeted gene knockout in chicken embryos in a reproducible, reliable, and specific way [40]. CRISPR/Cas9 can be combined with other methods of studying gene function in chicken and quail embryos, for example, with electroporation (in particular, for targeted editing of genes of the genitourinary birds' system) [41, 42].

Among other methodological approaches for virus-free transgenesis in birds, it is possible to note the production of chimeras when transplanting foreign cells into embryos. Early blastoderm cells or primordial cells are isolated,

foreign DNA is injected into their nuclei, then the cells are implanted in embryos, where they survive and divide [43-47]. Thus, Petitte *et al.* [43] obtained blastodermal cells from chicken embryos of the Barred Plymouth Rock line (it has a black pigment in the feathers due to a recessive allele at the I locus) and introduced them into the sub-germ cavity of embryos from the inbred Dwarf White Leghorns line with white feathers due to the dominant allele at the I locus. Six (11.3%) of 53 such embryos of the Dwarf White Leghorn line turned out to be phenotypic chimeras in feather color, one of them (cockerel) survived to hatch. In recipients, the distribution of black feathers varied and was not limited to any specific area, although in one case they prevailed on the head. This somatically chimeric rooster was cross-bred with several Barred Plymouth Rock hens to assess the extent to which donor cells were included in the testes. Two of received 719 chicks were Barred Plymouth Rocks by phenotype. It means that cells capable of turning into germ line cells were passed on to recipients. The DNA fingerprint technique from the blood and sperm of germ chimeras showed that both of these tissues differed from those of the inbred Dwarf White Leghorns line. Somatic chimeras and germ-line chimeras were obtained in the same way [43, 48, 49]. Chimeric individuals were raised and their offspring were removed from them. Some of the offspring were transgenic.

These and similar studies [20, 50-54] show that the isolation, transfer, and introduction of primordial germ cells can be used to produce transgenes in poultry farming. However, the described technology [43] is quite complex and expensive. Modern progress in isolation of primordial germ cells and cultivation opens up new opportunities for reproductive biotechnologies in poultry farming [55-57]. Methodological techniques for transferring foreign DNA with embryonic cells (including using CRISPR/Cas9) continue to be developed. For example, Nakamura *et al.* [58] showed that X-ray irradiation reduced the number of endogenous primordial germ cells and increased the transmission of transferred primordial germ cells in chimeric chickens. Reducing the number of endogenous primordial germ cells is also possible when using busulfan [59, 60]. Genetically modified primordial germ cells were transplanted directly into the testicles of sterilized adult roosters. Their ability to mature into functional spermatozoa and restore spermiogenesis was shown [61].

Transgenesis with spermatozoa as vectors for foreign genetic material to the zygote looks very tempting since artificial insemination is widely used in poultry farming. Studies have been carried out on the use of sperms as a vector for the delivery of foreign DNA to the zygotes of chickens. It has been shown that lipofectin interacts with DNA, forming lipid-DNA complexes that can connect to the plasma membrane of the cell, contributing to the ingress of DNA into the cell. In the case of lipofectin application, 51.6% of the sperm of the rooster showed the presence of exogenous DNA. When artificial insemination of chickens with sperms transfected using lipofectin was carried out, exogenous DNA was observed in the blastoderm of 67% of eggs. However, the presence of foreign DNA in the genome of hatched chickens was not detected, although its episomal integration was shown. To increase the efficiency of sperm-mediated gene transfer (SMGT), the method was supplemented with the use of restriction enzyme-mediated integration. Linear DNA, together with restriction, penetrates the target cell by lipofection or electroporation. Restrictase is expected to specifically cut genomic DNA to facilitate the integration of exogenous DNA with complementary sticky ends. Lipofection of plasmid DNA with a restriction enzyme is considered a highly effective method for obtaining transfected sperm for artificial insemination [62]. Using liposomes, chickens with the human granulocyte colony-stimulating factor gene were created by artificial insemination with

transfected sperm. The share of transgenic chickens was 33.3%, and the frequency of a foreign gene inheritance was 37.5% [63].

To use rooster sperms as vectors, the permeability of sperm membranes under various electroporation regimes was studied. The range of physical and chemical effects is determined, within which the formation of pores in the sperm shell is observed, and the sperms themselves retain motility. An increase in the amount of trypan blue dye deposited with rooster sperms after electroporation is shown, with an increase in the number of electrical pulses and a decrease in the osmolarity of the electroporation medium. Parameters that can be changed to increase the efficiency of the electroporation are determined [64]. However, the passive transfer of non-viral constructs via rooster sperm does not always lead to the appearance of transgenic individuals [65]. Although the successful transfer of an exogenous gene using spermatozoa has been shown in birds, there are few studies in which the expression of a foreign gene has been observed. It is assumed that if DNA is not internalized in the sperm nucleus, but only delivered in the ovicell cytoplasm by it, the DNA copies in the form of plasmids are found in early embryos, but not detected in chickens.

The inability to obtain stable and diverse modifications through SMGT has led to the need to look for other technologies. Wang *et al.* [66] successfully isolated haploid embryonic stem cells (haESCs) with the genome of highly specialized gametes. They can consistently maintain haploidy (through periodic cell sorting based on the amount of DNA), self-renewal, and pluripotency in the transplanted cell culture. In particular, haESCs derived from androgenetic haploid blastocysts (AG-haESCs) that carry only the sperm genome can provide generation of SC (semi-cloned) animals by injection into oocytes. It is noteworthy that after removing the imprinted control regions of H19-DMR (a differentially methylated section of DNA) and IG-DMR by double knockout (DKO) when using DKO-AG-haESCs blastocysts, it is possible to obtain SC-animals with high efficiency, i.e., DKO-AG-haESCs serves as the equivalent of sperm. It is important that DKO-AG-haESCs can be used for multiple rounds of in vitro gene modification to create generations of fertile animals with specified genetic characteristics. Thus, DKO-AG-haESCs ("artificial spermatozoa") modified using CRISPR/Cas technology can become a convenient fertilization agent for effectively obtaining genetically modified offspring and serve as a universal tool for analyzing the function of genes in vivo.

However, using sperms to deliver foreign DNA to ovicells in combination with CRISPR/Cas9 allows the creation of a first-generation transgenic bird, which significantly saves time and resources. In addition, the developed methods can be easily adapted to different types of birds [67].

The classic technology of virus-free transgenesis is the microinjection of DNA into the zygote. Its use in poultry is complicated due to the too large size of the oocyte. The possibility of incubating in vitro fertilized chicken ovicells extracted from the upper part of the protein section of the oviduct was studied [68]. Since the embryo is single-celled at this stage, it is theoretically possible to micro-inject foreign DNA into such an embryo. A fertilized chicken ovicell can be cultured in a shell with the protein of another egg [68]. The method includes three stages (taking into account the different needs of the embryo during development) and involves using the eggshell as a "vessel" for cultivation. The initial version involved culturing in glass vessels for the first 24 hours, followed by moving the embryos from vessel to vessel (from shell to shell) for cultivation between stages I and II, II and III. Further on, the method was simplified by culturing the embryo at the first stage of development in the shell, which eliminated the need to transfer it from the glass vessel to the shell. As a result, the number of

viable hatched chickens was 20%. The described cultivation or its separate stages can be used for various experimental purposes since this technique gives access to the developing embryo at all stages of its development [69].

As a method of creating a transgenic bird, the possibility of injecting DNA with further embryo cultivation was considered. The gene construct with the reporter gene β-galactosidase was injected. The expression of the gene construct in the first 7 days of development was studied histochemically by the manifestation of β-galactosidase activity in embryonic tissues. Cells with the β-galactosidase activity were observed at the stage of 250-500 cells in the center of the blastodisc. On Day 2, they were visible in large segments of the blastoderm, at later stages – in the corresponding smaller segments of extra-germ shells. β-galactosidase-positive cells were in most cases scattered around the primitive blastula band, but after gastrulation, they were observed in embryonic tissue in only 7% of embryos. This indicates transcriptional activity at the crushing stages and confirms data on the loss of foreign DNA during the early development of the chicken. The results of exogenous DNA injection at the stage of a single cell with further embryo cultivation showed that the inclusion of injected DNA in chicken chromosomes was a fairly rare event. The lack of integration into the genome can be hidden by the presence of extra-chromosomal copies of injected plasmids [68].

The approach successfully used in creating a transgenic bird [70] is an injection of a gene construct into the cytoplasm of a freshly fertilized chicken ovicell with its incubation before hatching. Ovicells for injection were extracted from the upper part of the protein section of the oviduct already covered with a thin layer of protein, but not the shell. The culture system has been improved to optimize the survival of the injected ovicell. The system is still three-stage, the eggshell is used as a vessel for cultivation, and the liquid protein diluted with a salt solution is used as a nutrient medium. At the first stage of cultivation, which lasts 24 hours, the injected ovicell is placed in a shell containing such an amount of nutrient medium that the germ disk is not immersed in it. At this stage, the embryo develops from one cell to the blastoderm (60 thousand cells), which is typical for laid fertilized eggs. Then, the shell is filled with diluted egg white until the embryo is completely submerged, and the window in the shell is sealed with tape. At this second stage, the embryo should develop a heart and extra-fetal blood circulation within 65 hours. At the third, the longest stage, which lasts until hatching, the contents of the shell are moved to another, larger shell. This is done to create the air space needed during the paraphytic period when the embryo begins to breathe with the lungs and prepares for hatching. For 1-2 days before hatching, with the beginning of pulmonary ventilation, the sealing film is perforated for free access of air to the shell. Further on, the film is loosened so that the chicken can come out of the shell. Thus, incubation at the first stage takes place in the surrogate oviduct – the incubator, where the bird's body temperature (42 °C), high relative humidity and high concentration of carbon dioxide are maintained. At the end of the first stage, there is a short period at room temperature to imitate egg-laying. The incubation conditions during stages II and III, equivalent to the incubation period of normal chicken eggs, are changed in comparison with traditionally used in poultry farming. The relative humidity is increased to about 75% (up to 18 days of incubation), then it is reduced to about 65% (before the hatching stage). During stage II, the frequency of rotations is increased (4 times per hour). At stage III, the angle of rotation is reduced so that the embryo does not touch tape that covers the window in the shell.

Creating transgenic chickens using the described method is quite time-consuming. Only 50% of injected eggs reach stage III, and the percentage of

hatched chickens is about 15%. However, the results of the analysis of embryos and chickens for transgenicity are encouraging. When using PCR, it was shown that the DNA of almost 50% of embryos and chickens that reached the last Day 12 of cultivation contained a transgene. Thus, two of the hatched chickens were found to have a transgene in the chorioallantoic membrane, rib pulp, and blood. In one of the roosters, the transgene was preserved until puberty. Its sperm contained a transgene (reporter gene) that was passed on to 3.4% of the offspring, i.e. 14 of the 412 chickens [70]. Thus, transgenic chickens were created by this method and the inheritance of the transgene was shown. All primary transgenic individuals were mosaic. In subsequent generations, the inheritance of the transgene corresponded to Mendel's laws. According to the authors' reports, about 60% of embryos [70] survived the hatching stage. However, when using this method in other laboratories, the same indicator did not exceed 3-10% [71].

Another variant of DNA microinjections in bird ovicells involves the formation of tertiary ovicell coats (protein, subshell, and shell) naturally in the bird's oviduct. To access the ovulated chicken ovicell, it is necessary to define the time when the abdominal cavity should be opened. In the bird used in the experiment (the work was carried out on White Leghorn chickens at the age of 180-300 days), ovulation occurred 20-25 minutes after egg-laying. Approximately 5 minutes after ovulation, the ovicell is located in the funnel of the oviduct completely, where it is fertilized. The time until the ovicell is completely moved to the protein section is a few minutes. An ovicell extracted from the abdominal cavity or the oviduct was injected and placed through the funnel into the protein section of the oviduct with or without a specially designed expander [72].

In the ovulatory process, the oviduct funnel is active in the chicken immediately before ovulation. In this state, it is sufficiently elastic and strong to place the transplanted ovicell in it. The active state of the funnel is observed for about 5 minutes, after which it becomes extremely loose and damaged when stretched. In such cases, either the ovicell was lost due to mechanical damage when trying to return it to the compressed oviduct funnel, or transplantation became impossible due to ruptures of the funnel itself. Only half of the implanted ovicells produced morphologically normal eggs.

To exclude the stages of ovulating ovicell extraction and its reimplantation into the oviduct, it was proposed to inject DNA into the blastodisc of the ovicells through a sufficiently transparent shell of the oviduct funnel. As a result, ovicell losses were reduced to almost zero, and the number of morphologically normal eggs was close to 100%. Thus, the effectiveness of the method has increased significantly [73, 74].

The techniques described above make it possible to obtain no more than one viable ovicell injected with foreign DNA from a single chicken in a single operation. An option that allowed a sharp increase in the number of such ovicells for each operation was an injection into the ovicells located in the ovary, in other words, into the follicles [75]. The chicken ovary has 4-5 large follicles, which are quite suitable for microinjections. However, this approach increases the technical difficulties. Surgical access to the ovary is somewhat more difficult than to the oviduct, and the follicle membranes are tougher and thicker than those of the oviduct funnel. Nevertheless, the positive aspects of the proposed option prevail. In addition, there is no need to set ovulatory cycles of poultry, which simplifies the preparation and conduct of the experiment, and the complexity of visual control during microinjections is easily overcome due to the use of modern ophthalmological and otolaryngological optics and microtechnics. Thus, hypothetically, it is possible to process and obtain 4-5 injected foreign DNA ovicells from each chicken for each operation, and without possible prob-

lems associated with non-fertilization of ovicells or abnormal formation of tertiary coats.

To assess the possibility of DNA microinjections into the follicles, it was necessary to make sure that an ovicell was formed from the injected follicle, and that a full-fledged egg was formed from it. To do it, a dye – ink or methylene blue was injected into the yolk of the follicles. Eggs were collected and opened from operated chickens for 12 days. The obtained results showed that eggs were formed from injected follicles. After 7 days, the dye in the eggs was never observed. It should be noted that surgery and injections can disrupt the order in the follicle hierarchy, according to which the largest follicle should ovulate first. Quite often, the dye was found in eggs laid not among the first, but later, although it was always injected into the three largest follicles. Autopsy of the chickens showed that the part of follicles has been reabsorbed. Quite often, only traces of the dye were observed in the eggs, which means that it is removed from the follicle during maturation. It is possible that some eggs marked as not containing dye came from injected follicles. Although the obtained results are not unambiguous enough for strict conclusions about the hierarchy of follicles, the order of their ovulation, etc., one can conclude that on average, at least 50% of eggs laid by chickens in the first 7 days after surgery come from injected follicles.

The developed surgical operations and methods of manipulating the eggs of chickens and quails allow making microinjections of DNA into the ovicell and ensure its further normal embryonic development. Using these technologies, transgenic chickens [76-78] with different gene structures and quail with the bovine somatotropin gene were created. At the same time, transgenic quails had obvious phenotypic differences from native quails. Integration of the bovine growth hormone gene into the quail genome increased their immune status, resulting in accelerated antibody production. The LaSota vaccine strain of the Newcastle disease virus was used as an antigen [79]. Another most notable phenotypic feature was large eggs laid by quails. The average weight of eggs in the experimental quail population was 20-50% higher than normal. The live weight of transgenic quails was 5-15% higher than that of both typical for the breed and the control group of native individuals. These phenotypic features were preserved in all 33 studied generations of transgenic quail offspring [80].

Thus, some progress has already been made in creating a transgenic bird based on several methodological approaches (retroviral and lentiviral vector systems, virus-free transgenosis using sperms, "artificial spermatozoa", microinjections of foreign DNA into a zygote, a freshly fertilized egg or follicles). However, the development of transgenesis in poultry farming is mostly still at an early stage, although all known genetic engineering technologies can be used in poultry, including actively developed methods of genomic editing based on CRISPR/Cas9, which are close to the natural mechanisms of action of these systems in cells. Modern leaders in successful genome editing developments are the United States, China, and the United Kingdom; such studies are also being conducted in Russia. This modern technology can be useful in solving a range of scientific and practical problems, including the creation of new breeds of poultry. However, it is not fully clear yet what are the consequences of bioengineering manipulations with the genome, including genomic editing to correct an existing feature or introduce a new one.

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