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—Original Article—

Is passive transmission of non-viral vectors through artificial insemination of sperm-DNA mixtures sufficient for chicken transgenesis?

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Abstract. DNA uptake in the post-acrosomal region of the spermatozoa takes place exclusively in immotile spermatozoa that are naturally unable to fertilize eggs. The present study aimed to assess whether passive transmission of non-viral vectors to the surrounding areas of chicken embryos could be an alternate mechanism in chicken sperm-mediated gene transfer. First, the presence of nucleases in rooster seminal plasma was evaluated. Semen ejaculates from five roosters were centrifuged and the supernatant was incubated with pBL2 for 1 h. A robust nuclease cocktail was detected in the rooster semen. To overcome these nucleases, plasmid-TransIT combinations were incubated with semen for 1 h. Incubation of exogenous DNA in the lipoplex structure could considerably bypass the semen nuclease effect. Then, intravaginal insemination of 1×10^9 sperm mixed with lipoplexes (40 µg pBL2:40 µl TransIT) was carried out in 15 virgin hens. Neither the epithelial tissue from the inseminated female reproductive tracts nor the produced embryos following artificial insemination showed the transgene. To remove any bias in the transgene transmission possibility, the plasmid-TransIT admixture was directly injected in close vicinity of the embryos in newly laid eggs. Nonetheless, none of the produced fetuses or chicks carried the transgene. In conclusion, the results of the present study revealed a nuclease admixture in rooster seminal plasma, and passive/active transmission of the non-viral vector into close vicinity of the chicken embryo was inefficient for producing transgenic chicks.

Key words: Chicken, *In ovo* injection, sperm-mediated gene transfer (SMGT), Sperm, Transgenesis

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Since the introduction of sperm-mediated gene transfer (SMGT) technology by Lavitrano *et al.* [1], it has been considered a controversial method for producing transgenic animals [2]. Thus far, many papers have been published regarding SMGT technology. However, only limited studies follow a step-by-step plan, from sperm-DNA interaction to producing transgenic animals. The main foundation for SMGT theory is that unlike dead spermatozoa, live/motile spermatozoa are able to take up exogenous DNA [1] at their post-acrosomal region [3]. Further studies revealed that the majority of DNA-interacted spermatozoa were dead [4, 5]. Initially, it was proposed that higher DNA uptake by motile spermatozoa triggered intra-cellular nucleases and subsequently resulted in sperm death [5, 6]. However, further clarification indicated that sperm death through SMGT is simply due to seminal plasma removal during the washing steps and sperm death is independent of the incubation with exogenous DNA [7]. Recently, it has been revealed that exclusively dead or disrupted membrane sperm cells can interact with exogenous DNA at the post-acrosome region [8, 9]. If we accept the inability of motile sperm to take up and transfer foreign DNA through fertilization [4, 9], alternate mechanisms, such as post-fertilization exogenous DNA uptake, could take place following artificial insemination (AI) of sperm-DNA mixtures.

Interestingly, unlike most mammalian reports on SMGT inefficiency (reviewed in [9]), the technology showed some promising results in chickens [10–13]. However, using radiolabeled spermatozoa, both live and dead spermatozoa reach the hen infundibulum within 15 min following intravaginal insemination [14]. In addition, because of the muscular movement of the hen reproductive tract, even the inseminated media can traverse the reproductive tract [14]. The presence of specialized tubular invaginations acting as sperm storage organs in hen reproductive tracts provide 3–4 weeks storage of sperm cells [15]. Therefore, lipoplex uptake by immotile sperm [4, 9] or the remaining lipoplexes with no interaction with spermatozoa can act as a transgene source in the tubular invagination or infundibulum and provide further interaction with ovulated eggs. In the current proof of principle study, we aimed to further clarify whether passive or active transmission of non-viral vectors could be efficient for chicken SMGT.

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Materials and Methods

Ethical statements

The semen collection, artificial insemination, and *in ovo* injection processes were carefully carried out to minimize any physical damage to the animals. Eggs that did not hatch by day 22 of incubation were cracked and their embryos were used for DNA extraction and PCR assays. Day-old chicks were humanely killed and used for DNA extraction. All the experimental procedures were assessed and approved by the Institutional Animal Care and Ethics Committee of Isfahan Branch, Islamic Azad University [16].

Nuclease activity of rooster seminal plasma

First, artificially ejaculated semen from five pre-adapted roosters were mixed and evaluated for sperm motility and concentration. Then, the ejaculated semen was centrifuged at 55 *g* for 10 min, and the supernatant was used for further evaluations. Two micrograms of pBL2 plasmid, containing human cytomegalovirus promoter/enhancer and luciferase CDS followed by a PolyA termination sequence, was incubated with TransIT (Mirus, Madison, WI, USA) at 1:1 and 1:3 (µg:µl) ratios for 1 h to form lipoplexes. A random sample of 300 µl seminal plasma supernatant was added to the DNA/TransIT mixture in 350 µl final reaction volume and incubated at 37.5°C for 30 and 60 min. Second, the chicken semen was centrifuged at 19,000 *g* for 10 min and the supernatant nuclease activity was evaluated following 1 h incubation of 300 µl supernatant with 2 µg pBL2 plasmid. The 1:1 ratio of pBL2:TransIT was also used for evaluation of the lipoplex protection property from the chicken seminal plasma nucleases during 60 min incubation period. In both experiments, following the incubation of seminal plasma and DNA, the mixture was precipitated using phenol-chloroform and ethanol in 50 µl distilled water. The eluted DNA was loaded on a 3% agarose gel and quantified by measuring the absorbance at 260/280 nm. Both experiments were repeated at least three times.

Artificial insemination of the sperm-DNA mixture

First, 40 µg pBL2 plasmid was incubated with 40 µl TransIT for 1 h at 25°C to form lipoplexes. Then, 1×10^9 sperm cells from the ejaculates of 5 pre-adapted roosters were incubated with the lipoplex complex at 37.5°C for 5 min. AI of hens that had not mated with any rooster were carried out in two groups; sperm cells only as a standard AI group and a sperm-lipoplex group (AI-SMGT), with 5 and 15 replicates, respectively. The inseminations were performed in the morning when all hens laid their daily eggs. Following the insemination date, all the collected eggs during 10 successive days from the AI-SMGT group, as well as a random sample of 30 eggs from the standard AI group, were incubated at 37.5°C and 65% relative humidity for 22 days. The DNA was extracted from the beak, blood, liver, heart, breast muscle, wings, and legs of all the hatched chicks. In the AI-SMGT group, eggs that did not hatch were cracked and the unhatched fetuses were used for DNA extraction. Moreover, after the last day of egg collection, the inseminated hens were killed and the epithelial tissue of the reproductive tracts were sampled and used for DNA extraction. To amplify a fragment of the luciferase transgene, polymerase chain reaction (PCR) was carried out on the extracted DNA. In addition, a segment of the chicken CHD1 gene on the Z/W chromosome was amplified as the chicken endogenous gene [16]. The details of all primer pairs that were used in the present study are presented in Table 1. It should be noted that due to plasmid contamination, some DNA samples from the negative control, standard AI, were PCR-positive for the transgene. Therefore, after a thorough cleaning of the utensils with water and 1-h exposure to ultraviolet light, DNA extraction and PCR reaction were repeated for all the PCR-positive DNA samples. Since the new round of PCR results did not show the transgene transfer, these results were considered as pseudo-positive [17].

Table 1. Primer pairs for amplification of luciferase and EGFP transgenes in pBL2 and pTn5 plasmids, respectively, as exogenous DNA and chicken CHD1 as an endogenous gene

Primer sequence	Target gene
FCHD1: 5′-AATAGCAACAAAACAGAATAC-3′ RCHD1: 5′-CAACAGAACTCGAGACTCA-3′	*CHD1*
FBL2: 5′-TTATTGGCATCTTGTACTGGG-3′ RBL2: 5′-GGAAGGCAGGGATTAATGG-3′	*Luciferase*
FTn5: 5′- ACGTAAACGGCCACAAGTTC -3′ RTn5: 5′- TGCTCAGGTAGTGGTTGTCG -3′	*EGFP*

Transfection of chicken embryos with in ovo injection

In the last experiment, 10 µg pTn5 plasmid, carrying the enhanced green fluorescent protein (EGFP) under the hCMV promoter and chicken beta-actin termination signal, was mixed with 10 µl TransIT and stored at 25°C for 1 h. To transfect chicken embryos, a sample of newly laid fertile eggs from Ross broiler breeders were purchased. The eggs were stored upright at 12°C for 24 h so that the embryo positioned beneath the tapering end of the egg. A small square hole, approximately 1 cm^2, was drilled at tapering end of the egg to reach the embryo. An *in ovo* injection was carried out using a Gastight 1700 Series syringe (100 µl, Model 1710 N SYR, Cemented NDL, 22s ga, 2 in, point style 5, Hamilton Robotics, Nevada, USA) and the treatment content was injected in the egg white closely surrounding the embryo. The experimental groups comprised sham injections containing phosphate buffer saline (PBS), pTn5 plasmid (10 µg), and pTn5:TransIT (1 µg:1 µl) groups. The separated egg shell was replaced and sealed, and the injected eggs were placed into the incubator at 37.5°C and 65% relative humidity for 22 days. DNA extraction was carried out from the above-mentioned organs of both hatched and unhatched chicks from the transgene injected groups followed by PCR to assess the transgene transfer.

Statistical analysis

For evaluation of semen nuclease activity, mean comparison was carried out using the least significant difference (LSD) test and P-value < 0.05 was considered as the basic significant level.

Results

Nucleases in chicken seminal plasma

Nuclease activity of the semen supernatants after 10 min cen-

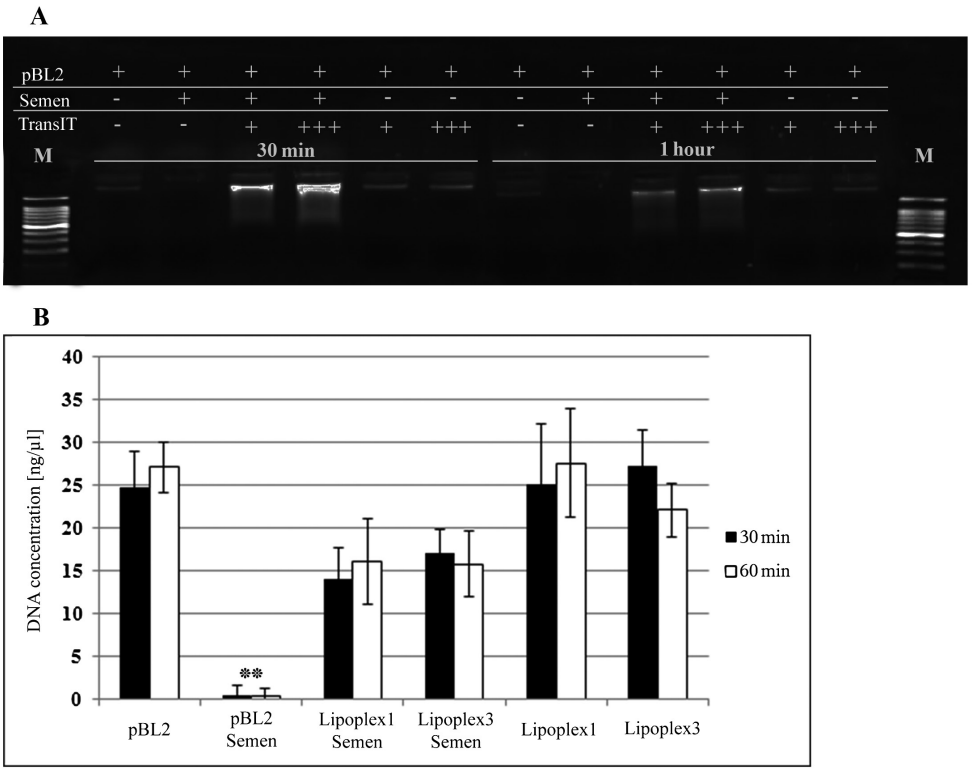

Fig. 1. Presence of a nuclease cocktail in chicken seminal plasma. Seminal plasma was separated from the sperm pellet after centrifugation at 55 *g* for 10 min. The nuclease activity was evaluated on the pBL2 plasmid in naked DNA and lipoplex structures following DNA-TransIT interaction in 1:1 and 1:3 (µg:µl) ratios which were designated as Lipoplex1 and Lipoplex3, respectively. The semen treated admixtures underwent phenol-chloroform and ethanol precipitation followed by loading on a 3% agarose gel (A) and quantification by a spectrophotometer (B). ** Means (± SD) showed significant differences with other groups (P-value < 0.01).

trifugation at 55 *g* was observed and quantified (Fig. 1). Incubation of the semen supernatants with pBL2 plasmid showed that almost the entire naked DNA was removed following 30 min incubation. The DNA concentration in the semen treated pBL2 group was significantly different from the semen treated lipoplexes and the untreated groups ($P < 0.05$). However, lipoplex formation of the plasmid could considerably improve the plasmid protection from the semen nucleases for 1 h incubation so that semen treated lipoplexes and the lipoplexes groups were not different (0.05 < P-value). There was no significant difference in DNA protection using 1 and 3 µl TransIT. In the second experiment, incubation of semen supernatants remained after 19,000 *g* centrifugation was carried out with naked DNA and lipoplexes (Fig. 2). The incubation results indicated that the plasmid started fragmentation at specific sites and produced two fragments with approximately 450 and 550 bp lengths. However, the nuclease activity was not significantly different between naked DNA and the lipoplex group.

Inefficient chicken transgenesis with AI-SMGT

Artificial insemination of the sperm-lipoplexes into the female reproductive tracts was carried out. Incubation of the collected eggs from the sperm-lipoplex inseminated females resulted in 20.7% hatchability, which was comparable to that of the standard AI group. Stringent DNA extraction (155 DNA samples) and PCR assays from various parts of the hatched chicks and unhatched fetuses could not reveal the transgene transfer in this group (Table 2). Genomic DNA of the epithelial layer of the reproductive tracts from all inseminated hens was analyzed using PCR. None of the evaluated tissues from the cloaca to infundibulum in any inseminated hens carried the luciferase transgene (Table 2).

Unsuccessful transgenesis through chicken embryo treatment with lipoplexes

Injection of the plasmid as naked DNA and lipoplexes were carried out at day 0 of the incubation. Hatchability ranged from 33.3 to 40% in different *in ovo* injection groups (Table 3). Extracted DNA from ectoderm, mesoderm, and endoderm tissues were assessed for transgene transfer. None of the hatched and unhatched chicks contained the EGFP transgene.

Discussion

Exogenous DNA can penetrate dead or disrupted-membrane sperm cells and can strongly protect it against DNase-I [4, 9], whereas the integrated cellular membrane of motile spermatozoa obstructs stable interactions with exogenous DNA [9]. In the present study, we devised

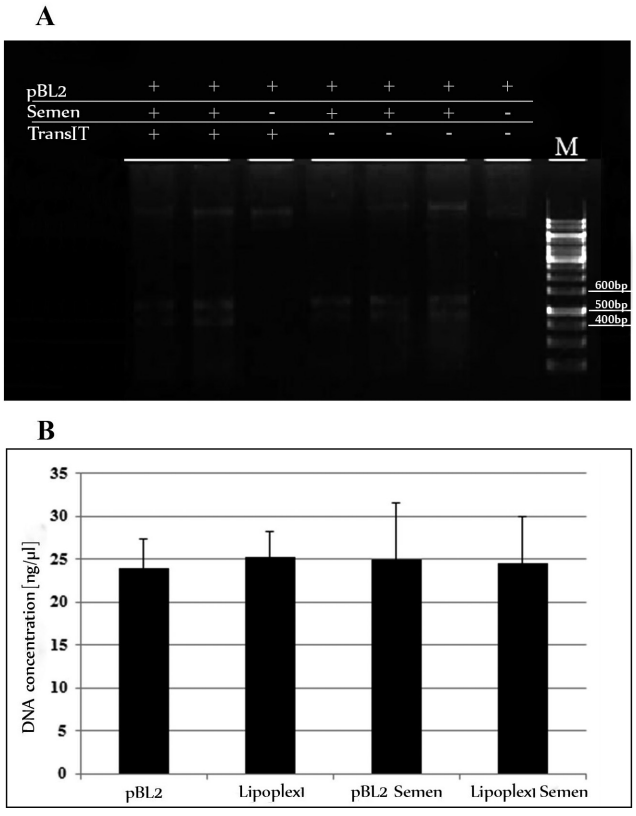

Fig. 2. Evaluation of rooster semen supernatants after centrifugation at 19,000 *g* for 10 min. Nuclease activity was evaluated on the pBL2 plasmid in naked DNA and lipoplex structures (1 µg pBL2:1 µl TransIT). The admixtures underwent phenol-chloroform and ethanol precipitation followed by loading on a 3% agarose gel (A) and quantification by a spectrophotometer (B). There was no significant difference between the mean (± SD) from different groups (P-value < 0.05).

a step-by-step technique to examine a set of factors that could be important in chicken SMGT. First, we evaluated chicken seminal plasma nuclease activity following centrifugation at 55 *g*, as this is the routine centrifugation speed for sperm washing [9, 10]. These results clearly indicated that there is a mixture of endonucleases and exonucleases in chicken semen that can completely remove the intact circular plasmid during incubation for half an hour. A higher speed centrifugation (19,000 *g*) for the separation of seminal plasma and sperm cells showed that the remaining fraction of nucleases in the seminal plasma supernatant contains specific endonuclease activity by which the circular DNA was quickly fragmented at specific sites. Comparison of semen nuclease activity after a high and low centrifugation clearly indicated that the majority of semen endonucleases and exonucleases have relatively high molecular weights and they sediment without the application of a high salt solution. However, there was a specific type of low molecular weight endonuclease that did not sediment at the high centrifugation speed. Even though nuclease activity of seminal plasma has been reported in other species [18], to our knowledge, this is the first study to report rooster semen nuclease activity. It should be mentioned that some sperm-DNA interaction inhibitory factors (IF) were reported in boar seminal plasma [19]. So far, the IF protein details and their mechanisms were unknown. The results from the present study confirm the importance of inhibitory proteins that interact with exogenous DNA and substantially reduce the sperm transfection efficiency. Therefore, it is possible that the IF proteins correspond to seminal plasma nucleases. To overcome these IF proteins, they suggested one- or two-step washing of sperm cells before incubation with transgenes [20]. However, seminal plasma removal has a robust adverse effect on sperm vitality and motility [7]. Therefore, we examined another strategy to eliminate the nucleases while maintaining the seminal plasma. Our results showed that incubation of the transgene in lipoplex form could considerably protect the DNA from semen nucleases. It has been reported that lipoplexes induce a strong resistance against DNase-I

Table 2. Artificial insemination of rooster sperm cells combined with pBL2:TransIT (40 µg:40 µl) complex

Group	No. of inseminated hens	No. of eggs	No. of hatched chicks (%)	No. of unhatched chicks (%)	No. of pseudo-positive luciferase samples (%)*	No. of transgenic chicks	No. of transfected female reproductive tract
Standard AI	5	30	5 (16.7)	0 (0.0)	4 out of 25 (16.0)	0	0
AI-SMGT	15	150	31 (20.7)	3 (2.0)	20 out of 155 (12.9)	0	0

* DNA was extracted from different organs of both hatched and unhatched chicks including the blood, breast muscle, heart, liver, beak, legs, and wings. The PCR result from each tissue sample was considered as pseudo-positive luciferase, unless it was confirmed after an extra repeat of the DNA extraction and PCR procedure.

Table 3. *In ovo* injection of pTn5 plasmid with/without TransIT for producing transgenic chicken

Group*	No. of eggs	No. of hatched chicks (%)	No. of unhatched chicks (%)	No. of pseudo-positive EGFP samples (%)**	Transgenic chicks
Sham Injection	30	12 (40.0)	8 (26.7)	0 out of 127	0
pTn5	30	10 (33.3)	7 (23.3)	0 out of 106	0
pTn5+TransIT	30	12 (40.0)	7 (23.3)	0 out of 120	0

* PBS was used as a sham injection vehicle, pTn5 plasmid (10 µg) containing EGFP transgene under human CMV promoter was used as the transgene, and TransIT was the cationic lipid for making lipoplexes. The pTn5:TransIT ratio was 10 µg:10 µl. ** DNA was extracted from nine organs of each hatched/unhatched chick including the blood, heart, liver, beak, breast muscle, wings, and legs. The PCR result from each tissue sample was considered as pseudo-positive EGFP, unless it was confirmed in an extra round of the DNA extraction and PCR procedure.

[9] and increase the stable interaction of sperm cells and foreign DNA [10]. We also assessed the DNA protection attributes of two ratios of DNA:cationic lipid for lipoplex formation. Results showed that considerable DNA protection was observed against semen nuclease for 1 h incubation even using the 1:1 ratio. We evaluated nuclease resistance of lipoplexes up to 1 h incubation because it has been verified that the requested migration time for the majority of live and dead sperm to reach the sperm storage organ and infundibulum is less than 1 h after chicken intravaginal insemination [14, 21].

Following confirmation of lipoplex resistance against rooster semen nucleases, we inseminated hens with a sperm-lipoplex admixture. The fetuses and hatched chicks produced from the inseminated semen supplemented with either the transgene or lipoplexes were used for the transgene detection assay. Implementation of a rigorous PCR approach for the desired transgene did not confirm the transgene transfer into the various organs of either the day-old chicks (n = 10 and 12) or the unhatched chicks (n = 7 and 7) in the plasmid and lipoplex injected groups, respectively. The results from the AI-SMGT in the present study agreed with previous results reported by Rottman (1992) [10] and Nakanishi and Iritani (1993) [11]. However, these results were inconsistent with Harel-Marknowitz *et al*. (2009) [12] and Collares *et al*. (2011) [13] that reported successful AI-SMGT in chickens. Lipofectamine-protected exogenous DNA was reported in 12 day-old chicken embryos, although there was no indication of transgene integration into the genome of embryonic cells [10]. Similar PCR positive results were reported in another liposome-mediated SMGT study with no transgene integration into chicken embryos [11]. Therefore, the PCR positive results in the previous study [10–12] could be related to utensil contamination with the propagated plasmid. Pseudo-positive transgenesis was observed in the current study as well as our previous experiences on bovine and ovine SMGT, although the experiments were carried out by a panel of experts in three separate laboratories [9, 17]. Even the main laboratory where successful SMGT was repeatedly reported (for review see [20]), longitudinal studies on transgenic piglets that were produced through AI-SMGT showed unrepeatability of PCR results [22]. The unrepeatability of the PCR results from transfected embryos or produced offspring could be due to utensil contamination [10] or the transient integration of the plasmid into the genome [22].

If we accept the inability of motile spermatozoa to stably and efficiently interact with exogenous DNA, post-fertilization transgene uptake by the embryo could explain the transgenic chicks. Passive transmission of the transgene via the muscular movement of the reproductive tract of female chickens [14] could provide a chance for the foreign DNA to be taken up by chicken embryonic cells following the fertilization. It is noteworthy that seminal plasma was removed before sperm-DNA incubation in both successful AI-SMGT in chickens [12, 13], whereas sperm cells were incubated and inseminated with seminal plasma in the current study. Therefore, the proportion of dead spermatozoa that can stably interact with exogenous DNA should be much higher in those studies compared with the current research, and this could increase the chance of transgenesis success in those reports.

Furthermore, it has been highlighted that differences in sperm preparation methods, fertilization procedures, plasmid quantity, and type of cationic lipids are important factors for successful SMGT [20]. Therefore, these factors could alter the amount of available transgenes around the embryo and subsequently cause the discrepancy in the chicken SMGT results. To overcome these issues, we directly injected lipoplexes in the close vicinity of day-0 chicken embryos. Unexpectedly, transgenesis was not detected in any of the hatched chicks or dead fetuses using PCR. More importantly, after stringent cleaning of the utensils in the AI-SMGT part of the study, no pseudo-positive embryo or chickens were detected from the *in ovo* injection phase of the study. Our results suggested that even if the incubated transgene at the time of AI could escape from chicken semen nucleases, pass through the female reproductive tract, be available around the newly fertilized oocyte, and still maintain the lipoplex structure to cross the embryonic cells, the AI-SMGT is not an efficient procedure to produce transgenic chicks. However, further experiments are needed to elucidate the mechanisms by which direct *in ovo* lipofection of embryos were ineffective for chicken transgenesis. The egg albumen nuclease activity may act as another barrier for transgene transfer into the chicken embryonic cells *in ovo* [23], although it has been suggested that avidin, as an abundant protein in chicken eggs, has protective properties for exogenous DNA [24]. Therefore, further *in ovo* and *in vitro* experiments are needed to elaborate on the practical importance of these factors for chicken transgenesis.

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