—Mini Review—

Animal Cloning by Nuclear Transfer Using Somatic Cells Recovered from Organs Frozen without Cryoprotectant

*Yoichiro Hoshino[1] and Kazuhiro Saeki[2]**

[1]Gifu Prefectural Livestock Research Institute, Gifu 506-0101, Japan
[2]Department of Genetic Engineering, Kinki University, Wakayama 649-6493, Japan

Abstract: *Cloning by somatic cell nuclear transfer (SCNT) can be used for the conservation of endangered and extinct animals and elite livestock, and their somatic cells have often been cryopreserved as genetic resources. However, viable cells are not always available when individuals are already dead or when their species have extinct. If the intact genome could be retrieved from such animals, cloned animals could be produced by SCNT technology. Recently, we have demonstrated that viable somatic cells can be obtained from certain bovine organs frozen without cryoprotectant for a decade and that viable cloned animals can be produced from the retrieved cells by SCNT technology. In this mini-review, we discuss recent attempts to rescue animal genetic resources from tissues, organs or bodies frozen without cryoprotectant for a long time.*

Key words: *Animal cloning, Somatic cell nuclear Transfer, Cryopreservation, Conservation of animal resources*

Introduction

Successful animal cloning by somatic cell nuclear transfer (SCNT) has been achieved in several mammalian species, such as sheep, mice, cattle, goats, and pigs [1–7]. The cloning technique is used to breed "elite" domestic animals in the livestock industry, and for the conservation of genetic resources of endangered wild animals. In the USA, cloned animals of elite livestock, such as beef bulls, breeding boars and top-performing horses, have been produced commercially (http://www.viagen.com/). It has been suggested that genome resource banks should be established for the cryopreservation of gametes, embryos and somatic cells of endangered animals [8], and clones have already been produced from several endangered mammalian species to maintain biodiversity [9–11].

For the cryopreservation of animal somatic cells, tissues are collected from live or postmortem animals within several hours after death, then they are cultured with appropriate culture media in an incubator to obtain cell cultures. In cases in which donor individuals are already dead or their species extinct, genetic resources such as viable cryopreserved cells are not always available. We have demonstrated that live and healthy cloned cattle can be produced from an organ frozen without any cryoprotectant for more than a decade [12]. In this review, we will discuss recent attempts to rescue animal genetic resources from non-viable cells or animal bodies, organs and tissues that have been frozen without cryoprotectant for a long time.

Cloning Animals from Non-frozen Dead Cells

There are two major methods of SCNT. One is a cell fusion method in which a somatic cell is fused with an enucleated oocyte by electro-stimulation. This fusion method was used for production of the first cloned sheep "Dolly" [1], and it is usually used for cattle [3] and sheep cloning. Theother method is the nuclear injection method in which a single denuded donor nucleus is directly injected into an enucleated oocyte. The nuclear injection method was used in the production of the first cloned mouse "Cumulina" [2], and it has also been applied to pigs [5] and goats [13]. For the fusion method, viable cells are generally needed as donor cells. However, previous reports of the successful production of cloned animals by the injection method clearly indicate that the genomic integrity of donor

Received: June 18, 2010
Accepted: July 21, 2010
*To whom correspondence should be addressed.
e-mail: saeki@waka.kindai.ac.jp

nuclei rather than the viability of donor cells is essential for the success of animal cloning. For example, Loi *et al.* produced live cloned animals from granulosa cells recovered from ovaries of two endangered sheep (mouflon) that were dead for 18 to 24 h. Trypan blue staining indicated that the granulose cells were dead, but cloned embryos produced by injection of their nuclei into enucleated goat oocytes developed to term [11]. Their report indicated that the genome of somatic cells recovered from dead animals is intact, at least shortly after death. Furthermore, Loi *et al.* also demonstrated that heat-denatured non-viable somatic cells that were subjected to temperatures of 55 to 75ºC for 15 min retain the potential to generate normal offspring following SCNT [14], suggesting that the structural integrity of nuclear and cytoplasmic proteins is not necessary for the development of SCNT embryos. Thus, these results indicate that cell death and/or cell denaturation does not always result in the loss of genomic integrity, and that cloned animals can be produced from non-viable cells if the genome is intact.

Intracytoplasmic Sperm Injection of Frozen Dead Sperm

Freezing organisms or cells without cryoprotectant results in a variety of harmful physical and chemical changes. When an organism is cooled to a temperature below the equilibrium freezing point of its own tissue fluids, the cells are damaged by the direct physical effects of ice crystals formed in the tissues and by the indirect chemical effects of salt [15]. The activity of DNase is largely influenced by divalent cations [16], and it is possible that the destruction of the cell membrane and sub-cellular compartments by ice-crystal formation alters intracellular ionic homeostasis, resulting in the activation of endogenous DNases, and subsequently DNA fragmentation.

Unlike somatic cells which are vulnerable to freezing damage, spermatozoa are tolerant to freezing because of their small size and low degree of hydration, and because their transcriptionally inactive DNA is tightly packed with protamines. Up to now, artificial insemination using frozen semen has played an important role in animal breeding. In addition, there have been many attempts to obtain offspring with microinjection of sperm frozen without cryoprotectant into oocytes, because simple freezing is easier and less costly method for sperm preservation.

There are several reports that mouse spermatozoa after freezing without cryoprotectant, and even after freeze-drying which severely damages plasma membranes making them dead in the conventional sense, were successfully used to fertilize mouse oocytes in intracytoplasmic sperm injection and that the resultant embryos developed to healthy mice [17, 18].

Ogonuki *et al.* have obtained live pups by intracytoplasmic injection of dead spermatozoa and spermatids from mouse testes which were recovered from dead mouse bodies stored at –20ºC in a freezer for 15 years without any cryoprotectant [19]. To avoid the activation of endogenous DNases which causes DNA damage, they used potassium-rich Ca^{2+} and Mg^{2+} free nuclear isolation medium (NIM) [20] for the suspension of the defrosted spermatozoa and spermatids. Although almost all spermatozoa and spermatids from the frozen mouse testes had extensively disintegrated cytoplasm, normal pups were produced after they were injected into oocytes, indicating that the genomes of the spermatozoa and spermatids can survive storage at –20ºC in a freezer for 15 years.

Cloning Animals from Frozen Dead Cells

If the integrity of the whole genome can be maintained in the nuclei of frozen dead somatic cells, cloned animals can be produced by SCNT technology. Can the genome of somatic cells withstand freezing under severe frozen conditions?

Loi *et al.* demonstrated that SCNT embryos produced from the nuclei of freeze-dried sheep granulosa cells develop to the blastocyst stage [21]. The granulosa cells were freeze-dried after adding trehalose as a cryoprotectant, and stored for three years at room temperature. Although these granulosa cells were dead and 60% of the nuclei showed obvious DNA damage, 16% of the SCNT embryos from the freeze-dried cells developed to the blastocyst stage, while the developmental rate of control SCNT embryos from fresh granulosa cells was 21%. This result suggests that damaged DNA in the donor nucleus is repaired by one or more unknown factors in the oocyte cytoplasm after introduction of freeze-dried cells into oocytes.

Li *et al.* established mouse embryonic stem (ES) cells from SCNT embryos, called nuclear transfer ES (ntES) cells. They were derived from the nuclei of mouse keratinocyte stem cells that were stored at –80ºC without cryoprotectant for 342 days. Although Li *et al.* failed to produce cloned mice from these NT embryos, they did produce germ-line chimeric mice using these ntES cells, and also produced clonal mice by injecting the ntES cells into tetraploid blastocysts [22].

Establishment of ntES cells might be a more efficient way of rescuing genomes from frozen-dead cells than direct generation of live cloned animals using frozen cells [23].

Interestingly, Wakayama *et al.* produced healthy cloned mice from somatic cell nuclei from a mouse body that had been frozen and kept at –20ºC [24]. They collected denuded nuclei from eleven organs of a mouse that had been stored at –20ºC for one week by homogenizing the organ tissues in NIM, and produced SCNT embryos by the direct injection of each nucleus into enucleated mouse oocytes. SCNT embryos produced using brain tissue showed the highest developmental competence and developed to cloned mice after embryo transfer. Cloned mice were also produced using cells recovered from brain tissues that had been kept at –20ºC for 1 month and 16 years [24]. In the latter case, a two-step nuclear transfer method was used for the production of SCNT embryos, which were produced from nuclei of ntES cells established using SCNT embryos produced from frozen mouse brain. However, fresh mouse brain cells were considered to be inappropriate as donor cells for mouse cloning [2].

These results indicate that some genomic integrity is maintained in certain organs or tissues even after 16 years at –20ºC. Different organs and cell types have different levels of cold tolerance [25], which might explain why some tissues maintain genomic integrity better than others after being frozen without cryoprotectant.

Resurrection of an Elite Bull from a Frozen Organ

The above-mentioned reports indicate that cloned animals can be produced from frozen and dead cells if the genomic integrity of the cells is maintained. However, it is almost impossible to examine whether the genomes of such cells are intact or not before nuclear transfer. If viable and proliferative cells can be obtained after being frozen without a cryoprotectant, the genomes of these cells should be intact. Our group demonstrated that viable cells can be obtained from animal organs frozen without cryoprotectant for more than a decade.

Yasufuku was a legendary bull of Japanese Black cattle (Wagyu). The beef of Yasufuku's offspring was valued for its very fine marbling and rich flavor. Today, it is estimated that 26% of Japanese Black cattle are progeny of Yasufuku. Yasufuku died of senility at 13.5 years of age in September 1993. His testicles were collected 12 hours after his death, then wrapped in aluminum foil and placed in a –80ºC freezer without cryoprotectant for 10 years. The testicles were then transferred to liquid nitrogen without cryoprotectant for another 3 years. It was expected that the somatic cells in these frozen testicles would be dead due to cryoinjury. In bovine cloning, live-cultured donor cells are commonly used to produce SCNT embryos. Thus, it was thought that cloning Yasufuku with dead cells from a frozen organ would be extremely difficult. However, we considered that if viable and culturable somatic cells from Yasufuku's frozen testicles could be retrived, it might be possible to produce cloned animals by SCNT technology.

Before experimenting with Yasufuku's testicles, we conducted preliminary experiments with fresh bovine castrated testicles. Testicles were collected from three 12- to 15-month-old bulls and were frozen at –80ºC without any special treatment in a freezer for 1 to 4 months. The frozen testicles were then dissected into different parts, caput epididymis, cauda epididymis, spermatic cords and testes. These were thawed quickly by putting them into saline at 42ºC. The thawed tissue was minced (5-mm in diameter) and incubated at 39ºC for 2 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% collagenase (Invitrogen, Carlsbad, CA, USA) and 0.2% dispase (Invitrogen, Carlsbad, CA, USA). After filtration of the resultant suspension through a 250-mm nylon mesh, the filtrate was centrifuged at 250 × g for 5 min. In our preliminary experiments, we used several kinds of culture media, such as DMEM or α-minimum essential medium (α-MEM) to obtain primary cultures for the frozen testicles. However, no cells grew from the thawed tissue. We conjectured that cells in the thawed tissue might have quite low proliferating activity. Therefore, we next selected MF-start™ medium (Toyobo, Osaka, Japan) that was developed to grow cells with low proliferating activity in primary culture. The precipitate was resuspended in MF-start™ medium and incubated at 38.5ºC in an atmosphere of 5% CO_2 in air with high humidity. After five days of culture, surprisingly, we found a number of attached cells from both the caput epididymis and the spermatic cords. Then medium was replaced with AmnioMAX™II complete medium (Invitrogen, Carlsbad, CA, USA) to induce the rapid proliferation of cells. Ten days after incubation, outgrowths had formed and the medium was replaced with MF-medium® (Toyobo, Osaka Japan). We found that active proliferated cells could be obtained from

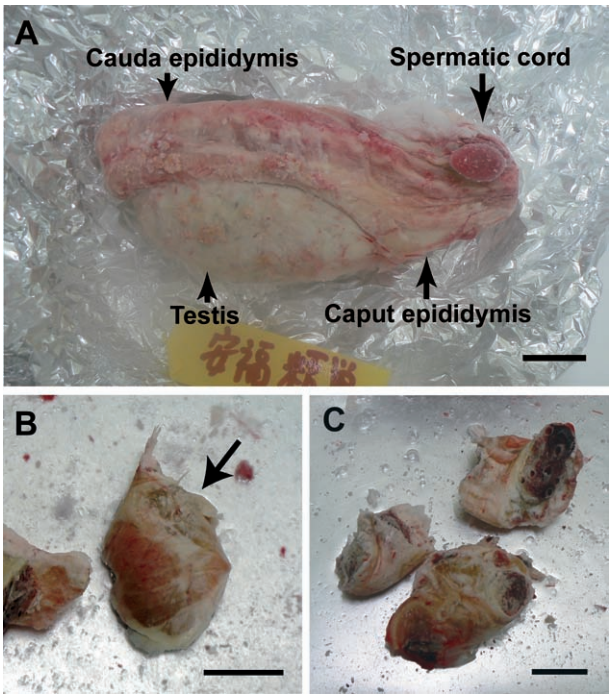

Fig. 1. (A) A frozen testicle after storage in a freezer for 10 years and in liquid nitrogen for 3 years. (B) A dissected part of the caput epididymis (arrow). (C) Spermatic cords that had been cut into three pieces. Scale bars represent 2 cm.

testicles frozen without cryoprotectant, suggesting that most of the genomes in these cells had not been severely damaged and that the plasma membranes of these cells were intact.

Next, we examined whether viable cells could be obtained from Yasufuku's frozen testicles (Fig. 1A). The caput epididymis (Fig. 1B) and the spermatic cords (Fig. 1C) of Yasufuku's testicle were isolated. They were cut into several small pieces thawed ane minced. Then, the pieces of tissue were digested as described above, and the precipitates were cultured. In the culture, a small number of cells were found attached to the bottom of the culture dishes, and they were allowed to proliferate (Fig. 2) until there were enough to use as donor cells for SCNT.

We produced SCNT embryos from Yasufuku's cells by the conventional cell fusion method. The SCNT blastocysts were transferred to 16 recipient females. Five of the recipients became pregnant and four of them delivered viable cloned calves (Fig. 3). One calf died of large offspring syndrome two days after birth and another died from peritonitis at age 5 months. The other two calves were healthy and remain healthy today, more than 2 years after birth (Fig. 4) [12]. The production efficiency of bovine clones (defined as the number of live calves over 6 months old / the number of

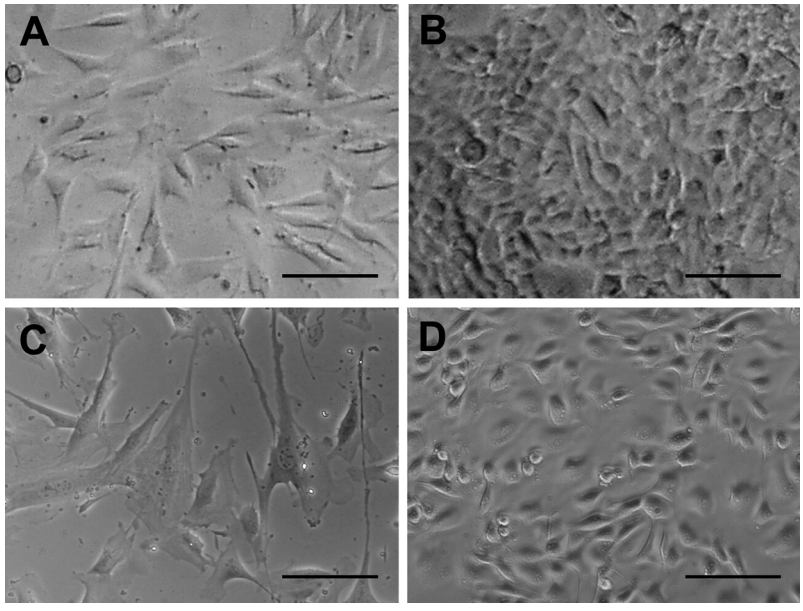

Fig. 2. Differential interference-contrast micrograph of cell populations established from Yasufuku's frozen testicles. A and C, fibroblast-like cells; B and D, epithelial-like cells. A and B were used for NT after primary culture, and C and D were cryopreserved and then subcultured with five passages. Scale bars represent 100 μm.

Fig. 3. Calves cloned from Yasufuku's frozen testicles. (A) A male calf derived from Yasufuku's testicles was born on 30 November 2007. The calf's birth weight was 18.5 kg and he remains healthy at the time of writing. (B) A male calf that was delivered by Caesarean section on 5 March 2008. The calf's birth weight was 47.5 kg; he died two days after birth. (C) The calf with ear tag "c95" was born on 22 July 2008. His birth weight was 32 kg. He died of peritonitis 169 days after birth. The calf with ear tag "c66" was born on 31 July 2008. His birth weight was 30 kg and remains healthy at the time of writing.

Fig. 4. Healthy cloned calves from Yasufuku's frozen testicles. (A) The first cloned calf of Yasufuku (30 months). We have collected its semen to produce the clone's progeny. (B) The fourth cloned calf of Yasufuku was castrated and is being fattened to examine the beef quality (22 months).

transferred embryos) in our experiment was 12.5%, which is higher than the values given in a recent report on cloning efficiency [26].

These results suggest that viable somatic cells can be obtained from mammalian organs or tissues that have been frozen without cryoprotectant, but have maintained the genomic integrity of cells.

Recovering Viable Cells from Frozen Adipose Tissues

The cloning of Yasufuku raises the possibility that a mammalian genetic resource can be maintained by simply freezing organs or tissues. We demonstrated that viable cells can be retrieved from frozen bovine testicles, although testicles are not the best source of an animal's genome because they can be obtained only from male animals. We recently proposed that adipose tissue may be more suitable for obtaining viable somatic cells after freezing without cryoprotectant [27].

Pieces of subcutaneous adipose tissue from twenty Japanese Black cattle were collected at a local slaughterhouse, individually packed in plastic sample bags, frozen in liquid nitrogen vapor and stored at –80ºC for over 4 months (Fig. 5A). The frozen adipose tissue was thawed at 39ºC (Fig. 5B). One gram of each sample was put into 5 ml of DMEM containing 0.1% collagenase and 0.2% dispase, and incubated in a water bath at 39ºC for 1 hour with agitation (Fig. 5C). After centrifugation, most of the cell debris and lipid floated to the top and cells were pelleted at the bottom (Fig. 5D). These cells were re-suspended with 500 μl of MF-start™ medium, and cultured in the wells of a 24-well dish. Many of the cells were in clumps (Fig. 6, d0).

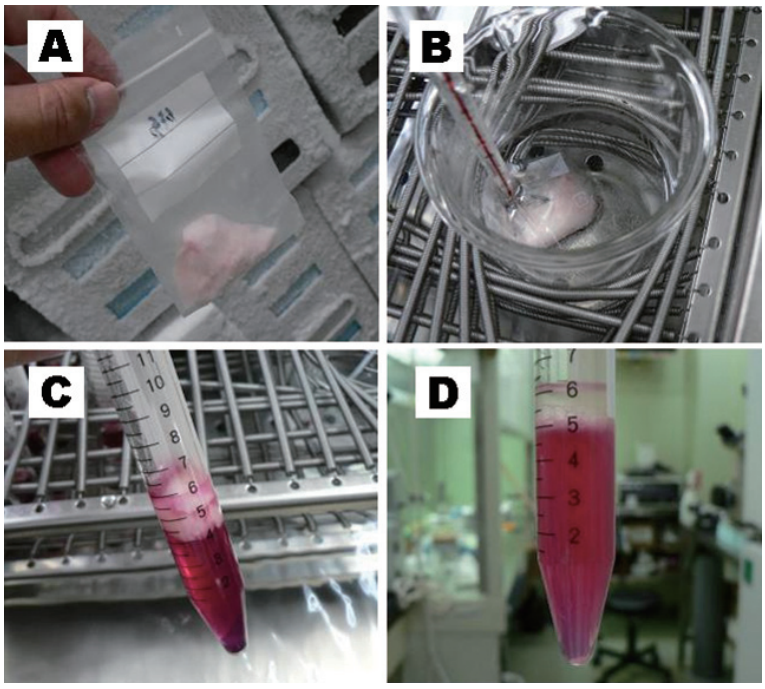

Fig. 5. (A) A piece of bovine adipose tissue packed in a plastic sample bag and stored at –80ºC. (B) Frozen adipose tissue was thawed by putting the plastic bags in saline at 39ºC. (C) One gram of thawed adipose tissue was put into 5 ml of digestion medium, and incubated in a water bath at 39ºC for 1 hour with agitation. (D) After centrifugation, culturable cells were collected from the bottom of tube.

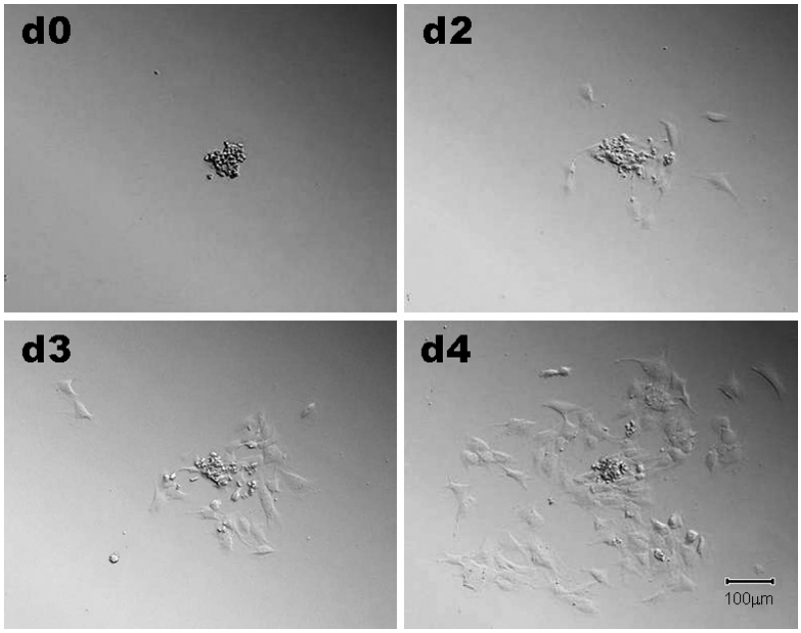

Fig. 6. A clump of cells that was collected from frozen bovine adipose tissue on day 0 (d0) and cells proliferating from the clump on days 2, 3 and 4 of culture (d2, d3 and d4, respectively).

Table 1. Confluence of cells derived from 1 g of frozen adipose tissues of 20 individual cattle after 7 and 14 days of culture

Sample	Confluence (%)	
	Day 7	Day 14
1	30	90
2	80	100
3	5	50
4	70	100
5	100	100
6	10	90
7	2	60
8	70	100
9	10	90
10	10	80
11	30	100
12	100	100
13	100	100
14	10	100
15	100	100
16	100	100
17	50	100
18	30	100
19	50	100
20	90	100

These cells did not appear to be adipocytes because no lipid droplets were observed in the cytoplasm. After seven days of culture, cells proliferated from the clumps in all samples, although the number of cells differed among samples (Table 1). We think these differences were caused by various factors. For example, the differences in the freezing and thawing speeds of adipose tissue might have affected the viability of the cells. Alternatively, there is a possibility that freeze-tolerant cells are located randomly in bovine adipose tissue. Finally, a sufficient number of cells for donor cells for cloning was obtained from all cultures after 14 days of culture.

We do not know why cells in adipose tissue are able to withstand cryoinjury in the absence of a cryoprotectant. Frozen, morselized human bone has also been found to contain live cells [28]. Further studies are needed to clarify the mechanisms by which small numbers of cells from adipose tissue can withstand or evade cryoinjury from ice crystals and/or osmotic stress.

Conclusion

The ability to clone animals from frozen animal bodies and organs indicates that intact nuclei can be stored after simple freezing. Our previous study [12] showed that when viable cells are retrieved from frozen organs or tissues, they can be used to clone animals by conventional SCNT. Wakayama *et al.* [24] showed that even if all cells are dead in frozen animals, cloned animals can be produced by injection of nuclei from dead cells into enucleated oocytes. Moreover, if cloned embryos fail to develop to term, it is possible to establish ntES cells from the cloned blastocysts, and to produce chimeric animals by injecting the ntES cells into tetraploid blastocysts, or to produce cloned animals by two-step SCNT.

Further studies are needed to determine the best organs or tissues for obtaining either viable somatic cells or intact nuclei after freezing without cryoprotectant. Freezing could be used for the conservation of threatened species. When the appropriate facilities for culture and cryopreservation of somatic cells are not available, we propose that storing adipose tissues at –80ºC without cryoprotectant would be the simplest way to cryopreserve animal genomes as viable somatic cells.

References

1) Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997): Viable offspring derived from fetal and adult mammalian cells. Nature, 385, 810–813.
2) Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998): Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature, 394, 369–374.
3) Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. (1998): Eight calves cloned from somatic cells of a single adult. Science, 282, 2095–2098.
4) Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W. and Echelard, Y. (1999): Production of goats by somatic cell nuclear transfer. Nat. Biotechnol., 17, 456–461.
5) Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. and Perry, A.C. (2000): Pig cloning by microinjection of fetal fibroblast nuclei. Science, 289, 1188–1190.
6) Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. and Campbell, K.H. (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature, 407, 86–90.
7) Betthauser, J., Forsberg, E., Augenstein, M., Childs, L., Eilertsen, K., Enos, J., Forsythe, T., Golueke, P., Jurgella,

G., Koppang, R., Lesmeister, T., Mallon, K., Mell, G., Misica, P., Pace, M., Pfister-Genskow, M., Strelchenko, N., Voelker, G., Watt, S., Thompson, S. and Bishop, M. (2000): Production of cloned pigs from in vitro systems. Nat. Biotechnol., 18, 1055–1059.

8) Corley-Smith, G.E. and Brandhorst, B.P. (1999): Preservation of endangered species and populations: a role for genome banking, somatic cell cloning, and androgenesis? Mol. Reprod. Dev., 53, 363–367.
9) White, K.L., Bunch, T.D., Mitalipov, S. and Reed, W.A. (1999): Establishment of pregnancy after the transfer of nuclear transfer embryos produced from the fusion of argali (Ovis ammon) nuclei into domestic sheep (Ovis aries) enucleated oocytes. Cloning, 1, 47–54.
10) Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D. and Damiani, P. (2000): Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. Cloning, 2, 79–90.
11) Loi, P., Ptak, G., Barboni, B., Fulka, J., Cappai, P. and Clinton, M. (2001): Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat. Biotechnol., 19, 962–964.
12) Hoshino, Y., Hayashi, N., Taniguchi, S., Kobayashi, N., Sakai, K., Otani, T., Iritani, A. and Saeki, K. (2009): Resurrection of a bull by cloning from organs frozen without cryoprotectant in a -80 degrees c freezer for a decade. PLoS ONE, 4, e4142.
13) Zou, X., Chen, Y., Wang, Y., Luo, J., Zhang, Q., Zhang, X., Yang, Y., Ju, H., Shen, Y., Lao, W., Xu, S. and Du, M. (2001): Production of cloned goats from enucleated oocytes injected with cumulus cell nuclei or fused with cumulus cells. Cloning, 3, 31–37.
14) Loi, P., Clinton, M., Barboni, B., Fulka, J., Cappai, P., Feil, R., Moor, R.M. and Ptak, G. (2002): Nuclei of nonviable ovine somatic cells develop into lambs after nuclear transplantation. Biol. Reprod., 67, 126–132.
15) Zachariassen, K.E. and Kristiansen, E. (2000): Ice nucleation and antinucleation in nature. Cryobiology, 41, 257–279.
16) Clark, P. and Eichhorn, G.L. (1974): A predictable modification of enzyme specificity. Selective alteration of DNA bases by metal ions to promote cleavage specificity by deoxyribonuclease. Biochemistry, 13, 5098–5102.
17) Wakayama, T. and Yanagimachi, R. (1998): Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat. Biotechnol., 16, 639–641.
18) Kusakabe, H., Szczygiel, M.A., Whittingham, D.G. and Yanagimachi, R. (2001): Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc. Natl. Acad. Sci. USA., 98, 13501–13506.
19) Ogonuki, N., Mochida, K., Miki, H., Inoue, K., Fray, M., Iwaki, T., Moriwaki, K., Obata, Y., Morozumi, K., Yanagimachi, R. and Ogura, A. (2006): Spermatozoa and spermatids retrieved from frozen reproductive organs or frozen whole bodies of male mice can produce normal offspring. Proc. Natl. Acad. Sci. USA., 103, 13098–13103.
20) Kuretake, S., Kimura, Y., Hoshi, K. and Yanagimachi, R. (1996): Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol. Reprod., 55, 789–795.
21) Loi, P., Matsukawa, K., Ptak, G., Clinton, M., Fulka, J., Nathan, Y. and Arav, A. (2008): Freeze-dried somatic cells direct embryonic development after nuclear transfer. PLoS ONE, 3, e2978.
22) Li, J. and Mombaerts, P. (2008): Nuclear transfer-mediated rescue of the nuclear genome of nonviable mouse cells frozen without cryoprotectant. Biol. Reprod., 79, 588–593.
23) Hochedlinger, K. and Jaenisch, R. (2006): Nuclear reprogramming and pluripotency. Nature, 441, 1061–1067.
24) Wakayama, S., Ohta, H., Hikichi, T., Mizutani, E., Iwaki, T., Kanagawa, O. and Wakayama, T. (2008): Production of healthy cloned mice from bodies frozen at -20 degrees C for 16 years. Proc. Natl. Acad. Sci. USA., 105, 17318–17322.
25) Fuller, B., Paynter, S. (2004): Fundamentals of cryobiology in reproductive medicine. Reprod. Biomed. Online, 9, 680–691.
26) Panarace, M., Agüero, J.I., Garrote, M., Jauregui, G., Segovia, A., Canè, L., Gutièrrez, J., Marfil, M., Rigali, F., Pugliese, M., Young, S., Lagioia, J., Garnil, C., Forte Pontes, J.E., Ereno Junio, J.C., Mower, S. and Medina, M. (2007): How healthy are clones and their progeny: 5 years of field experience. Theriogenology, 67, 142–151.
27) Hoshino, Y. and Saeki, K. (2009): 73. Viable cells can be derived from frozen fat without cryoprotection. Cryobiology, 59, 390.
28) Heyligers, I. C. and Klein-Nulend, J. (2005): Detection of living cells in non-processed but deep-frozen bone allografts. Cell Tissue Bank., 6, 25–31.