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Developmental rate and allocation of transgenic cells in rabbit chimeric embryos

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Summary

The objective of this study was to compare developmental capacity of rabbit chimeric embryos and the allocation of the *EGFP* gene expression to the embryoblast (ICM) or embryonic shield. We produced chimeric embryos (TR<>N) by synchronous transfer of two or three blastomeres at the 16-cell stage from transgenic (TR) into normal host embryos (N) at the same stage. In the control group, two to three non-transgenic blastomeres were used to produce chimeric embryos. The TR embryos were produced by microinjection of EGFP into both pronuclei of fertilized rabbit eggs. The developmental rate and allocation of EGFP-positive cells of the reconstructed chimeric embryos was controlled at blastocyst (96 h PC) and embryonic shield (day 6) stage.

All chimeric embryos (120/120, 100%) developed up to blastocyst stage. Using fluorescent microscope, we detected green signal (EGFP expression). In 90 chimeric (TR<>N) embryos (75%). Average total number of cells in chimeric embryos at blastocyst stage was 175 ± 13.10, of which 58 ± 2.76 cells were found in the ICM area. The number of EGFP-positive cells in the ICM area was 24 ± 5.02 (35%). After the transfer of 50 chimeric rabbit embryos at the 16-cell stage, 20 embryos (40%) were flushed from five recipients on day 6 of pregnancy, of which five embryos (25%) were EGFP positive at the embryonic shield stage.

Our results demonstrate that transgenic blastomeres in synchronous chimeric embryos reconstructed from TR embryos have an ability to develop and colonize ICM and embryonic shield area.

Keywords: Chimera, Embryo, Rabbit, Transgenic cells

Introduction

The developmental potential of a single blastomere from mammalian embryo is usually determined by its ability to form blastocyst with a visible inner cell mass (ICM) and a distinct trophoblast. It has been shown that isolated blastomeres from 4–8-cell-stage rabbit embryos are capable of regular *in vivo* development upon transfer to suitable recipients (Moore *et al*., 1968). However, fetal survival after transfer of embryos derived from blastomeres of cleavage stage embryos is poor and this may be attributed to a complete lack of, or an insufficient ICM participation in, a considerable proportion of the blastocysts. The proportion of cells in the blastocyst allocated to the ICM and trophectoderm (TE) is important for the future development. Although total cell counts and their number and distribution in the ICM and TE have been established for the rabbit embryos (Giles & Foote, 1995), the number and distribution of transferred transgenic cells in different development stage of rabbit chimeric embryos have not been determined.

Chimeric embryos have been used extensively for the study of embryology, development biology and some aspects of genetics (McLaren, 1975). It was established that when precompaction mammalian embryos are brought into contact with each other, the blastomeres from each embryos will participate together to produce a single chimeric embryos (Boediono *et al*., 1999).

Identification of transgenic cells allocation in the different stages of chimeric embryos can be done using the green fluorescent protein (*GFP*) gene. In mouse chimeric embryos, using GFP, it was possible to investigate development rate and allocation of transgenic blastomeres (Shimada *et al*., 1999). It was also reported in bovine chimeric blastocyst (Well & Powell, 2000).

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In this study the development rate and allocation of transgenic cells in chimeric rabbit embryos in the blastocyst and embryonic shield stage was evaluated.

Materials and methods

Gene constructs

An EGFP reporter gene was used for the microinjection into fertilized rabbit eggs after linearization of the plasmid pEGFP–N1 (Clontech) with *Cla*I. Aliquots of the DNA preparation were used for all injections.

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 to 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 Olympus microscope equipped with micromanipulation units (Alcatel) and microinjector Femto Jet (Eppendorf). The eggs were fixed by suction with a holding pipette and 5 μg/ml of the DNA (EGFP) in 1–2 pl was microinjected using air pressure (P_c – compensation and P_i – 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 8–16-cell stage for the analysis of transgene integration and subsequently for chimera's procedures.

Chimera production and embryo transfer

In the chimera production experiment, transgenic (TR) and *in vivo*-produced normal (N) embryos at 16-cell stage, were used. The zona pellucida of transgenic embryos was removed by the treatment with 0.5% pronase (Sigma). The rabbit zona-free embryos were incubated in PBS (Ca^{2+}- and Mg^{2+}-free) medium 5 min at 38 °C and disaggregated into single blastomeres by gentle pipetting through a fine glass pipette (40 μm inner diameter). Micromanipulation was conducted in CIM medium containing 10% FBS.

To produce synchronous rabbit chimera embryos, two to three transgenic (TR) blastomeres at 16-cell stage were injected (using pipette about 30 μm in inner diameter) into host embryos (N) at similar cell stage. Chimeric embryos were either cultured *in vitro* up to blastocyte stage with subsequent monitoring of the EGFP expression or they were transferred into oviduct of hormonally synchronized pseudopregnant recipients of the same breed. The number of transferred chimeric embryos per oviduct varied between five and six. Transferred reconstructed chimeric embryos at the embryonic shields stage (day 6 of gestation) were evaluated for developmental competency of transgenic cells according to EGFP expression.

Checking of EGFP expression

The EGFP expression in transgenic embryos was monitored under a fluorescence microscope (excitation 460–490 nm). On the day 6 of gestation the recipient females were laparotomized and the EGFP expression in the recovered embryonic shields was analysed.

Cell number and differential staining analysis

For the cell number determination, the blastocysts were stained for 20 min with 1 μg/ml of Hoechst 33342 (Sigma), mounted on microslide in Vectashield (Vector Laboratories) and analysed under Leica fluorescent microscope (excitation filter 340–380 nm). 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% 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 with bisbenzimide (Hoechst 33342, 10 μg/ml) for 30 min. After washing in PBS–BSA the embryos were incubated in 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 Leica fluorescent microscope (Fig. 1) using excitation filters 340–380 nm (for Hoechst 33342) and 515–560 nm (for PI).

Statistics

Development of rabbit embryos up to blastocyst stage and the EGFP expression were analysed using the chi-squared test. Cell numbers and percentages of allocated cells were processed using analysis of variance (ANOVA).

Results

Transgenic rabbit embryo production

The developmental rate of rabbit embryos microinjected with the *EGFP* gene construct up to 16-cell stage was significantly lower ($p < 0.01$) than of intact (non-microinjected) rabbit embryos (80 versus 98%, Table 1). Based on fluorescence analysis, the integration of

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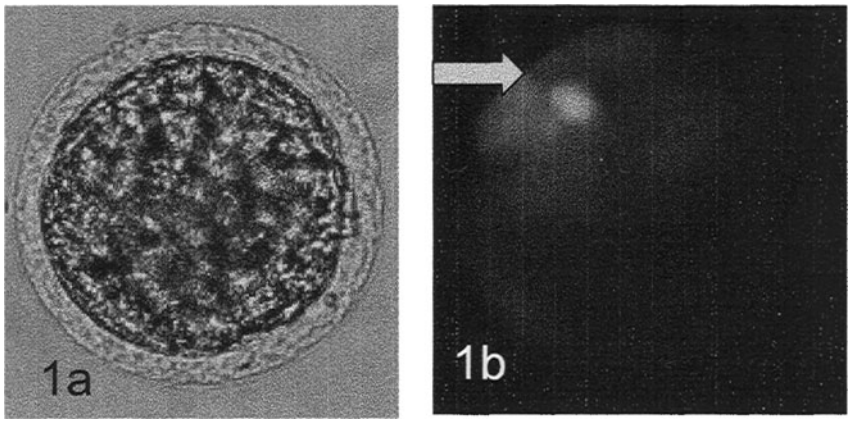

Figure 1 Representative results of rabbit transgenic embryos production at blastocyst stage (*a*); with *EGFP* gene expression (*b*).

EGFP gene was revealed in 30 16-cell stage embryos (15%). From these, six positive (green signal exhibited) embryos (20%) presented mosaic signal (two of them 50%, three 30% and one 15%), which was detected also at morula stage.

Rabbit chimeric embryo production

All chimeric embryos (120/120, 100%) cultured in *in vitro* conditions developed up to blastocyst stage (Table 2). The presence of rabbit transgenic (TR) blastomeres in chimeric embryos was monitored under a fluorescence microscope during preimplantation development at the stage of non-hatched blastocyst (96 hpc) and embryonic shield (day 6). In 90 chimeric embryos (75%) we detected green fluorescence (EGFP expression, Fig. 1*a*,*b*). After transfer of 50 chimeric rabbit embryos at 16-cell stage, 20 embryos (40%) were flushed from five recipients on day 6 of pregnancy, of which five embryos (25%) at embryonic shield stage were EGFP positive (Fig. 2 *a*,*b*).

Average total number of cells in chimeric embryos at blastocyst stage was 175 ± 13.10, of which 58 ± 2.76 cells were found in the ICM area. The number of EGFP-positive cells in the ICM area was 20 ± 1.02 (35%).

Discussion

Rabbit transgenic embryo production

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 higher (80%) developmental rate of microinjected embryos up to 16-cell stage, but lower *EGFP* gene expression (15%), when compared with previous study (66 and 35%, respectively). 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 *GFP* gene with different promoter was used (Chan *et al*., 1999, Rosochacki *et al*., 2003, Duszewska *et al*., 2004).

Table 1 *In vitro* survival of microinjected rabbit embryos and transgene integration rate

Group	No. of embryos injected (*N*)	No. of embryos advancing to 16-cell stage *n*/*N* (%)	EGFP positive embryos *n*/*N* (%)	Transgenic mosaic embryos *n*/*N* (%)
EGFP	250	200/250(80)[a]	30/200(15)	6/30(20)
Intact	50	49/50 (98)[b]	0/0	–

[a]versus [b]difference is significant at $p < 0.01$.

Table 2 *In vivo* and *in vitro* developmental rate of chimeric rabbit embryos and transgenic cell allocation

No. of chimeric embryos	No. of chimeric blastocysts *N* (%)	No. of blastocysts with expression *n*/*N* (%)	No. of blastocysts with EGFP in ICM *n*/*N* (%)	No. of flushed ICM embryonic shields *n*/*N* (%)	No. of embryonic shields with EGFP expression *n*/*N* (%)
120 (*in vitro*)	120 (100)	90/120 (75)	32/90 (35)	–	–
50 (*in vivo*)	–	–	–	20/50 (40)	5/20 (25)

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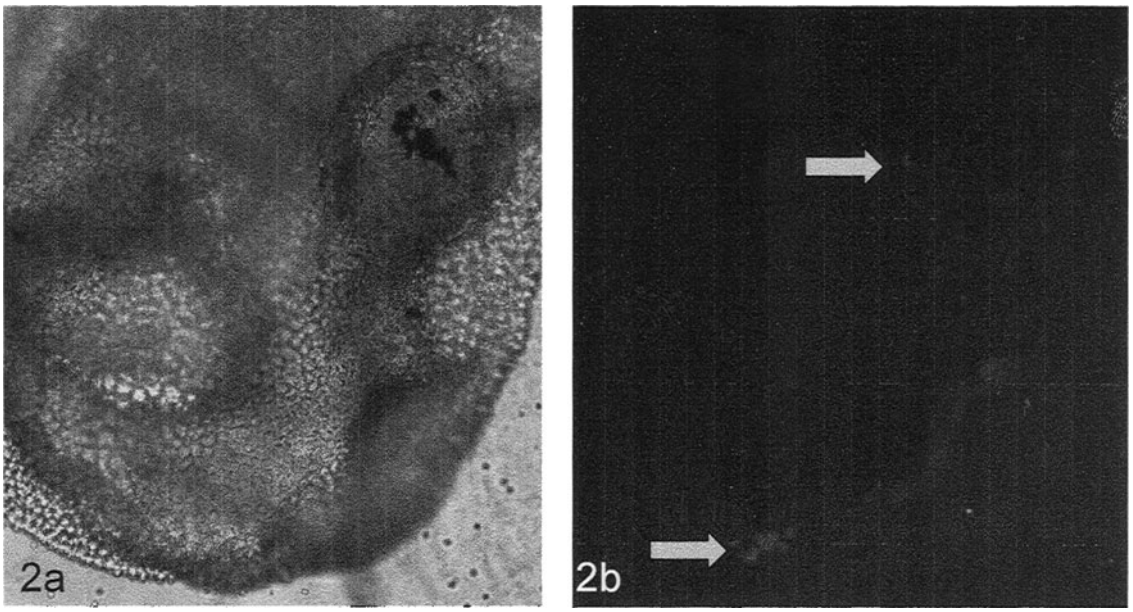

Figure 2 Representative results of rabbit chimeric embryonic shield production (*a*); with *EGFP* gene expression (*b*).

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 that means at the 8–16-cell stage. Mosaicism in our rabbit embryos may be explained by DNA integration related to cell cycle in embryos produced by microinjection (Chan *et al.*, 1999), but also that transgene is distributed randomly into every blastomere (Wang *et al.*, 2002). 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), what may explain mosaicism also in our experiment. Even though we detected some embryos with mosaicism, the *EGFP* gene allowed us to select also 100% positive rabbit embryos in early preimplantation (16-cell) stage, without any deleterious effect on their survival.

Rabbit chimeric embryo production

A synchronous aggregation of zona pellucida-free transgenic cells and nuclear transfer-derived transgenic cells for rabbit chimera production was reported previously (Chrenek & Makarevich, 2005). Developmental rate and allocation of the *EGFP* gene expression in chimeric embryos were examined only in *in vitro* conditions, because mucin coat necessary for implantation is not produced by zona pellucida-free rabbit embryo. In the study presented here, synchronous rabbit chimeric embryos were produced by the transfer of two to three transgenic (*EGFP* gene) blastomeres into intact host embryo at 16-cell stage. All chimeric embryos developed to blastocyst stage. Pregnancies obtained from transferred chimeric embryos showed that transgenic blastomeres were developmentally competent to form embryonic shields. Lower developmental rate and occupation of EGFP-positive cells was observed in postimplanted embryos (embryonic shield stage) compared with preimplanted embryos (blastocyst stage). The survival and allocation of transgenic blastomeres, in chimera, are probably influenced in a considerable extent by culture conditions. There is an evidence for metabolic cooperation between different cell types through permeable cell junctions that enables metabolic deficient cells to function in normal manner (Pitts & Burk, 1976). Developing intercellular junctions between blastomeres of different origin might play a role in communication and subsequently enhanced development of chimeric embryos (Ducibella, 1975).

We concluded that transgenic rabbit cells are able to develop and colonize the ICM and embryonic shield. These results demonstrate successful development of chimeric rabbit embryos after their transfer into recipient. Combination of the *EGFP* gene and chimeric production may be an efficient tool to disseminate transgenic cells among animals and therefore to increase efficiency of transgenesis.

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