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Efficient Production of Cloned Bovine Embryos from Transformed Somatic Cells*

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형질전환 체세포로부터 소 복제수정란의 효율적인 생산

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ABSTRACT

Human thrombopoietin (hTPO) is a cytokine that plays a central role in megakaryopoiesis. To direct hTPO expression in the mammary gland, an expression vector was constructed by combining the promoter of bovine beta-casein gene, cDNA of hTPO and neomycin resistance gene (pBT-L neo). Fibroblast cells derived from cow's ear skin tissue were transfected with the expression vector (pBT-L neo) using Lipofectamine. Transfected cells resistant to G418 treatment were cultured to form the colonies for more than 2 weeks. The transformed colonies identified by PCR were further expanded prior to nuclear transfer. Reconstructed oocytes with transformed cells were electrofused, activated using calcium ionophore and 6-DMAP, and cultured *in vitro* for 7 days. Of 35 cell colonies analyzed by PCR, 29 colonies (82.9%) were positive for the hTPO gene. Cleavage and developmental rates to the blastocyst stage of reconstructed embryos with the transformed cells were 65.1% and 23.8%, respectively. Of 29 blastocysts that developed from reconstructed embryos with the transformed cells, 27 embryos (93.1%) were transgenic. These results indicate that transgenic bovine embryos can be efficiently produced by somatic cell nuclear transfer using transformed cells.

(Key words : hTPO, Transfection, Nuclear transfer, Bovine)

Ⅰ. INTRODUCTION

In livestock, transgenic technology has been used to make animal bioreactors which produce a large amount of human proteins in the milk (Clark et al., 1989; Ebert et al., 1991; Krimpenfort et al., 1991; Wall et al., 1991; Velander et al., 1992a, van Berkel et al., 2002) and the generation of transgenic animals for xenotransplantation (Chen et al., 1999). So far, pronuclear injection method, which foreign

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DNA is injected into pronuclei of the fertilized egg, has been mainly employed for animal transgenesis. However, the overall efficiency of transgenesis in livestock is very low, e.g., 1% in pig (Hammer et al., 1985), and 0.8~1.9% in sheep (Damak et al., 1996) and cattle (Krimpenfort et al., 1991). In addition, foreign DNA is randomly integrated into chromosomes, thereby resulting in low expression level and germ line transmission of the transgene. Thus, a major drawback of pronuclear injection is inefficient transgenesis, eventually giving rise to high costs in the development of transgenic livestock. This inefficiency could be improved by using somatic cell nuclear transfer (NT). Transgenic clone animals are generated by NT technology using transformed cells as donor nuclei (Schnieke et al., 1997; Cibelli et al., 1998). Moreover, the gene targeted animals (knock-in and knock-out livestock) could be also produced using NT technology (McGreath et al., 2000; Lai et al., 2002). However, primary cells without drug-resistant gene (e.g., neo gene) also appear to be tolerant to the antibiotics (G418). It has been reported that some clone animals derived from transfected cells are non-transgenic (Betthauser et al., 2000; Denning et al., 2001; Bondioli et al., 2001; Echelard et al., 2002; Lai et al., 2002). The results may be due to a so-called bystander effects, where transgenic cells expressing the antibiotic-resistance gene affect nearby non-transgenic cells to survive in the antibiotics-containing medium either by secretion of the gene product into the medium or by direct cell-to-cell contact. In this context, it is speculated that both transgenic and non-transgenic cells grow together in the selection medium containing antibiotics. Therefore, to efficiently produce transgenic clone embryos or animals, a series of procedures including transfection of foreign DNA into primary cells, culture of single colonies and identification of the authentic transformed cells should be established prior to nuclear transfer.

In this study, hTPO expression vector was transfected into primary bovine fibroblast cells and then the transformed cell colonies were identified by PCR analysis. *In vitro* developmental competence of reconstructed embryos with the transformed cells were investigated.

Ⅱ. MATERIALS AND METHODS

Unless stated otherwise, all chemicals for this study were purchased from Sigma Chemical Company (St. Louis, MO).

1. *In Vitro* Maturation (IVM)

Bovine ovaries collected from the slaughterhouse were transported to the laboratory in 0.9% saline at 25~30℃. Cumulus-oocytes complexes (COC) were aspirated through an disposable 10 ml syringe with 18-gauge needle from follicles of 2~6 mm in diameter. COC with at least three layers of compact cumulus cells and homogeneous cytoplasm were selected and washed three times in TL-HEPES (1 mg/ml BSA, low carbonate TALP; Parrish et al., 1988). Approximately 50 COC were matured in 500 μl of the IVM medium in a 4-well dish (Nunc, Roskilde, Denmark) for 22 h at 38.5℃, 5% CO_2 in air. The medium used for oocyte maturation was TCM-199 supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), 1 μg/ml estradiol 17β and 1 μg/ml FSH-P (Schering-plough Animal Health Corp., Kenilworth, NJ).

2. Construction of hTPO Expression Vector

As a target vector we used pBT-L (Sohn et al, 1999), which is composed of bovine β-casein promotor, hTPO cDNA, and poly (A) adenylation site of bovine growth hormone (bGH). For selection of transformed cells, neo gene, which is resistant to

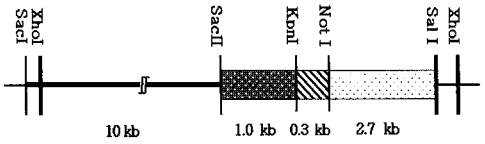

Fig. 1. The expression vector used in this study. This vector is composed of bovine β -casein promotor (10 kb), hTPO cDNA (1.0 kb), bovine growth hormone poly-adenylation sequences (0.3 kb) and neo gene (2.7 kb).

antibiotic neomycin, was taken from pMAMneo vector after treatment with BamH I restriction enzyme and then interposed in pBT-L vector. The construct was named as pBT-L neo vector (Fig. 1).

3. Transfection and Selection of Cells

pBT-L neo vector was transfected into bovine ear skin fibroblasts (bESF) using lipofectamine reagent (Gibco BRL). In a 6-well culture plate, primary bESF were seeded at the concentration of 1×10^5 cells per well in 2 ml of Dulbecco modified Eagle medium (DMEM, Gibco BRL) supplemented with 10% FBS, 100 μM MEM non-essential amino acid (Gibco BRL) and 500 μg/ml gentamicin sulfate. The cells were cultured at 37℃, 5% CO_2 in air until 70~80% confluence. For transfection, DNA-liposome complexes were formed by gently mixing 2 μg DNA and 13 μl Lipofectamine reagent into 100 μl serum-free medium per well, and then incubated at room temperature for 45 min. After complexes were formed, the cells were rinsed twice with 2 ml of serum-free medium and 0.8 ml serum-free medium were added to the tube containing the complexes overlaid onto the rinsed cells. Transfection was induced by co-incubation for 5 h and then 1 ml of the medium containing serum was supplemented to 6-well culture plate. In addition, the cells were cultured in the fresh medium at 24 h after transfection. Transfected cells were cultured and passaged from 6-well plate to 100 mm culture dish for at least 72 h to allow expression of the neomycin resistance gene. To select the transformed cells, transfected cells were incubated in the culture medium containing 600 μg /ml G418 (Gibco BRL). The single colonies with about 0.8 cm diameter were picked using cloning cylinders and transferred to 96-well plate. Antibiotic -resistant colonies were expanded to increase the cell population, culturing gradually in 96-well, 48-well, 24-well, 6-well, and 100 mm dish. After PCR analysis, transformed cells were equilibrated in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide, and then frozen in liquid nitrogen.

4. Karyotype of Donor Cells

Prior to nuclear transfer, it was examined whether the transformed cells have normal karyotypes. The cells were first cultured in 6-well culture plate until 70% confluence. After removal of culture medium, fresh medium supplemented with 0.5 μg/ml colcimid was added to the growing cells to be arrested in metaphase II for 8 hr. The cells were recovered by treatment with 0.25% trypsin-EDTA and then suspended in hypotonic solution (0.56% KCl) for 30 min at room temperature. Fixative (methanol : acetic acid = 3 : 1) was supplemented to cells additionally and centrifuged at 1,000 rpm for 5 min. This fixation process was repeated twice as described above. The cell pellet was re-suspended with 1 ml of fixative and dropped onto the slide on the hot plate with wet paper towels at 40℃. After air-drying, the slides were stained with 0.1% Giemsa (Fluka, USA) for 30 min, washed 3 times with DDW. Chromosome numbers were counted under 1,000 magnification on a microscope (Olympus, Japan).

5. Nuclear Transfer (NT)

Oocytes matured in vitro were treated with 0.1% hyaluronidase, followed by gently pipetting to remove cumulus cells. The denuded oocytes were enucleated with a glass pipette by aspirating the first polar body and part of cytoplasm containing M Ⅱ plate in TL-Hepes supplemented with 7.5 μg/ml cytohalasin B. Enucleation was confirmed by Hoechst 33342 staining and visualizing the aspirated portion of the cytoplasm under ultraviolet light. A single cell was inserted into the perivitelline space of the enucleated cytoplast. Oocyte-cell couplets were electrically fused on a fusion chamber with two stainless steel electrodes (1 mm apart) in fusion medium consisting of 0.3 M Mannitol, 0.5 mM Hepes, 0.01% BSA, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. A single direct current pulse of 1.6 kV/cm was applied for 20 μsec using an Electro Cell Manipulator 2001 (BTX, San Diego, CA). Activation of fused embryos was performed as a modified method described by Cibelli et al. (1998). At 4 h after electrofusion, fused embryos were activated with 5 μM ionomycin for 5 min, followed by treatment with 2 mM 6-dimethyl-aminopurine in CR1aa containing 10% FBS for 4 h at 38.5℃, 5% CO_2 in air. The reconstructed embryos were cultured in CR1aa supplemented with 3 mg/ml BSA (fatty acid free). After culture for 3 days, cleaved embryos were further cultured for 4 days in each drop of 50 μl CR1aa supplemented with 10% FBS and then blastocyst formation was observed.

6. PCR Analysis for the Transgene

Transformed colonies and transgenic embryos were identified by a nested PCR using the primers specific for hTPO gene. At the time of trypsinization and passage, 3～5 cells were placed into a tube containing 1 μl of lysis buffer, which consisted of 40 mM Tris (pH 8.9), 0.9% Triton X-100, 0.9% NP40, and 0.4 mg/ml proteinase K. Single embryos at the different developmental stages were individually transferred into a tube containing the lysis buffer. The reaction was carried out in PCR premix (Bioneer, Korea) on a Thermal Cycler with the following parameters; after 94℃ for 4 min, 30 cycles of 94℃ for 45 sec, 56℃ for 60 sec, 72℃ for 60 sec, and a hold at 4℃. The first primers used amplified a 500 base pair region and the second primers amplified a 300 base pair region of hTPO specific sequences. The first primers were 5'-GGAGCTGACTG- AATTGCTCCTCGT-3' and 5'-CCTGACGCAGAGGGTGGACCCTCC, and the second primers were 5'-GGAGCTGACTGAATTGC TCCTCGT and 3'-GAGACGGACCTCCAGAAAG CTG. PCR products were electrophoresized on 1.3% agarose gel, stained with ethidium bromide and then photographed.

Ⅲ. RESULT

1. Establishment of Transgenic Cell Lines

An expression vector with human thrombopoietin gene was transfected into bESF by using DNA-liposome mediated gene transfer. When the transfected cells were cultured in the selection media, colony formation was observed approximately 2 weeks post-transfection (Fig. 2A). Large colonies with more than 0.8 cm in diameter (Fig. 2B) were picked using cloning cylinder and then further

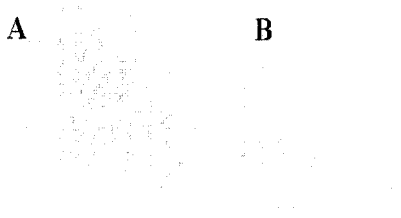

Fig. 2. The colony formation of transformed cells. A: early colony formation was initiated approximately 2 weeks after transfection. B: a large colony before picking.

proliferated to use as donor nuclei. The efficiency of transfection in the bovine primary cells was represented in Table 1. Of 94 colonies isolated, 35 colonies were expended to single 6-well dishes (37.2%). Thereafter, it was investigated by PCR analysis whether each colony is positive for the hTPO transgene (Fig. 3). Twenty -nine colonies (82.9%) were identified as transgenic cells. Of these transformed colonies, 10 lines (34.5%) were able to grow to 100 mm culture dish whereas the others failed to further proliferate.

To establish the transformed cell lines from somatic cells, the primary cells might be cultured until at least passages 10~12. These long term culture may give rise to chromosomal aberrancy of the cells. Prior to nuclear transfer, it was tested for one cell line (TS14) whether the transformed cells have normal karyotype. Of 47 metaphase spreads, 45 metaphases (95.7%) were normal (Fig. 4A) and 2 metaphases (4.3%) were aneuploid (Fig. 4B), having 58 and 59 chromosomes, respectively.

Table 1. Establishment of transgenic cell lines from bovine ear skin fibroblast cells after transfection

No. of colonies			No. (%) of cell lines obtained
Picked	Analyzed (%)	Identified (%)	
94	35 (37.2)	29 (82.9)	10 (34.5)

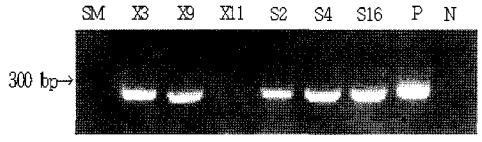

Fig. 3. Selection of transformed colonies by PCR analysis. X3, X9, X11 S2, S4 and S16 colonies were positive for hTPO gene. SM: DNA size marker (100 bp ladder), P: positive control, N: negative control.

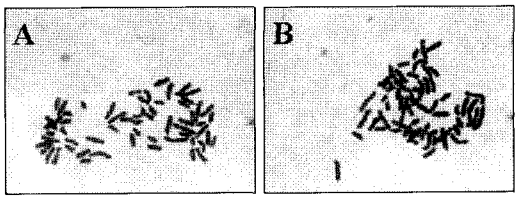

Fig. 4. Karyotype of transformed cells after a long-term culture (12 passages). A and B show normal and abnormal karyotypes, respectively.

2. *In Vitro* Development of NT Embryos

In vitro developmental rates of reconstructed oocytes with transgenic cells or non-transgenic cells are shown in Table 2. The rates of cleavage and development to the blastocyst stage of NT embryos with transgenic and non-transgenic cells were 65.1% (194/298) vs 78.0% (128/164) and 23.8% (71/298) vs 29.9% (49/164), respectively. No difference was observed in the preimplantation development of NT embryos between two groups.

3. Transgenic Efficiency of NT Embryos

During 7 days of culture, each other stage embryos were collected and transferred into the tubes containing lysis buffer prior to transgene -specific PCR analysis (Fig. 5). The proportion of eggs positive for hTPO gene was 48.3% in zygote, 75.0% in 2 cell, 62.1% in 4 cell, 69.8% in 8 cell and 93.1% in blastocyst stage. Thus, transgenic rates of reconstructed embryos were gradually increased from zygote (48.3%) to blastocyst (93.1%) as developmental stage advances (Table 3).

Table 2. *In vitro* development of reconstructed bovine embryos with transformed cells

Cell type	No. of embryos fused	No. (%) of embryos cleaved	No. (%) of blastocysts
Non-transformed	164	128 (78.0)	49 (29.9)
Transformed	298	194 (65.1)	71 (23.8)

Table 3. Transgenic efficiency of cloned embryos at the preimplantation developmental stages

No. (%) of transgenic/analyzed embryos				
1 cell	2 cell	4 cell	8 cell	Blastocyst
14/29 (48.3)	12/16 (75.0)	18/29 (62.1)	44/63 (69.8)	27/29 (93.1)

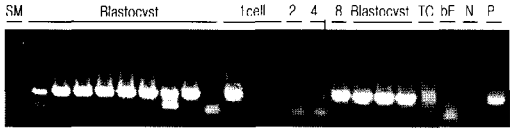

Fig. 5. Identification of transgenic embryos at various developmental stages by PCR analysis. SM: DNA size marker, TC: transformed cells with hTPO gene, bF: bovine fibroblast cells, N: negative control, P: positive control.

Ⅳ. DISCUSSION

In the generation of transgenic farm animals, somatic cell cloning offers many advantages as compared with pronuclear injection which has been mainly employed so far, although its efficiency is very low in that only less than 5% of offspring derived DNA-injected embryos are transgenic (Eyestorne, 1994; Pintado and Gutierrez-Adan, 1999). The foremost advantage of somatic cell cloning is a high fidelity transgenesis, demonstrating that almost all cloned offspring derived from the transformed donor cells are transgenic. However, there are still many impediments to use this technology to industrial application. Especially, to secure authentic transformed cells as donor nuclei is prerequisite for efficiently generating transgenic cloned animals. In this study, we have designed an expression vector to select the transformed cells and established a transfection system to introduce the transgene into primary bovine cells.

The transgenic animals, so-called animal bioreactors, secreting valuable proteins in their milk have become attractive because of high-level expression and low cost in the production of pharmaceutical proteins. So far, many human proteins such as α -anti-trypsin (Archibald et al., 1990), tissue plasminogen activator (Gordon et al., 1992) and protein C (Velander et al., 1992b) have been highly expressed in the milk of transgenic animals. Our previous data showed the expression and bioactivity of hTPO secreted in the milk of transgenic mice (Sohn et al., 1999). Human TPO, as a primary physiological regulator for production of platelet, plays a pivotal role in the proliferation and maturation of megakaryocytic progenitor cells and megakaryocytes (Bartley et al., 1994; De Sauvage et al., 1994; Sohma et al., 1994).

The hTPO expression vector was randomly introduced into bESF by using liposome-DNA mediated gene transfer. It was difficult to secure authentic transformed cell colonies to employ nuclear transfer as donor nuclei because the primary fibroblast cells after transfection frequently represented senescent state during expansion as well as antibiotic-resistance. In this study, of 94 colonies after transfection, 35 colonies were expanded and analyzed. Out of 29 colonies positive for the transgene, only 10 colonies were able to proliferate to the 100 mm petridish in diameter. This phenomenon was also reported by other researchers. A number (7/17) of transfected boar skin fibroblast cell colonies became senescence before cloning, randomly integrated colonies were only 2 clones and 8 colonies were negative (Bondioli et al., 2001). Only one colony (1/10) from randomly transfected goat fibroblast cells were obtained and other colonies reached replicative senescence (Zou et al., 2002). The non-dividing and non-transgenic colonies were also observed on the gene-targeted

event of sheep fetal fibroblast cells (Denning et al., 2001). Thus, characteristics of short life-span and by-stander effect in the primary fibroblast cells gives rise to a limitation to secure the transgenic cell line. Proliferation capability of transfected cells may go down during transfection, drug selection, and *in vitro* culture. Zakhatchenko et al. (2001) reported that transfected cells need culturing period for 2~3 weeks per passage until 60~70% confluent, suggesting that transfection of the transgene into primary cells may affect their viability resulting in the growth retardation and prolonged proliferation time. In the production of transgenic cloned animal, its efficiency may decrease by bystander effect which antibiotic resistance transfers to nearby non-transformed cells. The mechanism of bystander effect is unclear either by direct interaction between cells or elimination of the gene product into medium. We inserted neo gene into pBTL vector to resist the cytotoxic agent, G418, after transfection. In spite of culturing in G418 medium (600 μg/ml) for selection, non-trangenic colonies (17%, 6/35) were formed by by-stander effect whereas the other colonies (83%, 29/35) were transgenic (Table 1). In this study, transformed cells from single colonies were employed to the nuclear transfer experiments to enhance transgenic efficiency.

A long-term culture of primary cells may result in genetic or epigenetic defects, thereby leading to the reduction of NT embryo developmental potential. To validate this possibility, we examined the karyotype of transformed cells at 12 passages. No severe abnormalities were observed in the karyotype of the transformed cells (Fig. 4). Fetus-derived fibrblasts are generally used to generate transgenic cloned animals because of little differentiated status (Schnieke et al., 1997; Cibelli et al., 1998; McCreath et al., 2000). However, adult cells as donor nuclei showed a higher pregnancy rate of cloned embryos than fetal cells (Hill et al., 2000). In this study, fibroblast cells derived from ear skin were used to obtain transformed cells following a long-term culture.

Some cloned animals derived from antibiotic-resistant transformed cells are non-transgenic. Only 66.7% (4/6) of cloned calves derived from antibiotic-resistant CL53 cells were transgenic (Echelard et al., 2002), when they used the pooled cells after transfection. In the present study, only transgenic cells identified by PCR were individually transferred to enucleated oocytes and then the resulting embryos were detected for existence of hTPO gene at various to developmental stage by nested PCR. The NT embryos showed high transgenic rates of 93.1% at the blastocyst stage (Table 3). This proportion of transgenic embryos was higher than that reported by other laboratories (Zakhartchenko et al., 2001; Park et al., 2002; Chen et al., 2002).

In conclusion, our findings demonstrate that the transgenic cattle can efficiently produce by using nuclear transfer technique. In this study, we established a series of complex systems including transfection of expression vector into the primary fibroblasts, selection of single colonies, identification of transformed cell colonies and production of transgeic cloned embryos. These well-organized systems are very helpful for increasing the efficiency of transgenesis in the livestock.

V. 요약문

인체 트롬보포이에틴(hTPO)은 megakaryopoiesis 과정에 주요한 역할을 하는 사이토카인이다. 따라서 이러한 트롬보포이에틴을 유선조직에서 직접적으로 발현시키기 위하여 소 베타 카제인 프로모터, 인체 트롬보포이에틴 cDNA 및 네오유전자로 구성된 발현벡터를 구축하였다. 소 귀조직 세포로부터 유도된 섬유아세포에 lipofectamine을 이용하여 발현벡터(pBT-L n대)의 삽입을 유도하였다.

G418 저항성을 지닌 세포의 콜로니 형성을 유도하기 위하여 2주 이상 배양을 실시하였다. 형질전환 콜로니는 PCR에 의해 동정하였으며, 이들 콜로니를 핵치환 전까지 계속적으로 증식을 유도하였다. 형질전환 세포에 의해 재구성된 난자는 전기적인 융합과 calcium ionophore와 6-DMAP를 이용한 활성화를 실시하였으며, 체외에서 7일간 배양을 실시하였다. 총 35개의 콜로니를 PCR에 의해 분석한 결과, 이 중 29(82.9%)개가 형질전환된 콜로니였다. 형질전환된 세포로 재구성된 난자의 난할율 및 배반포로의 발달율은 65.1%와 23.8%로 나타났다. 형질전환된 세포로 재구성된 난자로부터 발달한 29개의 배반포 중 27개가 형질전환으로 확인되었다. 따라서 이러한 결과들은 형질전환 소 수정란을 형질전환된 세포를 이용한 체세포 복제 기법을 통해 효과적으로 생산할 수 있다는 것을 제시하고 있다.

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