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Production of Transgenic Pigs Harboring the Human Erythropoietin (hEPO) Gene Using Somatic Cell Nuclear Transfer

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Abstract. The production of transgenic pigs using somatic cell nuclear transfer (scNT) has been widely described, but a technique for removing nontransfected donor cells and for creating different founder animals has not yet been fully elucidated. In this study, four different expression vectors (pBC1hEPO, pMARBC1hEPO, pBC1hEPOwpre and pMARBC1hEPOwpre) were compared to determine the highest transgene expression, ideal conditions of enrichment of recombinant cells *in vitro* and efficiency of transgenesis following transfection into HC11 mammary epithelial cells. The highest protein expression in HC11 cells was obtained from the pMARBC1hEPOwpre expression vector. Next, we evaluated the efficiency of transgenic pig production by using geneticin (G418) selection alone or by using real-time PCR selection following G418 selection. Ideal enrichment of recombinant cells was obtained by a combination of real-time PCR and G418 selection; embryos reconstructed using donor cells selected by a combination of real-time PCR and G418 selection gave rise to nine piglets, all of which were transgenic. Among them, three founder transgenic pigs were established. Exogenous DNA fragments were shown to be integrated into chromosomes 1q2.4, 1p2.3 and 6q2.4, respectively, in these three pigs. However, the transgenic rate using G418 selection alone was only 33% (two of six pigs) and showed a very low efficacy compared with that of the combination of real-time PCR and G418 selection. Our results provide a valuable experimental model for applying and evaluating transgenic technology in pigs.

Key words: Erythropoietin, Geneticin (G418), Pig, Somatic cell nuclear transfer (SCNT), Transgenic

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Genetic manipulation, including transfer of foreign DNA into somatic cells and selection of transgenic somatic cells, is a crucial aspect of producing transgenic animals by somatic cell nuclear transfer (scNT). The low efficiency of scNT livestock production is a major obstacle to widespread use of this technology for production of transgenic animals. Most commercial companies producing transgenic livestock use scNT extensively only to produce transgenic founders. Microinjection of DNA into the pronuclei of fertilized oocytes has been the only practical means of producing transgenic livestock since the method was established in 1985 [1]. However, only a small proportion (approximately 5%) of animals integrate the exogenous DNA into their genome. Many transgenic lines do not provide sufficiently high levels of transgene expression because the integration site is random. Recently, the scNT technique has provided an alternative method for pronuclear microinjection as a means of transferring exogenous DNA to the germ line of an animal that allows for precise genetic modifications by gene targeting [2, 3]; Schnieke *et al.* [2] demonstrated that production of transgenic animals using the scNT technique improved overall efficiency to 2.5 times that of pronuclear microinjection. These previous studies were based on *in vitro* selection using antibiotics such as neomycin or hygromycin for selection of donor cells.

Recently, Schnieke *et al.* [2] demonstrated that nuclear transfer

from stably transfected somatic cells provides a cell-mediated method for producing transgenic livestock. Although the use of somatic cell donors for nuclear transfer in livestock offers many advantages over pronuclear microinjection, previous studies [4, 5] have shown that selection of transgenic cells using G418 was not sufficient to completely remove the nontransgenic cells from cultures, and scNT blastocysts as well as cloned animals derived from the donor cells were not always transgenic. As an alternative method to G418 selection, Watanabe *et al.* [6] showed that puromycin can be effectively used to select transgenic cells. However, it has also been reported that after puromycin selection, some nontransgenic cells are still puromycin-resistant, as reported for Betthausen *et al.* [7]. Collectively, these observations suggest that antibiotic selection, such as puromycin or G418, might require pre-screening of scNT embryos derived from the donor cells prior to embryo transfer into recipients.

Transfected cells that express green fluorescent protein (GFP) can be easily selected under fluorescence microscopy, and this means that these cells bypass the need for *in vitro* selection by drugs such as puromycin and G418 [8]. Therefore, GFP has become more popular as a selection marker of donor cells for production of transgenic animals [8–10]. However, in these studies, many cells constantly expressing GFP eventually died, indicating that GFP is generally toxic to cells, but the reason for this unknown. The only report of GFP-induced cell death suggests that exciting GFP intensely for extended periods may generate free radicals that are toxic to cells [11]. Although scNT has been employed

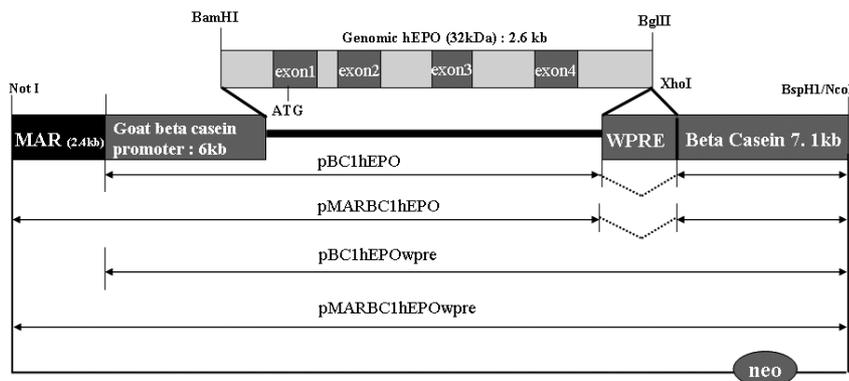


Fig. 1. A schematic illustration of the hEPO expression vectors used for production of transgenic pigs. The goat beta casein promoter gene is 6 kb in length, including the complete promoter sequence. Beta casein Poly A is 7.1 kb in length. The hEPO genomic DNA was inserted between the promoter and poly A sequences of the beta casein gene. See Material and Methods for details.

successfully to produce cloned transgenic pigs using GFP-expressing fibroblasts [9, 10], it has been suggested that some transgenes, such as those encoding GFP, are detrimental to early-stage cloned embryos [12]. Based on this observation, GFP may not be useful for commercial production of biopharmaceuticals using transgenic livestock.

Recently, we reported that the human erythropoietin (hEPO) yield from transgenic mouse milk is greater than that from an animal cell system such as STO cells [13]. In addition, the bioactivity and pharmacokinetics of milk-derived recombinant hEPO (rhEPO) are comparable to those purified from hEPO-expressing CHO cells. Since our previous study was restricted to a mouse model system, in the present study, we designed a system to produce rhEPO in pigs. In the present investigation, we constructed several hEPO expression vectors and transfected them into mammary gland-derived HC11 cells to evaluate the efficiency of expression of tissue-specific gene constructs. The present study was also designed to test the hypothesis that rapid selection of donor cells by using a combination of real-time PCR and G418 selection can be used as a reliable method for somatic cell-mediated gene transfer.

Materials and Methods

Construction of hEPO expression vectors

Human EPO genomic DNA (2.6 kb), a kind gift from Dr. R Sasaki (Kyoto University, Japan) was cloned into the pBluescript SK(+) vector (Stratagene, La Jolla, CA, USA). To generate pBC1hEPO and pBC1hEPOwpre expression vectors, DNA was isolated from the pBluescript SK(+) vector by digestion with BamHI and BglII [13, 14] and ligated into pBC1 expression vector (Invitrogen, Carlsbad, CA, USA). The posttranscriptional regulatory element from woodchuck hepatitis virus 2 genomic DNA (GenBank accession number M11082) was introduced according to the strategy of Zufferey *et al.* [15]; a PCR-amplified woodchuck hepatitis virus posttranscriptional regulatory element (WPRE fragment; nucleotides 1093–1684; GenBank No. J04514) was completely digested with XhoI and inserted into the unique BglII site located downstream of the hEPO stop codon. The pMARBC1hEPO and pMARBC1hEPOwpre expression vectors were constructed by inserting the matrix attachment region (MAR) within the upstream of goat beta casein promoter into the

pBC1hEPO and pBC1hEPOwpre expression vectors, respectively. To compare the expression efficiency of hEPO genes *in vitro* or *in vivo*, the hEPO expression vectors were classified according to the presence of their fragments as follows: (a) pBC1hEPO (the hEPO gene under the control of the beta casein promoter gene); (b) pMARBC1hEPO (the hEPO gene under the control of the beta casein promoter gene and including the MAR sequence); (c) pBC1hEPOwpre (the hEPO gene including the WPRE sequence under the control of the beta casein promoter gene); and (d) pMARBC1hEPOwpre (the hEPO/WPRE gene under the control of the beta casein promoter gene and including the MAR sequence). A schematic representation of the pBC1hEPO, pBC1hEPOwpre, pMARBC1hEPO and pMARBC1hEPOwpre expression vectors is shown in Fig. 1. The resulting 18.8-kb mammary gland-specific expression vector containing full-length genomic hEPO was isolated by SalI digestion and then used for somatic cell transfection.

Establishment of stable cell lines expressing pBC1hEPO, pBC1hEPOwpre, pMARBC1hEPO and pMARBC1hEPOwpre constructs

HC11 cells were grown in RPMI 1640 medium containing 5% fetal calf serum (HyClone, Logan, UT, USA) and 4 mg/ml insulin. They were transfected individually with the pBC1hEPO, pBC1hEPOwpre, pMARBC1hEPO or pMARBC1hEPOwpre constructs containing the neomycin gene (described above) or the pEGFP gene (Promega, Madison, WI, USA), as a positive control, using Effectene (Qiagen, Valencia, CA, USA), respectively. Briefly, HC11 cells were plated at a density of 2×10^5 cells per 35-mm dish in RPMI 1640 medium containing 5% FBS. Transfections were performed according to the manufacturer's instructions. Briefly, 1 μ g of each hEPO expression plasmid in TE buffer, pH 7.4, was diluted with a DNA-concentrating buffer, Buffer EC, to a total volume of 150 μ l, and 8 μ l of Enhancer was then added. The mixture was gently tapped for 5 sec and incubated at room temperature for 5 min. It was then centrifuged for a few seconds, and 5 μ l of Effectene transfection reagent was added and mixed by pipetting up and down five times. After incubation for 10 min at room temperature to allow transfection complexes to form, the transfection complexes were added to 1 ml of growth medium. The growth medium containing the transfection complexes was immediately added drop-wise onto cells in 35-mm dishes. Resistant cells after

G418 treatment were harvested and grown. To promote differentiation, confluent populations of cells were kept for 2 days in RPMI 1640 medium containing 5% fetal calf serum (HyClone), 4 $\mu\text{g}/\text{ml}$ insulin and 1 μM cortisol. Expression of hEPO was induced for 4 days in the same medium supplemented with 5 $\mu\text{g}/\text{ml}$ prolactin. When necessary, cells were kept frozen until use or subjected to Northern or Western assays.

Preparation of fetal fibroblast cells

Fibroblasts were isolated from pig fetuses (Berkshire) on Day 30 of gestation, as previously described [16–21]. Briefly, collected fetuses were washed three times with Ca^{2+} - and Mg^{2+} -free PBS (DPBS, Life Technologies, Gaithersburg, MD, USA). The heads and internal organs were removed using iris scissors and forceps. The remnants were washed twice in DPBS, minced with a surgical blade on a 100-mm culture dish and then dissociated with 0.25% (v/v) trypsin-EDTA (Life Technologies) containing Dulbecco's modified Eagle's medium (DMEM, Life Technologies) at 39 C for 1–2 h. After centrifuging three times at $300 \times g$ for 10 min, cell pellets were subsequently seeded onto 100-mm plastic culture dishes (Becton Dickinson, Lincoln Park, NJ, USA) and cultured for 6–8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 1% (v/v) nonessential amino acids (Life Technologies) and 10 $\mu\text{g}/\text{ml}$ penicillin-streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, subcultured at intervals of 5–7 days by trypsinization for 5 min using 0.1% trypsin and 0.02% EDTA and stored in freezing medium in liquid nitrogen at -192 C after two passages. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich), and 10% (v/v) FBS.

Transfection of donor cells into pig fetal fibroblast cells

For transfections, cells were grown on 24-well plates until 50–70% confluent and were then transfected with the pMARBC1hEPOwpre plasmid at 1 $\mu\text{g}/\text{well}$ complexed with the cationic commercial liposome Geneporter2 (GP; Qiagen), in serum- and antibiotic-free medium. In order to assess the cytotoxic effects of the cationic complexes, untransfected cells were used as a control for each experiment. Liposome was used at the ratio recommended by the supplier (DNA:liposome=1:4, w/w). At the end of a 2-h incubation, the cells were thoroughly rinsed with medium containing serum and further cultured for 2–3 days until confluency; they were then subjected to selection with G418 for 10 days.

Real-time PCR analysis

In order to detect the presence of the hEPO gene, real-time PCR was performed with selective primers in a Peltier Thermal Cycler 200 (MJ Research, Reno, NV, USA). The primers used in this study were designed to detect the pMARBC1hEPOwpre or WPRE genes. The primers used for human EPO cDNA detection were 5'-ATGGGGGTGCACGAATGTCC-3' (forward) and 5'-TCATCTGTCCCCTGTCTGC-3' (reverse). The primers used to detect WPRE gene were 5'-ATGCTGCTTTAATGCCT-3' (forward) and

5'-TGGGAGGTCCGCTGGATTGA-3' (reverse). Approximately 10 cells were directly subjected to PCR under the following conditions: denaturation for 3 min at 94 C; 35 amplification cycles of denaturation at 94 C for 30 sec and annealing at 55 C for 30 sec; and extension at 72 C for 1 min. A final extension step of 10 min at 72 C was performed to complete the PCR reaction.

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and were transported to the laboratory at 25–35C. Antral follicles (4–6 mm in diameter) were aspirated with an 18-gauge needle. Aspirated oocytes that had evenly granulated cytoplasm and were surrounded by at least three uniform layers of compact cumulus cells were selected and washed three times in HEPES-buffered NCSU-37 with 0.1% PVA. Oocytes were cultured for 20 h in four-well plates (Nunc, Roskilde, Denmark) that contained 500 μl of NCSU-37 medium supplemented with 10% porcine follicular fluid, 0.6 mmol/l cysteine, 1 mmol/l dibutyl cyclic adenosine monophosphate (dbcAMP, Sigma) and 0.1 IU/ml human menopausal gonadotropin (hMG, Teikokuzoki, Tokyo, Japan) per well. The oocytes were then cultured without dbcAMP and hMG for another 18–24 h, as previously reported [16].

Nuclear transfer

After thawing, donor cells were cultured in DMEM supplemented with 10% FBS to approximately 70% confluence (passages 7–9). For serum starvation, cells were cultured for 3 days in serum-starved DMEM supplemented with 0.2% FBS prior to scNT. Nuclear transfer was carried out as described previously [7, 16–21]. Briefly, matured eggs with a first polar body were cultured in NCSU-23 medium supplemented with 0.4 mg/ml demecolcine (Sigma) and 0.05 M sucrose for 1 h. The sucrose was used to enlarge the perivitelline spaces of the eggs. Treated eggs with a protruding membrane were transferred to medium supplemented with 5 mg/ml cytochalasin B (CB) and 0.4 mg/ml demecolcine, and the protrusion was removed with a beveled pipette. A single donor cell was injected into the perivitelline space of each egg and electrically fused by using two direct current pulses of 150 V/mm for 50 μsec in 0.28 M mannitol supplemented with 0.1 mM MgSO_4 and 0.01% polyvinyl alcohol. The fused eggs were cultured in medium with 0.4 mg/ml colcemid for 1 h before parthenogenetic activation and were then cultured in 5 mg/ml of CB-supplemented medium for 4 h. The reconstructed oocytes were activated by two direct current pulses of 100 V/mm for 20 msec in 0.28 M mannitol supplemented with 0.1 mM MgSO_4 and 0.05 mM CaCl_2 [16–18]. To obtain simultaneously activated oocytes, a group of oocytes were fused and activated with two 50- μsec pulses of 1.5 kv/cm. The activated eggs were then cultured in NCSU-23 medium for 7 days in an atmosphere of 5% CO_2 and 95% air at 39 C.

In vitro development of cloned embryos

We performed scNT using passage 7–9 donor cells, and the reconstructed embryos were then cultured in NCSU-23 medium for 7 days. Developmental ability and blastocyst formation were examined at the respective time points of cell stages up to 7 days. At the end of the culture period, nuclei in blastocysts were counted

Table 1. Determination of the number of hEPO copies in recombinant pig fibroblast cells by using real-time PCR*

| Cell number selected | EPO primer (relative %) | WPRE primer (relative %) |
|----------------------|---------------------------|-----------------------------|
| #4-1 | 27.59 ± 1.48 ^b | 33.25 ± 2.2 ^c |
| #4-2 | 1.54 ± 0.76 ^c | 1.63 ± 0.9 ^d |
| #4-3 | 1 ^c | 1 ^d |
| #4-4 | 35.16 ± 2.95 ^b | 87.23 ± 4.9 ^c |
| #4-5 | 44.37 ± 4.06 ^b | 115.05 ± 13.01 ^b |
| #4-6 | 97.9 ± 22.06 ^a | 463.7 ± 33.31 ^a |
| #4-7 | 47.6 ± 5.37 ^b | 210.13 ± 0.18 ^b |

* Copy numbers were determined by comparing with reference control (single-copy hEPO retroviral transgene).

after 5 µg/ml Hoechst staining.

Estrus synchronization and embryo transfer

Gilts (Duroc × Yorkshire) that were at least 8 months of age were served as recipients. Estrus synchronization of the recipients was established as reported previously [16–22]. One- or two-cell embryos reconstructed by scNT were then surgically transferred into the oviducts of the synchronized recipients. The pregnancy status of the recipients was determined by ultrasound between days 25 and 30.

Screening of transgenic pigs

Genomic DNA samples were obtained from the pigs' ears, and presence of the transgene was verified by PCR. The primers used were hEPO 5'-GTAGAAGTCTGGCAGGGCCT-3' (forward) and 5'-TCATCTGTCCCCTGTCCTGC-3' (reverse). Identification of transgenic pigs by PCR analysis were reconfirmed by Southern blot analysis. Briefly, 10 µg of genomic DNA was digested with HindIII and SmaI, electrophoresed in a 0.7% agarose gel and transferred onto a nylon membrane. Hybridization was carried out in an aqueous solution containing 6X SSC, 5X Denhardt's reagent and 1% SDS at 68 C using a random-primed full-length hEPO cDNA probe with 5 × 10⁶ cpm/ml activity. Final washes were performed with 1 × SSC containing 0.1% SDS at 65 C.

Analysis of transgene copy number by real-time PCR

Genomic DNA was isolated from cells transfected with hEPO gene using a QIAmp kit (Qiagen). The real time PCR analysis was carried out as previously described using primers specific for sequences located within the hEPO or WPRE region of the vector to determine the copy number [23]. The average copy number of the transgene in the genomic DNA isolated from transfected pig fetal fibroblast cells was determined using an ABI 7900 sequence detection system and TaqMan chemistries (Applied Biosystems, Foster City, CA, USA). In all real-time PCR analyses a single-copy hEPO retroviral transgene containing DNA sample from a cloned pig fibroblast cells was included as reference control.

Fluorescence in situ hybridization (FISH)

Pig blood metaphase chromosome spreads were prepared using a standard protocol for pig chromosomes [24, 25]. Porcine karyotyping was accomplished based on a proposed porcine

chromosome nomenclature [26]. FISH was performed by a standard method [22, 24, 25] using the entire transgene as a probe. Slides hybridized with a fluorescein isothiocyanate (FITC)-labeled probe were washed in 0.4 × SSC at 73 C for 2 min and rinsed in 2 × SSC/0.1% NP-40. Metaphase spreads were counterstained with 0.1 mg/ml propidium iodide. All slides were analyzed by a cooled CCD camera system attached to a fluorescence microscope with filters for FITC and the equipped Smart Capture software (CytoVision Chromofluor System; Applied Imaging, Calsbad, CA, USA).

Statistical analysis

All experimental data were presented as means ± SD. Each experiment was performed at least three times and was subjected to statistical analysis. For statistical analysis (Table 1), one-way analysis of variance (ANOVA) was performed to determine whether there were significant differences among all groups (P<0.05), and Duncan's multiple range procedure was performed to determine the significance of differences between pairs of groups. A P-value below 0.05 was considered significant.

Results

Different hEPO expression vectors express the hEPO gene to a different degree

To look for the highest level of expression among the various expression vectors, we transfected mammary epithelial-derived cells (HC11) with each expression vector (pBC1hEPO, pMARBC1hEPO, pBC1hEPOwpre, and pMARBC1hEPOwpre) individually using Effectene (Qiagen). To obtain a stable cell line expressing the hEPO gene, each transfected cell line was treated with G418. Since the level of human EPO expression in transfected HC11 cells varies from clone to clone, all colonies surviving after G418 treatment were directly examined for their levels of hEPO gene expression (Figs. 2A and B). The pBC1hEPO expression vector did not exhibit a significant hEPO mRNA level, whereas pMARBC1hEPO, pBC1hEPOwpre and pMARBC1hEPOwpre produced approximately 7-fold more hEPO mRNA in HC11 cells than pBC1hEPO. Unlike the hEPO mRNA expression, the highest level of hEPO protein expression in HC11 cells was observed in pMARBC1hEPOwpre compared with the other vectors. Therefore, we selected the pMARBC1hEPOwpre expression vector to test whether it possesses intrinsic hEPO

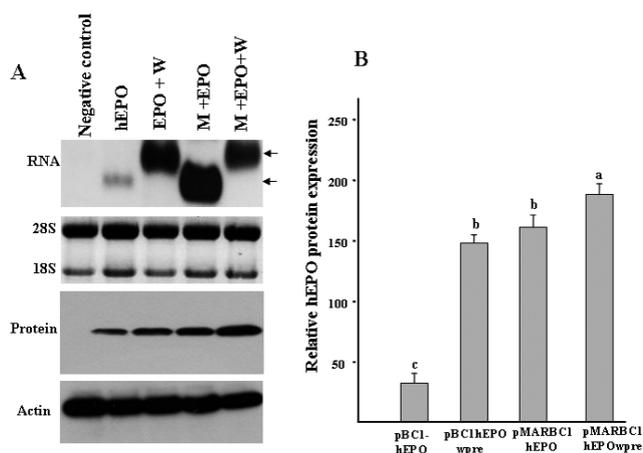


Fig. 2. Effect of hEPO expression in HC11 cells with each expression vector (pBC1hEPO, pMARBC1hEPO, pBC1hEPOwpre and pMARBC1hEPOwpre). (A) Northern and Western blots of hEPO in HC11 cells transfected with each hEPO expression vector. Twenty microgram total RNA and protein were loaded in each well. A positive band of the expected size was detected when full-length hEPO cDNA was used as a probe. (B) Electrophoretic comparison of hEPOs from mammary epithelial cell line HC11. In HC11 cells, WPRE and/or MAR had an additive effect on gene expression. Combination of the two elements (MAR and WPRE) significantly increased the basal level of hEPO expression.

expression *in vivo* for potential utilization as an animal bioreactor.

Real-time PCR and G418 selection can remove nontransfected cells efficiently

It is still unclear whether the higher efficiency of obtaining transgenic animals using somatic cell-mediated gene transfer is related to the high numbers of transfected donor cells or to more efficient removal of the nontransfected cells from the treated cells. To remove nontransfected pig-derived fibroblast cells, transfected cells with the pMARBC1hEPOwpre expression vector were treated with G418 at 10 days post-transfection. Colonies which appeared to be derived from single cells after G418 treatment were selected and transferred into 100 wells. These colonies were cultured in the absence of G418 and then replated. A few cells from each passage were directly subjected to real-time PCR to confirm the presence of hEPO. As shown in Fig. 3, transfected somatic cells were classified into four different patterns by real-time PCR. Pattern #1 showed a slight increase in hEPO DNA content in passage 9 (9p), after a significant decrease in passage 6 (6p), but the DNA content was still less than that at passage 3 (3p). Pattern #2 maintained low hEPO DNA content in 6p and 9p compared with that in 3p. Pattern #3 showed more significant fluctuation in hEPO DNA content following passages, which dramatically increased in 6p and then decreased in 9p. Ideal enrichment of recombinant cells was found in pattern #4, which was found in seven out of the 30 wells examined (23.3%); this pattern showed a significant increase in hEPO DNA content in a manner correlating with increase in the number of cell passages. These results suggest that pattern #4 fetal fibro-

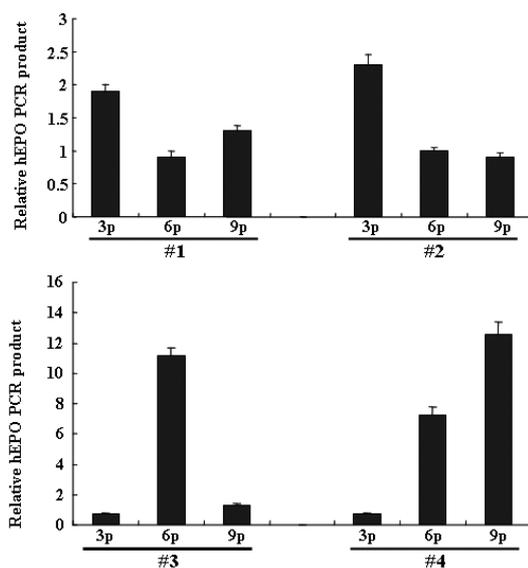


Fig. 3. Real-time PCR screening of hEPO recombinant cells after G418 treatment. Pig ear-derived fibroblast cells were transfected with a plasmid (pMARBC1hEPOwpre) that expressed the hEPO gene using liposome Geneporter2 (GP) (Qiagen). A single cell-derived colony was isolated after G418 treatment and then cultured, and a few cells were also directly subjected to real-time PCR to examine them for the presence of the hEPO gene. The resultant cells are classified into 4 patterns by real-time PCR. In patterns #2 and 3, the hEPO DNA contents were ultimately decreased in proportion to cell passage (p); however, pattern #1 showed an increase in hEPO DNA content (9p) after a slight decrease (6p). Ideal enrichment of recombinant cells was found in pattern #4, which showed an increase in hEPO DNA content in a manner correlating with increasing number of cell passages. The number of cell passages *in vitro* are indicated as 3p, 6p and 9p.

blast cells stably integrated the pMARBC1hEPOwpre expression vector into their chromosomes.

Previous reports have shown that, in transgenic mice, transgenes that integrated in a high copy number tended to be methylated and thus not expressed [27]. To select cells containing a low copy number of the pMARBC1hEPOwpre expression vector, 10 cells from the seven wells that showed pattern #4 were subjected to real-time PCR, and the number of copies was calculated (Table 1). Numbers 4–2 and 4–3 among the seven wells were estimated to harbor one copy per diploid, whereas the other five wells were estimated to harbor 27.59 ± 1.48 , 35.16 ± 2.95 , 44.37 ± 4.06 , 97.9 ± 22.06 and 47.6 ± 5.37 copies for the hEPO primer sets or 33.25 ± 2.2 , 87.23 ± 4.9 , 115.05 ± 13.01 , 463.7 ± 33.31 and 210.13 ± 0.18 copies for the WPRE primer sets, respectively, confirming the data obtained by real-time PCR analysis. Although the copy numbers calculated using the EPO or WPRE primer sets are slightly different, they can be divided into three statistical groups based on the EPO primer; donor cells with approximately 1–2 (group I), 27–48 (group II) or 49–98 (group III) copies were subjected to scNT for production of transgenic founder pigs.

To determine whether hEPO DNA copy numbers resulted in

Table 2. *In vitro* developmental competence of porcine preimplantation embryos reconstructed by somatic cells harboring hEPO gene under the control of goat beta casein promoter

| Copy numbers | No. of embryos | No. of embryos (%) | | | Molura | No. of blastocysts | No. of cells in blastocyst | No. of apoptotic cells |
|----------------------|----------------|--------------------|---------------|---------------|--------------|------------------------|----------------------------|------------------------|
| | | 2-cells | 4-cells | 8-cells | | | | |
| 1–2 (Group I) | 180 | 134 (74.4) | 114 (63.3) | 74 (41.1) | 44 (23.8) | 25 (14.4) ^a | 26.8 ± 2.2 ^a | 3.0 ± 1.1 |
| 27–48 (Group II) | 320 | 238 (74.3) | 199 (62.1) | 137 (42.8) | 64 (20.0) | 47 (14.6) ^a | 26.5 ± 2.7 ^a | 2.8 ± 1.1 |
| 49–98 (Group III) | 320 | 242 (75.0) | 212 (66.2) | 125 (39.0) | 63 (19.6) | 34 (10.6) ^a | 23.8 ± 2.3 ^b | 3.2 ± 1.2 |

^{a,b} Percentages with different superscripts within a column are significantly different ($P < 0.05$).

Table 3. Efficacy of transgenic pig production according to selection method for transfected cells *in vitro*

| Cell selection methods | No. of embryos transferred | Recipients | | No. of cloned piglets | No. of transgenic piglets |
|------------------------|----------------------------|--------------|------------|-----------------------|---------------------------|
| | | Pregnant | Delivered | | |
| PCR + G418 | 1,521 | 3/10 (30.0%) | 3/3 (100%) | 9 | 9/9 (100%) |
| G418 alone | 1,440 | 3/8 (33.0%) | 2/3 (67%) | 6 | 2/6 (33%) |

developmental damage of early preimplantation embryos, we analyzed the developmental potentials of scNT preimplantation embryos reconstructed using donor cells from groups I, II and III (Table 2). After electroactivation and cultivation in four-well dishes, 74.4.0% (134/180), 74.3% (238/320) and 75% (242/320) of the oocytes in groups I, II and III underwent cleavage on Day 2. When cells were cultured for 7 days, the cell numbers (23.8 ± 2.3) of the porcine scNT blastocysts were statistically lower in group III compared with those in groups I (26.8 ± 2.2 cells) and II (26.5 ± 2.7 cells), respectively. In addition, no significant changes were observed in either the development rates to the blastocyst stage or the apoptotic cell index (Table 2).

The combination of real-time PCR and G418 selection can increase transgenic efficacy

One- to four-cell-stage embryos reconstructed with donor cells selected by the combination of real-time PCR and G418 methods or G418 selection alone were surgically transferred to the oviducts of ten and eight recipient gilts, respectively. In the case of real-time PCR selection after G418 selection, three pregnant gilts among the 10 recipients developed to term and naturally delivered nine piglets (Table 3): One recipient produced two live piglets, another produced two live piglets and one stillbirth and the other produced three live piglets and one stillbirth. The average birth weight of the cloned live piglets was 1.2 kg (0.76–1.45 kg). In the case of G418 selection alone, three of the eight recipients became pregnant. Among them, two recipients gave birth to six piglets, including two stillbirths. When genomic DNA extracted from the scNT-derived piglets was subjected to Southern blot analysis, 33.3% (2/6) of those derived from G418 selection alone and 100% (9/9) of those derived from the combination of real-time PCR and G418 selection were transgenic, respectively (Table 3). The resultant transgenic pigs had approximately 1–40 copies of the exogenous hEPO gene, respectively (Fig. 4B). The chromosomal localization of the hEPO

gene was then determined by fluorescence *in situ* hybridization (FISH) using full hEPO genomic DNA as a probe; positive signals were detected in chromosomes 1q2.4, 1p2.3 and 6q2.4, respectively (Figs. 4C and D).

Discussion

The present study shows that appropriate donor cells for production of transgenic scNT pigs can be efficiently selected by the combination of G418 and real-time PCR selection methods. Of the 30 wells examined, approximately seven showed increased hEPO DNA content in a manner corresponding to a doubling of the cell population, indicating that the exogenous expression vectors had been stably integrated into the somatic cells' genome. As a result, approximately 94% (66/70) of the preimplantation embryos selected by using both G418 and real-time PCR were transgenic, whereas only 17% (14/84) of the preimplantation embryos were transgenic when donor cells were selected using G418 alone (data not shown). A previous study reported that efficiencies of transfection of fetal fibroblast cells with electroporation or lipofection are approximately 3–53% and 3–8%, respectively [28]; when porcine nuclear transfer (NT) was performed using G418 resistant cells as the donor cell, only 30–50% of the embryos were transgenic. For this reason, it has been suggested by many groups that somatic cells are often resistant to antibiotic treatment and that selection of transgenic cells using G418 alone is not sufficient to completely remove nontransgenic cells in culture [4, 5, 7]. Considering the definitive lifespan of primary fibroblast cells, strategies such as G418 selection may have limited merit for creation of scNT-derived transgenic animals.

The DNA constructs utilized in these experiments were designed to express the hEPO gene in the mammary gland and contained a geneticine (G418)-resistance gene for selection. When six scNT-derived piglets derived from G418 selection alone were analyzed,

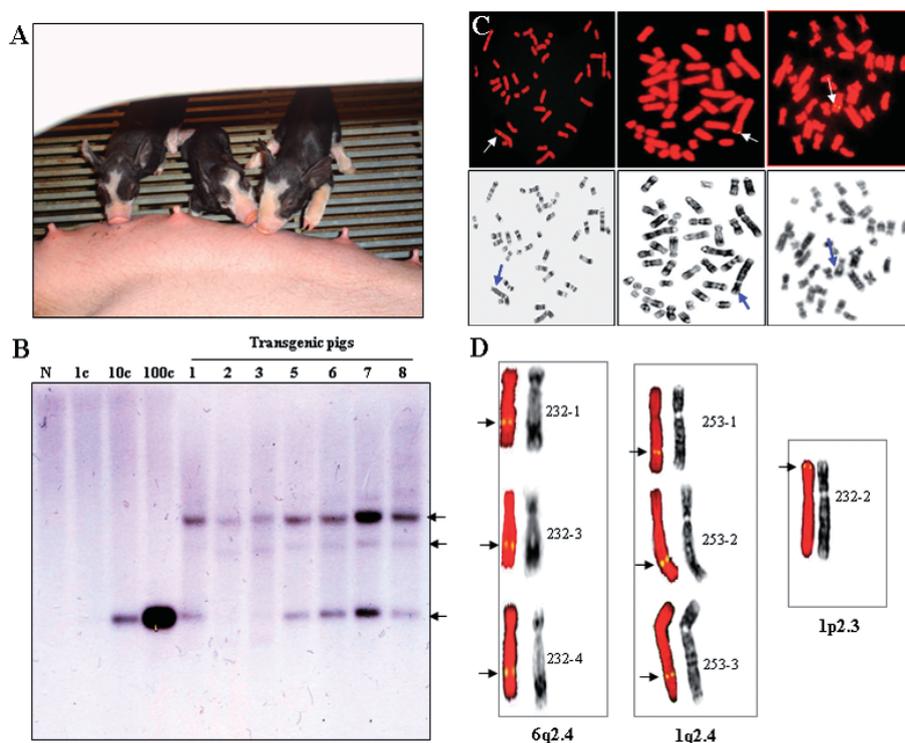


Fig. 4. Identification and localization of the hEPO gene in transgenic pigs. (A) Photo of a transgenic piglet taken at 4 day of age. (B) Transgenic pigs were identified by Southern blotting analysis. High molecular weight DNA extracted from the ear tissue of the transgenic piglets was digested with HindIII/SmaI and then transferred onto a membrane. Three positive bands (arrows) were detected when full-length human erythropoietin (hEPO) cDNA was used as a probe. N, 1c, 10c and 100c indicate the negative controls and 1, 10 and 100 copies, as a positive control, respectively. Copy numbers in transgenic pigs were determined by comparing with the 10c positive control. (C) The localization of the hEPO gene in the pig chromosomes is shown by arrows. (D) The integration sites of the hEPO gene are shown by arrows.

only two were transgenic. Previous studies have suggested that one explanation for the failure to detect an antibiotic-resistant gene after selection is the so called 'by-stander effect' [4, 7]; that is, expression of an antibiotic resistance gene in some cells could confer antibiotic resistance to nearby cells either by direct contact between cells or by secretion of the gene product into the medium. However, Watanabe *et al.* [6] have reported that puromycin can be used for selection of recombinant cells from non-cultured cells. The difference between the two groups' observations was the donor cells used; Betthausen *et al.* [7] used crossbred (Yorkshire/Landrace/ Berkshire cross)-derived donor cells, whereas Watanabe *et al.* [6] used Landrace-derived donor cells. We used adult ear-derived fibroblast cells from a gnotobiotic miniature pig and four commercial pigs, including three inbred (Duroc, Landrace and Berkshire) pigs and one crossbred pig (Duroc \times Landrace \times Berkshire). Among them, most of the spontaneously resistant cells after G418 treatment were obtained from commercially available crossbred pigs, and moderately resistant cells were obtained from inbred Duroc, Landrace and Berkshire pigs. However, all gnotobiotic miniature pig-derived fibroblast cells died 2 wk after G418 treatment (data not shown). This observation suggested that the

occurrence of spontaneously resistant cells after G418 treatment may be pig strain-specific. Most of the commercially available pigs were fed various antibiotics in their diet and were exposed to various diseases. Therefore, these pigs may have been subjected to antibiotic abuse, which may have promoted accumulation of antibiotics in their bodies. It is therefore possible that fibroblast cells derived from these pigs resulted in spontaneous resistance after G418 treatment.

To utilize a transgenic cell as a vector for gene transfer, we focused on the possibility of producing transgenic pigs with a low copy number of the hEPO gene. Previous reports have shown that, in transgenic mice, transgenes integrated at a high copy number tend to be methylated and silenced [27, 29]. It has recently reported that a transgene integrated at 200 copies can be expressed in transfected cells [30]. Therefore, to produce transgenic animals with the highest levels of transgene expression, it is necessary to pre-screen for a low copy number of the exogenous DNA in donor cells. Although the copy number estimates using real-time PCR and Southern blotting analysis were slightly different, our observations indicated that it is possible to manipulate cultured cells genetically *in vitro* and to screen recombinant cells with modest

gene expression. In the present study, we also tried to investigate the relationship between the copy number of hEPO in the cloned pig and hEPO expression in milk. However, all transgenic piglets died within 1 week after birth, except for one that grew up to adulthood but ultimately died on gestation day 60. Therefore, the relationship between copy number of transgene in cloned pigs and hEPO expression requires further study.

As the chromosomal location of integrated transgenes has been shown to influence their expression as a result of position effects [31, 32], we were interested in determining the chromosomal integration site of the pMARBC1hEPOwpre transgene. DNA FISH showed that there is a single integration site in a euchromatic region of chromosomes 1p2.3, 1q2.4, and 6q2.4 in each of the three transgenic pigs, respectively. We also tried to map the precise position of transgene insertion by chromosome walking. However, it is difficult to map the precise position of transgene insertions since a complete swine genome sequence has not yet been reported. In addition, we met some problems in carrying out our chromosome walking experiment. Although PCR for downstream DNA worked very well, PCR for upstream sequences did not work well, for an unknown reason. Therefore, the precise position of hEPO gene integration in the swine genome could not be mapped. In addition, no sequence homology was found for the swine sequence within the human or mouse genome sequences.

Biologically active rhEPO has been successfully expressed in the mammary glands of several transgenic animals, including rabbits and mice [33–38]. However, a number of deleterious side effects have been observed in rhEPO-transgenic animals, including premature death, polycythemia, splenomegaly and hepatomegaly [39, 40]. Furthermore, recent transgenic animals have not been proved to be useful for commercial production of rhEPO due to the low yield of animal milk [33, 37, 38]. The rate of development to term reported here is similar to those of other published studies for porcine somatic cell nuclear transfer [4, 7, 22]. In the present study, however, all transgenic piglets died within 1 week after birth, except for one that grew up to adulthood but ultimately died on gestational day 60. Although the reason for post-natal early death of hEPO transgenic pigs was not determined in the present study, the deleterious side effects observed in transgenic animals may be partially due to ectopic expression of transgene. Even if we used a hEPO expression vector containing goat beta casein promoter to induce exclusive expression in the mammary gland, goat beta casein promoter may not be an appropriate promoter system to induce specific hEPO expression in the pig mammary gland. Previously, we reported that goat beta casein promoter system could induce ectopic expression of rhEPO in the spleen, resulting in phenotypic disorder (splenomegaly) in transgenic mouse [13]. This suggests that ectopic expression or leakage of rhEPO from the mammary gland to the blood in transgenic pigs may produce deleterious side effects, leading to post-natal early death. Currently, we are attempting to overcome the side effects of ectopic expression of transgene by using either a pig-specific beta casein promoter system or a knock-in system of the hEPO gene within a pig casein gene and by testing for the possibility of selecting recombinant somatic cells with the hEPO knock-in system in beta- or α S1-casein loci by a combination of real-time PCR and G418 selection.

Taken together, this work shows that the combination of real-time PCR and G418 selection can be used for rapid selection of appropriate recombinant donor cells from *in vitro* transfected cells and that somatic cell-mediated gene transfer can be successfully employed for production of transgenic livestock.

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