Development of cloned embryos from porcine neural stem cells and amniotic fluid-derived stem cells transfected with enhanced green fluorescence protein gene

Yue-Mao Zheng, Hui-Ying Zhao, Xiao-E Zhao, Fu-Sheng Quan, Song Hua, Xiao-Ying He, Jun Liu, Xiao-Ning He and Hui Lin

College of Veterinary Medicine, Institute of Biotechnology, Northwest A&F University, 1-06#, Research Building, Yangling, Shaanxi 712100, People's Republic of China

Correspondence should be addressed to Y-M Zheng; Email: zhengyuemao@163.com

Abstract

We assessed the developmental ability of embryos cloned from porcine neural stem (NS) cells, amniotic fluid-derived stem (AFS) cells, fetal fibroblast cells, adult fibroblast, and mammary gland epithelial cells. The five cell lines were transfected with enhanced green fluorescence protein gene respectively using lipofection. NS and AFS cells were induced to differentiate *in vitro*. Stem cells and their differentiated cells were harvested for analysis of the markers using RT-PCR. The five cell lines were used for nuclear transfer. The two-cell stage-cloned embryos derived from each cell line were transferred into the oviducts of surrogate mothers. The results showed that both NS and AFS cells expressed POU5F1, THY1 and SOX2, and they were both induced to differentiate into astrocyte (GFAP⁺), oligodendrocyte (GalC⁺), neuron (NF⁺, ENO2⁺, and MAP2⁺), adipocyte (LPL⁺ and PPARG-D⁺), osteoblast (osteonection⁺ and osteocalcin⁺), myocyte (MYF6⁺ and MYOD⁺), and endothelium (PECAM1⁺, CD34⁺, CDH5⁺, and NOS3⁺) respectively. Seven cloned fetuses (28 days and 32 days) derived from stem cells were obtained. The *in vitro* developmental ability (morula–blastocyst rate was 28.26–30.07%) and *in vivo* developmental ability (pregnancy rate were 1.67–2.17%) of the embryos cloned from stem cells were higher ($P < 0.05$) than that of the embryos cloned from somatic cells (morula–blastocyst rate was 16.27–19.28% and pregnancy rate was 0.00%), which suggests that the undifferentiated state of the donor cells increases cloning efficiency.

Reproduction (2009) 137 793–801

Introduction

The development of nuclear transfer (NT) techniques have enabled researchers to generate cloned pigs (Betthausen *et al.* 2000, Polejaeva *et al.* 2000, Polejaeva 2001, Yin *et al.* 2002, Lee *et al.* 2003, Lagutina *et al.* 2006, Du *et al.* 2007). Although some believe that embryonic stem (ES) cells are not much better as the donor cell for NT (Obach & Wells 2002), the success rate for producing cloned mice from several ES cell lines appears to be higher than the rates for other differentiated donor cells (Wakayama *et al.* 1999, Rideout *et al.* 2000). The results suggest that the undifferentiated state of the donor cells may increase the birth rate of cloned animals. Some researchers found that the developmental stage of donor embryos affects the cloning efficiency (Cheong *et al.* 1993, Hiiragi & Solter 2005). In their results, cloned embryos receiving early-developmental-stage embryo nuclei developed to term, but those receiving late-stage embryo nuclei had seriously limited development. Their results also suggest that using undifferentiated donor nuclei is effective for generating cloned animals. Yamazaki *et al.* (2001) also reported that

cloned embryos develop to term with high efficiency (6%) in freshly isolated fetal immature neural cells transferred into enucleated oocytes. Thus, the undifferentiated state of the donor cell, such as tissue-specific stem cells, may increase the animal cloning efficiency even if the cell is derived from a somatic cell lineage. Investigating other somatic stem cells used in NT is necessary.

Neural stem (NS) cells can be cultured over the long term and are able to self-renew *in vitro* (Reynolds & Weiss 1992, 1996, Gage 2000, Philip *et al.* 2005). Amniotic fluid is known to contain stem cells (In't Anker *et al.* 2003, Prusa *et al.* 2004, Tsai *et al.* 2004). Human amniotic fluid-derived stem (AFS) cells can give rise to neurogenic, adipogenic, osteogenic, myogenic, and endothelial lineages, inclusive of all embryonic germ layers (De Coppi *et al.* 2007). The NS and AFS cells may increase cloning efficiency, if the undifferentiated state of the donor cells affects the success rate, as observed with ES cell donors.

Since first introduced as an expression marker by Chalfie *et al.* (1994), a fusion protein (green fluorescent protein, GFP) or a peptide tagged with GFP has been

used as a marker to follow *in vivo* gene expression and real-time protein localization (Rizzuto *et al.* 1996, Wacker *et al.* 1997). In pigs, enhanced GFP (EGFP) gene was successfully used as an indicator without any adverse biological effects on *in vitro* development of transfected embryos (Park *et al.* 2001a, 2001b). The success in selecting and producing transgenic offspring using GFP as a marker has paved the way for GFP use in transgenic experimentation. With the aid of this virtually ideal transgenic marker, progress in developing efficient gene delivery systems will be greatly accelerated. However, there are no reports on production of cloned embryos using porcine NS and AFS cells transfected with EGFP. This study was conducted to assess the developmental ability of cloned embryos derived from NS and AFS cells, and compared the developmental ability with that of embryos cloned from other donor cells such as fetal fibroblast (FF) cells, adult fibroblast (AF) cells, and mammary gland epithelial (MGE) cells in the porcine.

Results

Characterization of NS and AFS cells

After two days of culture, NS cells proliferated as neurospheres (Fig. 1A and B). We have established AFS cell lines with a typical doubling time of about 48 h and

Figure 1 EGFP gene transgenic cells viewed under green fluorescence microscope, 10 $\times$. (A and B) NS cells ((A) cultured for two days and (B) cultured for three days), (C) AFS cells, (D) MGE cells, (E) FF cells, and (F) AF cells. EGFP, enhanced green fluorescence protein; NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells; MGE cells, mammary gland epithelial cells; FF cells, fetal fibroblast cells; AF cells, adult fibroblast cells.

Figure 2 Markers of NS (B–D) and AFS (F–H) cells tested by RT-PCR. Positive control was β -actin. Products are shown with a 50-bp DNA ladder at the left of the gel and product sizes are under the margin. DNA marker is 50, 100, 150, 200, 250, 300, 350 (the brightest), 400, 450, 500, 550, and 600 bp. NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells; RT-PCR.

without need for feeder layers. Sub-confluent cells show no evidence of spontaneous differentiation (Fig. 1C). The neurospheres and AFS cells were positive for the undifferentiated cell markers, POU5F1, THY1, and SOX2 (Fig. 2B–D and F–H).

EGFP could be expressed in the cell lines

EGFP could be expressed in NS, AFS, FF, AF, and MGE cells (Fig. 1). EGFP also could be expressed in the differentiated cells derived from NS and AFS cells (Fig. 3).

Multilineage differentiation was characteristic of NS and AFS cells

NS and AFS cells are broadly multipotent. Under specific inducing conditions, they are able to give rise to lineages representative of the three embryonic germ layers. We found that NS and AFS cells were able to differentiate along neurogenic, adipogenic, osteogenic, myogenic, and endothelial pathways (Fig. 3). Both NS and AFS cells were differentiated into astrocyte (GFAP⁺), oligodendrocyte (GalC⁺), neuron (NF⁺, ENO2⁺, and MAP2⁺), adipocyte (LPL⁺ and PPARG-D⁺), osteoblast (osteocalcin⁺ and osteonectin⁺), myocyte (MYF6⁺ and MYOD⁺), and endothelium (PECAM1⁺, CD34⁺, CDH5⁺ and NOS3⁺; Fig. 4).

Developmental ability of embryos cloned from different donor cells

The results showed that the *in vitro* developmental ability (morula–blastocyst rate was 28.26–30.07%; Table 1) and *in vivo* developmental ability (pregnancy rate was 1.67–2.17%; Table 2) of the embryos cloned from stem cells were higher ($P < 0.05$) than that of the embryos cloned from somatic cells (morula–blastocyst rate was 16.27–19.28%, and pregnancy rate was 0.00%; Tables 1 and 2),

Figure 3 Differentiated cells derived from EGFP gene transfected NS (A1–F1) and AFS (A2–F2) cells viewed under green fluorescence microscope, 10 $\times$. (A1 and A2) neurogenic cells, (B1 and B2) adipogenic cells, (C1 and C2) osteogenic cells, (D1 and D2) myogenic cells, (E1 and E2) endothelial cells, and (F1 and F2) control group cells. NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells.

which suggests that the undifferentiated state of the donor cells increases cloning efficiency. Seven cloned fetuses (28 days and 32 days) derived from stem cells were obtained. EGFP gene could be expressed in the cloned fetuses (Fig. 5H–J).

Discussion

Production of cloned pigs by somatic cell NT (SCNT) has unlimited value for developing critical biotechnology such as xenotransplantation (Prather *et al.* 1999, Polejaeva *et al.* 2000, Richard *et al.* 2002). Various efforts have been made to establish this technology (Wolf *et al.* 1998, Boquest *et al.* 2002, Im *et al.* 2004), and live piglets have been delivered after transfer of SCNT embryos (Betthausen *et al.* 2000, Onishi *et al.* 2000, Polejaeva *et al.* 2000). However, the viability of porcine SCNT embryos is poor, with an extremely low rate of cloned piglet production. Studies have demonstrated that many factors are involved in the development of porcine SCNT embryos (Polejaeva 2001). The factors include donor cell types, recipient oocytes, fusion/activation methods, and *in vitro* culture system. The success rate for cloning mice from ES cells is relatively high compared with that for differentiated somatic cells (Wakayama *et al.* 1999, Rideout *et al.* 2000). This suggests that a cell in an undifferentiated state may be suitable as a donor cell for animal cloning. Although ES cells may be effective in NT in mice, this process is limited in other species, where definitive ES cells have not been

established. Investigation of other undifferentiated cell types, such as somatic stem cells, in NT is important for animal cloning techniques.

NS cells are a type of well-defined somatic stem cells that can proliferate and differentiate *in vitro* (Jingli *et al.* 2002, Philip *et al.* 2003, 2005). In this study, we isolated NS cells from porcine fetuses and the cells grown as spheres. The developmental potential differed between cloned embryos derived from neurospheres and from somatic cells, suggesting that the donor cells of the neurospheres we used were undifferentiated. We have demonstrated that stem cells can be obtained from porcine amniotic fluid. The AFS cells grow easily in culture. They are capable of extensive self-renewal, a defining property of stem cells. POU5F1 is expressed in totipotent ES and germ cells. A critical level of POU5F1 expression is required to sustain stem cell self-renewal and pluripotency. POU5F1 is not only a master regulator of pluripotency that controls lineage commitment, but is also the first and most recognized marker used for the identification of totipotent ES cells. THY1 can be used as a marker for a variety of stem cells (Chen *et al.* 1999). Ellis *et al.* (2004) show that SOX2 is expressed in multipotent NS cells at all stages of mouse ontogeny. In this study, porcine NS and AFS cells were positive for the undifferentiated cell markers, POU5F1, THY1, and SOX2 (Fig. 2B–D and F–H). These results also indicate that these cells were in an undifferentiated state. The surface marker profile of AFS cells and their expression

Figure 4 RT-PCR results of markers of the differentiated cells derived from NS (A1–O1) and AFS (A2–O2) cells. Products are shown with a 50-bp DNA ladder at the left of the gel and product sizes are under the margin. GFAP, NF, ENO2, MAP2, and GALC are neurogenic markers; LPL and PPARG-D are adipogenic markers; osteonectin and osteocalcin are osteogenic markers; MYF6 and MYOD are myogenic markers; PECAM1, NOS3, CD34, and CDH5 are endothelial markers. DNA marker is 50, 100, 150, 200, 250, 300, 350 (the brightest), 400, 450, 500, 550, and 600 bp. NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells.

of the transcription factor POU5F1 suggest that they represent an intermediate stage between pluripotent ES cells (Evans & Kaufman 1981, Martin 1981, James *et al.* 1998) and lineage-restricted adult stem cells.

AFS cells can serve as precursors to a broad spectrum of differentiated cell types. Siddiqui & Atala (2004) found that AFS cells were able to differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic pathways. We have assessed their multipotent character. This study shows that both NS and AFS cells are broadly multipotent, which were induced to differentiate into cell types representing each embryonic germ layer,

including cells of neuronal, adipogenic, osteogenic, myogenic, and endothelial lineages (Fig. 3). The induced differentiation to multiple fates could be documented by the expression of mRNAs for lineage-specific genes. Both NS and AFS cells could differentiate into astrocyte (GFAP⁺), oligodendrocyte (GALC⁺), neuron (NF⁺, ENO2⁺, and MAP2⁺), adipocyte (LPL⁺ and PPARG-D⁺), osteoblast (osteonectin⁺ and osteocalcin⁺), myocyte (MYF6⁺ and MYOD⁺), and endothelium (PECAM1⁺, CD34⁺, CDH5⁺, and NOS3⁺; Fig. 4).

A significant aspect of applying SCNT technology to generate transgenic embryos and/or offspring is the

Table 1 *In vitro* developmental ability of cloned embryos from different donor cells.

Type of donor cell	Reconstructed efficiency (%)	Two-cell rate (%)	Four- to eight-cell rate (%)	Morula–blastocyst rate (%)
Transgenic cells				
NS cells	82.97 (268/323) ^a	64.18 (172/268) ^a	45.90 (123/268) ^a	29.10 (78/268) ^a
AFS cells	81.48 (286/351) ^a	65.03 (186/286) ^a	45.10 (129/286) ^a	30.07 (86/286) ^a
FF cells	81.62 (191/234) ^a	53.40 (102/191) ^b	32.98 (63/191) ^b	18.32 (35/191) ^b
AF cells	79.70 (161/202) ^a	52.80 (85/161) ^b	32.30 (52/161) ^b	18.01 (29/161) ^b
MGE cells	78.67 (166/211) ^a	51.20 (85/166) ^b	28.92 (48/166) ^b	16.27 (27/166) ^b
Non-transgenic cells				
NS cells	82.88 (92/111) ^a	65.22 (60/92) ^a	44.57 (41/92) ^a	28.26 (26/92) ^a
AFS cells	80.83 (97/120) ^a	63.92 (62/97) ^a	44.33 (43/97) ^a	28.87 (28/97) ^a
FF cells	81.37 (83/102) ^a	50.60 (42/83) ^b	32.53 (27/83) ^b	19.28 (16/83) ^b
AF cells	80.19 (85/106) ^a	54.12 (46/85) ^b	34.12 (29/85) ^b	17.65 (15/85) ^b
MGE cells	79.00 (79/100) ^a	53.16 (42/79) ^b	29.11 (23/79) ^b	17.72 (14/79) ^b

Within the same column, values with same superscripts (a, b, c and d) were not significantly different ($P > 0.05$), and values with different superscripts (a, b, c and d) were significantly different ($P < 0.05$). Reconstructed efficiency is the number of cloned embryos divided by the number of enucleated oocytes. NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells; FF cells, fetal fibroblast cells; AF cells, adult fibroblast cells; MGE cells, mammary gland epithelial cells.

Table 2 *In vivo* developmental ability of cloned embryos from different donor cells.

Type of donor cell	Number of two-cell embryos transferred into surrogate mothers	Number of fetuses	Pregnancy rate (%)
Transgenic cells			
NS cells	92	2	2.17 (2/92)
AFS cells	96	2	2.08 (2/96)
FF cells	89	0	0.00 (0/89)
AF cells	86	0	0.00 (0/86)
MGE cells	93	0	0.00 (0/93)
Non-transgenic cells			
NS cells	95	2	2.11 (2/95)
AFS cells	60	1	1.67 (1/60)
FF cells	60	0	0.00 (0/60)
AF cells	60	0	0.00 (0/60)
MGE cells	60	0	0.00 (0/60)

NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells; FF cells, fetal fibroblast cells; AF cells, adult fibroblast cells; MGE cells, mammary gland epithelial cells.

origin/type of nuclear donor cells and the *in vitro* techniques utilized to obtain 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 (Uhm *et al.* 2000, Park *et al.* 2001a, 2001b, Bordignon *et al.* 2003), puromycin (Watanabe *et al.* 2005) and blasticidin S (Michalak *et al.* 2004), or EGFP (Arat *et al.* 2001, 2002, Zhang *et al.* 2006). Since it was first introduced by Chalfie *et al.* (1994), the EGFP-mediated genetic reporter system, which

was derived from the bioluminescent 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 (Murakami *et al.* 1999, Funahashi *et al.* 2001, Devgan *et al.* 2004, Zhang *et al.* 2006). 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 (Ono *et al.* 2001, Hyun *et al.* 2003, Gong *et al.* 2004). Porcine EGFP transgene-expressing blastocysts and/or offspring have been created from cultured somatic cells (Park *et al.* 2001a, 2001b, Lai *et al.* 2002, Roh & Hwang 2002, Lee *et al.* 2003, 2005, Zhang *et al.* 2006). Although cloned embryos from NS cells had been produced (Eiji *et al.* 2006), there are no reports focused on applying EGFP-transgenic stem cells to porcine SCNT technology. This is the first report on production of cloned embryos using porcine NS and AFS cells transfected with EGFP. Our results demonstrated that the embryos could be produced using NS and AFS cells transfected with EGFP, and EGFP could be expressed in the cloned embryos and fetuses. Our results showed that there were significant differences ($P < 0.05$) in developmental potential between the cloned embryos derived from stem cells (NS and AFS cells) and somatic cells (FF, AF, and MGE cells), which acted as donor cells. The developmental potential of the cloned embryos derived from stem cells (NS and AFS cells) were higher than that of the cloned embryos derived from somatic cells (Tables 1 and 2).

We conclude that porcine NS and AFS cells are broadly multipotent, which could be induced to differentiate into cell types representing each embryonic

Figure 5 Cloned fetuses derived from NS and AFS cells. (A–D, H and I) Fetuses (28 days) from NS cells, (E–G and J) fetuses (32 d) from AFS cells, and (H–J) photos viewed under green fluorescence microscope. NS cells, neural stem cells; AFS cells, amniotic fluid-derived stem cells.

germ layer, including cells of neuronal, adipogenic, osteogenic, myogenic, and endothelial lineages. The developmental potential of the cloned embryos derived from stem cells (NS and AFS cells) were higher ($P < 0.05$) than that of the cloned embryos derived from somatic cells (FF, AF, and MGE cells), which suggests that the undifferentiated state of the donor cell increases cloning efficiency. EGFP could be expressed in the cloned fetuses, which will contribute to obtaining EGFP-transgenic porcines in later research.

Materials and Methods

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co.

Animals

Porcine fetuses were obtained off-site from a pregnant sow placed under general anesthesia and terminated prior to waking, according to a protocol approved by the Institutional Animal Care and Use Committee. All tissues were acquired in compliance with National Institutes of Health and institutional guidelines.

Isolation of NS, AFS, FF, AF, and MGE cells

The isolation of porcine NS cells followed the following protocol. An embryonic day 30 (E30) fetal pig was obtained off-site from a pregnant sow placed under general anesthesia and terminated prior to waking. The craniums were removed, and the subventricular zone of the brain was dissected and minced with fine scissors. The brain tissue were cut up into smaller pieces, and treated with enzyme solution, light inhibitory solution, and heavy inhibitory solution respectively in a tube at 37 °C. Then the heavy inhibitory solution was removed and 5 ml of NSCs basal medium was added. After the tissue chunks were broken up, the appropriate amount of cells (1×10^6 cells/6-cm plate or 2×10^6 cells/10-cm plate) was added into a non-coated bacterial plate containing NSCs complete medium. The cells were incubated in 37 °C, 5% CO₂ incubator for several days. Once the spheres had formed, the medium was replaced every three days. NSCs were passaged when the neurospheres were sufficiently large. RNA was then extracted from the neurospheres for RT-PCR analysis of the NSCs markers. NSCs complete medium was composed of 10 ml NSCs basal medium, 10 µl EGF (100 ng/µl), and 10 µl bFGF (10 ng/µl; Gibco). NSCs basal medium was composed of 48.5 ml of NSCs medium, 1 ml B27 supplement, and 0.5 ml N2 supplement (Gibco). NSCs medium was composed of 500 ml neurobasal medium (Gibco), 5 ml 100× penicillin–streptomycin, and 5 ml 100× l-glutamine. Enzyme solution was composed of 20 ml dissociation medium, 6.4 mg cysteine, and 200 µl papain (Roche). Heavy inhibitory solution was composed of 6 ml dissociation medium, 60 mg BSA, and 60 mg trypsin inhibitor. Light inhibitory solution was composed of 9 ml dissociation medium and 1 ml heavy inhibitory solution. Dissociation medium was composed of 98 mmol/l

Na₂SO₄, 30 mmol/l K₂SO₄, 5.8 mmol/l MgCl₂, 0.25 mmol/l CaCl₂, 1 mmol/l HEPES, 20 mmol/l glucose, 0.001% phenol red, and 0.125 mmol/l NaOH.

The isolation of porcine AFS cells followed the following protocol. Amniotic fluid was collected from E30 fetal porcine, and the recovered cells were cultured in 24-well dishes. After expansion to confluence, a single-cell suspension was prepared by gentle trypsinization, and cells were harvested by trypsinization. AFS cells were grown in α-MEM medium (Gibco, Invitrogen) containing 15% ES-FBS, 1% glutamine, and 1% penicillin/streptomycin (Gibco), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA, USA) at 37 °C with 5% CO₂ atmosphere. AFS cells were subcultured routinely at a dilution of 1:4 to 1:8 and not permitted to expand beyond 70% of confluence. Clonal AFS cell lines were generated by the limiting dilution method in 96-well plates. Medium was replaced every three days. AFS cells were passaged, and RNA was then extracted for RT-PCR analysis of the AFS cells markers.

The techniques used for isolation of porcine FF cells, AF, and MGE cells used in this study were similar as described previously (Zheng *et al.* 2006).

Transfection of EGFP gene into NS, AFS, FF, AF, and MGE cells

The day before transfection, NS, AFS, FF, AF, and MGE cells (at passage 3–6) were trypsinized, counted, and plated into 35-mm culture dishes to reach 80% confluency on the day of transfection. Totally 40 µl (Invitrogen) of Lipofectamine 2000 and pEGFP-N1 of 3 µg (Clontech) were dissolved into 200 µl serum-antibiotics-free culture medium (SFCM) respectively in two tubes. The solution of the two tubes were mixed together, and then one 600 µl SFCM was added. Then, 2 ml Lipofectamine 2000–pEGFP-N1 mixture was ready to be used. Cells were rinsed with SFCM and cultured in 1 ml Lipofectamine 2000–pEGFP-N1 mixture for 20 h. Then, the mixture was removed and the cells were cultured with culture medium containing G418 (350 µg/ml). Expression of EGFP in the cells was monitored under an inverted u.v. microscope (TE2000-U, Nikon Inc., Tokyo, Japan).

Differentiation of NS and AFS cells in culture

The protocols used for differentiation of porcine NS and AFS cells used in this study were similar to those described by De Coppi *et al.* (2007). RNA was then extracted for RT-PCR analysis to confirm lineage-specific gene expression.

RNA extraction and RT-PCR

Analysis of mRNA expression by RT-PCR was carried out using standard protocols. Briefly, total RNA was extracted from stem and differentiated cells, using Purescript RNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Any residual genomic DNA was eliminated by treatment with DNase (DNA-free; Ambion, Inc., Austin, TX, USA). PCR was carried out in a final volume of 50 µl with 3 µl of cDNA template, 0.75 µl of forward and

reverse primers (0.5 µg/µl; Qiagen), and 1.25 units of Taq DNA Polymerase (Amersham) on a Genius thermocycler (Technique, Minneapolis, MN, USA). Initial denaturation for 5 min at 95 °C was followed by 40 cycles of 30 s at 94 °C, 15 s at the corresponding annealing temperature, and 45 s at 72 °C. The final step consisted of 5 min of extension at 72 °C. PCR products were run on 2% agarose gels and visualized with ethidium bromide against a 50-bp DNA ladder.

In vitro maturation of pig oocytes

Ovaries were retrieved from prepubertal gilts at a local slaughterhouse and transported to the laboratory in physiological saline at 30–35 °C within 2 h. Oocytes were aspirated from antral follicles (3–7 mm in diameter) and cultured in a 100 µl droplet of maturation medium (BSA-free NCSU23 with 10% porcine follicular fluid, 0.1 mg/ml cysteine, 1% MEM non-essential amino acid, and 0.2 mM pyruvate) with hormonal supplementation (2 g/ml Follitropin-V, Vetrepfarm, Ontario, Canada) in NCSU23 medium at 38.5 °C under 5% CO₂ in air for 44 h.

Preparation of donor cells

NS, AFS, FF, AF, and MGE cells were used for NT respectively. Immediately before whole-cell injection, donor cells were trypsinized, washed by centrifugation, and resuspended in injection medium of TL-HEPES and 10% polyvinylpyrrolidone solution at 1:1.

Enucleation and whole-cell injection

Recipient oocytes were prepared by centrifugation for 10 min in an Eppendorf centrifuge at 12 000 *g* in 200 µl TL-HEPES medium to allow detection of the first polar body. Only oocytes with excellent morphology and a visible polar body were selected for this experiment. For enucleation, groups of oocytes were transferred into droplets of TL-HEPES containing 5 µg/ml cytochalasin B (CB), which had previously been placed in the operation chamber on the microscope stage. In the initial experiments, enucleation was accomplished by aspiration of the first polar body and the metaphase II plate in a small amount (<15% of the oocyte volume) of cytoplasm. Successful enucleation was confirmed by examination after staining with 5 µg/ml Hoechst 33342. Successful enucleation was confirmed by staining the isolated cytoplasm. Whole-cell injection was conducted according to the following protocol. Briefly, donor cells were transferred to TL-HEPES containing 10% (w/v) polyvinylpyrrolidone and kept at room temperature. A microdrop (10 µl) of injection medium under light mineral oil was placed in the lid of a 60-mm sterile culture dish, which was positioned on an inverted microscope (Olympus) equipped with micromanipulators (Nikon). Individual donor cells were aspirated into the injection pipette with a sharp, beveled tip (inner diameter 10–12, 15–18, and 20–25 µm), and injected into an enucleated oocyte via the slit that was already made during the enucleation process. The cell expelled into the cytoplasm of the oocyte.

Activation of oocytes

Reconstructed embryos were washed and pre-incubated for 20 s in activation medium (0.25 M mannitol solution supplemented with 0.01% polyvinyl alcohol, 0.5 mM HEPES, 0.1 mM CaCl₂-H₂O, and 0.1 mM MgCl₂-6H₂O with pH 7.2) at room temperature. Electrical stimulation was delivered with a BTX Electro Cell Manipulator (Biotechnologies and Experimental Research, Inc., San Diego, CA, USA) to a chamber with two parallel platinum wire electrodes (200 µm outer diameter), spaced 1 mm apart overlaid with activation medium. The reconstructed oocytes were exposed to an electrical pulse for 10 s at 5 V AC followed by a 1 × 30 µs pulse at 2.2 kV/cm DC at room temperature. Non-manipulated, but u.v.-exposed, oocytes were activated 3 h after u.v. exposure as a control. Following somatic cell injections, oocytes were either immediately activated or then cultured in NCSU23 medium containing 10 µg/ml CB and cycloheximide for 5 h or left in NCSU23 medium at 38.5 °C under 5% CO₂ in air for 1.5, 3, and 6 h before electrical activation treatment.

In vitro culture of cloned embryos

After activation treatments, surviving cloned embryos were thoroughly washed and cultured in 50-µl drops of NCSU23 supplemented with 1% MEM non-essential amino acid and 0.4 mg/ml BSA for seven days at 38.5 °C in 5% CO₂ in air without a medium change. After 72–96 h of *in vitro* culture, cleavage-stage embryos were selected. Then 20–40 of cleaved embryos were cultured together in a 50-µl drop of NCSU23 medium supplemented with 10% FBS at 38.5 °C in an incubator with 100% humidity and 5% CO₂ in air for additional 72 h. At the end of the *in vitro* culture period (days 6–7), embryos were evaluated morphologically for blastocyst formation. Two-cell rate, four- to eight-cell rate and morula–blastocyst rate were evaluated under a stereomicroscope.

Surgical embryo transfer and pregnancy diagnosis

The two-cell stage-cloned embryos derived from each cell line (NS, AFS, FF, AF, and MGE cells) were transferred into the oviducts of the naturally cycling gilts on the first day of standing estrus. Examination of the ovaries during embryo transfer confirmed that none of the surrogates had completed ovulation. Non-return surrogates were checked for pregnancy by transabdominal ultrasound examination at day 25 after embryo transfer and at 2 weeks.

Statistical analysis

All data were pooled and then tested by χ^2 -analysis for significant differences. Differences between the experimental groups were considered to be significant at a level of $P < 0.05$.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported, and there is no any financial or other potential conflict of interest.

Funding

This work was supported by a grant from Northwest A&F University Research Foundation (07ZR001).

Acknowledgements

We thank everyone for assistance with this project.

References

- Arat S, Rzucidlo SJ, Gibbons J, Miyoshi K & Stice SL 2001 Production of transgenic bovine embryos by transfer of transfected granulosa cells into enucleated oocytes. *Molecular Reproduction and Development* **60** 20–26.
- Arat S, Gibbons J, Rzucidlo SJ, Respass DS, Tumlin M & Stice SL 2002 *In vitro* development of bovine nuclear transfer embryos from transgenic clonal lines of adult and fetal fibroblast cells of the same genotype. *Biology of Reproduction* **66** 1768–1774.
- Bethhauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R *et al.* 2000 Production of cloned pigs from *in vitro* systems. *Nature Biotechnology* **18** 1055–1058.
- Boquest AC, Grupen CG, Harrison SJ, McIlpatrick SM, Ashman RJ, D'Apice AJF & Nottle MB 2002 Production of cloned pigs from cultured fetal fibroblast cells. *Biology of Reproduction* **66** 1283–1287.
- Bordignon V, Keyston R, Lazaris A, Bilodeau AS, Pontes JH, Arnold D, Fecteau G, Keefer C & Smith LC 2003 Transgene expression of green fluorescent protein and germ line transmission in reconstructed calves derived from *in vitro*-transfected somatic cells. *Biology of Reproduction* **68** 2013–2023.
- Chalfie M, Tu Y, Euskirchen G, Ward WW & Prasher DC 1994 Green fluorescent protein as a marker for gene expression. *Science* **263** 802–805.
- Chen XD, Qian HY, Neff L, Satomura K & Horowitz MC 1999 Thy-1 antigen expression by cells in the osteoblast lineage. *Journal of Bone and Mineral Research* **14** 362–375.
- Cheong HT, Takahashi Y & Kanagawa H 1993 Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. *Biology of Reproduction* **48** 958–963.
- De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ *et al.* 2007 Isolation of amniotic stem cell lines with potential for therapy. *Nature Biotechnology* **25** 100–106.
- Devgan V, Rao MR & Seshagiri PB 2004 Impact of embryonic expression of enhanced green fluorescent protein on early mouse development. *Biochemical and Biophysical Research Communications* **313** 1030–1036.
- Du Y, Kragh PM, Zhang Y, Li J, Schmidt M, Bøgh IB, Zhang X, Purup S, Jørgensen AL, Pedersen AM *et al.* 2007 Piglets born from handmade cloning, an innovative cloning method without micromanipulation. *Theriogenology* **68** 1104–1110.
- Eiji M, Hiroshi O, Satoshi K, Nguyen VT, Takafusa H, Sayaka W, Mitsuko K, Eimei S & Teruhiko W 2006 Developmental ability of cloned embryos from neural stem cells. *Reproduction* **132** 849–857.
- Ellis P, Fagan BM, Magness ST, Hutton S, Taranova O, Hayashi S, McMahon A, Rao M & Pevny L 2004 SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. *Developmental Neuroscience* **26** 148–165.
- Evans MJ & Kaufman MH 1981 Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292** 154–156.
- Funahashi H, Ideta A, Konishi M, Urakawa M, Urano K, Aoyagi Y, Okabe M & Niwa K 2001 Nuclear transfer of blastomeres expressing EGFP-reporter gene may improve the efficiency of transgenic cattle. *Cloning Stem Cells* **3** 183–190.
- Gage FH 2000 Mammalian neural stem cells. *Science* **287** 1433–1438.
- Gong G, Dai Y, Fan B, Zhu H, Zhu S, Wang H, Wang L, Tang B, Li R, Wan R *et al.* 2004 Birth of calves expressing the enhanced green fluorescent protein after transfer of fresh or vitrified/thawed blastocysts produced by somatic cell nuclear transfer. *Molecular Reproduction and Development* **69** 278–288.
- Hiiragi T & Solter D 2005 Reprogramming is essential in nuclear transfer. *Molecular Reproduction and Development* **70** 417–421.
- Hyun S, Lee G, Kim D, Kim H, Lee S, Nam D, Jeong Y, Kim S, Yeom S, Kang S *et al.* 2003 Production of nuclear transfer-derived piglets using porcine fetal fibroblasts transfected with the enhanced green fluorescent protein. *Biology of Reproduction* **69** 1060–1068.
- Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A & Prather RS 2004 *In vitro* development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. *Theriogenology* **61** 1125–1135.
- Im't Anker PS, Scherjon SA, Kleijburg-Van Der KC, Noort WA, Claas Frans HJ, Willemze R, Fibbe WE & Kanhal Humphrey HH 2003 Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* **102** 1548–1549.
- James AT, Joseph IE, Sander SS, Michelle AW, Jennifer JS, Vivienne SM & Jeffrey MJ 1998 Embryonic stem cell lines derived from human blastocysts. *Science* **282** 1145–1147.
- Jingli C, Yuanyuan W, Takumi M, Jeanne LP, Mary TL, Kurt HA, Gerald JS & Mahendra SR 2002 Properties of a fetal multipotent neural stem cell (NEP cell). *Developmental Biology* **251** 221–240.
- Lagutina I, Lazzari G & Galli C 2006 Birth of cloned pigs from zona-free nuclear transfer embryos developed *in vitro* to blastocyst before transfer. *Cloning and Stem Cells* **8** 283–293.
- Lai L, Park KW, Cheong HT, Kühholzer B, Samuel M, Bonk A, Im GS, Rieke A, Day BN, Murphy CN *et al.* 2002 Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells. *Molecular Reproduction and Development* **62** 300–306.
- Lee JW, Wu SC, Tian XC, Barber M, Hoagland T, Riesen J, Lee KH, Tu CF, Cheng WTK & Yang XZ 2003 Production of cloned pigs by whole-cell intracytoplasmic microinjection. *Biology of Reproduction* **69** 995–1001.
- Lee GS, Kim HS, Hyun SH, Lee SH, Jeon HY, Nam DH, Jeong YW, Kim S, Kim JH, Han JY *et al.* 2005 Production of transgenic cloned piglets from genetically transformed fetal fibroblasts selected by green fluorescent protein. *Theriogenology* **63** 973–991.
- Martin GR 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *PNAS* **78** 7634–7638.
- Michalak E, Katska-Ksiazkiewicz L, Kalak R, Lipinski D, Wielgus K, Jarmuz M, Szalata M, Rynska B, Smorag Z & Slomski R 2004 *In vitro* transfection of fibroblasts and evaluation of transgenesis in long-term cell cultures. *Annals of Animal Science* **4** 233–240.
- Murakami M, Fahrudin M, Varisanga MD & Suzuki T 1999 Fluorescence expression by bovine embryos after pronuclear microinjection with the EGFP gene. *Journal of Veterinary Medical Science* **61** 843–847.
- Oback B & Wells D 2002 Donor cells for nuclear cloning: many are called, but few are chosen. *Cloning Stem Cells* **4** 147–168.
- Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H & Perry AC 2000 Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289** 1188–1190.
- Ono Y, Shimozawa N, Ito M & Kono T 2001 Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biology of Reproduction* **64** 44–50.
- Park KW, Cheong HT, Lai L, Im GS, Kuhholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN *et al.* 2001a Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. *Animal Biotechnology* **12** 173–181.
- Park KW, Kühholzer B, Lai L, Machaty Z, Sun QY, Day BN & Prather RS 2001b Development and expression of the green fluorescent protein in porcine embryos derived from nuclear transfer of transgenic granulosa-derived cells. *Animal Reproduction Science* **68** 111–120.
- Philip HS, Peter JB, Tannin JF, Hailing S, Diane KO & Henry K 2003 Isolation and characterization of neural progenitor cells from post-mortem human cortex. *Journal of Neuroscience Research* **74** 838–851.
- Philip HS, Hubert N, Ivan IK, Boback Z, Michael JY & Henry K 2005 Expression of neurodevelopmental markers by cultured porcine neural precursor cells. *Stem Cells* **23** 1286–1294.
- Polejaeva IA 2001 Cloning pigs: advances and applications. *Reproduction* **58** 293–300.

- Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL *et al.* 2000 Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407** 86–90.
- Prather RS, Tao T & Machaty Z 1999 Development of the techniques for nuclear transfer in pigs. *Theriogenology* **51** 487–498.
- Prusa A, Marton E, Rosner M, Bettelheim D, Lubec G, Pollack A, Bernaschek G & Hengstschläger M 2004 Neurogenic cells in human amniotic fluid. *American Journal of Obstetrics and Gynecology* **191** 309–314.
- Reynolds BA & Weiss S 1992 Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255** 1707–1710.
- Reynolds BA & Weiss S 1996 Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Developmental Biology* **175** 1–13.
- Richard JEA, Carrie BH, Pam T, Emma LR, Andrew R, Stephen BD, Anne ER & Roger AB 2002 The potential for circuit reconstruction by expanded neural precursor cells explored through porcine xenografts in a rat model of Parkinson's disease. *Experimental Neurology* **175** 98–111.
- Rideout WM, Wakayama T, Wutz A, Eggan K, Jackson-Grusby L, Dausman J, Yanagimachi R & Jaenisch R 2000 Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nature Genetics* **24** 109–110.
- Rizzuto R, Brini M, De Giorgi F, Rossi R, Heim R, Tsien RY & Pozzan T 1996 Double labelling of subcellular structures with organelle-targeted GFP mutants *in vivo*. *Current Biology* **6** 183–188.
- Roh S & Hwang WS 2002 *In vitro* development of porcine parthenogenetic and cloned embryos: comparison of oocyte-activating techniques, various culture systems and nuclear transfer methods. *Reproduction, Fertility, and Development* **14** 93–99.
- Siddiqui MM & Atala A 2004 Amniotic fluid-derived pluripotent cells: adult and fetal. *Handbook of Stem Cells* **2** 175–180.
- Tsai MS, Lee JL, Chang YJ & Hwang SM 2004 Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Human Reproduction* **19** 1450–1456.
- Uhm SJ, Kim NH, Kim T, Chung HM, Chung KH, Lee HT & Chung KS 2000 Expression of enhanced green fluorescent protein and neomycin resistant (NeoR) genes in porcine embryos following nuclear transfer with porcine fetal fibroblasts transfected by retrovirus vector. *Molecular Reproduction and Development* **53** 331–337.
- Wacker I, Kaether C, Kromer A, Migala A, Almers W & Gerdes HH 1997 Microtubule-dependent transport of secretory vesicles visualized in real time with a GFP-tagged secretory protein. *Journal of Cell Science* **110** 1453–1463.
- Wakayama T, Rodriguez I, Perry AC, Yanagimachi R & Mombaerts P 1999 Mice cloned from embryonic stem cells. *PNAS* **96** 14984–14989.
- Watanabe S, Iwamoto M, Suzuki S, Fuchimoto D, Honma D, Nagai T, Hashimoto M, Yazaki S, Sato M & Onishi A 2005 A novel method for the production of transgenic reconstructed pigs: electroporation-mediated gene transfer to non-cultured cells and subsequent selection with puromycin. *Biology of Reproduction* **72** 309–315.
- Wolf E, Zakhartchenko V & Brem G 1998 Nuclear transfer in mammals: recent developments and future perspectives. *Journal of Biotechnology* **65** 99–110.
- Yamazaki Y, Makino H, Hamaguchi-Hamada K, Hamada S, Sugino H, Kawase E, Miyata T, Ogawa M, Yanagimachi R & Yagi T 2001 Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer. *PNAS* **98** 14022–14026.
- Yin XJ, Tani T, Yonemura I, Kawakami M, Miya-moto K, Hasegawa R, Kato Y & Tsunoda Y 2002 Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. *Biology of Reproduction* **67** 442–446.
- Zhang Y, Pan D, Sun X, Sun G, Wang X, Liu X, Li Y, Dai Y & Li N 2006 Production of porcine cloned transgenic embryos expressing green fluorescent protein by somatic cell nuclear transfer. *Science in China. Series C, Life Sciences* **49** 164–171.
- Zheng YM, An ZX, Peng XR, Shi YQ & Zhang Y 2006 EGFP expression in goat mammary epithelial cells. *Chinese Journal of Agricultural Biotechnology* **3** 71–74.

Received 11 November 2008

First decision 27 January 2009

Accepted 4 March 2009