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Quality of rabbit vitrified/thawed transgenic embryos

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Summary

The aim of our study was to investigate the influence of vitrification on developmental rate and quality (total number of cells, number of blastomeres in inner cell mass (ICM) area, apoptotic index and embryo diameter) of transgenic (carrying an endogenous–*hFVIII* or exogenous–enhanced green fluorescent protein (*EGFP*) gene) rabbit embryos. *EGFP*-positive rabbit embryos were produced under *in vitro* conditions by the microinjection of foreign genes into the pronucleus of fertilized eggs. The transgenic rabbit embryos with the *hFVIII* gene were produced by mating homozygous transgenic rabbits and flushing at the single-cell stage. Developmental rate of vitrified/thawed transgenic embryos that reached hatching blastocyst stage (68.00% and 69.00%) and differed significantly ($p < 0.001$) from those in control embryos (100.00%). Significant difference ($p < 0.05$) was found in total cell counts between control (117.00 ± 36.00) and vitrified (141.00 ± 34.80) hFVIII-positive embryos. The higher proportion of ICM cells (32.00%) and greatest embryo diameter (130.85 ± 10.90) were found in the control group compared with the transgenic. Ratio of apoptotic cells was significantly higher ($p < 0.01$) in the control group (2.50%) and vitrified EGFP-positive embryos (2.90%) compared with the vitrified, hFVIII-positive group of embryos (0.70%). Our results demonstrate that neither gene microinjection itself, nor exogenous (EGFP) and endogenous (hFVIII) gene expression interferes with developmental rate and quality of rabbit embryos. However, a combination of microinjection and vitrification significantly decreases ($p <$ 0.001) the survival rate of rabbit embryos.

Keywords: Embryo, Quality, Rabbit, Transgenic, Vitrification

Introduction

One of the main aims of embryo research is the optimization of genetic manipulation techniques to minimize the rate of biological material destruction (Dobrinski, 2002). Microinjection of foreign DNA into the pronucleus or into both the pronuclei of fertilized egg is one of the techniques for production of transgenic organisms (Chrenek *et al.*, 2005). Therefore, the main problem in this field of biotechnology is to assure maximum embryo survival and viability. At present, vitrification–quick freezing in liquid nitrogen, is an important tool for preservation of mammalian embryos. At this temperature all biochemical activities that can lead to cell death are effectively stopped (Özkavukcu & Erdemli, 2002). Despite intensive research, procedures for cryopreservation still cause biochemical and morphological changes, which may result in loss in embryo viability and even induce cell death. Analysis of the viability and quality of vitrified/devitrified embryos is of great importance (Popelková *et al.*, 2005). Documentation of cell injury during or after cryoconservation provides convenient information for understanding the sensitivity of cells towards vitrification. This monitoring can lead to improvement of vitrification protocols and better understanding of embryology of domestic animals (Dobrinski, 2002). The developmental potential of a single blastomere from mammalian embryo is usually determined by its ability to form a blastocyst with a visible inner cell mass (ICM) and a distinct trophoblast (Chrenek *et al.*, 2008). The proportion of apoptotic cells has been considered one of the most important

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parameters for evaluating embryo health or culture conditions (Brison & Schultz, 1997; Hardy, 1999).

The aim of this study was to evaluate the quality of transgenic (carrying either *hFVIII* or *EGFP* gene) rabbit embryos following vitrification in comparison with intact embryos.

Materials and methods

Biological material

Three days before mating, New Zealand White rabbit donors (APRC Nitra, SK) were treated with pregnant mare serum gonadotrophin (PMSG) (Werfaser, 20 IU/kg of body weight, Austria) followed by hCG (Werfachor, 40 IU/kg of body weight, Austria) 72 h later (Chrenek *et al.*, 2002). At 19 to 20 h *post coitus*, the pronuclear stage eggs were flushed from the oviducts of the rabbit females with phosphate-buffered saline (PBS) (Sigma). The selection of flushed eggs was done in CIM medium with fetal bovine serum (FBS 10%; Gibco BRL). The rabbit embryos were cultured *in vitro* under the conditions (5% CO_2, 39°C, k-DMEM + 10% FBS, Gibco BRL) described previously (Chrenek *et al.*, 2005).

Gene constructs

For the microinjection into fertilized rabbit eggs the *EGFP* reporter gene after linearization of the plasmid (Clontech) with *Cla*I was used (Chrenek *et al.*, 2005). The second gene construct consisted of a 2.5 kb murine whey acidic protein promoter (mWAP), 7.2 kb cDNA of human clotting factor VIII (hFVIII), and 4.6 kb of 3′ flanking sequences of the *mWAP* gene. This gene was provided by Dr H. Lubon, American Red Cross, MD, USA (Chrenek *et al.*, 2005). The plasmid was digested with *Not*I to make the 14.3 kb insert and purified using a Qiaex II gel extraction kit (Qiagen).

Production of rabbit transgenic embryos *in vitro* (*EGFP* exogenous gene)

Following selection of the flushed ova, the eggs with both pronuclei were subjected to gene microinjection in CIM medium supplemented with 10% FBS using an Olympus microscope equipped with micromanipulation units (Alcatel) and microinjector (Eppendorf, Femto Jet), as reported earlier (Chrenek *et al.*, 2005). The eggs were fixed by suction with a holding pipette, and 1–2 pl of the DNA (EGFP) solution (4 μg/ml) were microinjected into the male pronucleus by single microinjection (SM) using air pressure. Swelling of pronuclei by 10% indicated successful microinjection. The eggs were cultured in k-DMEM medium supplemented with 10% FBS at 5% CO_2 and 39°C up to the blastocyst stage (96 hpc) (Chrenek & Makarevich, 2005). The embryos were analysed for the transgene integration at the morula stage using a Leica fluorescence microscope.

Production of rabbit transgenic embryos *in vivo* (*hFVIII* endogenous gene)

Transgenic rabbits (F4 generation) carrying the human factor VIII gene (gene construct mWAP-hFVIII; Chrenek *et al.*, 2005) were mated and transgenic embryos were then flushed out from the oviducts of transgenic donors at the 1-cell stage (20 hpc).

Vitrification (quick freezing) and devitrification of rabbit embryos

Rabbit embryos at the morula stage 48 h after flushing were put into a vitrification solution containing CIM medium + 20% fetal calf serum (FCS) (Gibco BRL) + 40% ethylene glycol (Sigma) + 18% Ficoll 70 (Sigma) + 0.3 M sucrose (Sigma) for 3–4 min and were then plunged into liquid nitrogen (Papis *et al.*, 2005). After 3 days the embryos were thawed. After removal from liquid nitrogen, the embryos were exposed to a devitrification solution containing CIM medium + 20% FCS + 0.3 M sucrose for 7–8 min and then transfered into conditional solution containing CIM medium + 20% FCS. The embryos were then cultured under *in vitro* conditions (5% CO_2 in air, 39°C, k-DMEM + 10% FCS) up to hatching blastocyst stage.

Differential staining

The embryos were incubated in freshly prepared 0.2% Triton X-100 in PBS containing 2 mg/ml bovine serum albumin (BSA) for 20 s, and immediately washed twice in PBS–BSA medium. The embryos were transferred into PBS–BSA containing 30 μg/ml of propidium iodide (PI) and incubated in the dark at 37°C in warm chamber for 5 min, and then washed twice in PBS–BSA medium. Then the embryos were incubated in 4% paraformaldehyde (PFA) containing 10 μg/ml bisbenzimide (Hoechst 33342, Sigma) for 30 min at room temperature and then washed twice in PBS-BSA medium. The embryos were incubated in a freshly prepared ice-cold solution of 0.1% Triton X-100 in 0.1% sodium citrate (v/v) for 5 min and washed twice in PBS–BSA medium. Washed embryos were covered with Vectashield mounting medium (Vector Laboratories), mounted on glass slides with coverslips and examined under a Leica fluorescence microscope (Mikro spol. s.r.o.; Fouladi-Nashta *et al.*, 2005).

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Analysis of apoptosis (TUNEL)

The embryos were removed from the culture medium, washed 3 × 5 min in PBS supplemented with polyvinylpyrrolidone (PBS–PVP, 4 mg/ml) and then fixed in 3.7% formalin for 5 min and in 70% ethanol for 10 min. For membrane permeabilization, the embryos were incubated in 0.5% Triton X-100 in PBS for 15 min. The embryos were processed for the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling assay (TUNEL) using a MEBSTAIN Direct Apoptosis Detection Kit (Immunotech) according to the manufacturer's instructions. Afterwards, the embryos were counterstained with PI (1 g/ml in PBS). After washing, the embryos were covered with 5 μl of Vectashield mounting medium (Vector Laboratories) and attached to microslide using small columns of nail polish (Makarevich *et al.*, 2005).

Embryo diameter

Embryo diameter including the zona pellucida was measured from the images on the screen of the monitor using a scale bar micrometer, which was previously calibrated on a ×40 objective and a ×10 eyepiece. The diameter of the embryos was the mean of two measurements made perpendicular to each other (Makarevich *et al.*, 2006).

Statistical analysis

Influence of microinjection and vitrification on the developmental rate and quality of rabbit transgenic and non-transgenic embryos were evaluated using the chi-squared test.

Results

The ability to reach hatching blastocyst stage under *in vitro* conditions was observed for each group of embryos (Table 1). The highest number of cells was recorded in the control group (142.00 ± 18.81), in comparison with the EGFP (135.00 ± 12.70) and the hFVIII (124.00 ± 10.57) groups of embryos. The proportion of ICM cells to the total cell count was similar for transgenic (26.00% for EGFP and 26.60% for hFVIII gene) and non-transgenic (25.00%) groups of rabbit embryos. Most of the apoptotic cells were detected in the EGFP group (6.00%) in comparison with non-transgenic (4.20%) and transgenic hFVIII (3.20%) groups. Embryo diameter in the control group (130.85 ± 10.90 μm) was not significantly different from both transgenic groups (124.42 ± 7.26 and 130.55 ± 8.50, respectively).

Developmental rate and quality of vitrified transgenic rabbit embryos are represented in Table 2.

Table 1 Developmental rate and quality of rabbit transgenic embryos

Type of embryos	Total no. embryos (*N*)	No. of HBl *n* (%)	Total cell no. (*N*) mean ± SD	No. of cells in ICM/embryo mean ± SD *n* (%)	No. of apoptotic cells/% mean ± SD *n* (%)	Diameter of embryos (μm) mean ± SD
Control	56	56 (100.00%)	142 ± 18.81	36 ± 2.35 (25)	6 ± 1.35 (4.2)	133.97 ± 10.93
EGFP	114	114 (100.00%)	135 ± 12.70	35 ± 2.80 (26)	8 ± 2.53 (6)	124.42 ± 7.26
hFVIII	135	135 (100.00%)	124 ± 10.57	33 ± 2.18 (26.6)	4 ± 2.18 (3.2)	130.55 ± 8.50

HBl, hatching/hatched blastocysts

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Significant differences ($p < 0.01$) in the developmental rate between control and vitrified EGFP-positive embryos (100.00% vs. 68.00%) and between control and vitrified hFVIII-positive embryos (100.00% vs. 69.00%) were observed. We found significant difference ($p < 0.05$) in total number of cells between the control group (117.00 ± 36.00) and the *hFVIII*-positive/vitrified group (141.00 ± 34.80) but not in the EGFP-positive/vitrified (135.00 ± 30.20) rabbit embryos. Proportion of the ICM cells to total cell counts in the control group was 32.00% whilst in transgenic/vitrified groups lower percentages of cells in the ICM area (23.70% and 25.50%, respectively) were found. We observed significant differences ($p < 0.01$) in apoptotic indices between both vitrified transgenic groups of embryos (2.90% and 0.70%, respectively) and between control and hFVIII-positive/vitrified embryos (2.50% vs. 0.70%). No significant differences were observed in embryo diameter between control and transgenic (EGFP vs. hFVIII) vitrified embryos.

Table 2 Development rate and quality of vitrified rabbit transgenic embryos

Type of embryo	No. of embryos Vi/DeVi (N)	No. of HBl *n* (%)	Total no. cells (*N*) mean ± SD	No. of cells in ICM/embryo mean ± SD *n* (%)	No. of apoptotic cells/embryo mean ± SD *n* (%)	Diameter of embryos (μm) mean ± SD
Control	35	35(100.00%)^{a}	117.00 ± 36^{c}	37.00 ± 7.50 (32)	3.00 ± 3.00^{e} (2.50%)	130.85 ± 10.90
Vi/deVi EGFP	25	17(68.00%)^{b}	135.00 ± 30.20	32.00 ± 6.50 (23.70%)	4.00 ± 1.85^{e} (2.90 %)	121.20 ± 7.20
Vi/deVi hFVIII	32	22(69.00%)^{b}	141.00 ± 34.80^{d}	36.00 ± 8.50 (25.50%)	1.00 ± 1.60^{f} (0.70%)	125.50 ± 7.55

Vi/deVi, vitrified/devitrified rabbit embryos.
a vs. *b* significant difference at $p < 0.001$; *c* vs. *d* significant difference at $p < 0.05$; *e* vs. *f* significant difference at $p < 0.01$.

Discussion

Microinjection, transgene integration and vitrification are factors that can affect the quality of manipulated embryos. In our experiments, we studied developmental rate and quality of vitrified transgenic (carrying exogenous *EGFP* or endogenous *hFVIII* gene) rabbit embryos. In the case of transgenic non-vitrified rabbit embryos, we did not found any reduction in developmental rate, because the embryos of all groups reached the hatching blastocyst stage. We did not detect differences between the transgenic embryos themselves, although *EGFP*-positive rabbit embryos were obtained by microinjection (*in vitro*) and *hFVIII*-positive embryos were derived from mating of transgenic rabbits (*in vivo*). Microinjection of the gene construct in the study of Makarevich *et al.* (2006) did not affect the cleavage rate of embryos, but blastocyst rate was significantly lowered compared with intact embryos. Popelková *et al.* (2005) reported that the ability to achieve the hatching in microinjected rabbit blastocysts (94.00%) is comparable with the control group of embryos (90.00%). Microinjection of a foreign gene into the pronucleus of eggs can result in chromosomal aneuploidy (Roychoudhury *et al.*, 2008), and DNA rearrangement during integration can cause decreased yield of blastocyst stage embryos (Voss *et al.*, 1990).

In our experiments we also investigated the influence of vitrification on the quality of transgenic rabbit embryos. Transgenic rabbit embryos, which have undergone vitrification process, had significantly reduced viability after thawing (68.00% vs. 69.00%)

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compared with the control group (100.00%). Decrease of embryo viability can be caused by the sort and concentration of the cryoprotectant, animal species, the genotype, developmental stage of the embryo and the time of exposure to cryoprotectant substances (Fabian *et al.*, 2005). Kasai *et al.* (1992) reported that 96.00% of vitrified rabbit morulae had intact zona pellucida after thawing. Popelková *et al.* (2005) used DMSO as a cryoprotectant and did not find significant difference in the developmental rate of rabbit embryos, because 57.00% of vitrified and 56.00% of intact rabbit embryos reached the hatching blastocyst stage. Results obtained by Papis *et al.* (2005) are also comparable with our study. All the available embryos were vitrified in a modified vitrification solution comprised of ethylene glycol (40%), Ficoll 70 (18%) and sucrose (0.3 M) in HEPES-buffered TCM medium, containing 20% FCS. The embryos were exposed to the vitrification solution for 3–4 min. They reported that the majority (71%) of thawed embryos survived vitrification and developed to the blastocyst stage. In contrast to our experiments, Papis *et al.* (2005) did not use transgenic embryos. Transgenesis may influence developmental rate of embryos. Makarevich *et al.* (2008) monitored the effect of the vitrification method on the developmental rate of rabbit embryos, using the vitrification medium with ethylene glycol and Ficoll 70. They showed significantly lower ability ($p < 0.05$) of hatching blastocyst formation when compared with intact (97.00%) or vitrified rabbit embryos (63.00%). Embryo diameter and cell number are non-invasive markers of embryo quality. Vitrification procedure used in our study caused a decrease in total cell numbers of vitrified hFVIII- positive embryos compared with the control group (117.00 ± 36.00 vs. 141.00 ± 34.80).

Procedure for gene microinjection of rabbit embryos, according to the study of Makarevich *et al.* (2006), did not affect embryo diameter. Our results concerning ICM cells are comparable with those of Fouladi-Nashta *et al.* (2005), who determined the proportion of cells in the ICM of bovine embryos (25.50%). Results in the case of vitrified transgenic rabbit embryos demonstrated that the ICM area cells and diameter were comparable with the non-vitrified/non-transgenic ones. In the study of Popelková *et al.* (2008) rabbit embryos had lower total cell number and embryo diameter in comparison to intact ones.

The presence of different molecular components of apoptotic cascade was confirmed in mouse, human and bovine preimplantation embryos (Warner *et al.*, 1998; Gutierrez-Adan *et al.*, 2004; Jurisicova & Acton; 2004). The percentage of apoptosis in mouse embryos was usually higher than in rabbit embryos (Fabian, 2007). The occurrence of apoptosis indicates suboptimal culture conditions or the effects of experimental treatments (Schwartzman & Cidlowski, 1993, Makarevich *et al.*, 2005; Fabian *et al.*, 2007). Higher proportion of apoptotic cells was observed in the EGFP-positive embryos in the case of non-vitrified as well as vitrified embryos. One of the reasons could be that GFP, derived from *Aequorea victoria*, induces apoptosis (Hsiao-Sheng *et al.*, 1999). Fabian *et al.* (2007) reported that apoptosis in intact embryos does not occur earlier than the 16-cell stage. In the case of early rabbit blastocysts, the proportion of apoptotic cells was 1.38%, but mouse embryos showed a higher proportion of apoptotic cells (6.60%) at that developmental stage. Makarevich *et al.* (2008) observed the influence of microinjection and vitrification on the presence of apoptotic cells in rabbit embryos. They recorded a lower proportion of apoptotic cells (5.70%) in the microinjection-derived embryos subjected to a combination of microinjection and vitrification (7.54%). At vitrification, using DMSO and EG as cryoprotectants, the highest proportion of apoptotic cells (10.35%) was measured. These results show that the quality of embryos after thawing depends on the cryoprotectants used.

In conclusion, our results demonstrate that neither microinjection of foreign gene, nor exogenous (*EGFP*) or endogenous (*hFVIII*) gene expression interfere with developmental rate and quality of rabbit embryos. Combination of microinjection and vitrification significantly decreases ($p < 0.001$) developmental rate and increases ($p < 0.01$) the proportion of apoptotic cells in rabbit embryos.

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References

Brison, D.R. & Schultz, R.M. (1997). Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including TGF-α. *Biol. Reprod.* **56**, 1088–96.

Chrenek, P. & Makarevich, A.V. (2005). Production of rabbit chimeric embryos by aggregation of zona-free nuclear transfer blastomeres. *Zygote* **13**, 39–44.

Chrenek, P., Vašíček, D., Makarevich, A., Uhrin, P., Petrovičová, I., Lubon, H., Binder, B.R. & Bulla, J. (2002). Integration and expression of the WAP-hPC gene in three generations of transgenic rabbits. *Czech J. Anim. Sci.* **47**, 45–9.

Chrenek, P., Vasicek, D., Makarevich, A.V., Jurcik, R., Suvegova, K., Parkanyi, V., Bauer, M., Rafay, J., Batorova, A. & Paleyanda, R.K. (2005). Increased transgene integration efficiency upon microinjection of DNA into both pronuclei of rabbit embryos. *Transgenic Res.* **14**, 417–28.

https://doi.org/10.1017/S0967199411000384 Published online by Cambridge University Press

Chrenek, P., Makarevich, A.V., Bauer, M. & Jurcik, R. (2008). Development rate and allocation of transgenic cells in rabbit chimeric embryos. *Zygote* **16**, 87–91.

Dobrinski, J.R. (2002). Advancements in cryopreservation of domestic animal embryos. *Theriogenology* **57**, 285–302.

Fabian, D., Gjorret, J.O., Berthelot, F., Martinat-Botté, F. & Maddox-Hyttel, P. (2005). Ultrastructure and cell death of in vivo derived and vitrified porcine blastocysts. *Mol. Reprod. Dev.* **70**, 155–65.

Fabian, D., Makarevich, A.V., Chrenek, P., Bukovská, A. & Koppel, J. (2007). Chronological appearance of spontaneous and induced apoptosis during preimplantation development of rabbit and mouse embryos. *Theriogenology* **68**, 1271–81.

Fouladi-Nashta, A.A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K.H.S. & Webb, R. (2005). Differential staining combined with TUNEL labeling to detect apoptosis in preimplantation bovine embryos. *Reprod. Biomed. Online* **4**, 497–502.

Gutierrez-Adan, A., Rizos, D., Fair, T., Moneira, P.N., Pintado, B., de la Fuente, J., Boland, M.P. & Lonergan, P. (2004). Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol. Reprod. Dev*. **68**, 441–448.

Hardy, K. (1999). Apoptosis in the human embryo. *Rev. Reprod*. **4**, 125–34.

Hsiao-Sheng, L., Ming-Shion, J., Chao-Kai, C.H., Ping-Hong, C.H. & Nir-Jihn, K. (1999). Is green fluorescent protein toxic to living cells? *Biochem. Biophys. Res. Comm.* **260**, 712–7.

Jurisicova, A. & Acton, B.M. (2004). Deadly decisions the role of genes regulating programmed cell death in human preimplantation embryo development. *Reproduction* **128**, 281–91.

Kasai, M., Hamaguchi, Y., Zhu, S.E., Miyake, T., Sakurai, T. & Machida, T. (1992). High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. *Biol. Reprod.* **46**, 1042–6.

Makarevich, A.V., Chrenek, P., Zilka, N., Pivko, J. & Bulla, J. (2005). Preimplantation development and viability of in vitro cultured rabbit embryos derived from *in vivo* fertilized gene–microinjected eggs: apoptosis and ultrastructure analyses. *Zygote* **13**, 125–37.

Makarevich, A.V., Chrenek, P. & Fl'ak, P. (2006). The influence of microinjection of foreign gene into the pronucleus of fertilized egg on the preimplantation development, cell number and diameter of rabbit embryos. *Asian-Aust. J. Anim. Sci.* **19**, 1–5.

Makarevich, A.V., Chrenek, P., Olexíková, L., Popelková, M., Turanová, Z., Ostró, A. & Pivko, J. (2008). Post-thaw survival, cell death and actin cytoskeleton in gene-microinjected rabbit embryos after vitrification. *Theriogenology* **70**, 675–81.

Özkavukcu, S. & Erdemli, E. 2002. Cryopreservation: basic knowledge and biophysical effects. *J. Ankara Med. Sch.* **4**, 187–96.

Papis, K., Sypecka, J., Korwin-Kossakowski, M., Wenta-Muchalska, E. & Bilska, B. (2005). Banking of embryos of mutated, paralytic tremor rabbit by means of vitrification. *Lab. Anim.* **39**, 284–9.

Popelková, M., Chrenek, P., Pivko, J., Makarevich, A.V., Kubovičová, E. & Kačmárik, J. (2005). Survival and ultrastructure of gene – microinjected rabbit embryos after vitrification. *Zygote* **13**, 283–93.

Popelková, M., Turanová, Z., Koprdová, L., Ostró, A., Toporcerová, S., Makarevich, A.V. & Chrenek, P. (2008). Effect of vitrification technique and assisted hatching on rabbit embryo developmental rate. *Zygote* **17**, 57–61.

Roychoudhury, S., Bulla, J., Curlej, J. & Chrenek, P. (2008). Hypodiploidy as a prominent attributor to chromosomal aneuploidy in transgenic rabbit embryos. *Czech J. Anim. Sci.* **53**, 388–97.

Schwartzman, R.A. & Cidlowski, J.A. (1993). Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocrine Rev.* **14**, 133–51.

Voss, A.K., Sandmoller, A., Suske, G., Strojek, R.M., Beato, M. & Hahn, J. (1990). A comparison of mouse and rabbit embryos for the production of transgenic animals by pronuclear microinjection. *Theriogenology* **34**, 813–24.

Warner, C.M., Exley, G.E., McElhinny, A.S. & Tang, C. (1998). Genetic regulation of preimplantation mouse embryo survival. *J. Exp. Zool.* **282**, 272–9.

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