Somatic-cell nuclear transfer (SCNT)

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Abstract: All animal cells come from stem cells. Somatic-cell nuclear transfer (SCNT) is a technique to create a viable embryo from the combining of a somatic cell and an egg cell. The technique is enucleate oocyte and implant a nucleus from a somatic cell into the enucleated oocyte. Dolly Sheep was the first successfully cloned mammal animal created by using SCNT technique. SCNT is the first step in the reproductive cloning practice.
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1. Introduction

Somatic-cell nuclear transfer (SCNT) is a technique to create a viable embryo from the combining of a somatic cell and an egg cell. The technique is enucleate oocyte and implant a nucleus from a somatic cell into the enucleated oocyte. Dolly Sheep was the first successfully cloned mammal animal created by using SCNT technique. SCNT is the first step in the reproductive cloning practice.

As the descibe above, the SCNT involves two different cells. The first is a female gamete (ovum, egg, or called oocyte), normally utilizing ovarian stimulation to get it. The second is a somatic cell from the human body that can be either of male a female. The nucleus of the donor egg cell is removed and discarded. The nucleus of the somatic cell is removed can used for the purpose of translanting into the enucleated oocyte. These 2 cells are fused by squirting the somatic nucleus into the enucleated oocyte. Now, the oocyte containing the somatic cell nucleus is stimulated with a shock and start to divide, and the egg is viable and able to grow as an adult organism containing whose genetic information is from the somatic cell neucleus. After inserted into the oocyte, the somatic cell nucleus is reprogrammed in the oocyte cell. After many mitotic divisions, it forms a blastocyst.

Stem cells can be obtained by the destruction of this clone embryo for use in therapeutic purpose. By this technique, it can obtain pluripotent cells from a cloned embryo. These cells genetically matched the neucleus donor and it can get the nucleus donor's specific pluripotent cells that could be used in therapies and researches.

Embryonic stem cells are undifferentiated stem cells that have the pluripotent potential because they have the ability to create all of the tissues in an adult organism. This ability allows stem cells to create any cell type, which could then be transplanted to replace damaged or destroyed cells.

Recently 2 groups have independently produced human embryonic stem-cell lines from embryos cloned from adult cells.

On April 17 of 2014, Young Gie Chung and Dong Ryul Lee et al at the CHA University in Seoul reported that they had cloned embryonic stem-cell (ES cell) lines made using nuclei from two healthy men, aged 35 and 75 in the *Cell Stem Cell* Journal. On April 17 of 2014 *Nature*'s website Dieter Egli at the New York Stem Cell Foundation Research Institute reported the experiment getting ES cells from a cloned embryo that contains the DNA from a 32-year-old woman with type 1 diabetes. The researchers also succeeded in differentiating these ES cells into insulin-producing cells.

To produce the cloned embryos, the nucleus from an animal's cell is placed into an unfertilized animal's egg whoese nucleus is removed before the nucleus tansfer. This reprograms the cell into an embryonic state. SCNT is the technique used to create the first mammal cloned from an adult cell in 1996 for the sheep Dolly.

Compared with iPS cells, ES cells are more useful. As the nucleus of the cloned embyo is from the pateint, the stem cells made from cloned adult cells are genetically matched to the patient and so are less chance of being rejected when transplanted.

There is ethically problem for the SCNT. It creates an embryo only for the purpose of harvesting stem cells and the embryo is destoyed in the process. Obtaining human eggs performs an invasive procedure on women who could be discomfort and possible certain feeling on the destoyed embryos. Another choice could be futher studied and choosed is the use an enimal's egg and patient's adult cell's nucleus.

iPS cell lines work well for drug screening and basic research, but it is unclear whether iPS cells or ES cells will work better in clincal therapies.

Another problem is that SCNT practice could be used to clone a human baby which is against by the

human minority.

The stem cells genetically from a patient have the same genetic background as the patient cells, which have no reject problem for the transplantation. If a patient donates his (or her) somatic cells, the stem cells resulting from the SCNT will have genes that contribute to his (or her) disease and has no immunologic rejection. Another application of SCNT stem cells is to use stem cells created by the SCNT to produce tissues or even organs for transplant into the specific patient. The resulting cells will be genetically identical to the somatic-cell donor and avoide the complications from immunologic rejection.

In 2005, South Korean sicentist Hwang Woo-suk claimed that his group had derived stem cell lines via SCNT, but is was reported that Hwang's report was with fabricated data. However, it has been proved that Hwang's group in fact realy created a stem cell line from a parthenote.

There is a problem in the human cells to progress past the eight cell stage of development and form a blastocyst. There is a technique problem to turn the somatic cell nucleus gene to the embryonic genes for proper development.

In April 2014, an Oregon research group were able to grow stem cells generated by SCNT using adult cells from two donors, aged 35 and 75. Late April 2014, the New York Stem Cell Foundation was successful in creating SCNT stem cells derived from adult somatic cells.

Anohter transfer is a father providing a sperm cell, a mother providing the egg nucleus and another mother providing the enucleated egg cell.

Interspecies nuclear transfer is to transfer the somatic cell nuclear of one species to the oocyte of another species. It is easier to get the successful is that the interspecies nuclear transfer utilizes a host and a donor of two different organisms that are closely related species (such as within the same genus). In 2000, Robert Lanza was able to produce a cloned fetus of a gaur, *Bos gaurus*, combining it successfully with a domestic cow, *Bos taurus*. Interspecies nuclear transfer can be used to facilitate the rescue of endangered species, or even to restore species after their extinction. Interspecies nuclear transfer shows the universality of the triggering mechanism of the cell nucleus reprogramming in the life world. NCSU23 medium, which is designed for in vitro culture of pig embryos, is able to support the in vitro development of cattle, mice, and chicken iSCNT embryos up to the blastocyst stage. Ovine oocyte cytoplast may be used for remodeling and reprogramming of human somatic cells back to the embryonic stage.

The sheep Dolly was born after 277 eggs were used for SCNT, which created 29 viable embryos. Only 3 embryos survived until birth, and 1 survived to adulthood (Dolly). The donor cell's mitochondria containing their own mitochondrial DNA are left after the SCNT. The hybrid cells retain these mitochondrial genes which belonged to the egg. As a consequence, clones are born from SCNT are not perfect copies of the donor's nucleus genes. There may be a immunoresponse to the non-self mtDNA after the transplant.

SCNT is a laboratory technique for creating an ovum with a donor nucleus. Briefly, in SCNT the nucleus of a somatic cell is removed and kept. At the same time, the nucleus of an egg cell is removed. The nucleus of the somatic cell is then inserted into the enucleated egg cell. After being inserted into the egg, the somatic cell nucleus is reprogrammed by the enucleated egg cell. The egg, now containing the nucleus of a somatic cell, is stimulated with a shock and will begin to divide. After many mitotic divisions in culture, this single cell forms a blastocyst (an early stage embryo with about 100 cells) with almost identical DNA to the somatic cell from the original organism. The following is an example for a SCNT protocol (Shoukhrat M. Mitalipov and Don P. Wolf., 2006; ScienceDaily, 2014**).**

2. Materials

1). Recombinant human follicle-stimulating hormone, luteinizing hormone (LH) and chorionic.
2). Antide.
3). Ketamine.
4). TH3 medium (1 L):
NaCl 6.660 g
KCl 0.239 g
$CaCl_2$-$2H_2O$ 0.294 g
$MgCl_2$-$6H_2O$ 0.102 g
Na_2HPO_4 0.048 g
Glucose 0.900 g
Na lactate 1.870 mL
Phenol red 0.010 g
$NaHCO_3$ 0.168 g
Gentamicin sulfate 0.050 g
HEPES 2.603 g
Na pyruvate 0.060 g
pH 7.2–7.4
Osmolarity 282
BSA 3 mg/mL before use or refilter.
Filter the medium using a 0.2-um filter and store for up to 1 month at 4°C.
5). Hamster embryo culture medium (HECM)-9 (1 L):
Polyvinyl alcohol (PVA) 0.100 g
NaCl 6.639 g
KCl 0.224 g
$CaCl_2.2H_2O$ 0.279 g
$MgCl_2.6H_2O$ 0.102
$NaHCO_3$ 2.1 g

Lactic acid, Na salt, 60% syrup 632 ⌈L
Gentamicin sulfate 0.010 g
pH 7.2–7.4
Osmolarity 277
Filter the medium using a 0.2-um filter and store for up to 1 week at 4°C.

6). 100X Amino acid–pantothenate stock (1 L):
Taurine 6.260 g
Asparagine 0.130 g
Cysteine 0.18 g
Histidine 0.21 g
Lysine 0.18 g
Proline 0.12 g
Serine 0.11 g
Aspartic acid 0.13 g
Glycine 0.08 g
Glutamic acid 0.17 g
Glutamine 2.92 g
Pantothenic acid 0.07 g
Filter the medium using a 0.2-um filter and store for up to 3 months at -20°C.

7). HECM-9aa medium: Add amino acid–pantothenate stock to HECM-9 base medium at a ratio of 1:100 before use. HECM-9aa is used to hold oocytes from the time of recovery until IVF, intracytoplasmic sperm injection (ICSI), or NT, as well as to culture embryos until the 4- to 8-cell stage. For extended culture (to the blastocyst stage), embryos are transferred at the 4- to 8-cell stage to HECM-9aa medium supplemented with 5% fetal bovine serum (FBS; HyClone, v/v). Embryos are transferred to fresh HECM-9aa + 5% FBS every other day.

8). D-Sorbitol fusion medium (1 L):
D-Sorbitol 46.378 g
Ca acetate 0.0158 g
Mg acetate 0.107 g
HEPES 0.119 g
Prepare fatty acid-free BSA 3 mg/mL before use and refilter
(Filter by 0.2-um filter, store for up to 1 month at 4°C).

9). Activation stocks: Ionomycin, Ca-salt: for 5 m*M* (1000X) stock, reconstitute 1 mg ionomycin in 267 uL of dimethyl sulfoxide, aliquot and store at –20°C.

10). TH1 medium: HEPES-buffered TALP medium, containing 1 mg/mL BSA.

11). TH30 medium: HEPES-buffered TALP medium, containing 30 mg/mL BSA 6-dimethylaminopurine (DMAP; Sigma D-2629) stock: for 200 m*M* (100X) stock reconstitute 1 g of DMAP in 30 mL Ca^{2+} and Mg^{2+} free Dulbecco's phosphatebuffered saline (DPBS) by heating the tube in near boiling water bath until all DMAP is dissolved. Separate into 0.5-mL aliquots and store at –20°C.

12). Hyaluronidase (Sigma H-3506, IN, USA) stock: for 10X stock reconstitute 50 mg in 10 mL of HEPES-buffered TALP medium. Separate into 0.5-mL aliquots and store at –20°C.

13). Polyvinylpyrrolidone (PVP; Irvine Scientific; Santa Ana, CA, USA): Reconstitute with 1 mL of HEPES-buffered TALP medium before use.

14). Ca^{2+} and Mg^{2+} free DPBS (Invitrogen, Carlsbad, CA).

15). Cytochalasin B (Sigma C-6762, 1 mg) stock: to prepare 5 mg/mL (1000X) stock, reconstitute 1 mg of cytochalasin B in 200 uL of dimethyl sulfoxide (Sigma). Aliquot at 5 uL per vial and store at –20°C.

16). Hoechst 33342 (Sigma B-2261): for 500X stock, reconstitute 1 mg in 1 mL of saline. Filter and aliquot 10 uL per vial. Store at –20°C; avoid light exposure.

17). Light paraffin oil (Zander IVF, Vero Beach, FL, USA).

18). High-viscosity silicon oil DC 200, 375 mPa (Fluka; Sigma-Aldrich, IN, USA).

19). Pronase (Protease, Sigma P-8811): to prepare 0.5% stock (1X), reconstitute. 100 mg of protease in 20 mL of HEPES-buffered TALP medium (no BSA), filter sterilize, aliquot 1 mL and store at –20°C.

20). Micropipets (Humagen, Charlottesville, VA, USA).

21). Cell strainers (70-um Nylon; Falcon; BD Biosciences, Bedford, MA, USA).

22). Patton Laparoscopic Catheter Introducer Set (Cook OB/GYN, Spencer, IN, USA).

23). Portable incubator (Minitube, Verona, WI, USA).

24). Ultrasonography equipment (Philips, USA).

25). Dissecting microscope (SZ-61, Olympus America, USA).

26). Restraint chair (Primate Products, Miami, FL).

27). Electrolyte cream (Reflux Creme, Hewlett Packard, Waltham, MA, USA).

28). S5 Square Pulse Physiological Stimulator (Grass Instruments, Quincy, MA, USA).

29). CCU 1 Constant Current Unit (Grass Instruments, USA).

30). Disposable electrodes made by folding 6-7 cm piece of ordinary light-weight aluminum foil 6 times length.

31). Inverted fluorescent microscope (IX-70 Olympus America)

32). Micromanipulators (Joystick Oil Hydraulic MO-202D and Coarse MMN-1, Narishige International).

33). Inverted and dissecting microscope heating stages (ThermoPlate, TOKAI HIT CO. Japan) to provide temperature control at 37°C during oocyte and embryo manipulations.

34). Cell fusion equipment (BTX Electro Square Porator T820, BTX Instrument Division Harvard Apparatus, Holliston, MA, USA).

35). Teflon® tubing (Inner diameter O.D. 0.9 mm, Outer diameter O.D. 2 mm, Narishige International).
36). Patton polyurethane transfer cannula (Cook OB/GYN).

3. Methods

3.1. Controlled Ovarian Stimulation

Protocols for controlled ovarian stimulation (COS) in rhesus monkeys with recombinant human gonadotropins:
1). Monitor cycling females for menstruation and 1–4 days after onset, administer 2 times per day intramuscular injections of 30 IU recombinant human follicle-stimulating hormone for 8 days.
2). Administer Antide at a dose of 0.5 mg/kg, subcutaneously once a day for 8 days to suppress pituitary function and prevent spontaneous LH surges.
3). On the last 2 days of stimulation (days 7 and 8), additionally administer 2 times per day injections of recombinant human LH (30 IU intramuscularly [IM]).
4). On day 8, anesthetize animals with ketamine (10 mg/kg body weight, IM) and examine ovarian morphology by ultrasonography. Typically, a responsive ovary will be enlarged from 6 mm to an average diameter of 10 mm or greater and will contain at least five large follicles of 2–4 mm in diameter.
5). On the day 9, inject monkeys meeting these criteria with recombinant human CG (hCG; 1000 IU IM) to induce oocyte maturation. Ovarian oocytes, which arrest at prophase I (germinal vesicle), resume meiosis in response to hCG and arrest again at metaphase II (MII). Approximately 20% of gonadotropin-treated female monkeys are discontinued at this time because of a lack of adequate response as judged by ultrasonography.

3.2. Laparoscopic Oocyte Recovery

Oocytes are collected by laporascopic follicular aspiration 27–33 hours after hCG injection via transabdominal needle aspiration of gravid ovarian follicles. Laparoscopy plays a prominent role in the IVF laboratory, with most surgical procedures accomplished by this methodology.
1). Anesthetize monkeys with isoflurane gas vaporized in 100% oxygen. Comprehensive physiological monitoring of animals should be conducted throughout the surgery, including electrocardiography, peripheral oxygen saturation, and end-expired carbon dioxide. Orotracheal intubation and mechanical ventilation to maintain expired CO_2 at less than 50 mmHg is mandatory.
2). Perform sterile skin preparation and draping after which the abdomen is insufflated with CO_2 at 15 mmHg pressure. Insert the viewing telescope via a small supraumbilical incision, with accessory ports placed in the paralumbar region.
3). Position the monkey in Trendeleburg, allowing the viscera to migrate in a cephalad direction exposing the reproductive organs.
4). Use a single small grasping forceps to stabilize the ovary for examination and needle aspiration.
5). After immobilization of the ovary, connect a 22-gage hypodermic needle to a source of continuous vacuum, and insert into individual follicles until all have been aspirated.
6). Reduce insufflation and close the incisions with interrupted absorbable suture in an intradermal pattern.
7). Place tubes containing follicular aspirates into a portable incubator at 37°C and transport quickly to the laboratory.
8). Add 10X hyaluronidase stock solution directly to the tubes containing aspirates at 1:10 ratio and incubate at 37°C for 30 second.
9). Gently agitate the contents with a serological pipet to disaggregate cumulus and granulosa masses and pour the entire aspirate onto a cell strainer.
10). Oocytes are retained in the mesh, while blood, cumulus, and granulosa cells are sifted through the filter.
11). Quickly backwash the strainer with TH3 medium and collect the medium containing oocytes in a Petri dish.
12). Rinse oocytes, which are now easily identified in TH3 medium.
13). Any remaining cumulus cells can be removed by manual clean up with a smallbore pipet (125 um in inner diameter).
14). Oocytes can be observed at higher magnification for determination of their developmental stage (germinal vesicle, MI, or MII). On average, 40 oocytes are collected per stimulation, with more than 70% matured or maturing (MII and MI stages).
15). After evaluation, transfer oocytes into chemically defined, protein-free HECM-9aa medium at 37°C in 5% CO_2, until further use. Most MI stage oocytes should mature to the MII stage within 3 to 4 h.

3.3 Collection of Spermatozoa

Penile electroejaculation provides a consisted, successful, and humane method for the collection of semen in the rhesus monkey. Pregnancyproven male monkeys assigned to electroejaculation must be evaluated on the basis of ease of restraint, number of attempts required to obtain a sample, and the animals' tolerance of the procedure.
1). Transfer animal to the restraining chair and secure by tying arms and legs with leather straps to the chair. The belly band restrainer can be useful on new animals to lessen animal movements.

2. Apply electrolyte cream to the entire shaft of the penis with the exception of the glans. Wrap one electrode around the base of the penis with the excess length folded to create a tab to which the negative stimulator lead is connected. Position the second electrode immediately behind the glans and connect to the positive stimulator lead.
3). With the electrodes attached, gently grasp the penis between the index and second finger, extend slightly, and position over a sterile 10-mL glass beaker.
4). Set the CCU 1 Constant Current unit output switch to NORMAL and the Current Adjustment dial to 0. At these settings, the animal receives approx 1 mA of current. This low current prepares the animal for the procedure, in a process called priming.
5). Adjust the S5 Square Pulse Stimulators Frequency setting to 17 pulses/s and a Duration setting of 17 ms with multiply switches on both setting at ·1. Set the maximum volt levels with multiply switch at·10.
6). Increase the Current Adjustment switch on CCU 1 gradually from 0 to a setting of 4 to 4.5 until collecting the sample. Never go beyond a setting of 5.
7). Continue to stimulate the animal until a sample is obtained, but never go beyond 20 s.
8). Turn off the Constant Current output by moving Output Adj I on CCU 1 Unit to the off position after obtaining a sample, or after a total stimulus time of 30–35 s (if priming time is added) per trial or less.
9). Allow the ejaculate to liquefy at room temperature for approx 15 min before processing.

3.4. Fertilization by ICSI and Embryo Culture

ICSI is a robust efficient fertilization procedure in the monkey resulting in high pronuclear formation rates (80–90%).
1). Wash collected spermatozoa twice by resuspending with TH3 medium followed by centrifugation of the liquid portion of the ejaculate for 7 min at 200 *g*.
2). Take an aliquot and determine motility and concentration before the final centrifugation and resuspension step.
3). Adjust sperm concentration to 1 · 106 motile spermatozoa per milliliter in TH3 medium and store for approx 3 h at room temperature before ICSI.
4). The ICSI procedure is conducted on an inverted microscope equipped with Hoffman optics, heating stage (set at 37°C), and micromanipulators.
5). Immobilize an oocyte using a holding pipet attached to a micropipet holder (Narishige) and controlled by air-filled Teflon tubing connected to a 20-mL plastic syringe (Becton Dickinson).
6). Fill approx half the holding micropipet with TH3 medium before the micromanipulation procedure.
7). Fill the ICSI micropipet completely with light paraffin oil and then attach it to a water filled Narishige pipet holder and Teflon tubing that extends to a 200-uL volume Hamilton microsyringe controlled by a microinjector (Narishige). The line, microsyringe, and ICSI micropipet must be completely free of air bubbles.
8). After setting up and positioning the micropipets, dilute a small aliquot of sperm with 10% PVP (1:4) and place a 5-uL drop in a micromanipulation chamber; usually the lid of a Falcon 1006 Petri dish.
9). Place a 30 uL drop of TH3 in the same micromanipulation chamber next to the sperm droplet and ensure both are covered with paraffin oil.
10). Place the oocytes into the micromanipulation drop and mount the chamber on the stage of the microscope.
11). Lower the ICSI pipet into the sperm drop and select a motile sperm, which is immobilized by striking the midpiece with the tip of the pipet, and slowly aspirated into the pipet tail first.
12). Move the injection pipet to the manipulation drop containing oocytes.
13). Lower the holding pipet into the manipulation drop and immobilize an individual oocyte with the polar body positioned.
14). Slightly lower the holding pipet with oocyte attached until it touches the bottom of the plate to stabilize the egg during injection.
15). Bring the ICSI pipet into sharp focus at the 3-o'clock position and slowly push the sperm to the pipet tip using the Hamilton microsyringe.
16). Insert the ICSI pipet through the zona pellucida and inject the sperm into the cytoplasm of the oocyte, away from the polar body, making sure that the pipet completely breaks through the plasma membrane and that the sperm is deposited with a minimal amount of medium.
17). After ICSI, place injected oocytes in four-well dishes containing pre-equilibrated HECM-9aa medium and culture at 37°C in 5% CO_2, 5% O_2, and 90% N_2. Maintain cultures under paraffin oil throughout the culture period.
18). Assess fertilization 12–14 h after injection by the presence of pronuclei.
19). At the eight-cell stage, transfer embryos to fresh dishes of HECM-9aa medium supplemented with 5% FBS and culture for a maximum of 7 d with observation/scoring and medium change every other day.

3.5. Enucleation

Enucleation procedures for rhesus monkey oocytes are similar to those described in the bovine.
1). Incubate MII oocytes for 5 min in Hoechst 33342 (5 ug/mL) for spindle visualization before enucleation.
2). Rinse oocytes two to three times in fresh drops of TH3 medium and place into a 30 uL of TH3

manipulation drop containing cytochalasin B (5 ug/mL) covered with paraffin oil.
3). The micromanipulation tool set up is similar to that described for the ICSI procedure except that a larger, beveled, enucleation pipet (25- to 27-um outer diameter) is used. Completely fill the enucleation pipet with high-viscosity silicon oil to improve control over aspiration and injection.
4). Immobilize an individual oocyte using the holding pipet with the first polar body positioned and lower the holding pipet with attached oocyte slightly until it touches the bottom of the plate to stabilize the egg during enucleation.
5). Bring the enucleation pipet into sharp focus at the 3-o'clock position of the oocyte with its beveled bore opening positioned toward the polar body.
6). Slowly insert the pipet through the zona pellucida without piercing the plasma membrane.
7). Once the zona is penetrated, withdraw the pipet slightly and slowly aspirate the first polar body and approx 15% of the underlying cytoplasm into the enucleation pipet.
8). Confirm the presence of the metaphase spindle in the pipet by a short (less than 10 s) exposure to an ultraviolet 2A fluorescent light.
9). If the spindle is still in the egg, navigate the enucleation pipet to the spindle under epifluorescence, ensuring that the tip of pipet and the spindle are brought to the same focal plane.
10). After aspirating the spindle into the enucleation pipet, turn the ultraviolet light source off and withdraw the pipet slowly from the slit in the zona pellucida.
11). Place enucleated oocytes in pre-equilibrated, HECM-9aa medium and culture at 37°C in 5% CO_2, 5% O_2, and 90% N_2 for 30–60 min for recovery before further manipulation.

3.6. Activation

Cytoplast activation is an essential component of the NT procedure since the introduction of the donor nucleus into the cytoplast bypasses fertilization and sperm-induced egg activation. Developmental rates to the blastocyst stage for embryonic cell NT embryos are higher when unsynchronized blastomeres are fused with pre-activated cytoplasts.
1). Add 1 uL of ionomycin stock to1 mL of prewarmed TH1 medium and mix thoroughly.
2). Place cytoplasts in ionomycin-containing TH1 medium for 2 min and then wash for 5 min in TH30.
3). Add DMAP stock to pre-equilibrated HECM-9aa medium at 10 uL/mL.
4). Transfer activated cytoplasts into DMAP medium and incubate at 37°C in 5% CO_2, 5% O_2, and 90% N_2 for 4 h.
5). After treatment with DMAP, rinse cytoplasts extensively (four to five times) in TH3 becaues residual DMAP may cause developmental arrest.
6). Alternatively, DMAP treatment can be replaced with either 50 u*M* roscovitine or 7.5 ug/mL cycloheximide combined with 5 ug/mL cytochalasin B.

3.7. Donor Cell Transfer

Blastomeres of 8- to 16-cell stage, ICSI-produced embryos are used as the source of donor nuclei.
1). Place embryos briefly (30–60 s) in 0.5% pronase to remove zonae pellucidae with constant monitoring under a dissecting scope.
2). Before the zonae disappear completely, move embryos quickly to TH3 medium and wash three times by gentle pipetting with movement from one drop of medium to another. During these washes, zonae will break up and completely disappear.
3). Incubate zona-free embryos in 0.5 m*M* EDTA in Ca^{2+}- and Mg^{2+}-free DPBS for 5–10 min to loosen cell-to-cell contacts.
4). Transfer donor embryo and recipient cytoplasts to micromanipulation medium containing 5 ug/mL cytochalasin B and incubate for 10–15 min before enucleation.
5). Mechanically disaggregate and then aspirate a single donor blastomere using a beveled transfer pipet (33- to 35-um outer diameter).
6). Immobilize individual cytoplasts with a holding pipet so that the hole in the zona made previously by the enucleation pipet is positioned.
7). Insert the transfer pipet through the hole in the zona and eject the donor cell into the perivitelline space.
8). Once all transfers are completed, wash NT couplets three times in TH3 and culture in HECM-9aa medium at 37°C in 5% CO_2, 5% O_2, and 90% N_2 medium until fusion.

3.8. Couplet Fusion

Fusion of NT couplets is induced by two 50-us direct current pulses of 2.7 kV/cm in D-sorbitol fusion buffer. The fusion chamber consists of two electrodes 0.5-mm apart placed into a 10-cm Petri dish taped to the stage of the dissecting microscope.
1). Set the BTX Electro Square Porator T820 controls as follows:

Mode—(HV) 99 ⌈s/3.0 kV
Pulse length—50
Voltage dial—0.13
Number of pulses—2

2). Pour prewarmed (37°C) fusion medium into the dish until electrodes are covered.

3). Transfer three to five NT couplets at a time first to the four-well dish with TH3 medium and then to the well containing equal volumes of TH3 and fusion medium.
4). Allow the couplets to settle to the bottom and transfer them to the well containing pure fusion medium.
5). Position couplets between electrodes of the fusion chamber and align them so that the donor cell is positioned toward the black (negative) electrode.
6). Initiate a fusion pulse and after a 20-s pause, transfer couplets back to TH3 medium in a reverse stepwise fashion.
7). Once all NT couples are electroporated, transfer to and culture in HECM-9 medium.
8). Evaluate fusion microscopically, 30–45 min after electroporation by noting the presence or absence of donor blastomeres in the perivitelline space.
9). Refuse unfused couplets as described previously.

3.9. Embryo Transfer

Adult, multiparous female monkeys monitored for menses are used as recipients. Daily blood samples are collected beginning on day 8 of the menstrual cycle and serum levels of estradiol are quantitated by radioimmunoassay. The day after the peak in serum estradiol is considered the day of ovulation (day 0).

The pregnancy success rate depends on the synchrony between the age of the transferred embryos, as measured by culture time in vitro, and the host endometrium, relative to the predicted day of ovulation. The optimal timing for blastocyst (day 6/7) transfer is into a day 4 uterine environment, whereas cleavage-stage embryos at a culture age of 1–4 d can be optimally transferred into a day 2 recipients. Recipient female monkeys within 1–4 d of ovulation are anesthetized with ketamine and prepared for laparoscopic embryo transfer using the same basic laparoscopic approach and anesthesia as described for follicular aspiration.
1). Examine the ovaries with a self-retaining microretractor inserted at a high paramedian position after insertion of the telescope and Trendeleburg positioning.
2). Transfer embryos preferentially into the oviduct with an ovulation site on the associated ovary.
3). Grasp the fimbria with a Patton retractor and place in traction.
4). Insert the Patton cannula transabdominally and advance through the fimbria into the oviduct to a distance of 1–3 cm.
5). Typically, transfer two ICSI or IVF embryos to the oviduct of the recipient. Given significantly lower developmental rates with NT embryos, the number of transferred embryos in this case has been increased to five to six.
6). Remove embryos from culture medium and transfer to a dish containing TH3 medium.
7). Connect the transfer catheter to a 1-mL syringe filled with about 0.01–0.02 mL of TH3 medium avoiding air bubbles.
8). Carefully load embryos near the catheter tip with a total volume not exceeding 0.03 mL.
9). Insert the catheter into the external orifice of the cannula and advance into the oviduct to a depth of 1–3 cm and deposit the embryos.
10). Carefully examine the catheter after transfer to ensure that all embryos have been transferred. If an embryo has been retained it can be subjected to a second transfer attempt.
11). The skin incision closure is identical to the follicle aspiration procedure described previously.
To detect pregnancy, serum levels of estrogen and progesterone are monitored every third day after embryo transfer. Pregnancy is confirmed by ultrasound approx 25 d after transfer and monitored periodically throughout gestation.

3.10. Somatic Cell Cloning Efforts in the Monkey

As noted previously, nuclear reprogramming is thought to be the limiting parameter in our monkey somatic cell cloning protocol. Obviously, the reprogramming required after SCNT is more extensive than the process that occurs during embryonic cell NT. For normal full term development of cloned embryos, genes normally expressed during embryogenesis, but silent in the somatic donor nucleus, must be reactivated in an appropriate temporal and spatial manner. Despite the fact that even terminally differentiated donor cell nuclei can be reprogrammed in egg cytoplasm acquiring the capacity to support full-term development, only a few NT embryos develop to term and, of those, many die shortly after birth in all species where cloning has been successful. The most likely explanation for such outcome is the inability of the cytoplast to reprogram the epigenetic profile of the somatic donor nucleus to that of the fertilized zygote. Our current research on somatic cell cloning in the monkey is focused on the effect of various cell types (younger fetal fibroblasts, cumulus, oviductal epithelial, embryonic stem cells) on NT development in efforts to screen for a more “reprogrammable” source of donor nuclei. Apparently, the primate egg cytoplasm lacks or contains insufficient levels of factors responsible for somatic chromatin remodeling; therefore, another logical approach is to induce nuclear remodeling in cultured cells before nuclear transfer.

The feasibility of such an approach is supported by the recent demonstration that denatured somatic

cells subjected to heat treatment can be reactivated after NT with development to blastocysts in vitro and to viable offspring in vivo. Such treatments destabilize high-order nucleoprotein complexes and, presumably, denatured chromatin is more readily accessible to the oocyte's reprogramming machinery. Often, fusion of such cells with recipient cytoplasts is not possible because pre-treatment of donor cells results in irreversible membrane damage. We have explored alternative methods of introducing donor chromatin involving direct injection without using piezo manipulators with results comparable to those achieved for cell fusion.

4. Notes

1). The percentage of "nonresponders" varies by season showing an increase during the summer months, reaching more than 35% in June and July. During summer, despite housing in controlled, constant environments, many female monkeys also become anovulatory, and it is impractical to attempt COS. Females can be recycled for COSs; however, the response to recombinant human gonadotropins is decreased gradually with increasing numbers of stimulations, apparently the result of an immune reaction. Practically, as many as three stimulations on average can be performed per female monkey, with the recovery of a reasonable number of high quality oocytes. The availability of monkey recombinant gonadotropins would allow the more efficient and extended use of females.

2). The time between aspiration and oocyte recovery should be minimized to avoid the detrimental effects of blood exposure, which usually contaminates the aspirates. The conventional approach of diluting aspirates with medium and searching for oocytes under a dissecting microscope is labor intensive often requiring two to three technicians. The recovery time can be minimized by sifting the aspirates through cell strainers.

3). Before assignment to the project, each animal receives a physical examination from the veterinary staff and is fitted with a special neck chain (using pole and collar method) to facilitate restraint. Only animals with sperm concentrations greater that 60 million per milliliter and with more that 70% motile cells with normal morphology are acceptable.

4). Priming is used on those animals that show little or no movement. If an animal is a mover in the chair, stimulation can begin immediately, moving the Current Adjustment dial without priming.

5). It usually takes approx 15 s for priming but when the Current Adjustment dial is moved up, the total time from post prime to the end of trial should be no more that 15–20 s. All samples will be collected within this time frame. If data are available from previous stimulations, the Current Adjustment dial is slowly adjusted to a level that had been previously successful for that particular animal. Total stimulus current time is left at this level for a maximum of 15–20 s during which ejaculation almost always occurs. If no sample is obtained, the stimulation is repeated within 1–1.5 min. No more that five consecutive stimuli attempts are made per animal on each trial. Each animal is given an abstinence period at least 48 h between sample collections.

6). Piercing the plasma membrane during the ICSI procedure is critical and often difficult to assess because the needle can grossly invaginate the membrane without breaking through. For that reason, the injection pipet should be inserted through the zona pellucida and "into" the oocyte across approx one-third of the egg's diameter. Cytoplasm is slowly aspirated into the ICSI pipet (as far back as the needle junction with the zona pellucida) until the plasma membrane brakes. A "pop" or sudden movement of cytoplasm into the pipet indicates the release of membrane tension. Once the membrane is penetrated, the sperm is expelled into the cytoplasm, visualized as a "dive" of the sperm. Frozen-thawed sperm can also be used for ICSI. We have previously reported that fertilization rates by ICSI with cryopreserved sperm injected shortly after thaw are significantly lower than with fresh sperm. However, preincubation times of more than 3 h after thaw were associated with ICSI fertilizing capacity similar to that seen with fresh sperm.

7). Preimplantation development of the primate embryo as in other mammals includes explicit developmental stages from the formation of the zygote after fertilization to cleavage, morula formation, compaction and finally cavitation with the formation of the blastocyst. In HECM-9aa + FBS, blastocysts containing 100 or so cells can be seen by day 6 or 7 in vitro. Admittedly, in vivo rates are probably somewhat faster than in vitro, which may reflect suboptimal culture conditions.

8). Monkey oocytes are more fragile than those of the cow and easily lyse during enucleation if the cytoskeleton organization has not been "softened" by cytochalasin. However, prolonged incubation in cytochlasin B during enucleation may cause excessive softening and swelling of the cytoplasm.

9). Penetration through the zona during enucleation is more difficult than in the ICSI procedure because of the larger size of the enucleation pipet, and in some cases, may require extending the pipet all the way to the other side of the egg.

10). As the pipet is pulled away from the egg, the plasma membrane will stretch and form a thin bridge between the egg and the material being removed. A

rapid movement of the enucleation pipet toward 6 o'clock will break this cytoplast bridge.
11). Various artificial activation treatments have been developed that mimic spermtriggered events and induce development of NT embryos. For example, ethanol, Pronuclear stage zygote with male and female pronuclei, 12 h after fertilization by intracytoplasmic sperm injection. **(B)** Day 1, two-cell stage embryo (day of fertilization = day zero). **(C)** Day 2, four-cell stage embryo. **(D)** Day 3, eight-cell stage embryo. **(E)** Day 4, morula stage embryo. **(F)** Day 5, compact morula. Note that individual blastomeres have maximized their intracellular contacts and compacted into a tight cell mass. **(G)** Day 6 or 7, expanded blastocyst with a single flat layer of trophectoderm surrounding a fluid filled blastocoel and the inner cell mass. Electroporation in calcium containing medium, ionomycin, or inositol 1,4,5-trisphosphate. However, M-phase-promoting factor activity at least in the young bovine and rabbit oocyte is quickly restored with recondensation of chromosomes and reentry of activated oocytes into a new M-phase arrest, known as MIII. This phase can be circumvented by additional treatments with protein synthesis (cycloheximide), protein phosphorylation (DMAP) or specific M-phase-promoting factor inhibitors (roscovitine). Thus, sequential approaches have evolved with ionomycin/DMAP, ionomycin/cycloheximide, or ionomycin /roscovitin that result in high activation rates in monkey NT embryos.
12). Stepwise transfer to fusion medium minimizes osmotic shock and reduces electrolyte contamination in the fusion chamber.
13). For information on optimal timing of embryo transfer.
14). A blunt transfer pipet (5–7 um outer diameter) is used to mechanically disrupt a single donor cell by aspiration in a 10% PVP drop. The nucleus is subsequently transferred into a conventionally prepared cytoplast via pipet passage through the hole in the zona and injection into the cytoplasm as described for the ISCI procedure.
The efficiency of direct injection is comparable to fusion with 80–90% survival rate and 60–70% pronuclear formation rate.

5. Discussion

The creation of a nuclear transferred human embryo is concerned. And, SCNT requires human eggs that only can be obtained from women.

To obtain an identical stem cell line to a specific patient, a cell nucleus from his/her cells will be transplanted by SCNT into an egg cell from an egg donor, creating a unique lineage of stem cells almost identical to the patient's own cells. If the patient is a woman who can produce eggs herself, the better way is to use her egg as the donor and all the cells from the hybrid cells will be totally identified. If the patient is a man or cannot have egg even he is a woman, the another choice is to get eggs from his/her closed relative who can donate the eggs.

As there could be millions of patients needing the stem cell therapy, and each patient could require a large number of donated eggs to successfully get a therapeutic stem cell line. This will need a large number of the donators. Another way is to create artificial eggs.

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