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Obtaining calves after transfer of embryos microinjected with the human interferon alpha (IFNα) gene, pbLGIFN-GFPBsd*

A.M. Duszewska[1,3,6], D. Lipiński[4,5], W. Gawron[2], E. Wenta-Muchalska[1], A. Rynkowska[1], R. Słomski[4,5] and Z. Reklewski[2]

Institute of Genetics and Animal Breeding, Polish Academy of Sciences,
[1]Department of Experimental Embryology,
[2]Department of Animal Sciences
Jastrzębiec, 05-552 Wólka Kosowska, Poland
[3]Warsaw University of Life Sciences, Division of Histology and Embryology,
Department of Morphological Sciences, Faculty of Veterinary Medicine
Nowoursynowska 159, 02-776 Warsaw, Poland
[4]Poznan University of Life Sciences, Department of Biochemistry and Biotechnology
Wołyńska 35, 60-637 Poznań, Poland
[5]Institute of Human Genetics, Polish Academy of Sciences
Strzeszyńska 32, 60-479 Poznań, Poland

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ABSTRACT

The objective of this study was to obtain calves with an integrated human interferon alpha (*IFNα*) gene. The transgene (pbLGIFN-GFPBsd) was introduced by microinjection into one of the zygote's pronuclei. The transgene (pbLGIFN-GFPBsd) contained the human interferon alpha (*IFNα*) gene, bovine lactoglobulin promoter (bLG), *gfp* (*green fluorescence protein*) and blasticidin resistance gene (*bsd*). After microinjection of 2107 zygotes, the proportion developing to the blastocyst stage (10.4%) was lower than for non-injected embryos (39.9%; $P<0.01$). Of the 2107 that were microinjected, 76 were GFP-positive blastocysts (3.6%). Fifty-seven GFP-positive blastocysts and 31 blastocysts from non-injected embryos were transferred singly to recipients. A lower rate of calving was observed after transfer of GFP-positive blastocysts (19.3%) as compared with non-injected embryos (48.4%; $P<0.05$). All newborn calves were healthy and of normal weight. PCR analyses indicated that none of the calves obtained after transfer of GFP-positive blastocysts carried the human interferon alpha gene.

KEY WORDS: human interferon alpha, microinjection, embryos, calves

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[6] Corresponding author: e-mail: duszewskaanna@hotmail.com

INTRODUCTION

The application of transgenic technology in cattle can bring genetic benefits in milk and meat production and health benefits in the form of enhanced immunity and the production of pharmacological products (Pinkert, 2002; Niemann et al., 2005). Achievements include transgenic cattle with a high level of β-casein and κ-casein in milk (Brophy et al., 2003), and others with resistance to mastitis (Wall et al., 2005). A great challenge is using cattle as bioreactors for production of pharmaceutical proteins in their milk. Notable successes are transgenic cattle that produce human lactoferrin (Van Berkel et al., 2002), albumin (Echelard et al., 2002) and growth hormone (Salamone, 2005).

Transgenic cattle can be created by microinjection of foreign DNA into zygotes (Krimpenfort et al., 1991). Other techniques are sperm-mediated DNA transfer (Sperandio et al., 1996), the use of transfected somatic cells for cloning (Cibbeli et al., 1998), and the use of viral vectors to introduce foreign DNA (Hofmann et al., 2004). Multiple modification in a single cell line, targeted insertions and microchromosome transfer have been used for creation of transgenic cattle (Wall et al., 2002; Robl et al., 2007).

The objective of this study was to obtain calves with integrated human interferon alpha (*IFNα*) gene. The transgene (pbLGIFN-GFPBsd) was introduced by microinjection into one of the zygote pronuclei. The introduced transgene pbLGIFN-GFPBsd (Figure 1) contained the human interferon alpha (*IFNα*) gene, bovine lactoglobulin promoter (bLG), a reporter gene, *gfp* (*green fluorescence protein*), and a selection gene, the blasticidin resistance gene (*bsd*). Using *gfp* allowed screening of preimplantation embryos to determine which of them had integrated the transgene (Chan et al., 2002). The pbLGIFN-GFPBsd transgene also contains the *bsd* gene (Lipiński et al., 2007). Only GFP-positive embryos were transferred to recipients. After birth all calves were tested to detect the integration of human interferon alpha (*IFNα*) into the host genome.

MATERIAL AND METHODS

In vitro maturation of bovine oocytes

Bovine ovaries were obtained from a slaughterhouse and transported within 2 h to the laboratory in phosphate-buffered solution (PBS) with 100 IU/ml streptomycin and 100 IU/ml penicillin at approximately 30°C. Cumulus-oocyte complexes (COCs) were collected by aspiration from follicles (2 to 6 mm in diameter) using a syringe with an 18-gauge needle and an air pump system. COCs were

washed 3 times in manipulation medium (MM): TCM199 buffered with 25 mM Hepes supplemented with 10% FBS, 50 µg/ml gentamicin sulphate, 100 IU penicillin and 50 µg/ml streptomycin, and adjusted to pH 7.4. A group of COCs (20 immature oocytes) was placed in one well of a 4-well dish and matured in 450 µl TCM199 buffered with 25 mM Hepes and supplemented with 10% FBS, 0.02 IU/ml pFSH, 1 µg/ml 17 β-oestradiol, 0.2 mM Na pyruvate and 50 µ/ml gentamicin sulphate, and adjusted to pH 7.4. The COCs were matured for 24 h at 38.5°C in 5% CO_2 in humidified air.

In vitro fertilization of bovine oocytes

Frozen spermatozoa obtained from a single bull were thawed in a water bath (37°C), centrifuged (200 *g*) for 10 min, and resuspended in 2 ml Sp-TALP medium containing 6 mg/ml BSA fraction V adjusted to pH 7.4. Spermatozoa were prepared by the *swim-up procedure*. After maturation, the COCs were washed 3 times in fertilization medium Fert-TALP supplemented with 6 mg/ml BSA FAF, 0.2 mM Na pyruvate and 50 µg/ml gentamicin sulphate. Groups of matured oocytes (10 COCs) were placed in 4-well dishes in 450 µl Fert-TALP supplemented with 6 mg/ml BSA FAF, 0.2 mM Na pyruvate, 50 µg/ml gentamicin sulphate, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 2 µg/ml heparin. Spermatozoa were used at a final concentration of $1x10^6$/ml. The COCs and spermatozoa were co-cultured for 18 h at 38.5°C in 5% CO_2 in humidified air.

Preparation of the pbLGIFN-GFPBsd gene construct

The pbLGIFN-GFPBsd construct contained a 1175 bp fragment of the bovine β-lactoglobulin promoter and a 973 bp human interferon alpha gene inserted in pTracer-EF/Bsd A plasmid vector (Invitrogen) (Figure 1). In the first step, the bovine β-lactoglobulin promoter (GenBank Accession X14710) was amplified by using the genomic sequence as the template and two primers: bLG-F

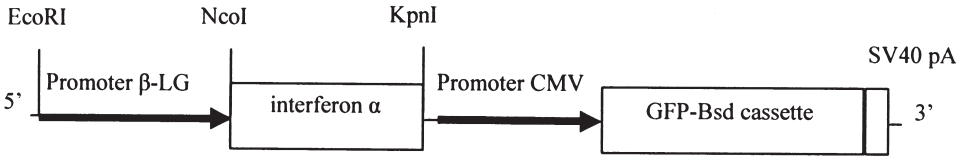

Figure 1. pbLGIFN-GFPBsd plasmid composed of the bovine β-lactoglobulin promoter fused to the human interferon alpha gene, followed by the GFP-Bsd cassette containing cycle 3-GFP gene fused to the blasticidin resistance gene (*bsd*) under the human cytomegalovirus (CMV) immediate-early promoter

(5'-TTGAATTCAGCCAGAGCTAGTCTAGGAG-3") and bLG-R (5' TTCCA TGGCTGCAGCTGGGGT-3'). PCR products were digested with *Eco*RI and *Eco*RV and cloned into pBluescript SK+ plasmid vector. The 5' end of the insert was modified by addition of an *Eco*RI restriction site. The 3' end contained an *Nco*I restriction site. In this way the ATG codon responsible for initiation of translation was introduced. In the next step, human genomic DNA isolated from peripheral blood lymphocytes was used as a template for interferon alpha gene amplification (GenBank Accession J00207). The sequence encoding human IFN alpha was amplified using IFN-F (5'-ATCACCATCACCTG GTTCCGCG TGGATCTATGGCCTTGACCTTTGCTT-3') and IFN-R (5'-AGAAAGC TTGCAAAAGTTCAATGAACAAC-3') primers. The 5' end of the gene was modified by addition of an *Nco*I restriction site, a sequence encoding six histidine residues, and the sequence recognized by thrombin. The 3' end of the gene was modified by addition of a *Hind*III restriction site. PCR products were digested with restriction enzymes (*Nco*I and *Hind*III) and ligated with the vector containing the bovine β-lactoglobulin promoter within *Nco*I and *Hind*III restriction sites. In the final step, the sequence containing the bovine β-lactoglobulin promoter and human interferon alpha gene was digested with *Eco*RI and *Kpn*I and recloned into 6.0 kb of pTracer-EF/Bsd A plasmid vector. The nucleotide sequence of the final pbLGIFN-GFPBsd gene construct was confirmed using a cycling sequencing kit and ALFExpress sequencer (Pharmacia Biotech).

Microinjection of the pbLGIFN-GFPBsd construct into bovine zygotes

At 18 h post-insemination the cumulus cells were removed and the zygotes were washed in manipulation medium (MM). Zygotes were transferred to a microcentrifuge tube into 50 µl of MM and centrifuged for 6 min at 12000 *g* at room temperature. They were then transferred to a manipulation chamber (15 µl drops of MM, covered with mineral oil). The manipulation chamber was placed under an Inverted Microscope with Nomarski contrast optics and micromanipulator (Leitz). The pbLGIFN-GFPBsd construct was diluted to 3 ng/µl in TE buffer (pH 8.0). Microinjections into one of the pronuclei were performed and the times of injections were noted by observing the swelling of the pronucleus. Microinjections were performed between 18-20 h post-insemination.

In vitro culture of bovine embryos

Non-injected and microinjected zygotes were washed in Menezo B2 medium (ART of CCD) supplemented with 10% FBS (GIBCO) and placed in 40 µl drops of Menezo B2 medium supplemented with 10% FBS under mineral oil. Zygotes from the microinjected group and non-injected group were co-cultured on Vero cell

monolayers (ATCC, Maryland, USA). Vero cells at a concentration of 2×10^3/10 µl were placed into a microdrop (40 µl) of Menezo B2 (Assisted Reproductive Technology of Laboratoire CCD, France) supplemented with 10% FBS under mineral oil. Zygotes were cultured until day 7 (168 h post-insemination), at 38.5°C in 5% CO_2 in a humidified incubator. During culture, the medium was renewed three times (at 48 and 72 h 20 µl of medium were removed and 20 µl of Menezo B2 supplemented with 10% FBS were added; at 144 h, 20 µl of medium were removed and 20 µl of Menezo B2 without serum were added). Development of the embryos was evaluated at 48 and 168 h post-insemination.

Detection of GFP expression by fluorescence analysis

At 48 h post-insemination, 357 embryos from the microinjected group and at 168 h post-insemination, 218 embryos from the microinjected group and 10 embryos from the non-injected group were examined individually using a Fluovert FS microscope (Leitz) with excitation of the chromophore at 488 nm and a standard GFP filter. The embryos were scored for GFP-positive blastomeres within each of them. A GFP-positive embryo was one in which at least one blastomere yielded green fluorescence.

Embryo transfer and offspring evaluation

The control and GFP-positive blastocysts were transferred to recipients. Oestrous synchronization of the recipients was induced by injecting 2 ml (0.5 mg) of the prostaglandin $F_{2\alpha}$-analogue Cloprostenol (Bioestrovet-Vetoquinol, Gorzów, Poland) every 11 days. Seven to eight days after standing heat, one fresh embryo was transferred to the uterine horn ipsilaterally to the ovary displaying a corpus luteum. Embryos were transferred to recipients in Embryo Transfer Medium (BioLife Transfer Medium, Agtech Inc., USA). Recipients were monitored daily for heat behaviour and examined by ultrasound after 5 weeks, and then monthly, to confirm pregnancy. After calving, offspring were weighed and subjected to veterinary examination.

Screening for the presence of pbLGIFN-GFPBsd transgene in the host genome

Samples of tissue from ears and of blood were collected from all newborn calves (both microinjected and non-injected groups). Cells of the ear tissue were lysed by incubating the samples in 0.05 M NaCl, 1 mM EDTA, 1% SDS, 250 µg/ml proteinase K, (pH 8.0) at 55°C overnight. Following lysis, DNA was purified by phenol-chloroform extraction, precipitated with isopropanol, and resuspended

in water. DNA from blood was isolated using the guanidinium isothiocyanate procedure.

PCR analyses of genomic DNA isolated from blood and ear cells were carried out for all newborn calves from the microinjected group and the non-injected group.

The screening procedure involved the genomic DNA from ear biopsies and blood samples and amplification of two PCR-fragments encompassing the promoter-gene junction. In the case of the pbLGIFN-GFPBsd gene construct, the forward primers were located in the β-lactoglobulin promoter region and the reverse primers in the region coding interferon alpha. The PCR product of 800 bp was amplified with bLGINF-F1 (5'-GAAGCCACCCTCCAGGACA-3') and bLGINF-R1 (5'-CCCCACCCCCTGTATCACA-3') primers, whereas the 398 bp fragment was obtained with bLGINF-F2 (5'-CGAATGGAAGAAGGCCTCCTA-3') and bLGINF-R2 (5'-ATGTCTGTCCTTCAAGCAGGA-3') primers. The reaction mixture contained: 100 ng DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 7.5 pmol of each primer and 0.7 U Taq polymerase (Sigma-Aldrich) in a final volume of 20 µl. PCR was performed using the following conditions: denaturation 94°C, 45 s; annealing 55°C, 45 s; synthesis 72°C, 90 s; 30 cycles. PCR products were fractionated in 1.5% agarose gel containing 1× TBE buffer.

Statistical analysis

Rates of cleavage and blastocyst formation per zygote and rate of development after transfer to recipients were analysed by Chi-square tests. Probabilities (P-values) of less than 0.05 were considered statistically significant and less than 0.01 highly statistically significant.

RESULTS

The results of the development of microinjected and non-injected zygotes were compared on day 2 (48 h post-insemination) and on day 7 (168 h post-insemination)

Table 1. *In vitro* development of microinjected and non-injected bovine embryos

Groups	Number of			
	zygotes/number of injected zygotes	cleaved embryos %	blastocysts %	GFP positive blastocysts after 168 h
Microinjected	2107/2107	357 ± 16.9[a]	218 ± 10.4[a]	76
Non-injected	720/0	591 ± 82.1[b]	287 ± 39.9[b]	-

[a,b] statistically significant difference between microinjected and non-injected groups at $P<0.05$

(Table 1). A higher percentage of cleaved embryos was observed in the non-injected group (82.1%) compared with the microinjected group (16.9%). A higher percentage of blastocysts was also found in the non-injectedgroup (39.9%) than in the microinjected group (10.4%). The difference between the groups was highly significant ($P<0.01$). At 48 h post-insemination two embryos from the microinjected group were GFP-positive (these embryos were removed from culture) whereas none in the non-injected group were positive. After 168 h post-insemination, 76 out of 2107 microinjected embryos were GFP-positive blastocysts (3.6%) among which: one was 100% GFP-positive and others were mosaic embryos: seven were 50% and sixty-eight, 25%. Only 57 GFP-positive blastocysts and 31 non-injected embryos were transferred to recipients (Table 2). The recipients were examined at 35 and 65 days after transfer. Although a lower rate of pregnancy between groups was

Table 2. Results for the transfer of GFP positive and non-injected embryos to recipients

Groups	No. of			
	transferred blastocysts	pregnancies on day 35 %	pregnancies on day 65 %	offspring, calving rate
Microinjected, GFP positive	57	16 ± 28.1	11 ± 19.3[c]	11 ± 19.3[c]
Non-injected, control	31	17 ± 54.8	15 ± 48.4[d]	15 ± 48.4[d]

[c,d] statistically significant difference between microinjected and non-injected groups at $P<0.01$

observed on day 35, the difference between the groups was not statistically significant. On day 65 the difference between the GFP-positive group (19.3%) and the non-injected group (48.4%) was statistically significant ($P<0.05$). The calving rate was lower in the GFP-positive group (19.3%) as compared with the non-injected group (48.4%). The difference between groups was statistically significant ($P<0.05$). Irrespective of group, all newborn calves were healthy and had a normal weight.

PCR analyses indicated that none of the calves obtained after transfer of GFP-positive embryos carried the pbLGIFN-GFPBsd transgene.

DISCUSSION

The objective of this study was to obtain calves with integration of the human interferon alpha (*IFNα*) gene introduced by microinjection into zygotes. Microinjection is more reliable than using sperm as a vector, and less technically demanding than cloning from transfected cells. The main disadvantage of pronucleus injection is the low efficiency of integration of the transgene into the host genome (Robl et al., 2007). Another problem is mosaicism, which is

connected with integration of the transgene after DNA replication in the first cell cycle or later (Chan et al., 1999).

The embryos from microinjected and non-injected groups were co-cultured with Vero cells which support embryo development better than other systems of culture used in our laboratory (Duszewska et al., 2000, 2003). Microinjection is a traumatic procedure for zygotes and can cause lysis or reduce early embryo development (Han et al., 2000; Chan et al., 2002). The results of the present study support this opinion in that, after microinjection, development to the blastocyst stage was only 10.4% compared with 39.9% for non-injected embryos (Table 1). This result is comparable with other studies in which the efficiency of embryo development to the blastocyst stage ranged between 6 and 14% (Eyestone, 1999; Behboodi et al., 2001). In our experiment, the negative effect of transgene introduction occurred in the first cell cycle because the microinjected embryos developed better (61.1%) from cleavage to the blastocyst stage than the control embryos (48.6%). This negative effect on zygote development may result from adverse influence of the high forces used for visualization of the pronuclei and opening of the plasma membrane during micromanipulation (Ito et al., 1998).

The pbLGIFN-GFPBsd transgene also contained a reporter gene, *gfp,* which was isolated from the jellyfish *Aequorea Victoria* (Prasher et al., 1992). The product of *gfp* expression is Green Flouorescence Protein that yields bright green fluorescence under UV light. In our study, expression of *gfp* was detected twice. First, after 48 h for elimination of those embryos in which even one blastomer gave green fluorescence, indicative of transient expression because, before maternal-zygotic transition (which occurs after 72 h), the embryo genome is switched off. Transient expression was detected in 2 out of 357 cleaved embryos and these were removed from *in vitro* culture. Transient expression can result from episomal expression of unintegrated DNA and is a major barrier in the generation of transgenic animals (Wall, 1997; Duszewska et al., 2004) The efficiency of production of GFP-positive embryos (3.60%) was lower than in our previous study, 6.57% (Duszewska, 2003) and also in comparison with other studies: 6% (Eyestone, 1999) and 14% (Behboodi et al., 2001).

Only one microinjected embryo was 100% GFP-positive and the others were mosaic embryos. The mosaicism in embryos is connected with the process of integration into the host genome. This process is time-limited and the frequency of integration would be expected to decrease with each successive cell division, which contributes to mosaicism. The non-integrated DNA can be degraded over time (Chan et al., 1999).

The 100% GFP-positive embryo, as well as some mosaic embryos, were transferred to recipients (Table 2). The lower rate of calving after transfer of GFP-positive embryos (19.30%) compared with the non-injected group (48.4%) confirms

the lower development potential of embryos after microinjection. This result indicates that the negative effect may occur both during early embryo development, as well as later, after transfer of these embryos to recipients. Similar results after transfer of microinjected embryos were obtained in other studies, 17% (Eyestone, 1999) and 21% (Han, 2000). All calves were born naturally and healthy. Independent of the group, the birth weights of all calves were similar.

All newborn calves from the microinjected group were obtained after transfer of mosaic embryos to recipients. Resorption was observed in the case of the 100% GFP-positive embryo. None of the calves obtained after transfer of GFP-positive embryos showed the presence of the human interferon alpha (*IFNα*) gene in either their blood or ear tissues, as tested by PCR. Perhaps the introduced transgene was integrated into other tissues which were not analysed.

CONCLUSIONS

Although the calves were born after transfer of pbLGIFN-GFPBsd microinjected embryos, none of them carried the human interferon alpha transgene.

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