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—Original Article—

Developmental Ability of Miniature Pig Embryos Cloned with Mesenchymal Stem Cells

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Abstract. The present study compared the developmental ability of miniature pig embryos cloned with fetal fibroblasts (FFs), bone marrow-derived mesenchymal stem cells (MSCs) and differentiated (osteocytes, adipocytes and chondrocytes) MSCs. MSCs were isolated from an approximately 1-month-old female miniature pig (T-type, PWG Micro-pig®, PWG Genetics Korea). MSCs were differentiated into osteocytes, adipocytes and chondrocytes under controlled conditions and characterized by cell surface antigen profile using specific markers. These differentiated or undifferentiated MSCs, as well as FFs of miniature pig, were transferred into enucleated oocytes of domestic pigs. Data from 10 replicates involving 1567 cloned embryos were assessed in terms of developmental rates. The *in vitro* development rate to the blastocyst stage of embryos cloned with undifferentiated MSCs was significantly ($P<0.05$) higher than that of embryos cloned with differentiated MSCs and FFs. Surgical transfer of 523 two-cell stage embryos cloned with undifferentiated MSCs into five synchronized domestic pig recipients resulted in 5 cloned miniature pig offspring (1 stillborn and 4 viable) from 2 pregnant recipients. The results imply that MSCs might be multipotent and that they can be used to produce viable cloned miniature pigs that cannot be easily reproduced with differentiated somatic cells.

Key words: Animal cloning, Differentiation, Mesenchymal stem cells, Miniature pig, Nuclear transfer

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The production of cloned animals by nuclear transfer (NT) has been increasingly studied as a potential approach to tissue and organ xenotransplantation with reduced immunogenicity for end-stage organ failure. Several studies have suggested that miniature pigs, rather than nonhuman-primate species, are suitable as a donor animal for human xenotransplantation [1, 2] because miniature pigs are superior in terms of similarities in gross anatomy and physiology to humans as well as in terms of ethical issues. The NT efficiency, however, remains relatively low due to abnormalities throughout pre- and post-implantation development regardless of the species or type of donor cell [3]. A number of factors affect NT efficiency, including the NT technical process, activation protocol, recipient oocyte age, cell cycle stage and type of donor cells, which has been implicated in the precise reprogramming of chromatin and genomic imprinting. Among these factors, incomplete selection of donor cells has also been considered a prime cause for NT inefficiency.

Transfer of embryonic stem (ES) cells to produce cloned embryos has resulted in consistently higher numbers of viable offspring compared with somatic cells in mice [4, 5]. Moreover, embryos cloned with porcine mesenchymal stem cells (MSCs) and their derivatives along the osteogenic lineage give rise to an increase in the rate of preimplantation development compared with adult fibroblasts [6]. Our previous study on gene expression profiles demonstrated that some genes in embryos cloned with MSCs closely resembled those of *in vivo* counterparts [7]. These findings indicate that undifferentiated or less differentiated genomes might be more efficiently reprogrammed to re-activate the expression of early embryonic genes to enhance NT efficiency [8].

The present study, therefore, was conducted to compare the *in vitro* developmental ability of embryos cloned with undifferentiated and differentiated (osteocytes, adipocytes and chondrocytes) MSCs and fetal fibroblasts (FFs). Furthermore, the *in vivo* development of embryos cloned with undifferentiated MSCs was observed after transfer into surrogate animals.

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Materials and Methods

Media and chemicals

All media were purchased from Gibco (Invitrogen Corporation, Grand Island, NY, USA) and all chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise specified. The cell culture medium was advanced Dulbecco's Modified Eagle's Medium (ADMEM) supplemented with 10% fetal bovine serum (FBS) and 1.0% penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively, Pen-Strep; Gibco). TCM199 containing 5% FBS, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 25 mM HEPES, 2.5 mM Na-pyruvate, 1 mM L-glutamine, 1.0% Pen-Strep with or without 0.5 μg/ml LH

and 0.5 μg/ml FSH (IVM+H and IVM-H, respectively) was used as the medium for *in vitro* maturation (IVM) of the cumulus-oocyte complexes (COCs). The embryo culture medium was porcine zygote medium-3 (PZM-3) containing 3 mg/ml bovine serum albumin (BSA, Fraction V), essential amino acids and non-essential amino acids [9]. Tyrode's albumin lactate pyruvate medium containing 2 mg/ml BSA, 12 mM sorbitol, 7.5 μg/ml cytochalasin B and 10 mM HEPES (HEPES-TALP) was used for manipulation. The pH of all media was adjusted to 7.2–7.4, and the osmolarity was adjusted to 285 mOsM.

Cell isolation and culture

MSCs and FFs were isolated from female miniature pigs (T-type, PWG Micro-pig®, PWG Genetics Korea). Gelatinous bone marrow was extracted from the femur of an approximately 1-month-old female to isolate the MSCs. The extracted bone marrow was mixed 1:1 (v/v) in phosphate-buffered saline (PBS), layered upon a Ficoll (density 1.077 g/ml, Amersham Biosciences, Uppsala, Sweden) gradient and centrifuged at 400 × *g* for 40 min at 20 C. The buffy layer cells on the interface were cultured at 38.5 C in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were gently removed 2 days after plating. Once confluent, the cells were dissociated with 0.25% (w/v) trypsin-EDTA solution and centrifuged at 500 × *g* for 5 min. The cells were passaged at a density of 1×10^4 cells/cm^2. FFs were isolated from a female fetus on approximately day 30 of gestation and obtained via hysterectomy from a pregnant miniature gilt. To isolate the fetal cells, the head, limbs and visceral organs of the fetus were removed, and the remaining tissues were washed in Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 10% FBS and treated with 0.05% (w/v) trypsin-EDTA solution for 5 min. After washing with ADMEM by centrifugation at 300 × *g* for 10 min, the cells were cultured at a final concentration of 2×10^5 cells/ml at 38.5 C in a humidified atmosphere of 5% CO_2 in air.

In vitro differentiation and cytochemical staining

Multilineage differentiation of MSCs and confirmation of their lineages were performed according to the methods previously explained [7, 10]. Briefly, cells were cultured in ADMEM until they reached 70–80% confluence in a 35-mm dish under conductive conditions for osteogenic, adipogenic and chondrogenic differentiation for 3 weeks. The osteogenic medium consisted of ADMEM, 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate and 10 mM β-glycerol phosphate.

The adipogenic medium consisted of ADMEM, 10% FBS, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin and 500 μM 3-isobutyl-1-methylxanthine (IBMX). The chondrogenic medium mainly consisted of TGF-β. The cells were then stained with Alizarin-red S solution and von Kossa for identification of the mineralized matrix, oil red O staining for accumulation of the lipid droplets and Alcian blue 8 GX solution staining for synthesis of glycosaminoglycans. The alkaline phosphatase (AP) activity of the cells was detected using BCIP/NBT (Promega, Madison, WI, USA).

Cell-surface antigen profile

Specific markers, CD13 (aminopeptidase N), CD29 (β1-integrin), CD44 (hyaluronate receptor), CD45 (leukocyte common antigen), CD105 (endoglin) and CD133 (prominin) antibodies (Santa Cruz Biotechnology, CA, USA), were used to characterize the cell-surface antigen profile of the MSCs. Cells were fixed using 3.7% formaldehyde in PBS for 30 min and incubated in PBS solution containing 0.3% Triton X-100 and 4 mg/ml BSA for 30 min. Samples were incubated with 5 μg/ml of primary CD antibodies and labeled with fluorescein isothiocyanate-conjugated secondary antibodies (1:100, Santa Cruz Biotechnology) for 30 min. Genomic DNA was labeled with 10 μg/ml propidium iodide solution in PBS for 15 min, and stained samples were observed using a fluorescence microscope at × 400 magnification (Nikon, Tokyo, Japan).

Nuclear transfer (NT)

The NT procedure was performed according to the previously described protocol [11] with minor modifications. Briefly, ovaries of domestic pigs were obtained from prepubertal gilts at a local slaughterhouse. COCs were aspirated from follicles 3–6 mm diameter with 19-G needle and 10-ml syringe. Sets of 100 COCs with uniform cytoplasm and multilayered cumulus cells were matured in 500 ml IVM+H medium for 22 h and further cultured for an additional 20 h in IVM-H medium at 38.5 C in a humidified atmosphere of 5% CO_2 in air. After removal of the cumulus cells, metaphase-II stage oocytes were selected for NT procedure. Oocytes which were enucleated their nuclei and the first polar bodies in manipulation medium were coupled with qualified nuclear donor cells (undifferentiated and differentiated MSCs and FFs). The couplets were oriented in a BTX Electro chamber (BTX, San Diego, CA, USA) filled with 0.28 M mannitol solution containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.1 mg/ml BSA and pulsed with 2.0 KV/cm direct current twice for 30 μsec using a BTX Electro Square Porator (ECM 830, BTX).

Embryo evaluation

Sets of 30 donor-cell-fused oocytes were cultured in 30 μl drops of PZM-3 and maintained for 7 days at 38.5 C in a humidified atmosphere of 5% CO_2 in air. The rates of cleavage and blastocyst development *in vitro* were assessed on day 2 and day 7, respectively. To compare total cell numbers, day 7 blastocysts were labeled with 5 μg/ml bisbenzimide and mounted onto a microscope slide, and their nuclei were counted under an inverted microscope equipped with epifluorescence.

Estrus synchronization and embryo transfer

The animal experiment was approved by the Animal Center for Medical Experimentation at Gyeongsang National University. To prepare the surrogate mother pigs, approximately 1-year-old female pigs (crossbreed of Landrace × Yorkshire) that were sexually mature and that had aborted by administration of 3 ml of a synthetic analogue of prostaglandin $F_2\alpha$ (Cyclix P, 92 μg/ml cloprostenol, Intervet, Netherlands) i. m. at day 30 of pregnancy were administered 1,000 IU eCG (Folligon, Intervet) and 2,500 IU hCG (Chorulon, Intervet) 72 h later to synchronize their estrus cycles.

General anesthesia of the animals was performed through an intravenous injection of 4 mg/kg of azaperone (Stresnil®, Janssen-Cilag, Belgium) and 1–2 mg/kg of tiletamine-zolazepam (Zoletil®, Virbac, France). Approximately 100 miniature pig NT embryos at the 2-cell stage were surgically transferred into the oviducts of each surrogate domestic pig with synchronous estrus. Pregnancy of the surrogate mothers was initially monitored using an ultrasound (MyLab™ 30, ESAOTE Pie medical, Netherlands) equipped with a 7.0-MHz convex transducer at approximately 28 days after transfer. Thereafter, pregnancy was monitored every 4 weeks until term.

Microsatellite DNA analysis

To clarify the offspring's genetic identity, polymorphic microsatellites from cloned piglets, surrogate mothers and MSCs that had been used as the nuclear donors were analyzed. Genomic DNA was extracted from the samples according to the manufactural instructions (GENE ALL™, General Biosystem, Korea). Eight highly polymorphic microsatellites (SW240, SW787, SW911, S0090, S0155, S0228, S0355, S0386) were selected for analysis. Polymerase chain reaction (PCR) amplification was performed using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA), 0.2 mM of each dNTP, 0.5 mM of each primer pair and 20 ng genomic DNA. PCR was performed with 40 cycles consisting of 94 C for 30 sec, 55 C for 30 sec and 72 C for 30 sec and analyzed using an ABI 1310 Genetic Analyzer and the GeneScan software (Applied Biosystems).

Statistical analysis

One-way analysis of variance (ANOVA; via SPSS) was used to analyze differences among the treatments. Duncan's and Tukey's multiple comparisons tests were used to compare the mean values of the treatments. Data are expressed as means ± standard error of the mean (SEM), and differences were considered significant when $P<0.05$.

Results

Characterization and differentiation of MSCs derived from bone marrow

The isolated MSCs appeared as stretched, single or spindle-shaped cells and formed colonies during the primary culture period (Fig. 1a and b). After the cells were subcultured 3 or 4 times, homogeneous adherent MSCs were observed in the culture dishes. The cell-surface antigen profile of the MSCs was characterized by immunofluorescence assay with specific markers, CD13, CD29, CD44, CD45, CD105 and CD133 antibodies. These MSCs were observed to be positive for CD13 (aminopeptidase N), integrin CD29 (β1-integrin), matrix receptor CD44 (hyaluronate receptor) and CD105 (endoglin; Fig. 2a–d), but hematopoietic markers such as CD45 and CD133 (data not shown) were not observed.

To differentiate MSCs into distinct mesenchymal lineages such as osteogenic, adipogenic and chondrogenic, MSCs were cultured under conductive conditions for 3 weeks (Fig. 2e–h). Following exposure to osteogenic medium, MSCs were differentiated to osteocytes by forming a mineralized matrix and expressed AP activity (Fig. 1c). Identification of the mineralized matrix was confirmed by staining with Alizarin-red S and von Kossa (Fig. 2e and f). Culture of MSCs in adipogenic differentiation medium led to the marked appearance and formation of lipid vacuoles that filled the whole cytoplasm, which were visualized by oil red O staining (Fig. 2g). The chondrogenic potential of MSCs was characterized by staining with Alcian blue 8 GX, which indicated accumulation of sulfated proteoglycans (Fig. 2h).

In vitro developmental potential of embryos cloned with fetal fibroblasts (FFs) and undifferentiated and differentiated (osteocytes, adipocytes and chondrocytes) MSCs

Table 1 shows the development rates and total cell numbers of embryos cloned with FFs, bone marrow-derived MSCs and differentiated (osteocytes, adipocytes and chondrocytes) MSCs. The cleavage rate was significantly ($P<0.05$) higher in the embryos cloned with undifferentiated MSCs than in those cloned with FFs and differentiated MSCs (85% *vs*. 64–75%). Similarly, the blastocyst rate of those FFs and differentiated with undifferentiated MSCs (47.7 ± 3.2%) was significantly ($P<0.05$) higher than in the embryos cloned with other MSCs. The blastocyst rate of the embryos cloned with FFs was significantly ($P<0.05$) lower than those cloned with differentiated osteocytes, adipocytes and chondrocytes (14.5 ± 4.3 *vs*. 34.5 ± 3.3, 31.1 ± 4.1 and 36.8 ± 3.5%, respectively). There were no significant ($P<0.05$) differences in the total cell numbers of embryos cloned with undifferentiated MSCs and differentiated osteocytes, adipocytes and chondrocytes (47.8 ± 4.6 *vs*. 40.5 ± 5.7, 40.3 ± 4.8 and 42.2 ± 6.2, respectively), but the total cell numbers of those embryos were significantly ($P<0.05$) higher than that of the embryos cloned with FFs (31.1 ± 3.8).

In vivo developmental potential of embryos cloned with undifferentiated MSCs

Transfer of 523 two-cell stage embryos cloned with undifferentiated MSCs into five domestic pig surrogates yielded four pregnancies at around 28 days after transfer. Two of the pigs had a miscarriage in the second month of pregnancy for unknown reasons. The two remaining pigs delivered 5 offspring (body weights: 190, 300, 320, 320 and 320 g) at 115 days of gestation; four were viable, and one was stillborn (Table 2). To clarify the offspring's genetic identity, eight polymorphic microsatellites (SW240, SW787, SW911, S0090, S0155, S0228, S0355, S0386) were analyzed from the cloned piglets, surrogate mothers and the MSCs used as the nuclear donors. There were 100% similarities between the genotypes of the piglets and the donor cells (Table 3).

Discussion

Miniature pigs continue to elicit interest as an animal model in the fields of biomedical research [12], transplantation organs [13, 14] and disease models [15, 16]. However, lack of comprehensive studies on miniature pigs and their cloning has made cloning in them less efficient than in other animals, including domestic pigs.

In the present study, the *in vitro* development of embryos cloned with undifferentiated MSCs derived from bone marrow was higher than that of embryos cloned with differentiated MSCs and FFs.

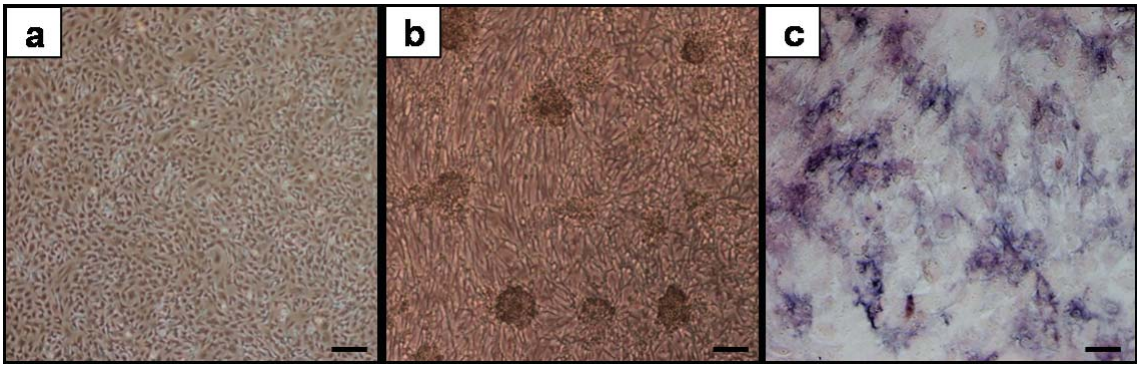

Fig. 1. MSCs derived from miniature pig bone marrow. Examination of morphology and AP activity in primary culture of MSCs revealed fibroblastic shape (a), homogeneous morphology (a) and colony formation (b). Staining with BCIP/NBT revealed alkaline phosphatase activity in MSCs that differentiated into osteocytes (c). Scale bars=100 μm.

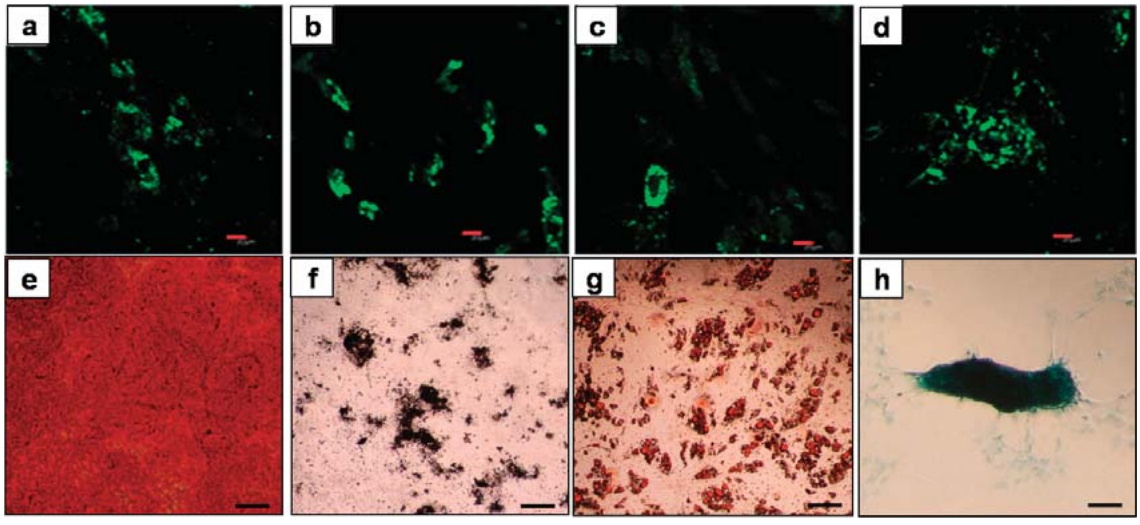

Fig. 2. Representative cell-surface antigen profiles (a–d) and images of differentiation potential (e–h) of MSCs. Green fluorescence indicates the positions of CD13 (a), CD29 (b), CD44 (c) and CD105 (d) as MSC specific markers. Cytochemical staining confirmed that MSCs differentiated into osteogenic, adipogenic and chondrogenic lineages. Osteogenic induction was assessed by Alizarin-red S solution (e) and von Kossa staining for identification of the mineralized matrix (f). Adipogenic induction was assessed by oil red O staining for accumulation of the lipid droplets (g) and Alcian blue 8 GX solution staining for synthesis of glycosaminoglycans in chondrocytes (h). Scale bars=20 μm (a–d) and 100 μm (e–h).

Following transfer of embryos cloned with undifferentiated MSCs, we generated four viable miniature pig offspring. These findings indicate that MSCs are more efficient nuclear donors for cloning in terms of enhancing the pre-implantation development of cloned pig embryos. It has been hypothesized that the genome of undifferentiated cells or partially differentiated multipotent progenitor cells can be easily reprogrammed by recipient oocytes [4, 6, 8, 17–19]. MSCs have been isolated from bone marrow extracts in various animal species and established for *in vivo* or *in vitro* applications. Multipotent MSCs that had originally been identified by their ability to differentiate into mesenchymal lineages, such as osteogenic [20], chondrogenic [21, 22] and marrow stromal lineages [23], have been considered to be promising tools for tissue engineering and cellular therapies [21, 24, 25]. In addition, MSCs have expanded *in vitro* without any apparent modification in phenotype or loss of multipotent function.

In the present study, adherent MSCs retained fibroblast-like morphology during primary culture, and after removal of non-adherent cells, a number of colonies formed and expressed AP activity that steadily increased as time progressed. All these findings were similar to earlier observations [26, 27]. Furthermore, the cells were observed to be positive for MSC-specific markers, such as CD13, CD29, CD44 and CD105 (Fig. 2a–d). However, CD45 and CD133, hematopoietic lineage markers, were not expressed

Table 1. Rates of development *in vitro* and total cell numbers of embryos cloned with various donor cells

Type of donor cell [1]	Reconstructed eggs	Development to (% mean ± SEM)		Total No. of cells [2] (mean ± SEM)
		Cleavage	Blastocyst	
FFs	334	216 (64.7 ± 4.1)[a]	48 (14.5 ± 4.3)[a]	31.1 ± 3.8[a]
MSCs	329	280 (85.1 ± 3.8)[c]	157 (47.7 ± 3.2)[c]	47.8 ± 4.6[b]
Osteocyte-MSCs	296	213 (72.0 ± 4.6)[b]	102 (34.5 ± 3.3)[b]	40.5 ± 5.7[b]
Adipocyte-MSCs	293	188 (64.2 ± 2.4)[a]	91 (31.1 ± 4.1)[b]	40.3 ± 4.8[b]
Chondrocyte-MSCs	315	237 (75.2 ± 2.8)[ab]	116 (36.8 ± 3.5)[b]	42.2 ± 6.2[b]

[1] FF: fetal fibroblasts. MSCs: mesenchymal stem cells. Osteocyte-MSCs: differentiation of MSCs into osteocytes. Adipocyte-MSCs: differentiation of MSCs into adipocytes. Chondrocyte-MSCs: differentiation of MSCs into chondrocytes. [2] The mean total number of cells in cloned blastocysts recovered at day 7. [a, b, c] Percentages with different superscripts within a column are significantly different ($P<0.05$). Ten replicates were performed.

Table 2. Pregnancy and offspring following transfer of embryos cloned with MSCs into surrogates

Surrogate pig [1]	No. NT embryos transferred [2]	Diagnosis of pregnancy (+/–) [3]	Maintaining gestation	No. offspring
A	110	–	–	–
B	92	+	Abortion	–
C	108	+	Full-term	4 (viable)
D	115	+	Full-term	1 (stillbirth)
E	98	+	Abortion	–
Total	523			

[1] One-year-old female (crossbred of Landrace × Yorkshire). [2] NT embryos derived from MSCs transferred into the uteri of estrus synchronized surrogate mother pigs. [3] Diagnosed at around 28 days after transfer using a ultrasound equipped with 7.0-MHz convex transducer.

Table 3. Microsatellite analysis of the genomes of the cloned offspring, donor MSCs and surrogate mothers

Markers	Donor MSCs	Offspring	Surrogate mothers
S0090	243/247	243/247	247/249
S0155	160/162	160/162	152/156
S0228	237/237	237/237	221/225
S0355	258/264	258/264	246/246
S0386	166/166	166/166	162/162
SW240	91/101	91/101	91/95
SW787	146/158	146/158	154/156
SW911	155/159	155/159	155/167

(data not shown). These observations show a consensus that MSCs do not express hematopoietic stem cell markers.

MSCs from 3 to 4 passages were induced to differentiate into osteogenic, adipogenic and chondrogenic lineages under specific culture conditions, and staining confirmed the multilineage differentiation of these three classical mesenchymal pathways (Fig. 2e–h). According to previous reports, >5 passage porcine MSCs retain multipotential capacity (the three principle lineages), but < 15 passage MSCs retain only adipogenic capacity [10] or capacity toward the osteogenic lineage [28]. In the present study, the MSCs at an early passage stage successfully differentiated into the three principle lineages. Based on the findings, the cells were confirmed to be multipotent [7, 26, 27] and were used as nuclear donors for NT.

According to numerous studies, the type of donor cell could be considered the prime cause affecting the developmental ability of cloned embryos, and the progressive decrease in cloning efficiency has been related to the differentiation status of the donor cell [29]. In general, due to rapid growth and the potential for multiple cell divisions, fetal fibroblasts have been commonly used as nuclear donors in pig embryo cloning. The blastocyst rates of pig embryos cloned with FFs are below the level of 20% [30–32]. These data are substantially in accordance with our data showing that 14.5% of embryos cloned with FFs developed to blastocysts. MSCs have been successfully established, and these cells have been further employed as nuclear donors for porcine NT [6–8, 33]. Similarly, pig embryos cloned with undifferentiated MSCs and their derivatives along the osteogenic lineage gave rise to consistently high development rates that are comparable to those of adult fibroblasts

[6]. In the present study, the highest rate of blastocyst formation of the embryos cloned with undifferentiated MSCs implied that differentiated cells as nuclear donors may decrease cloning efficiency. Furthermore, the higher total cell numbers in embryos cloned with undifferentiated MSCs and their derivatives suggested that embryos cloned with MSCs are of higher quality than embryos cloned with differentiated cells. These findings are similar to the observations of the above-mentioned studies. Our group has observed the expression profiles of genes involved in transcription, DNA methylation, histone deacetylation, apoptosis and embryonic growth at different developmental stages, and some genes in embryos cloned with MSCs closely resembled those of *in vivo* produced embryos [7].

The usefulness of miniature pigs as an animal model has been proven; but nevertheless, only a limited number of studies have reported on cloned miniature pigs derived from somatic donor cells [30, 31, 34–36]. Because of the comparison with domestic pigs, production of cloned miniature pigs using miniature pigs oocytes has limitations. The strain or origin of the recipient oocytes and surrogate mother could affect the developmental and pregnancy rates of the cloned animals. The difference in strain of the surrogate miniature pig affects the post-implantation development of miniature pig NT embryos [34]. On the other hand, viable cloned [30, 35] and transgenic [36] miniature pigs have been produced using domestic pig oocytes as recipient oocytes and surrogate mothers.

In the present study, transfer of domestic pig oocyte-miniature pig MSC complexes into 5 domestic pig surrogates yielded 2 pregnancies. Offspring (4 viable and 1 stillborn) were obtained by natural delivery after approximately 115 days of gestation. The body weights of the cloned offspring were similar to those of non-cloned miniature offspring, ranging from 190 to 320 g, and were comparable to the observations made in previous report [32]. To clarify the offspring's genetic identity, eight polymorphic microsatellites (SW240, SW787, SW911, S0090, S0155, S0228, S0355, S0386) were analyzed from the cloned piglets, surrogate mothers and MSCs used as the nuclear donors. There were 100% similarities between the genotypes of the piglets and the donor cells. These results strongly verified the donor cell line as the source of the genetic material used to produce the cloned piglet. Hence, for miniature pig cloning, domestic pigs might be suitable animals for supplying the recipient oocytes and surrogate mothers.

In conclusion, this study demonstrated that miniature pig embryos cloned with undifferentiated MSCs and their derivatives along the osteogenic, adipogenic and chondrogenic lineages give rise to consistently high development rates that are comparable to fibroblasts, with significantly higher cell numbers. In addition to enhanced *in vitro* development potential, embryos cloned with MSCs developed to full term *in vivo*. This evidence suggests that MSCs with a relatively undifferentiated genome could be more efficiently reprogrammed to reinitiate the expression of early embryonic genes and have greater ability as nuclear donors to support pre- and post-implantation development.

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