Pig Cloning by Microinjection of Fetal Fibroblast Nuclei

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Pig cloning will have a marked impact on the optimization of meat production and xenotransplantation. To clone pigs from differentiated cells, we microinjected the nuclei of porcine (Sus scrofa) fetal fibroblasts into enucleated oocytes, and development was induced by electroactivation. The transfer of 110 cloned embryos to four surrogate mothers produced an apparently normal female piglet. The clonal provenance of the piglet was indicated by her coat color and confirmed by DNA microsatellite analysis.

Live births of cloned sheep (1), cattle (2), and goats (3) have been achieved by somatic cell transfer, in which a nucleus donor cell is fused with an enucleated oocyte (4). The single report of pig cloning also used this method, with an undifferentiated early embryonic cell (the blastomere of a four-cell embryo) as the nucleus donor (5). Clonal propagation of selected porcine phenotypes is potentially important in meat production. In addition, genetic modification could be combined with cloning in the provision of potential donors for xenotransplantation to humans (6).

As a first step toward pig cloning from differentiated cells, we investigated parameters that might affect porcine embryogenesis in vitro. Mature oocytes were isolated from females of the Landrace breed (7) and were parthenogenetically stimulated to initiate embryonic development by one of two electroactivation protocols (8). Embryos were then exposed to the microfilament inhibitor cytochalasin B (to prevent chromosome loss by cytokinesis) and were incubated in one of three culture media under otherwise identical conditions (9) (Table 1). Fewer embryos developed after multiple pulses than after a single pulse at a slightly higher field strength (Table 1). In addition, development was influenced by the type of culture media, with the highest development to the blastocyst stage supported by culture in NCSU23 (Table 1).

Several reports of livestock cloning describe the use of fetal fibroblasts as nucleus donors (11–13). Moreover, these cells can be genetically modified before cloning (11–13). We therefore evaluated the ability of porcine fetal fibroblasts to support development to term after nuclear transfer. Fetuses derived from a Meishan × Meishan (black coat) fetal fibroblasts were each introduced into a single enucleated oocyte by piezo-activated microinjection (17, 19). Reconstructed preembryos were incubated at 38.5°C for 3 to 4 hours before electroactivation and culture of the resultant nuclear transfer embryos (8, 9).

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Table 1. Effect of different electroactivation protocols and culture media on in vitro porcine embryo development. Oocytes were matured in vivo. Percentages are of oocytes surviving electroactivation.

<table>
<thead>
<tr>
<th>Media</th>
<th>Electroactivating pulse</th>
<th>Number of oocytes surviving</th>
<th>Two-cell embryos</th>
<th>Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strength (kV/cm)</td>
<td>Duration (µs)</td>
<td>Number</td>
<td>Number</td>
</tr>
<tr>
<td>B ECM3</td>
<td>1.5</td>
<td>100</td>
<td>1</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>60</td>
<td>3</td>
<td>101</td>
</tr>
<tr>
<td>mW M</td>
<td>1.5</td>
<td>100</td>
<td>1</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>60</td>
<td>3</td>
<td>96</td>
</tr>
<tr>
<td>NCSU23</td>
<td>1.5</td>
<td>100</td>
<td>1</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>60</td>
<td>3</td>
<td>103</td>
</tr>
</tbody>
</table>

Although porcine embryos can develop well in vitro to the blastocyst stage, their subsequent development in utero after transfer to the uterus of surrogates is poor (20). Moreover, because pigs typically require at least four fetuses for a successful pregnancy, we reasoned that the presence of helper embryos produced by fertilization might assist the full development of cloned embryos. We therefore conducted two series of experiments to investigate the potential of helper (fertilized) embryos to assist in development in utero. Cloned embryos were transferred to surrogates after culture in vitro for 20 hours (series A, one-cell embryos) or 40 hours (series B, two- to four-cell embryos) (21). All offspring (9 from series A and 24 from series B) were white and therefore of nonclonal origin (Table 2). The failure of full-term development of cloned em-
Intracytoplasmic sperm injection was used to obtain four clones among these four term pregnancies may suggest that if the ratio of nuclear transfer to helper (fertilized) embryos is important, it was clearly not optimized in these experiments.

A third experimental series (series C) was conducted in which 110 cloned embryos were transferred to the two- to eight-cell stage (Fig. 1A) between four surrogates that did not harbor helper (fertilized) embryos (Table 2). The cloned embryos were derived from fibroblast cultures at passages two to six. Three of the four surrogates returned to estrus 27, 35, or 61 days after transfer. The delayed resumption of estrus is expected since the cloned embryos were not derived from fibroblast cultures at passage two. One of the embryos developed to term, and the resulting piglet, named Xena, was delivered by natural birth on 2 July 2000, with birth and placental weights of 1.2 and 0.3 kg, respectively (both in the normal range for noncloned offspring). Her associated placenta was apparently anatomically normal. Some cloned cattle exhibit placental abnormalities (22, 23), and the placentas of mice cloned by nuclear microinjection are invariably larger than those of nonclones (24, 25).

R E PORTS

Table 2. Development of cloned embryos in vitro and to term following transfer to surrogate mothers.

<table>
<thead>
<tr>
<th>Experiment series</th>
<th>Number of oocytes surviving</th>
<th>Number of survivors activated</th>
<th>Number of embryos developed</th>
<th>Number of embryos transferred</th>
<th>Number of surrogates</th>
<th>Number of offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>108</td>
<td>102</td>
<td>99</td>
<td>3</td>
<td>3</td>
<td>9 White</td>
</tr>
<tr>
<td>B</td>
<td>120</td>
<td>107</td>
<td>44</td>
<td>24</td>
<td>3</td>
<td>24 White</td>
</tr>
<tr>
<td>C</td>
<td>210</td>
<td>188</td>
<td>78</td>
<td>50</td>
<td>4</td>
<td>1 Black</td>
</tr>
</tbody>
</table>

Fig. 2. Representative PCR analyses of microsatellite markers in genomic DNA from the Landrace surrogate mother (m), the cloned piglet Xena (c), and her progenitor fibroblast culture derived from a single female Meishan × Meishan fetus (f). Each panel shows data for a randomly selected microsatellite-specific primer pair: (A) SW1311, (B) SW1327, (C) SWR414, and (D) SW717. Traces were produced on a 373A Autosequencer (26). Sizes are in base pairs.

Fig. 1. (A) Embryos clonally derived by microinjecting the nuclei of Meishan × Meishan (black coat) fetal fibroblasts into Landrace (white coat) enucleated oocytes. These embryos are at the four-cell stage after 40 hours of culture in vitro. Phase contrast microscopy is ×100. (B) Xena, the cloned piglet, at 24 hours, showing her black coat color; her white-colored Landrace surrogate mother is in the background.

References and Notes

7. Oocytes were collected from Landrace (white coat) or Landrace × Large White pigs crossed with Duroc boars (which would give animals that were white with black spots). Mature oocytes were from postpubertal (≥6 months old) and prepubertal gilts. Preparation for ovulation was after the induced abortion of (postpubertal) pregnant gilts at E21 to E40 (E0 is the day of insemination) by intramuscular (im) injection with 0.2 mg of the prostaglandin F2α analog, (−)-cloprostenol (Planate, Osaka, Japan). This was followed 24 hours later by im injection of 0.2 mg of cloprostenol and 1500 international units (IU) of equine chorionic gonadotrophin (eCG). Preparation of prepubertal gilts was by a single im injection of 1500 IU of microinjection selectively removes much of the donor cell cytoplasm so that it is relatively dilute in the early embryo.

Random integration and gene targeting of cells in vitro, followed by clonal derivation, has been used to introduce germ line mutations in cattle (12) and sheep (11, 13). These findings raise the prospect that this approach can also be applied to pigs. Such an approach is particularly promising because pig embryonic stem cells have not been cultured. Porcine genome manipulation will also be assisted by the replication of individuals harboring desired genotypes, occurring either through conventional breeding or transgenesis. Our results, together with the recent report of intracytoplasmic sperm injection in the pig (27), indicate the potential of microinjection to facilitate porcine cloning.
eCG followed 72 hours later by 500 IU of injection of hCG. Gilts were slaughtered 45 hours after hCG administration, and oocytes were recovered by flushing oviducts with Ca-free, Mg-free Dulbecco’s phosphate-buffered saline (PBS) supplemented with 0.1% (v/v) bovine serum albumin (BSA). Oocytes were held in culture medium at 38.5°C in a water-saturated atmosphere of 5% CO₂ in air until required.

8. Electroactivation was typically 54 to 55 hours after hCG injection (3 to 4 hours after nucleus microinjection) in an activation medium containing 280 mM D-mannitol, 0.05 mM CaCl₂, 0.1 mM MgSO₄, and 0.01% (v/v) BSA. Pulses were delivered to cells in the chamber of an SSH-2 somatic hybridizer (Shimadzu, Kyoto, Japan). The number, intensity, and duration of pulses are detailed in Table 1. After the final pulse, oocytes were transferred for culture in medium supplemented with cytochalasin B (5 μg/ml) for 2 hours to prevent cytokinesis, after which culture was continued in fresh medium lacking cytochalasin B.

9. Embryo culture was in 100-μl droplets of medium under mineral oil at 38.5°C in a water-saturated atmosphere of 5% CO₂ in air. Unless stated otherwise, the culture medium was NCSU23 [R. D. Metters and K. D. Wells, J. Reprod. Fertil. 48 (suppl.), 61 (1993)] supplemented with 0.4% (v/v) BSA and 0.01% (v/v) cysteine. Two other media were used in preliminary studies: Beltsville embryo culture medium (BECM3) [J. R. Dobrinsky et al., J. Anim. Sci. 71, 1561 (1993)].


21. Preparation of surrogate mothers (surrogates) was as follows. Pregnant Landrace × Large White × Duroc crosses inseminated by Landrace boars were terminated at E21 to E40 by im injection of 0.2 mg of cloprostenol as described in (7), except that the second injection was accompanied by a reduction in eCG to 1000 IU. Induction of estrus in surrogates by im injection of hCG (500 IU) was 72 hours before activation. Seven of the ten surrogates used in this study were artificially inseminated with Landrace boar semen 24 hours after hCG injection to generate “helper” embryos, and one side of the oviduct was flushed at the time of embryo transfer in preparation for the cloned embryos. The cloned embryos were transferred to oviducts 48 or 68 hours after hCG injection, 20 or 40 hours after electroactivation, respectively. One of the four noninseminated surrogate carrying embryos 40 hours after activation gave birth to the cloned offspring Xena.


25. T. Wakayama, personal communication.

26. PCR analysis of genomic DNA in ear-punch biopsies of the surrogate mother, the cloned piglet Xena, and her fetal fibroblast progenitor culture was performed with 23 porcine-specific microsatellite markers [G. A. Rohrer et al., Genome Res. 6, 371 (1996)]. Samples were processed blind by means of a 373A Autosequencer supported by GenoType software (PE Biosystems, Foster City, CA). The following markers were used: SW286, SW840, SW957, SW133, SW274, SW373, SW491, SW839, SW742, SW1327, SW1311, SW122, SW435, SW540, SW942, SW1021, SW1339, SW249, SW426, SW524, SW414, and SW717. Data from three marker sets (SW133, SW274, and SW1021) were inconclusive. The characteristics of all remaining marker sets were shared between genomic DNA from the cloned piglet Xena and the fibroblast culture but were distinct from those of the Landrace surrogate mother.


28. This work was supported in part by a Grant-in-Aid (Bio Cosmos Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan. We are grateful to T. Wakayama and E. Nemeth for incisive comments and suggestions during manuscript preparation.

17 July 2000, accepted 27 July 2000