O-08

Cats Cloned from Fetal Fibroblast Cells by Nuclear Transfer

X. J. Yin[1], H. S. Lee[1], Y. H. Lee[1], W. S. Hwang[2] and I. K. Kong[1]

[1]Department of Animal Science & Technology, College of Agriculture & Life Science, Sunchon National University, 315 Maegok-dong, Sunchon, JeonNam Province, 540-742, S. Korea.

[2]Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, San 56-1, Shilim-Dong, Kwanak-Gu, Seoul, 151-742, S. Korea.

Corresponding should be addressed to I.K. Kong;

E-mail: ikong@sunchon.ac.kr

Abstract

This work was undertaken in order to study the developmental competence of nuclear transfer cat embryo with fetal fibroblast and adult skin fibroblast as donor nuclei. Oocytes wererecovered by mincing the ovaries in Hepes-buffered TCM199 and selected the cumulus oocyte complexes (COCs) with compact cumulus cell mass and dark. Homogenous ooplasm were cultured for maturation in TCM199 + 10% fetal bovine serum (FBS) for 12 hours and used as a source of recipient cytoplast for exogenous somatic nuclei. In Experiment 1, we evaluated the effect donor cell types on the reconstruction and development of cloned embryos. Fusion, first cleavage and blastocyst developmental rate was not different between fetal fibroblast and adult skin cell (71.2 vs. 66.8; 71.0 vs. 57.6; 4.0 vs. 6.1%, $P<0.05$). In Experiment 2, cloned embryos were surgically transferred into the oviducts of recipient queens. One of seven recipient queens was delivered naturally 2healthy cloned cats and 1 stillborn from fetal fibroblast cell of male origin after 65 days embryo transfer. One of three recipient queens was delivered naturally 1 healthy cloned cat from adult skin cell of female after 65 days embryo transfer. The

cloned cats showed genotypes identical to the donor cell lines, indicating that adult somatic cells can be used for feline cloning.

Introduction

Many animal species including sheep (Wilmut *et al.* 1997), cattle (Kato *et al.* 1998), goat (Baguisi *et al.* 1999), mice (Wakayama *et al.* 1998), pig (Yin *et al.* 2002), rabbit (Chesne *et al.* 2002), mule (Woods *et al.* 2003), horse (Galli *et al.* 2003) and rat (Zhou *et al.* 2003) have been successfully cloned using somatic cells. Cats have been considered up to now as difficult to clone. Indeed, although one group reported the birth of a cloned cat with donor cumulus cell (Shin *et al.* 2002), no group has obtained feline cloningusing fetal and skin fibroblast as nuclear donor. We report here the successful cloning of cats from male fetal fibroblasts and female adult somatic cells. Four cats were naturally delivered from the two cell types. Our results will contribute to the rescue the endangered feline species.

Results

Experiment 1: *In vitro* development of nuclear transfer embryos

When matured eggs with a first polar body were treated with demecolcine for 1 h, more than 70% of the eggs had a membrane protrusion and the chromosomemass migrated to a cortical location. Enucleation of the eggs with a small volume of cytoplasm was easy and the enucleation rate was high (85%; 123 of 145).

The *in vitro* development of nuclear transfer embryos reconstructed with fetal and adult fibroblast cells. Fusion rates were not different between fetal and adult fibroblast cells (71.2 vs. 66.8%; $P < 0.05$). The proportion of reconstructed embryos that cleaved was higher in the fetal group compared with the adult group (71.0 vs. 57.6%; $P < 0.05$). Development to the blastocyst stage was not different using either type of karyoplast (4.0 vs. 6.1%). Total cell numbers for the five blastocysts produced using fetal fibroblasts were 23, 31, 17, 12 and 28. The blastocyst produced in the adult fibroblast had a total cell number of 20 and 27.

Experiment 2: Cloned embryo transfer into recipient queens

A donor fibroblast cell line was established from a five week-old male fetus and passaged one to nine times for expansion in culture. These cells were cultured in DMEM+0.5% serum for 3 additional days before use of nuclear transfer and fused with enucleated oocytes devoid ofmetaphase chromosomes by micromanipulation as described (Yin *et al.* 2002, 2003). Cloned embryos at the one cell stage (120 and 140 eggs) or 2 to 4-cell stage (n=30-90 eggs) were transferred into seven synchronized recipients approximately 30 hours after hCG treatment. One recipient received 140 cloned embryos, of which were reconstructed with 9 passaged cells and maintained the pregnancy to term. Pregnancy was confirmed by X-ray photography one day before parturition. Two live kittens and one stillborn fetus were delivered naturally 65 days after embryo transfer. The birth weight of the cloned live cats at delivery was 96 and 84 g and the stillborn offspring was 56 g. We suspected the stillborn fetus was related to the palpation used to confirm pregnancy. Two cloned cats are still healthfully alive for 4 month (Fig. 1a). An adult somatic cell line was established from a 12 month-old female cat as described above. One hundred forty five cloned embryos at the 2 to 4-cell stage were transferred into 3 recipients.

Figure 1. Cloned kitties with surrogate mother and nuclear-donor, fetus and cat. a, Surrogate mother and 2 cloned kitties, and nuclear donor fetus in small box, b, Surrogate mother and a cloned kitten, and nuclear donor cat in small box.

One recipient was confirmed pregnant after receiving65 reconstructed embryos derived from one passaged cells and then produced one kitten (Fig. 1b) by natural delivery 65 days after embryo transfer. The birth weight of the cloned kitten was 102 g. The kitten was vigorous at birth and appeared to be completely normal, but died 3 days post-parturition as a result of starvation from refusal of colostrum sucking. Pathological analysis of the cloned cat did not detect any abnormalities. Microsatellite analysis reveals that the donor cells were the source of the genetic material used to produce the newborn cats (Table 1).

Table 1. Analysis of feline genetic markers

Feline markers	Experiment I (tail tissue) Fetal cell	Surrogate queen	Kitty I	Kitty II	Kitty III	Experiment II (tail tissue)		
FCA229	ND	ND	ND	ND	ND	Skin cells	Surrogate queen	Kitty I
FCA290	213/215	213	213/215	213/215	213/215	170	163/170	170
FCA441	150/154	150/159	150/154	150/154	150/154	213	213	213
FCA201	151/155	151/153	151/155	151/155	151/155	150/154	ND	150/154
FCA224	175	153/175	175	175	175	151	138	151
						162/175	162	162/175

* Values represent the sizes (in base pairs) of the two versions of the amplified microsatellite DNA markers in each sample. ND: not determined.

Discussion

In the present study, we investigated the possibility of producing normal cloned cats by using fetal and adultsomatic donor cells. This is the first time to our knowledge that produced normal cloned cats by using fetal donor cells. The results of our experiment showed that fusion, cleavage and developmental rate to blastocyst were similar to that reported previously. However, the quality of blastocysts, based on the cell number, was poor in comparison to other reported.In this study we cultured in TCM199 supplemented with 0.3% BSA for 6 days. *In vitro* culture system in this study seems to be not proper for culturing of cloned cat embryos.

A major difference between the methods described here and that used to produce cloned kittens in the previous report (Shin *et al.* 2002) is the use of chemically assisted removal of maternal chromosomes, as in pig cloning (Yin *et al.* 2002) and the transfer of large number embryos. Matured cat eggs treated with demicolcine had a membrane protrusion in which the condensed chromosome mass was located. Although the mechanisms of action of demicolcine are not clear, the appearance of the protrusion might be related to the condensation of maternal chromosomes. Such protrusions are observed in demicolcine-treated pig (Yin *et al.* 2002). This simple, chemically assisted method to remove maternal chromosomes makes it possible to produce a large number of nuclear-transferred eggs and to efficiently produce cloned cats.

To verify the genetic overlap between donor and cloned kittens, we performed parentage analysis with feline microsatellite makers among the cloned kittens, surrogate mother and control cats. Analysis of five unlinked, high polymorphic, feline-specific microsatellite loci confirmed that the kittens were clonal.

Although the coat color of two cloned males (gold on white) resembledeach other, the coat color of a cloned female (gold on white coat) was different from the donor female cat (gold and tan spots on white coat). We attribute this to the pigmentation pattern in multicolored animals is not only related to genetic factors, but also to developmental factors that are not controlled by genotype. The mitochondria inherited from the recipient oocyte would have a major influence over functions that depend on mitochondria gene expression (Holt *et al.* 2004).

In conclusion, we produced the cloned cats from fetal fibroblast cells of a male donor and adult somatic cells of a female by natural delivery. These results will haveimportant applications in domestic cat breeding as well as endangered felid species cloning, and will contribute to extending the use of cat models for biotechnological applications.

References

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ,

Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW & Echelard Y 1999 Production of goats by somatic cell nuclear transfer. Nature Biotechnol 17 456-461.

Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L & Renard JP2002 Cloned rabbits produced by nuclear transfer from adult somatic cells. Nature Biotechnol 20366-369.

Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R & Lazzari G 2003 A cloned horse born to its dam twin. Nature 424 635.

Holt WV, Pickard AR & Prather RS 2004 Wildlife conservation and reproductive cloning. Reproduction 127 317-324.

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H & Tsunoda Y1998 Eight calves cloned from somatic cells of a single adult. Science 282 2095-2098.

Shin TY, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L & Westhusin M 2002 A cat cloned by nuclear transplantation. Nature 415 859.

Wakayama T, Perry AC, Zuccotti M, Johnson KR & Yanagimachi R 1998 Full term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394 369-374.

Wilmut I, Schnieke AE, McWhir J, Kind AJ & Campbell KHS 1997 Viable offspring derived from fetal and adult mammalian cells. Nature 385 810-813.

Woods GL, White KL, Vanderwall DK, Li GP, Aston KI, Bunch TD, Meerdo LN & Pate BJ 2003 A mule cloned from fetal cells by nuclear transfer. Science 301 1063.

Yin XJ, Tani T, Yonemura I, Kawakami M, Miyamoto K, Hasegawa R, Kato Y & Tsunoda Y2002 Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. Biol Reprod 67 442-446.

Zhou Q, Renard JP, Fries GL, Brochard V, Beaujean N, Cherifi Y, Fraichard A & Cozzi J2003 Generation of fertile cloned rats by regulating oocyte activation. Science 302 1179.