AGRICULTURAL BIOLOGY,
2020, V. 55, № 2, pp. 306-314
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:575.2.084:577.21:57.086.86

doi: 10.15389/agrobiology.2020.2.306eng
doi: 10.15389/agrobiology.2020.2.306rus

GENETIC MODIFICATION OF ROOSTERS' GERM CELLS USING VARIOUS METHODOLOGICAL APPROACHES

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The authors declare no conflict of interests

Acknowledgements:

Supported financially by Russian Foundation for Basic Research, grant No. 18-29-07079 and the Ministry of Science and Higher Education of Russia, theme No. AAAA-A18-118021590132-9

Received January 13, 2020

Abstract

Gonad germ cells of male farm birds are considered as promising target cells for introducing recombinant DNA for the purpose of targeted genetic modification of their genome. Germ cell precursors (primordial germ cells and spermatogonia cells) are of most interest for modifications. The transplantation of these transformed cells and their successful colonization in the gonads of recipient individuals would allow a population of transformed mature germ cells — sperm — to be obtained, which can be used for female insemination in order to obtain transgenic offspring. The results of studies on the genetic modification of rooster germ cells by transfection of primordial germ cells in embryos and spermatogenous cells in the testes were presented in this work. The novelty of the research consists in the development and optimization of individual stages of local transformation in roosters' spermatogenic cells in vivo to obtain a genetically modified sperm. Our aim was to evaluate the effectiveness of the genetic transformation in roosters' germ cells using various methodological approaches. The study was carried out with poultry (*Gallus gallus domesticus*) of the Russian White breed. Primordial germ cells were isolated from 6-day-old embryos. The resulting culture of PGCs was transformed by electroporation using the Neon system (Thermo Fisher Scientific, USA). For transfection, the ZsGreen1-N1 plasmid (Addgene, USA) with the *ZsGreen* gene under the CMV promoter was used. Transformed cells in the amount of 400, 700 and 1000 were introduced into the dorsal aorta of 2.5-day-old embryos. The embryos of the control group were injected with DMEM growth medium in the dorsal aorta. To transform spermatogenic cells in vivo, a viral preparation was used, which was injected directly into the testes of roosters by multiple injection. The introduction of the viral drugs was carried out once at the age of 3 or 4 months and twice at the age of 3 and 4 months. The viral preparation at a concentration of 1×10^7 CFU/ml was introduced at the rate of 0.5 ml per testis. The lentiviral vector contained the *ZsGreen* reporter gene under the CMV promoter. Histological sections of the testes from experimental males were obtained and analyzed to assess the efficiency of colonization and development of donor primordial germ cells (PGCs) in the gonads of recipients, as well as to evaluate the effectiveness of spermatogenic cell transformation in vivo. As a control, we used histological sections of the testes from non-transgenic roosters, selected on the basis of analogues (age, breed). The fertilizing ability of the sperm from experimental roosters and the proportion of embryos with *ZsGreen* gene expression were evaluated. The transformation efficiency of target cells was determined by expression of the *ZsGreen* reporter gene using a Nikon Ni-U microscope (Nikon, Japan). The chicken embryonic cell culture obtained in the first stage of the experiment consisted of the several types of cells. The proportion of PGCs did not exceed 3%. The percentage of PGCs in the cell suspension increased to 81% after separating the different types of embryonic cells by adhesion. The PGCs culture transformation efficiency was 12 %. The presence of fluorescent spermatogenic cells in the testes seminiferous tubules was established both with the introduction of transformed donor PGCs and with a lentiviral vector. With the introduction of donor PGCs at a concentration of 400, 700 and 1000 cells per embryo, the percentage of chickens with transformed germ cells was 16%, 23% and 26%, respectively. With the twofold introduction of the viral drug into the testes at the age of 3 and 4 months, the highest transformation efficiency of spermatogenic testicular cells in vivo was established, which amounted to 10%.

With a single injection of the viral drug, this indicator was 2 times lower. The possibility of using the obtained individuals with transformed germ cells to obtain transgenic offspring is shown. The efficiency of obtaining transgenic embryos is 6-10%.

Keywords: roosters, embryos, primordial germ cells, spermatogenic cells, lentiviral vector, transgenesis

Male germ cells can be used for targeted delivery of recombinant DNA to generate genetically modified individuals [1, 2]. The success of this largely depend on the improvement of artificial insemination method, which greatly enhance potential of using genetically modified semen to generate population of individuals with desirable traits. The genetic transformation of male germ cells makes it possible to purposefully affect specific target cells, reducing the risks of transgenic mosaics unable to produce transgenic offspring. Primordial germ cells (PGCs, the germ cell precursors) [3, 4], spermatogonia (undifferentiated male germ cells) [5, 6] and sperm (mature male germ cells) [7] can be used as targets in genetic transformation of male germ cells of farm birds.

PGCs and spermatogonia are the most effective in producing transgenic and chimeric individuals, since these cells, during differentiation, can form a significant population of transformed mature germ cells [8, 9]. An in vitro culture of poultry germ and spermatogenic cells allows the number of techniques to be involved in delivering recombinant DNA into target cells with the use of liposomal transfection [10], electroporation [11, 12], transposon-mediated manipulations [13), cationic polymers [14], lentiviral (15) and retroviral vectors [16, 17].

PGCs and spermatogonia donor cells, after isolation and transformation, should be transplanted into the gonads of recipients to subsequently generate offspring with the acquired trait. Colonization of donor germ cells during their transplantation into the gonads of male recipients have been initially shown in laboratory animals [18, 19]. There are reports on efficiency of donor germ cell transplantation in various farm animals, i.e. in pigs [20, 21], sheep [22], goats [23], and bulls [24]. For poultry, works have been done on roosters [25, 26] and quails [27].

The novelty of this research is the development and optimization of particular stages of local transformation of rooster spermatogenic cells by transfecting PGCs of embryos and testicular spermatogenic cells during early differentiation.

Our goal was to evaluate the effectiveness of various techniques in genetic transformation of rooster's germ cells.

Materials and methods. Primordial germ cells were isolated from 6-day-old embryos of Russian White chicken (*Gallus gallus domesticus*). The embryos were subjected to mechanical dissection/dissociation and enzymatic dissociation with a 0.05% trypsin solution. The resulting cell suspension was cultured in Petri dishes in DMEM growth medium (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific, USA) with high glucose concentration (4.5 g/l), bovine fetal serum (10%), glutamine (2 mM), 2-mercaptoethanol (10^{-6} mM) and gentamicin (50 μg/ml).

PGCs transformation was carried out by electroporation (a Neon system, Thermo Fisher Scientific, USA). ZsGreen1-N1 plasmid (Addgene, USA) with *ZsGreen* gene under the control of a CMV promoter was used for transfection. Transformed cells (400, 700, and 1000 cells for test groups I, II, and III, respectively) were injected into dorsal aorta of 2.5-day-old embryos, which were incubated until hatching (RCOM Maru 190 Deluxe MAX, Rcom, South Korea). The chicks, when aged 1.5-2-month, were slaughtered, and the reproductive organs were sampled to assess the effectiveness of germ cell transformation. In the control, DMEM growth medium was injected into the dorsal aorta of embryos.

For in vivo transformation of spermatogenic cells, a lentiviral vector preparation (1×10^7 CFU/ml) was injected directly into the testes of roosters (0.5 ml per testis), once at the age of 3 months (group I), once at the age of 4 months (group II), and twice at the age of 3 months and 4 months (group III). The lentiviral vector contained *ZsGreen* reporter gene under the control of a CMV promoter. Upon reaching maturity, testicular tissues were sampled, and histological sections were prepared (a Shandon Cryotome E cryostat, Thermo Fisher Scientific, USA). Non-transgenic roosters of the same age and breed were control.

The roosters with donor germ cells (group I with transforrmed PGCs introduced into embryos) and their own transformed germ cells (group II with the viral vector injected into the testes) were used to inseminate the females. The fertilizing ability of the semen and the proportion of embryos with *ZsGreen* gene expression were evaluated to assess the transgenic offspring production.

Target cell transformation efficiency was determined by expression of the *ZsGreen* reporter gene. Analysis of cytological and histological preparations was carried out using a Nikon Ni-U microscope with NIS-Elements imaging software (Nikon, Japan).

Statistical analysis was performed with Microsoft Excel 2016 software (*t*-test). The tables show arithmetic means (*M*) and mean errors (±SEM). Differences were deemed statistically significant at $p < 0.01$

Results. The obtained chicken embryonic cell culture consisted of several types of cells, mainly fibroblasts, with PGCs portion not exceed 3%. Separation of different types of embryonic cells by adhesion allowed us to increase the PGCs percentage in cell suspension up to 81% (Fig. 1, A, B). This cell culture was used for transformation with recombinant DNA. The efficiency of PGCs transformation was 12%. This culture was used as donor cells for injection into embryos (see Fig. 1, C, D).

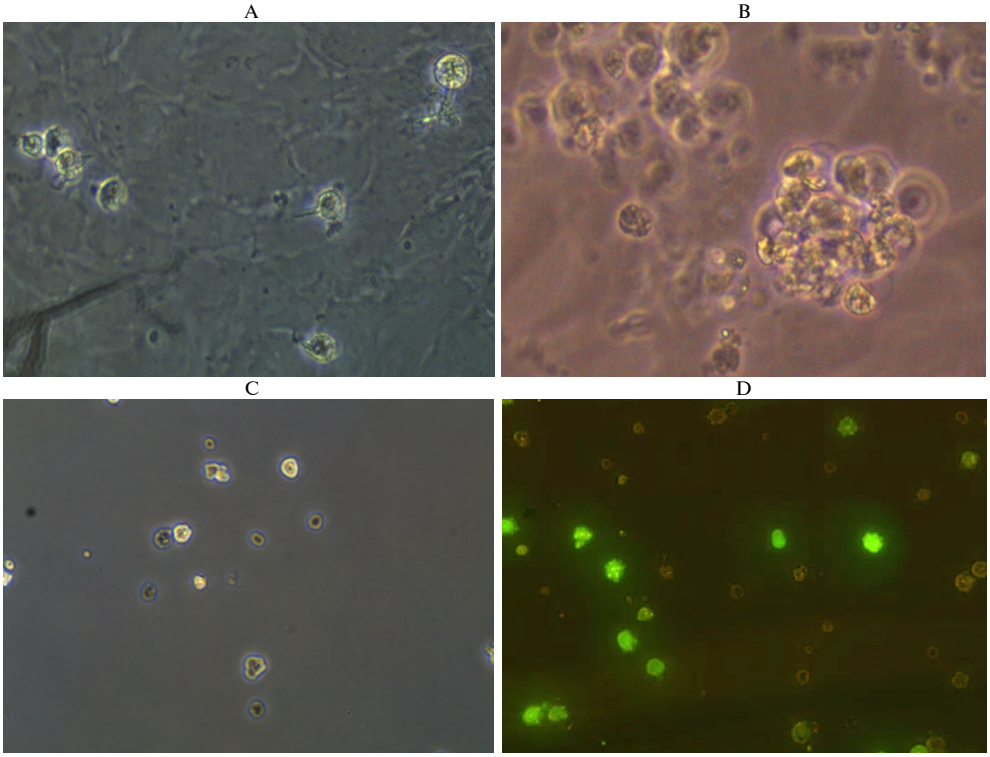

Fig. 1. Culture of primordial germ cells of Russian White chickens: A — day 2 of culture, B — day 7

of culture (colonies of primordial germ cells — PGCs), C — native suspension of PGCs before transformation, G — PGCs suspension after transformation (transformed cells fluoresce). Light microscopy, phase contrast (A-C); fluorescence microscopy (D) (magnification ×400, Nikon Ni-U microscope, Nikon, Japan).

A culture of transformed donor PGCs in various concentrations was introduced into the dorsal aorta of 2.5-day recipient embryos (152 embryos in total). The number of the introduced donor cells did not significantly affect development of the embryos. The proportion of developed embryos varied between test groups from 78 to 85%, 83% value in the control group. In the test groups, a stop in the embryo development occurred mainly on days 6 to 10 and at the end of incubation, while in the control group at the end of incubation. Increased embryonic mortality in the test groups during early embryogenesis may be associated with the genetic engineering manipulations.

Colonization efficacy and the development of donor PGCs in recipient chickens were investigated in birds aged 1.5-2 months. Fluorescence microscopy reveled green-fluorescent spermatogenic cells in the seminiferous tubules of testicle (Fig. 2, A). The number of chickens with transformed germ cells varied depending on the number of administered donor cells, being 16% for group I (400 PGCs), 23% for group II (700 PGCs), and 26% for group III (1000 PGCs) (Table 1).

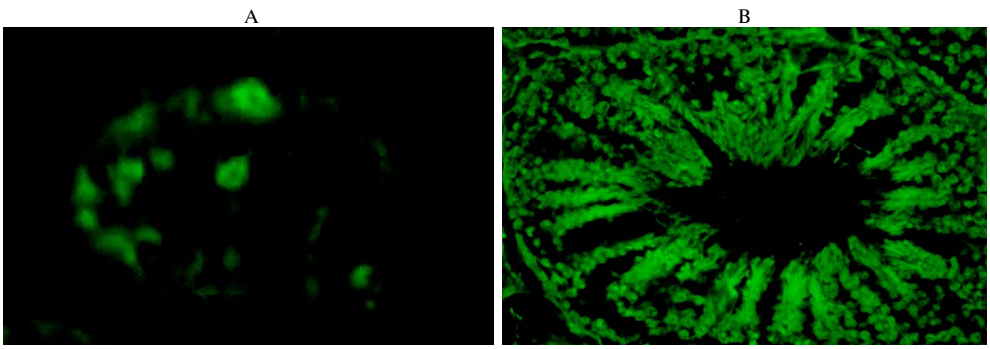

Fig. 2. Expression of transferred *ZsGreen* gene in gonad cells of Russian White roosters of different age: A — 1.5 month of age (after injection of 400 transformed primordial germ cells to a recipient embryo)**, B — 9 month of age** (in vivo viral-vector mediated testis gene transfer). Fluorescent spermatogenic cells at different stages of differentiation are visible inside the seminiferous tubules of the testes (A, B), including spermatozoa in the lumen of the transformed tubule (B). Fluorescence microscopy (A — magnification ×400, B — magnification ×160, Nikon Ni-U microscope, Nikon, Japan).

1. Colonization efficacy of transformed donor primordial germ cells (PGCs) **in recipient Russian White chicken embryos**

Indicator	Control	Group I	Group II	Group III
Embryos per group, *n*	30	50	52	50
Donor cells, *n*		400	700	1000
Developed embryos, *n* (%)	25 (83)	40 (80)	44 (85)	39 (78)
Hatched chickens:				
in total, *n* (%)	18 (60)	31 (62)	33 (63)	29 (58)
with genetically modified germ cells, *n*		8	12	13
Efficacy of transgenesis, %		16	23	26

N o t e. For a description of the groups, see the Materials and methods section. Efficacy of transgenesis is the ratio of the individuals with transformed germ cells to the total number of injected embryos.

Along with transplantation of transformed donor PGCs, a technique was developed for the in vivo transformation of spermatogenic cells in rooster's testes to generate genetically modify sperm. The efficiency of transformation of spermatogenic cells depended on the scheme of the lentiviral vector administration

(see Fig. 2, B, Table 2). In the roosters upon one injection of the vector at 3-month age (group I), the percentage of seminiferous tubules with transformed spermatogenic cells varied from 3 to 8% and averaged 5.8±0.4%. The efficiency of single administration of the lentiviral vector to roosters aged 4 months (group II) was less effective than in group I and did not exceed 4.0±0.6% on average. A 2-fold administration of the viral preparation (group III) contributed to a reliable 1.7-fold and 2.4-fold ($p < 0.01$) increase in the efficiency of in vivo transformation as compared to group I and group II, respectively.

2. Efficiency of in vivo viral-vector mediated gene transfer to testicular spermatogenic cells of Russian White roosters (*M*±SEM)

Indicator	Group I	Group II	Group III
Roosters per group, *n*	5	5	5
Age of lentiviral vector administration	3 months	4 months	3 and 4 months
Seminiferous tubules per rooster, *n*	201±1	252±13	257±24
Seminiferous tubules with transformed spermatogenic cells, *n*	12±1	10±1	24±2[a, c; b, c]
Efficacy of transgenesis, %	5.8±0.4	4.0±0.6	9.6±0.8[a, c; b, c]

N o t e. For a description of the groups, see the Materials and methods section. Efficacy of transgenesis is the ratio of the number of seminiferous tubules with transformed spermatogenic cells to total number of seminiferous tubules в.

[a, c] Differences between group I and group III are statistically significant at $p < 0.01$.

[b, c] Differences between group II and group III are statistically significant at $p < 0.01$.

The percentage of live sperm cells in the semen of transgenic roosters was slightly lower compared to non-transgenic analogues at less than 2% differences (Table 3). In the test groups, the number of morphologically abnormal spermatozoa was 3% higher as compared to the control. Defects of sperm flagella were the most frequent in both control and test roosters.

Sperm fertilizing ability of transgenic roosters was 5-6% lower compared to the control. The expression of reporter *ZsGreen* gene was detected in 10% of embryos in group I and 6% of embryos in group II.

3. Sperm quality and fertilizing ability in non-transgenic and transgenic Russian White roosters produced by transplanting genetically transformed donor primordial germ cells and in vivo viral-vector mediated gene transfer of spermatogenic cells (*M*±SEM)

Indicator	Control	Group I	Group II
Sperm motility, %	84±1	82±1	82±2
Proportion of live spermatozoa, %	88±1	87±2	86±2
Proportion of sperm with abnormal morphology, %	7±1	9±1	10±1
Fertilizing ability:			
incubated eggs, *n*.	50	50	50
developed embryos on day 6 of incubation, *n* (%)	43 (86)	41 (80)	42 (81)
Embryos with *ZsGreen* gene expression, *n* (%)		5 (10)	3 (6)

N o t e. For a description of the groups, see the Materials and methods section.

Various techniques have now been suggested for transformation of PGCs and spermatogenic cells. As per J. Macdonald et al. [13], an efficiency of chicken PGCs transfection with piggyBac and Tol2 transposons was 5.4 and 25.5%, respectively. Sawicka et al. [29] and Tyack et al. [10] effectively transfected chicken PGCs using transposon vectors in combination with lipofection. Naito et al. [28] derived cell culture from chicken PGCs and transformed it by nucleofection with an efficiency of 10%. The cultured GFP-positive PGCs were transferred into the bloodstream of recipient embryos. Test mating revealed one chimeric chicken, which produced one donor-derived offspring (with detected reporter GFP) of 270 examined. These data are consistent with 12% efficiency of chicken PGCs transformation we obtained in our research by electroporation.

Min et al. [30] and Li et al. [14] used direct injection of SofastTM cationic polymer in combination with a genetic construct into the testis parenchyma for in

vivo transformation of rooster spermatogenic cells. Min et al. [30] conducted research on the production of chickens resistant to avian influenza virus. The transformation efficiency of spermatogenic cells was 72.2%. The transgene was found in 10% of sperms and in blood of 7.8% of the resulting F_1 progeny. In the experiments of B. Li et al. [14] the efficiency of spermatogenic cell transformation did not exceed 12-19%.

We used the lentiviral vector carrying *ZsGreen* reporter gene for in vivo transfection of roosters' testicular spermatogenic cells. As a result, GFP gene expression was detected in 10% of the spermatogenic cells and in 6% of embryos after the sperm of the transgenic roosters was used for insemination.

In conclusion, our findings confirm that male primordial germ cells (PGCs) and spermatogonia are good targets for recombinant DNA transfer. Resultant male recipients with genetically modified germ cells in the gonads can generate transgenic offspring with certain traits. In our research, when 1000 donor PGCs after genetic transformation were transferred to a recipient embryo, the transgenesis efficiency was the highest: 26% of chickens with GFP expression in gonads vs. 16% and 23% for 400 and 700 PGCs per embryo, respectively. A 2-fold administration of the lentiviral vector to males at the age of 3 and 4 months provided 10% in vivo modification of testicular spermatogenic cells which was 2 times higher compared to a single injection of the lentiviral vector. Six to ten percent of offspring from the roosters with genetically transformed germ cells were transgenic.

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