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).

Subsequently, a single 100-μs activating pulse of 1.5 kV/cm was employed with culture in NCSU23. Under these conditions, we determined the developmental potential of mature versus in vitro–matured oocytes (*10*). Of 167 in vitro–matured oocytes subjected to this protocol, 116 (69.5%) cleaved within 48 hours, but only 4 (2.4%) developed to blastocysts. This value (2.4%) is significantly lower ($P = 0.001$, χ^2 test) than the corresponding value for mature oocytes (31.2%) (Table 1). The subsequent experiments therefore used mature oocytes that had developed in vivo (*7*).

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) cross were killed at embryonic day 24 (E24) and used to establish primary cell cultures after dispersal by trypsinization (*14*). Cultures were established by plating cells at high density for two to six passages, after which they were allowed to reach confluence. Culture was continued for 16 days without media replenishment, producing cells at cell cycle phase G_0 (*14, 15*). Polymerase chain reaction (PCR) analysis (*14*) of the fibroblasts revealed the sex of the progenitor fetus in each case (*16*). Consistent with their fibroblast origin, the cultured cells were negative for cytokeratin and stage-specific embryonic antigen–1 but were strongly positive for the mesodermal marker vimentin (*16*).

Recently, mice were cloned from adult somatic cells by a distinctive nonfusion method in which donor nuclei were selectively introduced into enucleated oocytes by piezo-actuated microinjection (*17*). We adopted this method for porcine nuclear transfer. Oocytes from white (Landrace) or white-black spotted (Landrace × Large White × Duroc) gilts were depleted of metaphase II chromosomes and the first polar body by piezo-actuated micromanipulation in NCSU23 containing cytochalasin B (*18*). The nuclei of Meishan × Meishan (black coat) fetal fibroblasts were each introduced into a single enucleated oocyte by piezo-actuated 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*).

Although porcine embryos can develop well in vitro to the blastocyst stage, their subsequent development in utero after transfer to the uterine horns 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-

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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.

Media	Electroactivating pulse			Number of oocytes surviving	Two-cell embryos		Blastocysts	
	Strength (kV/cm)	Duration (μs)	Number		Number	Percentage	Number	Percentage
BECM3	1.5	100	1	105	73	69.5	16	15.2
	1.3	60	3	101	78	77.2	11	10.9
mWM	1.5	100	1	101	61	60.4	4	4.0
	1.3	60	3	96	60	62.5	1	1.0
NCSU23	1.5	100	1	109	99	90.8	34	31.2
	1.3	60	3	103	89	86.4	22	21.4

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

Experiment series	Number of oocytes surviving	Number of survivors activated	Number of embryos developed				Number of embryos transferred	Number of surrogates	Number of offspring	Coat color
			1 cell	2 cell	4 cell	8 cell				
A	108	102	99	3	0	0	96	3	9	White
B	120	107	44	24	20	19	63	3	24	White
C	210	188	78	50	58	2	110	4	1	Black

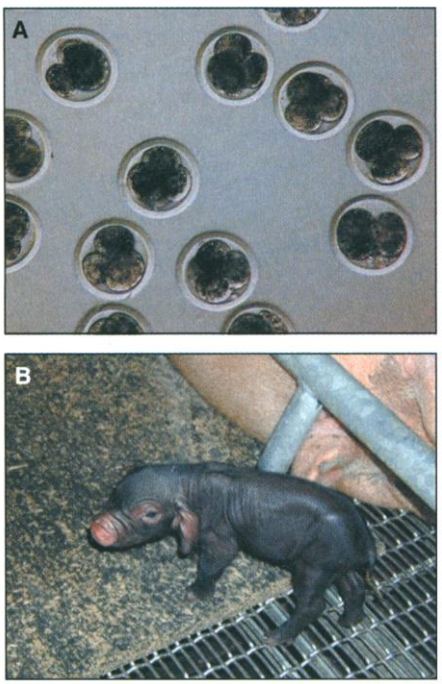

Fig. 1. (**A**) Embryos clonally derived by microinjecting the nuclei of Meishan × Meishan (black coat) fetal fibroblasts into Landrace (white coat) enucleated oocytes. These embryos are at the four-cell stage after 40 hours of culture in vitro. Phase contrast microscopy is ×100. (**B**) Xena, the cloned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

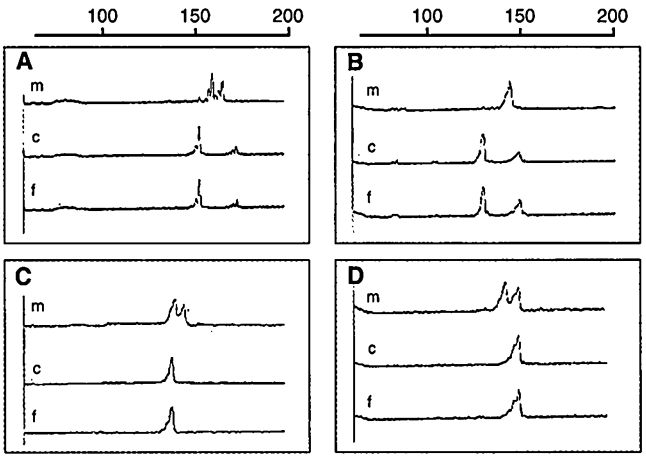

Fig. 2. Representative PCR analyses of microsatellite markers in genomic DNA from the Landrace surrogate mother (m), the cloned piglet Xena (c), and her progenitor fibroblast culture derived from a single female Meishan × Meishan fetus (f). Each panel shows data for a randomly selected microsatellite-specific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

bryos 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 at 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 suggests that each surrogate had become pregnant; the porcine estrous cycle lasts 21 days. Because embryo-uterine attachment occurs at E13 to E14 in the pig, it is likely that development terminated after placentation in two of the pregnancies. We do not know the cause(s) of the terminations.

A fourth surrogate in series C maintained pregnancy after the oviductal transfer of 36 cloned embryos that were 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*).

Xena (Fig. 1B) is a healthy female with a black coat, as predicted by the clonal derivation of her Meishan × Meishan nuclear genome. To corroborate this derivation, we subjected genomic DNA from Xena, her Landrace surrogate mother, and the fibroblast cell culture from which Xena was derived to porcine strain-specific microsatellite analysis, with 23 marker sets. The analysis was performed "blind" in another laboratory (*26*). These analyses (Fig. 2) confirm that Xena shares the genome of her Meishan fibroblast progenitor, distinct from that of her Landrace surrogate.

We have here shown that pigs can be cloned by microinjection of somatic cell nuclei into enucleated oocytes. We do not know all of the reasons for our success but speculate that it may derive in part from the rapidity of micromanipulation afforded by piezo-actuation of the micropipette. Furthermore, it is possible that success in porcine cloning is sensitive to contamination by nucleus donor cell cytoplasm. This would favor nuclear transfer by microinjection as opposed to fusion (cell transfer); unlike fusion, 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.

References and Notes

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7. Oocytes were collected from Landrace (white coat) or Landrace × Large White gilts 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 coinjection of 0.2 mg of cloprostenol and 1500 international units (IU) of equine chorionic gonadotrophin (eCG). Superovulation was induced 72 hours after eCG by an im injection of 500 IU of human chorionic gonadotrophin (hCG). Preparation of prepubertal gilts was by a single im injection of 1500 IU of

eCG followed 72 hours later by 500 IU of im 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% (w/v) bovine serum albumin (BSA). Oocytes were held in culture medium at 38.5°C in a water-saturated atmosphere of 5% CO_2 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 $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/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% CO_2 in air. Unless stated otherwise, the culture medium was NCSU23 [R. M. Petters and K. D. Wells, *J. Reprod. Fertil.* **48** (suppl.), 61 (1993)] supplemented with 0.4% (w/v) BSA and 0.01% (w/v) cysteine. Two other media were used in preliminary studies: Beltsville embryo culture medium (BECM3) [J. R. Dobrinsky *et al.*, *Biol. Reprod.* **55**, 1069 (1996)] and modified Whitten's medium (mWM) [C. R. Youngs *et al.*, *J. Anim. Sci.* **71**, 1561 (1993)].
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14. Fibroblasts for nuclear transfer were obtained from fetuses produced by a Meishan × Meishan (black coat) cross 24 days after insemination. Fetuses were removed, and the tissue was cut into small pieces with fine scissors. Cells were dispersed by incubation for 3 hours at 4°C in PBS containing 0.25% (w/v) trypsin and 1 mM EDTA, after which they were washed once in Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco). Fetal cultures were established independently, without mixing, by plating at high density in DMEM supplemented with 10% (v/v) FCS. Culture was for two to six passages; with splits of 1:2 at each passage, we estimate that this represents 3 to 12 population doublings. Nucleus donor cell cultures were allowed to reach confluence and were cultured for a further 16 days without replenishment. Cultures were positive for proliferating cell nuclear antigen (PCNA) after 3 days but lost PCNA immunoreactivity after 10 days, suggesting that the cells were at G_0 after 16 days. PCR based on *ZFY*- and *SRY*-specific primers [E. Aasen and J. F. Medrano, *Biotechnology* **8**, 1279 (1990); D. Pomp *et al.*, *J. Anim. Sci.* **73**, 1408 (1995)] revealed the sex of fibroblast progenitors in each case. A normal karyotype for each cell culture was confirmed by Giemsa staining. Cultures derived from a single fetus were used in this study.
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18. Oocytes with a pronounced first polar body were orientated so that the position of the polar body was at 12, 3, or 6 o'clock. Removal of metaphase II chromosomes and the first polar body to generate enucleated oocytes for nuclear microinjection was by gentle aspiration with a beveled pipette of 25- to 30-μm external diameter, driven by a piezo-actuated unit (Prime Tech, Ibaraki, Japan). Piezo-actuation of the pipette permitted rapid penetration of the zona pellucida. Enucleation was in batches of 10 to 15 oocytes, at room temperature in NCSU23 medium containing cytochalasin B (5 μg/ml). Confirmation that the chromosomes had been completely removed was by staining with the DNA-specific dye Hoechst 33342 (Sigma) and examination under ultraviolet light. After successful enucleation, batches of oocytes were washed thoroughly in NCSU23 medium lacking cytochalasin B and returned to 38.5°C in a water-saturated atmosphere of 5% CO_2 in air until required.
19. Nucleus donor cells were resuspended from washed (three times in PBS) confluent cultures of nonreplenished fetal fibroblasts by gentle treatment with 0.25% (w/v) trypsin. The reaction was quenched by the addition of DMEM containing 10% (v/v) FCS, and the cells were pelleted. After a final wash in NCSU23, they were resuspended in 10 ml of NCSU23. A 10-μl aliquot was removed immediately before microinjection, mixed with 100 μl of NCSU23, and placed onto an inverted microscope stage. Batches of 10 to 15 enucleated oocytes were placed in the droplet for nuclear transfer. Each nucleus donor cell was approached by a flush-ended microinjection pipette (external diameter of 7 to 10 μm), and its plasma membrane was broken by gentle aspiration of the entire cell into and out of the pipette. The nucleus was gathered into the pipette and moved toward an enucleated oocyte. Piezo-actuation of the pipette again enabled rapid penetration of the zona pellucida and microinjection of the donor nucleus with minimal damage to the enucleated oocyte plasma membrane. Between manipulations, injection pipettes were washed in a droplet of NCSU23 containing 15% (w/v) polyvinylpyrrolidone (average relative molecular mass of 40,000). After donor nucleus microinjection, reconstituted preembryos were incubated at 38.5°C in a water-saturated atmosphere of 5% CO_2 in air for 3 to 4 hours before activation.
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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 after the eCG injection. Six 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 surrogates carrying embryos 40 hours after activation gave birth to the cloned offspring Xena.
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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 GenoTyper software (PE Biosystems, Foster City, CA). The following markers were used: SW286, SW840, SW957, SW133, SW274, SW373, SW491, SW741, SW839, SW742, SW1327, SW1311, SW122, SW435, SW540, SW942, SWR1021, SW1339, SW249, SWR426, SW524, SWR414, and SW717. Data from three marker sets (SW133, SW274, and SWR1021) 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.
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Twists in Catalysis: Alternating Conformations of *Escherichia coli* Thioredoxin Reductase

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In thioredoxin reductase (TrxR) from *Escherichia coli*, cycles of reduction and reoxidation of the flavin adenine dinucleotide (FAD) cofactor depend on rate-limiting rearrangements of the FAD and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) domains. We describe the structure of the flavin-reducing conformation of *E. coli* TrxR at a resolution of 3.0 angstroms. The orientation of the two domains permits reduction of FAD by NADPH and oxidation of the enzyme dithiol by the protein substrate, thioredoxin. The alternate conformation, described by Kuriyan and co-workers, permits internal transfer of reducing equivalents from reduced FAD to the active-site disulfide. Comparison of these structures demonstrates that switching between the two conformations involves a "ball-and-socket" motion in which the pyridine nucleotide–binding domain rotates by 67 degrees.

TrxR from *E. coli* is a member of the pyridine nucleotide–disulfide oxidoreductase family of flavoenzymes. These enzymes use an active-site dithiol-disulfide to transfer reducing equivalents from FAD to external substrates (Fig. 1). The NADPH and FAD binding domains that constitute the TrxR monomer are related to corresponding domains in glutathione reductase, the prototype for this family of flavoenzymes (*1*). However, the organization of functional elements is different between *E. coli* TrxR and glutathione reductase. The active-site disulfide loop is in the NADPH domain in TrxR and in the FAD domain in glutathione reductase. Thus, the mechanisms

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