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EFFICIENCY OF LOCAL TRANSGENESIS OF THE OVIDUCTAL CELLS IN CHICKEN AS INFLUENCED BY HORMONAL STIMULATION

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Abstract

One of the promising areas of biotechnology is to create transgenic chicken bioreactors. However, despite notable successes in avian transgenesis, the creation of transgenic chickens now is a particular problem. Searches and development of alternative methods of directed gene transfer are required, one of which is genetic transformation of certain organs, in particular chicken oviduct (somatic transgenesis). This can significantly reduce the expenditure of time and materials in the preparation of transgenic organisms than using other cellular targets for targeted delivery of DNA (cell blastoderm, primordial germ cells, embryonic stem cells) because the genetically engineering manipulation is possible to be conducted only on embryonic material. The aim of this study was to investigate the effectiveness of the delivery of recombinant DNA into the chicken oviduct cells in vivo and development of methodological approaches to increase the efficiency of transgenesis. In the present work a retroviral vector pLN-GFP was used, based on the Moloney murine leukemia virus (Mo-MuLV), in which a sequence of GFP (green fluorescent protein) marker gene has been cloned. Packaging cell line GP+envAM12 has been used to package retroviral vector pLN-GFP. To determine the optimal duration of administration of gene constructs a proliferative activity was analyzed in the oviduct cells at the age of 1, 2, 3, 4, 4.5 and 5 months, and at the age of 2 months within 24 and 48 hours after hormonal stimulation by 0.1 % sinestrol solution. The introduction of the retroviral vector was performed by injecting the solution of the gene construct directly into the protein part of the oviduct of hens at the age of 4 months without hormonal pre-stimulation (group I) and at the age of 2 months within 24 h after sinestrol injection (group II). Analysis of the integration and expression of recombinant DNA in chick oviduct cells was performed at the age of 6 months. As a result of histological investigations it was found that the maximum proliferative activity in the protein part of oviduct (magnum) was observed in a period between 4 and 4.5 months: the relative DNA content of the cells at this period increased by 3.4 rel. units, which was equivalent to an increase of this parameter over the entire preceding period from 1 to 4 months. The analysis of histological sections of the oviduct in 2-month-old chickens 24 hours after sinestrol injection revealed a significant change in the structure of the oviduct, typical for histological structure of the magnum portion of the oviduct at the 4 month age, and after 48 hours the oviduct histological structure corresponded to that of mature chicken. An average efficiency of the hen oviduct cell transformation assessed as a percentage of the transformed cells to the total cell number of this type in the oviduct, in group I was 17.2±3.1 % whereas in group II it was 57.3±6.3 %. Thus, the use of hormone treatment has allowed a 3.3-fold increase in the effectiveness of local transgenesis of the oviductal cells in chickens.

Keywords: hens, retroviral vectors, transfection, transgenic animals.

Using transgenic poultry as bioreactors is one of the alternative methods of producing recombinant proteins [1-9]. Poultry is immune to potential therapeutic proteins (e.g., human erythropoietin), the expression of which can adversely affect the health of transgenic mammals when used as a commercial producers of drugs. Furthermore, it is possible to significantly reduce the cost of recombinant products compared to other production methods based on the use

of *Escherichia coli*, yeast or mammalian cells as the production platform [10].

Despite significant progress in poultry transgenesis, the efficiency of producing transgenic chickens is limited by the factors that result from the characteristics of their reproduction and ontogenesis. Due to the early initiation of embryo development, an embryo in female reproductive organs includes an average of 60,000 cells [11]. This greatly reduces the efficiency of the conventional method of introducing exogenous DNA into animal cells (microinjections), which limits the use of transgenic technology in poultry.

Genetic transformation of certain organs, in particular chicken oviduct (somatic transgenesis) may be considered as an alternative methodological approach. This can significantly reduce the expenditure of time and materials in the preparation of transgenic organisms compared to other cellular targets for targeted DNA delivery (cell blastoderm, primordial germ cells) as genetic engineering manipulations are possible to be conducted only on embryonic material. Vector systems based on recombinant retroviruses, including lentiviruses are considered as a promising delivery system for recombinant DNA in chicken cells in vivo [12-21].

We first studied the effect of sex hormones on the proliferative activity and histological features of the cells of the protein portion of oviduct in immature Ptichnoe cross chickens in connection with the possibility of their genetic transformation in vivo using retroviral vectors and demonstrated that hormone treatment improved the effectiveness of oviduct cell local transgenesis by 3.3 times.

Our goal was to optimize the conditions and determine the effectiveness of the delivery of recombinant DNA into chicken oviduct cells in vivo based on on the developed retroviral vector system using hormonal stimulation.

Techhnique. Studied chickens of Ptichnoe cross received standard compound feed and was kept in the conditions of the physiological yard of the L.K. Ernst All-Russian Research Institute of Animal Husbandry according to the necessary sanitary and technical requirements. Oviduct samples (n = 30) for histological examination were collected at the chicken age of 1; 2; 3; 4; 4.5, and 5 months. Sinestrol solution (0.1 % in the amount of 0.1 ml per chick) was used as estrogen for hormonal stimulation of oviduct cells. The agent was administrated subcutaneously in 2-month aged chickens, then, in 24 and 48 hours, samples of the magnum tissue (the protein part of oviduct) were collected for histological examination. They were fixed in 10 % formalin solution for 24 hours and embedded in paraffin after dehydration by standard technique [22-23].

Histological sections 4.5 μm thick were prepared using a rotary microtome. Preparations were examined microscopically (Nikon, Japan; lens ×40, eyepieces ×10, ×16) using the Image Scope computer program (LLC «Systems for Microscopy and Analysis», Russia). DNA was detected in cell nuclei by Feulgen staining [22]. At least 30 nuclei of each specimen were analyzed. Relative amount of DNA (N) in cell nuclei was determined using the following formula: $N = 100 \times (1/B) \times S$, where B was average nuclei brightness; $100 \times (1/B)$ was relative nuclei density; S was nucleus area. The intensity of staining and the area of nuclei of chicken oviduct cells were evaluated microscopically.

In this study, retroviral vector pLN-GFP based on the Moloney murine leukemia virus (Mo-MuLV) with a sequence of *GFP* (green fluorescent protein) marker gene cloned was used. Packaging cell line GP+envAM12, with the advantages of the lack of wild-type virus and the ability to introduce cloned genes into numerous host cells, was used to package retroviral vector pLN-GFP.

Delivery of retroviral vector (viral preparation) was performed in vivo by injecting the agent into the protein part of oviduct in chickens aged 4 months without hormonal pre-stimulation (group I, n = 3) and aged 2 months within 24

hours after sinestrol injection (group II, $n = 3$). Surgery was performed out in compliance with the rules of asepsis and antisepsis, similar general anesthesia and post-operative therapy were applied in all chickens. Surgical access to the magnum part of oviduct was performed through an incision below the last rib and in parallel to it. During 24-36 hours prior to the surgery, the birds were completely deprived of feed with continued access to water. Before the surgery, the chickens were fixed in a lateral position. The feathers were pulled out and the skin was treated with iodized alcohol in the area of intended incision line. Access was opened behind the costal wall, a 3-4 cm long incision from the contour eye muscle was made in 0.4-0.5 cm from the last rib. Skin was pre-shift slightly. After the dissection of the skin and muscle layers, the wound edges were put aside using a retractor. Then, peritoneum was opened using a gavage or injection needle, and the intestine was moved down to gain access to the oviduct. Gene constructs were injected into the wall of chiken oviduct. The surgery was terminated by the imposition of a one-storey Sadovsky-Plakhotin suture.

Oviduct tissues for the analysis of integration and expression of recombinant DNA were collected from 6-month-old chickens. DNA was isolated by the salting-out method [24]. Presence of recombinant DNA was determined using PCR [12]. Expression of recombinant proteins in epithelial cells was studied by immunohistochemistry using the first antibodies specific of the identified protein (GFP). To detect the immunohistochemical reaction product 3,3-diaminobenzidine (DAB) tetrachlorate («Sigma», USA) was used. To visualize immunochemically stained oviduct histological structure, studied preparations were additionally stained with hematoxylin. At least 30 sections 4-5 μm thick were studied per chicken.

Statistical processing was performed by variation statistics methods using Microsoft Excel.

Results. Intensive growth and high proliferative activity of the epithelial layer cells were observed in the age from 4 to 5 months (Table 1). Within this period, cell height increased by 65 %, and the amount of DNA in them increased by 25 %. Considering that a sharp increase in DNA content was observed in the age of 4-4.5 months, this age period was chosen as the optimum one for introducing retroviral vectors.

Delivery of gene constructions into oviduct at an early chicken age, when the oviduct is small, is one of the techniques that can improve the efficiency of transgenesis. However, in this age, epithelial cells of the magnum had low proliferative activity, which limited the effectiveness of the use of retroviral vectors for the recombinant DNA transfer (see Table 1). It is known that proliferation of the gland epithelial layer cells occurs 24 hours after the initiation of estrogen administration, but in such cells the lysozyme and ovalbumin synthesis reaches the required level only 2-3 days after the hormonal therapy. The function of tubular glands depends on the constant presence of estrogen, and the lack of the latter results in a rapid suppression of the oviduct function and subsequent involution which is followed by weight loss and a decrease in lysozyme synthesis [25].

We observed considerable changes in the oviduct structure 24 hours after the 0.1 % sinestrol injection, g.e. the epithelial layer was enlarged, folds appeared, the cells migrated into the submucosal layer, which corresponded to the histological structure of the protein part of oviduct in chickens aged 4 months (Fig. 1, B). Microscopic examination of chicken oviduct 48 hours after estrogen administration revealed numerous tubular glands, which corresponded to the oviduct histological structure in mature chicken (see Fig. 1, B).

1. Morphological and functional indicators of the cells of oviduct epithelial layer in Ptichnoe cross chickens of different age (*M±m*; L.K. Ernst All-Russian Research Institute of Animal Husbandry, Moscow Province).

Age, months	Height of epithelial layer, μm	Area. μm²		Nuclear/cytoplasmic ratio	DNA content per cell, relative units
		nucleus	cytoplasm		
1	9.50±0.16	7.56±0.26	7.12±0.43	1.14±0.12	11.5±0.3
2	12.64±0.15	8.34±0.34	8.91±0.44	0.95±0.03	13.0±0.5
3	14.18±0.17	12.14±0.17	13.09±0.11	0.93±0.01	13.7±0.4
4	16.95±0.16	15.91±0.22	20.29±0.19	0.78±0.01	14.9±0.3
4.5	17.37±0.26	14.77±0.31	21.44±0.67	0.70±0.02	18.3±0.4
5	27.93±0.52	23.01±0.61	49.05±0.64	0.47±0.01	18.6±0.2

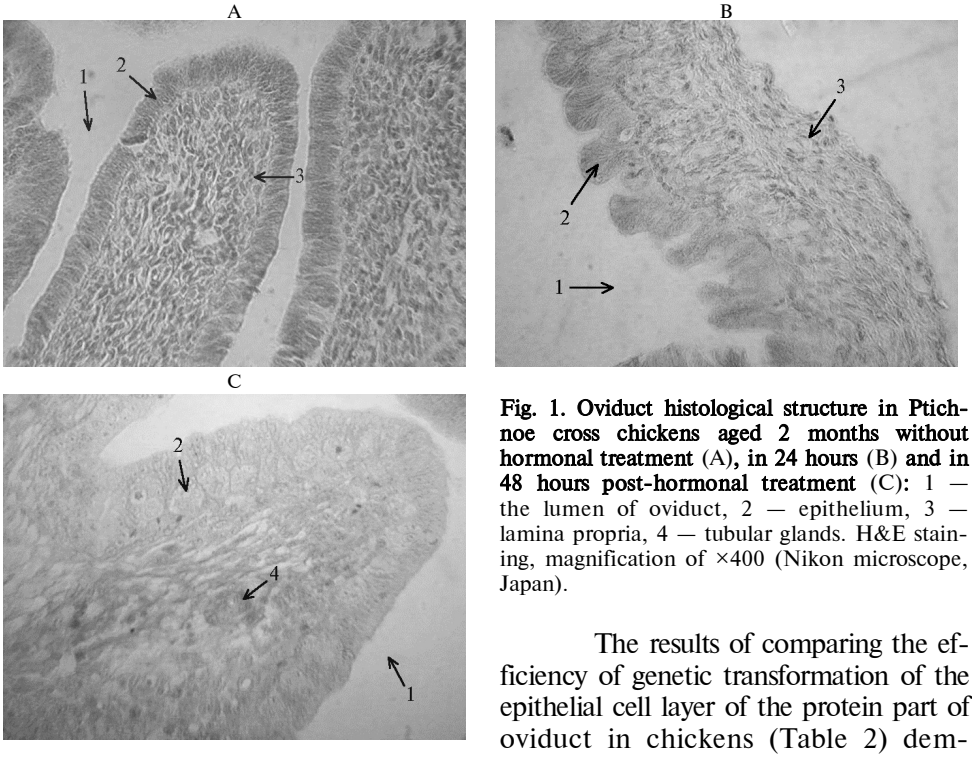

Fig. 1. Oviduct histological structure in Ptichnoe cross chickens aged 2 months without hormonal treatment (A)**, in 24 hours** (B) **and in 48 hours post-hormonal treatment** (C)**:** 1 — the lumen of oviduct, 2 — epithelium, 3 — lamina propria, 4 — tubular glands. H&E staining, magnification of ×400 (Nikon microscope, Japan).

The results of comparing the efficiency of genetic transformation of the epithelial cell layer of the protein part of oviduct in chickens (Table 2) demonstrated that in group I the frequency was relatively low and varied from 3.6±2.4 to 19.1±6.4 % which was likely due to the introduction of the gene constructs in chicken in the period of oviduct active growth and considerable increase in the area of target cells.

2. Effectiveness of oviduct cell genetic transformation in Ptichnoe cross chickens in vivo by pLN-GFP gene construct (*M±m*, L.K. Ernst All-Russian Research Institute of Animal Husbandry, Moscow Province)

Parameter	No hormonal stimulation	Hormonal stimulation
Age at retroviral vector introduction, months	4	2
Studied chickens	3	3
Studied sections per chicken, *n*	20	20
Proportion of sections with transformed cells[1] in %:		
minimum	30	75
maximum	55	90
average in group	43.3±7.3	83.3±4.4
Efficiency of oviduct cell transformation[2] in %:		
minimum	3.6±2.4	49.9±6.8
maximum	19.1±6.4	65.2±9.5
average in group	17.2±3.1	57.3±6.3
Studied DNA samples per chicken, *n*	10	10
Proportion of positive PCR samples in %	54.3±2.4	83.3±6.7

N o t e. 1 — ratio of the number of sections with transformed oviduct cells to the total number of sections studied in %; 2 — ratio of the number of transformed oviduct cells to the total number of cells of this type examined in %.

Introduction of gene constructions into the oviduct at an early age after hormonal stimulation (group II) contributed to a significant increase in cell transgenesis efficiency (Fig. 2). The number of oviduct sections with transformed cells increased 1.9 times. The effectiveness of transgenesis of the cells of epithelial layer of the protein part of oviduct estimated against the number of transformed oviduct cells to the total number of cells examined, increased 3.3 times (see Table 2).

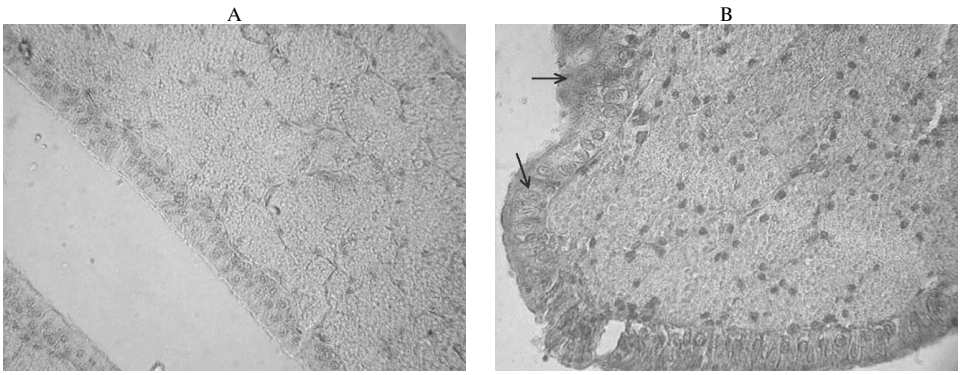

Fig. 2. Protein part of oviduct (magnum) of a Ptichnoe cross chicken aged 6 months after genetic cell transformation in vivo by pLN-GFP gene construct expressing GPF green fluorescent protein: A — without hormonal treatment (control), B — with hormonal pre-treatment. Arrows mark transformed cells. To visualize histological structure after immunohistochemical staining (3,3-diaminobenzidine chromagen, «Sigma», USA) preparations were additionally stained with hematoxylin, magnification of ×400 (Nikon microscope, Japan).

Thus, the prospects of the use of retroviral vectors for genetic transformation of oviduct cells in adult poultry in vivo was confirmed. Analysis of histological structure and proliferative activity demonstrated that chicken age of 4-4.5 months may be considered optimal for genetic engineering manipulations with oviduct cells. However, the effectiveness of transgenesis with introducing retroviral vector in oviduct during this period was relatively low (17.2±3.1 %) due to the relatively large size of this organ. Hormonal stimulation of immature 2-month old chickens with 0.1 % sinestrol solution activated proliferation and, consequently, the effectiviness of target cell transgenesis increased 3.3 times (up to 57.3 6.3 %).

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