Efficient production of transgenic bovine/cat by microinjection and cloning technology of early embryos

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Objectives

Transgenic livestock have been developed for a variety of purposes, including improvement of food products or disease resistance, production of valuable therapeutic proteins in milk, and as models of human diseases (6, 8, 10, 11, 25, 26). However, the cost of transferring microinjected embryos to recipients which do not generate transgenic offspring is a major constraint to this approach. Therefore, for the production of large transgenic animals such as cattle, detection of the transgene at the pre-implantation stage would be desirable, considering the long gestation period and limited number of offspring. In bovine studies, although several non-invasive methods using firefly luciferase as a marker (13, 14, 17) or neomycin resistance gene (2) have been reported for selection of the transgenic embryos, the former method requires a step for loading the substrate (luciferin) inside the cells, and the substrate is known to be toxic, whereas the latter requires the presence of neomycin in the culture medium.

In the last few years, green fluorescent protein (GFP), a protein of 238 amino acids found in jellyfish *Aequorea victoria*, has been applied for various objectives as a useful marker for the monitoring of gene expression in situ (7). GFP absorbs blue light and emits green fluorescence without any need for exogenous substrates or co-factors, and this characteristic is a great advantage of GFP as a marker. Since no preliminary steps are required for the detection of GFP, cells or organs can be observed at any time in their viable and intact from by simple use of a fluorescence microscope. Therefore, the present study was conducted as a preliminary experiment aimed at evaluating the applicability of this convenient marker for in vitro selection of transgenic bovine/cat embryos. The expression of fluorescence by pre-implantation expression by bovine/cat embryos was observed after pronuclear microinjection or cloning with an enhanced GEP (EGFP; S65T + F64L) gene construct (18).

Materials and methods

Experiment 1:The methods used for in vitro maturation, in vitro fertilization (IVF), and subsequent culture in this experiment were modifications of the procedure described by Boediono et al.(1).

Briefly, cumulus-oocyte complexes (COCs) were aspirated from the follicles (2-7 mm in diameter) of cow ovaries collected at a local abattoir and cultured in maturation medium [25 mM Hepes TCM-199 with Eagle's salts (Gibco, Grand Island, NY) supplemented with 5% superovulated cow serum (SCS), 0.01 mg/ml follicle stimulating hormone (FSH, Denka, Kawasaki, Japan), 20 mM taurine (Wako, Osaka,

Japan), and 50 μg/ml gentamicin (Sigma, St. Louis, MO) at 38.5°C under 5% CO_2 in air. After 20-22 hr of culture, the COCs were fertilized in vitro with frozen-thawed sperm for 5 hr in Brackett and Oliphant medium (3) containing 2.5 mM caffeine (Sigma), 3 mg/ml bovine serum albumin (BSA, fraction V; Sigma), 20 mg/ml heparin (Shimizu Pharumaceuticals, Japan) and 20 mM taurine, and then cultured in vitro in TCM-199 supplemented with 5% SCS, 5 μg/ml insulin (Sigma), 20mM taurine, and 50 μg/ml gentamicin.

After 17 hr of IVF, these presumptive zygotes were incubated with 300 IU/ml hyaluronidase (Sigma) for 20 min, and their adherent cumulus cells were removed by repeated pipetting. The denuded zygotes were centrifuged at 16,000 g for 20 min in the presence of 5 μg/ml cytochalasin B (Sigma) to visualize the pronuclei [9], and transferred to a 100ml drop of Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} [DPBS (-); Gibco] supplemented with 3 mg/ml BSA, 5 μg/ml cytochalasin B, and 50 μg/ml gentamicin. After transfer, the pronuclei of the zygotes were microinjected with approximately 2 *p*l of the buffer solution (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.5) containing 1.5 μg/ml EGFP cDNA fragment under control of the chicken beta-actin promoter and cytomegalovirus enhancer (18) using an injection capillary (0.5 μm in diameter) attached to the micromanipulator (Leitz, Wetzlar, Germany). These injected zygotes were cultured in vitro for an additional 8 days (until day 9; IVF=day 0) to examine their developmental competence and fluorescent expression.

The green-light emission by the injected embryos was detected under a fluorescence microscope with DM 505 filters (EX 450-490 and BA 520; Nikon, Tokyo, Japan).

The developmental stage of the fluorescent embryos was recorded on every second day after injection until day 9.

Experiment 2:The application of nuclear technology using blastomeres of early bovine embryos has been reported (9, 20). The donor nuclei were prepared from the 8 cell stage embryos which were observed for fluorescence expression.

The recipient cytoplasts were prepared from in vitro matured oocytes, which were enucleated at 20-22 hr after the onset of in vitro maturation. The enucleation process was done by push out the first polar body and a small amount of cytoplasm after cutting the zona pellucida with a sharp glass needle. The successful of enucleation was confirmed by culturing the oocytes in the medium containing Hoechest 33342 (2 μg/ml) for 20 min, and subsequently were exposed into ultraviolet light for a few seconds. All manipulations were done in 20 μl drops of PBS supplemented with 5 μg/ml cytochalasin B and 3 % BSA covered by mineral oil.

The embryos that were reconstructed by fusing donor cells and presumptive M-phase cytoplasts, the transfer and fusion were accomplished immediately after the enucleation of the metaphase plate of the recipient oocytes.

The fusion was initiated by a single DC pulse of 1 kv/cm for 50 μsec (delivered by BTX 2001, San Diago, CA) in Zimmerman medium. The fused couple were then parthenogenetically activated by exposed into culture medium (CR1aa supplemented with 3% BSA and 5% FCS) containing calcium ionophore (10μg/ml) for 5 min followed by cultured in the medium containing cycloheximide (10 μg/ml) for 5 hr. After the activation, five to ten reconstructed embryos were cultured in the 100 μl drop

of culture medium covered by mineral oil at 38.5°C in humidified chamber and 5% CO_2 in air for 7 d. The developmental stage of the fluorescent embryos was recorded on every second day after fusion.

Experiment 3: The methods used for nuclear technology using foetus cell lines (5, 22, 23, 24) and subsequent EGFP gene transfer in this experiment were modifications of previous report (16, 18).

The donor nuclei (somatic cells) were prepared from bovine and cat foetus cell lines, which were derived from frozen-thawed foetus that had been frozen at –20°C for about three months. Seven to twelve passaged of the cell lines were used as donor nuclei in this study.

We conducted to transfer the EGFP gene fragment into the bovine or cat fetus fibloblasts using polybrene or electrophoration as follows;

(1)Culture the cells with 100ng-1mg DNA+rhAT gene (releasing solidification of blood) and 10µg/ml polybrene in DMEM containing 10% FCS for 6hrs.
(2)Treat the cells with the medium containing 30% DMSO for 4 min.
(3)Wash the cells twice and culture for 2-7 days.
(4)Observation of the green cells under a fluorescence microscope.
(5)Selection of the green cells using a micromanipulator, and transfer them to the other culture dish.
(6)After several weeks of culture, colonies of the green cells might be formed.
(7)Use as donors for nuclear transfer using bovine or cat enucleated oocytes

The cells were in DME medium supplemented with 0.5% serum for 4-5 d before being used as donor nuclei. The procedure of cloning process and observation of fluorescence expression of embryos was done by same as experiment 1 and 2. The developmental stage of the fluorescent embryos was recorded on every second day after injection until day 9. However, the number of fluorescent embryos that were observed to include non-fluorescent blastomeres were recorded as the criteria of mosaic expression, regardless of the brightness of light-expression by the embryos.

The rate of development to blastocysts and expanded/hatched blastcocysts was compared between the gene-transfer and non-treatment groups, and the rate of fluorescence expression was compared between the whole and mosaic groups using the X^2 test.

Results and Discussion

In the first experiment, a total of 310 zygotes were microinjected with the EGFP gene constract into the pronuclei. As a first step, the developmental competence in vitro of the gene-injected embryos was examined, and the results are summarized. Forty-six (14.8%) zygotes degenerated within 24 hr after injection. The cleavage rate for the injection group was lower than that of the non-treatment group. However, statistical comparison between the two groups was not made because the oocytes in the non-treatment group had not been selected for the presence of pronuclei. The rate of development to balstocycts was calculated for the cleaved embryos in order to facilitate comparison betwenn the injection and non-treatment groups. The rate of development to blastocysts in the injection group was significantly lower ($p<0.01$) than that of the non-treatment group. Among these balstocysts, the rate of development to

expanded/hatched blastocysts was significantly ($p<0.05$) lower in the injection group than the non-treatment group [56.8% (25/44) and 73.3% (121/165), respectively].

After gene-injection, the fluorescence expression was observed in a total of thirty seven (11.9%) embryos. However, although the fluorescent embryos at the blastocysts stage were detected in 2.9% of the injected embryos, six out of nine fluorescent embryos (66.7%) showed mosaic expression in their inner cell mass (ICM) and trophectoderm. Furthermore, the rate of fluorescent embryos that showed mosaic expression after injection was significantly ($p<0.05$) higher than the embryos of whole expression (8.4% and 3.5%, respectively).

The developmental competence of bovine zygotes decreased after pronuclear microinjection with the EGFP gene. The centrifugation treatment for the polarization of intracellular lipids, as performed herein, has been shown to maintain the normal in vitro developmental capacity of bovine zygotes in our previous experiment (15). It has been demonstrated that pronuclear microinjection with either water or buffer decreased bovine embryonic development significantly, and inclusion of DNA in the injection buffer decreased the development even more drastically (19). Therefore, the developmental restriction of zygotes found in this experiment was considered attributable to the treatment was due to mechanical damage resulting from embryo manipuration or influence of the microinjected DNA itself could not be determined. In addition, the frequent observation of the embryos under the excitation light might have some detrimental effect on their development.

The previous studies have been shown that the expression of EGFP was detected in pre-implantation bovine embryos following pronuclear microinjection with the modified GFP (S65T) gene constructs (4, 23). However, it has been indicated that some GFP-positive morulae became weekly positive or almost negative at the balstocyst stage (23). In the present study, such a loss in fluorescence expression was not detected in the light-emitting embryos during the observation carried out after injection of the EGFP (S65T + F64L) gene. On the contrary, some non-fluorescent morulae were observed to emit green-light when they developed to the blastocyst stage [2/310 (0.6%)]. We could not clarify the cause of the difference in fluorescence expression by the embryos. However, in the study of EGFP transgenic mouse (18), humanized modification of the codon usage in the EGFP sequence was assumed to be responsible for their increased efficiency of fluorescence expression.

The fluorescence was expressed by the injected embryos at various intensities throughout their development up to blastocyst stage. However, only 1.0% of the injected embryos developed to blastocysts that emitted green-light in their whole blastomeres. Moreover, the majority of the fluorescent embryos showed mosaic expression [(26/37) 70.3%]. It has been indicated that the mosaic was detectable commonly approximately half of the fluorescent embryos when the EGFP gene construct used herein was injected into mouse zygotes (M. Okabe, personal communication).Although the exact reason for this phenomenon is unknown, Lewis-Williams et al. (12) has demonstrated that the incidence of transgenic mosaics in mouse embryos increased significantly with time after microinjection of a target DNA, using fluorescence in situ hybridization (FISH) analysis. They indicated that most integration evens seems to occur after the first cleavage, generating a majority of mosaics among the transgenic offspring. It has also been revealed that delayed integration of microinjected DNA into the embryo genome often results in mosaic

founder animals (21).

In the experiment 2, the rate of survival and fluorescence expression of the reconstructed embryos were 69.4% (25/36) and 52% (13/25), respectively. These results indicated that the rate of expression of EGFP gene with the nuclear transfer embryos is higher than that of microinjected embryos. In the experiment 3, the results was almost same compared to the experiment 2.

EGFP gene will be linked with any pharmaceutical protein during culture with fibroblast cells using polybrene or electroporation procedure. What genes should be added or removed? One gene of interest, particularly rhAT gene that is a factor of releasing solidification of blood for human.

Additional advantages of nuclear transfer combined with transgenics become obvious when it is compared with microinjection transgenics. One advantage is the time and expense saved with nuclear transfer transgenics. With nuclear transfer all of the initial offspring are transgenic. They are all transgenic because the gene of interest is inserted into the fibroblast cells prior to making the nuclear transfer embryos. Then only nuclei from transgenic cell lines are used in the process. Also, the sex of the transgenic offspring can be predetermined, in this case female, by using nuclei from lines of cells in which the sex is known. This alone saves two years or several months of development time by eliminating one generation of cattle or cat, respectively. Since these are all genetically identical females, testing for expression and herd expansion can be performed rapidly.

In the present study, we used the cat for gene transfer using cloning technology. This technology is very efficiency because the gestation period of cat is only 60 days. Therefore, the isolation of the efficiency protein from milk should lead to a safer and more cost-effective product.

Conclusion

In summary, the present results suggest the feasibility of EGFP-gene for non-invasive selection of transgenic bovine embryos at the pre-implantation stage using the fluorescence microscope, by linking the marker gene contact with a desired gene. However, these injected embryos showed impaired development and high rate of the mosaic expression. Therefore, we conducted to transfer the EGFP gene fragment into the bovine or cat fetus fibroblasts using polybrene for expressing bright green fluorescence in the whole part ICM. EGFP is a great advantage as a marker, cells or organ can be observed at any time in their viable and intact and easily connected with pharmaceutical protein in vitro. Additional advantages of nuclear transfer combined with transgenics become obvious when it is compared with microinjection techniques. In the future, we produce the pharmaceutical proteins from cow or cat using nuclear transfer with EGFP gene.

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