

COMPARISON OF TRANSFECTION METHODS FOR RABBIT ZYGOTES

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Abstract

The aim of the study was to compare the efficiency of standard DNA microinjection procedure and a new DNA lipomicroinjection method, used for transfection of rabbit zygotes. Comparison of transfection (transgenesis) efficiency leads us to conclude that it was much better when the standard DNA microinjection technique was used. The proportion of “transgenic” blastocysts obtained from zygotes transfected using standard DNA microinjection was more than twice that of “transgenic” blastocysts obtained using lipomicroinjection. Because the study was conducted under *in vitro* conditions, we cannot state conclusively which method will produce a lower percentage of mosaic individuals. The DNA lipomicroinjection method is much simpler to perform and causes lower loss of transfected material.

Key words: pig, transgenesis, DNA microinjection, DNA lipomicroinjection, efficiency

The production of transgenic animals is a complex procedure. The process as a whole is affected by several factors, which result in the production of a transgenic animal with modified genotype. Despite increasingly sophisticated technologies, the efficiency of the process remains disproportionately low compared to the labour inputs and costs. In rabbits, transgenesis efficiency using standard DNA microinjection, does not exceed 4% in terms of the number of zygotes subjected to microinjection (Jura et al., 2003; Jura and Jurkiewicz, 2006).

Cloning using transfected somatic cells or their nuclei to produce transgenic animals is even less efficient. This is due to the inefficiency of the cloning process, the complexity and limitations of which have been well documented in the literature (Kruip and Den Daas, 1997; Melo et al., 2007).

In the near future, application of the new-generation lentiviral vectors, whose transfection efficiency is increasingly promising, will likely improve the efficiency of the production of transgenic animals, including transgenic rabbits. However, this

will take place only when researchers have developed commercially available vectors using safer types of lentiviruses, which will enable their use in laboratories with lower security levels (Jura and Jurkiewicz, 2006).

Likewise, technologies that use transfected stem cells to produce transgenic mammals are still undergoing improvement and standardization in the case of farm animal species. The main constraint to full use of stem cell technology in transgenesis of rabbits and other farm animal species is the availability of pure stem cell germ lines suitable for transfection.

As already mentioned, transgenesis is a complex process and factors related to reproduction physiology as well as embryological and molecular factors have a significant effect on its efficiency (Jura et al., 1998; Jura et al., 2000; Fana and Watanabe, 2003; Lipiński et al., 2003; Jura and Jurkiewicz, 2006). A key role is played by method of introducing exogenous genetic information. The efficiency of the process is low when standard DNA microinjection technique is used. Therefore, there is an urgent need to develop a less complex transfection method, whose efficiency will surpass or match the efficiency of DNA microinjection (Jura and Jurkiewicz, 2006; Shen et al., 2006; Smorağ and Jura, 2006; Skrzyszowska et al., 2006; Melo et al., 2007).

The objective of this study was to determine the efficiency of lipomicroinjection, a new zygote transfection method, in comparison with standard DNA microinjection. The comparative tests also accounted for the “molecular factor”, or gene constructs (vectors) used to carry genetic information to induce certain changes in the genotype, which plays a key role in the transgenesis process. The gene constructs were equipped with reporter genes, which enable the procedure efficiency to be determined under *in vitro* conditions and transgenic embryos to be selected before transfer to synchronized recipients.

The paper presents the results of transfection of rabbit zygotes obtained using standard DNA microinjection in comparison with a new, alternative method of transfection named lipomicroinjection.

Material and methods

New Zealand White does aged 5–7 months at 3–4 kg body weight were used as donors of zygotes for transfection.

Collection of zygotes

Donor does were superovulated by intramuscular injection of 100 IU of PMSG (Serogonadotropin, Biowet, Poland). Stimulated for superovulation, donor does were mated or inseminated 72 h after PMSG administration and 100 IU of HCG (Biogonadyl, Biomed, Poland) was intravenously injected. Zygotes were recovered surgically 20–22 h after HCG administration or postmortem by flushing oviducts with PBS containing bovine albumin (Sigma, USA). The eggs obtained were subjected to morphological evaluation under a stereoscopic microscope. Only morphologically normal eggs were used for transfection.

Lipomicroinjection

Morphologically normal eggs were placed in a micromanipulation chamber filled with PBS containing 20% fetal calf serum (FCS) (Sigma, USA). The procedure was carried out under an inverted microscope equipped with Nomarski optics and two micromanipulator units. The liposome/gene construct complex was inserted under the zona pellucida into the perivitelline space. After lipomicroinjection, transfected zygotes were transferred to a small vessel containing PBS and albumin and again subjected to morphological evaluation. Only zygotes with an intact cytoplasmic structure were selected for culture.

DNA microinjection

Eggs with intact cytoplasm and two visible polar bodies were placed in a micromanipulation chamber filled with PBS containing 20% fetal calf serum (FCS) (Sigma, USA). The procedure was carried out under an inverted microscope equipped with Nomarski optics and two micromanipulator units. The gene construct was inserted into male pronuclei up to the moment when its size increased by 50%. After DNA microinjection, transfected zygotes were transferred to a small vessel containing PBS and albumin and again subjected to morphological evaluation. Only zygotes with an intact cytoplasmic structure were selected for culture.

For transfection (microinjection and lipomicroinjection) *CMV RFP* (pcDNAZC3H-rfp) reporter gene construct, containing the *Red Fluorescent Protein* reporter gene, was used.

***In vitro* culture**

Transfected zygotes were transferred to four-well dishes filled with culture medium. Cultures were performed in Upgraded B2 INRA Medium (Laboratoire C.C.D. Paris, France), in a laminar flow incubator (39.5°C, 5.0% CO₂, maximum humidity) (Jura et al., 2000). After 4 days of culture, embryos were assessed morphologically by choosing morulae/blastocyst embryos for *in vivo* assessment. Selected embryos were transferred to PBS (Sigma, Germany) supplemented with 20% FCS (Sigma, Germany) and mounted in groups of 5 on a depression slide.

***In vivo* assessment of transfection efficiency**

After 4 days of *in vitro* culture, embryos were evaluated under a fluorescence microscope (Nikon, Japan) using 510–560 nm UV filter. Embryos that reached morulae/blastocyst stage and showed marked red luminescence, were classified as positive (transgenic).

Results

Twenty-eight does were superovulated to obtain zygotes for lipomicroinjection and standard microinjection. A total of 415 zygotes were obtained. Lipomicroinjection and standard DNA microinjection were performed on 160 zygotes each. The

control group were 95 non-transfected zygotes, *in vitro* cultured to the morulae/blastocyst stage (Table 1). As a result of culture, zygotes transfected using lipomicroinjection produced 6 (5.82%) morulae/blastocyst stage transgenic embryos. After the standard DNA microinjection 17 (22.07%) transgenic morulae/blastocyst stage embryos were produced (Table 1, Figure 1).

Table 1. Comparison of rabbit zygote transfection results obtained using lipomicroinjection and standard microinjection methods

Transfection method	No. of transfected zygotes	No. of embryos obtained (%)	No. of transgenics (%)
Lipomicroinjection			
CMV-RFP	160	103 (64.37)	6 (5.82)
Standard DNA Mi			
CMV-RFP	160	77 (48.12)	17 (22.07)
Control	95	64 (67.36)	

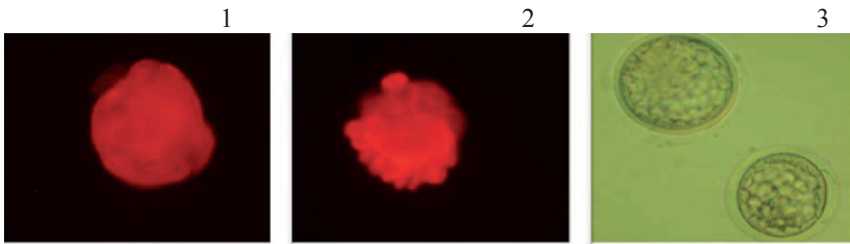


Figure 1. 1. Transgenic blastocyst obtained after lipomicroinjection of CMV-RFP vector 2. Transgenic blastocyst obtained after standard microinjection of CMV-RFP vector 3. Blastocysts from control

Discussion

Efficiency of DNA microinjection, the most common method used to date to produce transgenic rabbits, is low. Other popular transfection techniques used to produce transgenic mice are either unavailable for this species or highly complex. In a search for an alternative zygote transfection method, which would result in comparable or better efficiency of transfection (and thus transgenesis) in rabbits, an attempt was made to use modified lipofection for transfection of rabbit zygotes. Lipofection is a popular method for transfection of somatic cells. It is a highly efficient procedure of DNA transfection using liposomes. In this method, exogenous DNA (gene construct) is carried by monolayer or multilayer liposomes, made of cationic lipids and spontaneously interacting with DNA. In addition, they are capable of penetrating cell membranes and this capacity is used to transport exogenous DNA into the nucleus. Zygote transfection by conventional lipofection, which is used to transfect somatic

cells, where the carrier (liposomes) linked to exogenous DNA comes into direct contact with cell membranes surrounding the cytoplasm, is not feasible for zygotes, which are surrounded by the zona pellucida. To enable contact between DNA/liposomes carrier complex and cell membranes surrounding the zygote, a method for microinjection of the liposome/DNA complex into the perivitelline space, named lipomicroinjection, was used. The present study showed that injection of the complex under the zona pellucida of the zygote is a straightforward procedure. What is more, a much greater volume of the DNA mixture can be deposited in the perivitelline space than is possible with direct injection of DNA mixture into the pronucleus (about 300 pl into perivitelline space, 5 pl into pronucleus). In theory, a greater number of insert copies should offer a greater chance for integration of exogenous DNA with the host genome. Furthermore, unlike standard DNA microinjection, lipomicroinjection causes no damage to intracytoplasmic or pronuclear structures. Insertion of the injection pipette into the cytoplasm or pronuclei itself causes about 10–15% of the transfected zygotes to die. Compared to standard DNA microinjection, lipomicroinjection turned out to be a less invasive method, which clearly translates into the number of blastocysts obtained from transfected zygotes cultured *in vitro* (Table 1). In our study, the type of gene construct used for transfection had no substantial effect. In addition, the type of the reporter gene used did not influence the clarity and quality of the *in vivo* evaluation of insert expression (Figure 1). Furthermore, the use of reporter gene enabled transfection efficiency to be assessed rapidly (about 48 h after *in vitro* culture) and effectively. Comparison of transfection (transgenesis) efficiency leads us to conclude that it was much better when the standard DNA microinjection technique was used. The proportion of “transgenic” blastocysts obtained from zygotes transfected using standard DNA microinjection was more than twice that of “transgenic” blastocysts obtained using lipomicroinjection (Table 1).

Because the study was conducted under *in vitro* conditions, we cannot state conclusively which method will produce a lower percentage of mosaic individuals. It is known that this proportion is relatively high for standard DNA microinjection.

The DNA lipomicroinjection method is much simpler to perform and causes lower loss of transfected embryos. The more complex DNA microinjection technique makes it possible, at least at this stage, to obtain a greater percentage of “transgenic” embryos. Standard DNA microinjection has reached its limits as it cannot be modified to improve method efficiency. Lipomicroinjection is an alternative method and a number of parameters have to be fine-tuned (e.g. optimum parameters for the liposome/DNA complex have to be determined). Another advantage is that new and increasingly efficient and capacious generations of carriers (liposomes) are being developed.

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Porównanie metod transfekcji zygot króliczych

STRESZCZENIE

Celem pracy było porównanie efektywności standardowej metody mikroiniekcji DNA w odniesieniu do nowo zaproponowanej metody transfekcji zygot króliczych – lipomikroiniekcji. Porównując efektywność transfekcji (transgenozy) należy stwierdzić, że wypadła ona dużo lepiej przy zastosowaniu metody standardowej mikroiniekcji DNA. Odsetek „transgeniczných” blastocyst uzyskanych z zygot transfekowanych metodą standardowej mikroiniekcji DNA był ponad dwukrotnie większy w porównaniu z odsetkiem „transgeniczných” blastocyst uzyskanych po zastosowaniu lipomikroiniekcji. Ponieważ badania prowadzone były w warunkach *in vitro*, nie można jednoznacznie stwierdzić, która z metod będzie skutkowałą mniejszym odsetkiem uzyskanych osobników mozaikowych. Metoda lipomikroiniekcji DNA jest dużo prostsza w wykonaniu i powoduje niższe straty w transfekowanym materiale.