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Production of human lysozyme-transgenic cloned porcine embryos by somatic nuclear transfer

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Abstract

Due to their physiology and organ size, pigs have significant potential as human disease models and as organ transplantation donors. Genetic modification of pigs could provide benefits for both agriculture and human medicine. In this study, five fetal pig fibroblast cell lines from two species (Wuzhishan and Landrace pigs) were transfected using double-marked human lysozyme (HLY) plasmids (pBC1-HLY-GFP-NEO) by a liposome-mediated method. The ratio of green fluorescent protein (GFP)-expressing cells was >95% in sw7, sw8, slw3 and slw6 cell lines, but only 49.3% in slw9 cells. Cells from the four highly transgenic lines were used as nuclear donors to construct embryos, which were then cultured after fusion and activation by electric stimulation. The rate of cleavage was 76.7%, 48 h after activation. After 7 days, 18.5% of cleaved eggs had developed to the blastocyst stage and 93.3% of blastocysts were GFP-positive. These results indicate that transgenic fetal pig fibroblast cell lines could be obtained by a liposome-mediated method, though the transfection efficiency varied between cell lines. Reconstructed embryos derived from transgenic cells could successfully develop into blastocysts, most of which were GFP-positive.

Keywords: EGFP; Porcine; Nuclear transfer; Fetal fibroblast; Human lysozyme (HLY)

1. Introduction

The control of pig diseases has always been an important issue in the pig-rearing industry. Vaccination is a key means of preventing acute virus infections, while the control of bacterial diseases depends mainly on antibiotics. Due to the limited antimicrobial spectrum and the occurrence of drug resistance, the cost of pig production has increased, and sick animals cannot always be treated effectively. This is detrimental to the pig industry, especially in terms of piglet production. Both morbidity and mortality of piglets can be high because of their young age and poor disease resistance. It is necessary to establish an economic, safe and efficient method for pig disease control.

Lysozyme is a natural nonspecific immunity substance, which can break-down microbial cell walls and kill them. The microbial population of pig alimentary tracts has been investigated, which could help in improving their immunity [1]. It was reported that the addition of lysozyme to piglets' diet could improve performance, decrease the incidence of diarrhea and improve the feed conversion ratio [2–4]. Lysozyme is widely distributed in many organisms, including humans and other animals, as well as some plants and microorganisms [5]. The content of lysozyme in human milk is about 0.5 mg/ml, which is 1500–4000 times higher

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than that in cattle, sheep and goat milk. The antibacterial activity of human lysozyme (HLY) is about 300 times greater than that of cattle milk lysozyme. The production of a transgenic pig whose galactophores could express a reconstructed human lysozyme gene would have the potential to greatly reduce the incidence of pig diseases. The secretion of lysozyme with high antibiotic activity in pig milk could protect piglets from infection. The successful cloning of Mini-pigs [6] and Northeast Mini-pigs [7] has been reported, but there have not yet been any reports of successful production of transgenic cloned embryos or piglets with functional gene insertions, except for marker gene transfer [8]. HLY-transgenic cloned mice [9–13], goats [14–17] and cattle [18,19] have been produced, but no reports on HLY-transgenic pigs or pig embryos have been published.

In this study, five different fetal pig fibroblast cell lines derived from two species were transfected using double-marked HLY plasmids (pBC1-HLY-GFP-NEO), using a liposome-mediated method. After screening, transgenic cells were used as nuclear donors to construct cloned embryos. The object of this study was to investigate a technique for producing HLY-transgenic embryos by a somatic nuclear method. This will provide valuable information for the future production of HLY-transgenic pigs.

2. Materials and methods

Unless otherwise stated, chemicals were all purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.1. In vitro maturation of porcine oocytes

Ovaries of prepubertal gilts were collected at a local slaughterhouse and were transported to the laboratory in physiological saline supplemented with antibiotics at 30 °C, within 2 h of slaughter. Cumulus–oocyte complexes (COCs) from 3 to 6 mm diameter follicles were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. Compact COCs with at least three layers of cumulus cells were washed 3 times with TCM199 supplemented with 20 mM HEPES and 10% fetal bovine serum (FBS). COCs were then cultured with TCM199 supplemented with 10% porcine follicular fluid (pFF, self-made), 10% estrus calf serum (ECS, self-made), 10 ng/ml epidermal growth factor (EGF), 10 IU/ml pregnant mare serum gonadotrophin (PMSG) and 10 IU/ml human chorionic gonadotrophin (hCG) in 4-well dishes (NUNC). Each well contained about 100 COCs and was filled with 400 μl maturation medium and covered with 400 μl mineral oil and incubated for 40 h at 39 °C and 100% humidity under 5% CO_2 in air.

The pFF was aspirated from 5 to 8 mm diameter follicles and was centrifuged at 1600*g* for 30 min in 50 ml centrifuge tubes. The supernatant was collected, filtered using 0.45 μm syringe filters and was stored at −20 °C in 15 ml tubes until use.

2.2. Donor cell culture, transfection and selection of donor cells

The fetal pig fibroblast lines, sw7, sw8 (from Wuzhishang pigs) and slw3, slw6 and slw9 (from Landrace pigs), were established and cultured as described previously [8]. Genetic transfection of the cell lines was performed in 6-well dishes, in a culture medium consisting of DMEM supplemented with 10% FBS. The fibroblast cells were transfected with linearized double-marked pBC1-hLY-GFP-NEO plasmids using Lipofectamine2000 (Invitrogen) when they reached 50–60% confluence. After selection with 1000 μg/ml G418 for 14–20 days, the cells expressing GFP were detected under a fluorescence microscope. Cultures with >90% GFP-positive cells were passaged twice then stored in liquid nitrogen.

The sw8, slw3 and slw6 cell lines were thawed and cultured for 5 days before nuclear transfer. The cells were further cultured for 1–2 days after they reached 100% confluence, and were then used as donors. Cells were digested with 0.1% trypsin for 2 min, then collected after centrifuging and stored at room temperature until use.

2.3. Enucleation and microinjection

Cumuli were removed from COCs with 1 mg/ml hyaluronidase dissolved in DPBS after *in vitro* maturation. The oocytes with homogeneous cytoplasm and first polar bodies were selected for micromanipulation. The operating medium was HEPES-buffered TCM199 supplemented with 7.5 μg/ml cytochalasin B (CB). Mature eggs were collected in a drop of operating medium in a pipette (outer diameter 100–120 μm). Oocyte spindles were excluded by pressure. The cells with smoothly refracting membranes and ~20 μm in diameter were transferred into the perivitelline space. The micromanipulation of each group of oocytes was completed within 30 min.

2.4. Fusion and activation of reconstructed embryos

The reconstructed embryos were fused and activated simultaneously. The fusion medium consisted of 0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.01% PVA. After 3–5 min in the fusion medium, five reconstructed embryos from each group were transferred into a chamber (BTX, San Diego, CA) containing fusion medium. The couplets were adjusted to make the interface of the somatic cell-oocyte parallel with the electrode. Two DC pulses of 1.6 kV/cm for 100 μs each with a 1 s interval were delivered by a CUY-21 square wave electroporator (Japan) to induce fusion and activation. Eggs were picked out and washed three times with PZM3 [19]. After being cultured in the droplets of PZM3 with 10 μg/ml CB and 10 μg/ml cycloheximide (CHX) for 4 h to chemically assist activation, fusion of reconstructed couplets was examined under a stereomicroscope.

2.5. Embryo culture and fluorescence observation

The fused embryos were picked out from the medium and were washed three times for 5 min each. They were then transferred into PZM3 medium and were cultured at 39 °C, 5% CO_2, 7% O_2, 88% N_2 and 100% humidity. The rates of cleavage and blastocyst formation were examined at 48 and 168 h, respectively. The GFP-positive embryos were identified by the emission of green light during exposure to blue excitation light.

2.6. Molecular detection of transgenic cells

The molecular detection of HLY-transgenic cloned porcine embryos was conducted by a PCR [9]. PCR primer sequences were 5′ TTA TAC ACA CGG CTT TAC 3′ and 5′ CAG CAT CAG CGA TGT TAT CT 3′. The PCR amplification conditions were 94 °C 5 min, then 94 °C 40 s, 53 °C 40 s, 72 °C 40 s, 72 °C 7 min, 4 °C 1 h. A total of 30 cycles were performed. The length of the expected amplification fragment was 750 bp.

2.7. Statistical analysis

The rate of GFP-positive cells, the developmental rate of reconstructed embryos, and the percentage of transgenic embryos were all analyzed using *chi*-square tests. Differences between groups were considered significant when $P < 0.05$.

3. Results

3.1. Cell transfection with pBC1-hLY-GFP-NEO plasmid

sw7, sw8, slw3, slw6 and slw9 cells were screened with G418 from the second day of transfection. Some cells began to die on day 3, and many dead cells were evident by day 4. Only a few surviving single cells adhered to the bottom after treatment for 6 d. After further screening, 7–10 separate clones were identified in each well (Fig. 1). On days 11–13 of drug treatment, the cells were passaged, and the ratio of GFP-positive cells was calculated. The results of transfection of the five cell lines are shown in Table 1. slw9 cells showed a lower transfection efficiency than the other four cell lines, using the same methods of transfection and screening. A proportion of GFP-negative cells remained and demonstrated G418 resistance after passage. The highly efficient transgenic cell lines, sw7, sw8, slw3 and slw6, were stored in liquid nitrogen. PCR was conducted to determine if the HLY gene had integrated into the cell genome. The results are shown in Fig. 2: all four cell lines expressed a band corresponding to the positive control plasmid, compared with a wild-type negative control cell.

3.2. In vitro developmental ability of reconstructed embryos from transgenic HLY cells

The *in vitro* development of reconstructed embryos derived from sw8-HLY, slw3-HLY and slw6-HLY cells is shown in Table 2. There were no significant differences in cleavage rate or blastocyst formation rate between these three transgenic cell lines ($P > 0.05$). Almost all the blastocysts emitted green fluorescence under the blue light of a fluorescence microscope (Fig. 3).

4. Discussion

In this study, five different fetal pig fibroblast cell lines were transfected using a liposome-mediated method. After screening with G418, only GFP-positive cell clones survived in most cell lines (80%). The reconstructed embryos derived from GFP-positive cells underwent normal cleavage and developed *in vitro* with a high blastocyst formation rate of 18.5%. The HLY-positive band was also amplified clearly by PCR analysis. This result provides a sound basis for the production of HLY-transgenic pigs.

The ease of culture, long life span and good viability for nuclear embryo transfer [20–22] means that fetal pig fibroblast cell lines are one of the most commonly used donor cells for porcine somatic nuclear transfer. For the production of genetically modified pigs, it is necessary to first transfect the cells and then introduce the foreign gene into the donor cell genome. Fetal fibroblasts are likely to con-

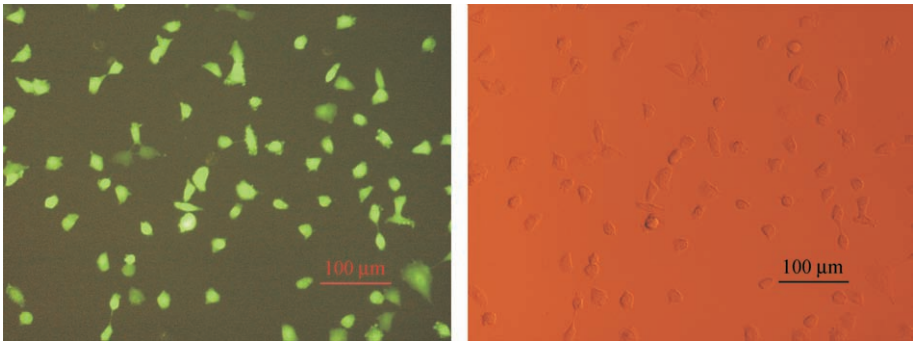

Fig. 1. Transgenic cell clones after selection for 10 d with G418 (Bright-field pictures of cells on the right; corresponding images under a fluorescence microscope on the left).

Table 1
Results of transfection of cell lines with pBC1-HLY-GFP-NEO plasmid.

Cell lines	Some dead cells	Many dead cells	Survived cell clones on day 10	Rate of GFP^+ (%)[a]
sw7	Day 3	Day 4	GFP^+	96.3 (289/300)
sw8	Day 3	Day 4	GFP^+	95.3 (286/300)
slw3	Day 3	Day 4	GFP^+	98.0 (294/300)
slw6	Day 3	Day 4	GFP^+	95.7 (287/300)
slw9	Day 3	Day 4	GFP^+ and GFP^-	49.3 (148/300)

slw9 had significantly fewer GFP-positive cells than the other four lines ($P < 0.05$).
[a] The data in parentheses indicate positive cell numbers for every 300 cells in different visual fields.

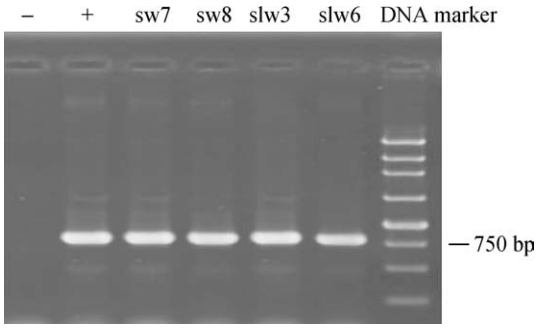

Fig. 2. Molecular detection of HLY-transgenic cell lines transfected with pBC1-HLY-GFP-NEO plasmid.

tinue to be the first choice as donor cells until porcine embryonic stem cell techniques are well established [23–29]. Cell transfection is the key process in the production of transgenic cloned animals, but because of the cell morphology, cell density and sensitivity to drugs, it has always been difficult to get satisfactory transfection results. In this study, five cell lines were transfected, but transgene-positive cells survived in only four of the cell lines. These results show that transgenic cell lines can be obtained with high purity, with no abnormalities in cell shape or multiplication. This indicates that the cell transfection and screening methods were feasible. The reason for the low transfection rate in cell line slw9 was possibly due to the differences in characteristics between the cell lines. The minimum lethal dose of G418 against slw9 cells, the cell density, number of generations and experiment time were all the same as those in the other four cell lines. The reasons for the difference in transfection efficiency need further study.

Somatic nuclear transfer was performed in three transfected cell lines. Compared with reconstructed embryos produced from cells in our laboratory without transfection, the cleavage rate and the rate of blastocyst formation in this study were about 75% and 20%, respectively. Similar results were found by Gong et al. [30]. Some papers have reported that the somatic cell cloning efficiency of transgenic cells declined significantly with long periods of drug

Table 2
In vitro development of reconstructed embryos which used transgene-positive cells as nuclear donors.

Donor cells	Number of reconstructed embryos	Cleavage (%)	Blastocyst (%)	GFP^+ blastocyst (%)
sw8-HLY	226	168 (74.5)[a]	39 (17.3)[b]	37 (94.9)[c]
slw3-HLY	98	79 (80.7)[a]	18 (18.4)[b]	17 (94.4)[c]
slw6-HLY	319	246 (77.1)[a]	62 (19.4)[b]	57 (91.9)[c]
Total	643	493 (76.7)[a]	119 (18.5)[b]	111 (93.3)[c]

Same letter superscripts in the same column indicate no significant difference ($P > 0.05$).
sw8-HLY indicates fetal fibroblast cells of Wuzhishan pig with integrated pBC1-HLY-GFP-NEO plasmid; slw3-HLY and slw6-HLY were transgenic cells from Landrace pigs.

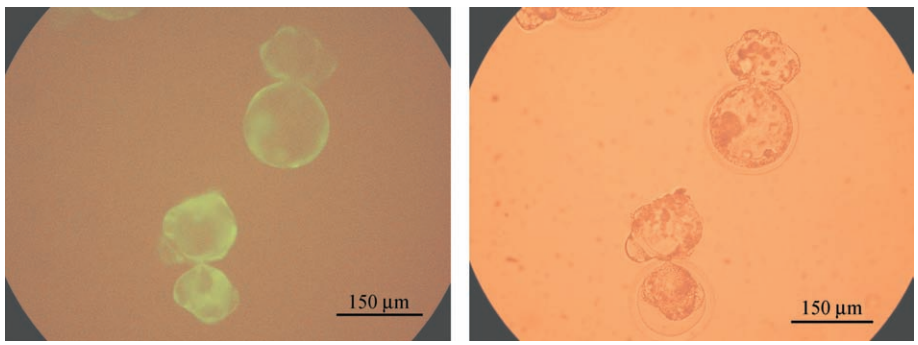

Fig. 3. GFP-positive embryos expressing the transgene from pBC1-HLY-GFP-NEO plasmid (Bright-field picture of embryos on the right; corresponding images under the fluorescence microscope on the left).

screening [31,32]. This might be due to the differences between cell lines from different laboratories. GFP is a commonly used reporter gene, which has the advantage of being safe and easily tested, and it is used, together with antibiotic resistance genes, to construct a gene carrier, as in this study. The embryos constructed here from transgenic cell lines showed very high rates of GFP-positivity. In theory, the rate of HLY-positive transgenic embryos should be the same as the rate of GFP-expressing embryos, and previous reports have shown the accuracy of this marker gene [33]. This study introduced the HLY gene into the pig cell genome for the first time, and successfully produced transgenic blastocysts, which developed *in vitro*. This transgene has been shown to be expressed successfully in mouse mammary glands, with the same activity as HLY. It is therefore expected that the implantation of the reconstructed pig embryos could result in the production of HLY-expressing transgenic pigs. These transgenic pigs could then continue to reproduce as a new species, so increasing effective utility of sows.

In conclusion, the results of this study indicated that fetal fibroblast cells from both Mini-pigs and Landrace pigs could be transfected using a liposome-mediated method. A high rate of HLY-transgene positive cells expressing GFP gene could be achieved. Differences existed between different fetal fibroblast cell lines in terms of transfection efficiency. The reconstructed embryos successfully developed to the blastocyst stage *in vitro*.

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