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Development of porcine transgenic nuclear-transferred embryos derived from fibroblast cells transfected by the novel technique of nucleofection or standard lipofection

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Abstract

The aim of our study was to determine the in vitro developmental potential of porcine nuclear-transferred (NT) embryos that had been reconstructed with *Tg*(*pWAPhGH-GFPBsd*) transgene-expressing fibroblast cells. The gene construct was introduced into fibroblast cells by the novel method of nucleofection or standard lipofection. NT oocytes derived from foetal and adult dermal fibroblast cells were stimulated by either simultaneous fusion and electrical activation (Groups IA and IB) or sequential electrical and chemical activation (Groups IIA and IIB). The percentages of cloned embryos that reached the morula and blastocyst stages were 152/254 (59.8%) and 77/254 (30.3%) or 139/276 (50.4%) and 45/276 (16.3%) in Groups IA or IB, respectively. The rates of NT embryos that developed to the morula and blastocyst stages were 103/179 (57.5%) and 41/179 (22.9%) or 84/193 (43.5%) and 27/193 (14.0%) in Groups IIA and IIB, respectively. In conclusion, the in vitro developmental competences of porcine transgenic NT embryos that had been reconstructed with the *Tg*(*pWAPhGH-GFPBsd*) gene-transfected fibroblast cells were relatively high. Further, the nucleofection efficiency of all the porcine fibroblast cell lines as estimated by intra-vitam fluorescent evaluation based on the index of reporter eGFP transgene expression was nearly 100%. However, PCR analysis for transgene screening confirmed the absence of *Tg*(*pWAPhGH-GFPBsd*) fusion gene in some of the nucleofected cell lines. To our knowledge, the novel method of nucleofection is the first to transfect nuclear donor cells in the production of transgenic cloned embryos.

Keywords: Porcine; Fibroblast cell nucleofection; Nuclear transfer; Oocyte activation; Transgenic embryo

1. Introduction

Swine embryo engineering (somatic cell cloning combined with transgenesis) is a particularly important research field within assisted reproduction technologies, and may relate to both xenotransplantology [1,2] and the creation of animal bioreactors for the production of biopharmaceuticals [3,4]. Nonetheless, the overall efficiency involving the generation of viable transgenic embryos and/or offspring with the aid of somatic cell cloning remains relatively low.

A significant aspect of applying somatic cell nuclear transfer (SCNT) technology to generate transgenic embryos and/or offspring is the origin/type of nuclear donor cells and the in vitro techniques utilized to obtain

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viable donor nuclei for cloning procedures. Genetically transformed cells can be selected in vitro, and only the cells with stable, integrated transgenes are used as a source of nuclear donor cells to reconstruct the enucleated oocytes through somatic cell cloning. For the establishment of transgenic cell lines, various selection markers with different actions and advantages have been employed, including either antibiotics such as neomycin/geneticin G418 [5–7], puromycin [8] and blasticidin S [9], or enhanced green fluorescent protein (eGFP) [10–12]. Although antibiotics have been used successfully in generating genetically modified cells, antibiotics induce cellular damage, a shortening of the life-span of cultured somatic cells, a diminishment of the frequency of cell population doublings, replicative senescence and chromosomal abnormalities after the long-term selection of somatic cells ranging from 8 to 14 days of in vitro culture [5,8,13,14]. Since it was first introduced by Chalfie et al. [15], the eGFP-mediated genetic reporter system, which was derived from the biochemiluminescent jellyfish *Aequorea victoria*, is now emerging and provides us with a valuable xenogeneic selection marker because of its expression in a broad range of organisms and its lack of reported obvious adverse biological (i.e., cytotoxic) effects [12,16–18]. So far, the 238 amino acid residue eGFP protein has been applied for various purposes as a useful marker among others for the vital monitoring of the efficiency of somatic cell transfection and selection of in vitro cultured transgenic cells [19–21]. It has already been used for visual transgene screening and the controlling/assessment of its expression in situ in the preimplantation embryos of different mammalian species [22–25]. Although it has been shown in several studies that eGFP transgene overexpression determined by the brightest eGFP protein-derived fluorescence emission may reduce to a very limited degree the viability of transfected cells, no deleterious effects of eGFP at faint-moderate expression levels on the development of SCNT-derived transgenic embryos to the blastocyst stage have been reported [10,16,26,27].

In mice and cattle, eGFP reporter vectors have been effectively used to select transgenic embryos produced by intrapronuclear microinjection [16,18,24,28]. Furthermore, intra-vitam eGFP-mediated selection of nuclear donor cells has been applied to generate transgenic embryos and/or offspring by SCNT in mice [21], cattle [17,19,29,30], sheep [31,32] and goats [14]. Finally, porcine eGFP transgene-expressing blastocysts and/or offspring have been created from cultured fibroblast cells that were most often transfected with gene constructs (fusion genes) by replication-defective viral vectors or liposome carriers [4,25,33,34]. But to our knowledge, the production of porcine cloned embryos using somatic cells undergoing the novel method of nucleofection has not yet been reported. The nucleofection strategy – a combination of lipofection and electroporation – enables targeted transduction of the gene construct directly into the nucleus of the somatic cell, mediated through liposome carriers. Moreover, compared to the standard transfection methods for somatic cells, such as lipofection or electroporation, the nucleofection technique allows a considerable shortening of the time needed to verify transgenesis efficiency. This verification is performed through live-eGFP reporter gene expression controlling, from 24 to 48 h and up to even 4–6 h after the transfection procedure [35,36].

This is also the first report in which the effect of different protocols of oocyte artificial activation on both the preimplantation development of porcine transgenic cloned embryos and eGFP expression rates and patterns in the blastocysts generated were determined. This determination was depended on the type of lipofected or nucleofected nuclear donor fibroblast cells. To date, there are few studies focused on applying eGFP transgenic cells to porcine SCNT technology. Among them, foetal fibroblast cells have been used for the generation of genetically transformed embryos/offspring because of their rapid growth rate and potential for multiple mitotic cell divisions before replicative senescence under in vitro culture conditions [4,37–39]. In turn, among adult somatic cells, only ear skin-derived fibroblast cells have been utilized in the production of porcine eGFP gene-expressing cloned embryos and/or offspring [3,23,39,40]. However, none of the studies on swine somatic cell cloning have compared the preimplantation developmental capability and intra-vitam eGFP expression profiles between nuclear-transferred (NT) embryos derived from oocytes reconstituted with different types of transgenic fibroblast cells and stimulated by using different activation treatments. Therefore, in this study we investigated the effect of oocyte activation protocols on the in vitro developmental abilities of porcine cloned embryos that had been reconstituted with *Tg(pWAPhGH-GFPBsd)* gene-transfected foetal and adult dermal fibroblast cell nuclei.

2. Materials and methods

2.1. Preparation of the Tg(pWAPhGH-GFPBsd) gene construct

The PCR primers (TibMolBiol) were designed on the basis of human growth hormone gene (Accession

M13438) and rat whey acidic protein (WAP) promoter (Accession X01153) sequences available in GenBank at NCBI. In the first step, the rat WAP promoter (987 bp) was amplified by using the genomic sequence as a template. The PCR products were digested with EcoRI and EcoRV and cloned into pBluescript SK+ plasmid vector (Stratagene). The 5′ end of the insert was modified by the addition of an EcoRI restriction site. The 3′ end contained a NcoI restriction site. In this way, the ATG codon that was responsible for initiation of translation process was introduced. In the next step, human genomic DNA, which had been isolated from peripheral blood lymphocytes, was used as a template for growth hormone gene (1563 bp) amplification. The 5′ end of the gene was modified by the addition of a NcoI restriction site. The 3′ end of the gene was modified by the addition of a HindIII restriction site. PCR products were digested with restriction enzymes (NcoI and HindIII), and ligated with the vector containing the rat WAP promoter within the NcoI and HindIII restriction sites. In the final step, the sequence consisting of the rat WAP promoter and human growth hormone gene was digested with EcoRI and HindIII and recloned into 6.0 kb of pTracer-EF/Bsd A plasmid vector (Invitrogen). The vector contained a cyclic 3-GFP, an enhanced GFP gene for non-invasive in vivo detection. The eGFP gene was fused to the blasticidin S resistance gene for selection in mammalian cells under the human cytomegalovirus (CMV) immediate-early promoter. The nucleotide sequence of the gene construct was determined using a cycling sequencing kit and ALFExpress sequencer (Pharmacia Biotech).

2.2. Establishment and transfection of porcine fibroblast cell lines

Foetal fibroblast cells were isolated from conceptuses (9.0 cm in length), following the removal of their heads and internal organs. The foetuses were obtained from a slaughterhouse; therefore, the age and breed of the foetuses were unknown. Adult fibroblast cells were collected from an ear skin biopsy obtained from 6-month-old gilt. Foetal body-retrieved or adult dermal tissue samples were cut into small pieces using a tissue chopper (0.5 mm), and tissue explants were placed in a culture flask with a small volume of Dulbecco's Modified Eagle Medium (DMEM, Gibco Invitrogen Co., UK). This volume was enough to wet the bottom of the flask, but not too wet so as to cause the tissue pieces to float. For the first 2–3 days of incubation, a few more drops of medium were added every 2–3 h, and then gradually more medium was added when pieces had firmly attached. Cultures were replenished 2–3 times per week. For the primary cultures of dermal fibroblasts, modified Dulbecco's Minimum Essential Medium, which had been supplemented with 10% foetal bovine serum (FBS, Sigma, St. Louis, MO), 2 mM non-essential amino acids (NEAA, Sigma), 2 mM L-glutamine (Sigma), 0.36 mM sodium pyruvate (Sigma) and 1% antibiotic–antimycotic solution (Sigma), was used. After removal of the explants (Days 5–6), monolayers of fibroblast cells were harvested using DMEM supplemented with 0.25% trypsin–EDTA (Sigma). Cells were subsequently cultured up to a total confluency, and then passaged at least three times. Cells harvested from flasks by trypsinization were washed in 10 mL HEPES-buffered Tissue Culture Medium 199 (TCM 199-HEPES, Sigma) with 10% FBS and centrifuged at $200 \times g$ for 10 min. The cell pellet was then suspended in FBS containing 9% dimethyl sulfoxide (DMSO, Sigma) before freezing in a Minicool freezer. Cryopreserved donor cells were thawed at 37 °C and 200 μL of FBS was added. The suspension was kept at room temperature for 10 min, and then 800 μL of cell culture medium was added before the cells were centrifuged at $300 \times g$ for 5 min. The supernatant was removed, and 50 μL of manipulation medium was added. Frozen foetal or adult dermal fibroblast cell lines were thawed and cultured in vitro up to 95%-confluency state (in the medium supplemented with 10% FBS) prior to their transfection.

The subconfluent adherent fibroblast cell lines were subjected to one of two methods of transfection: either nucleofection or lipofection. For nucleofection, the *Tg*(*pWAPhGH-GFPBsd*) gene construct was purified by using the UltraMobius Plasmid Kit (Novagen), digested to linear form with SacI and used for transfection by the nucleofection method. The nucleofection samples consisting of $0.5–1 \times 10^6$ foetal fibroblast cells or $4–5 \times 10^5$ adult ear skin-derived fibroblast cells per 100 μL Dermal Fibroblast Nucleofector™ Solution (Amaxa Biosystems) combined with 3.5 μg of highly purified linear DNA in 2 μL of Tris/HCl–EDTA buffer were transferred into amaxa certified cuvettes. The cuvettes were inserted into the Nucleofector™ apparatus, in which AC pulses were generated using the U-20 or U-23 program for high transfection efficiency of foetal or adult dermal fibroblast cells, respectively. Positively selected transgenic fibroblast cells, which had been evaluated by live-eGFP fluorescence excitation, were in vitro cultured up to a total confluency and then used for somatic cell cloning.

In the case of lipofection, 8 μL (8 μg) of *Tg(pWAPhGH-GFPBsd)* plasmid and 20 μL of Lipofectamine 2000 (Gibco BRL) were separately diluted with 500 μL FBS-deprived DMEM per each transfection sample. After 5 min of equilibration, both the prepared solutions were mixed to a final volume of 1028 μL and incubated together for 20 min to enable the plasmid DNA and Lipofectamine 2000 molecules to be associated. Afterwards, the DNA–Lipofectamine 2000 mixture was added into 2 mL of cell culture medium. The somatic cells were incubated for 24 h at 37 °C in an atmosphere of 5% CO_2, and the medium was replaced for the remainder of the incubation. Two days later, the culture medium was supplemented with 5 μg mL^{-1} blasticidin S (Invitrogen) to select for stable and permanent cell lines of positively transfected fibroblast cells. Following 8-day antibiotic-mediated selection, the generated clones were expanded and frozen in liquid nitrogen. The efficiency of cell transgenesis was verified by both visual assessment of eGFP-reporter transgene expression under blue light as well as by molecular screening of the whole *Tg(pWAPhGH-GFPBsd)* gene construct. Before use for SCNT, the cryopreserved transgenic clonal cell lines were thawed and then cultured up to a total confluency state in order to synchronize their mitotic cycle at the G1/G0 stages through contact inhibition of their migration and proliferative growth.

2.3. Screening for presence of the Tg(pWAPhGH-GFPBsd) transgene

The screening procedure for the presence of the *Tg(pWAPhGH-GFPBsd)* transgene involved the isolation of genomic DNA from porcine foetal and adult ear skin-derived fibroblast cells and the amplification of two PCR-fragments encompassing the promoter-gene junction. Forward primers were located in the WAP promoter region and reverse primers in the region coding the growth hormone. The PCR product of 313 bp was amplified with WHGH2F (5′-AGTCTTCCTCCT-GTGGGTC-3′) and WHGH2R (5′-TCTCTCTCCATC-CCTCCAG-3′) primers, whereas the 524 bp fragment was obtained with WHGH1F (5′-GTCCCAACCCAAC-CATTC-3′) and WHGH1R (5′-TGGCGATACTCA-CATTCAGA-3′) primers. The PCR reaction mixture contained: 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 7.5 pM of each primer and 0.7 U of Taq DNA polymerase (Sigma) in a final volume of 20 μL. The PCR reaction was performed using the following conditions—denaturation: 94 °C, 45 s; annealing: 55 °C, 45 s; synthesis: 72 °C, 90 s; 30 cycles. The PCR products were fractionated on a 1.5% agarose gel.

2.4. Fluorescent in situ hybridization (FISH) analysis

In vitro cultured fibroblast cells, which had undergone contact inhibition of their migration and proliferative growth after reaching the total confluency state, were collected for FISH probing by harvesting with DMEM supplemented with 0.1% trypsin (Sigma) and 0.2% EDTA (Sigma). The cells were then suspended in a hypotonic solution (0.075 M KCl) and fixed with a cold acetic acid/methanol solution (ratio 1:3). The *Tg(pWAPhGH-GFPBsd)* gene construct was used as a molecular probe for FISH after a labeling reaction with biotin-16-dUTP (Roche). The slides with interphase cell nuclei were treated with RNase and pepsin, and then dehydrated in an ethanol series and denatured by an incubation in 70% formamide in 2× SSC solution at 70 °C for 3 min. After dehydratation in the ethanol serial dilutions, the denatured probe was applied onto slides, covered with cover slip and incubated overnight at 37 °C. The next day, post-hybridization washes were performed and the signal was detected using a combined layer of FITC–avidin, goat anti-avidin and FITC–avidin antibodies (Vector Laboratories). Slides were stained in DABCO/DAPI (Vector Laboratories) and analyzed using a fluorescence microscope (Zeiss, Axiovert 200).

2.5. Oocyte collection and in vitro maturation

Slaughterhouse ovaries were collected from prepubertal and postpubertal gilts and sows. Cumulus–oocyte complexes (COCs) were recovered by aspiration of follicular fluid from 2- to 6-mm antral ovarian follicles. The COCs, with evenly granulated ooplasm and several uniform layers of compact cumulus cells, were washed three times in HEPES-buffered Tissue Culture Medium 199 (TCM 199-HEPES; Gibco BRL, Life Technologies Inc., Grand Island, NY) with the addition of 4 mg mL^{-1} bovine serum albumin (fraction V; BSA-V). The COCs were selected for in vitro maturation under atmospheric conditions. The maturation medium comprised 25 mM HEPES and 26 mM sodium bicarbonate-buffered TC 199 medium (Gibco BRL, Grand Island, NY), supplemented with 10% porcine follicular fluid (pFF), 0.6 mM L-cysteine, 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP) and 0.1 IU mL^{-1} human

menopausal gonadotropin (hMG). Approximately 50–60 COCs were cultured in the db-cAMP- and hMG-supplemented medium for 20 h at 39 °C in a 100% water-saturated atmosphere of 5% CO_2 and 95% air. The oocytes were then cultured for 22–24 h in fresh maturation medium that did not contain db-cAMP and hMG. After maturation, expanded cumulus cells and corona cells were completely removed by vigorous pipetting of the COCs in the presence of 0.1% hyaluronidase in 500 μL of HEPES-buffered TCM 199 for 1–2 min. The metaphase II-staged oocytes, which had been selected on the basis of accepted morphological criteria involving evenly granulated, dark ooplasm and the presence of distinctly expelled first polar bodies, provided a source of recipient cells for exogenous cell nuclei in the somatic cell cloning procedure.

2.6. Production of nuclear-transferred porcine embryos

Cumulus-denuded oocytes were incubated in the maturation medium supplemented with 0.4 μg mL^{-1} demecolcine (DMCC) and 0.05 mM sucrose for 1 h at 39 °C. Afterwards, the treated oocytes were transferred into a glass micromanipulator chamber filled with TCM 199 containing 4 mg mL^{-1} BSA-V, 5 μg mL^{-1} cytochalasin B (CB) and 0.4 μg mL^{-1} DMCC. Maternal chromosomes (metaphase plates), which had been allocated into the chemically induced protrusion of the plasma membrane, were removed microsurgically. Enucleation was accomplished by gently aspirating the ooplasmic cone, which contained the condensed chromosome mass, with the aid of a beveled micropipette of 20–25 μm external diameter. Following enucleation, the resulting cytoplasts were washed extensively in HEPES-buffered TCM 199/BSA and held in this CB- and DMCC-free medium until microinjection of donor nuclei. Reconstruction of enucleated oocytes was achieved by electrofusion of whole (foetal or adult dermal) fibroblast cells with ooplasts. Single nuclear donor cells were inserted into the perivitelline space of previously enucleated oocytes. The resulting somatic cell-ooplast couplets were transferred to a fusion/activation chamber filled with electroporation medium. Nuclear transfer-derived oocytes were artificially stimulated with the use of one of the two experimental protocols: simultaneous fusion and electrical activation (SF–EA) or sequential (combined) electrical and chemical activation. In the first activation protocol, the complexes of enucleated oocytes and fibroblast cells were simultaneously fused and activated by application of two successive DC pulses of 1.2 kV cm^{-1} for 60 μs, which were delivered with the use of a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). The fusion/activation medium consisted of 0.3 M mannitol supplemented with 1.0 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.2 mg mL^{-1} fatty acid free BSA (FAF-BSA). In turn, the second activation protocol included the SF–EA followed by an additional treatment of the reconstituted oocytes with chemical factors (ionomycin and cycloheximide). The SF–EA were induced using the DC pulses of the same technical parameters as for the first activation protocol. Nevertheless, the process of SF–EA occurred in the dielectric solution with a standard level of extracellular calcium cations, i.e., with the minimal threshold dose of 50.0 μM $CaCl_2$. Two-grade chemical activation of clonal cybrids was initiated after a 1.5–2-h delay. The nuclear–cytoplasmic hybrids were exposed to 15 μM calcium ionomycin in NCSU-23 medium for 5–7 min, and were then washed three times in 500 μL of NCSU-23 medium containing 0.4% BSA. The hybrids were then incubated in 10 μg mL^{-1} cycloheximide (CHXM) and 5 μg mL^{-1} cytochalasin B for 1.5–2 h at 39 °C in humidified air with 5% CO_2 in NCSU-23 medium supplemented with 4 mg mL^{-1} BSA. Afterwards, reconstituted zygotes were transferred to the same medium supplemented only with 10 μg mL^{-1} CHXM for an additional 1–1.5 h.

2.7. In vitro culture of reconstructed embryos

Cloned embryos were cultured in 50-μL droplets of NCSU-23 medium supplemented with 4 mg mL^{-1} BSA-V that had been overlaid with light mineral oil. The number of embryos per droplet of culture medium ranged from 20 to 30. After 72–96 h of in vitro culture, cleaved embryos were transferred into a 50-μL drop of NCSU-23/BSA medium supplemented with 10% FBS for an additional 72 h. The reconstructed embryos were incubated at 39 °C in a 100% water-saturated atmosphere of 5% CO_2 and 95% air. At the end of the in vitro culture period (Days 6–7), embryos were evaluated morphologically for morula/blastocyst formation rates.

2.8. Visual monitoring of eGFP expression in transgenic cloned blastocysts

Assessment of transcriptional and translational activity for eGFP transgene in all the blastocysts that had originated from NT embryos reconstructed with eGFP transgene-expressing foetal or adult ear skin-derived fibroblast cells was carried out in the dark on an

epi-fluorescent microscope (Olympus IMT-2, Tokyo, Japan). Blue light (λ_{max} = 488 nm) was used to excite the recombinant xenogeneic eGFP molecules that were synthesized and accumulated in the cytoplasm of inner cell mass and trophectoderm cells. The embryos that emitted a bright green or yellowy-green fluorescence were classified as eGFP transgenic.

2.9. Statistical analysis

In order to compare the number of successfully reconstructed oocytes, the number of cleaved embryos and the number of embryos at morula and blastocyst stages between different groups, including the NT-derived oocyte activation method and nuclear donor cell type, the Chi-square test was used.

3. Results

The transgenic fibroblast cell lines were generated by transfection of a linearized gene construct encompassing the rat WAP promoter and the human growth hormone coding sequence in the pTracer-EF/Bsd A vector by nucleofection and lipofection methods. Sequence analysis revealed that cloned DNA sequences were 100% identical with the sequences provided by a BLAST search.

A screening procedure involving 313 and 524 bp PCR products was applied to identify transgenic fibroblast cell lines. One individual cell line after nucleofection (1/5) and three cell lines after lipofection (3/5) were found to have the transgene incorporated into the nuclear genome. All of them were eGFP-positive. The results of screening for the *Tg(pWAPhGH-GFPBsd)* transgene are presented in Fig. 1. Moreover, the FISH analysis, which had been performed on interphase cell nuclei, confirmed the presence of the integrated *Tg(pWAPhGH-GFPBsd)* transgene in all the lipofected cell lines (Fig. 2). For lipofection, the

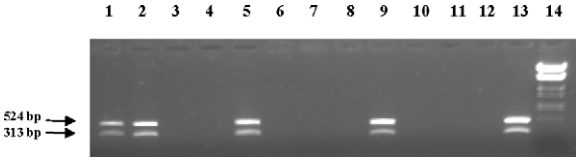

Fig. 1. Analysis for the presence of *Tg(pWAPhGH-GFPBsd)* transgene in transfected fibroblast cells. Screening for the transgene was performed by PCR encompassing 313 and 524 bp DNA fragments. Lanes 1–5, foetal fibroblast cells after lipofection; lanes 6–10, foetal fibroblast cells after nucleofection; lane 11, negative control (without DNA); lane 12, negative control (porcine DNA); lane 13, positive control (vector with transgene); lane 14, size marker (λ DNA/HindIII, EcoRI). The PCR probe was confirmed by DNA sequencing.

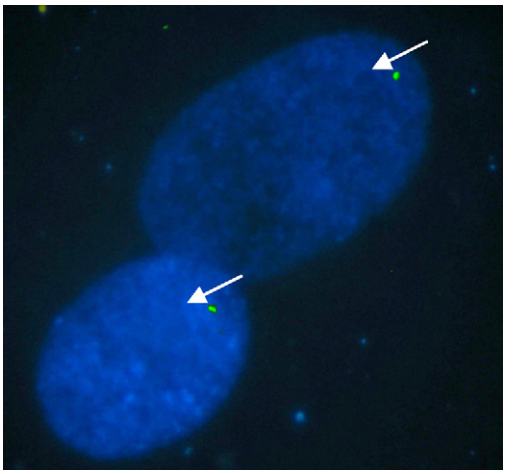

Fig. 2. FISH results on interphase cell nuclei of porcine lipofected foetal fibroblasts. The arrows indicate the single hybridization signals confirming the presence of one copy of the *Tg(pWAPhGH-GFPBsd)* transgene. Original magnification 1000×.

transgene integration was correlated with visual eGFP expression (Fig. 3). All of the transgenic cell lines were eGFP-positive and non-transgenic cell lines were eGFP-negative. For nucleofection, all the fibroblast cell lines were eGFP-positive, but only one of them had transgene incorporated into the genome (Table 1). Nonetheless, it appeared to us that the survival rates and proliferative activities of the nucleofected cell lines (both transgenic and non-transgenic) were considerably higher than in the lipofection group. Furthermore, the nucleofection efficiency of in vitro cultured porcine foetal and adult dermal fibroblast cells as estimated by nuclear donor live-fluorescent evaluation based on the expression index of the eGFP transgene reporter, was nearly 100% (Fig. 4a and c). The eGFP expression in PCR negative cell lines may result from transient expression or unstable transgene integration. But, only such foetal or adult dermal fibroblast cell lines, which had been found to be positive for the *Tg(pWAPhGH-GFPBsd)* fusion gene as a result of both PCR analysis and the monitoring of visual eGFP expression, were selected for the somatic cell cloning procedure.

In our studies, the preimplantation developmental abilities of porcine NT embryos derived from eGFP-selected donor cells were compared according to different oocyte activation treatments. Only fibroblast cell lines with insertion of the *Tg(pWAPhGH-GFPBsd)* gene, which had been confirmed through both molecular analysis methods (PCR and FISH; Figs. 1 and 2) and visual controlling of eGFP expression (Figs. 3 and 4a and c), were applied as a source of nuclear donor cells for reconstruction of enucleated

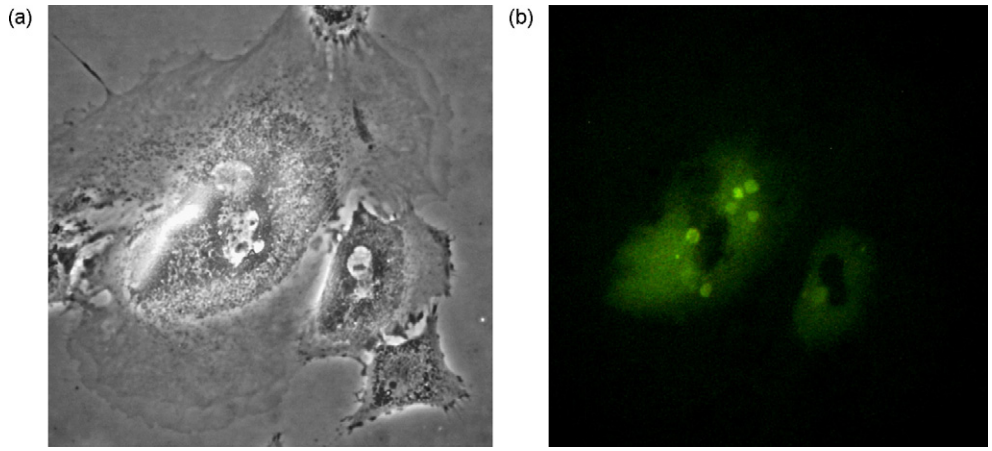

Fig. 3. Cultured porcine foetal fibroblast cells lipofected with the *Tg(pWAPhGH-GFPBsd)* gene construct. (a) The cells were evaluated under light microscopy. (b) The eGFP-positive signaling was detected following blue light excitation. Original magnification 1000×.

Table 1
Insertion of the *Tg(pWAPhGH-GFPBsd)* gene construct into in vitro cultured cell lines of porcine foetal and adult ear skin-derived fibroblasts

Cell line	Transfection method	Survival after selection/ number of experiments	eGFP-positive	PCR positive
Foetal fibroblasts	Lipofection	1/3	1/3	1/3
Adult dermal fibroblasts	Lipofection	2/2	2/2	2/2
Foetal fibroblasts	Nucleofection	3/3	3/3	1/3
Adult dermal fibroblasts	Nucleofection	2/2	2/2	0/2

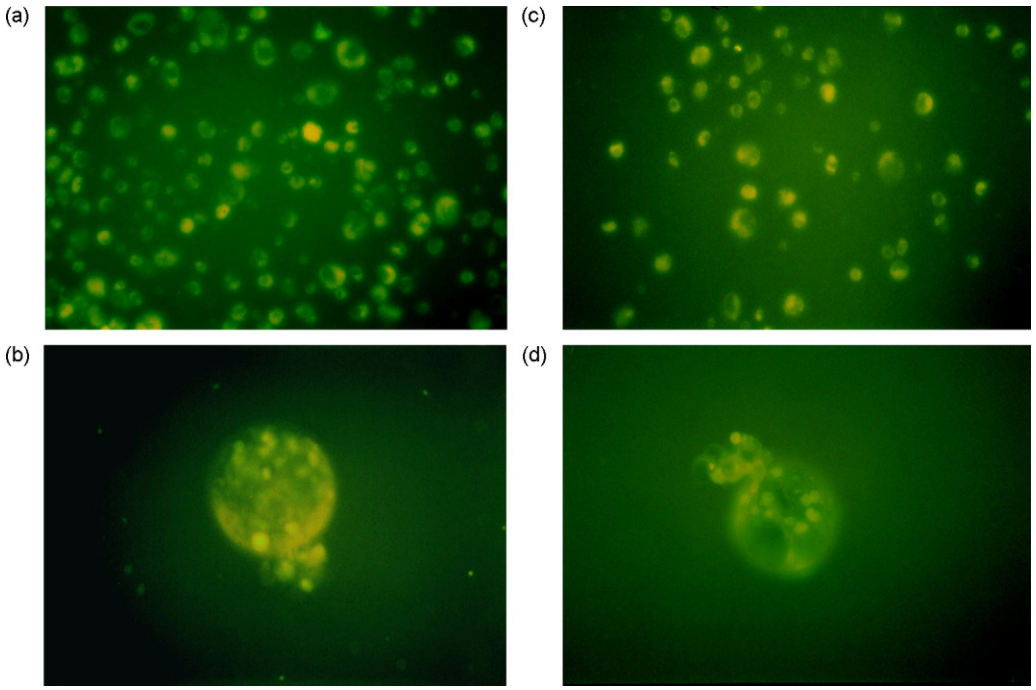

Fig. 4. Expression of eGFP fluorescence in nuclear donor fibroblast cells and cloned blastocysts. (a) Transgenic foetal fibroblasts. (b) Nuclear-transferred embryo at hatching blastocyst stage derived from *Tg(pWAPhGH-GFPBsd)* fusion gene-transfected foetal fibroblast cell. (c) Transgenic adult ear skin-derived fibroblast cells. (d) Nuclear transfer embryo at hatching blastocyst stage derived from *Tg(pWAPhGH-GFPBsd)* fusion gene-transfected adult dermal fibroblast cell. Original magnification 200×.

Table 2
Effect of activation treatments on the in vitro developmental potential of nuclear-transferred oocytes reconstructed with transgenic foetal fibroblast cells

Activation protocol	Number of oocytes/embryos			Development to	
	Enucleated	Fused (%)	Cleaved (%)	Morulae (%)	Blastocysts (%)
SF–EA	292	254 (86.98)[a]	207 (81.49)[a]	152 (59.84)[a]	77 (30.31)[a]
Sequential electrical and chemical	202	179 (88.61)[a]	131 (73.18)[b]	103 (57.54)[a]	41 (22.90)[a]

Values with different superscripts (a and b) within the same column differ significantly, $P < 0.05$. Chi-square test. Number of replicates ≥6.

Table 3
Effect of activation treatments on the in vitro developmental potential of nuclear-transferred oocytes reconstructed with transgenic adult dermal fibroblast cells

Activation protocol	Number of oocytes/embryos			Development to	
	Enucleated	Fused (%)	Cleaved (%)	Morulae (%)	Blastocysts (%)
SF–EA	322	276 (85.71)[a]	190 (68.84)[a]	139 (50.36)[a]	45 (16.30)[a]
Sequential electrical and chemical	231	193 (83.54)[a]	109 (56.47)[b]	84 (43.52)[a]	27 (13.98)[a]

Values with different superscripts (a and b) within the same column differ significantly, $P < 0.01$. Chi-square test. Number of replicates ≥7.

Table 4
Effect of the type of transgenic nuclear donor fibroblast cells on the in vitro developmental potential of cloned embryos derived from oocytes undergoing the SF–EA

Type of fibroblast cells	Number of oocytes/embryos			Development to	
	Enucleated	Fused (%)	Cleaved (%)	Morulae (%)	Blastocysts (%)
Foetal	217	189 (87.10)[a]	154 (81.48)[a]	110 (58.20)[c]	56 (29.63)[a]
Adult dermal	238	202 (84.87)[a]	139 (68.81)[b]	102 (50.49)[d]	35 (17.33)[b]

Values with different superscripts (a and b) within the same column differ significantly, $P < 0.001$. Chi-square test. Number of replicates = 6. Values with different superscripts (c and d) within the same column differ significantly, $P < 0.05$. Chi-square test. Number of replicates = 6.

oocytes. The overall developmental rates of cloned embryos from two activation groups are presented in Tables 2 and 3. The effect of the activation protocol on the rate of in vitro development to the morula and blastocyst stages was not significant. However, the competences of foetal fibroblast cell nuclei to direct the development of cloned transgenic embryos to the morula/blastocyst stages were significantly higher than the competences of adult dermal fibroblast cell nuclei (Tables 2–4). The expression of eGFP fluorescence was detected in all the blastocysts generated, as analyzed by vital eGFP fluorescence excitation (Fig. 4b and d). It was also shown that SCNT-derived blastocysts exhibited an approximately 100% index of transcriptional and translational activity for xenogeneic eGFP gene (Fig. 4b and d).

4. Discussion

The nucleofection method, which was applied in our study to transfect the porcine fibroblast cell lines, turned out to be rather an efficient strategy for producing both *Tg*(*pWAPhGH-GFPBsd*) transgenic nuclear donor cells and genetically transformed cloned embryos. Nevertheless, although the nucleofected cell lines were characterized by considerably higher survival rates and proliferative activities than the lipofected cell lines, it was revealed that only one out of five genotypically analyzed cell lines that had undergone the nucleofection were positive for the *Tg*(*pWAPhGH-GFPBsd*) transgene signaling/screening compared to three out of five cell lines subjected to the lipofection. In the other cell lines diagnosed, the transgene PCR screening turned out to be negative. The possible explanation of this phenomenon may be that the transgene integration was lost during the in vitro culture of fibroblast cells and the establishment of clonal cell lines. The other elucidation why the several cell lines obtained were not genetically engineered may be that the *Tg*(*pWAPhGH-GFPBsd*) gene construct was improperly, i.e., partially or unstably, incorporated/built into the genomic DNA. From that reason transcriptional and

translational activity of only the reporter eGFP transgene was visually confirmed by the vital fluorescent evaluation of nuclear donor fibroblast cells and cloned blastocysts. It cannot be excluded that the pattern of transient episomal (extrachromosomal) expression of whole *Tg*(*pWAPhGH-GFPBsd*) fusion gene might have occurred in the transfected somatic cells and NT embryos. Therefore, considering the long-term stability of the process of transgene integration with the somatic cell-derived nuclear genome, the novel technique of nucleofection may not have an advantage over the conventional method of lipofection. More extensive investigations should also be performed to establish whether the use of nucleofection method adapted in our laboratory for the purposes of somatic cell cloning can lead to improving the incorporation efficiency of whole gene constructs, and not only the eGFP-reporter transgene, into the genomic DNA of nuclear donor fibroblast cells. It is also reasonable that the characterization of more parameters that affect the incomplete, i.e., discontinuous, integration of *Tg*(*pWAPhGH-GFPBsd*) fusion gene with the nuclear genome of foetal or adult dermal fibroblast cells can help the inventive strategy of nucleofection to identify its potential disadvantages and to address what should be done to eliminate them.

However, using the nucleofected fibroblast cell nuclei, the percentage of porcine cloned embryos that were able to reach the morula and blastocyst stages under in vitro culture conditions was relatively high compared with the percentage of morulae and blastocysts obtained by others [20,23], and ranged from approximately 44% to 60% versus 18% to 27% and 14% to 30% versus 9.5% to 16%, respectively. The integration of the eGFP-reporter transgene in the NT embryos was not determined by using the molecular analysis methods (PCR and/or FISH), but only was assumed by controlling the visual expression of eGFP at the blastocyst stage. However, the screening for the presence of transgene in the nuclear donor cells that had been positively selected by blasticidin S and detection of eGFP-derived fluorescence was performed through complex PCR and FISH diagnostics. Nonetheless, only such fibroblast cell lines, which had been found to be positive for the *Tg*(*pWAPhGH-GFPBsd*) fusion gene as a result of both the PCR and FISH analysis methods and the monitoring of vital eGFP expression, were selected to the SCNT procedure. It has been shown that all the fluorescently diagnosed blastocysts, which had developed from cloned embryos reconstructed with the cell nuclei of nucleofected or lipofected foetal or adult ear skin-descended fibroblast cells, were positive for the eGFP transgene signaling. The expression of eGFP fluorescence was detected without mosaicism in all the NT-descended blastocysts, regardless of the type of nuclear donor fibroblast cells, the method of transfection of cultured somatic cells or the protocol of clonal cybrid activation. This finding, which was based on the lack of interaction between the effects of the activation protocol and the method of *Tg*(*pWAPhGH-GFPBsd*) gene construct transfection into the foetal fibroblast cells on the pattern of visual eGFP expression in the SCNT blastocysts, was confirmed through the results of the study by Martinez Diaz et al. [27]. In contrast to the results of the current investigation and to the study by Martinez Diaz and co-workers, Lee et al. [4] reported that following simultaneous electrical fusion and activation of oocytes that had been reconstituted with the cell nuclei of genetically transformed foetal fibroblast cells the proportion of embryos expressing eGFP transgene in all the produced NT-derived blastocysts ranged from only 64% to 67% as estimated by visual indication of eGFP protein. Similarly to the results of our study, the 100% index of eGFP transcriptional/translational activity in the porcine cloned blastocysts developing from oocytes that received transgenic foetal fibroblast cell nuclei was demonstrated in the experiments of Park et al. [23], Martinez Diaz et al. [27] and Watanabe et al. [8]. Bovine NT blastocysts, which were generated from embryos reconstituted with lipofected foetal fibroblast cells, also exhibited a total [5,11] or above 90% visual eGFP expression pattern [13]. Moreover, Arat et al. [11] have shown that bovine cloned blastocysts, which were produced from the oocytes receiving genetically transformed adult dermal fibroblast cell nuclei, were also 100%-positive for eGFP fluorescent vital screening. On the contrary, Park et al. [23] reported that nearly 60.0% of porcine NT blastocysts derived from eGFP-expressing adult dermal fibroblast cells exhibited not a total, but a mosaic pattern of transcriptional/translational activity for a xenogeneic eGFP-reporter transgene. But, approximately 24.0% of cloned blastocysts were characterized by a 0% index of eGFP gene expression. However, it has been shown that bovine SCNT produces a threefold higher percentage of blastocysts that are 100%-negative for eGFP-reporter transgene expression [29].

We have also found that the methods applied to artificially activate the porcine SCNT-derived oocytes do not affect the in vitro developmental capability of *Tg*(*pWAPhGH-GFPBsd*) transgenic cloned porcine embryos. Although the genetically transformed fibroblast cells had a slightly higher cloning competence

following the SF–EA of reconstituted oocytes compared to the competence of cells undergoing sequential electrical and chemical activation, the differences between the two activation groups were not statistically significant. Yin et al. [41] also reported that the in vitro developmental abilities of NT embryos, which had been reconstructed with non-transgenic foetal or adult heart tissue-retrieved cells, do not differ significantly between the two protocols for oocyte stimulation. These different protocols call for simultaneous fusion/activation and post-activation and use physical, chemical or physico-chemical activating agents. However, in the experiments by Hyun et al. [20], porcine cloned embryos, which had been derived from eGFP transgenic foetal fibroblast cell nuclei and oocytes undergoing sequential electrical and chemical activation with the use of DC pulses and calcium ionomycin/6-DMAP, exhibited significantly lower blastocyst formation rates than those for SCNT embryos originating from oocytes simultaneously fused and activated by electric pulses. Nevertheless, using the combination of electrical and chemical activation to stimulate the NT-derived oocytes reconstructed with genetically modified foetal fibroblast cells, Hyun et al. [20] reported considerably lower blastocyst yields (ranging from 12% to 16%) than those obtained by us (approximately 23%). But, in our experiments, the cloned embryos reconstructed with cell nuclei from the *Tg(pWAPhGH-GFPBsd)* transgenic foetal fibroblasts and oocytes stimulated with the use of DC pulses and subsequently calcium ionomycin/CHXM, developed to blastocyst stage at a similar rate as those in the study by Boquest et al. [37]. Boquest et al. applied chemical post-activation to porcine non-transgenic NT-derived oocytes treated with ionomycin and 6-DMAP. In contrast, the cavitation rate of embryos that had developed from oocytes that received nuclei from the eGFP gene-expressing adult dermal fibroblasts and that were sequentially activated with DC pulses before ionomycin/CHXM treatment was twofold higher in our study (approximately 14% versus 6%) than in other studies [40,42,43]. In these other studies, the oocytes were activated following the application of electric pulses only or in combination with 6-DMAP.

Additionally, we have shown that regardless of the methods used to artificially activate the reconstructed oocytes, the abilities of cell nuclei of the *Tg(pWAPhGH-GFPBsd)* transgenic foetal fibroblasts to direct the in vitro development of porcine cloned embryos to morula/blastocyst stages were considerably higher than those for adult dermal fibroblast cell nuclei. Similarly, Lee et al. [39] reported that following the SF–EA of reconstructed oocytes the cloning competence of non-transgenic foetal fibroblasts, as measured with blastocyst formation rate, was twofold higher than the competence of adult dermal fibroblasts (approximately 16% versus 8%). Nevertheless, using the same activation protocol, a considerably higher percentage of blastocysts originating from NT embryos reconstituted with either *Tg(pWAPhGH-GFPBsd)* transgenic foetal or adult ear skin-retrieved fibroblast cells has been generated by us (approximately 30% and 16%, respectively). On the contrary, in the experiments by Park et al. [23], NT embryos, which had developed from oocytes reconstituted with eGFP-transgenic foetal fibroblast cells and were stimulated by the SF–EA, were characterized by a blastocyst formation rate similar to that for cloned embryos derived from adult dermal fibroblasts (9.5% versus 10.0%).

In conclusion, the in vitro developmental capability of porcine transgenic NT embryos, which had been reconstructed with the *Tg(pWAPhGH-GFPBsd)* gene-transfected foetal or adult dermal fibroblast cells, was relatively high. We also show that the nucleofection efficiency of all the porcine fibroblast cell lines as estimated by intra-vitam fluorescent evaluation based on the index of reporter eGFP transgene expression was nearly 100%. Moreover, both the survival rates and proliferative activities of the fibroblast cells following the nucleofection were considerably higher than those following the lipofection procedure. However, PCR analysis for transgene screening confirmed the absence of the *Tg(pWAPhGH-GFPBsd)* fusion gene in some of the nucleofected cell lines. Therefore, further studies are required to establish the reliability and feasibility of the nucleofection strategy for more efficient integration of whole gene constructs, and not only the reporter eGFP transgene, with the genomic DNA of nuclear donor fibroblast cells. Nonetheless, to our knowledge, the nucleofection method was used for the transfection of nuclear donor cells and subsequent production of porcine transgenic cloned embryos for the first time. Future experiments are also needed to establish whether the use of nucleofection method adapted in our laboratory for the purposes of SCNT can result in producing viable transgenic cloned piglets.

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