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Nuclear Transplantation in Early Pig Embryos[1]

RANDALL S. PRATHER,[2] MICHELLE M. SIMS, and NEAL L. FIRST[3]

Department of Meat and Animal Science
College of Agricultural and Life Sciences
University of Wisconsin-Madison
Madison, Wisconsin 53706

ABSTRACT

Nuclear transfer was evaluated in early porcine embryos. Pronuclear stage embryos were centrifuged, treated with cytoskeletal inhibitors, and subsequently enucleated. Pronuclei containing karyoplasts were placed in the perivitelline space of the enucleated zygote and fused to the enucleated zygote with electrofusion. The resulting pronuclear exchange embryos were either monitored for cleavage in vitro (9/13 cleaved and contained 2 nuclei after 24 h, 69%) or for in vivo development. In vivo development after 3 days resulted in 14/15 (93%) of the embryos transferred cleaving to the ≥4-cell stage and after 7 days 6/16 (38%) reaching the expanded blastocyst stage. A total of 56 pronuclear exchange embryos were allowed to go to term, and 7 piglets were born.

A similar manipulation procedure was used to transfer 2-, 4- or 8-cell nuclei to enucleated, activated meiotic metaphase II oocytes. Enucleation was effective in 74% (36/49) of the contemporary oocytes. Activation was successful in 81% (37/46) of nonmanipulated but pulsed oocytes versus 13% (4/31) of control oocytes ($p<0.01$). After 6 days in vivo, 9% (1/11) of the 2-cell nuclei, 8% (7/83) of the 4-cell nuclei, and 19% (11/57) of the 8-cell nuclei transferred to enucleated, activated meiotic metaphase II oocytes resulted in development to the compact morula or blastocyst stage ($p<0.01$). A total of 88 nuclear transfer embryos were transferred to recipient gilts for continued development. A single piglet was born after the transfer of a 4-cell nucleus to an enucleated, activated metaphase II oocyte and subsequent in vivo development. Therefore 4-cell nuclei are capable of directing development to term after transfer to an enucleated, activated meiotic metaphase II oocyte.

INTRODUCTION

The transfer of nuclei between the cells of early mammalian embryos has been accomplished in a variety of species, including mice (McGrath and Solter, 1983a; Robl et al., 1986), sheep (Willadsen, 1986), cattle (Prather et al., 1987; Robl et al., 1987), and rabbits (Stice and Robl, 1988), and has been described briefly for pig embryos (Robl and First, 1985). Nuclear transfer studies have served to describe nuclear versus cytoplasmic inheritance (McGrath and Solter, 1983b, 1984a; Mann, 1986; Robl et al., 1988) and imprinting during gametogenesis (Surani et al., 1986; Barra and Renard, 1988), as well as to determine the extent of nuclear differentiation in early development (Willadsen, 1986; Prather et al., 1987; Stice and Robl, 1988). Interestingly, the results obtained to date for all mammalian embryos other than mouse embryos suggest that nuclei from early cleavage stages can be reprogrammed to behave as 1-cell embryos if transferred to enucleated, activated meiotic metaphase II oocytes (reviewed by Prather and First, 1989).

Since all mammalian nuclei do not respond to nuclear transfer as mouse nuclei do, it is important to determine the affects of nuclear transfer in mammals other than the mouse. Here we report data showing that pronuclei can be exchanged successfully between porcine zygotes and result in normal offspring, and that some cleavage-stage nuclei are capable of directing complete development after transfer to an enucleated, activated meiotic metaphase II oocyte.

MATERIALS AND METHODS

Source of Embryos

Crossbred (Yorkshire × Landrace) gilts were monitored twice daily for signs of estrus. Oocytes were collected from nonmated animals 36 or 48 h after first-detected estrus, and pronuclear, 2-cell, 4-cell, and

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[2]Present address: Department of Animal Sciences, University of Missouri-Columbia, Columbia, MO 65211.
[3]Reprint requests.

8-cell embryos were collected from mated animals at 48–72 h after the onset of estrus. Embryos were collected by flushing the oviducts and/or uteri with N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-buffered Tyrode's media (HbT; Bavister et al., 1983) supplemented with bovine serum albumin (3 mg/ml, Sigma, St. Louis, MO) and gentamicin sulfate (50 μg/ml; Sigma).

Macromanipulation

Micromanipulation of pronuclear embryos was completed as described for the bovine by Robl et al. (1987). Briefly, pronuclear stage embryos were centrifuged at 15,000 × *g* for 3 min to allow visualization of the pronuclei (Wall et al., 1985; Robl et al., 1987). Centrifuged pronuclear embryos were placed in HbT containing 7.5 μg/ml cytochalasin B (Sigma) and 0.1 μg/ml demecolcine (Sigma) for 5 min prior to macromanipulation. A single embryo was held in place by a holding pipette attached to a Narshigie micromanipulator. Next a 30–33 μm (external diameter, O.D.), beveled, sharpened, glass pipette, attached to another Narshigie micromanipulator, was inserted into the embryo and moved adjacent to the pronuclei. The pronuclei were then aspirated into the pipette, and the pipette was removed. This permitted the removal of the pronuclei within a membrane bound karyoplast. This karyoplast was transferred to an enucleated zygote. The membranes were then fused as described below.

Nuclear transfer procedures were carried out as described by Prather et al. (1987) in cows. Briefly, after embryos and oocytes were exposed to HbT containing cytochalasin B, but not demecolcine, oocytes were prepared by aspirating the first polar body and cytoplasm directly underneath (presumably containing the metaphase chromosomes) with a 30–33 μm (O.D.) pipette. A karyoplast from a 2-cell, 4-cell, or 8-cell stage embryo was then aspirated into the transfer pipette and expelled into the perivitelline space of the enucleated oocyte. Electrically induced membrane fusion was conducted as described below. Some oocytes were enucleated, stained, and evaluated for the presence of meiotic metaphase II chromosomes. Other oocytes were not manipulated but were exposed to similar conditions and then to the electrofusion pulse, or were sham-pulsed and cultured 24 h and examined for the presence of a single pronucleus.

Membrane Fusion

Membranes were fused by electrofusion (Prather et al., 1987; Robl et al., 1987). The nuclear transfer embryos were placed between two platinum electrodes 1 mm apart in a solution of 0.3 M mannitol (Sigma). The embryos were manually oriented so that the fusion plane was parallel to the electrodes, exposed to a 5 V AC (1000 KHz, 20% duty on) field for 5–10 s, and a 30-μs, 120 V/mm (DC) pulse was then applied. The AC field was programmed to decrease to 0 V over 5 s following the DC pulse. Power was provided by a Zimmermann Cell Fusion™ instrument (Model Z1000, GCA/Precision Scientific Group, Chicago, IL).

Culture

In vitro culture was conducted in 50-μl drops of TCM 199 supplemented with 10% heat-treated fetal bovine serum (Gibco, Grand Island, NY) under paraffin oil at 39°C under an atmosphere of 100% humidity and 5% CO_2 in air. Nuclear transfer embryos that had fused and were destined for in vivo culture were transferred to the oviducts of synchronized recipient gilts. In some gilts, pregnancy was terminated at slaughter and embryos were recovered by flushing the oviducts or uteri with HbT. These embryos were evaluated by phase-contrast microscopy or after fixation and staining with aceto-orcein and phase-contrast microscopy. Some of the blastocyst-stage embryos were transferred to the uteri of synchronized recipient gilts. In some cases, the recipient gilts were bred to a Hampshire boar to aid in maintaining the pregnancy by providing enough conceptuses while providing a color marking to identify offspring. Estrus was subsequently monitored twice daily with the aid of a boar.

Data were analyzed by chi-square (Snedecor and Cochran, 1980).

RESULTS

Pronuclear Exchange

Pronuclear exchanges were conducted to determine if the procedures developed for nuclear transfer were detrimental to continued development. Electrofusion was successful in 76% (89/117) of the manipulated zygotes. After 24 h of in vitro cultures, 9/13 (69%) of the pronuclear exchange embryos had cleaved and had 2 nuclei. After 3 days in vivo, 14/15 embryos contained 4 or more cells; these 15 embryos were subsequently transferred to another recipient gilt. Thirty-eight percent (6/16) of the pronuclear exchange embryos cultured in vivo for 7 days were recovered as expanded blastocysts:

four of these embryos were subsequently transferred to another recipient gilt. This resulted in a total of 56 pronuclear exchange embryos transferred to 6 recipient gilts that were allowed to continue to term (Table 1). One of these animals returned to estrus on Day 16, one on Day 50, and one on Day 93. The remaining 3 gilts farrowed a total of 32 pigs, 7 of which were derived from the transfer of 35 pronuclear exchange embryos.

Cloning

An examination of the steps involved with the methods for cloning revealed that 74% (36/49) of contemporary meiotic metaphase II oocytes were in fact enucleated. The pulse required for fusion activated 81% (37/46; as judged by the presence of a single pronucleus after 24 h of in vitro culture) of meiotic metaphase II oocytes that were not manipulated, whereas sham-pulsed oocytes activated 13% (4/31) of the time ($p<0.01$). Electrofusion rates were not significantly different between donor cell stages (2-cell, 59/77, 77%; 4-cell, 115/138, 83%; 8-cell, 71/83, 86%; $p>0.25$; this is a retrospective analysis).

In vitro development showed that 55% (11/20) of the 2- and 4-cell nuclei transferred, participated in a cleavage division (2-cell, 7/15; 4-cell, 4/5) after 24 h. After 6 days in vivo, 9% (1/11) of 2-cell donor nuclei, 8% (7/83) of 4-cell donor nuclei, and 19% (11/57) of the 8-cell donor nuclei ($p<0.01$) transferred to an enucleated, activated meiotic metaphase II oocyte had developed to the compact morula or expanded blastocyst stage (4 of the 4-cell donor nuclei-derived blastocyst-stage embryos were subsequently transferred to another recipient gilt; the other three were not transferred because a synchronized recipient was not available). A total of 42 nuclear transfer embryos were transferred to 4 nonbred recipients and allowed to continue pregnancy. One had a normal cycle, one had a 28-day cycle, and 2 had cycles of 52 days or more (Table 2). A total of 46 nuclear transfer embryos were transferred to 5 bred recipients. One 4-cell nuclear transfer piglet and forty-eight control piglets were born from these 5 gilts (Table 2).

TABLE 1. Establishment and maintenance of pregnancy after the transfer of pronuclear exchange embryos to recipient gilts.

Number transferred	Recipient	Pregnancy result
9	25[a]	estrus detected Day 16
8	1–4[a]	estrus detected Day 50
4[b]	17[c]	estrus detected Day 93
8	32[a]	4 pronuclear exchange pigs born 10 control pigs born
12	51[a]	2 pronuclear exchange pigs born 7 control pigs born
15[b]	7–7[a]	1 pronuclear exchange pig born 8 control pigs born

[a]Gilt was bred to a color-marked boar.

[b]Embryos were retransferred to a secondary recipient after collection from a primary recipient gilt.

[c]Gilt received 2 mg estradiol on Days 12 and 13 to maintain pregnancy (Pope et al., 1987).

TABLE 2. Pregnancy establishment and maintenance after the transfer of 2-, 4- or 8-cell nuclear transfer embryos to recipient gilts.

Number transferred	Donor cell stage	Recipient	Pregnancy result
10	2	25	extended cycle, 28 days
12	2	6809521	extended cycle, 52 days
11	2	509[a]	13 control piglets born
7[b]	4	55[a]	6 control piglets born 1 nuclear transfer pig born
4	4	234[c]	normal cycle
16	4	7352	extended cycle, 72 days
7	4	230[a]	11 control piglets born
17	8	46[a]	10 control piglets born
4	8	7372[a]	8 control piglets born

[a]Gilt was bred to a color-marked boar.

[b]Embryos were retransferred to a secondary recipient after collection from a primary recipient gilt.

[c]Gilt received 2 mg estradiol on Days 12 and 13 to maintain pregnancy (Pope et al., 1987).

DISCUSSION

The results presented in this paper show that pig zygotes can tolerate the conditions necessary for pronuclear exchange and continue development to term. The results further show that oocytes in meiotic metaphase II can be enucleated, activated, fused with a 4-cell karyoplast, and subsequently direct development to the blastocyst stage and to term.

Pronuclear exchange has been used to study the nuclear versus cytoplasmic inheritance of cell surface antigens (SSEA-3; McGrath and Solter, 1983b), lethal mutations (T^{hp}: McGrath and Solter, 1984a), imprinting (Surani et al., 1986), and control of early development (Robl et al., 1988). These studies were all conducted in mouse embryos, but the application of the techniques developed by McGrath and Solter (1983a) to the cow embryo (Robl et al., 1987) and now to the pig embryo permit similar investigations in these domestic species.

Nuclear transfer for cloning has been successful in embryos from amphibians (reviewed by Gurdon, 1986;

DiBerardino, 1987; Prather, 1989) as well as in embryos from sheep (Willadsen, 1986), cattle (Prather et al., 1987), rabbits (Stice and Robl, 1988), and mice (Tsunoda and Shioda, 1988), although it is not clear if any nuclear reprogramming has resulted from this nuclear transfer (McGrath and Solter, 1984b; Barnes et al., 1987; Howlett et al., 1987). A major developmental difference between these animal embryos is the timing of the transition from maternal control of development (relying upon maternally stored RNA) to zygotic control of development (relying upon zygotically produced RNA). The major transition appears to occur at the mid-blastula stage for *Xenopus* embryos (~4000-cell stage; Newport and Kirschner, 1982), 8- to 16-cell stage for sheep embryos (Calarco and McLaren, 1976; Crosby et al., 1988), 8- to 16-cell stage for cow embryos (Camous et al., 1986; Barnes, 1988; King et al., 1988) and 2-cell stage for mouse embryos (Bolton et al., 1984). Why nuclear transfer for cloning is successful in species other than the mouse is not known, but it is interesting to note that the transition to zygotic control of development for the mouse occurs at an earlier time than in the other species. Pig embryos appear to fall between cow, sheep, and mouse embryos and make a transition by the 8-cell stage (White et al., 1987; Prather et al., 1989b); they may begin producing rRNA by the 4-cell stage, since this is the time at which nucleoli are first seen to begin reticulating (Norberg, 1970).

In vivo development to the morula/blastocyst stage was lower for the 2-cell and 4-cell donor nuclei versus the 8-cell donor nuclei ($p<0.01$). However, because of the limited number of replications, high degree of variability between replications, and the fact that these are retrospective data, no firm conclusions should be inferred.

The fusion percentage was not significantly different for the different donor cell stages and resembles that found for the 2-cell to 8-cell stage bovine blastomeres (Prather et al., 1987).

A major complicating factor in conducting these experiments is the lack of tight control over the stage of the cell cycle at which the embryos are collected and manipulated. This should not present a problem for the pronuclear exchanges, because the zygotes should be close to the same stage of the cell cycle–probably all in S. However, for cloning, a single embryo collection may contain cells of 2 different cleavage stages, "late" 4-cell and "early" 8-cell embryos, e.g. 4-cell embryos and 5- to 8-cell embryos. In this paper, these late 4-cell donor nuclei were pooled with early 4-cell donor nuclei, e.g. a single collection containing 2-cell and 4-cell stage embryos. Although differences in stage of the cell cycle for nuclear transfer are not important for early amphibian nuclei (Ellinger, 1978), such effects of stage of the cell cycle are important for more slowly dividing cells (Von Beroldington, 1981), but this has not been evaluated in mammals. Future studies of ours will attempt to evaluate the differences between early and late cell stage donor nuclei.

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