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Evaluation of porcine urine-derived cells as nuclei donor for somatic cell nuclear transfer

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ABSTRACT

Background: Somatic cell nuclear transfer (SCNT) is used widely in cloning, stem cell research, and regenerative medicine. The type of donor cells is a key factor affecting the SCNT efficiency.
Objectives: This study examined whether urine-derived somatic cells could be used as donors for SCNT in pigs.
Methods: The viability of cells isolated from urine was assessed using trypan blue and propidium iodide staining. The H3K9me3/H3K27me3 level of the cells was analyzed by immunofluorescence. The *in vitro* developmental ability of SCNT embryos was evaluated by the blastocyst rate and the expression levels of the core pluripotency factor. Blastocyst cell apoptosis was examined using a terminal deoxynucleotidyl transferase dUTP nick end-labeling assay. The *in vivo* developmental ability of SCNT embryos was evaluated after embryo transfer.
Results: Most sow urine-derived cells were viable and could be cultured and propagated easily. On the other hand, most of the somatic cells isolated from the boar urine exhibited poor cellular activity. The *in vitro* development efficiency between the embryos produced by SCNT using porcine embryonic fibroblasts (PEFs) and urine-derived cells were similar. Moreover, The H3K9me3 in SCNT embryos produced from sow urine-derived cells and PEFs at the four-cell stage showed similar intensity. The levels of Oct4, Nanog, and Sox2 expression in blastocysts were similar in the two groups. Furthermore, there is a similar apoptotic level of cloned embryos produced by the two types of cells. Finally, the full-term development ability of the cloned embryos was evaluated, and the cloned fetuses from the urine-derived cells showed absorption.
Conclusions: Sow urine-derived cells could be used to produce SCNT embryos.

Keywords: Pig; urine; somatic cell nuclear transfer; embryonic development

INTRODUCTION

Somatic cell nuclear transfer (SCNT) has great potential for animal husbandry, regenerative medicine, and the conservation of endangered animals. Moreover, SCNT technology is essential for gene editing, establishing animal disease models, and investigating xenotransplantation. Since "Dolly" the sheep was born in 1997 [1], more than twenty mammalian species have been cloned through SCNT [2]. The type of donor cells is a critical

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factor that affects the cloning efficiency because each cell type has a distinct epigenome and different degrees of differentiation [3]. In pigs, the most common donor cells used in SCNT are fibroblasts. Usually, the fibroblasts are isolated from fetal (porcine embryo fibroblast, PEF) or adult tissues. Although this suggests that only a minimal injury to the body (i.e., ear tissue) is needed to collect donor cells, there is a risk of damage caused by the need to restrain the animal. In particular, breeds, such as wild boar, mini-pigs, and some disease model animals (such as hemophilia or immune deficiency model), are sensitive to restraining stress and injury. In addition, the less invasive and convenient the procedure, the more likely the animals' owner will allow it. Therefore, donor cells that can be collected wholly in a noninvasive and straightforward manner are an attractive source.

Finding a way to collect donor cells non-invasively without harming the animal is desirable. For example, cows can be cloned from mammary gland epithelial cells derived from milk [4,5]. On the other hand, milk collection is limited to female animals in the lactation period. By contrast, urine contains several types of somatic cells [6], such as squamous epithelial cells from the urethra and bladder and renal tubular cells, which can be cultured after collection. Induced pluripotent stem (iPS) cells have been established from human urine-derived cells [7-10], suggesting that urine-derived cells are a good candidate donor for NT [11]. In addition, the successful cloning of cattle and mice by urine-derived cells has improved confidence in the cloning of pigs from this type of cell. [12,13]. On the other hand, unlike mice, there is limited information on pig urine-derived cells. Moreover, the genitourinary tract anatomy and urine composite of pigs are different from other animals. Therefore, this study aimed to classify the characteristics of porcine urine-derived cells to determine if the urine-derived cell was suitable for SCNT.

MATERIALS AND METHODS

Collection of urine-derived cells

Urine samples were collected from healthy sows or boars in 50 mL tubes containing 1 mL 2% penicillin and streptomycin obtained during natural urination. All pigs were raised under the same management conditions and received the same nutrition with an average age of 15–24 months. The urine samples were stored at 4°C and transferred to the laboratory immediately. The urine samples were centrifuged at 1,500 rpm for 20 min to isolate the somatic cells by a standard procedure. The pellet was then washed three times with the washing medium (PBS, supplemented with 1% gentamicin, 100 IU/mL penicillin, 100 mg/mL streptomycin, 2.5 eggs/mL amphotericin B). For the Percoll procedure, the pellet was further resuspended in 2 mL of the washing medium, then loaded onto a gradient of Percoll (v/v 90%, v/v 50%, v/v 30%, and v/v 10%) in a 15 mL Falcon tube and centrifuged at 1,500 rpm for 20 min. The contents of the 30% and 50% layers were collected and washed twice with PBS and used for cell analysis. For the cell culture, the contents of the 30% and 50% layers were washed twice with the culture medium (DMEM/F12, 15% FBS, 10 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 μg/mL gentamicin, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B) and cultured in the same medium in a CO_2 incubator (5% CO_2 in air) at 39°C for seven days. The medium was changed with fresh medium every 48 h.

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PEF isolation and culture

Porcine embryonic fibroblasts were isolated from an E32 fetus. The body wall was digested in 0.25% trypsin, and the cells were cultured in DMEM (Gibco, USA) for four passages with 20% FBS at 38.5°C in 5% CO_2, 95% air, and saturation humidity. DMEM containing 10% FBS was used for culture beyond the first passage.

Collection of oocytes and *in vitro* maturation

Oocyte maturation is described elsewhere [14]. Briefly, porcine ovaries were collected from a slaughterhouse of Harbin Haxincheng Food Co., Ltd., in Harbin city, Heilongjiang province. After exposure, the ovaries were placed in physiological saline with antibiotics at 37°C and transported to the laboratory. The follicles were aspirated, and the follicular contents were washed with HEPES-buffered Tyrode's lactate. The cumulus-oocyte complexes (COCs) were recovered and cultured in a maturation medium. After 42 h, the COCs were vortexed in hyaluronidase for 30 sec to remove the cumulus cells. Only oocytes with a visible polar body, regular morphology, and a homogenous cytoplasm were used in subsequent experiments.

SCNT

The procedure for porcine SCNT was performed as described elsewhere [14]. After maturation culture for 42 h, the oocytes were treated with 1 mg/mL hyaluronidase to remove the cumulus cells. Oocytes with an extruded first polar body were selected as the recipient cytoplasts. Cumulus-free oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm with a glass pipette, 25 μm in diameter, in TCM199-Hepes plus 0.3% BSA and 7.5 μg/mL Cytochalasin B. A single donor cell was injected into the perivitelline space and fused electrically using two direct pulses of 120 V/mm for 30 uses infusion medium. Combined eggs were cultured in a porcine zygote medium-3 (PZM-3) medium for six days in an atmosphere containing 5% CO_2 and 95% air at 39°C. After activation, the cleavage and blastocyst rates were assessed at 48 h and 6 days. All processes were performed according to guidelines for the ethical treatment of animals and were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (NEAUEC20190102).

In vitro fertilization (IVF)

The spermatozoa were resuspended and washed three times in DPBS supplemented with 0.1% (w/v) BSA by centrifugation at 1,500 × g for 4 min. The spermatozoa concentration was measured using a hemocytometer, and the proportion of motile sperm was determined. The spermatozoa were diluted to the optimal concentration with a modified Tris-buffered medium (mTBM). Cumulus-free oocytes were washed three times in mTBM. Approximately 30 oocytes were inseminated in 50 μL drops of mTBM at a final sperm concentration of 3 $\times 10^5$/mL for 6 h. The embryos were then washed and cultured in PZM-3 in an atmosphere containing 5% CO_2 at 39°C.

Immunofluorescence staining for epigenetic markers in cells and pluripotency factors in embryos

The global levels of trimethylation of lysine 9 at histone 3 (H3K9me3) and trimethylation of lysine 27 at histone 3 (H3K27me3) were determined in PEF and urine-derived cells by immunofluorescence staining, as described earlier [15]. The antibodies used in the study were as follows: rabbit anti-H3K9me3 (abcam, ab8898), rabbit anti-H3K27me3 (Millipore, 07-449), and goat anti-rabbit-488 (Sigma, A9169). The cells were counterstained with Hoechst 33342 (Sigma–Aldrich, USA).

The H3K9me3 level of embryos at the four-cell stage was analyzed on day 2. For the immunofluorescence evaluation of embryos, the samples with an intact zona pellucida were exposed to the embryonic manipulation medium with 5 mM HCl for approximately 5 sec to remove the zona pellucida. They were then fixed with 4% paraformaldehyde in PBS for 40 min at room temperature. The embryos were permeabilized with 1% Triton X-100 (Sigma–Aldrich) in PBS for 5 h at 4°C and blocked with 1% bovine serum albumin (BSA) in PBS for 1h at room temperature. The samples were then incubated with primary antibodies in PBS containing 0.01% Triton X-100 and 0.1% Tween 20 overnight at 4°C. The antibodies used in the study were as follows: goat anti-Sox2 (Santacruz, sc-17320), goat anti-Oct4 (Santa Cruz, sc8628), goat anti-Nanog (Sigma, SAB2500670), and rabbit anti-goat (abcam, ab6741). After incubation, the embryos were washed three times for 5 min, and then with 0.01% Triton X-100 and 0.1% Tween 20 in PBS at room temperature, followed by incubation with the secondary antibodies diluted at 1:1,000 in 0.01% Triton X-100 and 0.1% Tween 20 in PBS for 1 h at room temperature. Finally, the embryos were counterstained with Hoechst 33342 (Sigma-Aldrich), mounted, and at least 10 blastocysts were analyzed for each pluripotency marker. NIS-element essential research image processing software (Nikon) equipped with a microscope was used for image acquisition and quantitative measurements of the mean pixel intensity emitted by each nucleus.

Cells and embryos apoptosis detection

The cells were collected and stained with trypan blue (0.04%, Gibco) and propidium iodide (PI) (2 mg/mL; Molecular Probes, Inc., USA). Embryo apoptosis was detected using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) BrightRed Kit (Vazyme, A113) according to the manufacturer's instructions. Briefly, the embryos were washed three times in PBS with 0.1% polyvinylpyrrolidone and fixed in 4% paraformaldehyde solution for 40 min at room temperature. The embryos were then permeabilized in PBS with 0.1% Triton X-100 (v/v) for 1 h at room temperature. They were then incubated in a TUNEL reaction medium for 1 h in a humidified atmosphere at 37°C in the dark. The reaction mixture contained 45 mL of a TUNEL Label with 5 mL of TUNEL Enzyme, mixed before use. As a negative control, 50 mL of the TUNEL Label only was used. Labeled embryos were washed and counterstained with Hoechst 33342 (10 mg/mL in PBS) for 10 min at room temperature in the dark. Finally, the embryos were washed three times with PBS (1% Tween 20) and mounted on slides. The blastocyst cells were then observed and counted under an epifluorescence microscope.

Embryo transplantation and pregnancy check

The reconstructed embryos produced by SCNT using PEF and urine-derived cells were surgically transplanted into the gilts oviducts on the first day of spontaneous estrus (0 d = standing reaction). Approximately 250 reconstructed embryos were transferred to each surrogate gilt (Landrace). The first ultrasonic examination for the non-recycled recipients was performed on the 28–30th days. Subsequently, the recipients were examined ultrasonically weekly.

Statistical analysis

The data were analyzed using SPSS software (IBM Corp., USA). The significance was set at $p < 0.05$ unless specified otherwise. The results are expressed as mean ± standard deviation. A student's *t*-test was performed to assess the difference in the embryonic development parameters, fluorescence intensity, and apoptotic parameters.

RESULTS

Information of urine samples and cell concentration

Based on the standard purification method, the results showed many urine crystals in the samples, particularly in boar urine. Percoll was used to improve the purified outcome. Most of the urine crystals can be removed using Percoll (**Fig. 1**). The cell number in urine from boars and sows were measured. The results showed that the cell concentration in the boar urine was higher than in sow urine. The pH and osmolality in the boar urine were higher than in sow urine (**Supplementary Table 1**, **Supplementary Fig. 1**). In addition, the boar urine-isolated cells were much larger than those isolated from sows' urine (**Fig. 1**). The viability of isolated cells was determined by staining the cells with trypan blue and PI. The staining results showed that most cells did not take the trypan blue and PI dye for the sow urine-isolated cells, suggesting that most cells were viable (**Fig. 2A-C**). On the other hand, most somatic cells isolated from the boar urine showed intense trypan blue and PI staining (**Fig. 2D-F**).

Culture of pig urine-derived cells

The sow and boar urine was cultured and the cells were propagated. For sow urine, single, small, compact "rice-grain" like cells were observed 3–5 days after initial seeding (**Fig. 3A and B**). These cells formed clones within an additional seven days (**Fig. 3C**). For boar urine, however, some urinary crystals could not be removed from the somatic cells. On the other hand, although there were more somatic cells in sow urine samples (**Table 1**), the cells derived from boar urine did not stand for culture (**Fig. 3D-F**).

NT procession using urine-derived cells

For the sow urine-derived cells, the cells were slightly larger than PEF and could be used for NT (**Fig. 4A-C**). For boar urine, the somatic cells purified from boar urine could not be used in NT because most cells with a large size (diameter > 40 um) (**Fig. 4D**). Although a few cells can be injected into the perivitelline space, it is not enough to construct embryos for further development.

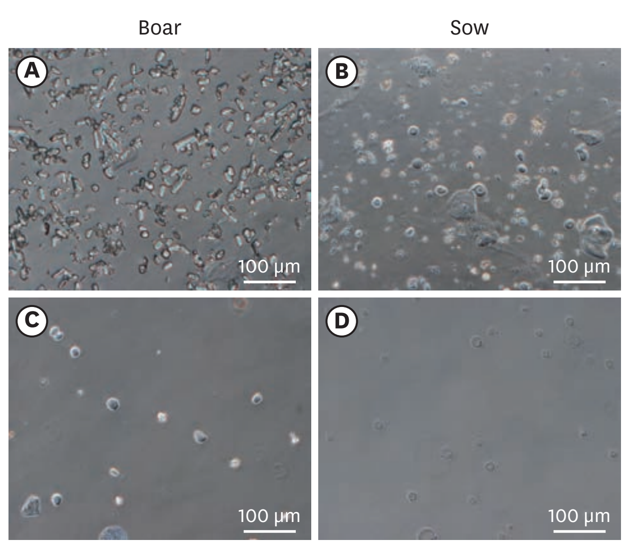

Fig. 1. Cells isolated from urine samples. (A, C) Cells isolated from boar urine with or without using Percoll. (B, D) Cells isolated from sow urine with or without using Percoll.

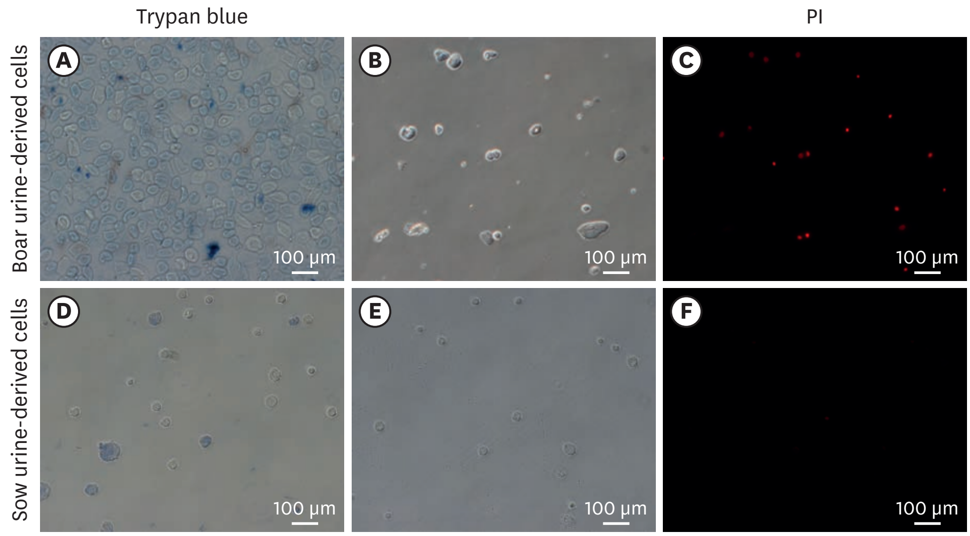

Fig. 2. Viability of cells isolated from pig urine samples. (A-C) Cells isolated from boar urine and stained with trypan blue and PI. (D-F) Cells isolated from sow urine and stained with trypan blue and PI.
PI, propidium iodide.

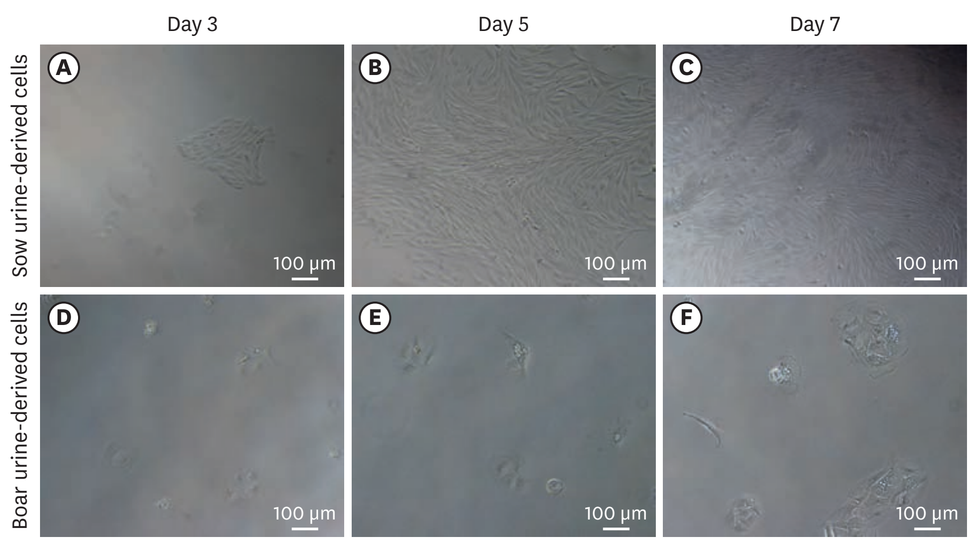

Fig. 3. Culture of somatic cells isolated from urine samples of sows. (A) At day 3 of culture; (B) day 5 of culture; and (C) day 7. Somatic cells isolated from urine samples of boar. (D) at day 3 of culture; (E) day 5 of culture; and (F) day 7.

Table 1. Developmental competence of blastocysts produced by SCNT using PEF and urine-derived cells

	No. of oocytes	Fusion rate (%)	Cleavage rate (%)	Blastocyst rate (%)	Blastocyst cell number
PEF	318	85.1 ± 4.9	69.4 ± 2.9	20.3 ± 2.3	37.3 ± 3.64
Urine-derived cells	270	88.8 ± 4.5	66.3 ± 0.75	19.0 ± 3.1	35 ± 6.72

SCNT, Somatic cell nuclear transfer; PEF, porcine embryonic fibroblast.

Level of H3K9me3/H3K27me3 in urine-derived cells from the sow, PEF, and SCNT embryos derived from two types of cells

The aberrant reprogram during NT is related to the somatic epigenetic modification pattern of the donor nuclei. The main epigenetic barrier of SCNT reprogramming is high H3K9me3 and H3K27me3 modification. The IF results showed that both H3K9me3 and H3K27me3 modification in urine-derived cells were similar to PEF (**Supplementary Fig. 2**). Furthermore,

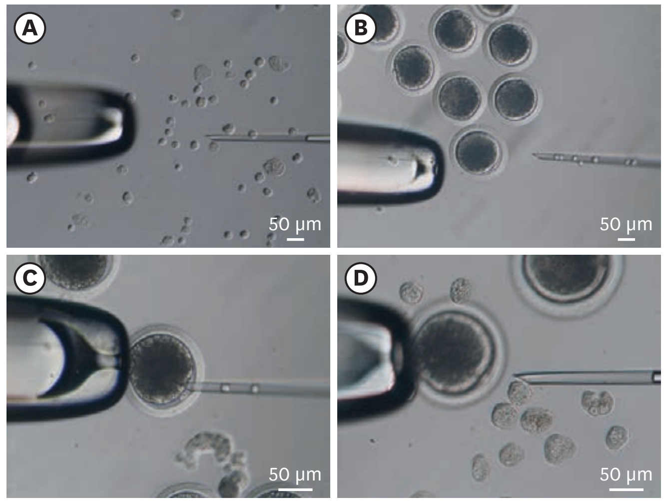

Fig. 4. SCNT using urine-derived cells. (A) Cells were collected from the urine of the sow with a micropipette and a micromanipulator. (B, C) The collected sow urine-derived cells were placed into M199 medium until used for SCNT. (D) Cells collected from the urine of the boar.
SCNT, Somatic cell nuclear transfer.

the H3K9me3 level was also evaluated in the reconstructed embryos at the four-cell stages. The IF results showed that those two types of embryos had a similar intensity of H3K9me3 modification (**Supplementary Fig. 3**).

Development competence and quality of embryos produced by SCNT using sow urine-derived cells as nuclei donors

The cleavage rate and blastocyst rate did not show a significant difference when the urine-derived cells were used as nuclei donors (cleavage rate 69.4% ±2.9% vs. 66.3% ± 0.75%; blastocyst rate 20.3% ± 2.3% vs. 19.0% ± 3.1%) (**Table 1**, **Fig. 5**). The blastocyst cell number between PEF and urine-derived cells did not differ significantly. Furthermore, multiple pluripotency genes, including Nanog, Oct4, and Sox2, were determined using IF at the blastocyst stage. The results showed that the Nanog, Oct4, and Sox2 expression levels were similar in two types of blastocyst (**Fig. 6**).

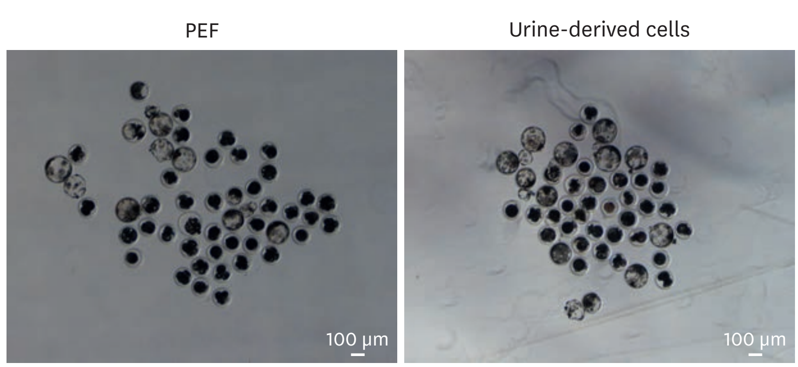

Fig. 5. Blastocysts produced by nuclear transfer using PEF and urine-derived cells (blastocysts at day 6).
PEF, porcine embryonic fibroblast.

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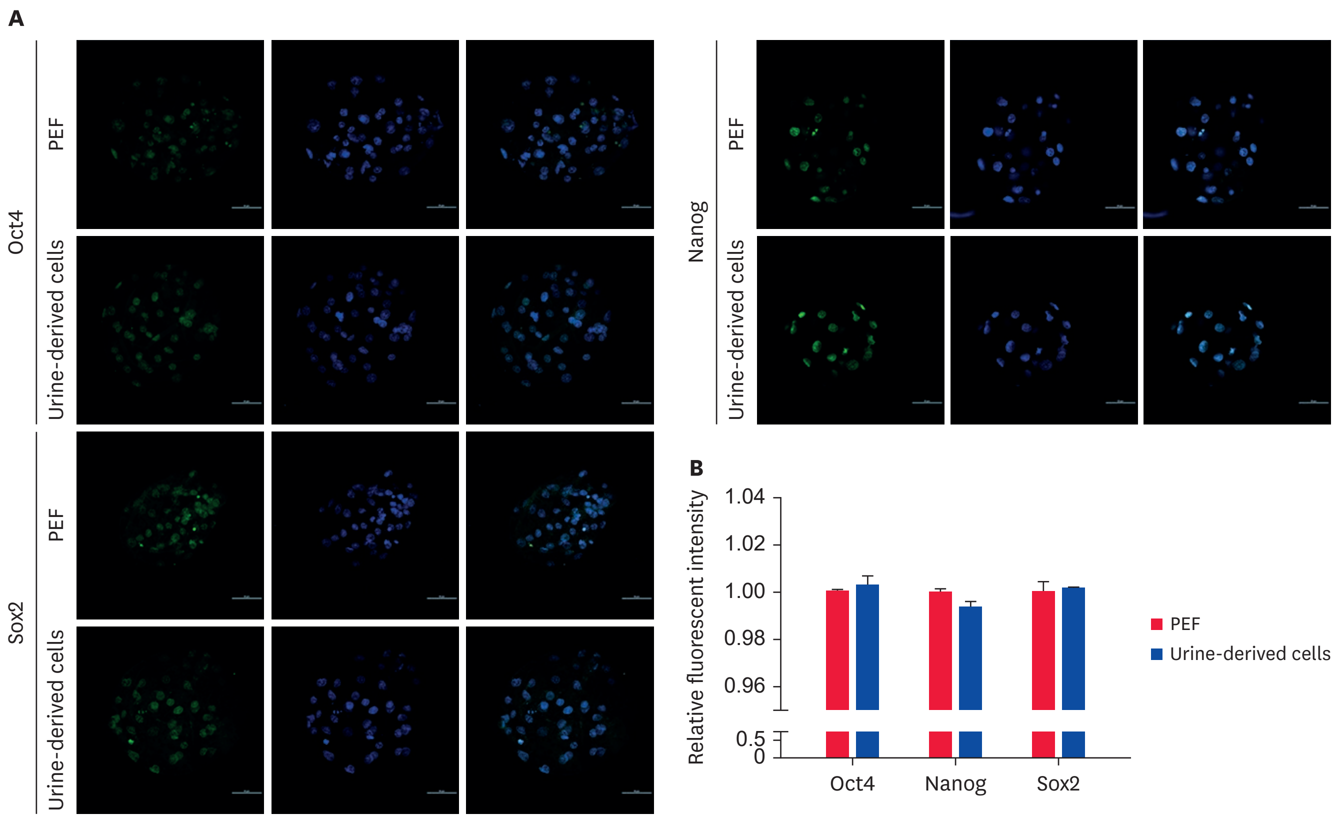

Fig. 6. Immunofluorescence staining of pluripotency factors in two types of cloned embryos at blastocysts stage. (A) Immunofluorescence assays show Oct4, Nanog, and Sox2 in day 6 blastocysts of two groups. (B) The intensity of Oct4, Nanog, and Sox2 of two groups of blastocysts at day 6. Data are the mean ± SD. PEF, porcine embryonic fibroblast.

Apoptosis detection of blastocyst produced by nuclear transfer using PEF and urine-derived cells

The apoptosis level of the cloned embryos constructed using the urine-derived cells from sows was further investigated. The TUNEL assay of the cloned embryos showed a similar positive rate in the blastocysts produced using PEF and urine-derived cells (**Fig. 7**).

In vivo development ability of embryos produced by SCNT using urine-derived cells

The *in vivo* development and birth rate of embryos produced by SCNT using urine-derived cells and PEFs were evaluated (**Table 2**). Seven hundred and twenty-two SCNT embryos of urine-derived cells were transferred into three surrogates. One of these surrogates was pregnant, but the fetuses were absorbed (shown in **Supplementary Fig. 4**).

DISCUSSION

The PEFs are commonly used to construct NT embryos in studies of reprogramming mechanisms or generation of disease models [3,4]. Ear tissue-derived fibroblasts are usually isolated and cultured for animal cloning for breed conservation. On the other hand, isolation of PEF and adult fibroblasts is generally invasive. Moreover, these methods are unsuitable for wild boar, some disease model animals, or mini-pigs, which are usually sensitive to restraint or injury. Several methods of collecting donor cells from animals non-invasively have been

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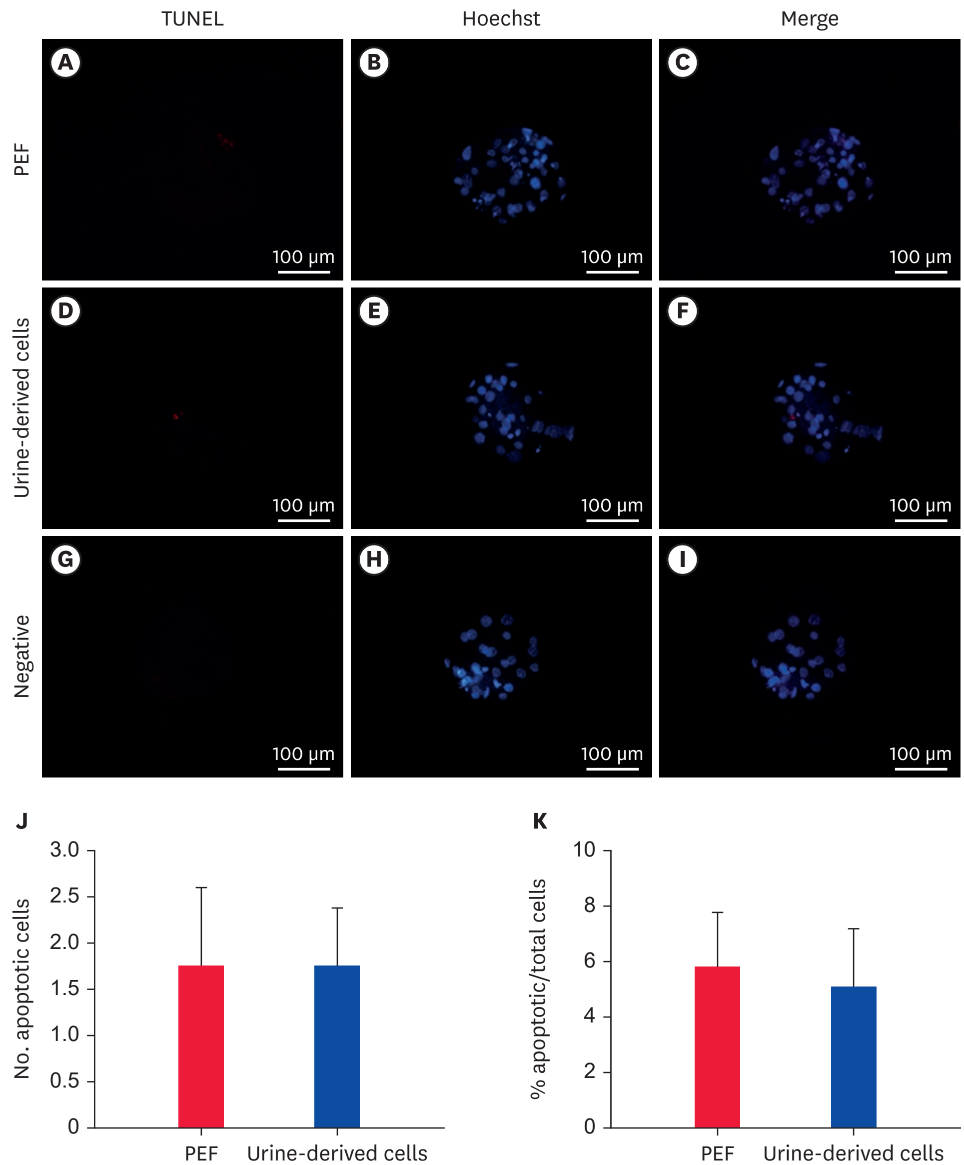

Fig. 7. Apoptosis detection of blastocysts produced by nuclear transfer using PEF and urine-derived cells. (J) Several apoptotic cells in day 6 blastocysts and (K) the ratio of apoptotic cells to total cells in day 6 blastocysts, based on the TUNEL assay, in the two types of blastocysts. Data are the mean ± SD.
TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PEF, porcine embryonic fibroblast.

Table 2. *In vivo* development and birth rate of SCNT embryos using PEF and urine-derived cells as donor cells

Donor cell type	No. of embryo transplanted	Pregnancy check	Birth	Birth rate
PEF	253	+	0	
PEF	246	-	2 live + 1 dead	1.22
PEF	276	-		
Urine-derived cells	236	-		
Urine-derived cells	249	+	Absorbed	0
Urine-derived cells	237	-		

SCNT, Somatic cell nuclear transfer; PEF, porcine embryonic fibroblast.

reported [5]. For example, the cells can be obtained from urine [16-18]. Approximately 100,000–200,000 cells can be detached from the urinary system daily and collected in human urine [19,20]. In the present study, the cells isolated from boar and sow urine showed some differences [21]. First, the cell concentration in sow urine is lower than boar urine. For sows, the urine contains a high proportion of cells with a small size. Moreover, most of the sow urine-derived cells survived until used, showing a low trypan blue ratio and PI staining. In contrast, most urine-derived cells from boars have considerable size and a high risk of death. The small and round cells with a soft surface could be chosen for nuclear transfer immediately after collection, but the number of cells was too small to construct sufficient embryos in boar urine. The reason for the high proportion of cell death might result from the high osmolality in boar urine [22,23].

The number of primarily purified cells is generally insufficient to generate SCNT embryos. The pig SCNT reprogramming is very inefficient. Therefore, it is essential to culture and propagate urine cells before NT. For cell isolation and propagation from sow urine, EGF and hydrocortisone were supplemented in the culture medium, and the cells can be cultured easily. On the other hand, the attempts to establish cells of boar urine in culture failed repeatedly because of the few viable cells and fungi contamination. In particular, the problems were not overcome even when combined with cytokines supplementation and high concentrations of antibiotics for the initial seven days of culture. Frequent contamination is probably due to pathogen storage in the diverticulum preputial. Another problem in isolating urine cells in boar is the contamination of the urine crystals, even by utilizing Percoll gradient centrifugation to enrich the cell population. Mizutani et al. obtained male and female cloned mice using urine-derived cells [12,24,25], suggesting that urine-derived cells from both male and female mice are sufficiently viable to be used as nuclear donors. For farm animals, it was reported that one cloned buffalo was generated from urine-derived cells [13]. In that study, however, the donor urine-derived cells were collected from two female buffaloes. Therefore, the status of urine-derived cells in pigs is much different from rodents.

Urine is not optimal for cell survival because the osmolality is high, and uric acid and ammonia are toxic [26]. The extreme environment would adversely affect cell survival and nuclear integrity. Furthermore, the epigenome might be affected by environmental factors. Each cell type at different degrees of differentiation might be reflected in a different pattern of epigenetic modifications. Several studies have shown that the aberrant H3K9me3 during zygote gene activation is the key obstacle for somatic cell reprogramming in each species [27-30]. In the present study, H3K9me3 staining in two types of NT embryos at the four-cell stage showed a similar intensity but much higher than IVF embryos. Moreover, sow urine-derived cells and PEF also had similar modifications levels of H3K9me3 and H3K27me3. The above results suggested that the urine-derived cells are potentially optimal nuclei donors. Furthermore, this speculation was proven by the similar blastocyst rate and blastocyst cell numbers between the two types of embryos. Moreover, the developmental competence of blastocysts, as indicated by the expression level of pluripotency-related factors (Oct4, Nanog, and Sox2) and TUNEL-staining, was similar between the cloned embryos produced from urine-derived cells and PEF.

Most importantly, the *in vivo* development of embryos produced by SCNT using urine-derived cells was evaluated. The cloned embryos constructed from urine-derived cells and PEFs showed a similar implantation rate, even though the fetuses of the urine-derived cells group eventually absorbed. For implanted cloned embryos derived from other types of somatic cells, such as mammary gland epithelial cells derived from colostrum, most also suffer growth retardation during pregnancy [31]. In summary, the boar and sow urine-derived cells were isolated for the first time and used in SCNT. This study suggests that sow urine, not boar urine, is a good source of donor cells for SCNT.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Informations of urine and cell concentration from sow and boar

Click here to view

Supplementary Fig. 1

Evaluation of pH and osmolality in pig urine.

Click here to view

Supplementary Fig. 2

Evaluation of H3K9me3 and H3K27me3 modifications of sow urine-derived cells and PEF.

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Supplementary Fig. 3

H3K9me3 level in two types of cloned embryos at the four-cell stage.

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Supplementary Fig. 4

Cloned fetuses produced from urine-derived cells showed arrested development.

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