4 Cloning The Laboratory Mouse by Nuclear Transfer

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4.1 Introduction

The development of the fertilized zygote into a complex organism has traditionally been thought of as a unidirectional process, with cells in the developing fetus becoming gradually more committed to a specific tissue type. The recent development of mammalian cloning by nuclear transplantation (NT) demonstrates that the mammalian oocyte has the remarkable ability to relieve the constraints imposed by cellular differentiation and return an adult nucleus to a totipotent, embryonic state (Wilmut et al. 1997; Gurdon and Colman 1999). Thus, cloning by NT provides a unique opportunity to elucidate the molecular and cellular mechanisms by which an adult cell can be returned to an undifferentiated state, a process termed nuclear reprogramming (Rideout et al. 2001).

Due to the prevalence of the laboratory mouse as a genetic and embryological model organism, it is a desirable system for the study of cloning and nuclear reprogramming. However, the mouse remains one of the more difficult mammals to clone, with transfer of NT technology from laboratory to laboratory occurring at a slow rate. With the technical difficulties of mouse NT in mind, we have assembled a chapter that describes the basic methodologies we use for the generation of cloned mouse embryos. The methodology we describe here is a modified version of the "Honolulu technique", first developed in the laboratory of R. Yanagimachi at the University of Hawaii; this technique relies on direct microinjection of the donor nucleus into the recipient oocyte (Fig. 4.1A,B) (Wakayama et al. 1998).

This chapter has three principal sections: (1) a discussion of the parameters influencing cloning efficiency and nuclear reprogramming, (2) a discussion of equipment set-up and methods for NT, and (3) an in-depth, step-by-step protocol for the NT procedure.

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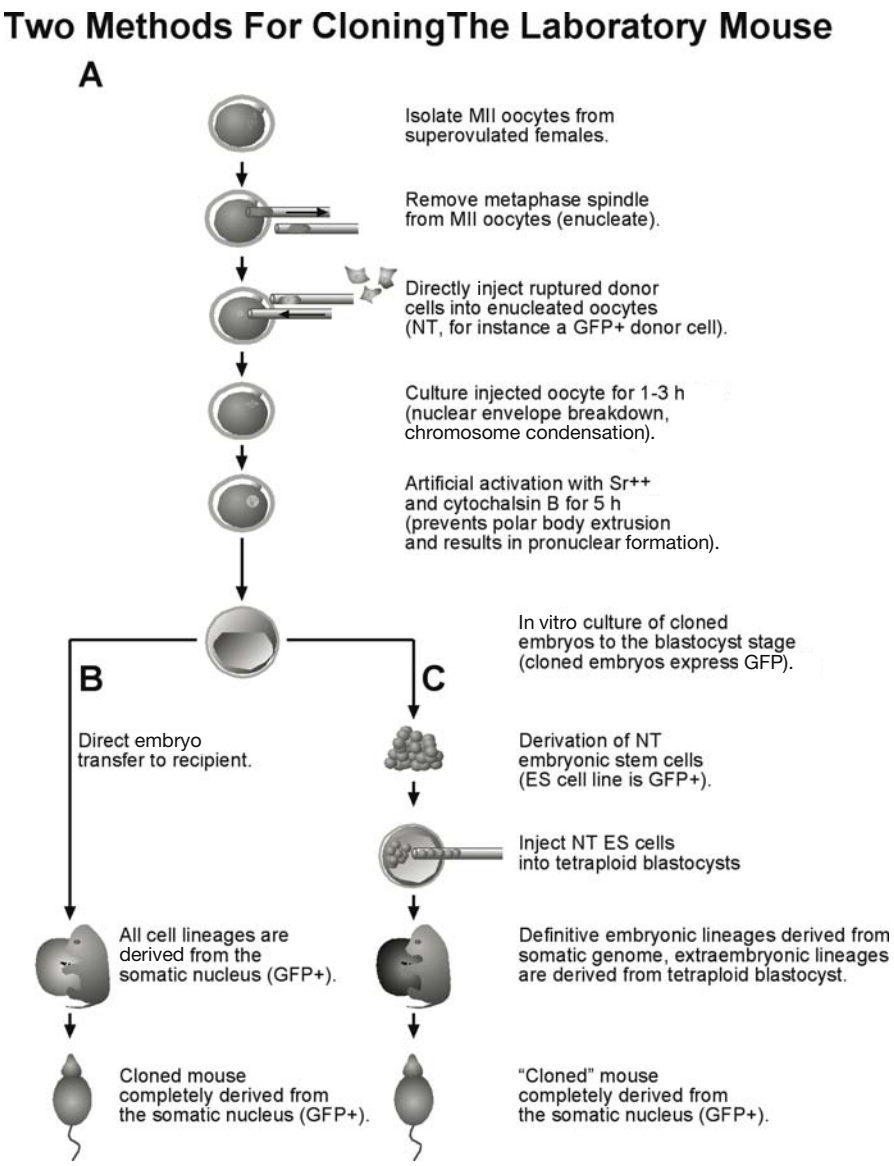

Fig. 4.1. A–C Cloning by direct injection nuclear transfer

In contrast to cloning procedures in other mammals, where the donor nucleus is introduced into the egg by cell-cell fusion, nuclear transfer in the mouse has been successful by the physical injection of an isolated nucleus into an enucleated egg. Therefore, we will concentrate on this procedure as used in mouse cloning. The chapter is written for an audience already skilled in handling and micromanipulating preimplantation mouse embryos, as required for the production of transgenic and mutant mice. For general information on embryo culture, mouse husbandry, tetraploid embryo complementation and embryo transfer surgery, we recommend Chaps. 4 and 11 of this book, and the more extensive descriptions that can be found elsewhere (Nagy et al. 2002).

4.2 Factors Influencing Cloning Success

A wide array of animals have been have been cloned through the introduction of somatic nuclei into enucleated oocytes (Campbell et al. 1996; Cibelli et al. 1998; Onishi et al. 2000; Polejaeva et al. 2000; Baguisi et al. 1999; Chesne et al. 2002; Wakayama et al. 1998; Shin et al. 2002). However, cloning by NT remains very inefficient, and cloned animals display a variety of embryonic, perinatal and postnatal phenotypes. These phenotypes include, but are not limited to, early developmental failures, dramatic fetal and placental overgrowth, neonatal respiratory failure, obesity, and premature death (Wakayama et al. 1998; Eggan et al. 2001; Tamashiro et al. 2002; Ogonuki et al. 2002). The biological basis of these phenotypes and the inefficiency of cloning by NT remain poorly understood.

Unfortunately, the universally low efficiency of NT experiments has made it difficult to dissociate technical difficulties from biological phenomena in cloning research. However, as research interests have shifted from whether cloning is merely possible to the parameters that influence its success, some of the variables that determine a successful cloning outcome have begun to emerge. To familiarize the reader with important technical aspects of cloning by NT, these parameters and the experiments that suggest they are critical will be described. Emphasis has been placed on results from the mouse, as it is the experimental system pertinent to this chapter. However, data from other animals are included to illustrate general issues of nuclear cloning.

4.2.1
Cell Cycle Status of the Donor Cell

Cloning experiments with a variety of cell types has demonstrated a correlation between the efficiency of NT embryo development to the blastocyst stage and the proportion of the donor cell population in the G1 phase of the cell cycle. When cumulus cells (Wakayama et al. 1998) and serum-starved fibroblasts (Wakayama and Yanagimachi 1999), primarily in a G1 state, were used for NT, the majority of activated embryos developed to the blastocyst stage. In contrast, when rapidly cycling embryonic stem (ES) cells were used as nuclear donors (Wakayama et al. 1999; Eggan et al. 2001), only a small percentage of NT embryos completed cleavage development. Consistent with the interpretation that differences in cell-cycle state caused this effect, culture conditions that force a higher proportion of ES cells into the G1 phase of the cell cycle, such as partial serum withdrawal, increase the potential of ES cell NT embryos to reach the blastocyst stage (Wakayama et al. 1999).

The importance of the donor nucleus cell-cycle state is linked directly to compatibility with the recipient oocyte cytoplasm. In the MII oocyte, metaphase/maturation promoting factor (MPF) levels are high (reviewed by Fulka et al. 1996). High MPF levels in the oocyte cytoplasm lead to somatic cell nuclear envelope breakdown and premature chromosome condensation after NT (Wakayama et al. 1998). The S phase of the cell cycle, characterized by DNA replication and a specific chromatin conformation, is likely to be incompatible with this condensation, leading to DNA damage and zygotic arrest. As most ES cells in a given population are in S phase, this is likely the cause of the poor in vitro development of ES-cell-derived embryos after NT.

4.2.2
Genetic Influences on the Cloning Process

When ES cells derived from several mouse strains were used as nuclear donors, inbred 129, C57/B6 and a variety of F1 ES cell lines all gave rise to newborn clones (Wakayama et al. 1999; Rideout et al. 2000; Eggan et al. 2001). However, clones from the inbred ES cell lines died shortly after birth due to respiratory failure (Wakayama et al. 1999; Rideout et al. 2000; Eggan et al. 2001). In contrast, most clones derived from five different F1 ES cell lines survived to adulthood (F1 here refers to ES cell lines derived from embryos produced by intercrossing parents with different inbred genetic backgrounds; (Rideout et al. 2000; Eggan et al. 2001)).

Neonatal lethality has also been reported in mice entirely derived from inbred ES cells injected into tetraploid blastocysts (Fig. 4.2) (Nagy et al.

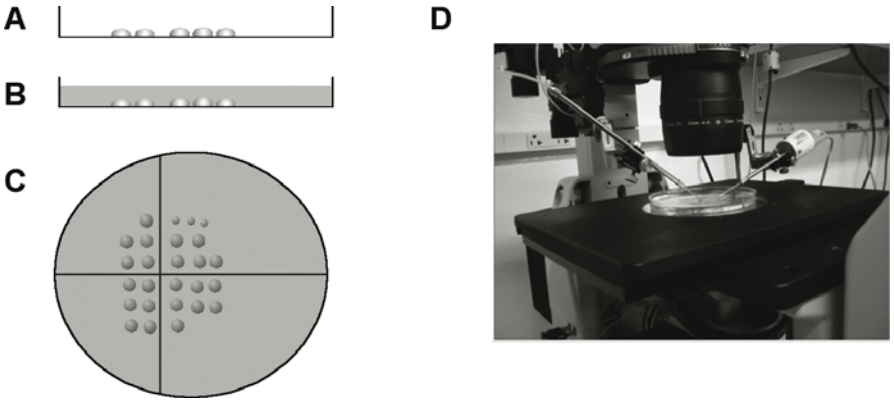

Fig. 4.2. A–D Setting up Petri-dish lid, micromanipulation chamber and micromanipulators, and inverted microscope set for piezo micromanipulation. Inverted microscope with Hofman modulation contrast set for nuclear transfer using the lid of a Petri dish as a micromanipulation chamber. Particularly, note the angle of inclination for the piezo micromanipulator, which is at about 20°

1990, 1993). Like inbred clones, ES cell-tetraploid neonates derived from inbred ES cell lines died shortly after delivery with signs of respiratory distress (Nagy et al. 1990; Eggan et al. 2001). In contrast, most ES cell-tetraploid neonates derived from six F1 ES cell lines, developed into fertile adults (Eggan et al. 2001). These results suggest that the death of inbred ES cell clones is not a direct result of the NT procedure itself but instead is due to the intrinsic character of inbred ES cells. It is possible that the decreased developmental potential and respiratory failure observed in inbred ES NT embryos could be due to delayed developmental timing relative to their F1 counterparts. However, experiments demonstrating that NT newborns derived from both F1 (Wakayama et al. 1998) and inbred 129 cumulus cells (Wakayama and Yanagimachi 2001) survive from birth to adulthood at equal frequency seem to argue against this conclusion.

It is possible that inbred ES cells may suffer some ill effects due to long-term cell culture, which ultimately leads to the death of ES-cell-derived offspring. Experiments suggesting that prolonged in vitro passage of ES cells can further aggravate these phenotypes in ES-cell-tetraploid-derived offspring lend further support to this hypothesis (Nagy et al. 1993). Interestingly, an F1 genetic background also seems to protect against these detrimental effects, since offspring from all F1 genotypes tested survived to adulthood, even at high passage (Eggan et al. 2001).

Cloned animals of other species, including sheep, also often display signs of respiratory distress at birth, especially following prolonged donor cell in vitro culture (McCreath et al. 2000). Thus, it could be that the ill-

effects of long-term in vitro culture of donor cells, which are modified by genetic factors in mice cloned from ES cells, may be generally relevant to the survival of other cloned animals derived from cultured cells.

4.2.3 Intrinsic Developmental Potential of the Donor Cell

Comparing the efficiency of NT experiments using both somatic and embryonic cells has suggested that the intrinsic developmental potency of the donor cell plays a role in the cloning outcome. When mouse cumulus (Wakayama et al. 1998) and fibroblasts cells (Wakayama and Yanagimachi 1999) were used as nuclear donors, only 1–3% and 0.5%, respectively, of embryos transferred to surrogate mothers developed to term. In contrast, 5–25% of blastocysts generated by NT with ES cell (Wakayama et al. 1999; Rideout et al. 2000; Eggan et al. 2001) or blastomere (Tsunoda and Kato 1997) nuclei survived until birth.

These observations have sparked debate as to whether all nuclei have the capacity to direct embryogenesis after NT. One viewpoint is that surviving clones are derived from rare cells with intrinsic developmental potential that are present at a low frequency in the donor cell population, such as somatic stem cells (Clarke et al. 2000; Hochedlinger and Jaenisch 2002b). Thus, the great inefficiency of cloning might reflect the rare nature of donor cells with the developmental capacity to direct development after NT. Experiments using mature lymphocytes (Hochedlinger and Jaenisch 2002a) and neurons (Eggan et al. 2004) as nuclear donors have now demonstrated that bona fide differentiated cells can give rise to ES cell lines and cloned mice. However, in both of these cases a two-step cloning process including an ES cell intermediate was used (Fig. 4.1C), raising the question of whether complete reprogramming of a terminally differentiated nucleus requires passage of the genome through the ES cell state. It is possible that the embryo and the oocyte alone can directly reprogram the epigenetic state of a terminally differentiated cell to give rise to a cloned embryo after direct embryo transfer, but this may be exceedingly inefficient and has not been achieved as yet (Fig. 4.1B).

4.2.4 Cellular Identity of the Donor Cell

Several lines of evidence suggest that the identity of the donor cell nucleus may influence both gene expression and phenotypes in cloned embryos and animals. First, in classical NT experiments using the amphibian Rana pipiens it was observed that, when endoderm nuclei were used

as nuclear donors, cloned embryos showed normal development of endodermal derivatives but abnormal development of mesodermal and ectodermal tissues (Briggs and King 1952, 1957). Complementary phenotypes were observed in cloned embryos derived from neural ectoderm nuclei, and these phenotypes were subsequently termed endoderm and ectoderm syndrome (DiBerardino and King 1967).

Similarly, there is mounting evidence suggesting the donor cell type might influence the phenotype of cloned mice. When cumulus cells were used as nuclear donors, many cloned mice became obese (Tamashiro et al. 2002). In contrast, mice derived from sertoli cells of the same genetic background did not become obese, but instead died prematurely with some signs of tumorigenesis and kidney failure (Ogonuki et al. 2002). Our own analysis of placental transcriptional profiles from cloned mice revealed abnormalities in gene expression that were common to all cloned animals. However, we also detected gene expression abnormalities that were present in all animals cloned from cumulus cells but that were not found in animals cloned from ES cells, which had their own set of common abnormalities that were not found in cumulus clones (Humpherys et al. 2002). Together, these results suggest there may be incomplete cell-type specific reprogramming of the donor nucleus, and that these cell-type specific effects may have important influences on the development and phenotypes of cloned mice.

4.2.5 Epigenetic Reprogramming after Nuclear Transfer

The most intriguing issue in cloning by NT is the problem of epigenetic reprogramming (Gurdon and Colman 1999). In order for clones to complete development, genes normally expressed during embryogenesis, but silent in the somatic donor cell, must be reactivated. To date, the efficiency of deriving live cloned animals has been low, independent of the cell type used as nuclear donor, with two notable exceptions. Nuclei isolated from ES cells and from embryonic blastomeres generated viable cloned animals with a significantly higher efficiency than any somatic donor cell type (reviewed in Rideout et al. 2001). As stated above, this observation is consistent either with the notion that only a limited number of somatic cells, those with sufficient developmental potential, are competent nuclear donors, or that the genome of pluripotent embryonic cells is more easily reprogrammed than that of somatic cells.

In normal development, reprogramming of the genome occurs during gametogenesis, a complex process that assures that, when combined at fertilization, the genome of the two gametes can faithfully activate early

embryonic genes (Barton et al. 1984; Kafri et al. 1992). In cloning, reprogramming presumably must occur in the short interval between transfer of the donor nucleus into the egg and the onset of cellular differentiation at the blastocyst stage, a cellular context dramatically different from that of normal fertilization. The challenge has been to identify the epigenetic abnormalities, arising either as a result of faulty reprogramming or during donor cell in vitro cultivation and aging, that are responsible for the inherently inefficient nature of cloning and the phenotypes of cloned animals. The hope has been that by understanding these abnormalities we might determine the nature of epigenetic information that either is, or is not, reprogrammed after NT, and that we might use this information to pinpoint the time at which reprogramming occurs, allowing identification of the molecular machinery responsible.

A major focus of this research has been to determine the identity of epigenetic information that either is, or is not, reprogrammed after NT. For instance, X chromosome inactivation and telomere length adjustment are rapidly and robustly reprogrammed after NT, indicating that disturbances in these epigenetic states are not likely impediments to the development of clones (Eggan et al. 2000; Tian et al. 2000; Betts et al. 2001; Lanza et al. 2000; Young et al. 1998). Epigenetic information encoding mono-allelic expression of imprinted genes, however, is not restored to a functional state after NT, and disturbances in the expression of these genes may lead to the severe over-growth observed in many cloned animals (Bartolomei and Tilghman 1997; Humpherys et al. 2001; Inoue et al. 2002). Finally, emerging evidence suggests that the reactivation of developmentally regulated gene expression may occur incorrectly, or incompletely, after NT, hinting at a root cause for the early developmental arrest of many cloned embryos (Boiani et al. 2002; Bortvin et al. 2003).

4.3 Methods, Equipment and Techniques

4.3.1 Embryo Culture Media and Common Stock Solutions

Preimplantation embryos are very sensitive to organic solvents, detergents and perturbations in pH, thus we recommend using disposable plastic or dedicated glassware for media preparation. We use ultrapure H_2O, such as that available from Specialty Media (Phillipsburg, NJ). KSOM or CZB embryo culture media used for NT are produced using a master salt mix as a base (Chatot et al. 1990).

4.3.2
Mouse Strains and Animal Husbandry

We purchase 4- to 6-week-old B6D2F1 female oocyte donors from Charles River labs (www.criver.com). These females are housed in our facility for a minimum of 1 week before superovulation. To produce pseudopregnant recipients for embryo transfer, we mate Swiss Webster females, weighing between 28 and 40 g, to vasectomized Swiss Webster males. Mice are housed on a 12 h day/night cycle. (For additional detail, see Chap. 11 on Ancillary Techniques, this manual.)

4.3.3
Preparation of Cumulus Cells for Nuclear Transfer

Cumulus cells for NT are isolated from cumulus complexes in parallel with MII oocytes as previously described ((Wakayama et al. 1998); also see below). After 5 min of hyaluronidase treatment, cumulus cells are aspirated into a transfer pipette with a minimal amount of medium and deposited in a 500 μl drop of HCZB under mineral oil in a small dish. This dish is then placed on ice until needed for NT.

4.3.4
Preparation of Tail-tip Cells for Nuclear Transfer

We prepare tail-tip donor cells as described for the production of the first male cloned animals (Wakayama and Yanagimachi 1999). Euthanise the donor mouse by approved means and then amputate one-half of the tail. After amputation, the biopsy is placed into Wescodyne for 2 mins, washed several times through Hepes-buffered saline (HBS) and the outer layer of skin is removed. After several more washes through HBS, and finally through Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal calf serum (FCS), the tail is chopped into very small pieces with a scalpel on the lid of a Petri dish. The pieces of tail are then split equally among two to three wells of a six-well tissue culture dish. The wells of the tissue culture dish are then filled with DMEM supplemented with 15% FCS. After 3 days of culture in 5% CO_2 at 37 °C the medium is changed and fibroblasts should be observed growing on the bottom of the dish. These cultured cells are best used 1–2 weeks after generation. Around 24–48 hour prior to NT, DMEM with 15% FCS is replaced with DMEM with 0.05% FCS in order to force the donor cells to exit the cell cycle. To prepare tail-tip cells for NT, they are trypsinized, washed twice in DMEM with 15% FCS, and placed on ice until needed.

4.3.5
Culture and Preparation of ES donor cells for Nuclear Transfer

ES cell culture is carried out essentially as previously described (Hogan et al. 1994). ES cells are cultured in ES cell medium [ESCM; DMEM with 15% fetal calf serum (Hyclone), 0.1 mM non-essential amino acids (Gibco, Rockville, MD), 2 mM L-glutamine, 50 IU Penicillin, 50 IU Streptomycin (Gibco) and 0.1 mM beta-mercaptoethanol (Sigma, St. Louis, MO), 1,000 U/ml leukemia inhibitory factor (LIF)] on gelatinized tissue culture ware (Falcon; www.bdbiosciences.com) pre-plated with a monolayer of gamma-irradiated primary mouse embryo fibroblasts (MEFs). Recently we have begun supplementing our ES cell media with the MEK kinase inhibitor PD98059 (Cell Signaling Technology, www.cellsignal.com) at a final concentration of 50 μM; this compound has been reported to inhibit differentiation of mouse ES cells (Burdon et al. 1999). For nuclear transplantation, ES cells are cultured in ESCM with 5% FCS for 24–48 h to slow down their cell cycle. Following starvation, ES cells are trypsinized, resuspended in DMEM and pre-plated on a standard 10 cm tissue culture dish for 30 min to remove feeder cells and debris. Following pre-plating, trypsinized ES cells are washed once in ESCM and placed on ice until NT.

4.3.6
Microscope Set-up

All micromanipulation required for NT is carried out with standard hydraulic micromanipulators (Narishige, M188NE or equivalent, www.narishige.co.jp) on an inverted microscope (such as Nikon TE200) with Hofman modulation contrast optics (Fig. 4.2). The lid of a Petri dish is routinely used as a micromanipulation chamber (Fig. 4.2). For NT by direct injection – the "Honolulu" method – we use the piezo micromanipulator made by Primetech [Ikabari, Japan, distributed in the United States by Brinkmann (www.brinkmann.com)]. To cope with the significant backpressure generated during piezo microinjection, we recommend the IM6–2 microinjector (Narishige). This manipulator is also suitable for the precise micromanipulation procedures required for successful NT.

4.3.7
Micromanipulation Instruments for Nuclear Transfer

Flat-tipped microinjection pipettes are used for NT. The method used to produce microinjection instruments is identical to that used to produce

instruments for piezo-assisted blastocyst injection with ES cells, as described in Chap. 3. For enucleation, a pipette with an internal diameter of 8 μm is used. For NT, a microinjection pipette just smaller than the diameter of the donor cell is used (ES cells and cumulus cell 6 μm, tail-tip cell 8–10 μm). For NT, 3–5 μl mercury is loaded into the back of the pipette (a 1 ml syringe fitted with silicon tubing is appropriate for this task). Great care should be taken while back loading mercury into the pipette as the back pressure generated is substantial and can cause the mercury to be expelled suddenly from the back of the pipette. After the pipette has been loaded with mercury it is affixed to the pipette holder (Fig. 4.2).

4.3.8 Isolation of Metaphase II Oocytes for Nuclear Transfer

A detailed description of superovulation and microdrop embryo culture can be found elsewhere (Hogan et al. 1994). Briefly, all embryo culture should be carried out in 20–30 μl drops of medium (unless otherwise noted) on a standard Petri dish flooded with mineral oil (see Fig. 4.2). Ovulated MII oocytes for NT are isolated from super-ovulated 8- to 10-week-old B6D2F1 females. Superovulation is induced by intraperitoneal (IP) injection of 5 IU pregnant mare's serum (PMS) (Calbiochem, San Diego, CA) performed between 6 and 7 pm; 46–48 h after PMS, 5 IU human chorionic gonadotropin (HCG) (Calbiochem) are injected IP.

At 14–15 h following the administration of HCG, female oocyte donors are sacrificed and the oviducts removed. After dissection, oviducts can be placed directly into 200 μl drops of HCZB placed on a Petri dish and covered with mineral oil. One of the droplets is supplemented with bovine testicular hyaluronidase at a final concentration of 0.1% w/v (Sigma). The oocytes are isolated from the oviducts by tearing the ampullae with forceps, releasing small drops of oviductal fluid containing the oocytes into the mineral oil. Once all cumulus complexes have been removed from the oviducts they can be quickly and simultaneously moved into the drop of HCZB containing hyaluronidase. After 2–3 min, the hyaluronidase will begin to dissociate the cumulus complexes; 5–10 min after the oocytes were placed in hyaluronidase, they should be washed, using a mouth-controlled aspirator assembly, through the other drops of HCZB medium, eliminating any hyaluronidase and remaining cumulus cells.

Following cumulus cell removal, oocytes should be transferred into MCZB medium that has been pre-equilibrated for 20–30 min at 37 °C under 5% CO_2 in air. Alternatively, we now also use KSOM with amino acids (Specialty Media) for long-term embryo culture. Oocytes should be

washed through several drops of medium to remove residual HZCB. Recipient oocytes should be used for enucleation within 2 h of isolation.

4.3.9
Enucleation of MII Oocytes

After their isolation, groups of 15–20 oocytes are placed on the microscope stage in a micromanipulation chamber prepared as described in Fig. 4.2, containing HCZB with 5 μg/ml cytochalasin B (Sigma). The oocytes should rest in cytochalasin-containing medium for 5–10 min to allow actin-cytoskeletal depolymerization to occur. Cytoskeletal depolymerization allows the metaphase spindle to be removed from the oocytes with minimal chance of lysis. Following cytoskeletal depolymerization, enucleation can be performed as described in Fig. 4.3. After enucleation, oocytes should be washed through several drops of KSOM or MCZB and returned to one of these media in the incubator. This process can then be repeated until the desired number of enucleated oocytes has been collected. Enucleation of a single group of embryos should take no longer than 15–20 min.

4.3.10
Nuclear Transfer

For nuclear transfer, donor cells are diluted 1:10 into a drop of HCZB containing 11% w/v polyvinylpyrrolidone (PVP). It is critical that the cells are mixed thoroughly with the PVP to ensure that they are not damaged by the ionic gradient between the two media with and without the volume-excluding PVP. Just before donor nucleus isolation, a group of 10–20 enucleated oocytes should be placed on the stage in HCZB containing 5 μg/ml cytochalasin B. Donor nuclei are isolated by aspirating intact cells into the injection pipette and slowly working them back and forth in the needle, while applying gentle pulses with the piezo micromanipulator. In general, 3–10 nuclei are isolated for NT at one time. Following donor nucleus isolation, the instruments are moved to the drop of medium containing the enucleated oocytes and direct injection NT can be performed as described in Fig. 4.3.9. After NT, reconstructed embryos are washed through several drops of KSOM or MCZB and returned to the incubator.

4.3.11
Oocyte Activation and Subsequent Culture of Cloned Embryos

We activate reconstructed NT embryos 1–3 h after NT in Ca^{++}-free MCZB medium containing 10 mM Sr^{++} and 5 μg/ml cytochalasin B. Oocytes are

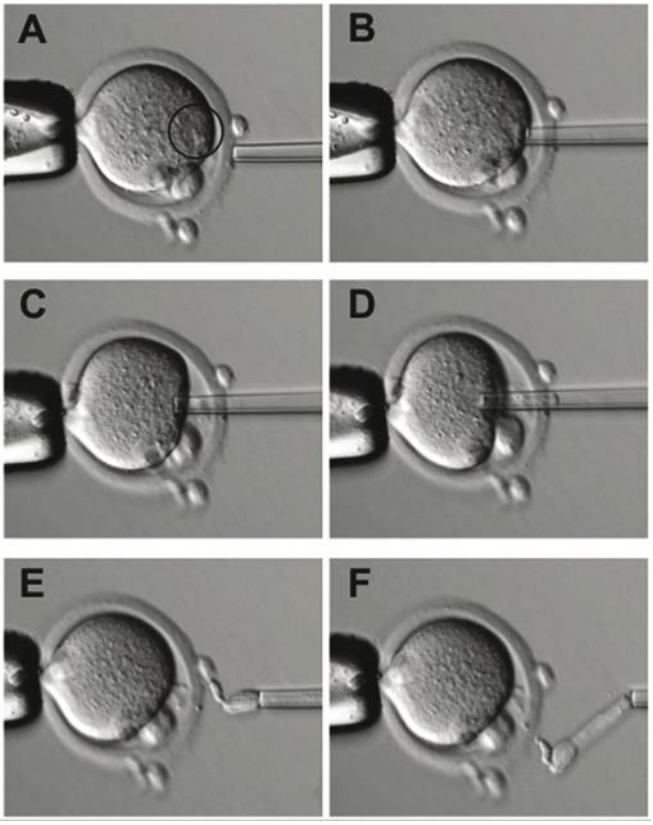

Fig. 4.3. A–F Piezo micromanipulator enucleation of metaphase II (MII) mouse oocyte. **A** Mouse MII oocyte immobilized with a standard holding pipette. The MII spindle has been circled. Note that the spindle has an optical nature different from the rest of the oocyte and can be seen as a more refractive portion of the cytoplasm. **B** Application of piezo micromanipulator combined with gentle suction to the enucleation pipette leads to zona "drilling", which allows access of the blunt needle to the perivitelline space. **C** Before aspirating the spindle into the enucleation pipette it is often worthwhile to touch the spindle with the end of the pipette. If the spindle moves, one can be confident that the needle is in the correct location and that the spindle will be removed with a minimum of cytoplasm. **D** After the pipette is in the correct location, suction is applied to the enucleation needle, leading to aspiration of the spindle into the pipette. Note that the refractive portion of the cytoplasm is being drawn into the pipette. **E** Once the spindle is two-thirds to three-quarters inside the pipette, the pipette should be drawn away from the oocyte. If done properly the spindle will remain in the pipette, removing the minimum of cytoplasm. **F** To confirm that enucleation was complete, the spindle can be observed after expulsion from the enucleation pipette. Unlike the rest of the ooplasm, which will form a sphere when discarded from the needle, the spindle has a rigid character and will remain in a bar-shaped karyoplast. Often the karyoplast will have a distinct "dog-bone" appearance

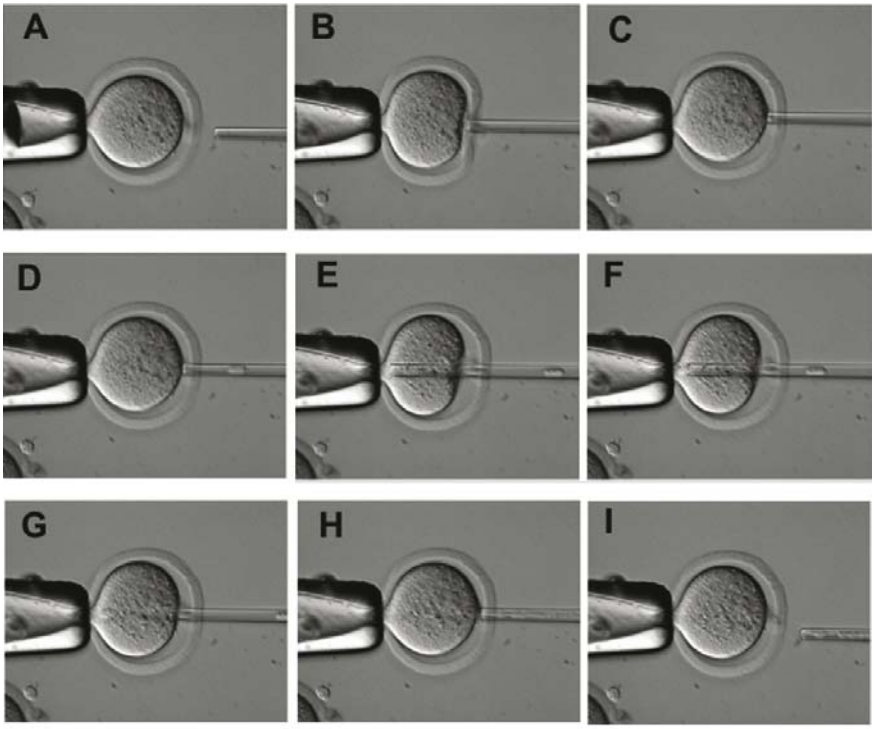

Fig. 4.4. A–J Piezo micromanipulator direct injection nuclear transfer with hole removal method. **A** After cell pickup, the instruments are returned to the culture drop containing enucleated oocytes. The oocyte to be injected should be immobilized with the largest portion of the perivitelline space facing the injection pipette. **B** The blunt injection pipette cannot enter the perivitelline space without use of the piezo. **C** Once the piezo element is applied, the zona can be drilled. Zona drilling will cause a plug of zona to be aspirated into the needle. After drilling, this piece of zona should be expelled into the perivitelline space. **D** Expelling medium into the perivitelline space brings the donor nucleus towards the oocyte. **E** Just before the donor nucleus reaches the end of the pipette, the pipette should be moved forward into the oocyte, forming a channel of ooplasmic membrane around the injection pipette. **F** A brief pulse of the piezo breaks the oolema, creating a small hole, allowing the donor nucleus to be deposited inside the oocyte. Note nucleus just outside of injection pipette, proximal to the opening of the holding pipette. **G** Immediately after nucleus drop off, the needle should be quickly withdrawn just to the opening of the channel created in the membrane. **H** Once at the opening of the channel, suction should be applied. Proper suction will aspirate the membrane into the injection pipette. If this is done correctly, the hole created by microinjection is aspirated into the needle. **I** If the needle is removed carefully and quickly, the fluid ooplasmic membrane with pinch off from the end of the needle, topologically removing the hole from the membrane! **J** After hole removal, the remaining cytoplasm should be expelled from the injection needle

activated under these conditions for 5.5–6 h. It is critical that NT embryos be carefully and thoroughly washed in this medium prior to incubation, to remove the calcium present in KSOM. Failure to wash embryos can result in lysis. We generally culture a maximum of five NT embryos per 20 μl drop of activation medium. Oocyte death during activation, which can lead to Sr^{++} precipitation and further oocyte death, is not uncommon. Keeping a minimal number of oocytes in each drop lowers this risk to any particular NT embryo. After activation, NT embryos are washed through several drops of KSOM or MCZB. Following washing, embryos are cultured in KSOM or MCZB until embryo transfer.

4.3.12 Derivation of Nuclear Transfer ES Cells

The methods we use for derivation of ES cell lines from NT embryos are similar to those previously reported (Hogan et al. 1994) with several modifications (Hochedlinger and Jaenisch 2002a; Eggan et al. 2004). Following oocyte activation, NT embryos are cultured in vitro in KSOM for 3–4 days. Embryos reaching the morula or blastocyst stage after this time are used for ES cell derivation. NT morulae and blastocysts are exposed to acid Tyrode's solution (Specialty Media) to remove the zona pellucida and then transferred into individual wells of a four-well dish pretreated with 0.1% gelatin and seeded with gamma-irradiated primary MEFs. The embryos are cultured in standard ESCM (see above) supplemented with PD98059 (Cell Signaling Tech, www.cellsignal.com). PD98059 is a MEK kinase inhibitor, which, in addition to inhibiting the differentiation of mouse ES cells, increases the efficiency with which ES cell lines are derived.

Once the zona-stripped embryos are placed on MEFs they are left undisturbed for 48 h. After 48 h, the embryo explants must be monitored every day. At 72 and 96 h following explantation, a few drops of ESCM with PD98059 are added to each well of the dish. Once the embryos attach to the dish, one-half of the medium can be removed from the well and replaced with fresh medium every 24 h. Special attention should be paid to not switching the medium too soon as this can result in embryo loss. Attached embryos should be monitored every 24 h to assess outgrowth of the inner cell mass (ICM). In our experience, ICM outgrowth can take much longer for NT embryos than for fertilized embryos, and can be observed anytime between 5 and 15 days following embryo explantation. Actively growing ICMs should be picked with a mouth pipette, rinsed in hepes buffer and dispersed in 0.1% trypsin-EDTA for 10 min. Following physical dispersal of the picked ICM, cells should be plated on fresh MEFs in ESCM with PD98059. Initial ES cell colonies can often be observed 48–

72 h following ICM dispersal. Following derivation, NT ES cell lines are maintained by standard methods.

4.3.13 Embryo Transfer of Cloned Embryos

For production of cloned mice, we have had best success transferring NT embryos at the two-, four- or eight-cell stage to the oviducts of 0.5 days post coitum (dpc) pseudopregnant females. Although it does work in our hands, we have not found transfer of NT morula/blastocyst stage embryos to the uteri of 2.5 dpc pseudopregnant as consistently successful as oviduct transfer (Wakayama et al. 1998). The oviduct transfer method we use is a variation of that described elsewhere (Hogan et al. 1994, Chap. 11 of this manual). For oviduct transfer, mice are anesthetized with the barbiturate Avertin. In the meantime, NT embryos are retrieved from the incubator and moved from microdrop culture under mineral oil, to microdrop culture under air. This transfer prevents oil droplets, which may cling to the transfer pipette, from entering the reproductive tract. To access the oviduct, a small incision is made one inch ($\sim$ 2.5 cm) from the dorsal midline, at the clear depression in the back of the animal. After cutting through the skin, another incision is made in the body cavity, being careful to avoid red blood vessels and white nerves. The ovary and oviduct should be clearly visible (see Hogan et al. 1994, for a diagram of the ovary and oviduct, and Chap. 11 of this manual). After the second incision is made, a pair of forceps can be used to reach into the body cavity and carefully withdraw the ovary and oviduct. Under increased magnification it should be possible to locate the end of the oviduct as it enters the ovarian bursa. Once the end of the oviduct has been identified, immobilize the ovary with a haemostat.

To transfer the embryos, pick up 5–10 μl embryo culture medium in the transfer pipette, followed by two small air bubbles and then 10–15 NT embryos in a similar volume of medium. Holding the transfer pipette and a syringe with a 30.5 gauge needle in the same hand, use the needle to make a small hole in the oviduct wall, just outside the ovarian bursa. Special effort should be made not to rupture blood vessels in the oviduct. After making the hole, drop the syringe, adjust your grip on the transfer pipette and place the tip of the transfer pipette into the hole made by the syringe. Lastly, blow the embryos and the air bubbles into the oviduct. The air bubbles and medium should push the embryos into the swollen ampulla. After transfer, carefully replace the ovary back into the body cavity, close the hole in the body wall with a single suture, and staple the wound closed with two or three autoclips. Repeat on the other oviduct or proceed to another

recipient. Note that we find this transfer method less invasive and more reliable then the oviduct transfer method described elsewhere (Hogan et al. 1994) where the bursa is ripped from the ovary and the embryos introduced via the infundibulum.

4.3.14
Cesarean Section and Cross Fostering of Cloned Animals

We routinely carry out cesarean section in the morning of recipient 19.5 dpc (see Chap. 3, section 3.3.10). After sacrifice of the recipient, full-term pups are quickly but carefully removed from the uterus, the umbilicus is carefully cut from the pups and the placentas arrayed in such a way that their identities can be maintained. After removal from the uterus, neonates are quickly wiped off with a Q-tip or Kim-wipe, paying special attention to airways. Pups are kept under a heat lamp until cross-fostering. Generally we prefer to cross-foster to BALB/c foster mothers that have delivered the previous day. To cross foster, remove the BALB/c litter from the cage with a little bedding. Mix pups to be fostered and bedding thoroughly until pups have taken on the smell of the bedding. We generally leave 6–8 pups per foster mother, i.e., number of NT pups to be fostered + X BALB/c pups = 6–8 in total.

4.4
Protocol for Direct Injection Nuclear Transfer

4.4.1
Production of Embryo Culture Medium, Reagents and Mice

4.4.1.1
Master Salts

- Begin with 980 ml ultrapure H_2O in a sterile 1 l bottle.
- Add dry components:

NaCl	4,760 mg	Sigma S-5886
KCl	360 mg	Sigma P-5405
$MgSO4 \cdot 7H_2O$	290 mg	Sigma M-2773
KH_2PO_4	160 mg	Sigma P-5655
EDTA 2NA	40 mg	Sigma E-6635
Glucose (D)	1,000 mg	Sigma G-6152

- Add liquid components:

Na-lactate (lactic acid)	5.3 ml	Sigma 44263
Pen'Strep (100x TC)	10 ml	Gibco 15140–122

- Two primary media types are made from these master salts: bicarbonate-buffered MCZB medium for long-term culture at 37 °C with 5% CO_2, and HCZB medium for short-term culture and manipulation at room temperature in air. To prepare these media, the master stock is subdivided and supplemented to produce MCZB and HCZB stocks salts.

4.4.1.2
MCZB Stock Salts

- Sterile filter 500 ml master salts into a sterile container.
- MCZB stocks can be used for up to 3–4 months if stored at 4 °C.

4.4.1.3
HCZB Stock Salts

- Start with 500 ml master salts.
- Add 50 mg PVA (cold-soluble; Sigma P-8136).
- Stir for 30–60 min and sterile filter.
- Store for up to 3 months at 4 °C.
- These CZB stocks are used as a base for MCZB and HCZB media, which must be prepared every 2 weeks and should be stored at 4 °C.

4.4.1.4
MCZB For Long-Term Embryo Culture in 5% CO_2

- Start with 99 ml MCZB stock salts.
- Add:

$NaHCO_3$	211 mg	Sigma S-5761
Na-pyruvate (pyruvic acid)	3 mg	Sigma P-4562
L-Glutamine	15 mg	Sigma G-8540
Bovine serum albumin (BSA)	500 mg	Sigma A-3311

- Add liquid:

128 mM $CaCl_2$	1 ml	Sigma C-7902

- Swirl until dissolved, sterile filter. KSOM medium purchased from Specialty Media can be substituted for MCZB.

4.4.1.5
Ca^{++}-Free MCZB For Oocyte Activation in 5% CO_2

- Start with 99 ml MCZB stock salts.
- Add:

$NaHCO_2$	211 mg	Sigma S-5761
Na-pyruvate (pyruvic acid)	3 mg	Sigma P-4562
L-Glutamine	15 mg	Sigma G 8540
BSA	500 mg	Sigma A-3311

- Swirl until dissolved, sterile filter.

4.4.1.6
HCZB (use for micromanipulation in air)

- Start with 99 ml HCZB stock salts.
- Add:

Hepes-Na	520 mg	Sigma H-3784
$NaHCO_3$	42 mg	Sigma S-5761

- Add liquid:

128 mM $CaCl_2$	1 ml	Sigma C-7902

- adjust pH to 7.5 with 1 N HCl.
- Swirl until dissolved, sterile filter.

4.4.1.7
Cytochalasin B (100× stock)

500 μg/ml cytochalasin B (Sigma) in DMSO. Store at −20 °C.

4.4.1.8
Strontium chloride (10× stock)

100 mM mM SrCl in H_2O. Store at room temperature.

4.4.1.9
HCZB with 11% w/v PVP

For nuclear transfer, donor cells are suspended in an HCZB solution containing 11% w/v PVP. We have found that the composition and preparation of this solution is one of the most critical factors in cloning success. HCZB medium (20 ml) should be placed in a 50 ml conical tube or beaker. Place 2.2 g PVP (MW 360,000, ICN Biochemicals, Aurora, OH) on top of the liquid. The conical tube should then be closed or the beaker sealed, placed

at 4 °C and left undisturbed for 72–96 h. After 96 h, the PVP will have entered solution. The HCZB/PVP solution should then be passed through an 8 μm filter and stored at 4 °C.

4.4.1.10 Mice

Mice should be superovulated with PMSG (3 days earlier) and HCG (1 day before NT) but not mated. For instance, PMSG Friday afternoon (∼ 6:00 p.m.), HCG Sunday afternoon (∼ 6:00 p.m.), egg collection Monday morning 8:00–9:00 a.m. (+14–15 h after HCG, +16 h is too late).

4.4.2 Oocyte (Egg) Collection

Prepare Petri dishes 1–5 in advance.

- Petri dish 1:
  - One drop of HCZB with hyaluronidase (HCZB is based on HEPES buffer and does not require CO_2 for pH-equilibration).
  - Several (four) drops of HCZB (to wash eggs after hyaluronidase).
  - Cover with oil.
- Petri dish 2:
  - Many small drops of KSOM (20 μl) to wash hyaluronidase/HCZB from oocytes. One or two large (100 μl) drops of KSOM in which to keep washed oocytes.
  - Many small drops of KSOM to keep enucleated oocytes.
  - Cover with oil, place at 37 °C.
- Petri dish 3 (enucleation and nuclear transfer dish):
  - For this chamber use the lid of a Petri dish.
  - Draw four quadrants on the outside of the lid (Fig. 4.2).
  - Mix 10 μl cytocholasin B (100× stock in DMSO) with 990 μl HCZB in a microcentrifuge tube; this is enucleation and microinjection medium.
  - Make many small drops of enucleation medium in three out of the four quadrants.

  - In the remaining quadrant, make several small drops of HCB-PVP medium (prevents needles from becoming clogged, also to use for isolation of nuclei).
  - Cover with oil, place on the micromanipulation stage.
- Petri dish 4 (culture dish for oocyte activation):
  - Prepare activation medium:

10 µl	Cytocholasin B (1:100 dilution, use once)
100 µl	100 mM Sr^{2+} (10× stock)
890 µl	Ca^{2+}-free MCZB
1,000 µl	Total volume

  - Place two large drops activation of medium on the dish. These will be used to wash KSOM from reconstituted eggs before activation.
  - Use many small drops of activation medium for NT embryo culture at activation stage. Culture 5–10 reconstituted embryos per drop.
  - Several drops of KSOM-to wash eggs after activation (to free from cytocholasin B, Sr^{2+} etc.).
- Petri dish 5 (long term culture):
  - Make several drops of KSOM or MCZB medium for long-term culture.
  - Cover with oil, place at 37 °C.

4.4.2.1 Isolate ovaries and oocytes

1. Sacrifice female oocyte donors by accepted method and dissect out oviducts.
2. Put oviducts in oil (dish 1), tear ampula(e), eggs spill into oil (oocytes surrounded by cumulus cells).
3. Simultaneously, push cumulus/oocyte complexes into hyaluronidase.
4. Let oocytes rest at room temperature 2–5 min (maximum 10 min) until cumulus cells start to fall away.
5. Wash oocytes through HCZB (3–4 times) to remove hyaluronidase.
6. Move oocytes to dish 2 and wash through two KSOM drops to remove HCZB.
7. Place dish 2 back in 37 °C incubator.

4.4.3 Enucleation

Note: Before starting, make sure the micromanipulator lines are filled with H_2O. Air bubbles in the micromanipulation lines will prevent the manipulators from responding properly.

4.4.3.1 Setup

1. Fill piezo pipette needle with mercury:
   - Pipette 3–5 μl mercury into an insulin syringe fitted with a piece of silicon tubing in place of the needle.
   - Use the syringe to backfill the enucleation pipette with mercury. The mercury should be advanced nearly to the tip of the enucleation needle.
2. Attach the needle to the right-hand micromanipulator:
   - Position the instrument so that it can be lowered into the drop of HCZB-PVP medium.
   - Carefully lower the enucleation pipette into the PVP medium.
   - See Fig. 4.2.
   - Using the microinjector syringe, expel microscopic drops of mercury from the small tip of the enucleation needle and then aspirate in PVP.
   - Pipette the mercury up and down in the enucleation pipette (this will clean the pipette and coat the needle with PVP, preventing stickiness on its inner and outer surfaces.
   - After washing the pipette with PVP, rinse the outside of the needle through a drop of injection/enucleation medium while retaining PVP medium inside the pipette.
   - PVP is toxic to the oocytes in large quantities and smaller amounts of PVP may affect egg viability. Therefore, avoid moving PVP on the instruments directly into drops of enucleation/injection medium containing oocytes.
   - Once the enucleation pipette is in place, the holding pipette can be similarly brought into position. Never expose the holding pipette to the medium containing PVP. Its large surface area is difficult to rinse

and can introduce large amounts of the chemical into microdrops that contain oocytes.

- Turn on the piezo drill and set the instrument at a moderate power for "zona drilling" (speed 3–5, intensity 3–5).

4.4.3.2 Begin enucleation

1. For beginners, retrieve 5–15 eggs from dish 2 and place them on the microscope stage in a drop of enucleation medium on dish 3.
2. Let eggs rest for 5–7 min in enucleation medium before beginning micromanipulation.
3. Cytocholasin B disrupts the actin cytoskeleton within the egg, allowing the ooplasm and membrane to move more fluidly. This will allow enucleation without lysis of the oocyte.
4. Do not keep oocytes on the stage for more than 20–25 minn. Oocytes should be manipulated and returned to the incubator within this timeframe.
5. Under a 20× or 40× objective, identify the metaphase spindle of the oocyte.
6. See Fig. 4.3 for photographic depiction of enucleation.
7. Rotate the egg with the injection pipette until you see the differently refractive metaphase spindle. Depending on the orientation of the egg, the spindle will appear either as a circular structure or as a blunt rod.
8. Position the oocyte on the holding pipette so that the spindle and the zona are in the same focal plane, with the spindle positioned close to the enucleation pipette.
9. "Drill" through the zona pellucida using the piezo micromanipulator.
10. Ensure there is sufficient gap between the zona and egg membrane, otherwise the pipette may lyse the egg.
11. Aspirate the metaphase plate into the pipette without taking much of the cytoplasm. A good rule of thumb here is to not begin aspirating until you can see that the pipette can move the spindle.
12. Once 60–70% of the spindle is in the pipette, slowly pull the needle away from the oocyte. Rapid movement or aspiration of the spindle into the needle too quickly can cause oocyte lysis.

13. As the pipette is pulled away from the oocyte, the fluid membrane will close behind it. The membrane is never compromised.
14. The spindle is more rigid than the rest of the ooplasm. One guideline for whether the spindle has been successfully removed is to expel the karyoplast from the enucleation pipette. When the spindle karyoplast is expelled from the needle it will be more rigid then cytoplasm alone and will often take on a characteristic "bone" shape.
15. Use the smallest enucleation needle possible, thus reducing the amount of cytoplasm taken up with the karyoplast during enucleation.
16. When finished with all oocytes, or after 20–25 min, move enucleated oocytes back into KSOM in Petri dish 2.
17. Wash enucleated eggs through three consecutive drops of KSOM.
18. Place enucleated oocytes in a fourth drop of KSOM.
19. Repeat enucleation in batches of oocytes that can be completed within these time constraints, keeping un-enucleated and enucleated oocytes in dish 2 at 37 °C.
20. Complete enucleation after no longer than 2 h.

4.4.4 Donor Nucleus Isolation

1. Remove enucleation needle.
2. Fill injection/NT needle with mercury as for enucleation pipette.
3. Attach to manipulator and lower into PVP as described for the enucleation needle.
4. Using a mouth-controlled pipette or a P20 Pipetteman (Gilson), place a few thousand donor cells into a clean drop of PVP medium.
5. Leave the remaining donor cells on ice.
6. Mix cells thoroughly with PVP. Cells may begin to die after an hour and a new drop of donor cells should frequently be made, particularly if the cells become sticky.
7. Place a group of oocytes into a drop of enucleation medium (again a number of oocytes that can be manipulated in less the 25 min).
8. Observe donor cells under 40× objective and aspirate donor cells into injection pipette. As the cells are picked up into the pipette, they should

be aspirated back and forth several times through the opening of the needle.

9. The cell should be distorted by the pipette when it is picked up. If the pipette is too large the cell will not be broken, if it is too small, the nucleus will be damaged.
10. Collect no more than 5–10 cells at a time.

4.4.5 Nuclear Transfer

1. Once the cells (nuclei) have been collected, move the injection pipette to the drop of medium containing the oocytes.
2. See Fig. 4.3.9 for photographic depiction of NT.
3. Immobilize the first oocyte to be injected on the holding pipette.
4. Drill through zona.
5. Expel zona "plug" into space between zona and oocyte.
6. Bring the injection pipette almost to the opposite side of the enucleated oocyte, close to the holding pipette, again making a deep furrow in the egg (like pushing one's finger into a balloon).
7. Engage a single pulse of the piezo to break the oocyte membrane, suck in a very small amount of egg cytoplasm and then immediately eject it together with the donor nucleus.
8. Leave as little PVP behind as possible.
9. Rapidly withdraw the needle from the oocyte while aspirating at the cytoplasmic membrane at the right end of the furrow.
10. By simultaneously withdrawing the needle and aspirating, it should be possible to close the hole left behind by NT. This "hole removal" technique will greatly reduce the number of oocytes that lyse during NT.
11. Each batch of enucleated oocytes should be reconstructed within 30 min or less and then returned back to KSOM at 37 °C.
12. When returning the NT embryos to the incubator, wash them three times through KSOM, and place into a fresh drop of KSOM.
13. If the donor nucleus is exposed to normal enucleation medium it will becomes sticky immediately and should be discarded.
14. 90–95% of reconstructed eggs can survive this procedure.

4.4.6
Oocyte Activation and Long-Term Culture

1. Allow the reconstructed eggs to rest in KSOM, 37 °C, for 1–3 h.
2. Pick up NT embryos in batches and transfer them to activation medium in Petri dish 4.
3. Wash NT embryos through three drops of activation medium to remove residual KSOM.
4. Place small groups of embryos (5–10) into the small drops of activation medium.
5. Return dish to incubator and culture for 5–6 h at 37 °C.
6. Following activation, wash the embryos through KSOM medium 6–7 times to remove cytochalasin B and place embryos in KSOM (Petri dish 5).
7. Culture at 37 °C until the two-cell stage for oviduct transfer or the blastocyst stage for ES cell derivation and uterine transfer.

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