Development and Transgene Expression of Porcine Nuclear Transfer Embryos Using Somatic Cells

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INTRODUCTION

The successful production of offspring derived from NT embryos in mammals has important implications not only for multiplication of valuable domestic animals, but also for elucidation of genomic totipotency of donor nuclei. Especially, cloning from transformed cells has other advantages for making transgenic farm animals (Anderson and Seidel, 1998). Cloned animals including sheep (Schnieke at al., 1997; Wilmut et al., 1997), goats (Baguisi et al., 1999), mice (Wakayama et al., 1998), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999) and pig (Onishi et al., 2000; Polejaeva et al., 2000) have been successfully produced by nuclear transfer using somatic cells. A variety of somatic cells such as cumulus, oviduct (Wakayama et al., 1998; Kato et al., 1998), granulosa (Wells et al., 1999), mammary epithelium (Wilmut et al., 1997), fetal fibroblast (Schnieke et al., 1997; Cibelli et al., 1998), fetal germ cells (Zakhartchenko et al., 1999b) and ear skin cells Zakhartchenko et al., 1999a) have been used as sources of donor nuclei.

Artificial activation is one of important factors in nuclear transfer procedures. Ionophore A23187 was suggested to be an activator for mammalian eggs (Steinhardt et al., 1974). It induces cortical granule exocytosis, second polar body extrusion, and pronuclear formation in the oocytes of several species (Marcus, 1990; Liu et al., 1998; Wang et al., 1998). Porcine oocytes treated with the ionophore could form pronuclei and parthenogenetically develop to the blastocyst stage (Funahashi et al., 1994; Wang et al., 1999). Electrical stimulation is also known to be a potent activator of porcine oocytes (Prather et al., 1989; Jolliff and Prather, 1997; Wang et al., 1998). When porcine NT eggs with fibroblast cells were activated and fused simultaneously by electrical stimulus, a few embryos developed to blastocyst stage (Du et al., 1999). Recently, we suggested that activation of porcine oocytes reconstructed with fibroblast cells by electrical

stimulation was appropriate 2 h after electrofusion (Han et al., 2000).

Somatic cell nuclear transfer has become a powerful tool for making transgenic animals, especially transgenic livestock. Cloned transgenic animals have been produced from transformed fibroblast cells (Schnieke et al., 1997, Cibelli et al., 1998). To generate transgenic pigs by using nuclear transfer technique, genetic modifications should be first done in somatic cells.

The aim of this study was to determine the optimal activation conditions for porcine NT eggs using somatic cells. The developmental abilities of porcine NT eggs induced by different electrical voltages or activation times after electrofusion were examined. Thereafter, developmental competence of NT embryos was compared to parthenogenetic and in vitro fertilized embryos. Then, in this study developmental ability of porcine NT embryos with transformed cells was compared to that of NT embryos with non-transformed cells. It was also examined whether the transgenes express in the porcine NT embryos during early development.

RESULTS

1. Effect of Different Activation Treatments on In Vitro Development of NT Embryos

It was examined whether different activation treatments (A23187, A23187/6-DMAP and electrical pulse) after electrofusion affect in vitro development of porcine NT embryos with nuclei of cumulus cells to the blastocyst stage. Higher cleavage rate (74.9±7.5%) and development rate to blastocysts (11.0 ±4.1%) were observed in the electric pulse group when compared to A23187 and A23187/6-DMAP treatment groups (58.1±13.9 and 60.7±6.3 for cleavage rate; 2.2±2.8 and 2.2±1.5 for developmental rate, respectively; $P<0.05$). However, no difference was detected in the mean nuclei number of blastocysts among groups. The results demonstrate that electrical pulse could induce a higher development of NT embryos than chemical treatments in vitro.

2. Effect of Electrical Field on *In Vitro* Development of NT Embryos

To evaluate if electrical fields affect in vitro development of NT embryos, the fused embryos were activated at different voltages (120 and 150 V/mm DC) after electrofusion and then cultured in vitro for 6 days. The rate of blastocyst development (11.6±1.6%, 14/121) of NT embryos in field strength of 120 V/mm was significantly high as compared to 150 V/mm group (6.5±2.3%, 8/121) ($P<0.05$). However, mean cell numbers of blastocysts were similar between two

groups (29.5±11.3 and 27.6±10.5, respectively) (P>0.05). For subsequent experiments, the activation was performed with a single 120 V/mm DC pulse.

3. Effect of Activation of NT Embryos at Different Times After Electrofusion

This experiment was carried out to determine the optimal activation timing after fusion. The cleavage rates of NT embryos in 2 h and 4 h groups were higher than those of 6 h group. A higher proportion of NT embryos (11.6±2.9%, 20/187) activated 2 h after electrofusion developed to the blastocyst stage, as compared to 4 h (6.6±2.3%, 11/190) and 6 h (8.1±3.3%, 14/184) groups. Mean cell numbers of NT embryos in 2, 4 and 6 h groups were 30.4±10.4, 24.6±10.1 and 16.5±7.4, respectively. A significant difference was observed between 2 and 6 h groups (P<0.05). These results suggest that activation timing after electrofusion can affect developmental potential of porcine NT eggs.

4. Developmental Competence of Porcine NT Embryos

Developmental potential of NT porcine oocytes with cumulus (NT†) or/and fibroblast cells (NT‡) was compared to IVF-derived embryos. Fusion rates of enucleated oocytes reconstructed with cumulus or fibroblast cells after electrofusion were 67% (257/374) or 69% (273/396), respectively. Thus, there was no difference in the cleavage rate of NT embryos between 2 donor cell types. NT embryos from both cell types, however, showed lower developmental rate to the blastocyst stage than IVF-derived embryos (P<0.01). Mean nuclei numbers of IVF-derived, NT† and NT‡ blastocysts were 38.6±10.4 (ranging from 23 to 65), 28.9±11.4 (ranging from 17 to 51) and 30.2±9.9 (ranging from 18 to 51), respectively. Thus, NT embryos were comprised of number of nuclei smaller than IVF-derived embryos (P<0.05).

5. Comparison of Developmental Ability of Porcine NT Embryos to IVF-Derived or Parthenogenetic Embryos

NT embryos using fibroblast cells showed a lower developmental rate (9.4± 0.9%, 20/289) to the blastocyst stage than IVF-derived (21.4±1.9%, 43/201) or parthenogenetic embryos (22.4±7.2%, 43/189) (P<0.01), although there was no difference in the cleavage rate of the embryos among experimental groups. After 6 days of culture, most blastocysts (16/20) from NT embryo were hatched, although the mean number of nuclei was smaller than that of IVF-derived blastocysts. The reason for early hatching of NT blastocysts seems to be due to the partial dissection of zona pellucida during the enucleation procedure. The mean cell number of NT embryos (28.9±11.5, ranging from 14 to 61, n=18) was

smaller than that of IVF-derived blastocysts (36.2±9.7, ranging from 18 to 67, n=22) ($P<0.05$), while similar to that of parthenogenetic embryos (29.9±12.1, ranging from 13 to 50, n=24). These results represent that the porcine NT embryos using fibroblast cells could develop to blastocysts in vitro, although having lower developmental competence when compared to IVF-derived embryos. However, it remains to be examined whether the porcine NT embryos using nuclei of somatic cells can normally develop to term.

6. GFP Expression of NT embryos

Finally, it was examined whether transformed cells have in vitro developmental potential following nuclear transfer in the pig. Porcine fibroblast cells were introduced with exogenous -actin promoter/GFP gene by retroviral infection, and surviving colonies were obtained after treatments of G418 for approximately 2 weeks. GFP expression in transfected fibroblast cells was confirmed by epifluorescence microscopy. Fusion rate (67%, 195/292) of NT oocytes with transformed fibroblast cells was similar to that (71%, 197/277) of non-transformed cells. Developmental rates of NT embryos to blastocysts were also similar between transformed and non-transformed cells. All NT embryos derived from transformed cells showed strong GFP expression. The results indicate that porcine NT embryos with transformed cells have a similar developmental potential to those with non-transformed cells.

DISCUSSION

It has been shown that calcium is the primary intracellular signal responsible for initiating the activation process in mammalian eggs. The activation must occur to induce the subsequent development of NT embryos to preimplantation stage or to term. To alter the intracellular calcium concentration of bovine NT eggs with nuclei of somatic cells, several chemicals such as Ca-ionophore/6-DMAP (Cibelli et al., 1998), ionomycin/6-DMAP (De Sousa et al., 1999; Dominko et al., 1999) or cycloheximide/cytochalasin B (Zakhartchenko et al., 1999c) have been used. Only a few porcine NT eggs developed to blastocysts when treated with thimerosal and dithiothreitol (Tao et al., 1999), and no development to term has been reported. Development to blastocyst stage of porcine oocytes activated with calcium ionophore was lower compared to those by electric stimulation (Wang et al., 1998; Nussbaum and Prather, 1995). Electrical pulses are to induce parthenogenesis in the mouse (Tarkowski et al., 1970; Witkowska et al., 1973)

and pig (Prochazka et al., 1992; Sun et al., 1992). A single electrical pulse was sufficient to induce cortical granule exocytosis and resumption of oocyte meiosis in the pig, although it induced only a single Ca2+ oscillation (Sun et al., 1992). The present study demonstrated that porcine NT eggs exposed to only a single electrical pulse developed to the blastocyst stage in vitro.

In cattle, NT embryos activated at 4 to 8 h after fusion of donor cells with MII cytoplasts showed improved embryonic development (Stice et al., 1996; Wells et al., 1998). A prolonged exposure of transferred nuclei to oocyte cytoplasmic factors facilitated nuclear remodeling and reprogramming (DiBerardino et al., 1984; Ware et al., 1989). Hence, it may be required several hours to facilitate nuclear remodeling or reprogramming of the fused embryos prior to activation, although reliable evidences have not been reported. In the present study the porcine NT embryos activated at 2 h after fusion showed improved development as compared with the longer periods (4 and 6 h). This difference may be due to the timing of genomic activation between species. The activation of embryonic genome activation occurs at the 4-cell stage in the pig, while during the 8- to 16-cell stage in sheep and cattle (Kopsency, 1989). In subsequent experiments, rates of blastocyst formation in reconstructed embryos were 2.1 (2/97), 3.2 (2/85) and 10.4% (11/106), respectively, when they were activated at 0, 30 min and 1 h after electrofusion. Thus, this study demonstrated that the electrical activation of reconstructed porcine oocytes is most effective 1 to 2 h after electrofusion. These results also suggest that the time required for the remodeling or reprogramming of donor nuclei in recipient cytoplasts after fusion may differ among species.

Approximately 70% of reconstructed embryos using pig fibroblasts failed to divide when activated with thimerosal/dithiothreitol treatment (Tao et al., 1999). As shown in our data, the cleavage rate of porcine NT eggs activated by a single electric stimulus was similar to those of parthenogenetic and IVF-derived embryos. This result indicates that the activation procedure applied in our experiments was appropriate. Porcine NT eggs with somatic cells have a lower blastocyst development than parthenogenetic or IVF-derived embryos. Porcine NT embryos reconstituted with 8- to 16-cell nuclei have developed to morula stage (Terlouw et al., 1992), and recently, it has been reported that a few blastocysts could develop from porcine NT eggs using fibroblast cells (Du et al., 1999; Tao et al., 1999). Thus, the developmental competence of porcine NT embryos using nuclei of somatic cells is still low, although improved in this study. Many factors involved in the nuclear transfer procedure could result in the low developmental competence of porcine NT embryos. First, the lower development of porcine NT eggs may come from inappropriate cell cycle of donor nuclei, as they could have

received cells that were not in G0 or G1 phase of the cell cycle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999), and a consequence of inadequate nuclear reprogramming from using nonquiescent cells. Second, it may be essential to control the ploidy of the reconstructed embryo after activation stimulus is applied. In order to proceed normal development, bovine NT embryos have been incubated in medium supplemented with 6-DMAP following exposure to ionomycin or ionophore (Cibelli et al., 1998; De Sousa et al., 1999). It has been known that 6-DMAP, protein kinase inhibitor, may inhibit the phosphorylation necessary for the spindle apparatus (Susko-Parrish et al., 1994), and therefore prevent micronuclei formation known to occur when fusion precedes activation (Czolowska et al., 1984). In this study the porcine NT eggs were activated b

It has been suggested that developmental ability of NT embryos with somatic cells could be different according to a variety of cell types in mammal (Kato et al., 1999, Zakhartchenko et al., 1999a). The present study shows that porcine NT embryos regardless of donor cell types (cumulus or fibroblast cells) have developmental potential to the blastocyst stage. However, in vitro development of porcine NT embryos with somatic cells still remains relatively low when compared to IVF-derived embryos. Terlouw et al. (1992) also reported that porcine NT embryos reconstituted with 8- to 16-cell stage nuclei had poor developmental ability to morula stage. To improve development of porcine NT embryos, further studies on activation and in vitro culture systems are needed in the future. Our results indicate that NT porcine blastocysts have a smaller mean nuclei number than IVF-derived embryos. Tao et al. (1999) also reported that NT porcine blastocysts derived from fibroblast cells had low nuclei number. This might be caused by reduced amount of cytoplasm during enucleation procedure (Peura et al., 1998, Dominko et al., 2000).

NT blastocysts had a smaller cell number than IVF-derived blastocysts. This difference might arise from the reduced cytoplasm volume removed during the enucleation procedure (Peura et al., 1998). The low pregnancy rates and high abortion rates in NT embryos may be attributable simply to the reduced cell numbers (Yang et al., 1993). Tao et al. (1999) reported that two porcine NT blastocysts using nuclei of fibroblast cells contained 14 and 11 nuclei, respectively. Our data showed the increased nuclei number of NT blastocysts, although numbers were still small compared to IVF-derived blastocysts. Even IVF-derived porcine embryos, blastocysts that developed in vitro had lower cell numbers than the embryos grown in vivo (Han et al., 1999). Culture of NT embryos in vivo rather than in vitro may result in blastocyst-stage embryos that have at least twice the number of nuclei (Machaty et al., 1998). Further studies

should be carried out to examine developmental potential of porcine NT embryos using somatic cells to term.

It has been reported that using the donor nucleus in G1 or G0 phase (pre-S phase) of the cell cycle is beneficial for nuclear transfer (Prather et al., 1992; Campbell et al., 1994). However, the phase of the cell cycle resulting in better development after nuclear transfer is still controversial. Wilmut et al. (1997) suggested the donor cell for nuclear transfer to be in G0 phase of the cell cycle, a quiescent state. Nuclei of serum-starved fibroblasts supported the development of reconstructed embryos to the blastocyst stage significantly better than those of non-starved fibroblasts (Zakhartchenko et al., 1999c). Alternatively, cycling cells could be successfully used for nuclear transfer in cattle (Cibelli et al., 1998). In the present study porcine fibroblast cells used as donor nuclei were normally cultured until confluent without a serum starvation. The proportion of G1/G0 phases of porcine fetal fibroblast cells at confluency was as high as the cells serum-starved for 5 or 10 days (Boquest et al., 1999).

The successful achievement of somatic cell nuclear transfer in the pig might be applicable to develop the transgenic pigs for xenotransplantation or disease models. Genetic modification of somatic cells should be preceded prior to nuclear transfer. Though the transformed cells used in this study were undergone multiple passages during selection, in vitro development of NT embryos derived from transformed cells was similar as compared to non-transformed donor cells of early cell passage. This result indicates that developmental ability of NT embryos is not affected by cell passage number (Kubota et al., 2000).

Our results suggest that electrical stimulus for activation of NT porcine oocytes is more effective than chemical treatments, and that both cumulus and fetal fibroblast cells used as donor nuclei could support the development of NT porcine embryos to the preimplantation stage. The present study suggests that a single electrical stimulation 1 to 2 h after electrofusion was sufficient to activate NT oocytes in pigs. This study also shows that NT embryos with transformed cells have developmental competence and express the transgenes during early development, demonstrating that somatic cell nuclear transfer is useful for making transgenic pigs. Furthermore, development of somatic cell nuclear transfer procedures in the pig will be applicable to multiply high-valued pigs and to produce transgenic pigs for xenotransplantation or disease models. However, the efficacy of the techniques in the production of NT embryos in pigs is still remained to be improved in future studies.