The transgenic chickens obtained by microinjection of DNA in the ovarian follicles

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Abstract. The technology of the transgenesis applied is based upon a surgery providing external access to the ovary and subsequent natural deposition of membranes resulting in eggs suitable for incubation. The microinjection of foreign DNA directly into the blastodiscs of large pre-ovulated follicles within the ovarian hierarchy improved the operation rate in compare to microinjections of ovulated follicles through the wall of the infundibulum. Though the access to ovary is more difficult than to infundibulum, and follicular membrane is harder and thicker than infundibular wall, this technique has certain important advantages including the lack of necessity in the determination of ovulatory cycle timing and ovulation times for every follicle to be injected. The technique gives 2-3 injected follicles per surgery per hen without possible problems related to the irregular deposition of tertiary membranes. The trials were performed on White Leghorn chicken; the injected construct was linearized plasmid pSMTHG9 containing metallothionein promoter and gene of growth hormone (GH). 45 chickens were operated; 120 follicles were injected; 56 whole injected eggs were obtained and incubated; 40 day-old chicks were hatched and studied for the transgenicity. GH was found in the blood of 10 chicks in concentrations of 0.5-1.0 ng/ml; in 2 chicks the nucleotide sequences of GH were found in the DNA of whole blood. It can result from the mosaicism of the obtained transgenic birds.

Key words*:* transgenesis, microinjection of DNA, chicken.

Introduction

Transgenesis is one of the possible approaches to the genetic modification of poultry. The research related to different methods of induction of transgenesis in poultry is continuing since the emergence of transgenic mouse in 1980s. First manipulations with chicken zygote have been ineffective for the routine application; however, these studies paved the way for modern more effective technologies of the transgenesis [1-4].

Microinjections of foreign DNA into the zygote is still a classic technology of non-viral avian transgenesis. The technology of the transgenesis applied in our study is based upon a

surgery providing external access to the ovary and subsequent natural deposition of membranes resulting in eggs suitable for incubation [5, 6].

Purpose of the study – to develop a technique for microinjecting DNA into chicken ovarian follicles in order to create transgenic individuals.

Materials and methods

The trials were performed on White Leghorn chicken (180-300 days of age) kept in individual cages in the Institute's vivarium and inseminated artificially. The introduction of foreign gene was performed in two replicates. The laparotomy was performed with local anesthesia (Novocain) of abdominal area above the left thigh to get an open access to the ovary (since only left ovary and left oviduct are well developed in chicken).

Microinjections into ovarian follicles were made with a micropipette of Pyrex glass with a tip of 2-4 microns, made of a glass blank with a diameter of 1 mm. The volume of injected DNA in various experiments varied from 200 to 1000×10^{-12} L. The injected construct was linearized plasmid pSMTHG9 containing metallothionein promoter and gene of growth hormone (GH).

The samples of comb, skin, skeletal muscles, liver, and blood were biopsied from the hatched chicks. The samples of other tissues were obtained at the post mortem examination of naturally dead birds. DNA was isolated by phenol-chloroform deproteinization after the treatment of the homogenates of tissues by pronase E. The analysis of DNA by blot hybridization was performed with Zet-Probe (Bio-Rad, USA) membranes according to the protocol recommended by the producer.

Results and discussion

At the first step of the trial a preliminary test was performed to be sure that injected follicles really can form an oocyte and egg suitable for incubation. The stain (methylene blue or ink) was injected into the follicle's yolk; then eggs from the hens with injected follicles were collected for 12 days and broken to determine the presence of the stain. The main results of this preliminary test are presented in Table 1.

Table 1. The presence of stain in eggs laid by hens with stain-injected follicles

Number of stain-injected follicles	Time after the surgery, days						
	1	2	3	4	5	6	7
3		○	●		○	●	○
4		●	○	●		○	○
5			○	●	●	●	
3		●		○	●	○	●
4			○	●	●		○

4	○	○	●	○	●	○

○ – egg without stain; ● – egg with stain

Since 8 days after the surgery the stains disappeared from the eggs. It should be noted that the surgery can disturb the follicular hierarchy where the largest follicle should ovulate first. The stains often were nor found in the first and largest follicles and appear later, although three largest follicles were always stained during the surgery. The post mortem examination of hens revealed that certain follicles can dissolve before the ovulation. In some eggs only the traces of the stains were found indicating the intense clearance of the stains from the maturating follicles. Certain eggs where the stains were nor found resulted from the stained follicles. The results of the preliminary test are therefore insufficiently clear and unambiguous to make strict conclusions on the effects of injections on the follicular hierarchy, the order of ovulations, etc. However, a solid conclusion can be made that not less than 50% of eggs laid by operated hens during the subsequent 7 days are originated from the injected follicles.

The next step of the trial was the injection of gene constructs into the follicular blastodiscs. During the surgery 3-4 follicles in average were injected in each operated hen. Then the eggs from the operated hens were collected for 7 days excluding the eggs laid next day after the surgery since these eggs were already in the oviduct at the moment of the surgery and were not injected. Then the eggs were incubated.

45 chickens were operated; 120 follicles were injected; 56 whole injected eggs were obtained and incubated (**Table 2**); 40 chicks were studied for the transgenicity. GH was found in the blood of 10 chicks in concentrations of 0.5-1.0 ng/ml; in 2 chicks the nucleotide sequences of GH were found in the DNA of whole blood (**Table 3**).

Table 2. Injection of DNA into the follicular blastodiscs

Experiment	Hens operated	Follicles injected	Eggs laid		Grown chickens
			total	standard	
1	11	33	19	19	8
2	34	87	39	37	32

Table 3. Analysis of the transgenicity in chicks developed from injected follicles

Chick No	Sex	hGH in blood serum		hGH in DNA from blood
		1	2	
3108	♀	–	+	–
5897	♀	–	+	+
5899	♂	+	+	–

5900	♂	–	+	–
5958	♀	+	+	–
5968	♀	–	+	–
5969	♀	+	–	–
5970	♀	+	–	–
5972	♀	+	–	–
5975	♀	+	+	+

The analysis of DNA in hatched chicks by blot hybridization revealed the nucleotide sequences of the injected plasmid. The radioautograph of the analysis of samples of different tissues of chick No 5975 is presented on **Fig. 1**. Together with fragments sized 2800 and 2500 base pairs (bp) expectable for the gene construct used (marked with arrows on **Fig. 1**) there was a fragment sized 1600 bp (the most intense strip) that can be formed in the tandem transgene integration, typical of the transgenic animals obtained by DNA microinjections into the ovicells. The fragments sized 850 and 3000-3500 bp are apparently the flanking sequences for the tandem transgene sequence. The size of 850 bp fragment indicates that the integration took place in 3'-end position near the Rst I of site of the genomic DNA. Since flanking sequences are well seen and are close in the intensity to the internal fragments of the tandem the latter contains only several repeats. The lesser intensity of large restrictive fragments of the transgene compared to small fragments is supposedly related to the incomplete transfer of large fragments from the gel onto the membrane.

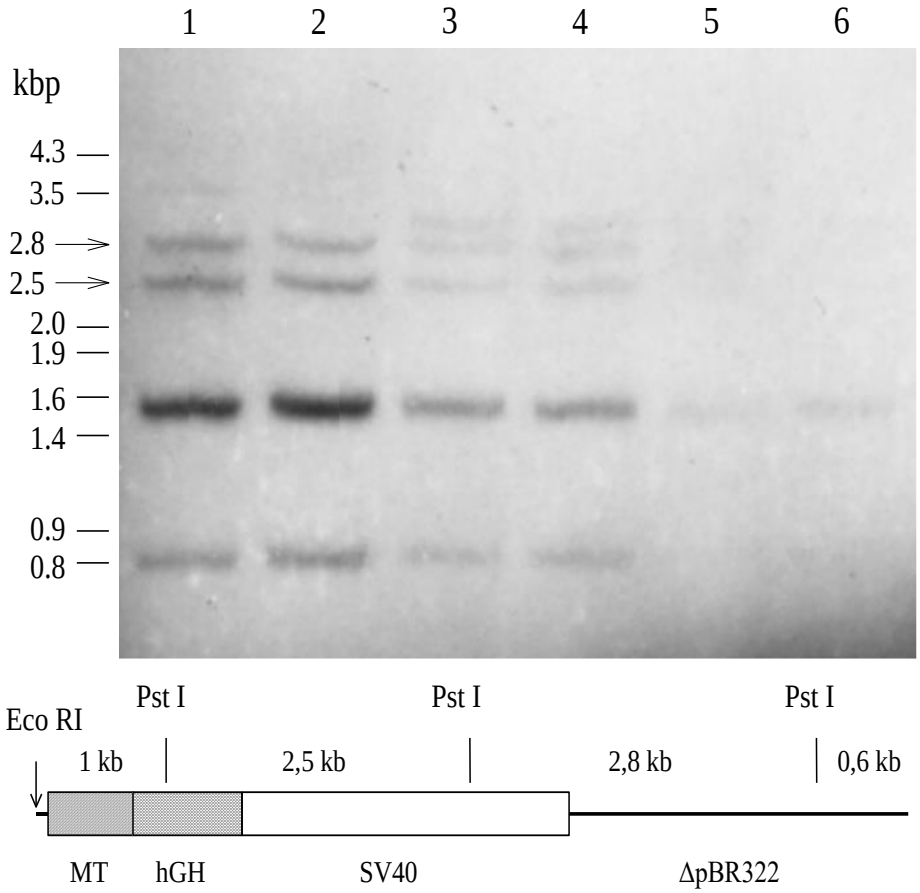

Figure 1. Hybdridization analysis of the integration of human somatotropin gene into the DNA of chick No 5975 isolated from the intestine (1,2), lungs (3,4), gizzard (5,6); restriction Pst I.
At the left: approximate position of marker fragments of lambda phag DNA (EcoR I + Hind III) and their sizes in kbp.

The hens (F0) grown from the transgenic chicks were crossed with the intact cockerels to obtain the first generation F1 (**Table 4**).

Table 4. The analysis of the heritability of the transgenicity in chicks injected with human GH gene

Chick No	Number of offsprings studied	Nos. of transgenic offspring	Sex	hGH in blood serum	hGH in DNA from blood
F0		**F1**			
5897	39	301407	♀	+	+
		1512	♂	–	+
5975	23	1591	♀	+	+
		2608	♂	+	+
		3905	♀	–	+
F1		**F2**			

1591	21	0418	♂	+	+
		0818	♀	–	+
		3006	♀	–	+
		3011	♂	–	+
		3014	♀	–	+
2608	29	1518	♂	–	+
		2808	♀	+	+

The transgenic F1 birds were, in their turn, crossed with intact cockerels. The yield of transgenic birds in F1 (62 birds totally) was 5 birds (or 8%). In F2 the frequency of the transgenesis was 14%. This fact can be explained by low livability of the embryos from the transgenic hens. The hatchability of eggs from these hens was 20% vs. 90% in non-operated control; the embryonic deaths occurred predominantly at the early stages of development, apparently as a result of non-controlled expression of the transgene in the embryonic tissues resulting in the lethal disturbances of embryogenesis.

Conclusions

The microinjection of foreign DNA directly into the blastodiscs of large pre-ovulated follicles within the ovarian hierarchy improved the operation rate in compare to microinjections of ovulated follicles through the wall of the infundibulum. Though the access to ovary is more difficult than to infundibulum, and follicular membrane is harder and thicker than infundibular wall, this technique has certain important advantages including the lack of necessity in the determination of ovulatory cycle timing and ovulation times for every follicle to be injected. The technique gives 2-3 injected follicles per surgery per hen without possible problems related to the irregular deposition of tertiary membranes.

References

1. Sang H. Prospects for transgenesis in the chick // Mechanisms of Development. – 2004. – V. 121. – N 9. – P. 1179-1186. doi:10.1016/j.mod.2004.05.012
2. Park T.S., Han J.Y. piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens // Proc. Natl. Acad. Sci. USA. – 2012. – V. 109. – N 24. – P. 9337-9341. doi:10.1073/pnas.1203823109
3. Han J.Y., Lee B.R. Isolation and Characterization of Chicken Primordial Germ Cells and Their Application in Transgenesis // Meth. Mol. Biol. – 2017. – V. 1650. – P. 229-242. doi:10.1007/978-1-4939-7216-6_15

4. Trefil P., Aumann D., Koslova A., Mucksova J., Benesova B., Kalina J., Wurmser C., Fries R., Elleder D., Schusser B., Hejnar J. Male fertility restored by transplanting primordial germ cells into testes: a new way towards efficient transgenesis in chicken // Sci. Rep. – 2017. – V. 7. – N 1. – P. 14246. doi:10.1038/s41598-017-14475-w
5. Korshunova L.G., Karapetyan R.V., Fisinin V.I. Methods for genetic modification in poultry // Agricultural bioloby. – 2013. – № 6. – P. 3-15.
6. Korshunova L.G., Karapetyan R.V., Ziadinova O.F. Modification of chicken genome by interferon gene // Russian Agricultural Sciences. – 2014. – V. 40. – N 5. – P. 379-381. doi:10.3103/s1068367414050140