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In vivo Testis Transfection Efficiently Produced Transfected Sperm Cells in Ram but not Rooster

Research Article

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ABSTRACT

We report a method for gene transfer via *in vivo* testis-mediated gene transfer (TMGT) in sheep. A non-viral vector, pDB2, which carried an enhanced green fluorescent protein (EGFP) transgene under control of a human cytomegalovirus (CMV) promoter, was mixed with the TransIT transfection reagent. The lipoplex mixture was injected intra-testicularly or into the cauda epididymis of 12 and 5 rams, respectively, as well as the intra-testis of 5 mature roosters. Each injected rooster was crossed with four virgin hens and their hatched chicks were assessed for the presence of the EGFP transgene. After 60 days, both rams and roosters were investigated for the transgene. Unlike roosters, polymerase chain reaction (PCR) analysis of sperm cells which were collected from the epididymis and seminiferous revealed that more than 50% of sperm samples from the intratesticular rete-injected group were PCR-positive for the EGFP transgene. Transgene uptake was also observed in seminiferous tubules and epididymis of the intratesticular rete and the cauda epididymis groups, respectively. In conclusion, the combined approach of TMGT and lipofection can lead to ovine sperm transfection. This approach has potential to be combined with the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas technology and used for on-farm gene editing of sheep species.

KEY WORDS chicken, sheep, sperm-mediated gene transfer, testis-mediated gene transfer, transgenesis.

INTRODUCTION

Improving the genetic make-up of farm animals has been the main approach for enhancing various attributes of livestock to meet human food demands (Laible *et al.* 2015). Unlike classical genetic selection strategies that need long-term breeding plans, transgenesis approaches could achieve similar genetic improvements within a single generation. Apart from its agricultural applications, farm animal transgenesis has also been employed in biomedicine such as gene pharming and xenotransplantation. So far, great milestones have been achieved for precise genome engineering using the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology in farm animals (Whitelaw *et al.* 2016). However, there is still a large room to improve the efficiency of gene transfer technologies in farm animals (Laible *et al.* 2015). Gamete transfection is one of the most attractive approaches in animal transgenesis. The main advantage of this system is the lack of mosaic patterns of gene integration and expression at the G_0 generation (Esponda and Esponda, 2005). Direct transfection of sperm cells has been considered as a promising strategy to deliver transgenes in a straight-forward pathway in large animals (Lavitrano *et al.* 2002). However, its reproducibil-

ity has been a challenging issue for transgenesis in various species (Eghbalsaied *et al.* 2013; Chaparian *et al.* 2016; Barkhojasteh *et al.* 2020). As an alternative, intracytoplasmic sperm injection (ICSI) of sperm-DNA mixture could successfully produce transgenic embryos in mammalian (Chan *et al.* 2000; Eghbalsaied *et al.* 2013). On the other side, *in vitro* transfection of chicken primordial germ cells (PGCs) followed by *in vivo* transplantation into the testis of surrogate roosters (Lee *et al.* 2006; Schusser *et al.* 2013), as well as viral transduction (McGrew *et al.* 2004) and non-viral transfection (Eghbalsaied and Keus, 2020) of early embryos are the main avian transgenesis methods. However, these technologies are multi-step technologies and therefore time-, cost-, and personnel-consuming. Although a puncture-free transfection method has been introduced for chicken embryos (Amini *et al.* 2017), the transfection efficiency of this method is still low compared to the before-mentioned technologies. To put the genetic engineering in farm animals in practice, attempts have been made to devise on-farm technologies for genome editing (McFarlane *et al.* 2019).

Among these on-farm technologies, direct transfection of spermatogonial stem cells inside of the testis or testis-mediated gene transfer (TMGT) is a straightforward alternative for the transfection of spermatozoa (Sato *et al.* 1994). It does not need any *in vitro* transfection step, microinjection device, and expert technician. This technique needs healthy sires, either DNA-lipoplexes or an electroporation device, and an injection syringe. Thus far, TMGT success has been limited to the murine species. In this study, we examined on-farm testis transfection of two farm animals, sheep as a small ruminant and chicken as an avian species using lipoplexes.

MATERIALS AND METHODS

Animals

In this study, we used male lambs (4-month old) and roosters (30-week old) at the early age of puberty. For the transgene injection into the testis, the animals were locally anesthetized based on the standard protocol and the intra-testis injection was carried out using 100 µL Hamilton syringes (Model 1710N SYR, Hamilton Robotics, USA) with a 22 gauge cemented needle, 2-inch length needles, and point style 3 and 1 mL insulin syringes and 26-gauge needle in lambs and roosters, respectively. Two months after the transfection, the 6-month old lambs and 52-week old roosters were euthanized and humanely killed, and their testes were used for the analysis of the transgene transfer. All the experimental procedures were assessed and approved by the Institutional Animal Care and Ethics Committee in Isfahan Branch of Islamic Azad University. The project was approved by the identification code of 51755920814006, October 2013. All national and institutional guidelines were appropriately followed.

Transfection reagents

In this study, we used pDB2 plasmid which carried an enhanced green fluorescent protein (EGFP) gene under the control of the human CMV promoter. The injection solution consisted of 50 µL of the circular plasmid (100 ng/µL) and 50 µL of TransIT transfection reagent (MirusBio LLC, USA). The plasmid-liposome mixture was incubated for 30 min at room temperature to allow the formation of lipoplexes. Before transfecting the construct through *in vivo* systems, the EGFP expression was verified via transfection of ovine fibroblast cells with the plasmid and TransIt transfection reagent (Figure 1). Our previous results on the transfection of chicken spermatozoa using pDB2 and TransIT evidenced an acceptable rate of sperm motility and fertilization after the transfection assay (Chaparian *et al.* 2016).

Intra-testis injection of lipoplexes

Initially, in a pilot experiment, the deep injection of Trypan blue into slaughterhouse-collected testis which was wrapped in the scrotoma confirmed the in-deep deposition of the injection solution into the seminiferous tubules and rete testis (data not shown). In this experiment, either the intra-testicle or the caudal epididymis was the injection site for the lipoplexes. The scrotum was locally anesthetized using 0.1 mg/kg xylazine (Nolan *et al.* 1986). Each injection comprised of 100 µL transfection solution into either four sites of each testicle or one site on the epididymis using a 100 µL Hamilton syringe; each testicle and epididymis received 20 and 5 µg of exogenous DNA, respectively. Both right and left testes of each animal underwent the injection procedure. Twelve and five male lambs received seminiferous and caudal epididymis injections, respectively.

Five 30-week old roosters were used for the intra-testis injection of the lipoplexes. Testis injections were carried out by an operation in the caudal-dorsal part of the body, between the thigh attachment site and the last rib (Hasebe *et al.* 1998). Briefly, feathers were removed from the operation site, roosters were locally anesthetized using xylazine (5 mg/kg), (Mostachio *et al.* 2008) carefully underwent a 2-3 cm incision between the thigh and the last rib, and the testicles were accessed for the injection (Hasebe *et al.* 1998). Then the lipoplex mixture was randomly injected into four parts of each testis using an insulin syringe (each testicle received 20 µg of the transgene). Both testicles received the lipoplexes. Finally, the operation site was sutured quickly. Total surgery time was an average of 20 min (Hasebe *et al.* 1998).

Transfection analysis

After 60 days of the transfection process, at 6-month of age, the testis tissues were evaluated for the presence of the EGFP transgene. This 60-day interval was to ensure that the transfected spermatogonial stem cells already involved in the sperm production phase (Cardoso and Queiroz, 1988). Each injected rooster was mated with four virgin hens for two weeks. The collected eggs were incubated at 37.5 ˚C and 65% humidity for 21 days. Afterwards, the hatched chicks were assessed for the presence of the transgene. After two months of injection, all rams were killed. Then, the testes were decapsulated and the epididymis was separated from the testis. Each tissue was sliced thoroughly in a separate 12 mm Petri dish. Sperm cells were collected from the sliced tissues in PBS (Eghbalsaied *et al.* 2009). The sliced tissues were washed successively in PBS to completely remove the remnant of sperm cells. Then, a small fraction of the tubules from different sites of the tissue was mixed and used for DNA extraction. The collected samples from different locations of the testis and epididymis increased the chance of including a broader range of the tissue for the PCR (polymerase chain reaction) analysis.

Genomic DNA was extracted using the standard method of phenol–chloroform from the tissues of the epididymis and seminiferous as well as the sperm cells which were derived from seminiferous or epididymis tissues (Eghbalsaied *et al.* 2009). In addition, spermatozoa were collected from injected roosters by the abdominal massage method (Burrows and Quinn, 1935). The spermatozoa from the ejaculated semen as well as the hatched chicks which were sired by the intra-testis injected roosters were evaluated for the transgene.

The following primer pairs were used for PCR analysis of the EGFP transgene: 5'-ACGTAAACGGCCACAAGTTC-3' and 5'-TGCTCAGGTAGTGGTTGTCG-3' as forward and reverse primers, respectively (Chaparian *et al.* 2016). The PCR program comprised of 95 ˚C for 4 min¸ 35 cycles of 94 ˚C for 45 s, 58 ˚C for 35 s, 72 ˚C for 45 s¸ and a final elongation at 72 ˚C for 4 min. The PCR products were analysis by electrophoresis on a 1.5% agarose gel.

RESULTS AND DISCUSSION

Following injection of plasmid DNA/lipofection reagent complexes in the testicle or the caudal epididymis, we aimed to assess if the EGFP transgene had been taken up and maintained during the process of spermatogenesis in rams. The results of lamb testis transfection are presented in Table 1. The presence of the EGFP transgene was assayed both in tissue and sperm derived from the two injection sites. In the intra-testicular injection group, the majority of seminiferous tissue (56.3% and 75.0%) was positive for the transgene.

In addition, a major fraction of these sixteen positive seminiferous tissues contained positive sperm cells that carried the EGFP transgene (13 out of 16 or 81.2%). In this intra-testicular group, the epididymis tissue was not positive for the transgene, but it contained transfected sperm cells (Figure 2). On the other side, in the epididymis injection group, sixty days after the transfection, 60.0% of the epididymis tissues carried the transgene. Additionally, in one out of six positive tissues, positive sperm cells were also observed.

However, assessment of the seminiferous tissue in this group showed that neither the tissue nor sperm cells carried the EGFP transgene. In this experiment, the lipoplex injection was carried out in the intra-testis tissue of the roosters. None of the injected roosters carried the transgene in their sperm cells derived from either ejaculated semen or seminiferous tubules (Table 2). The roosters were used to inseminate non-transgenic virgin hens. The PCR results of the hatched chicks which were sired by the injected roosters showed that none of them carried the EGFP transgene.

Direct transfection of spermatozoa has been a challenging issue for transgenesis in different species (Lavitrano *et al.* 1989; Eghbalsaied *et al.* 2013). As an effective alternative, *in vivo* transfection of spermatogonial stem cells or TMGT have been shown to be straightforward procedures in mice (Sato *et al.* 1994; Sato *et al.* 2002). In the current study, we attempted to execute TMGT in sheep and chicken. In this study, lipofection of testis cells was carried out in two farm animal species, chicken and sheep.

Table 1 Testis transfection of ovine lambs using enhanced green fluorescent protein (EGFP) transgene and TransIT transfection reagent[1]

Transfection site	Assessed target	No. of animals	Derivation source			
			Seminiferous		Epididymis	
			Right	Left	Right	Left
Intra-testicular	Tissue	12	9 (75.0)	7 (56.3)	0	0
	Sperm cells	12	7 (58.3)	6 (50.0)	6 (50.0)	6 (50.0)
Caudal epididymis	Tissue	5	0	0	3 (60.0)	3 (60.0)
	Sperm cells	5	0	0	0	1 (8.3)

[1] The number (percentage) of positive PCRs is presented from sperm cells as well as their tissue of origin (seminiferous or epididymis tissues).

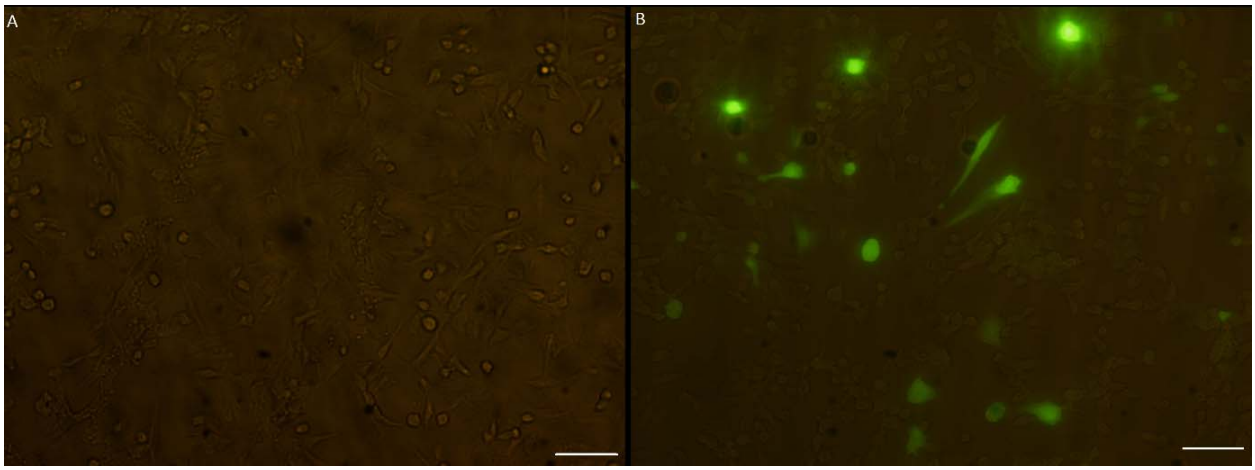

Figure 1 Transfection of ovine cells with pDB2 plasmid (1 µg) and TransIT (1 µL) reagent
A and B are negative control and transfected cells with TransIt transfection reagent, respectively
Bar indicated 400 µm

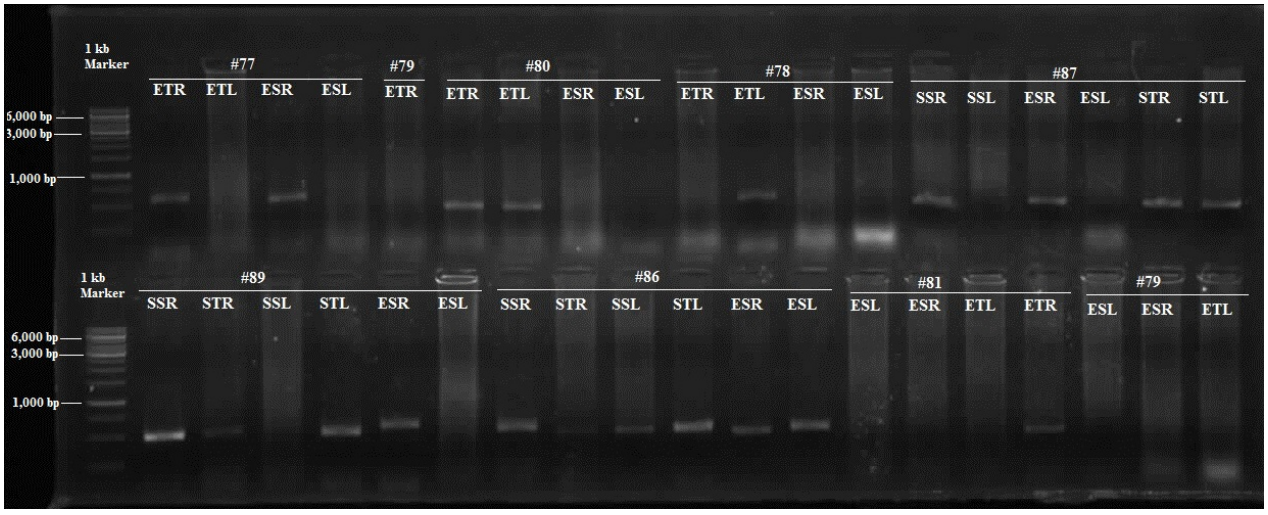

Figure 2 PCR results from intratesticular- and caudal epididymis-injected rams
Lipoplexes of EGFP-encoding plasmid and TransIT were injected in either intra-testicular or caudal epididymis of 4-month lambs
Numbers 77-80 are from caudal epididymis- and numbers 86 88, and 89 are from intra-testicular injection group
ETR and ETL are epididymis tissues right and left, ESR and ESL are sperm from the right or left epididymis, STR and STL are seminiferous tissues right and left, and SSR and SSL are sperm cells from the right and left seminiferous
The 1kb marker is Gene Ruler 1 kb DNA ladder (#SM0311, Thermofisher Scientific)

Table 2 Transfection of rooster's testis using enhanced green fluorescent protein (EGFP) transgene and TransIT[1]

Assessed animals	No. animal	Assessed sample	Transfection rate
Roosters	5	Testis	0
	5	Ejaculated spermatozoa	0
Offspring (hatched chicks)	12	Testis	0
	12	Heart	0
	12	Liver	0
	12	Muscles	0
	12	Brain	0
	12	Eyes	0

[1] The transgene was intra-testicularly injected and assessed in the testis tissue and the ejaculated spermatozoa as well as the hatched chicks which were sired by the intratesticular-injected roosters.

In rams, irrespective of the injection site, the tissue cells, seminiferous or epididymis, were transfected. We carefully separated the seminiferous tubules, and collected sperm cells from these tubules. Our results clearly showed the transgene transfer into the sperm cells and seminiferous tubules. The collected sperm cells from the epididymis of the deep-injected testis also showed that the sperm cells carried the transgene, whereas the epididymis tissue was non-transected. The sperm transfection efficiency was more than 50% when the intra-testicular injection was carried out. It has been verified that TMGT is an efficient technology for mouse transgenesis (Sato *et al.* 1994; Wang and Cao, 2013). However, compared to mouse testis, livestock testis has a larger ratio of volume-to-surface, thicker tunica albuginea, and smaller seminiferous tubules (Honaramooz *et al.* 2002; Rodriguez-Sosa *et al.* 2006). Although these attributes disfavor TMGT efficiency in livestock, *in vitro* electroporation of goat testis showed that seminiferous tubules are transfectable (Raina *et al.* 2015). In addition, microinjection of DNA-lipofection complex into the pig testis could transfect male germ cells in 25% of the seminiferous tubules (Kim *et al.* 1997). By using a 2-inch length Hamilton syringe, we could carry out a deep injection, which could surpass the scrotum and reach the rete testis of four-month-old lambs (Salhab *et al.* 2001). It should be noteworthy that we injected roughly 70% of the liposome reagents into the deep site of the testis in a fixed position, while the remaining 30% was infused when the injection needle was pulled out gradually from the testis. Therefore, a cross-section of the testis tissue was interacted with the lipoplexes throughout each injection process. Moreover, the injection was carried out in four distant sites on the same testicle. It could provide a higher chance for transfection of seminiferous tubules which were disrupted by the Hamilton needle. Direct access to seminiferous tubules of livestock through rete testis is also important for transplantation of male germ cells into the surrogate sires (Hill and Dobrinski, 2005). Injection of germinal stem cells into the rete testis of pre-pubertal goats via the testis surface could successfully lead to the transplantation of fluorescent-labelled germinal cells in the entire injected testis (Honaramooz *et al.* 2003). Moreover, a higher transplantation rate was observed when the rete testis was accessed through extra-testicular rete or cauda epididymis of sheep and pig, respectively (Honaramooz *et al.* 2002; Rodriguez-Sosa *et al.* 2006). Therefore, the results of these *in vivo* studies on germinal cell transplantation agree with the idea that seminiferous tubules are accessible through direct injection from the surface of livestock testis.

Our attempt was not successful to implement TMGT in rooster. Neither the testis tissue nor the ejaculated sperm cells carried the transgene after two months from the injection date. These results agreed the previous unsuccessful try on *in vivo* transfection of rooster testis (Hasebe *et al.* 1998).

It should be considered that we couldn't use the Hamilton syringe for the rooster testis because the needle length and diameter was not appropriate for the intra-testis injection in chicken. Instead, the injection was carried out by an insulin syringe which was much narrower than the Hamilton syringe. Therefore, it might not efficiently interact with the seminiferous tubules of the injected area. Also, the observed discrepancy between ram and rooster TMGT could be due to the species differences in the histology of testis tissue. Seminiferous tubules in chickens are anastomosis and wrapped by a large basal lamina (Deviche *et al.* 2011).

In the current study, we transfected the gonads at the early stages of puberty, at which gonad circumference is smaller than the mid-puberty ages, aiming to have a higher chance of transfection. However, the proportion of interstitial tissue, as well as leydig and sertoli cells to the seminiferous tubules vary significantly from the pre-puberty to the late puberty periods in chicken (González-Morán *et al.* 2008). Therefore, transfection of chicken testis at the pre-puberty and mid-puberty stages is yet to be explored.

CONCLUSION

In conclusion, the current study documented *in vivo* transfection of ram testis. This is the first report showing the feasibility of sperm transfection based on *in vivo* lipofection of TMGT in sheep although the transgene transfer into the next generation is yet to be achieved. Nonetheless, in the parallel experiment on chicken, we couldn't detect any evidence supporting the transgene transfer into the spermatozoa. Further researches are required to look into the physiological reasons for the inefficiency of TMGT in chicken.

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