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ORIGINAL PAPER

A new method to efficiently produce transgenic embryos and mice from low-titer lentiviral vectors

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Abstract Vector injection into the perivitelline space has emerged as the standard delivery method to transduce lentivirus to mammalian oocytes or one-cell embryos, but its application is limited by the need for high titers of lentivirus. Herein we developed a new method by using a Piezo impact micro-manipulator for injecting low titer of lentivirus into the subzonal space of two-cell embryos or the perivitelline space of one-cell embryos that were shrunk with a highly concentrated sucrose solution. The survival rate of embryos was greater than 98% using this micromanipulation strategy, which was increased compared to the normal one-cell embryo injection method. More than 90% of injected embryos were GFP positive after subzonal injection of a lentivirus vector carrying the *GFP* gene with titers of 2×10^8 I.U./ml. Even when a low titer of lentivirus (2×10^6 I.U./ml) was used, 53.26% and 40.85% transgenic embryos were obtained after two-cell embryonic injection and one-cell sucrose treated embryonic injection, respectively. The GFP-positive rates were also greater than in the conventional method of injecting one-cell embryos (25.39%). In addition, blastocysts from the two-cell embryo injection group displayed stronger GFP fluorescence than the one-cell embryo injection groups treated with or without the sucrose solution. Increased expression of GFP suggests that the embryos obtained from this injection method have higher exogenous gene expression levels compared to previous methods. Therefore, in contrast with the traditional injection method, we have demonstrated a simplified and efficient lentivirus-mediated gene transfer method based on a low-titer virus preparation.

Keywords Lentiviral vectors · Transgenic embryos · Low titer · Mouse

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Introduction

Transgenic animals provide an outstanding in vivo model for biomedical research because they can be used to generate preclinical models of human

diseases and to develop gene therapy strategies. The lentiviral vector has recently emerged as an attractive alternative method for delivering exogenous genes into cells because human immunodeficiency virus (HIV)-derived gene delivery vehicles can efficiently mediate the integration of their cargo into zygotes or other cell types, even during a quiescent phase. Based on developing lentiviral vectors for gene therapy (Pfeifer 2004; Pfeifer and Hofmann 2009), its ability to efficiently deliver a gene into the germ line has been repeatedly demonstrated in mice (Lois et al. 2002; Pfeifer et al. 2002), rats (Lois et al. 2002), chickens (McGrew et al. 2004), pigs (Hofmann et al. 2003; Whitelaw et al. 2004), cattle (Hofmann et al. 2004) and monkeys (Chan et al. 2001). However, there are some limitations in lentiviral vectors' extensive utilization, especially concerning the necessity for high lentiviral titers.

Due to the makeup of the mammalian embryo, lentiviruses must penetrate the zona pellucida, a protective outer layer of the embryo. The zona pellucida is a 5- to 10-μm-thick spherical shell mainly composed of glycoproteins that serves as a physical barrier to prevent lentivirus penetration. The preferred method of delivery utilizes subzonal lentivirus injection, which introduces the lentivirus into the perivatella space (Lois et al. 2002), allowing the virus to gain access to the membrane of either the oocyte or the zygote. Considering the small volume of perivitelline space between the cytoplasmic membrane and the zona pellucida, only 50–100 pl of lentivirus vector can be injected. As a result, high titers of the lentivirus vector are needed for this traditional transfer method, and in practice, titers of 10^9 infectious units (I.U.)/ml are required to achieve a satisfactory level of efficiency (Lois et al. 2002; Ritchie et al. 2007). High titers of lentivirus are difficult to acquire and require complex procedures that may raise safety risks. An alternative delivery method is to co-incubate the lentivirus with denuded single-cell embryos using either pronase or a micro-drill. This method does not require high titers of lentivirus, but has drawbacks due to impaired embryonic developmental potential and a low rate of implantation after embryonic transfer, which may be due to the 'sticky' nature of the zona pellucida-denuded embryos and technical damage (Lois et al. 2002; Ritchie et al. 2005; Ewerling et al. 2006). Although some efforts, such as the repeated injection of a low-titer lentiviral vector and the back pressure method, can resolve the problem, need of high titers of lentivirus, and improve the transgenic efficiency (Ritchie et al. 2007), these methods also increased the micromanipulation difficulty and decrease the embryonic survival rate.

The objective of this study was to develop a new subzonal injection method for delivering lentiviruses to increase the transgenic efficiency using two-cell embryonic injection and one-cell sucrose-shrunk embryonic injection. Mouse zona pellucida can be punctured via a piezo-driven pipette without the pipette being too deeply inserted, a Piezo impact micro-manipulator (PMM) should be used to penetrate the zona pellucida which results in less injury (Kimura and Yanagimachi 1995). Since using a hyperosmotic solution to shrink the cytoplasm can also decrease the incidence of mechanical damage when manipulating the zona pellucida (Nakagata et al. 1997; Anzai et al. 2006), embryos at the two-cell stage have a larger perivitelline space and a greater distance between the zona pellucida and the vitelline membrane than one-cell embryos without the hyperosmotic solution treatment. These distinguishing features may let more virus suspension fill the subzonal space and theoretically allow the embryos to avoid the pulse injury that can be caused by PMM. Consequently, most injections in this study were carried out in two-cell embryos. When one-cell embryos were used, they were shrunk with a 0.5 M sucrose solution to expand the subzonal space and increase the distance between the zygote and its zona pellucida.

Materials and methods

Lentivirus production

Plasmids for the generation of the FUGW human polyubiquitin C promoter (UbiC) enhanced green fluorescent protein (EGFP), the delta8.9 plasmid and the VSVG plasmid (Lois et al. 2002) were kindly provided by Dr. Carlos Lois (Division of Biology, California Institute of Technology, Pasadena, CA). All viruses were produced as described previously (Lois et al. 2002; Tiscornia et al. 2006). Briefly, 293T cells were infected with the vectors, packaging plasmids, and a plasmid coding for the G protein of

the vesicular stomatitis virus using the calcium phosphate method. The supernatant was harvested 60 h after infection and concentrated by ultracentrifugation (70,000*g*, 2 h). The viral titer was determined by infecting 293T cells.

Embryo collection

Mature mice (Kunming White outbred strain) were caged in a controlled environment with a 12 h light: 12 h dark cycle. The adult females were superovulated via injecting 10 IU of pregnant mare serum gonadotropin (PMSG), followed by injection of 10 IU of human chorionic gonadotropin (hCG) 48 h later; then, the females were mated overnight with wild type males. To collect one-cell embryos, the females were sacrificed by cervical dislocation 20–24 h after hCG injection, and the embryos were released from the oviducts. The cumulus cells were removed via hyaluronidase (Sigma) treatment and pipetting. Two-cell embryos were harvested 44–46 h after hCG treatment. After collection embryos were further selected and assigned to appropriate samples based on their morphology. Recovered embryos were incubated in M2 medium (Sigma). After perivitelline injection, the embryos were cultured in M16 (Sigma) microdrops under mineral oil (Sigma) in an atmosphere of 5% CO_2 at 37°C.

Lentivirus injection, determination of reporter gene activity and transgenic embryo transfer

Three groups of embryos were injected: one-cell embryos (OEI group), two-cell embryos (TEI group), and one-cell sucrose shrunk embryos (OESI group). The OESI group was put into a 0.5 M sucrose solution to shrink the cytoplasm and avoid PMM-induced cytoplasmic damage (Anzai et al. 2006). One-cell embryos were injected with lentivirus by the traditional method, which serve as the control group. Three additional groups of embryos (one of each treatment) were cultured directly after collection without injection. These one-cell and two-cell stage embryos and these sucrose-shrunk one-cell embryos were cultured to assess culture conditions.

Injection of lentivirus into the perivitelline space was performed under an inverted microscope (Olympus IX70) equipped with a hydraulic micromanipulation system (ON2-99D-3 Microinjector, Narishige, Japan) and a PMM (PMM-150FU, Prime Technology, USA), using the backpressure method (Ritchie et al. 2007). During manipulation, the zona pellucida swelled immediately after the pipette was pushed into the perivitelline space and remained in that state for 5–10 s, so the perivitelline space could be filled with lentivirus. The holding and injection pipettes were hand-made using a micropipette puller (P-97, Sutter Instruments, USA) and a micro-forge (MF-830, Narishige, Japan). After subzonal lentivirus injection, embryos were washed with M16 medium three times and then cultured in the same medium. In addition, embryos from the OESI group were returned to their original size in M2 medium with normal osmotic pressure so that they could be cultured in vitro. Each treatment was repeated at least five times.

Following 72 h of in vitro culture, GFP expression was apparent in blastula- or morula-stage embryos developing from the infected embryos. GFP fluorescence was detected using ultraviolet light with an excitation filter operating between 455 and 495 nm.

For the embryonic transfer procedure, we transferred 3.5 day postcoital (dpc) blastocysts into the uteri of 2.5 dpc pseudopregnant Kunming female mice.

Statistical analysis

Difference in the rates of embryo survival post-injection, blastocyst formation and formation of GFP-positive blastocysts among the experimental groups were analyzed using the PROC GLM procedure with the SAS software package (SAS Inst. Inc., Cary, NC, USA: SAS Institute, 1988). All percentage data were subjected to arcsine transformation before statistical analysis. Duncan's multiple range tests and chi-squared tests were used to compare the values of each individual treatment. Differences with $P < 0.05$ were considered statistically significant.

Results and discussion

Lentiviral injection into two-cell embryos increases the rates of embryo survival, blastocyst formation and GFP-positive embryos

Virus titration showed that a 1-ml suspension contains 2×10^8 viral I.U. Lentivirus titers of 2×10^6 I.U./ml

were obtained by dilution to mimic low-titer lentiviral vector preparations.

Subzonal injection relies on PMM to penetrate the zona pellucida, the physical barrier of viral infection. Although high survival rates have been acquired using the conventional method, in which transductions are made to oocytes or zygotes, approximate 10% of embryos died due to mechanical damage, as determined when embryonic survival rates were evaluated immediately after micromanipulation (Table 1). With lentivirus titers of 2×10^8 I.U./ml, embryonic survival rates in the TEI group were greater than in the OEI group (99.71 vs. 92.53%, $P < 0.05$). The same trend was observed when the lentiviral titer was 2×10^6 I.U./ml. This might be due to the distance between the zona pellucida and the vitelline membrane, but an additional advantage in the TEI group was the injection was parallel to the membrane instead of being injected perpendicularly to it (Fig. 1). Using this injection strategy may greatly decrease the injury caused by the PMM pulse.

When the perivitelline injection was performed in two-cell embryos, 82.02 and 84.74% of the embryos reached the blastula stage when the lentiviral titers were 2×10^8 and 2×10^6 I.U./ml, respectively. Blastocyst rate was not significantly different among the two-cell stage group that was cultured without injection and the two-cell stage injection groups with different titers of lentivirus. Similar results were also acquired in the two OEI groups in comparison with their respective noninjected control group, and the two OESI groups in comparison with their control

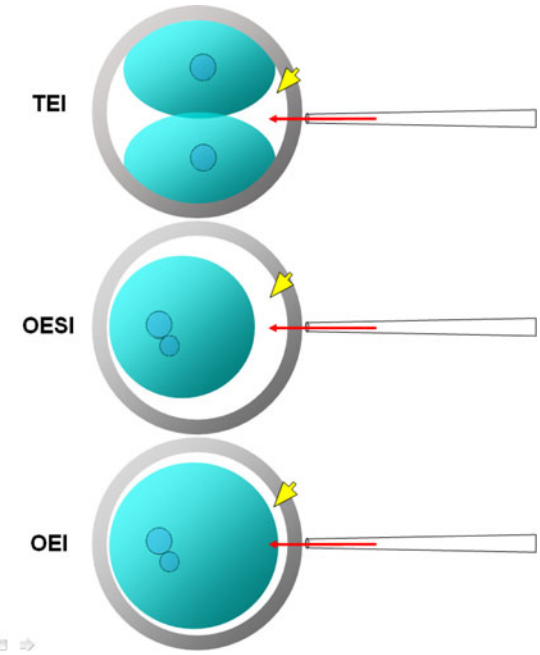

Fig. 1 Schematics of injections at the two-cell stage (TEI), the one-cell stage with sucrose treatment (OESI), and the one-cell stage (OEI). The *yellow arrow head* indicates the perivitelline space of the embryos. The *red arrow* indicates the direction of the pulsating strength of PMM to the zygote or blastomeres

Table 1 Embryos infected by lentiviral perivitelline injection

Injection stage of embryos	Lentiviral titer (I.U./ml)	Total no. oocytes injected	No. (% ±SD) embryos survived after injection	No. (% ±SD) blastocysts	No. (% ±SD) GFP-positive blastocysts
2-cell stage (TEI)	2×10^8	272	271 (99.71 ± 0.88%)^a	227 (82.02 ± 4.91%)^a	221 (95.20 ± 4.96%)^a
1-cell stage + sucrose (OESI)	2×10^8	210	210 (100 ± 0.00%)^a	134 (74.29 ± 5.87%)^b	125 (93.42 ± 2.19%)^{a,b}
1-cell stage (OEI)	2×10^8	259	240 (92.53 ± 3.91%)^b	171 (71.74 ± 4.66%)^b	154 (90.19 ± 8.85%)^b
2-cell stage (TEI)	2×10^6	263	263 (100 ± 0.00%)^a	228 (84.74 ± 8.70%)^a	118 (53.26 ± 7.96%)^c
1-cell stage + sucrose (OESI)	2×10^6	210	207 (98.53 ± 2.22%)^a	136 (74.09 ± 3.30%)^b	48 (40.85 ± 6.01%)^d
1-cell stage (OEI)	2×10^6	305	294 (95.55 ± 2.32%)^b	206 (69.34 ± 5.18%)^b	52 (25.39 ± 5.74%)^e
2-cell stage	Without injection	159	–	134 (84.03 ± 10.64%)^a	–
1-cell stage + sucrose	Without injection	245	–	185 (74.32 ± 5.05%)^b	–
1-cell stage	Without injection	180	–	127 (71.40 ± 7.23%)^b	–

Values in the same column with different superscripts are significantly different ($P < 0.05$)

group. When considering TEI and OEI/OESI together, greater blastocyst rates were obtained when the injection was performed on two-cell embryos at each concentration of lentiviral titers. These results suggest that micromanipulation and lentiviral titers have no effect on the developmental rates of the embryos and that two-cell embryos have a greater developmental potential than one-cell embryos. A possible explanation for this difference may be that embryos at the two-cell stage have passed the "two-cell block." Alternatively, the one-cell embryo groups may contain oocytes that were not fertilized.

Although delivery of the lentiviral vector at 2×10^8 I.U./ml resulted in more than 90% of the embryos demonstrated GFP in the TEI and the OEI groups (95.20 and 90.19%, respectively), results were greater ($P < 0.05$) from the TEI group compared to the OEI group. The percentage of GFP-positive embryos between these groups was also different when applying low titers of lentivirus (2×10^6 I.U./ml). Injection of two-cell embryos resulted in greater positive rates than OEI (53.26 vs. 25.39%, respectively, $P < 0.01$). This is likely due to the increased volume of perivitelline space in two-cell embryos compared to one-cell embryos, which allowed a greater volume of lentivirus to infect the embryos after injection.

We know that no genes were transcribed at the zygotic stage, but genes increased their expression levels during embryonic development. This revealed the patterns of maternal RNA degradation and zygotic gene activation, especially since the first wave of transcription corresponded to zygotic genome activation in the two-cell stage (Hamatani et al. 2004). Lentiviral vectors integrate preferentially into genomic regions that are either transcriptionally active or poised for activation (Bushman et al. 2005; Sauvain et al. 2008). This means that high rates of lentiviral integration may occur when the injections are carried out on two-cell embryos, which have more transcriptionally active regions. Some embryos were transfered in our study and six healthy and normal transgenic mouse have been obtained by TEI method, this result indicated that the TEI has no negative effect on the late embryonic development. In addition, it is noteworthy that there are two blastomeres in two-cell embryos, which means virus infect the cells in different styles. Therefore, mosaic embryos maybe generated using TEI methods.

Sucrose treatment of one-cell embryos increases embryonic survival and GFP positivity embryos after lentiviral injection

In order to simulate the morphology and validate the advantage of the two-cell embryos, one-cell embryos were placed into 0.5 M sucrose solution to shrink the cytoplasm when the injection was carried out. As expected, the embryonic survival rate of one-cell stage injection could be increased significantly by the sucrose treatment regardless of lentiviral titers (Table 1). These results indicate that OESI is less harmful than OEI. OESI resulted in similar survival rates as the TEI groups. This is likely due to decreased injury and damage of PMM's mechanical stimulus, and this may be due to the larger distance between the zona pellucida and the vitelline membrane in zygotes that had undergone sucrose treatment to shrink the cytoplasm or were at the two cell stage. These data also help explain the high embryonic survival rates of the TEI groups.

When considering blastocyst rates after injection and development, there was no difference between the OESI and the OEI groups, and no difference was observed between the OESI groups and the control cultured directly group. This suggests that the sucrose treatment had no effect on embryonic developmental potential.

Applying the lentivirus vector at a concentration of 2×10^8 I.U./ml resulted in 93.42% of OESI embryos expressing GFP. This was similar to both the TEI and the OEI groups. However, the percentage of GFP-positive embryos was decreased in both the TEI and the OESI group compared to the TEI groups when low titers of lentivirus were applied (2×10^6 I.U./ml). One-cell embryos injected with sucrose treatment increased the GFP-positive rate (40.85%) compared to the OEI group ($P < 0.05$). This increase in GFP positive embryos may be a result of the sucrose shrinking the cytoplasm, which resulted in a larger perivitelline space similar to the morphology of the two-cell stage embryos. This allowed for a greater volume of lentivirus injection. Conversely, the perivitelline space of the OESI group embryos decreased to its previous volume, and the embryos returned to their former size in the M2 medium. Concurrently, the lentiviral suspension could escape from the perivitelline space via the injection hole, which may explain why the GFP-positive rate of the

OESI group was decreased in comparison to the TEI group.

Although we conclude that the huge perivitelline space of the two-cell stage embryos is a major cause for the high rates of GFP-positive embryos observed here, more than one difference exists between the TEI and the OESI groups, so we cannot neglect the more transcriptionally active regions of the two-cell embryos that may contribute to the high integrative rates. Nevertheless, although we suggest that the transcriptional activity of the two-cell embryos is another major cause for the high GFP-positive rates, the mechanisms involved have yet to be elucidated.

The blastocysts derived from two-cell embryonic injection displayed stronger GFP fluorescence

Delivering the virus by injection into the perivitelline space yielded transgenic embryos with high efficiency; however, the number of integrated proviruses in the genome varied substantially from animal to animal, ranging from none to more than 20 (Lois et al. 2002). As Fig. 2 shows, the blastocysts displayed stronger GFP fluorescence after two-cell embryonic injection compared to traditional injection (OEI), with delivery of the lentiviral vector at a concentration of 2×10^8 I.U./ml. Intensity was positively correlated with copy number, as estimated qualitatively (Lois et al. 2002), which may indicate that embryos had a greater expression level of the foreign gene after TEI. The greater expression may be due to the larger volume of perivitelline space and the high transcriptional activity of the two-cell embryos, as mentioned above.

Low-titer lentivirus can efficiently infect embryos using the two-cell embryonic injection

Although applying increased back pressure to the pump system allowed a low-titer lentiviral vector preparation to efficiently generate transgenic embryos, only 12% of embryos was positive when

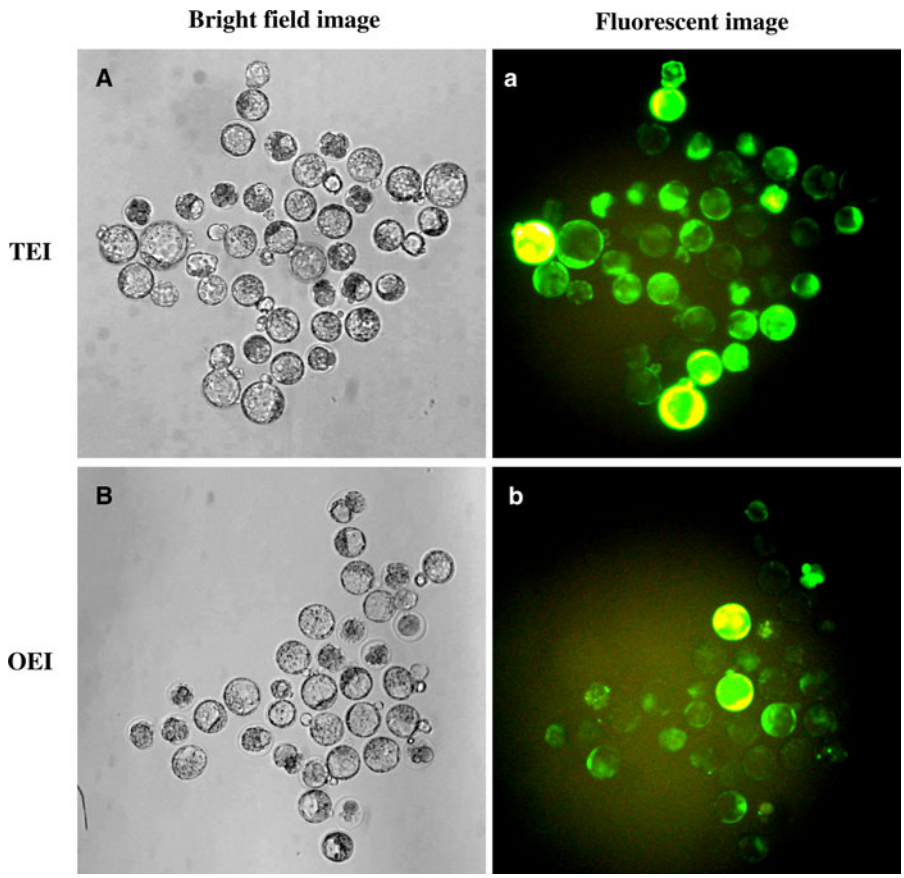

Fig. 2 Blastocyst after TEI and OEI, with delivery of the lentiviral vectors at 2×10^8 I.U./ml. Images from the two-cell embryonic injection groups (TEI; photo A and a), and the one-cell embryonic injection groups (OEI; photo B and b)

the lentiviral vector was delivered at 2.4×10^6 I.U./ml (Ritchie et al. 2007). In addition, only 12.5% of denuded embryos carried the transgene after incubation with 0.8×10^6 I.U./ml lentiviral vectors (Lois et al. 2002). Using the TEI method an unprecedented high efficacy rate of 53.26% was obtained, even when low titer 2×10^6 I.U./ml was used.

Conclusion

This project has demonstrated a new method to efficiently produce transgenic embryos using a low titer of lentiviral vectors, by which Piezo-impacted injection was carried out in either two-cell embryos or one-cell embryos shrunk with a hyperosmotic sucrose solution. These methods did not impair embryonic developmental potential and enabled the efficient generation of transgenic embryos, even when a low titer of lentivirus (2×10^6 I.U./ml) was applied. Furthermore, it is important that these greater blastocyst rates and high expression levels of the exogenous gene were acquired using the two-cell embryonic injection strategy, compared with the traditional one-cell embryonic injection method.

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