Production of a Cloned Buffalo (*Bubalus bubalis*) Calf from Somatic Cells Isolated from Urine

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Abstract

This study was aimed at isolation of cells from urine and skin on the ventral part of the tails of healthy adult female buffaloes (*Bubalus bubalis*), an area rarely exposed to solar radiation, establishment of the cells in culture, and their use as donor cells for production of buffalo embryos by handmade cloning (HMC). The blastocyst rate and total cell number of urine- and tail skin-derived embryos were similar to those of control embryos derived from ear skin cells; however, their apoptotic index was lower ($p < 0.05$) than that of control blastocysts. The global level of histone H3 acetylated at lysine 9 (H3K9ac) was similar in the three types of donor cells and in urine- and tail skin-derived HMC blastocysts and *in vitro*-fertilized (IVF) blastocysts (controls). The global level of histone H3 trimethylated at lysine 27 (H3K27me3) in the cells was in the order ($p < 0.05$) urine $\geq$ tail skin $>$ ear skin-derived cells, whereas in blastocysts, it was higher ($p < 0.05$) in urine- and tail skin-derived HMC blastocysts than that in IVF blastocysts. The expression level of *CASPASE3*, *CASPASE9*, *P53*, *DNMT1*, *DNMT3a*, *OCT4*, and *NANOG*, which was similar in HMC blastocysts of three the groups, was lower ($p < 0.05$) than that in IVF blastocysts, whereas that of *HDAC1* was similar among the four groups. Following transfer of urine-derived embryos ($n = 10$) to five recipients (two embryos/recipient), one of the recipients delivered a normal calf that is now 5 weeks old.

Introduction

SOMATIC CELL NUCLEAR TRANSFER (SCNT) is a technique in which the nucleus of a somatic cell is transferred into an enucleated metaphase II oocyte for the generation of a new individual that is genetically identical to the somatic cell donor. Although SCNT has the potential of being applied to a number of related reproductive technologies, such as stem cell production, multiplication of genetically elite animals, production of transgenic animals, production of organs or tissues for xenotransplantation, conservation of endangered species, etc., its applicability is severely limited by the low rate of live offspring which, at $< 5\%$, is much lower than that of more than 40% obtained with embryos produced by *in vitro* fertilization (IVF) (Campbell et al., 2007).

The success of SCNT is believed to be dependent on several biological factors, among which choosing the right nuclear donor is perhaps the most important, not only because the cloned animal will be a genetic copy of the donor cell genome but also because the epigenetic status of the donor cell influences its capability to be reprogrammed by the oocyte cytoplasm. The very wide variety of donor cells

from different origins and various tissues that have been used for SCNT with observed differences in overall efficiency include fibroblasts from skin, ovarian cumulus cells, mammary epithelial cells, skin cells from internal organs, Sertoli cells, macrophages, oviduct epithelial cells, granulosa cells, muscle cells, neural stem cells, blood leukocytes, lymphocytes, natural killer T cells, mature B and T cells, neural and embryonic stem cells, uterine epithelial cells, and olfactory cells (for review, see Campbell et al., 2007).

Comparison of the cloning efficiency of different cell types is difficult in view of the differences in many parameters, such as nuclear transfer (NT) procedures, species, age and genotype of the donor cells, and quality of recipient oocytes among various studies. However, certain patterns have emerged following such comparisons. In comparison to adult or terminally differentiated cells, embryonic and fetal cells have been reported to be more successful for NT in cattle (Chavatte-Palmer et al., 2002; Heyman et al., 2002) and buffalo (George et al., 2011; Shah et al., 2009). Among adult cell types, cumulus cells appear to be the best cells because they offer the highest cloning efficiency and the proportion of animals born with abnormalities is minimal

(Shah et al., 2009; Tian et al., 2003). Cumulus cells have been perhaps the most successfully used cell types to produce NT calves (Kato et al., 1998, 2000; Wells et al., 1999).

The choice of cell type cannot, however, be determined by cloning efficiency alone. Another factor that cannot be ignored if NT is to be applied extensively for producing elite cloned farm animals on a large scale is the complexity of the procedure involved in collection of donor cells from high-genetic-merit animals. The more invasive the procedure, the more is the likelihood of resistance from the owner to allow an expensive farm animal to be subjected to it. Therefore, donor cells that can be collected in a completely noninvasive and simple manner, such as cells isolated from milk, urine, or saliva, have an edge over other cell types, such as cumulus cells, which require invasive procedures like ultrasound-guided transvaginal oocyte retrieval for their collection. There is an urgent need to evaluate the efficacy of cells that can be obtained through noninvasive or minimally invasive procedures, in terms of their ability to act as nuclear donors for producing good-quality cloned embryos.

The present study was carried out to explore the possibility of isolating somatic cells from sources other than the conventional ones and to use them for production of buffalo embryos by handmade cloning (HMC). We chose urine as a source of somatic cells for NT because it can be collected in a completely noninvasive manner. In addition, we chose skin cells obtained from the ventral part of the buffalo's tail, which has minimal exposure to sunlight, as exposure to solar radiation has been reported to cause damage to the skin cell DNA (Hoffmann-Dörr et al., 2005; Kielbassa et al., 1997; Pflaum et al., 1998; Schuch and Menck, 2010).

Here we report the isolation and establishment of cells from urine and tail skin and production of HMC embryos using these cells as donors. We compared the developmental competence of cloned embryos, as indicated by the blastocyst rate, and their quality, as indicated by the total cell number (TCN) and apoptotic index (AI), using ear skin cells as controls. We also compared the global level of acetylation of histone H3 at lysine 9 (H3K9ac) and global level of histone H3 trimethylated at lysine 27 (H3K27me3), and relative expression levels of pluripotency- (*OCT4* and *NA-NOG*), apoptosis- (*CASPASE3*, *CASPASE9*, and *P53*), and epigenetics-related genes (*DNMT1*, *DNMT3a*, and *HDAC1*) in the three cell types and the cloned embryos produced from them. IVF embryos were used as controls.

Materials and Methods

All of the chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA); the disposable plasticware was from Nunc (Roskilde, Denmark), and the media were from GIBCO (Grand Island, NY, USA) unless otherwise mentioned. Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Animal experiments were carried out after approval by the Animal Ethics Committee (National Dairy Research Institute, Karnal). Three types of somatic cells (ear skin-, tail skin-, and urine-derived cells) were obtained from the same animal. Two healthy adult female buffaloes (Mu-5345 and Mu-5365) available at the Animal Farm, NDRI, Karnal, were used in the present study. Somatic cells between passages 3 and 8 were used for HMC for each cell type.

Isolation and culture of urine-, tail skin-, and ear skin-derived somatic cells

For isolation of somatic cells, urine samples collected in a sterile beaker were immediately transferred to the laboratory and centrifuged at $400 \times g$ for 10 min. The pellet was then washed six to eight times with the washing medium [Dulbecco's modified Eagle medium (DMEM)/F12, 10% FBS, 100 $\mu\text{g}/\text{mL}$ gentamicin, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 2.5 $\mu\text{g}/\text{mL}$ amphotericin B]. The final pellet, resuspended in 2 mL of the washing medium, was loaded onto a gradient of Percoll (90%, 50%, 30%, and 10%) in a 15-mL Falcon tube and centrifuged at $400 \times g$ for 20 min. The contents of the 30% and 50% layers were washed twice with the culture medium [DMEM/F12, 20% FBS, 10 ng/mL epidermal growth factor (EGF), 5 $\mu\text{g}/\text{mL}$ insulin, 0.5 $\mu\text{g}/\text{mL}$ hydrocortisone, 100 $\mu\text{g}/\text{mL}$ gentamicin, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 2.5 $\mu\text{g}/\text{mL}$ amphotericin B] and cultured in the same medium in a CO_2 incubator (5% CO_2 in air) at 38.5°C for ~ 7 days. The medium was changed with fresh medium every 48 h. Cell colonies appeared after ~ 7 days, after which the culture medium was changed to DMEM/F12, 20% FBS, 10 ng/mL EGF, 5 $\mu\text{g}/\text{mL}$ insulin, 0.5 $\mu\text{g}/\text{mL}$ hydrocortisone, 50 $\mu\text{g}/\text{mL}$ gentamicin, 50 IU/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 2.5 $\mu\text{g}/\text{mL}$ amphotericin B. The cells were cultured until they reached 70–80% confluence, after which they were trypsinized and subcultured.

For obtaining tail skin-derived cells, the skin tissue biopsy was taken from the ventral side of the tail, just above the anal region, using an ear notcher. Ear skin biopsies were taken in a similar manner. Primary cell culture and donor cell preparation for HMC were performed as reported earlier (Selokar et al., 2012a).

Immunocytochemical characterization of somatic cells

The cells were cultured in 96-well plates (approximately 2000 cells/well) until they attained 70–80% confluence, following which they were fixed for 1 h in 4% paraformaldehyde [in Dulbecco's Phosphate-Buffered Saline (DPBS)]. The cells were permeabilized by treatment with 0.5% Triton X-100 for 30 min, blocked with 3% bovine serum albumin (BSA), incubated for 1 h with the primary antibody (mouse anti-cytokeratin, 1:500, SC-32329, Santa Cruz Biotechnology; anti-keratin, 1:500, MAB1611, Millipore; mouse anti-vimentin 1:500; V6630, Sigma) diluted in the blocking solution, and then with the secondary antibody [goat anti-mouse/rabbit immunoglobulin G (IgG)] conjugated with fluorescein isothiocyanate (FITC) for 1 h. Positive controls used for testing the staining protocol were from each respective cell culture and were labeled with mouse anti-tubulin (1:500; T8328, Sigma), whereas the addition of the primary antibody was omitted in the negative controls. The cells were incubated for 10 min in 10 $\mu\text{g}/\text{mL}$ Hoechst 33342 to stain the nuclei. The cells were examined by epifluorescence microscopy to detect fluorescence. The images were merged using Adobe Photoshop CS 8.0.1 software (Adobe Systems Inc., San Jose, CA, USA).

Examination of relative cell viability of cells

The metabolic activity of the donor cells of the three types was determined as described earlier (Selokar et al., 2012a). Briefly, donor cells between passages 8 to 10 were

seeded in 96-well plates and cultured in DMEM/F12 and 20% FBS for 72 h. The cells were then incubated with 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 37°C for 2 h after which dimethyl sulfoxide (DMSO) (diluted 1:1 with the culture medium) was added and the reactants were mixed until the formazan crystals were completely dissolved. The optical density of dissolved formazan was measured at 570 nm using a Multiskan Ascent Microplate Reader. Absorbance ratio of treated to nontreated control cells was calculated and presented as relative cell viability.

Production of IVF embryos

Cumulus–oocyte complexes (COCs) collected from abattoir buffalo ovaries were subjected to *in vitro* maturation (IVM) and IVF as described earlier (Sharma et al., 2011). For *in vitro* culture (IVC), the presumed zygotes were washed several times with Research Vitro Cleave Medium (K-RVCL-50, Cook®, Queensland, Australia) supplemented with 1% fatty acid-free BSA and were cultured in this medium for up to 8 days after insemination in a CO₂ incubator at 38.5°C.

HMC and assessment of embryo development and quality

Somatic cells were synchronized in the G₁ stage of the cell cycle by growing them in culture to full confluence for contact inhibition, as described previously (Selokar et al. 2012a). HMC, which included IVM, cumulus/zona removal, manual enucleation, fusion, and activation, was performed as described earlier (Selokar et al. 2012b). The blastocyst rate recorded on day 8 of IVC was taken as a measure of the developmental competence of embryos. For examining the quality of blastocysts, their TCN and level of apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, as described earlier (Selokar et al., 2014). Cell counting was performed from the digital images obtained on an inverted Nikon fluorescence microscope. Each experiment was repeated at least three times. AI = (number of TUNEL-positive nuclei in the blastocyst/total number of nuclei counted in that blastocyst) × 100.

Immunofluorescence staining for epigenetic markers in somatic cells and embryos

The global levels of H3K9ac and H3K27me3 were examined by immunofluorescence staining in the three types of somatic cells and the blastocysts derived from them, as described earlier (Selokar et al., 2014). NIS-Elements Basic Research image-processing software (Nikon, Tokyo, Japan) equipped with a microscope was used for image acquisition and quantitative measurements of the mean pixel intensity emitted by each individual nucleus. At least 10 images (50 nuclei from each image) were analyzed for each epigenetic marker.

Gene expression analysis in donor cells and embryos

Quantitative real-time PCR (qPCR) was performed as described earlier (Singh et al., 2014). Briefly, RNA was isolated from the three types of donor cells and from pools of blastocysts ($n=10$ each) using an RNAqueous-Micro Kit (Ambion, Austin, TX, USA) as per the manufacturer's

protocol. Following DNase treatment, a reverse transcriptase (RT) reaction was performed for cDNA preparation using Superscript Reverse Transcriptase III (Invitrogen). Quantification of mRNA was carried out by qPCR using a CFX96 C1000 Thermal Cycler Real-Time System (BioRad). The reaction mixture (10 μ L) contained 5 μ L of SYBR Green Master Mix (Maxima SYBR Green Mastermix, Thermo Scientific), 0.2 μ L of 10 μ M of each primer, and cDNA diluted 2 $\times$. Thermal cycling conditions consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 15 sec at 95°C, 15 sec at the corresponding annealing temperature, and 15 sec at 72°C, followed by 95°C for 10 sec (see Table S1) (Supplementary Data are available at www.liebertpub.com/cell/).

All of the primer pairs used were confirmed for their PCR efficiency, and specific products were checked by melt curve analysis and for the appropriateness of size by 2% agarose gel electrophoresis. Primer sequences are provided in Table S1. The expression data were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and were analyzed with CFX Manager software (BioRad). In all of the experiments, three trials were carried out, each in duplicate.

Embryo transfer and detection of pregnancy

Cycling buffaloes possessing a functional corpus luteum were treated with prostaglandin F_{2 α} (PGF_{2 α}) analog (cloprostenol sodium, 500 mg) intramuscularly. Those exhibiting estrus 72 h after the treatment were selected as recipients. Day 7/8 blastocysts ($n=4$) produced using donor cells obtained from urine were transferred, two to each recipient. Pregnancies were examined by transrectal palpation between days 60 and 90.

Experimental design and statistical analysis

In experiment 1, somatic cells were isolated from urine, tail skin, or ear skin and were established in culture and characterized. In experiment 2, the relative cell viability of the three types of cells was compared by the MTT assay. In experiment 3, the three types of cells were used as donor cells for producing cloned embryos, which were compared for their developmental competence, as indicated by the blastocyst rate, and quality, as indicated by TCN and AI. In experiment 4, the global levels of H3K9ac and H3K27me3 were compared among donor cells of the three types and in blastocysts produced from them using immunofluorescence staining. In experiment 5, the relative mRNA abundance of some apoptosis- (*CASPASE 3*, *CASPASE 9* and *P53*) and epigenetics-related genes (*DNMT1*, *DNMT3a*, and *HDAC1*) was compared among donor cells of the three types.

Also, the relative transcript level of these genes and of pluripotency-related genes (*OCT4* and *NANOG*) was compared among blastocysts produced from the three types of donor cells using IVF embryos as controls. Statistical analysis was carried out using Sigma Stat (v. 3.1, Aspire Software International, VA, USA). The datasets were analyzed by one-way analysis of variance (ANOVA) followed by the Holm–Sidak test. Percentage values were subjected to arcsine transformation prior to analysis. The differences were considered to be statistically significant at $p < 0.05$. Data were presented as mean $\pm$ standard error of the mean (SEM).

Results

The morphological appearance of urine-, tail skin-, and ear skin-derived somatic cells is presented in Figure 1. Somatic cells from urine and tail skin were found to express vimentin, keratin, and cytokeratin, whereas those from ear skin were found to express vimentin but not keratin and cytokeratin (Fig. 2). In experiment 2, the relative cell viability of urine- and tail skin-derived cells was found to be higher ($p < 0.05$) than that for the ear skin-derived cells (Fig. 3).

In experiment 3, the cleavage and blastocyst rates of embryos produced using donor cells isolated from urine and tail skin were found not to be significantly different from those of embryos produced using ear skin cells (controls) (Table 1). The AI was lower ($p < 0.05$) for the blastocysts produced from urine- and tail skin-derived cells than that for blastocysts produced from ear skin-derived cells. However, the TCN was similar among the blastocysts of the three groups.

In experiment 4, the immunofluorescence staining for H3K9ac and H3K27me3 of donor cells of different types and cloned blastocysts produced using these cells is shown in Figure S1. The global level of H3K9ac, which was found to be similar in urine-, tail skin-, and ear skin-derived somatic cells, was not significantly different in blastocysts produced from urine- or tail skin-derived cells from that in IVF blastocysts, whereas it was higher ($p < 0.05$) in ear skin-derived blastocysts than that in tail skin-derived or IVF blastocysts (Fig. 4). The global level of H3K27me3 was significantly higher ($p < 0.05$) in urine- than that in tail skin-derived cells which, in turn, was higher ($p < 0.05$) than that in ear skin-derived cells. In the blastocysts, it was higher ($p < 0.05$) in those produced from urine- and tail skin-derived cells than that in IVF blastocysts, which had a global level of H3K27me3 similar to that in ear skin-derived blastocysts.

Gene expression analysis in donor cells and embryos

Among apoptosis-related genes, the relative transcript levels of *CASPASE3*, *CASPASE9*, and *P53* were similar in the urine- and tail skin-derived cells and significantly higher ($p < 0.05$) than that in the control ear skin-derived cells (Fig. 5A). However, in the cloned blastocysts produced using these three types of cells, the relative transcript levels of these genes were not significantly different, but were lower ($p < 0.05$) than that in IVF blastocysts (Fig. 5B).

Among the epigenetics-related genes, the relative transcript levels of *DNMT1* and *DNMT3a* were not significantly different among the three types of donor cells. They were similar in the cloned blastocysts produced from them, which was lower ($p < 0.05$) than that in IVF blastocysts. The expression level of *HDAC1* was lower ($p < 0.05$) in the urine- than that in the tail skin- and ear skin-derived cells, whereas it was similar in the cloned blastocysts produced from the three types of donor cells and that in IVF blastocysts. Among pluripotency-related genes, the expression of *OCT4* and *NANOG* could not be detected in the donor cells. The relative transcript levels of these two genes were similar in cloned blastocysts produced from the three types of cells, which were lower than that in IVF blastocysts. Following transfer of urine-derived embryos ($n = 10$) to five recipients (two embryos per recipient), one of the recipients delivered a normal healthy calf that is now 5 weeks of age (Fig. 6). The parentage of the cloned calf was confirmed by DNA microsatellite analysis (Table S2).

Discussion

To our knowledge, this is the first report in any species of the production of a cloned animal using donor cells isolated from urine. We also produced NT embryos from cells

FIG. 1. Somatic cells isolated from urine (A) at the time of seeding; at day 7 of culture (B), and in confluent monolayer (C). Somatic cells isolated from tail skin in primary explant culture (D) and in confluent monolayer (E). Somatic cells isolated from ear skin in confluent monolayer. Magnifications, A–D, 200 $\times$; E and F, 40 $\times$).

FIG. 2. Expression of cell-specific markers in cells isolated from urine (A), tail skin (B), and ear skin (C). Scale bar is 100 μm .

FIG. 3. Relative cell viability of cells isolated from urine, tail skin, and ear skin. Bars with different superscripts differ significantly ($p < 0.05$).

isolated from skin on the ventral side of tail, which is rarely exposed to solar radiation. The developmental competence, as indicated by the blastocyst rate and the TCN, and the expression profile of many important apoptosis- (*CASPASE3*, *CASPASE9*, and *P53*), epigenetics- (*DNMT1* and *HDAC1*), and pluripotency-related (*OCT4* and *NANOG*) genes was similar among cloned embryos produced from urine- and tail skin-derived cells and those produced using ear skin-derived cells (controls), which are the most commonly used types of donor cells. The cloned blastocysts produced from urine- and tail skin-derived cells scored over those produced from ear skin-derived cells in terms of better quality, as indicated by lower AI, and being closer to IVF blastocysts (controls) in their global level of H3K9ac.

We explored the clonability of two new types of yet unexplored donor cells. Urine was chosen as a source of somatic cells because it can be collected in a noninvasive manner. Our attempts to isolate cells from urine and establish them in culture failed repeatedly because of inability to remove high levels of contamination present frequently in the urine samples, despite several washings, and failure of collected cells to attach to the substrate and survive the initial culture, probably due to low cell density. Both of these problems were overcome by combining Percoll gradient centrifugation for enriching the cell population and the use of high concentrations of antibiotics for the initial 7 days of culture. In addition, supplementation of the culture medium with EGF, insulin, and hydrocortisone enabled the cells to proliferate and form colonies with 7 days of being seeded.

FIG. 4. Mean pixel intensity of H3K9ac and H3K27me3 examined by immunofluorescence staining in donor cells of different types and in cloned blastocysts produced using these cells. Bars with different superscripts differ significantly ($p < 0.05$).

Although skin fibroblasts are perhaps the most commonly used types of donor cells for NT, to our knowledge, there is no report on the use of skin fibroblasts obtained from the part of the body that is not exposed to sunlight. Therefore, we took a biopsy of skin tissue from the ventral side of the tail, just above the anal region, which is not exposed to sunlight. Somatic cells isolated from urine and tail skin expressed vimentin, keratin, and cytokeratin, indicating that they could be a mixed population of fibroblasts and epithelial cells, whereas those from ear skin expressed vimentin but not keratin and cytokeratin, indicating that they were of fibroblast origin. The relative cell viability of urine- and tail skin-derived cells was found to be higher ($p < 0.05$) than that for the ear skin-derived cells between passages 8 and 10, indicating their robustness in culture.

We found that not only the urine- and tail skin-derived cells were capable of being reprogrammed and forming cloned embryos but also that the developmental competence

TABLE 1. DEVELOPMENTAL COMPETENCE AND QUALITY OF EMBRYOS PRODUCED BY HANDMADE CLONING USING DIFFERENT TYPES OF DONOR CELLS

	Reconstructs (n)	Cleaved n (%)	Blastocysts n (%)	Blastocysts examined for TUNEL assay (n)	Total cell number	Apoptotic index
Ear skin (control)	257	243 (94.0 ± 1.96)	101 (40.2 ± 3.61)	25	206.8 ± 23.27	5.51 ± 0.68 ^a
Tail skin	226	217 (95.8 ± 0.92)	108 (47.8 ± 3.47)	25	237.0 ± 21.96	2.89 ± 0.34 ^b
Urine	284	271 (95.2 ± 1.63)	148 (50.4 ± 4.18)	22	267.1 ± 29.43	3.61 ± 0.44 ^b

Data from 13 trials.

Values are mean ± standard error of the mean (SEM).

Values with different superscripts within the same column differ significantly ($p < 0.05$).

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

FIG. 5. Relative mRNA abundance of some important genes in donor cells of different types (A) and in cloned blastocysts produced using these cells (B). Bars with different superscripts differ significantly ($p < 0.05$). IVF, in vitro fertilization.

of embryos produced from these cells, as indicated by the blastocyst rate, was higher than that of embryos produced from ear skin cells, which were used as controls, although the differences were not statistically significant. The blastocysts produced from urine- and tail skin-derived cells were, however, of superior quality compared to those produced from ear skin cells, in terms of a lower AI. This could be due to a higher level of apoptosis in ear skin cells because the AI has been reported to be higher in bovine blastocysts derived from annexin-positive apoptotic cells than in those derived from annexin-negative nonapoptotic cells (Miranda Mdos et al., 2009).

However, a lower level of apoptosis was not reflected in the relative transcript levels of proapoptotic genes *CASPASE3* and *CASPASE9* and in the expression level of *P53*, which were found to be similar among the blastocysts produced from the three types of cells. It is important for NT embryos to have a lower level of apoptosis because the higher apoptotic morphology and/or TUNEL labeling re-

ported in NT bovine embryos compared to that in IVF embryos (Cui et al., 2011; Selokar et al., 2013) is believed to be an important reason behind the lower conception rate obtained with NT embryos.

What makes a good donor cell is currently not fully understood. Very little is known about the nature of biological factors that determine the “clonability” of cells. However, the following three principal reasons have been attributed to the differences in the clonability of different types of donor cells: (1) Genetic or epigenetic errors in the donor genome, (2) faulty or incomplete genetic or epigenetic reprogramming, or (3) a combination of both (Obach and Wells, 2002). It is believed that all the three factors may be important, but their relative roles depend on the choice of the nuclear donor.

The solar radiation reaching the earth’s surface includes ultraviolet B (UVB, wavelength 280–320 nm) and UVA (wavelength 320–400 nm) radiation; of the short wavelength part of the spectrum emitted by the sun, wavelengths shorter than 290 nm (UVC) do not reach the earth’s surface because

FIG. 6. A cloned calf, produced from somatic cells isolated from urine, at 5 weeks of age.

they are efficiently absorbed by the atmospheric ozone layer. Besides UV, infrared (IR) radiation and especially the near-IR range (wavelength 760–1440 nm) also reaches the earth's surface. The skin is the primary target organ for solar radiation. Ionizing and UV radiation cause cell damage or death by several mechanisms, including production of reactive oxygen species (ROS) and reactive carbonyl species (Jacobson et al., 2001), formation of pyrimidine dimers (Sutherland et al., 1992), induction of genotoxic oxidative DNA damage in cells (Hoffmann-Dörr et al., 2005), induction of transcription of many genes (Bender et al. 1997), increase in immunoreactivity of p53 (Hall et al., 1993) and c-Fos and c-Jun in the epidermis (Gillardon et al., 1994; Lu et al., 1994), and photoaging of the skin (Calles et al. (2010). Our results suggest that the adverse effects of sunlight on skin cells may influence their clonability as well as the quality of cloned embryos produced, as indicated by the lower AI of blastocysts produced from tail skin cells compared to that of blastocysts produced from ear skin cells. Although the level of apoptosis is an important factor related to embryo quality, there are many other factors, such as gene expression levels, epigenetic status, and especially the live offspring rate, that must be examined before the superiority of cells not exposed to sunlight can be established.

DNA methylation patterns have been found to be abnormal and aberrant in NT embryos in cattle (Kremensky et al., 2006) and pigs (Deshmukh et al., 2011). It is believed that DNA methylation and specific histone modifications influence each other and that the relationship can work in both directions: Histone methylation can help to direct DNA methylation patterns, and DNA methylation might serve as a template for some histone modifications after DNA replication (Cedar and Bergman, 2009). Histone modification and DNA methylation can have different roles in gene silencing, with histone modifications providing labile transcriptional repression and DNA methylation being a highly stable si-

lencing mark that is not easily reversed (Cedar and Bergman, 2009). H3K27me3 is an important histone modification that has substantial influence on regulation of gene expression through alteration in DNA methylation. H3K9ac is another most well-known epigenetic marker, the level of which is enriched in the promoter region of activated genes and is strongly correlated to gene expression (Nishida et al., 2006). Both acetylation and trimethylation of H3K27, the profiles of which vary between NT and normal embryos (Breton et al., 2010; Zhou et al., 2014), are considered important epigenetic markers. The repressive marker H3K27me3 has been reported to be actively antagonized by H3K27ac, leading to activation of the Polycomb group protein silencing genes during mouse embryonic stem cell differentiation (Pasini et al., 2010).

In our study, no clear pattern emerged in the epigenetic status of urine- and tail skin-derived blastocysts in terms of their global levels of H3K9ac and H3K27me3 compared with those of IVF blastocysts. Whereas the urine- and tail skin-derived blastocysts were found to be closer to those produced by IVF in terms of the global level of H3K9ac, which was similar among these three groups of blastocysts, an opposite pattern was observed for H3K27me3, the global level of which was higher in urine- and tail skin-derived blastocysts than that in IVF blastocysts. The global level of H3K27me3, but not that of H3K9ac, in the cloned blastocysts reflected that in the donor cells.

The methylation pattern of the DNA is established and maintained by DNA methyltransferases (DNMTs). DNMT1 is responsible for maintenance of DNA methylation (Bestor, 2000), whereas DNMT3a and b are thought to be responsible for the establishment of *de novo* methylation during gametogenesis and early embryonic development (Okano et al., 1999). We found that the relative transcript levels of *DNMT1* and *DNMT3a* were not significantly different among the three types of donor cells. The expression levels of these genes and of pluripotency-related genes *OCT4* and *NANOG* were similar in cloned blastocysts produced from the three types of cells, but were lower than those in IVF blastocysts. These results agree with those of Beyhan et al. (2007), who found *DNMT3a* and *OCT4* expression levels in NT bovine embryos to be lower than that in IVF embryos.

Acetylation status of a particular chromatin domain is maintained by opposing activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). We found that the expression level of *HDAC1* was lower ($p < 0.05$) in the urine-derived cells than that in the tail skin- and ear skin-derived cells but was similar in cloned blastocysts produced from the three types of cells and that in IVF blastocysts.

The ultimate test of the quality of any NT embryo is its ability to result in pregnancy leading to birth of normal offspring. We transferred urine-derived embryos ($n = 10$) to five recipients, two embryos to each recipient, to examine their *in vivo* developmental competence. One of the recipients, which was found to be pregnant at day 60, delivered a normal healthy calf which is now 5 weeks of age.

In conclusion, it was demonstrated in the present study that urine- and tail skin-derived somatic cells can be successfully used for producing NT embryos. The developmental competence, as indicated by the blastocyst rate, and quality, as indicated by the TCN, and expression profile of

many important apoptosis- (*CASPASE3*, *CASPASE9*, and *P53*), epigenetics- (*DNMT1* and *HDAC1*), and pluripotency-related (*OCT4* and *NANOG*) genes were similar among cloned embryos produced from urine- and tail skin-derived cells and those produced using ear skin-derived cells. The cloned blastocysts produced from urine- and tail skin-derived cells scored over those produced from ear skin-derived cells in terms of lower AI and being closer to IVF blastocysts in their global level of H3K9ac.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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