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Quality of transgenic rabbit embryos with different *EGFP* gene constructs

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Summary

The aim of this study was to compare the quality of rabbit transgenic embryos obtained upon microinjection of gene constructs containing different promoters and green fluorescent proteins (CMVIE–EGFP, PGK–EGFP and CMVIE–hrGFP). Developmental rate, total cell number in hatching blastocyst stage, number of apoptotic cells, diameter of embryos, transgene integration and transgenic mosaicism were investigated.

The rate of rabbit embryos microinjected with the different gene constructs developed up to morula stage was significantly lower ($p < 0.05$) than that of intact (non-microinjected) rabbit embryos (66–74vs. 98%). The highest efficiency of transgene integration (15%) was found when the CMVIE–EGFP (*Drd*I) gene construct was used, however a significantly higher transgenic mosaicism (60%) was found in rabbit embryos using this gene. The lowest cell number was counted in rabbit transgenic embryos with CMVIE–rhGFP linearized by *Sca*I (115.0 ± 8.20), the highest cell number (134.0 ± 35.00) was detected in rabbit transgenic embryos carrying PGK–EGFP (*Not* I*)* gene. The highest number of apoptotic cells (2.6 ± 0.33) was recorded in rabbit transgenic embryos with the integrated CMVIE–EGFP (*Cla*I) transgene.

Based on these results a more suitable gene marker for rabbit transgenic embryos production and selection is the CMVIE–EGFP (*Cla*I) gene construct. Prior to using microinjected embryos (for embryo transfer, vitrification or ESC isolation) it is necessary to pre-select microinjected embryos with evident transgenic mosaicism.

Keywords: Embryo, GFP, Mosaicism, Quality, Rabbit, Transgenic

Introduction

The production and application of genetically modified (transgenic) embryos and animals, despite its high cost, is still important. New methods are needed to increase efficiency of transgenesis (gene integration and expression) and decrease the final cost. One of the most promising approaches is to use GFP (green fluorescent protein) reporter genes for preimplantation-stage screening of embryos. Detection of GFP expression is possible using a fluorescence microscope (592 nm wavelength) with no deleterious effect on embryo vitality (Ikawa *et al*., 1995, Chrenek *et al*., 2005). On the other hand the controversial aspect of using GFP as a marker for the production of transgenic animal has been reported (Duszewska *et al*., 2004).

It was shown that the production of transgenic embryos by microinjection of foreign gene into pronuclei of fertilized eggs decreases survival of microinjected embryos (Makarevich *et al*., 2005). The other important factor limiting the efficiency of transgenic animal production is the quality of transgenic embryos resulting in poor development and low transgene integration and expression rates (as a result of random integration) or transgenic mosaicism. In transgenic mosaic embryos or animals not all cells carry the transgene (Wang *et al*., 2001; Duszewska *et al*., 2004).

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Biological material developed in *in vitro* conditions differs in quality and vitality from that developed *in vivo*. Although the efficiency of the embryo production *in vitro* increased in the last decade, the quality of these embryos is still different from *in vivo* derived embryos, particularly in morphology and metabolism (Khurana & Niemann, 2000), chromosomal abnormalities (Shi *et al*., 2004) and ultrastructural changes (Popelkova *et al*., 2005). Cleavage-stage arrest of microinjected embryos related to apoptosis was also documented in rabbit embryos (Makarevich *et al*., 2005).

The aim of this study was to compare the quality of rabbit transgenic embryos obtained by microinjection of four *GFP* gene-bearing constructs. Developmental rate, total cell number in hatching blastocyst stage, number of apoptotic cells, diameter of embryos, transgene integration and transgenic mosaicism were examined in our study.

Materials and methods

Gene constructs

The first GFP gene construct (CMVIE–EGFP, *Cla*I) consisted of full length (4.7 kb) plasmid pEGFP–N_1 (Clontech, USA) linearized by *Cla*I and used for microinjection into fertilized rabbit eggs. The second gene construct (CMVIE–EGFP, *Drd*I) contained the same expression cassette CMVIE–EGFP–SV40 poly A on a short 1.99 kb *Drd*I fragment of pEGFP–N_1. The expression cassette of this construct was flanked by short (ca 180 bp) vector backbone sequences on both sides. The third construct (PGK–EGFP) was a *Not*I linearized 6.3 kb plasmid PGKNEO PGK EGFP (INRA), and the fourth gene construct (CMVIE–hrGFP) containing humanized renilla GFP gene was *ScaI* linearized 5.0 kb pIRES–hrGFP–2a (Stratagene, USA). All DNA fragments were agarose gel-purified and diluted in endotoxin-free TE buffer for all microinjections at a final concentration of 4 μg/ml.

Embryo manipulation and microinjection

Three days before mating, New Zealand White rabbits were treated with PMSG (Werfaser) followed by hCG (Werfachor) 72 h later. At 19–20 h after mating, the pronuclear stage eggs were flushed with PBS from the oviducts of the animals. After the evaluation of flushed ova, the eggs with both pronuclei were subjected to microinjection in CIM medium + 10% fetal bovine serum (FBS, both from Gibco BRL) using an Olympus microscope equipped with micromanipulation units (Alcatel) and microinjector FemtoJet (Eppendorf). The eggs were fixed by suction with a holding pipette, and 4 μg/ml of the DNA (EGFP) in 1–2 pl was microinjected using air pressure (Pc – compensation and Pi – injection pressure, with injection time) into both pronuclei (Chrenek *et al*., 2005). Swelling of the pronuclei by 10% indicated successful microinjection. The eggs were cultured in k-DMEM medium supplemented with 10% FBS (Gibco BRL) at 5% CO_2 and 39°C up to the morula stage for the analysis of transgene integration and transgenic mosaicism or up to the blastocyst stage for the diameter and number of cells.

The zona pellucida from rabbit transgenic embryo at morula stage was removed by treatment with 0.5% pronase (Sigma, USA). The rabbit zona-free embryos were incubated in PBS (Ca^{2+}- and Mg^{2+}-free medium) for 5 min at 38°C and separated into single blastomeres by gently pipetting through a fine glass pipette (20–30 μm inner diameter, Chrenek & Makarevich, 2005).

Fluorescence analysis of EGFP expression

The EGFP expression in microinjected embryos at the morula or blastocyst stage was monitored using a Leica fluorescence microscope (excitation filter at 450–490 nm), as described by Chrenek *et al*. (2005).

Cell number counting and differential staining

For the cell number determination, the blastocysts were stained for 20 min with 1 μg/ml of Hoechst 33342 (Sigma), mounted on a microslide in Vectashield (Vector Laboratories) and analysed under a Leica fluorescence microscope (excitation filter 340–380 nm) as was early reported by Chrenek *et al*. (2005). For the ICM allocation, the blastocysts were differentially stained according to Fouladi-Nashta (2005). Briefly, embryos were incubated in 0.2% Triton X-100 diluted in PBS with 0.2% BSA for 20 s. After twice washing in PBS solution with 0.2% BSA the embryos were stained in propidium iodide (30 μg/ml) diluted in PBS–BSA for 5 min. Following washing in PBS–BSA the embryos were fixed and stained in 4% paraformaldehyde containing bisbenzimide (Hoechst 33342, 10 μg/ml) for 30 min. After washing in PBS–BSA the embryos were incubated in a cooled solution of 0.1% Triton X-100 and 0.1% sodium citrate for 5 min, washed, covered with glycerol and mounted under coverslip. The embryos were examined under a Leica fluorescence microscope using excitation filters 340–380 nm (for Hoechst 33342) and 515–560 nm (for PI).

TUNEL assay of embryos

The embryos were washed three times for 5 min in washing solution PBS with 4 mg/ml polyvinylpyrrolidone (PBS–PVP, Sigma, USA). Then the embryos were fixed in 3.7% neutrally buffered formalin for 5 min and in 70% ethanol for 10 min.

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Table 1 Development rate and transgenic mosaicism of rabbit embryos

Gene construct	No. MI embryos N	Development rate in morula n/N (%)	GFP-positive morula n/N (%)	Total blastomeres in morula, mean ± S.D.	No. GFP-positive blastomeres, mean ± S.D. (%)
CMVIE–EGFP (*Cla*I)	230	165/230 (72)	15/165 (9)	35.5 ± 2.5	28 ± 4.5 (89)[a]
CMVIE–EGFP (*Drd*I)	229	170/229 (74)	25/170 (15)	29.8 ± 5.0	10.4 ± 3.5 (40)[b]
PGK–EGFP (*Not*I)	235	155/235 (66)	20/155 (13)	29.3 ± 7.1	15.3 ± 6.1 (60)[b]
CMVIE–hrGFP (*Sca*I)	209	144/209 (69)	6/144 (4)	28.9 ± 2.5	12.0 ± 2.0 (55)[b]
None (non-injected)	200	178/200 (98)	–	34 ± 4.0	–

[a]vs[b] significant differences at $p < 0.05$.

Table 2 Apoptosis, embryo diameter and total cell number in rabbit embryos

Gene construct	No. transgenic hatching blastocysts N	Embryo diameter (μm)	Total cell number per embryo, mean ± S.D.	No. apoptotic cells per embryo, mean ± S.D.
CMVIE–EGFP (*Cla*I)	35	151.0 ± 17.10	131.8 ± 16.40	2.6 ± 0.33
CMVIE–EGFP (*Drd*I)	35	133.5 ± 11.82	131.3 ± 24.00	2.3 ± 0.36
PGK–EGFP (*Not*I)	30	128.8 ± 18.20	134.0 ± 35.00	1.7 ± 0.45
CMVIE–rhGFP (*Sca*I)	25	140.7 ± 7.44	115.0 ± 8.20	2.3 ± 0.50

Permeabilization was carried out by 15 min incubation of embryos in 0.5% Triton X-100 in PBS. The embryos were processed for TUNEL using MEBSTAIN Direct Apoptosis Detection Kit (IM3171, Immunotech) according to the product's manual. Briefly, fixed and permeabilized embryos were incubated in TdT-labelling mixture (TdT buffer, FITC–dUTP and TdT) at 37 °C for 1 h. Following the incubation the TUNEL-reaction was stopped by three-times washing of embryos in PBS–PVP solution. Then the embryos were transferred onto a coverslip and covered with 5 μl of Vectashield anti-fade mounting medium, containing DAPI stain. The coverslip was fixed to a microslide using nail polish. The samples were stored at −20°C until fluorescence analysis (Makarevich *et al*., 2005).

Embryo diameter

Embryo diameters, excepting zona pellucida, were measured from the same images on the screen of the monitor using a scale bar micrometer (Leica, Germany), which was previously calibrated on a ×10 or ×20 objective and ×10 eyepiece. The diameter of the embryo without the zona pellucida was the mean of two measurements made perpendicularly to each other.

Statistics

Development of rabbit embryos up to blastocyst stage and the GFP expression were analysed using the chi-squared test. Cell numbers and TUNEL cells in embryos were calculated using analysis of variance (ANOVA).

Results

Developmental rate and transgenic mosaicism

The developmental rate of rabbit embryos, microinjected with different GFP gene constructs, up to morula stage was significantly lower ($p < 0.05$) than in intact (non-microinjected) rabbit embryos (from 66–74% vs. 98%, Table 1). No significant differences in total cell number at morula stage among the groups were found.

Based on fluorescence analysis, GFP gene expression was detected at the morula stage. The highest portion of GFP-positive embryos (15%) was found using the CMVIE–EGFP–N1 (*Drd*I) gene construct, the lowest one (about 4%) was exhibited using CMVIE–hrGFP (*Sca*I) gene construct. Significantly higher transgenic mosaicism (60%) was found in rabbit embryos microinjected with CMVIE–EGFP–N1 (*Drd*I) gene construct, as was determined by fluorescence analysis of individual dissociated blastomeres.

Apoptosis, embryo diameter and total cell number

No significant differences in total cell number and diameter of transgenic rabbit embryo with different *GFP* gene constructs at hatching blastocyst stage were found (Table 2). The lower cell number was recorded in rabbit transgenic embryos with CMVIE–hrGFP linearized by *Sca*I (115.0 ± 8.20), the higher cell number was detected in rabbit transgenic embryos at hatching blastocyst stage with PGK–EGFP linearized by *Not*I (134.0 ± 35.00). The diameter of rabbit transgenic embryos was in the range of 128.8–158.4 μm.

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Significant difference ($p < 0.05$) was observed in the number of apoptotic cells between transgenic rabbit embryos with different *GFP* gene constructs (Table 2). The higher number of apoptotic cells (2.6 ± 0.33) was detected in rabbit transgenic embryos at hatching blastocyst stage carrying the CMVIE–EGFP (*Cla*I) transgene.

Discussion

In our previous study (Chrenek *et al*., 2005), we showed that a double microinjection technique increased transgene integration rate in rabbit embryos with a significant difference in blastocyst survival rate between double and single microinjection. Our present results demonstrate a higher (80%) developmental rate of microinjected embryos up to the morula stage, but lower GFP gene expression (15%), when compared with our previous study (66% and 35%, resp.). Generally, GFP has become more popular as a living marker for positively transfected clones in many studies, but variation in the levels of GFP expression has been shown (Liu *et al*., 1999). Variation of GFP expression in transgenic embryos was also reported. In about 20% of positive embryos mosaic green signal (some blastomeres were without signal) was found. Higher mosaicism (up to 50% of microinjected embryos) was reported, when the *GFP* gene was used with different promoters (Rosochacki *et al*., 2003). In our study, the CMVIE promoter (Clontech) was used for the expression of EGFP. The protein could be visualized in microinjected rabbit embryos after maternal/zygotic genome transition i.e., at the 8–16-cell stage. Transgenic mosaicism in our rabbit embryos may be explained by DNA integration related to cell cycle in embryos produced by the microinjection (Chan *et al*., 1999), but also that the transgene is distributed randomly into every blastomere (Wang *et al*., 2001). The major factors influencing successful transgene integration and expression in all blastomeres of transgenic embryos are competency of DNA repair system, replication and transcriptional activity of target cells (Chan *et al*., 1999), which may also explain transgenic mosaicism in our experiment. Even though we detected some embryos with mosaicism, the use of *GFP* gene enabled us to also select 100% positive rabbit embryos at early preimplantation (morula) stage, without any deleterious effect on their survival. Based on our results and some literature reports we may conclude that it is necessary to preselect microinjected embryos with evident transgenic mosaicism. These embryos are not suitable for embryo transfer, because transgenic mosaic animals will be born. It will not be a problem if transgene integration of transgenic mosaic animal is integrated in germinal tissues, as this could provide transgene transmission on to the next generation. In our unpublished results we received nine transgenic mosaic rabbits exhibiting different levels of GFP mosaicism, but with no transgene transmission to a new generation.

PCR analysis of microinjected embryos at several developmental stages has repeatedly shown that DNA construct persists in most of the morula stage embryos (Krisher *et al*., 1994). In morula and blastocyst stage embryos, the proportion of surviving embryos, in which detectable levels of microinjected DNA were maintained, dropped to 25% (O'Neill, 1995). In our previous *in vitro* experiments we detected the hFVIII transgene in 38% of single microinjected embryos and in 43% of double microinjected embryos at blastocyst stage (Chrenek *et al*., 2005). Although Page *et al*. (1995) obtained a 13% transgene frequency in microinjected embryos using polylysine–DNA mixtures, so far no live transgenics have been reported by cytoplasmic microinjection of DNA alone.

The embryo diameter and cell number are non-invasive markers of embryo quality, as their determination does not require destructing the embryo, when vital dye staining is used (Makarevich *et al*., 2006). Although embryo diameter is assumed to be a potential marker for the viability testing of bovine expanded blastocyst (Mori *et al*., 2002), this statement has not been confirmed in rabbit transgenic embryos. In the study of Shu-Zhen Liu *et al*. (2005) the total cell number in rabbit cloned embryos was significant lower than in *in vivo* derived rabbit embryos.

Apoptosis (programmed cell death) is an active physiological process and the result of this process is elimination of abundant, damaged or harmful cells. This process is genetically controlled (Schwarzman & Cidlowski, 1993). The presence of various molecular components of the apoptotic cascade has been proved in mouse, human and bovine preimplantation embryos (Warner *et al*., 1998; Gutierrez–Adan *et al*., 2004; Jurisicova & Acton, 2004). Liu *et al*. (1999) reported that the link exists between expression of GFP and induction of apoptosis. Our results confirmed the result of Makarevich *et al*. (2005), that apoptosis is not always the primary cause of the decrease in embryo cell number. Apoptotic processes at earlier stages of preimplantation development showed obvious dissimilarities with apoptotic processes in the blastocyst. In this case, the occurrence of apoptosis was sporadic and its presence was noted only after reaching particular developmental stages. Percentage of apoptotic cells in mouse embryos was usually higher than in rabbit embryos (Fabian *et al*., 2007). Occurence of apoptosis *in vitro* was indicative of suboptimal culture conditions or influence of experimental procedures (Schwarzman & Cidlowski, 1993; Makarevich *et al*., 2005). Significant differences between both transgenic

groups found in our study can be explained by the fact that transgenic GFP rabbit embryos were produced by microinjection into the pronucleus of eggs. Therefore the higher proportion of apoptotic cells in transgenic EGFP embryos can be caused by many factors associated with the microinjection itself, for example, mechanical damage by microinjection pipette, exposure of the zygote to a microscope light of a higher intensity, or their combination (Chrenek *et al*., 2005; Makarevich *et al*., 2005).

Conclusion

Basing on developmental rate, transgenic mosaicism, embryo diameter and number of cells, a more suitable gene marker for rabbit transgenic embryos production and selection seems to be the CMVIE–EGFP (*Cla*I) gene construct.

Moreover, before further use of microinjected embryos (for embryo transfer, vitrification or ESC isolation) it is necessary to pre-select microinjected embryos with evident transgenic mosaicism. These embryos are not suitable for embryo transfer, because transgenic mosaic animals will be born.

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