Springer Protocols

Shirley Pease
Thomas L. Saunders *Editors*

Advanced Protocols for Animal Transgenesis

An ISTT Manual

Shirley Pease • Thomas L. Saunders
Editors

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ISBN 978-3-642-20791-4 e-ISBN 978-3-642-20792-1
DOI 10.1007/978-3-642-20792-1
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2011936518

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Cover illustration: Laser assisted injection with a beveled injection pipette; see Fig. 11b in Chap. 17 “Combining ES cells with Embryos”, Elizabeth Williams, Wojtek Auerbach, Thomas M. DeChiara, and Marina Gertsenstein (Photo by Michael Brown)

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Chapter 13

Mouse Cloning by Nuclear Transfer

Sayaka Wakayama, Nguyen Van Thuan, and Teruhiko Wakayama

Abstract

A new mouse cloning method using nuclear injection with a piezo impact drive unit can aid in the bypass of several steps of the original cell fusion procedure. This approach has made it possible to create not only live cloned mice, but also ES cell lines from adult somatic cells via nuclear transfer. It is important to note that these techniques potentially may also be applied to the preservation of genetic material from any mouse strain instead of preserving embryos or gametes. At present, this is the only technique available for the preservation and propagation of valuable genetic resources from mutant mice that are infertile or old, or recovered from carcasses, without the use of germ cells. This technique will greatly help not only in mouse cloning, but also in other forms of micromanipulation such as intracytoplasmic sperm injection into oocytes (ICSI) or embryonic stem (ES) cell injection into blastocysts. Moreover, the piezo unit simplifies pipette preparation, as it allows one to use blunt tipped pipettes without any additional modification.

Abbreviations

EF medium	Embryonic fibroblast medium
ES cell	Embryonic stem cell
NIM	Nuclear isolation medium
NKT cell	Natural Killer T cell
NT	Nuclear transfer
ntES cell	Nuclear transfer embryonic stem cell
SCR	Scriptaid
TSA	Trichostatin A

13.1 Introduction

A new mouse cloning method using nuclear injection with a piezo impact drive unit (hereafter termed "piezo unit") [1] can aid in the bypass of several steps of the original cell fusion procedure.

S. Pease and T.L. Saunders (eds.), *Advanced Protocols for Animal Transgenesis*, Springer Protocols,
DOI 10.1007/978-3-642-20792-1_13, © Springer-Verlag Berlin Heidelberg 2011

Until now, cutting the zona pellucida, inserting a donor cell into the perivitelline space, moving cells and enucleated oocytes to an electrofusion machine, applying electrofusion and later confirmation of cell fusion have been the standard approach. Utilization of the piezo method presents an alternative to many of these steps. Surprisingly, this piezo injection method can result in the production of normal mice from dead cells or even from frozen mouse bodies stored in a freezer for long periods. The challenge in this method lies in the fact that this is technically a very difficult procedure and likely to take most individuals some time to develop sufficient skill before being able to obtain useful data. Without focused practice at these procedures, production of cloned mice is impossible. However, once the piezo unit is properly set up, it will greatly help not only in mouse cloning, but also in other forms of micromanipulation such as intracytoplasmic sperm injection into oocytes (ICSI) [2, 3] or embryonic stem (ES) cell injection into blastocysts [4]. Moreover, the piezo unit simplifies pipette preparation, as it allows one to use blunt tipped pipettes without any additional modification.

This approach has made it possible to create new types of embryonic stem (ES) cell lines from adult somatic cells via nuclear transfer (ntES cell lines) [5, 6]. We have shown that such ntES cell lines have the same differentiation potential as ES cells from fertilized blastocysts [7]. Moreover, cloned mice can be obtained from these ntES cell lines using a second nuclear transfer procedure, [6, 8] which can be used as a backup for the donor cell genome and help increase the overall numbers in mouse cloning [8]. It is important to note that these techniques potentially may also be applied to the preservation of genetic material from any mouse strain instead of preserving embryos or gametes [5, 8]. At present, this is the only technique available for the preservation and propagation of valuable genetic resources from mutant mice that are infertile or old, or recovered from freeze-dried cells or carcasses, without the use of germ cells [9–12]. Here we describe our improved approaches for the production of cloned mice and establishment of ntES cell lines from somatic cells.

13.1.1. Applications

Currently, cumulus cells [1], tail tip cells (probably fibroblasts) [13], Sertoli cells [14], fetal cells [15, 16] and ES cells [17] have been used to produce cloned mice. NKT cells [18], primordial germ cells [19], hematopoietic stem cells [20], keratinocyte stem cells [21], fetal neuronal cells [22] and newborn neuronal stem cells [23, 24] have also been used (*see* review [25, 26]). The genetic background of the mouse strain used as oocyte donors is very important [16, 27]; usually cloning hybrid mouse donor cells is much easier than cloning the cells of inbred mouse strains. Therefore, each researcher must choose the donor cell type carefully according to the need. For example, ES cells are the most

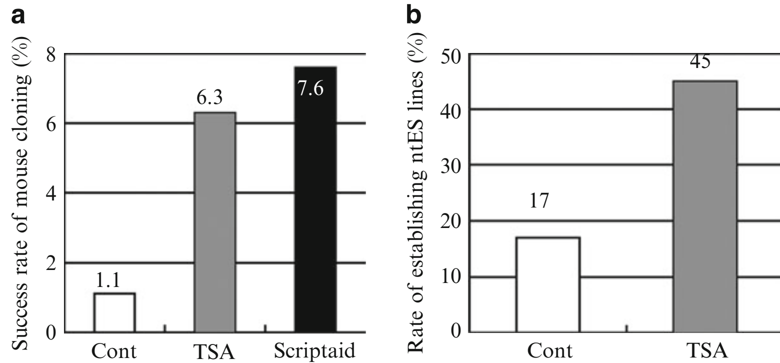

Fig. 13.1 Effects of trichostatin A (TSA) and Scriptaid on mouse nuclear transfer for full-term development and establishment of nuclear transfer embryonic cell (ntES) lines. The success rate of mouse cloning and rate of establishment of ntES cell lines increased up to sevenfold for cloning (**a**) and threefold for ntES cell derivation (**b**) by adding this histone deacetylation inhibitor into the oocyte activation medium. Key: *Cont* controls.

popular for nuclear transfer experiments because, to date, they produce the best results for production of full-term offspring. However, these are pluripotent cells, not differentiated somatic cells, so they are not appropriate for genomic reprogramming experiments. Recently, we increased the efficiency of mouse cloning and ntES cell establishment by up to sixfold by adding the histone deacetylation inhibitor (HDACi), such as trichostatin A (TSA) or scriptaid (SCR), to the oocyte activation medium (Fig. 13.1) [28–32]. This new protocol allowed us to generate cloned mice from inbred strains [28, 33]. Moreover, recently we succeeded in recovering normal live mice from frozen carcasses by using a combination of cloning and ntES cell techniques. These results suggest that even with a low success rate, mouse cloning from murine cells in any given condition is already possible.

13.1.2. Outline of the Procedure (Illustrated in Fig. 13.2)

Put donor cells into culture.

Day 3 – Hormone prime oocyte donors (PMSG).

Day 1 – Hormone prime oocyte donors (HCG). Mate foster mothers (nt pups are recovered by cesarian section).

Prepare media and microinjection needles and equipment.

Day of injection:

1. Collect oocytes
2. Enucleation
3. Prepare single donor cells
4. Nuclear injection
5. Oocyte activation and culture
6. Mate recipient females to vasectomized males

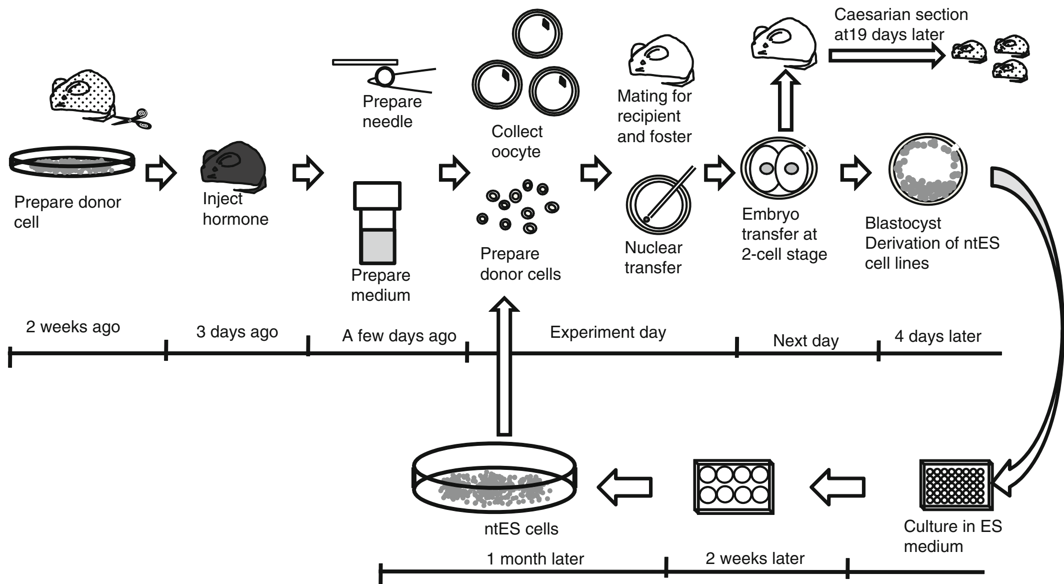

Fig. 13.2 Outline of the procedure.

Subsequent days:

7. Embryo transfer
8. Cesarian section
9. Establishment of ntES cells

13.2 Materials

13.2.1. Equipment

Inverted microscope with Hoffman optics from Olympus (Tokyo, Japan; model IX71)

Micromanipulator set from Narishige (Tokyo, Japan; model MMO-202ND, http://www.narishige.co.jp/)

Microforge (Narishige MF-900)

Warm plate (Tokai Hit, http://www.ivf.net/ivf/tokai_hit_thermo_plate-o738.html)

Piezo impact drive system from Prime Tech Ltd. (Ibaraki, Japan; model MM-150FU, http://www.primetech-jp.com/en/01products/index.html)

Pipette puller (P-97) and glass pipette (B100-75-10) from Sutter Instrument Co. (Novato, CA, USA; http://www.sutter.com)

37°C, 5% CO_2, humidified incubator

Tissue culture hood

Centrifuge

13.2.2. Mouse Strains

Normally B6D2F1 (C57BL/6 × DBA/2) mice (about 2–3 months old) are used for the provision of donor cells and oocytes. Other hybrid strains, such as BCF1 or B6129F1, can also be used as donors. Inbred stains such as C57BL/6 or C3H/He may also be used, but it should be expected that success rates for the production of cloned mice will be lower. The donor mouse strain is important for several reasons; B6D2F1 oocytes are very translucent and the metaphase spindle is easy to find; oocytes from hybrid mice are stronger for in vitro manipulation and culture and somatic cells from hybrid mice provide better donor cells for the production of cloned mice. We use the ICR (CD-1) strain of mouse for production of pseudo-pregnant surrogates, for lactating foster mothers and for vasectomized males.

13.2.3. Culture Media

13.2.3.1. Stocks

CB Stock Solution: Cytochalasin B (1 mg, Sigma-Aldrich, St Louis, MO, USA; C6762). Add 2-mL DMSO to a vial with 1-mg cytochalasin B (500 μg; 100× CB stock solution). Divide into small tubes (10–20 μL) and store at 80°C.

TSA Stock Solution: Trichostatin A (TSA; 1 mg, Sigma-Aldrich; T8552). Add 3.311-mL DMSO to a vial with 1-mg TSA (1 mM) and then take 2 μL of this stock and dilute with 198 μL of DMSO (10 μM, 200× TSA stock solution). Divide into small tubes (10–20 μL) and store at −80°C.

SRC Stock Solution: Scriptaid (SRC; 1 mg, Sigma-Aldrich; S7817). Add 3.064-mL DMSO to a vial with 1-mg SCR (1 mM) and then take 5 μL of this stock and dilute with 95 μL of DMSO (50 μM, 200× SCR stock solution). Divide into small tubes (5–10 μL) and store at −80°C.

$SrCl_2$ Stock Solution: $SrCl_2$ $6H_2O$ (Sigma-Aldrich S0390) is dissolved in distilled water (DW) at 100 mM and stored in aliquots at room temperature (10× stock solution).

EGTA Stock Solution: EGTA (Sigma-Aldrich; E8145) is dissolved in DW at 200 mM and stored in aliquots at 4°C (100× stock solution).

Equine chorionic gonadotrophin (eCG or PMSG, Sigma-Aldrich; G4527) is dissolved in normal saline at 50 IU per mL and stored in aliquots at −20°C.

Human chorionic gonadotrophin (hCG, Sigma-Aldrich; C8554) is dissolved in normal saline at 50 IU per mL and stored in aliquots at −20°C.

PMSF Stock: Phenylmethanesulfonyl fluoride (Sigma-Aldrich P7626) is dissolved in ethanol at 50 mM and stored in aliquots at 4°C (100× stock solution).

13.2.3.2. Oocyte and Embryo Culture Media

KSOM medium (Millipore, Temecula, CA, USA; MR-106-D) (http://www.millipore.com)

M2 medium (Millipore; MR-015)
M2 + hyaluronidase (Millipore; MR-051)
Acid Tyrode's solution (Millipore; MR-004-D)

13.2.3.3. Fibroblast Cell Culture Media

Tail tip or embryonic fibroblast cells are cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich D6429) supplemented with 10% fetal bovine serum (termed EF medium).

13.2.3.4. ntES Cell Establishment Medium

CultiCell Medium (Dainippon Sumitomo Pharma, Tokyo, Japan; S2211101) (http://www.ds-pharma.co.jp/english/index.html) or Stem Cell Sciences KK, Kamiarai, Tokorozawa, Saitama, Japan (http://www.scskk.com/English/product_service/Culti-Cell.html) is used for ntES cell establishment.

13.2.3.5. ntES Cell Maintenance Medium

Complete ES cell media (Specialty Media; ES-101-B) or in-house prepared mouse ES cell media.

Phosphate-buffered saline (PBS) Ca/Mg free (Millipore; BSS-1006-B).

Trypsin solution (Millipore; SM-2003-C).

13.2.3.6. Enucleation Media

Add 2 μL of CB stock solution to 198 μL of M2 medium (M2+CB medium).

The final concentration of CB is 5 μg/mL.

13.2.3.7. PVP Medium

12% PVP in M2: Add 1.2-g PVP to 8.8-mL M2 and place at 4°C overnight. Mix the solution and sterile filter it on the next day and store 1-mL aliquots at 4°C.

13.2.3.8. Oocyte Activation media

Add 10 μL of $SrCl_2$ stock solution (final 10 mM), 2 μL of EGTA stock solution (final 2 mM), 2 μL of CB stock solution (final 5 μg/mL) and 1 μL of TSA or SCR stock solution (final 50 or 250 nM, respectively) to 185 μL of KSOM medium [34].
Note: If ES cells are used as sources of donor nuclei, TSA does not work well. If G2/M phase ES cells are used as donor nuclei, cytochalasin B must be omitted from the medium.

13.2.3.9. Reprogramming Enhancement Media

For reprogramming enhancement, cloned embryos should be cultured for an additional few hours with TSA or SCR. Add 1 μL of TSA or SCR stock solution (final 50 or 250 nM, respectively) to 199 μL of KSOM medium.

13.2.3.10. Nuclear Isolation Media

The 123.0-mM KCl, 2.6-mM NaCl, 7.8-mM NaH_2PO_4, 1.4-mM KH_2PO_4, 3-mM EDTA disodium salt and 0.5-mM PMSF.

The pH is adjusted to 7.2 by addition of a small quantity of 1-M KOH.

13.2.4. Other Reagents

Distilled water (DW, Sigma-Aldrich, St Louis, MO, USA; W1503)

DMSO (Sigma-Aldrich; D2650)

Mercury (Sigma-Aldrich; 215457)

Mineral oil (Sigma-Aldrich; M5310)

Polyvinylpyrrolidone (PVP; 360 kD) (Irvine Scientific, Santa Ana, CA, USA; 99311) (http://www.irvinesci.com)

13.3 Nuclear Donor Cell Sources

13.3.1. Donor Cell Preparation 1 (from Cells Maintained in Culture)

13.3.1.1. Tail Tip Fibroblasts

Tail tip cells must be prepared at least 2 weeks before NT. Cut the tail into sections at least 2-cm long and wash in 70% ethanol. In a sterile tissue culture hood, remove the skin and cut the tail into many small pieces on a 6-cm plastic dish. Culture the fragments in 10-mL DMEM under 5% CO_2 in air until used. There is no need to passage the cells.

13.3.1.2. ES Cells

ES cell should be passaged 2 days before an experiment, or thawed at least 2 days before an experiment and cultured in six-well dishes. We do not recommend the use of cells within 1 day of passage. ES cells are the most popular cell type for NT experiments because they have been seen to give the best rate of success in the production of full-term offspring. However, each individual ES cell line, even if from the same genetic background, will lend itself to this procedure to different degrees, resulting in varying results for full-term pups produced. The number of passages of ES cell lines will also affect the rate of success. Note that these are pluripotent cells, not differentiated somatic cells, so they are not appropriate for genomic reprogramming experiments.

13.3.1.3. Method

1. Prepare cells in culture for use: remove any culture medium from the dish and wash them in PBS (Ca^{++}, Mg^{++} free). Remove the PBS, add trypsin medium and then incubate 5–20 min in the incubator. Add culture medium (including serum) and triturate the cells to produce a single-cell suspension. Spin down the cells in a centrifuge at $200 \times g$ for 10 min and wash cells with PBS by centrifugation at least three times, as above. ***Note:*** *As trypsin is very toxic at the time of nuclear injection, the donor cells must be washed thoroughly.*

2. Make a very concentrated cell suspension in EF medium. The final volume should be less than 10 μL. If the final concentration is too low, it is difficult to find an appropriate donor cell and the delay can be detrimental to the recipient oocytes awaiting NT.

13.3.2. Donor Cell Preparation 2 (Primary Cells, Directly from In-Vivo Collection)

13.3.2.1. Cumulus Cells

Cumulus cells are the easiest to prepare as nuclear donors because they can be used immediately after collection without washing, with no need to remove hyaluronidase from the medium.

13.3.2.2. Sertoli Cells

Sertoli cells are testicular sustentacular cells and the male counterparts of cumulus cells. Adult Sertoli cells are inappropriate donor cells because of their large size, but those collected from neonatal mouse testes (immature Sertoli cells) are small enough for injection and usually give better results than cumulus cells [14, 27]. Thus, the age of donors will affect the success rate. We recommend the use of newborn males at less than 6 days of age.

13.3.2.3. Method

Wash in vivo collected cells in PBS and centrifuge at 100 rpm or $3 \times g$ for 10 min, at least three times, in order to remove any enzymes that may be present. Cumulus cells are an exception and may be used without washing.

Prepare concentrated cell suspension as described in Subheading 13.3.1.3.

13.3.3. Donor Cell Preparation 3 (from Frozen Mice)

13.3.3.1. Brain

Collect the brain from the frozen body and break it into small pieces (1–2 mm^3) on the dry ice. Homogenize it under 500 μL of Nuclear isolation media (NIM) in 1.5-mL tube by homogenizer pestle, then filter it by cell strainer (BD Falcon 352235) and collect the supernatant. This medium contains many naked nuclei. Introduce a few microliters of suspension into NIM on the manipulation chamber instead of PVP medium.

13.3.3.2. Blood

Collect 1–2 μL of blood cells from the frozen-thawed mouse tail and add to 500 mL of NIM. Make a condensed cell suspension by centrifugation and introduce it into NIM in the manipulation chamber instead of PVP medium.

13.4 Protocol

13.4.1. Oocyte Production

13.4.1.1. Hormone Priming for Superovulation

Inject equine chorionic gonadotrophin (eCG or PMSG; 5 IU) into the abdominal cavity 3 days before an experiment and then inject human chorionic gonadotrophin (hCG, 5 IU) 48 h later (1 day before an experiment). Usually we inject mice at 5–6 p.m.

13.4.1.2. Collection of Oocytes

1. Collect oocyte–cumulus cell complexes from the oviduct ampullae at 14–15 h after hCG injection (usually we collect oocytes at 8–9 a.m.; Fig. 13.3) and move them into M2+ Hyaluronidase medium.
2. After 5 min, pick up the good oocytes, wash them in M2 medium three times and place them in the KSOM medium prepared as above.
3. Place oocytes as drops. The number of oocytes in a drop depends on each person's skill or type of experiment. We recommend that all oocytes in one drop must be manipulated within 15 min. Therefore, place 10–30 embryos per drop, according to skill level.

13.4.2. Preparation of Enucleation and Injection Pipettes

13.4.2.1. Micropipettes

Micropipettes can be ordered from several companies (e.g., Prime Tech Ltd. http://www.primetech-jp.com/en/01products/psk.html). Alternatively, they may be made in the lab. For the holding pipette, the outside diameter (OD) should be smaller than that of the oocyte (e.g., OD 80 μm; inner diameter (ID) 10 μm). The ID

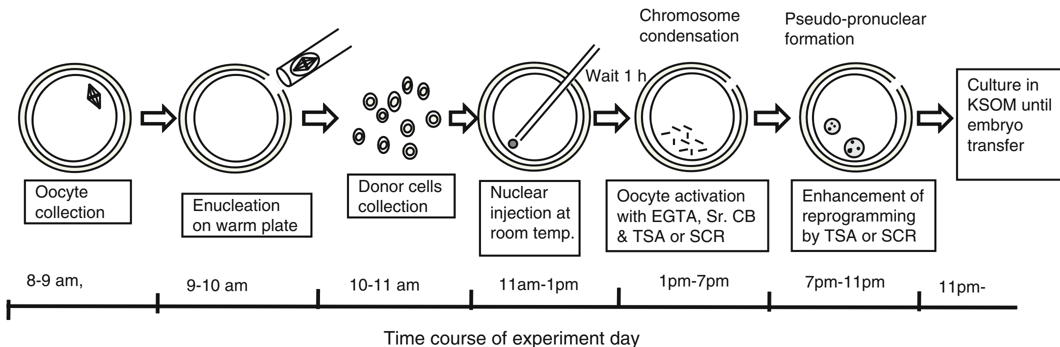

Fig. 13.3 Outline of the experimental procedure. Without diligent practice, it is very difficult to complete this procedure within the allotted time. This method is referred to as the "Honolulu method" because it was developed in a laboratory in Honolulu in 1998 [1].

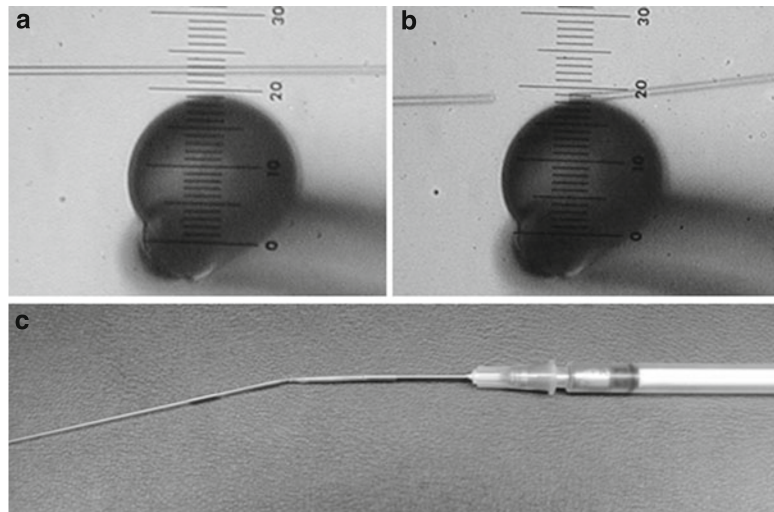

Fig. 13.4 Making pipette. The micropipette is positioned above the grass bead (**a**) and the filament is heated for less than 1 s. The pipette is fused to the glass bead, and when filament was cool down, pipette was broken (**b**) at the point of contact. The size of outer diameter must be adjusted depending on the cell size. (**c**) Load a small amount of mercury from back of pipette using 1-mL syringe and 30-gauge needle or 26-gauge needle with fine tubing.

of the enucleation pipette is 7–8 μm. The ID of the injection pipette depends on nuclear donor cell type: 5–6 μm for cumulus cells and 6–7 μm for fibroblasts or ES cells (Fig. 13.4a, b). If you are purchasing pipettes, ask the supplying company to bend all pipettes close to the tip (about 300 μm back) at 15–20° using a microforge.

13.4.2.2. Inserting Mercury into the Pipette

Backload a small amount (about 3-mm-long column) of mercury into the enucleation/injection pipette using a 1-mL syringe and 30-gauge needle (Fig. 13.4c). Fill the syringe part way with mercury, insert the needle into the back of the pipette and inject a mercury droplet into the needle. Store in 10-cm dish at room temperature.

Note: Mercury is toxic if absorbed by breathing or through the skin. At a minimum, wear appropriate gloves and always use mercury in a working fume hood. Use appropriate safety handling conditions, as recommended by your Institutional Safety Office.

13.4.3. Preparation of Media Dishes for Manipulation

1. Place many 15-μL droplets of KSOM medium on a 6-cm cell culture dish and cover this with mineral oil and place in incubator. This medium can be used from oocyte collection to activation (Fig. 13.5a). This dish must be prepared before starting any other part of the procedure.
2. Place ~15-μL droplets of three different media (M2, M2+CB (enucleation media) and PVP) on the lid of a 10-cm dish as shown in Fig. 13.5b and then cover this with mineral oil. This

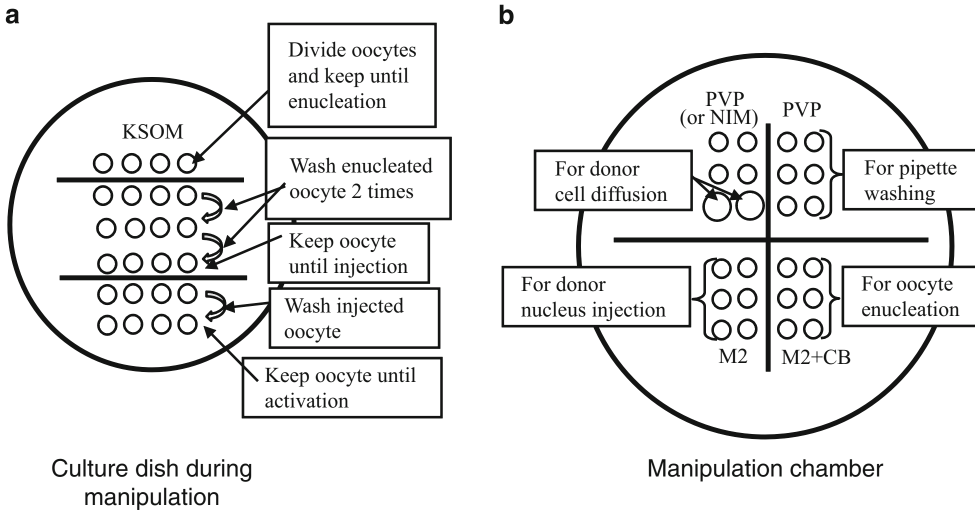

Fig. 13.5 Manipulation dish and chamber. Oocytes are kept from the time of collection to just before activation in KSOM medium in a 6-cm dish (**a**). The lid of a 10 cm dish is used as a micromanipulation chamber (**b**). Key: *PVP* polyvinylpyrrolidone, *NIM* nuclear isolation medium.

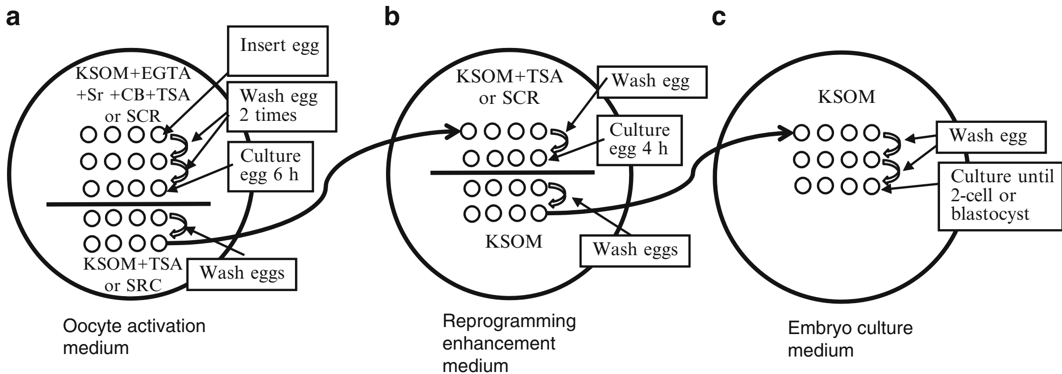

Fig. 13.6 Oocyte activation, enhancement of reprogramming and long-term culture. Reconstructed oocytes are exposed to the activation medium (**a**) for 6 h. Move embryos to the reprogramming enhancement medium (**b**) and culture for a few hours. Move embryos to the long-term culture dish (**c**) until transfer into a recipient female.

chamber can be used for both enucleation and microinjection. Draw a line on the dish to distinguish these media.

3. Place ~15-μL droplets of oocyte activation media: KSOM+EGTA+Sr+CB+TSA (use SCR when somatic cell nuclei donors are derived from inbred mice [33]), re-programming enhancement media: KSOM+TSA or SCR and embryo culture media: KSOM, on the 6-cm dish as shown in Fig. 13.6a–c and then cover this with mineral oil. Draw a line on the dish to distinguish these media.

13.4.4. Setting Up the Micromanipulator

1. Attach the enucleation pipette to the pipette holder of the piezo unit. The top of the pipette holder must be screwed in tightly. Position the piezo unit on the micromanipulator.
2. Expel any air and oil and a few drops of mercury from the enucleation pipette in the PVP medium. Wash both the inside and outside of the pipette using PVP medium.
3. Adapt and fit the pipette to the piezo unit. While expelling the air and mercury from the pipette in the PVP droplet, the piezo unit must be applied with high power (more than 10 units) and high speed (more than 10 units) for at least 1 min continuously.
4. Attach the holding pipette on the opposing side of the micromanipulator.

13.4.5. Enucleation of Oocytes

1. Place one group of oocytes in an M2+CB droplet into the micromanipulation chamber and wait at least 5 min before starting enucleation. The cytochalasin B makes the oolemma more flexible and reduces lysis.
2. Locate the metaphase II spindle inside the oocyte. It can be recognized without any staining using Nomarski or Hoffman optics. Rotate the oocyte to place the spindle at between 2 and 4 o'clock or between 8 and 10 o'clock and then attach the oocyte firmly to the holding pipette (Fig. 13.7a).
3. Cut through the zona pellucida using a few piezo pulses. To avoid damaging the oocyte, ensure that there is a large space between the zona pellucida and the oolemma, approximately as wide as the thickness of the zona pellucida.
4. Insert the enucleation pipette into the oocyte without breaking the oolemma and remove the metaphase II spindle by aspiration with a minimal volume of cytoplasm. The oocyte membrane and spindle must be pinched off slowly, draw the needle out carefully until the oolemma seals and do not apply piezo pulses to cut the membrane (Fig. 13.7b). The MII spindle is harder than the cytoplasm, so you can feel its consistency through the micromanipulator.
5. Wash the enucleated oocytes twice in KSOM to remove the cytochalasin B completely, and return to the incubator in KSOM medium under oil for at least 30 min before starting donor cell injection (Fig. 13.5a). If the CB is not completely washed out, many oocytes will lyse after injection.
6. If you feel tired at this point, take a short break before starting the next step. From the next step, there is no respite and the procedure requires intense concentration.

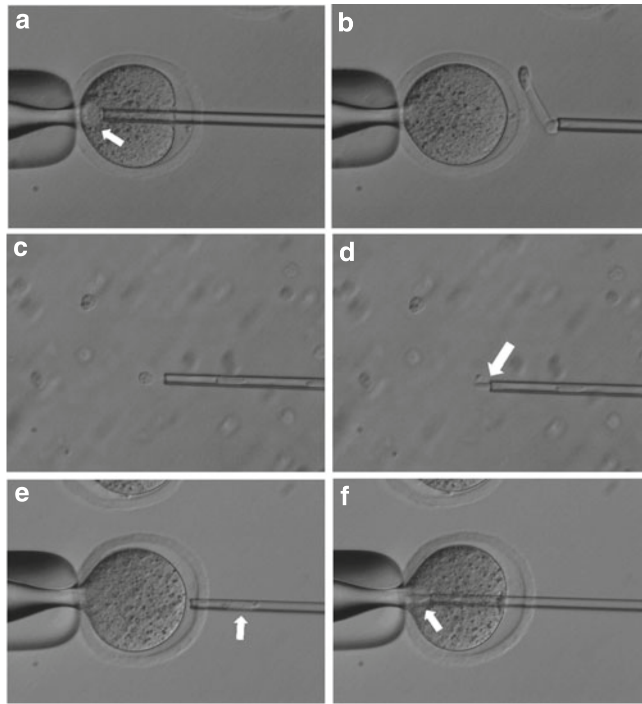

Fig. 13.7 Nuclear transfer using the piezo unit. (**a**) Rotate the oocyte, locate the metaphase II spindle and place it between the 8 o'clock and 10 o'clock position, or between the 2 o'clock and 4 o'clock position (see *arrow*). Then stabilize the oocyte on the holding pipette. (**b**) Remove the spindle by suction without breaking the plasma membrane and gently pull the pipette away from the oocyte. (**c**) and (**d**) Donor nuclei are gently aspirated in and out of the injection pipette until their nuclei are largely devoid of visible cytoplasmic material (*arrow*). (**e**) Hold the enucleated oocyte and cut the zona pellucida using piezo pulses (*arrow*). (**f**) Insert the injection pipette into the enucleated oocyte. Apply a single piezo pulse to break the membrane, and then inject the donor nucleus immediately (*arrow*). Gently withdraw the injection pipette from oocyte.

13.4.6. Donor Nucleus Injection

1. Pick up 1–3 μL of condensed donor cell suspension and put the cells in a PVP medium droplet in the micromanipulation chamber (Fig. 13.5b). Mix the donor cells with PVP medium gently but completely, using sharp forceps. Do not scratch the bottom of the chamber.
2. Place about 10–20 enucleated oocytes into M2 medium. The number of oocytes per droplet depends on each individual's skill level. Each group should be finished within 15 min.
3. Remove the donor nuclei from the cells by gently aspirating them in and out of the injection pipette until each nucleus is clearly separate from any visible cytoplasmic material (Fig. 13.7c, d). Take up a few nuclei into the injection pipette.
4. Stabilize an enucleated oocyte using a holding pipette. Cut the zona pellucida using a few piezo pulses (Fig. 13.7e).

5. Reduce the power level of the piezo unit (power levels 1–2 and speed 1). The oolemma is weaker than the zona pellucida and the survival rate of oocytes after injection will be better with this reduced power.
6. Push one nucleus forward until it is near the tip of the pipette and advance the pipette until it almost reaches the opposite side of the oocyte's cortex (Fig. 13.7f). Do not apply the piezo unit's power until the pipette reaches the opposite side. If the piezo power is applied with the tip of the pipette in the middle of the oocyte, the oocyte will die after injection.
7. Apply one weak piezo pulse to puncture the oolemma at the pipette tip. This is indicated by a rapid relaxation of the oocyte membrane (Fig. 13.7f). Expel the donor nucleus into the enucleated oocyte cytoplasm immediately with a minimal amount of PVP medium. Gently withdraw the injection pipette from the oocyte.
8. Wash the injection pipette with PVP medium by expelling some mercury and applying power from the piezo unit. This washing step is essential to prevent the pipette from getting sticky.
9. Keep the injected oocytes in this drop for at least 10 min then transfer them into KSOM medium (Fig. 13.5a) and culture for at least 30 min in the incubator before activation.

Note: The process of nuclear transfer should be performed at room temperature (25–26°C). Do not use warm plate for injection process.

13.4.7. Activation and Embryo Culture

1. Prepare the oocyte activation medium (Fig. 13.6) at least 30 min before use and equilibrate in a CO_2 incubator.
2. Transfer and culture each group of oocytes into drops of activation medium and wash twice, then culture for 6 h in a 5% CO_2 incubator at 37°C (Fig. 13.6a).
3. All embryos must be washed twice in TSA or SCR-KSOM medium to remove the cytochalasin B completely (Fig. 13.6a, below the line). Examine the rate of oocyte activation. If NT and activation are done properly, those oocytes should each possess two or three pseudo-pronuclei after 6 h in culture as above [1].
4. Move the cloned embryos to a different dish. Because some of the chemicals used for activation can diffuse to other drops through the mineral oil and are embryotoxic, all embryos should be moved to different culture dishes for long-term culture. Some batches of mineral oil are toxic. So it is advisable to test all new batches of oil by culture of embryos in drops of

KSOM, overlaid with test oil. Always set up a control group of embryos cultured under oil of known quality.

5. Additional culture of cloned embryos in TSA-KSOM for 10 h or SCR-KSOM for up to 24 h will enhance the reprogramming (Fig. 13.6b, [33]).
6. Wash the cloned embryos and move them to another dish (Fig. 13.6c); culture them to the two-cell stage (next day) or to the blastocyst stage (3 days later).

13.4.8. Embryo Transfer and Cesarian Section

1. Mate estrous ICR female mice with normal males on the same day or 1–2 days before the experiment; these will be used as foster mothers for receipt of NT pups derived by cesarian section at E19.5. Foster mothers are needed for NT pups because NT litters are too small to stimulate lactation in the surrogate mothers.
2. Mate estrous ICR female mice with vasectomized males on the same day as the experiment; these will be used as pseudo-pregnant (surrogate) mothers.
3. Transfer the two-cell (24 h after NT) or four- to eight-cell (48 h) cloned embryos into oviducts of 0.5 days post-copulation (dpc), or morulae/blastocysts (72 h) or blastocysts (96 h) into the 2.5-day post-coitum (dpc) pseudo-pregnant female mice, respectively [35].
4. A cesarian section is required to recover the cloned mice fetuses securely (*see* point 9 in Subheading 13.6). Euthanize the surrogate mother at 18.5 or 19.5 dpc. Remove the uterus from the abdomen and dissect out the cloned pups with their placentas. Wipe away the amniotic fluid from the skin, mouth and nostrils and stimulate the pups to breathe by rubbing the back or pinching them gently with blunt forceps. Warm to 37°C.
5. To transfer the cloned pups to the cage of a naturally delivered foster mother, first remove the mother from the cage. Take some soiled bedding from the cage and nestle the cloned pups in the bedding material so that they take on the odor of the bedding. Keep the pups warm while allowing them to remain in the bedding for a few minutes. Remove some pups from the foster mother's litter and then mix the cloned pups with the foster female's pups. Return the mother to the cage and leave the animal room quietly.

13.4.9. Establishing ntES Cell Lines from Cloned Embryos

1. The preparation of mouse embryonic fibroblasts for use as ES cell feeder cells is described in Chapter 14. Prepare a 96-well plate of mitotically inactivated feeder cells at least 1 day before ntES cell establishment.

2. Change the medium of the dish from DMEM to at least 200 μL of CultiCell medium before plating cloned embryos. The CultiCell medium does not contain fetal calf serum. Serum may contain potential differentiation factors. Therefore, it is important to use serum-free medium for establishment of new ntES or ES cell lines. Alternatively, you can use the new ES cell establishment "3i medium" which inhibits GSK3, MEK and FGF receptor tyrosine kinases and enhances the ES cell establishment rate significantly [36]. *See* Chapter 19, Rat and Mouse ES cell Derivation by 2i/3i Method.
3. Remove the zona pellucida from cloned blastocysts (Day E 3.5) using acidic Tyrode's solution. The zona pellucida will dissolve within 30 s and prolonged exposure to acid Tyrode's solution will decrease the quality and survival of embryos. Therefore, before dissolving the zona pellucida completely, pick up the embryos and wash them several times in M2 medium. The remaining thinned zonae are easily broken by repeated pipetting.
4. Plate each cloned blastocyst onto the feeder cell of 96-well multidishes one by one (Fig. 13.8a).
5. Culture the multidish for 10–14 days in an incubator without changing the medium. During this period, the cloned

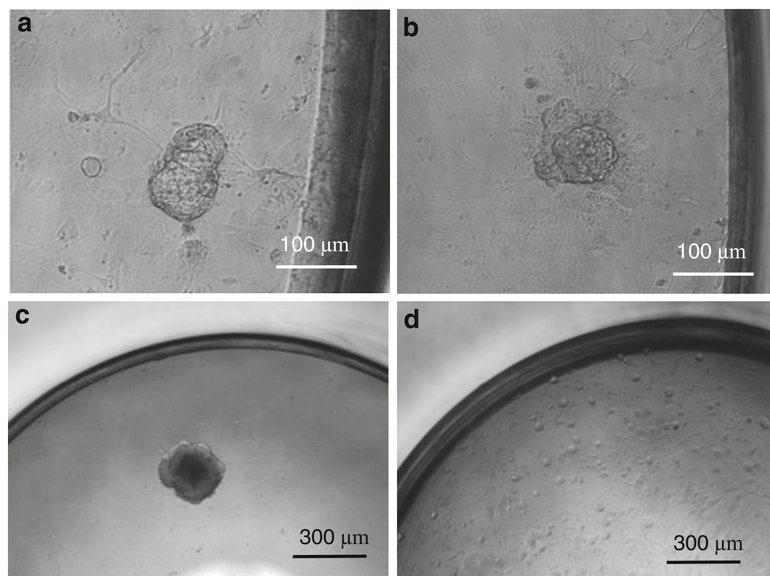

Fig. 13.8 Establishment of ntES cell lines. (**a**) Zona-free cloned blastocysts are plated on 96-well dishes. (**b**) Cloned blastocysts are attached onto the feeder cells, then the trophoblast cells spread and the inner cell mass (ICM) appears 7–9 days after plating. (**c**) The ICMs grow to almost 5–10 times as large as the original at 11–14 days after plating. (**d**) Two days after trypsinization, some wells show newly established ntES cell lines.

blastocyst will attach to the surface of the feeder layer and the inner cell mass (ICM) can be seen to grow (Fig. 13.8b, c).

6. Some of the wells should develop ICMs, which form large clumps. When those clumps appear, treat them with trypsin and disaggregate the cells using a 200 μL pipette. Then re-plate the suspension into another well (pre-plated with feeders) of the same multidish.

7. When ES-like cell colonies dominate the well (Fig. 13.8d), the cells should be expanded in a clonal manner, gradually to 48 well, 24 well, 12 well and then 12.5 cm^2 flask and 25 cm^2 flask by repeated passages several times. As these ES cell lines are to be used for nt at a later date, we do not use feeder cells to support ES cell culture from 48-well dishes onwards. After the cell numbers have increased, the cells should be cryopreserved in the manner usual for ES cells [37].

13.4.10. Production of Cloned Mice from ntES Cell Nuclei

Nuclear transfer may be accomplished by using ntES cells as donors (Fig. 13.2), repeating the procedure from section 13.3 onwards. Unlike somatic cells, ntES cells (like ES cells in general) [7] will divide indefinitely, so they offer the possibility for use without limitation, unlike somatic cells that have a limited life in cell culture. Moreover, the overall success rates of cloning from individuals are increased when ntES cell lines are used as intermediate nuclear donors [8].

13.5 Results

The genetically modified mouse is a powerful tool for research in medicine and biology. However, in one large-scale study on ethyl-nitrosourea (ENU) mutagenesis, infertility was listed as a phenotypic trait in more than half of the mutants described [38]. Overcoming this infertility is a challenge worth undertaking, as the ability to maintain such types of mutant mice as genetic resources would afford numerous advantages for research in human infertility and reproductive biology. Unfortunately, the success rate of somatic cell cloning is very low. Even when cloning a phenotypically sterile mouse is successful, it will still be necessary to clone all subsequent generations. This represents another significant barrier, as the success rate of repeated cloning from cloned mice decreases for each successive generation after the first NT [39]. On the other hand, the ntES cell establishment rate is nearly ten times higher than the success rate of cloned mice, even from the so-called "unclonable" mouse strains (Fig. 13.9). Therefore, we recommend the establishment of ntES cell lines at the same

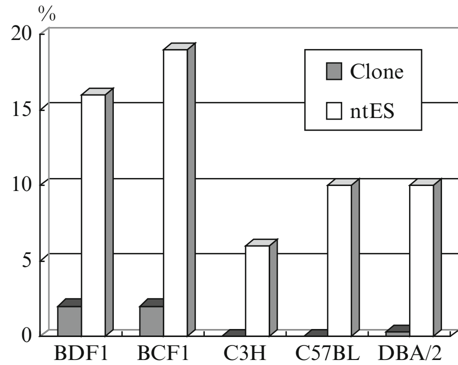

Fig. 13.9 Comparison of the success rate of cloned mice and the rates of establishment of ntES cell lines from different mouse strains.

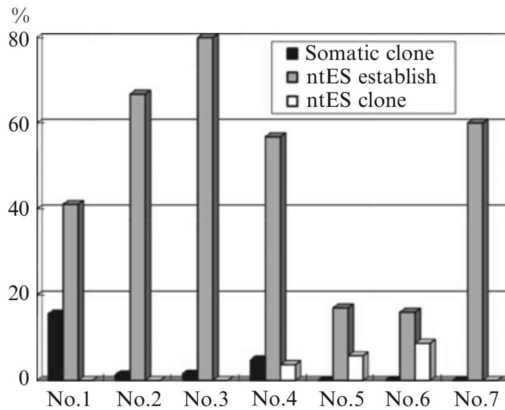

Fig. 13.10 Comparison of the success rates of producing cloned mice, establishing ntES cell lines and producing cloned mice from the ntES cell nuclei. Six out of seven donor mice could produce clones using either technique [8].

time as cloning to preserve the donor genome because these lines can then be used as an unlimited source of donor nuclei for subsequent rounds of NT. For example, because of the generally low success rate of cloning, we were able to generate cloned animals from only four out of seven somatic cell donor mice. We were ultimately able to obtain cloned mice from six out of seven individuals by using either somatic cells or ntES cells [8] (Figs. 13.10). Senescent mice are often infertile and the cloning success rate decreases with age, making it almost impossible to produce cloned progeny directly from such animals. We succeeded in establishing ntES cell lines from all aged mice, regardless of sex or strain. The ntES cells were then used to generate cloned mice by a second NT. In addition, healthy offspring were obtained from all aged donors via germline transmission of the ntES cells in chimeric mice [9]. For example, we found a mutant, hermaphrodite and sterile mouse in our ICR strain mouse-breeding colony.

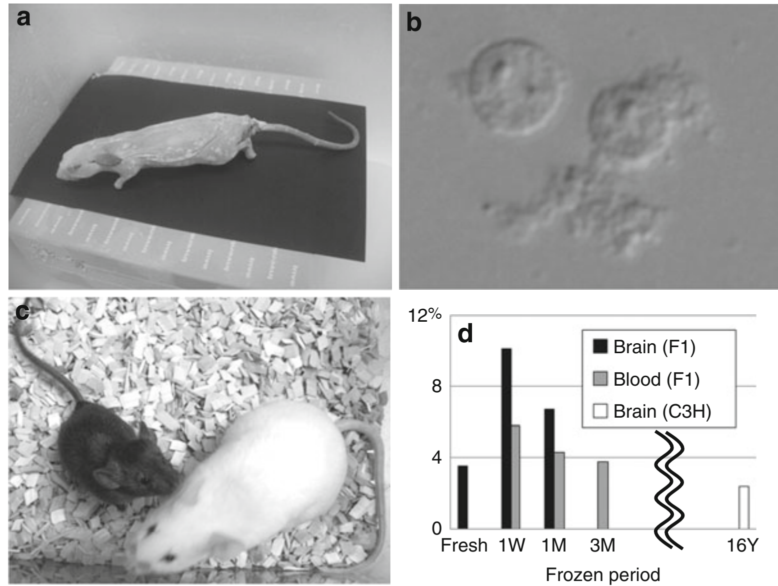

Fig. 13.11 Production of cloned mice from bodies frozen for 1 week, 1 month, 3 months or 16 years without cryoprotectant [12]. (**a**) A frozen mouse. (**b**) Donor nuclei derived from frozen brain by simple homogenization. (**c**) Cloned mouse and its foster mother. (**d**) Rate of establishing ntES cell lines. F1: B6D2F1 mice; C3H: C3H/He mice were used as donors, respectively.

Unfortunately, ICR is one of the most difficult strains to clone from [28, 40]. However, ntES cell lines from a tail tip fibroblast of this particular mouse were established successfully. Although this mouse was lost soon after tail-tip biopsy, we attempted to make cloned mice from its ntES cell nuclei, but we could not obtain full-term offspring. Finally, using tetraploid complementation [41], we obtained two cloned mice. Using the diploid chimera method, most of the mutant mouse genes were transmitted to the next generation via the ntES cells. As a second example, recently we developed new NT techniques, which allowed us to resurrect normal mice from bodies kept frozen at −20°C for up to 16 years without any cryoprotection (Fig. 13.11a) [12]. Although we could not produce cloned offspring from the somatic cell nuclei, several ntES cell lines were established from the cell nuclei of most organs. Finally, healthy cloned mice were produced from these ntES cells by a second round of nuclear transfer (Fig. 13.11c).

Thus, this technique is applicable for the propagation of a variety of animals, regardless of age or the potential for fertility. If a valuable mouse is unexpectedly lost, then it is clearly possible, now, to generate live copies of such animals from tissue that was otherwise considered lifeless.

13.6 Troubleshooting

1. *Cannot make a hole using the piezo drill?*

If you cannot cut the zona pellucida, check the connection between the pipette and pipette holder. The top of the pipette holder must be screwed in tightly. Expel all oil inside the pipette, as oil may have reduced the piezo power transmission. There should be a slight negative pressure inside the pipette to enhance the piezo power.

2. *Sticky pipettes?*

PVP will cover both the inside and outside of the pipette to keep the surface slick. Washing the pipette in PVP medium is very important and will affect not only oocyte survival rate, but also embryo development after NT. Without this step, the pipette soils rapidly and needs to be changed.

3. *Difficulty in finding the oocyte spindle?*

The room temperature is very important as the spindle microtubules will disperse and become unclear at room temperature. However, the spindle will become visible again if the oocytes are cultured at 37°C for 30 min before enucleation. Oocyte transparency also depends on the mouse strain; B6D2F1 is better than some others.

4. *Donor cells aggregate in PVP medium?*

Mix the donor cells with PVP medium using sharp tweezers for at least 30 s. If the donor cells are not mixed sufficiently in PVP medium, they will aggregate and it will be difficult to isolate single cells. ES cells are especially sensitive and fragile and it is better to make new ES cell suspension drops every 30 min.

5. *Difficulty in releasing donor nuclei from the pipette?*

Probably the pipette is too dirty. It must be washed frequently using PVP medium by expelling some mercury and applying power from the piezo unit.

6. *Oocyte lysis after nuclear injection?*

Either you used too large a pipette, the room temperature is too high or the pipette insertion was too shallow. A large pipette or warm temperatures increase the rate of oocyte lysis. The injection pipette must be inserted very deep into the oocyte before applying the piezo pulse. In addition, you need practice. If you are a beginner, all oocytes will lyse immediately after injection. One month after starting practice, about half of your oocytes might survive.

One year later, about 80% of oocytes will survive, if you continue to practice diligently. If oocytes are transferred to KSOM medium immediately after injection, 10–20% of them undergo lysis from the damage of injection. The oocyte membrane must be allowed to recover before transfer to KSOM; this takes about 10 min.

7. *Oocytes die during activation?*

The activation medium must be checked before use, using intact oocytes. During strontium treatment, up to 10% of the oocytes will die and the medium will become dirty. This is normal and the surviving oocytes are usually undamaged.

8. *No pseudo-pronuclear formation after oocyte activation?*

There are several reasons why oocytes do not form pronuclei. Usually it is because of failure to break the donor cell membrane or failure of oocyte activation. The injection pipette must be smaller than the donor cell. If the donor cell has a tough cell membrane (e.g., tail tip fibroblasts), apply piezo power to break the donor cell membrane at the time of cell pickup.

9. *Cloned neonate dies at birth?*

All cloned mice allowed to go to full term gestation to date have been born with abnormal and hypotrophic placenta and often die immediately after birth from respiratory failure. At this point, there is no way to avoid this lethal phenotype.

10. *Pups do not develop to full term?*

If you have no success in getting full-term development, change the donor cell type from somatic to ES cells, or other hybrid mouse strains. Also, try making up new embryo culture medium. However, the most important solution is to keep practicing. Technical skill is essential. Do not give up!

References

1. 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
2. Kimura Y, Yanagimachi R (1995) Intracytoplasmic sperm injection in the mouse. Biol Reprod 52:709–720
3. Wakayama T, Yanagimachi R (1998) Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat Biotechnol 16:639–641
4. Kawase Y, Iwata T, Watanabe M, Kamada N, Ueda O, Suzuki H (2001) Application of the piezo-micromanipulator for injection of embryonic stem cells into mouse blastocysts. Contemp Top Lab Anim Sci 40:31–34
5. Wakayama S, Ohta H, Kishigami S, Thuan NV, Hikichi T, Mizutani E, Miyake M, Wakayama T (2005) Establishment of male and female nuclear transfer embryonic stem cell lines from different mouse strains and tissues. Biol Reprod 72:932–936
6. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292:740–743
7. Wakayama S, Jakt ML, Suzuki M, Araki R, Hikichi T, Kishigami S, Ohta H, Van Thuan