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

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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 (osteonectin$^+$ 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.

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Introduction

The development of nuclear transfer (NT) techniques have enabled researchers to generate cloned pigs (Betthauser 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 than the donor cell for NT (Oback & 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 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 (osteonectin+ and osteocalcin+), 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),
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 (Betheauser 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 3 Differentiated cells derived from EGFP gene transfected NS (A1–F1) and AFS (A2–F2) cells viewed under green fluorescence microscope, 10 X. (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.
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+, 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.

<table>
<thead>
<tr>
<th>Type of donor cell</th>
<th>Reconstructed efficiency (%)</th>
<th>Two-cell rate (%)</th>
<th>Four- to eight-cell rate (%)</th>
<th>Morula–blastocyst rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS cells</td>
<td>82.97 (268/323)a</td>
<td>64.18 (172/268)a</td>
<td>45.90 (123/268)a</td>
<td>29.10 (78/268)a</td>
</tr>
<tr>
<td>AFS cells</td>
<td>81.48 (286/351)a</td>
<td>65.03 (186/286)a</td>
<td>45.10 (129/286)a</td>
<td>30.07 (86/286)a</td>
</tr>
<tr>
<td>FF cells</td>
<td>81.62 (191/234)a</td>
<td>53.40 (102/191)b</td>
<td>32.98 (63/191)b</td>
<td>18.32 (35/191)b</td>
</tr>
<tr>
<td>AF cells</td>
<td>79.70 (161/202)a</td>
<td>52.80 (85/161)b</td>
<td>32.30 (52/161)b</td>
<td>18.01 (29/161)b</td>
</tr>
<tr>
<td>MGE cells</td>
<td>78.67 (166/221)a</td>
<td>51.20 (85/166)b</td>
<td>29.82 (48/166)b</td>
<td>16.27 (27/166)b</td>
</tr>
<tr>
<td>NS cells</td>
<td>82.88 (92/111)b</td>
<td>65.22 (60/92)b</td>
<td>44.57 (41/92)b</td>
<td>28.26 (26/92)b</td>
</tr>
<tr>
<td>AFS cells</td>
<td>80.83 (97/120)b</td>
<td>63.92 (62/97)b</td>
<td>44.33 (41/97)b</td>
<td>28.87 (28/97)b</td>
</tr>
<tr>
<td>FF cells</td>
<td>81.37 (83/102)b</td>
<td>50.60 (42/83)b</td>
<td>32.53 (27/83)b</td>
<td>19.28 (16/83)b</td>
</tr>
<tr>
<td>AF cells</td>
<td>80.19 (85/106)b</td>
<td>54.12 (46/85)b</td>
<td>34.12 (29/85)b</td>
<td>17.65 (15/85)b</td>
</tr>
<tr>
<td>MGE cells</td>
<td>79.00 (79/100)b</td>
<td>53.16 (42/79)b</td>
<td>29.11 (23/79)b</td>
<td>17.72 (14/79)b</td>
</tr>
</tbody>
</table>

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.
developmental ability of cloned embryos from different
donor cells.

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

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.

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 (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
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 porcine 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 × 10⁶ cells/6-cm plate or 2 × 10⁶ 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 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% 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
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, Vetrepharm, 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 χ2-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.
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