Production of Cloned Mice by Nuclear Transfer of Cumulus Cells

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Abstract

Background: Over the past several years, mammals have been successfully cloned by either the splitting of an early stage embryo or nuclear transfer of adult somatic cells (NT) into oocytes. Although it has been 15 years since the generation of the first cloned mammals from somatic cells by NT, the success rate for producing live offspring by this technique is low regardless of the cell type and animal species used. However, these techniques have the potential to be important tools for future research in basic biology. In the present study, we described our experiences in producing successfully cloned mouse using NT method and piezo-actuated micromanipulator.

Methods: B6D2F1 mice, 8-12 weeks old, were superovulated with injections of 5 IU of pregnant mare serum gonadotropin and 5 IU of human chorionic gonadotropin administered 48 hr apart. Enucleation and donor nuclei cumulus cell injection were performed with a piezo-actuated micromanipulator after which activation and trichostatin A treatment were used for reconstructed oocytes. Two-cell stage cloned embryos that developed in the mWM medium were transferred into the oviducts of pseudopregnant NMRI mice.

Results: Of 367 oocytes collected, 131 (69%) developed into 2-cell stage embryos. Of these, 5 (1%) live pups were successfully delivered. We used NMRI foster mother to raise the pups by lactation. One adult cloned mouse was mated, after which she delivered and raised normal offspring.

Conclusion: For mouse cloning, the present study also successfully tested the capability of somatic cell nuclear transfer SCNT using a piezo unit.

Keywords: Mouse, Nuclear transfer techniques, Trichostatin A

Introduction

Reproductive cloning is defined as the production of a group of individuals with the same genotype by asexual reproduction, a natural occurrence in numerous plants and animals 1. Over the past several years mammals have been successfully cloned by the splitting of an early embryo or Nuclear Transfer (NT) of adult somatic cells into oocytes. It is difficult to pinpoint the origin of cloning as this technology has been used by numerous scientists. In 1894, Dreisch successfully cloned a sea urchin and several years later Hans Speman successfully cloned a salamander. Briggs and King used the NT technique to clone frogs. In 1985, Steward isolated cells from the roots of a mature carrot, which resulted in the
creation of a full clone. Steward also proved that it was possible to generate clones by using somatic cell NT \(^2\). Cloning experiments have also been undertaken by other scientists such as Gurdon (\textit{Xenopus laevis}) \(^3\) and Willadsen (sheep) \(^4\).

Nuclear transfer techniques for the cloning of domestic animals have undergone rapid development. On July 5, 1996, Wilmut et al used Roslin method to clone the first mammal, a sheep named Dolly. In their method, somatic cells were permitted to grow and divide. They were deprived of nutrients and induced into a suspended or dormant stage. An oocyte with removed nucleus was placed in adjacency to a somatic cell and then both cells were shocked by an electrical pulse. Both cells were fused and the oocyte was permitted to grow into an embryo. The embryo is then transferred into a surrogate mother. The birth of Dolly was considered a turning point in the history of cloning in that it showed the feasibility of producing live offspring by somatic cell NT \(^5\).

In 1998, Wakayama et al used Honolulu method to inject enucleated oocytes with cumulus cell nuclei and produced the first cloned mouse, Cumulina, which survived \(^6\). They removed the nucleus from a somatic cell and injected it into an oocyte with removed nucleus. The oocyte was bathed in a chemical solution and cultured. The growing embryo was then transferred into a surrogate mother and permitted to develop. Following this success, other animals such as cattle \(^7\), goats \(^8\), dogs \(^9\), domestic cats \(^10\), rabbits \(^11\), horses \(^12\), rats \(^13\), ferrets \(^14\) and zebra fish \(^15\) have been successfully cloned.

In Iran, cloning experiments were performed by Kazemi Ashtiani and colleagues at Royan Institute. They produced the first cloned sheep in Iran and the Middle East \(^16\).

Wakayama et al developed a mouse somatic cell NT method by injecting donor nuclei into enucleated oocytes using a piezo unit \(^6\). For other researchers, obtaining results with piezo unit was difficult. Currently, numerous laboratories have reported the continual reproduction of cloned mice, including cumulus cells \(^6\), tail-tip cells \(^17\), Sertoli cells \(^18\), fetal cells \(^19,20\) and chimeric mice \(^21\).

Cloning technology has numerous applications in industry, agriculture and medicine. This technology is considered as a research tool in basic biology \(^22\) and is a tremendous tool for the study of processes such as nuclear reprogramming, imprinting genes and gene activation \(^23\). The adaptability of the mouse has been introduced as an experimental model of choice, which can be used in the development of new applications \(^24\).

Currently, progress in biological research is dependent on the capability of researchers to skillfully perform techniques in the field of stem cell and NT. In the present study, we described the successful cloning of a mouse by using piezo-assisted NT.

**Materials and Methods**

**Preparation of media, stock solutions and pipettes**

All media, reagents and stock solutions were prepared according to a protocol by Kishigami \textit{et al} \(^25\), with the exception of mWM medium (modified Whitten's medium) which was prepared as a gift from the Department of Genetic Engineering and Animal Resources, Center for Developmental Biology, Riken Institute, Kobe, Japan.

**In vitro development of fertilized embryos: evaluation of culture media and reagents**

We evaluated the media quality, their supplements, and mineral oil by culturing the fertilized embryos in order to ensure optimal \textit{in vitro} culture conditions. The rates of murula and blastocyst formation in mWM medium were approximately 80% (data not shown).

**Animals**

B6D2F1 (C57BL/6×DBA/2) mice were used to prepare oocyte and somatic-cell (cumulus cell) donors. Surrogate females were NMRI females mated with vasectomized males of the same strain. All mice were used in accordance with the Guidelines for the Care and Use of Laboratory Animals by Kurdistan Uni-
versity of Medical Sciences. Mice were given food and water *ad libitum* and maintained under controlled temperature and lighting conditions during the experiments.

**Oocyte collection**

Mature oocytes were collected from the oviducts of 8-12 week-old female mice. Mice were superovulated with injections of 5 IU of pregnant mare serum gonadotropin (PMSG; Serotrophin, Teikoku Hormone Mfg. Co., Tokyo, Japan) and 5 IU of human chorionic gonadotropin (hCG; Serotrophin, Teikoku Hormone Mfg. Co., Tokyo, Japan) administered 48 hr apart. We collected the oocytes 14-15 hr following the hCG injection. Oocytes were placed in M2+ hyaluronidase ((M2+Hy; Specialty Media) until the cumulus cells dispersed. The oocytes were then placed in KSOM that contained non-essential amino acids and essential amino acids (KSOM AA; Specialty Media) supplemented with 1 mg/ml bovine serum albumin (BSA, Sigma, Japan), covered with mineral oil (Sigma, Japan), and stored at 37°C, in 5% CO2 in air.

**Preparation of donor cells**

Cumulus cells were freshly isolated from recipient oocytes of B6D2F1 mice. After oocyte collection, cumulus cells were dispersed in droplets of M2 medium that contained 0.1% hyaluronidase. After 15 min, the cumulus cells were transferred to 1.5 ml tubes and centrifuged at 200 g for 5 min. The supernatant was removed and the cells suspended in M2 medium.

**Enucleation of oocytes**

B6D2F1 oocytes were freed from the cumulus oophorus and held in M2 medium (Specialty Media) at 37°C under 5% (v/v) CO2 in air until use. The cover of a tissue culture dish was used as a micromanipulation chamber. Each ten oocytes were placed into a M2+CB (Cytochalasin B) droplet in the micromanipulation chamber and after approximately 7 min, enucleation started. Using relative contrast optics the metaphase II spindle was located inside the oocyte and identified as a translucent region. The oocyte was rotated to place the spindle between the 8 and 10 o’clock position, and then it was firmly attached to the holding pipette. The zona pellucida was "cored" following the application of several piezo-pulses (Prime Tech Ltd., Japan) to an enucleation pipette. To avoid damage to the oocyte, we maintained a large space approximately equal to the thickness of the zona pellucida that was located between the zona pellucida and the oolemma. The enucleation pipette was inserted into the oocyte without breaking the oolemma. We removed the metaphase II chromosome-spindle complex by aspiration with a minimal volume of cytoplasm. To verify enucleation, the nucleus was pushed out. It was felt solid and harder than the cytoplasm (Figure 1). The enucleated oocytes were washed three times in KSOM to remove the CB completely after which they were maintained in KSOM medium for at least 30 min in the incubator before beginning donor cell injection. The inner diameters of the enucleation pipettes were approximately 8 µm.

**Donor nuclei injection**

A very concentrated cumulus cell suspension was created in M2 medium. We picked...
up 3 μl of condensed donor cell suspension and introduced into a M2+PVP (Polyvinylpyrrolidone) droplet on the micromanipulation chamber. The donor cells were gently and completely mixed with M2+PVP medium using sharp forceps. The nuclei were removed from the donor cells by gentle aspiration in and out of the injection pipette until each nucleus was separated from any visible cytoplasmic material. The donor nuclei, along with a small amount of cytoplasm, were injected deep inside the ooplasm using the piezo-driven micropipette. One weak piezo pulse was applied to puncture the oolemma at the pipette tip. The donor nucleus was immediately expelled into the enucleated oocyte cytoplasm along with a minimal amount of PVP medium. The injection pipette was gently withdrawn from the oocyte. The inner diameters of the injection pipettes were 5-6 μm.

**Oocyte activation**

The injected oocytes were maintained in the M2 drop for at least 10 min, then transferred into KSOM medium and cultured for at least 30 min in an incubator at 37°C prior to activation. Next, groups of oocytes were placed in Ca²⁺-free CZB that contained 5 μg/ml CB, 5 nM, TSA 10 mM SrCl₂ (strontium chloride) for 6 hr.

Using intact oocytes, we tested the activation medium prior to its use. Intact oocytes were maintained in activation medium for 6 hr in a 5% CO₂ incubator at 37°C. During strontium treatment, the medium became turbid due to the death of approximately 5% of the oocytes.

Following activation, all resultant embryos were transferred to KSOM+TSA (5 nM, trichostatin A, Sigma) and allowed to incubate for at least 4 hr in a 5% CO₂ incubator at 37°C.

Finally, all embryos were washed several times in mWM medium to completely remove the chemicals. For oviduct transfer, the embryos were cultured overnight until they developed to the 2-cell stage in mWM medium.

**Embryo transfer**

We transferred 2-cell stage cloned embryos to the oviducts of females on day 1 of pseudopregnancy (0.5 dpc). To assess development, recipients were evaluated at 19.5 days of gestation and pups were recovered from the uterus. Live pups were raised by lactation by two NMRI foster mothers.

**Results**

**Pre- and post-implantation development of enucleated oocytes injected with cumulus cell nuclei**

When we felt that we had enough experience for doing cloning experiment by NT method, we performed that seven times. Of 367 (M: 52±21) oocytes collected, 309 (85%, M: 44±14) were successfully enucleated and 299 (81%, M: 42±13) were survived. From 299 survived enucleated oocytes, 236 (81%, M: 33±9) survived following donor nuclei injection. Approximately 19% of injected oocytes died after they were transferred to activation medium. The success rate of oocyte activation was 81%. During strontium treatment, the medium became turbid due to the death of the oocytes (Figure 2). The majority of these oocytes obtained one to three pseudo-

![Figure 2](http://www.ajmb.org)
pronuclei. Of 191 oocytes, 131 (69%, $M$:
18±6) developed into two-cell stage embryos
(Figure 2). Of these, 5 (1%, $M$: 0.7±1) live
pups were successfully delivered, one died on
the first day and two survived for two weeks.
Another pup survived for one month; howev-
er the final pup survived for fourteen months.
According to morphology or microanatomy of
the genital system, we determined that all
pups were female. We were unable to deter-
mine the color of the first mouse that died,
however the remaining mice were black. The
surviving cloned mouse was mated and deliv-
ered normal offspring, which she raised. Al-
though the cloned mouse appeared obese, its
offspring were of normal size.

**Discussion**

In this study, we reported the production of
live mouse pups by a stable NT method in
which donor nuclei from cumulus cells of
B6D2F1 mice were directly injected into enu-
cleated oocytes using a piezo-actuated mi-
cromanipulator. The resultant clones derived
from cumulus-cell nuclei were not contami-
nated, as the transferred embryos were not the
result of in vitro fertilization. Pseudopregnant
mice (NMRI, albino) were allowed to mate
with vasectomized males (NMRI, albino) which
were confirmed to be infertile. In the rare
event of fertilization by a vasectomized male,
the newborns would be albino. We transferred
two-cell embryos into the oviducts of foster
mothers. The surviving four mice had black
cloth colors. Where possible, assumed clones
were sexed and determined to be female. Ad-
ditionally, we tested all culture media and re-
agents, and determined their correctness.

Pioneers in the field of mouse cloning have
stated that microinjections using the piezo
unit, as a new technique, are of greater benefit
in comparison with other micromanipulation
techniques, such as ES cell injection into blas-
tocysts or pronuclear DNA injection. How-
ever, personnel will need several months to
achieve an adequate level of skill in the re-
quired techniques. Much practice is necessary
to produce cloned mice; otherwise it is impos-
sible. According to reports, when a highly
skilled person performs mouse cloning exper-
iments using cumulus cell nuclei, 99-100% of
the oocytes will be enucleated successfully
and 80-90% will survive nuclear injection. Of
these, 70-80% should survive activation, 60-
70% show pseudo-pronuclear formation and
50-60% will cleave to the two-cell stage. The
rate for the production of full-term offspring
ranges from 0 to 10%.

In the current study, with adequate tech-
nical expertise of more than one year, we
could get the acceptable results in all stages of
our experiment. The rate of two-cell stage de-
velopment for reconstructed oocytes in the
current study was higher than previous studies
that used different culture media such as CZB
and KSOM. Therefore, we have shown
that mWM is an appropriate medium to sup-
port the development of cloned mouse em-
byros.

In the current study, the donor cells were
cumulus cells. There was one foster mother
with five fetuses in our study. In this regard,
our result is similar to the result obtained by
Satoshi et al. They have reported that fol-
lowing oocyte activation, treatment of oocytes
with 5-50 nM TSA increased *in vitro* devel-
opment of somatic cloned embryos from 2- to
5-fold, which was dependent on the donor
cells, including cumulus cells. The success
rate of mouse cloning from cumulus cells was
increased by over 5-fold without any obvious
abnormalities. In their study, treatment with 5
or 50 nM TSA led to multiple conceptions. In
one case, five fetuses of one foster mother
were born full term. However in this study,
the percent of live offspring was approximately
1%. In the study by Satoshi et al., it was
6%. Most likely, additional experience is nec-
essary in order to improve the cloning exper-
iment results in our laboratory. Since accura-
cy of our experiment was detected and con-
firmed by comparison of sex and coat color of
cloned mice with source of donor cells, we
didn’t perform any genetic test for more eval-
uation.

The adaptability of mice as a small mam
mal has resulted in its use as an appropriate experimental model for the study of new cloning applications and strategies. The information available on their reproduction, development and genetics is unmatchable to other laboratory animals. At present, numerous advanced techniques have been established by the use of mouse embryos. The mouse has a short generation period, which could be advantageous for the examining of long-term genetic effects that occur during biological manipulation, such as cloning.

Conclusion
Our study resulted in the production of cloned mice by somatic cell NT using piezo-actuated micromanipulator. This accomplishment is another important event in the technology of cloning in Iran.

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Conflict of Interest
There is no conflict of interest in this study.

References


