Video Article

Nuclear Transfer into Mouse Oocytes

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Abstract

Nuclear transfer into an unfertilized oocyte can restore developmental potential to a differentiated cell. This demonstrates that the processes underlying development, differentiation and aging are epigenetic rather than genetic processes. The reversibility of these processes opens exciting perspectives in basic research, and in the more distant future, in regenerative medicine. In the mouse, embryonic stem cells can be derived from cloned preimplantation stage embryos. Such embryonic stem cells have the ability to give rise to all cell types of the adult organism. Importantly, these cells are genetically identical to the donor. If applicable to human, this would allow the derivation of stem cells from a patient. These cells could then be differentiated into the affected cell type of the patient and studied in vitro, or used to replace the damaged or missing cells. The study of nuclear transfer in the mouse remains important as it can inform us about the principles of nuclear reprogramming. This movie and the accompanying protocol are intended to help learning nuclear transfer in the mouse, a method initially developed in the group of Prof. Yanagimachi (WAKAYAMA et al. 1998).

Video Link

The video component of this article can be found at https://www.jove.com/video/116/

Protocol

Preparations:

1. A detailed description of superovulation and microdrop embryo culture can be found elsewhere [2].
2. Prepare a petri dish with microdrops of embryo culture medium (e.g. KSOM, Chemicon). Cover with mineral oil (mineral oil batches should be tested for compatibility with embryo culture). Equilibrate at 37°C in air plus 5% CO_2. (In Thermo-Electron 3110 water jacketed incubator or equivalent)
3. Prepare a dish for the isolation of oocytes: Place drops of hepes buffered CZB & hyaluronidase (0.1% w/v, Sigma) in one half of the dish and HCZB in the other half. Cover with mineral oil. Keep the dish warm on a heated stage of a dissection microscope.
4. Prepare the microscope: Place a holding pipette of an outer diameter of about 50μm (slightly smaller than the diameter of the oocyte), and an opening of about 20μm in one pipette holder. Holding pipettes are easy to make with the necessary equipment, a needle puller (Sutter Instruments) and a microforge (Narishige), but they may also be purchased from Humagen.
   Load 3-5 μl mercury into the tail of a micropipette (inner diameter 8μm). Avoid mercury spills. Prepare a dish with several small drops each of 11% w/v PVP (polyvinylpyrrolidone) in HCZB and 5μg/ml cytochalasin B in HCZB. Use the lid of a Petri dish rather than the dish itself as the lid has a short rim that will not interfere with the movement of the holding and the enucleation pipettes. Align the holding pipette and the enucleation pipette in an HCZB drop. Aspirate a little HZCB into the tip of the holding pipette.
5. Preparation of donor cells depends largely on the cell type and the experiment the investigator intends to perform. In general, donor cells should be treated with a proteolytic enzyme such as trypsin and kept on ice to reduce stickiness until transfer.
6. Humanely euthanize mice 14-15 hours after the administration of hCG. Harvest oviducts and dissect them in the warm HCZB-hyaluronidase drops. After approx. 5 min, oocytes will be mostly free from cumulus cells. They should then be washed several times in HCZB, then washed further in the pre-equilibrated KSOM drops, and kept in the incubator until enucleation.

Enucleation

Manipulations are done with hydraulic micromanipulators, (Narishige) on an inverted microscope, such as the Nikon TE200. A piezo micromanipulator (Primetech) is used for drilling the zona pellucida. Flat-tipped enucleation and transfer pipettes can be purchased from Humagen. Clean and lubricate the enucleation pipette in the PVP drops by expelling microscopic mercury droplets and by aspirating and expelling PVP. To prevent stickiness of the needle, the same procedure should be done between each group of oocytes that are either enucleated or transferred.

Place a small group of oocytes into the HCZB-cytochalasin B drop. The size of the group should be adapted to the level of experience of the investigator. Oocytes should not be kept on the stage for longer than 20-30 min. Move the instruments to the HCZB-cytochalasin B

drops. The holding pipette and the enucleation pipette should be brought into alignment with the equatorial plane of the oocyte. This will be possible if the holding pipette has an outer diameter that is slightly smaller than the oocyte itself. Rotate an oocyte until the differently refractive metaphase spindle can be seen. If a beginner cannot see the spindle, the metaphase plate can be identified using the DNA dye Hoechst and UV illumination, however, that is not compatible with efficient embryonic development. Position the metaphase spindle at 3 o'clock and hold it well with the holding pipette. Apply piezo pulses to penetrate the zona pellucida. Touch the metaphase plate with the enucleation pipette. Once the resistance of the metaphase spindle can be 'felt', aspirate the spindle and withdraw the needle. The oocyte cytoplasm is fluid, the metaphase spindle however moves as a clump when touched with the pipette. Try to reduce the amount of cytoplasm that is removed with the spindle. After a group of oocytes has been enucleated, wash them through embryo culture medium and place them into the incubator until transfer. Some enucleated oocytes should not be transferred but should be activated and serve as a control for enucleation. These oocytes will fragment within several hours, indicating successful enucleation.

Nuclear transfer

Place cells into PVP solution (11% PVP in HCZB). If many clones arrest at the 1-cell stage, the PVP concentration should be lowered to 5% or less. Mix cells well with PVP. Pick up cells with a pipette with an inner diameter slightly smaller than the diameter of the cell. The membrane of the cell should break upon aspiration. In some cases, repetitive expelling and aspiration as well as the application of gentle piezo pulses can be used to break the donor cell. A new drop of donor cells should be made with each group of oocytes being transferred, as donor cells become sticky after some time. Inject the broken donor cell shortly after breaking the membrane. For the transfer, focus on the equatorial plane of the oocyte itself rather than the equatorial plane of the zona pellucida, and position the transfer and the holding pipette in the same plane. Hold the oocyte including a part of its cytoplasm firmly. Penetrate the zona pellucida using piezo pulses. Bring the donor nucleus to the tip of the pipette, then push the pipette tip almost to the opposite side of the enucleated oocyte, close to the holding pipette, making a deep furrow in the oocyte. Aspirate a very small amount of oocyte cytoplasm into the pipette, apply a single weak piezo pulse to break the oocyte membrane, eject the donor nucleus, withdraw the needle rapidly while aspirating at the cytoplasmic membrane at the right end of the furrow. By simultaneously withdrawing the needle and aspirating, it should be possible to close the hole left behind by NT. This 'hole removal technique will reduce the number of oocytes that lyse during NT. Wash the reconstructed oocytes in KSOM and return them to the incubator for 1-3 hours.

Oocyte activation

Prepare one half of a dish with droplets of calcium free MCZB and the other half with droplets of calcium free MCZB plus 10mM Sr^{2+} and 5μg/ml cytochalasin B to inhibit polar body extrusion. Equilibrate for 30 min. under 5% CO^2 in air. Wash NT embryos well in calcium-free MCZB and then place them in small groups in different drops of calcium-free MCZB. Return the dish to the incubator and culture for 5-6 hours. To control for the artificial activation protocol and for embryo culture conditions, non-enucleated oocytes should be used. They will develop as parthenogenetic embryos. Following activation, wash the embryos through KSOM and place embryos in the incubator for long-term culture. Pronuclei should be visible at this time point, indicating successful transfer.

Embryo Culture Media Cookbook

Master Salts

Begin with 980 mL ultrapure H_2O in sterile 1 L bottle

Add dry components:

NaCl	4760 mg (81mM)	Sigma S-5886
KCl	360 mg (5mM)	Sigma P-5405
$MgSO_4 \cdot 7H_2O$	290 mg (1.18mM)	Sigma M-2773
KH_2PO_4	160 mg (1.17mM)	Sigma P-5655
EDTA 2NA	40 mg (0.1mM)	Sigma E-6635
Glucose (D)	1000 mg (5.5mM)	Sigma G-6152

Add liquid Components

Na-lactate (lactic acid)	5.3 mL	Sigma 44263
Pen'Strep	10 mL	Gibco 15140-122

For MCZB Stock Salts:

Filter sterilize 500 mL master salts (good for 3-4 months; store at 4° C)

For 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 (good for 3 months; store at 4° C)

Ca^{++} Free MCZB (for oocytes activation in 5% CO_2)

Start with 99 mL MCZB stock salts

Add dry components:

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

Swirl until all components are dissolved; sterile filter.

HCZB (for micromanipulation in ambient atmosphere)

Start with 99 mL HCZB stock salts

Add dry components:

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

Add liquid components:

128 mM $CaCl_2$ (18.8g/l)	1 mL	Sigma C-7902

Adjust pH to 7.5 with 1 *N* HCl

Swirl until dissolved; sterile filter.

Discussion

Good luck!

Disclosures

The authors have nothing to disclose.

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