Recovery of cell nuclei from 15,000 years old mammoth tissues and its injection into mouse enucleated matured oocytes

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Abstract: Here, we report the recovery of cell nuclei from 14,000-15,000 years old mammoth tissues and the injection of those nuclei into mouse enucleated matured oocytes by somatic cell nuclear transfer (SCNT). From both skin and muscle tissues, cell nucleus-like structures were successfully recovered. Those nuclei were then injected into enucleated oocytes and more than half of the oocytes were able to survive. Injected nuclei were not taken apart and remained its nuclear structure. Those oocytes did not show disappearance of nuclear membrane or premature chromosome condensation (PCC) at 1 hour after injection and did not form pronuclear-like structures at 7 hours after injection. As half of the oocytes injected with nuclei derived from frozen-thawed mouse bone marrow cells were able to form pronuclear-like structures, it might be possible to promote the cell cycle of nuclei from ancient animal tissues by suitable pre-treatment in SCNT. This is the first report of SCNT with nuclei derived from mammoth tissues.

Keywords: mammoth, SCNT, cytochrome b, ancient DNA (aDNA)

Introduction

Since reports of “Dolly”1) and “Cumulina”,2) cloned animals have been successfully produced by somatic cell nuclear transfer (SCNT) in many species.3)–9) In these reports, cultured or freshly collected cells were used as nuclear donor cells in SCNT. Meanwhile, there were reports of the production of mice by intracytoplasmic sperm injection (ICSI) with the freeze-dried spermatoozoa10) or with the spermatoozoa derived from frozen whole body kept at −20 °C for 15 years without cryoprotectant.11) Furthermore, there were reports of the birth of offspring from SCNT with the nuclear donor cell frozen at −80 °C for 342 days,12) with the nuclear donor cell derived from frozen whole body kept at −20 °C for 16 years without cryoprotectant13) and with the nuclear donor cell derived from frozen organ kept at −80 °C for 10 years without cryoprotectant.14) These reports suggest that if tissues or cells are cryopreserved postmortem without cryoprotectant, the resurrection of the postmortem animal individual might be possible by SCNT.

In cases of extinct or endangered species, it is difficult to use oocytes derived from same species as the recipient cytoplasm in SCNT. However, recently, there were some reports on successful production of offspring by SCNT with oocytes derived from closely related species.15)–19) These results suggest that if oocytes from closely related species are available as the recipient cytoplasm, it will be possible to resurrect extinct or endangered species by SCNT.

Woolly mammoth (Mammuthus primigenius) is a very famous animal existed during ice ages and became extinct at the end of the last ice age.20) Its relics including soft tissues like skin, muscle and other inner parts have been excavated from Permafrost.20) Recently, the prospects of the resurrection of woolly mammoth by SCNT has heightened.21),22) However,
even if the soft tissue of woolly mammoth is excavated, it is not clear whether its cell nuclei still keep their biological characteristics for more than several thousands years. In this paper, we report the collection of cell nuclei from 14,000–15,000 years old mammoth tissues and the injection of its cell nuclei into mouse enucleated matured oocytes as the nuclear donor in SCNT.

Materials and Methods

1. Excavation of ancient animal sample. Frozen mammoth legs were excavated from Northeast Siberian Permafrost on the bank of Maxuniokha River (Fig. 1-A, at the point of 71° 34′ 56.9″ N and 141° 37′ 37.6″ E). This excavation was performed as one of the International Science and Technology Center (ISTC) projects funded by Gifu Prefecture, Japan. Excavated frozen mammoth legs were transported to Museum of Mammoth in Yakutsk under frozen condition. Pieces of skin, muscle, bone and bone marrow samples (Fig. 1-B, C and D) were collected from the frozen mammoth leg and transported to our laboratory under frozen condition. In our laboratory, all samples were kept at −80°C.

2. The radiocarbon age analysis of the sample. A small piece of skin sample was cut out from frozen skin and washed by ultrasonic cleaner for 10 minutes, then in 0.5N HCl for 1.2 hours, 0.1N NaOH for over night and 0.5N HCl for 2.6 hours to remove all organic substances derived from Permafrost. After washing, the skin sample was soaked in chloroform: methanol (2:1) for over night to remove all lipids. Then the skin sample was washed in distilled water (DW) and collagen was extracted by heating at 90°C for 12 hours in DW. Extracted collagen was oxidized to CO2 and graphitized with iron catalyst. The mixed graphite-iron powder was pressed into an aluminum cathode holder, and analyzed by accelerator mass spectrometry (NIES-TERRA, Japan).

3. Histological analysis of mammoth sample. Thin sections of skin, muscle, bone and bone marrow samples were made, stained with hematoxylin-eosin double staining method and observed by phase-contrast microscope (Fig. 2-A-D).

4. DNA retrieval and amplification by PCR. DNA was extracted from about 1 g each of bone marrow and muscle. Small pieces of bone marrow and muscle samples were washed 3 times in sterile
DW, then once in 0.5% NaClO and 3 times in sterile DW again. After washing, samples were minced with a surgical razor blade under sterile condition and the minced tissue was dissolved in SSC buffer using proteinase K (0.45 mg/ml) and SDS (0.5%) by keeping at 55 °C for 3 hours, then, at 37 °C for over night. DNA solution was treated once with phenol, 4 times with phenol: chloroform (1:1) following once with chloroform. DNA solution was concentrated 50 times using Microcon-YM100 (MILLIPORE, USA).

**Fig. 2.** Thin sections of skin (A × 100), muscle (B × 400), bone and bone marrow (C × 100 and D × 400) samples stained by hematoxylin-eosin double staining method. There were many cell nuclei in the muscle (Fig. 2-B). There were many foam shaped structure in the medullary cavity of the bone marrow (Fig. 2-C) and blood cells or epithelial cells in the bone (Fig. 2-D).

**Fig. 3.** Recovered cell nucleus-like structures (arrows) in bright field (A) and dark field (B). In Fig. 3-B, cell nucleus-like structures showed the red fluorescent from PI. The diameter of recovered cell nucleus-like structures was about 3 μm.
b gene was amplified by semi-nested or nested PCR with primers designed from previously reported mitochondrial cytochrome b gene of woolly mammoth\textsuperscript{23} (GenBank accession number: D50842). For bone marrow sample derived DNA solution, the primers for 1st PCR, 5'-AATATCATTCTGAGGGGCAA-3' and 5'-GAATGGAGCATATTTGGCCG-3'; and for 2nd PCR, 5'-AATACATTCTGAGGGGCAA-3' and 5'-TGTAGTTGTCGGGGTCCTCCT-3' were used. For muscle sample derived DNA solution, the primers for 1st PCR, 5'-CGAAAATCTCACCCCTACT-3' and 5'-TGTAGTTGTCGGGGTCCTCCT-3'; and for 2nd PCR, 5'-TCACTACTAGGACTGACCATGCCT-3' and 5'-GTGATTACGGTTGCCG-3' were used. Amplified DNA fragment was sub-cloned into pGEM\textsuperscript{R-T} Easy Vector (Promega, USA), and sequenced with ABI PRISM\textsuperscript{TM} 310 Genetic Analyzer (Applied Biosystems, USA).

5. Recovery of cell nuclei from skin and muscle tissue. Cell nuclei from cryopreserved samples were prepared according to Wakayama \textit{et al.}\textsuperscript{13} Briefly, small pieces of cryopreserved (−80°C) skin or muscle samples were thawed at 37°C, minced with scissors and put into a glass homogenizer containing 500–1,000 μl of cold NIM (nuclear isolation medium).\textsuperscript{24} Homogenized samples were suspended with NIM followed by filtration through 100 and 40 μm mesh to remove debris. The suspensions were washed twice with NIM by centrifugation at 400 × g for 5 minutes at 4°C and resuspended with 100 μl of NIM. In case of the muscle tissue, after washing, samples were treated with collagenase type IV (0.1%) and dispase (0.2%) at 32°C for 10 minutes and resuspended with 100 μl of NIM. For nuclear staining, skin and muscle samples were stained with propidium iodide (PI), washed and resuspended with 20 μl of NIM.

6. Injection of collected nuclei into enucleated mouse matured oocytes. The experimental design and handling of laboratory animals were performed according to the guidelines for animal experiments at Kinki University. For recipient cytoplasm, enucleated matured mouse oocytes derived from BDF1 matured female mice (Japan SLC, Inc., Japan) were used. In this experiment, bone marrow cell nuclei derived from frozen (−80°C)-thawed mouse thighs were also injected into enucleated matured mouse oocytes as the control treatment. Cell nuclei derived from mammoth skin or muscle samples were collected under the fluorescent microscope. Cell nuclei with the red fluorescence by PI were collected (Fig. 3). Nuclear injection was performed according to Wakayama \textit{et al.}\textsuperscript{2} At 1 hour after nuclear injection, the disappearance of the nuclear membrane and premature chromatin condensation (PCC) was checked under the microscope in bright field. Oocyte activation treatment was also performed according to Wakayama \textit{et al.}\textsuperscript{2} At 7 hours after nuclear injection, oocytes with pronuclear-like structure were counted as activated oocytes. Then all oocytes were whole mounted on slide glasses and checked for the fluorescence derived from PI and DAPI.

Results

The conventional radiocarbon age of the sample after 13C correction was 13,100–12,800 BC or 12,400–11,800 BC. This meant that these samples were derived from a woolly mammoth existed about 14,000–15,000 years ago. This is at the end of Pleistocene.

There was no obvious structure in the skin sample (Fig. 2-A), however, in the muscle sample, cell nuclei stained with hematoxylin were observed (Fig. 2-B, arrows). In bone and bone marrow tissues, many foam shaped adipocytes (Fig. 2-C, arrows) were observed in the medullary cavity, and blood cells or epithelial cells (Fig. 2-D, arrows) were observed in the bone.

From bone and bone marrow samples, a 320 bp DNA fragment was amplified by PCR, sub-cloned and successfully sequenced. The homology of the sequence amplified from sample DNA was checked by comparing DNA sequences with those registered to GenBank by BLASTN search (nr-nt). This 320 bp DNA fragment showed highest homology (310/321, 96%) to the cytochrome b gene sequence of the registered woolly mammoth (GenBank accession number: FJ753553) and also next (309/321, 96%) to the cytochrome b gene sequence of the registered woolly mammoth (GenBank accession number: EU155210). From the muscle sample, a 300 bp DNA fragment was amplified by PCR, sub-cloned and successfully sequenced. This 300 bp DNA fragment showed highest homology (296/302, 97%) to the cytochrome b gene sequence of the registered woolly mammoth (GenBank accession number: EU153444) and also next (292/302, 96%) to the registered woolly mammoth (GenBank accession number: MPU79411).

From both skin and muscle samples, cell nucleus-like structures identified by PI staining were successfully recovered (Fig. 3). Although efficacy of
the recovery of cell nuclei was low, the number of nuclei recovered was sufficient for SCNT experiment. The number of recovered cell nuclei was about 10 times higher from mammoth muscle samples than those recovered from mammoth skin samples.

After nuclear injection, more than half of the oocytes injected with nuclei derived from mammoth tissues survived (55%). All oocytes injected with mammoth skin or muscle derived nuclei showed the red fluorescence derived from PI. In the case of oocytes injected with nuclei derived from frozen-thawed mouse bone marrow tissue, many nuclei lost the red fluorescence after injection by extruding almost all PI through nuclear membrane and 27% of the oocytes were able to form PCC (Table 1, Fig. 4-C). In the case of oocytes injected with nuclei derived from mammoth skin and muscle tissues, no disappearance of nuclear membrane or PCC was observed at 1 hour after nuclear injection (Table 1, Fig. 4-D and E and Fig. 5, upper and middle panels). At 7 hours after nuclear injection, 46% of oocytes injected with nuclei derived from frozen-thawed mouse bone marrow tissue showed pronuclear-like structure (Table 1, Fig. 4-F and Fig. 5, lower panels). On the other hand, there was no oocyte with pronuclear-like structure in oocytes injected with nuclei derived from mammoth skin or muscle (Table 1, Fig. 4-D and E and Fig. 5, upper and middle panels).

**Discussion**

From the results of the radiocarbon age analysis and the DNA sequence analysis amplified by PCR, it was confirmed that samples used in this experiment was derived from a woolly mammoth that existed about 14,000–15,000 years ago, at the end of Pleistocene. In Siberia, it was thought that the mammoths disappeared about 10,000 years ago. In the case of skin and muscle samples, the tissue was easily fragmented into small pieces while making thin-section (Fig. 3-A and B). Probably, this fragmentation was caused by severe dehydration that occurred while the sample was preserved in Permafrost. The average temperature of Permafrost is about −5°C and at this temperature, the ice crystal is not stable and develops. As the result of the activity of ice crystal, mammoth samples would be severely dehydrated. In the case of bone marrow, in medullary cavity, many foam shaped adipocytes were observed (Fig. 3-C). At the time of the birth of an animal, in medullary cavity, there is much hemopoietic tissue (red bone marrow). After the animal becomes an adult, the hemopoietic tissue transforms into the adipose tissue (yellow bone marrow). This meant that the tissue samples used in this experiment were taken from an adult mammoth.

When DNA was amplified by PCR, about 700 bp length of DNA fragment was successfully amplified. Usually, in the case of ancient DNA (aDNA), derived from fossil samples or museum samples, DNA was fragmented into about 100–200 bp. And in the case of aDNA amplification by PCR, the success rate of amplification declines with increasing average temperature in the area from which the sample originated. Samples used in this experiment were freshly excavated from Permafrost and after excavation, all samples were preserved in the frozen condition. From this point, samples used in this experiment were preserved comparatively in good condition until the time of experiment.

There was large difference in the number of nu-

### Table 1. Early development of oocytes injected with nuclei derived from different tissues in mammoth and mouse

| Origin of nuclei      | No. of oocytes used | No. of oocytes survived (%) | No. of oocytes with PCC (%) | No. of oocytes with pronuclear-like structure (%) |
|-----------------------|--------------------|----------------------------|-----------------------------|--------------------------------------------------|
| Mammoth skin          | 18                 | 12 (67)                    | 0 (0)                       | 0 (0)                                            |
| Mammoth               | 131                | 72 (55)                    | 0 (0)                       | 0 (0)                                            |
| Mouse bone marrow     | 80                 | 26 (33)                    | 7 (27)                      | 12 (46)*                                         |

\[ e = \frac{b}{a} \times 100, \quad f = \frac{c}{b} \times 100, \quad g = \frac{d}{b} \times 100. \]

\[ ^\dagger : \text{Oocytes were evaluated 1 hour after nuclear injection.} \]

\[ ^\ddagger : \text{Oocytes were evaluated 7 hours after nuclear injection.} \]

\[ ^* : \text{Among the oocytes with pronuclear-like structure, 11 oocytes showed single pronuclear-like structure and 1 oocytes showed 2 pronuclear-like structures.} \]
Fig. 4. A-C: Oocytes injected with nuclei derived from mammoth skin (A), mammoth muscle (B) and mouse bone marrow (C) at 1 hour after nuclear injection. Injected nuclei were visible (arrows). D-E: Oocytes injected with nucleus derived from mammoth skin (D), mammoth muscle (E) and mouse bone marrow (F) at 7 hours after nuclear injection. In D and E, injected nuclei without any change were still visible (arrows). Meanwhile, oocytes injected with mouse bone marrow derived nucleus transformed into 2 pronuclear-like structure (arrows).

Fig. 5. Fluorescent images of oocytes injected with nucleus derived from mammoth skin (1st row), mammoth muscle (2nd row) and mouse bone marrow (3rd row) at 7 hours after nuclear injection under dark field with UV. Left column: oocytes stained with DAPI, Center column: oocytes stained with PI, Right column: Merged figure of DAPI and PI figures. Injected nucleus in each oocyte shown with an arrow. The other nucleus beside the injected nucleus stained only with DAPI was the nucleus of the 1st polar body.
clei recovered from skin or muscle tissue. Probably, this difference was caused by preservation condition due to the characteristics of the tissues. Muscle cell is the multinuclear cell. The sample relatively preserved its structure and contained many nuclei (Fig. 2-B). Skin tissue has much collagen fiber and less numbers of nuclei. In addition, skin sample might be much damaged as shown in Fig. 2-A.

There are several reports of SCNT with postmortem nuclear donor cell. Such experiments can be divided into experiments with 2 types of nuclear donor cells. These are; 1) cells still survived even postmortem cell collection and after culture of cells, cells proliferated again and living cells were available for nuclear donor in SCNT;14,18 and 2) cells lost their viability and no living cell was available.12,13,16 In the latter case, when cells were collected within a day postmortem, an offspring was successfully produced by one-step SCNT.16 However, when cells were collected about a year or 16 years postmortem, it was necessary to repeat SCNT via the production of ntES (nuclear transferred embryonic stem) cells.12,13 The reason for the difference between these 2 types of SCNT with postmortem nuclear donor cell would depend on the DNA integrity of the nuclear donor cell. Loi et al. reported on the SCNT with the lyophilized nuclear donor cell.27 In this report, when nuclear donor cells were lyophilized without trehalose, the genome was damaged extensively and after SCNT with the lyophilized nuclear donor cell, irregular pronuclei with fragmented DNA were observed. These results suggest that cryopreserved nuclear donor cell without cryoprotection for long period damaged its DNA integrity and to retrieve the damage, it is necessary to repeat SCNT via the production of ntES cells. DNA samples collected in this report showed severe damage when the gel electrophoresis of collected DNA samples was performed (data not shown). To have successful embryonic development after the SCNT with mammoth cells, it would be necessary to repeat SCNT to retrieve mammoth genome.

In this experiment, we succeeded in recovering cell nuclei from about 15,000 years old mammoth tissues. We also succeeded in transferring them into mouse enucleated oocytes, but the structure of injected nuclei remained at 1 and 7 hour after injection. Because of for a considerable length of time preservation of mammoth tissue, the biological characteristics of the nucleus might be extremely changed or the ability of the nucleus to respond to factors existing in recipient cytoplasm might be lost. Or there were other possibilities that nuclei recovered from mammoth tissues were enclosed with or contained denatured proteins and such denatured proteins might interrupt the interaction between the chromatin and factors existing in recipient cytoplasm. Besides, as in case of control group, pronuclear-like structures were formed without the disappearance of nuclear membrane or PCC in oocytes injected with nuclei derived from frozen-thawed mouse bone marrow tissue, the compatibility of mammoth donor nucleus and mouse recipient oocytes might possibly be unsuitable for nuclear change.

Finally, we demonstrated the recovery of cell nuclei from for a considerable length of time postmortem animal tissues and the transplantation of those nuclei into oocytes. This report is the first step to resurrect ancient animal and further research is required to improve conditions to promote cell cycle of the transplanted nuclei for having successful resurrection of the ancient animal.

Acknowledgements

We would like to express our gratitude to Mr. H. Ichihashi of Gifu Prefecture and Mr. S. Teemev of ISTC for coordinating the excavation of mammoth, Mr. V. Plotnikov of Museum of Mammoth, Institute of Applied Ecology of the North and Dr. V.E. Repin, Dr. O.S. Taranov and Dr. V.G. Pugachov of Vector State Research Center of Virology and Biotechnology for helping the excavation of mammoth and Dr. D.D. Savvinov of Academy of Sciences of Sakha Republic (Yakutia) for supporting the excavation of mammoth. This study was funded in part by Gifu Prefecture, Japan. The accession numbers of cloned mammoth’s mitochondrial cytochrome b sequences in this experiment are FJ966075 and FJ966076.

References

1) Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H.S. (1997) Viable offspring derived from fetal adult mammalian cells. Nature 385, 810–813.
2) Wakayama, T., Perry, A.C.F., Zuccotti, M., Johson, 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. et al. (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., Destrempe, M.M., Cammuso, C. et al. (1999) Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17, 456–461.

5) Oonishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T. et al. (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. et al. (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407, 86–90.

7) Chesne, P., Adenot, P.G., Vibletta, C., Baratte, M., Boulanger, L. and Renard, J.P. (2002) Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat. Biotechnol. 20, 366–369.

8) Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L. et al. (2002) A cat cloned by nuclear transplantation. Nature 415, 857.

9) Zhou, Q., Renard, J.P., Le Fricc, G., Brochard, V., Beaujanc, N., Cherifi, Y. et al. (2003) Generation of fertile cloned rats by regulating oocyte activation. Science 302, 1179.

10) Wakayama, T. and Yanagimachi, R. (1998) Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat. Biotechnol. 16, 639–641.

11) Ogonuki, N., Mochida, K., Miki, H., Inoue, K., Fray, M., Iwaki, T. et al. (2006) 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.

12) 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.

13) Wakayama, S., Ohta, H., Hikichi, T., Mizutani, E., Iwaki, T., Kanagawa, O. et al. (2008) Production of healthy cloned mice from bodies frozen at −20 °C for 16 years. Proc. Natl. Acad. Sci. USA 105, 17318–17322.

14) Hoshino, Y., Hayashi, N., Taniguchi, S., Kobayashi, N., Sakai, K., Otani, T. et al. (2009) Resurrection of a bull by cloning from organs frozen without cryoprotectant in a −80 °C freezer for a decade. PLoS ONE 4, e4142.

15) Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin C.E. et al. (2000) Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. Cloning 2, 79–90.

16) Loi, P., Ptak, G., Barbonti, B., Fulk, J. Jr., 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.

17) Sansinena, M.J., Hylan, D., Hebert, K., Dennis, R.S. and Godke, R.A. (2005) Banteng (Bos javanicus) embryos and pregnancies produced by interspecies nuclear transfer. Theriogenology 63, 1081–1091.

18) Oh, H.J., Kim, M.K., Jang, G., Kim, H.J., Hong, S.G., Park, J.E. et al. (2008) Cloning endangered gray wolves (Canis lupus) from somatic cells collected post mortem. Theriogenology 70, 638–647.

19) Folch, J., Cocero, M.J., Chesne, P., Alabart, J.L., Dominguez, V., Cognie, Y. et al. (2009) First birth of an animal from an extinct subspecies (Capra pyrenaica pyrenaica) by cloning. Theriogenology 71, 1026–1034.

20) Lister, A. and Bahn, P. (2007) Mammths: Giants of the Ice Age. 3rd ed., University of California Press, Berkeley and Los Angeles, California.

21) Nicholls, H. (2008) Darwin 200: Let’s make a mammoth. Nature 456, 310–314.

22) Fulk, J. Jr., Loi, P., Ptak, G., Fulk, H. and John, J.S. (2009) Hope for the mammoth? Cloning Stem Cells 11, 1–4.

23) Noro, M., Masuda, R., Dubrovo, I.A., Yoshida, M.C. and Kato, M. (1998) Molecular phylogenetic inference of the woolly mammoth Mammuthus primigenius, based on complete sequences of mitochondrial cytochrome b and 12S ribosomal RNA genes. J. Mol. Evol. 46, 314–326.

24) 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.

25) Pääbo, S. (1989) Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. Proc. Natl. Acad. Sci. USA 86, 1939–1943.

26) Pruvost, M., Schwarz, R., Correia, V.B., Champlot, S., Braguier, S., Morel, N. et al. (2007) Freshly excavated fossil bones are best for amplification of ancient DNA. Proc. Natl. Acad. Sci. USA 104, 739–744.

27) Loi, P., Matsukawa, K., Ptak, G., Clinton, M., Fulk, J. Jr., Nathan, Y. et al. (2008) Freeze-dried somatic cells direct embryonic development after nuclear transfer. PLoS ONE 3, e2978.

(Received May 12, 2009; accepted May 22, 2009)