Video Article

Transnuclear Mice with Pre-defined T Cell Receptor Specificities Against *Toxoplasma gondii* Obtained Via SCNT

Oktay Kirak[1], Eva-Maria Frickel[1], Gijsbert M. Grotenbreg[1,2], Heikyung Suh[1], Rudolf Jaenisch[1,3], Hidde L. Ploegh[1,3]

[1]Whitehead Institute for Biomedical Research

[2]Departments of Microbiology and Biological Sciences, National University of Singapore

[3]Department of Biology, Massachusetts Institute of Technology

Correspondence to: Oktay Kirak at kirak@wi.mit.edu, Rudolf Jaenisch at jaenisch@wi.mit.edu, Hidde L. Ploegh at ploegh@wi.mit.edu

URL: https://www.jove.com/video/2168
DOI: doi:10.3791/2168

Keywords: Developmental Biology, Issue 43, SCNT, immunology, TCR, BCR, mouse model, transnuclear

Date Published: 9/30/2010

Citation: Kirak, O., Frickel, E.M., Grotenbreg, G.M., Suh, H., Jaenisch, R., Ploegh, H.L. Transnuclear Mice with Pre-defined T Cell Receptor Specificities Against *Toxoplasma gondii* Obtained Via SCNT. *J. Vis. Exp.* (43), e2168, doi:10.3791/2168 (2010).

Abstract

Lymphocytes, such as T cells, undergo genetic V(D)J recombination, to generate a receptor with a certain specificity[1]. Mice transgenic for a rearranged antigen-specific T cell receptor (TCR) have been an indispensable tool to study T cell development and function. However, such TCRs are usually isolated from the relevant T cells after long-term culture often following repeated antigen stimulation, which unavoidably selects for T cells with high affinity. Random genomic integration of the TCR α- and β-chain and expression from non-endogenous promoters can lead to variations in expression level and kinetics.

Epigenetic reprogramming via somatic cell nuclear transfer provides a tool to generate embryonic stem cells and mice from any cell of interest. Consequently, when SCNT is applied to T cells of known specificity, these genetic V(D)J rearrangements are transferred to the SCNT-embryonic stem cells (ESCs) and the mice derived from them, while epigenetic marks are reset. We have demonstrated that T cells with pre-defined specificities against *Toxoplasma gondii* can be used to generate mouse models that express the specific TCR from their endogenous loci, without experimentally introduced genetic modification. The relative ease and speed with which such transnuclear models can be obtained holds promise for the construction of other disease models.

Video Link

The video component of this article can be found at https://www.jove.com/video/2168/

Protocol

This method was used in the research reported in Kirak *et al.*, Science 328 (5975), 243-248 (2010).

1. Isolation of Donor Cells

Before specific T or B cells can be used as donor cells to generate transnuclear mouse models, mice need to be infected with a pathogen of interest or immunized with an antigen of interest. Since we have used CD8+ cells specific for *Toxoplasma gondii*, the following protocol will describe the generation and isolation of these cells and needs to be adapted according to personal interest.

1. Cysts derived from a mouse brain homogenate (about 40) are injected intra-peritoneally into BL/6 x Balb/c F1 (B6CF1) mice, which enables isolation of both H-2^b and H-2^d restricted CD8+ T cells.
2. At the peak of the immune response, the infected mouse is euthanized, the spleen isolated and placed into a dish containing 6 mL red blood cell (RBC) lysis buffer.
3. Using the frosted sides of two cover slides, the spleen is carefully ground and incubated in the RBC lysis buffer for 5 min at RT.
4. Add 14 mL PBS, filter the cell suspension through a cell strainer (40 or 70 µm) into a 50 mL falcon tube and centrifuge for 5 min at 300g.
5. Remove supernatant, resuspend the cell pellet in 1 mL PBS and transfer into a 1.5 mL Eppendorf tube. Centrifuge for 5 min at 300g and remove the supernatant afterwards.
6. Resuspend the cell pellet in 50 µL PBS, add fluorescently labeled antibodies to identify the cell type of interest (e.g. CD3, B220, CD8, and CD4), and fluorescently labeled MHC-I tetramer (MHC-II tetramer for CD4 T cells) loaded with the peptide of interest to the cell suspension and incubate for 30 min at 4°C.
7. Add 1 mL PBS, centrifuge for 5 min at 300 g, resuspend pellet in 3 ml PBS, and filter the cell suspension through a cell strainer (40 or 70 µm) into a tube.
8. Perform FACSorting by setting the gates stringently (Figure 1B).

2. Somatic Cell Nuclear Transfer

There are a few protocols for Somatic Cell Nuclear Transfer. The one described here makes use of oocytes arrested at Metaphase-II and the inhibition of the second meiotic division using Cytochalasin B. Therefore donor cells are needed which are in G0/G1 phase.

1. Preparation of oocytes
    1. Female mice of BDF1 background are injected intra-peritoneally with 5IU PMS per mouse between 5PM and 7PM.
    2. About 47 hours later, each mouse is injected intra-peritoneally with 5IU HCG.
    3. Prepare a culture dish (KSOM-AA drops under oil) for the oocytes on the same day as the HCG injection and place them into an incubator 37°C, 5% CO_2. Also, prepare the needles necessary for SCNT (7µm for enucleation and 4 or 5µm for nuclear transfer of lymphocyte nuclei) by loading them with mercury.
    4. Euthanize mice and isolate oviducts about 13h after HCG (approximately 8AM). Collect the oviducts in 1-2 drops of HCZB.
    5. One-by-one move the oviducts into a drop containing M2 w/Hyaluronidase. Nick the oviduct carefully with a forcep and move the oocyte-cumulus-complex into the drop. After all oocyte-cumulus-complexes have been isolated, the dish is incubated at 37°C.
    6. After 2-5 min (the exact time depends on the batch of Hyaluronidase) collect the oocytes, wash in HCZB and plate them into KSOM-AA drops.

2. Enucleation of oocytes
    1. Use the lid of a petri dish to prepare the SCNT plate. Set up the microscope, and the micromanipulator.
    2. Wash the enucleation needle (7 µm) in PVP before starting with enucleation.
    3. For enucleation, place a group of oocytes (10-30) into a drop of HZCB with Cytochalasin B (5 µg/mL). While incubating (min. 5 min) line-up the oocytes horizontally.
    4. Take one oocyte and place it in front of the holding needle. Fix the oocyte to the holding needle by applying a vacuum. Using the enucleation needle, turn the oocyte until the chromosome-spindle-complex (CSC, often called the "nucleus") is in the 3 o'clock position.
    5. Using piezo-pulse penetrate the zona pellucida carefully without damaging the oocyte. Place the opening of the needle adjacent to the Metaphase-II spindle and apply vacuum. The "nucleus" should slowly move into the needle, and the membrane should seal itself once it is removed completely.
    6. Transfer the enucleated oocytes back into the KSOM-AA drops, and wash them multiple times. Repeat the enucleation steps with a new group of oocytes. Continue until all eggs are enucleated or until about 11AM.

3. Nuclear Transfer
    1. For the nuclear transfer, exchange the enucleation needle (7 µm) with a nuclear transfer needle (4 or 5 µm) and wash it with PVP.
    2. Place your cells of interest (e.g. CD8+ T cells) into a drop with PVP, mix well and incubate for a minimum of 10 min.
    3. Place a group of enucleated oocytes (10-30) into a drop of HCZB with Cytochalasin B (2.5 µg/mL). While incubating (min. 5 min) line-up oocytes horizontally by using the nuclear transfer needle.
    4. Pick up a donor cell from the PVP-cell suspension and aspirate it in-and-out until the outer membrane has broken down (fragments of the membrane and cytosol should be visible). If necessary a piezo-pulse can be applied. Once a nucleus has been isolated, move it a little bit up in the needle, and continue to pick up nuclei until you have 10-30.
    5. Move to the drop containing the aligned enucleated oocytes. Fix one oocyte to the holding needle by applying vacuum. Place the nuclear transfer needle (the nuclei should be away from the opening) adjacent to the zona pellucida and apply piezo-pulse until the needle has gone through the zona pellucida. Carefully move one nucleus to the tip of the needle, push the needle into the oocyte until the tip of the needle is 2/3 inside. Apply a small negative pressure, so that a little bit of the oocyte membrane is in the needle.
    6. The next step is very critical, since it is the only time when the oocyte is actually "open". Apply a single pulse of piezo, push the nucleus out of the needle by applying positive pressure, carefully pull back the needle so that the tip is about 1/3 in the oocyte, and then apply negative pressure and continue to pull back the needle to seal the oocyte. Repeat this step with each oocyte.
    7. After all oocytes of a group have received a nucleus, they are washed and cultured in KSOM-AA media. Repeat this procedure with all oocytes.
    8. After the last group has been cultures in KSOM-AA for a minimum of 30 min, the SCNT-embryos are transferred into drops of Ca-free activation media containing SrCl2.
    9. After 6 hours of activation, the amount of pseudo-pronuclei (PPN)-positive SCNT-embryos is determined. The embryos are then washed and cultured in KSOM-AA drops for additional 3.5 days until they have reached the blastocyst stage.
    10. Drugs or chemicals of choice (such as Trichostatin A) can be added to the activation and culture media.

3.Derivation of embryonic stem cells

The SCNT-blastocysts can be used to either transfer them into pseudo-pregnant females (also known as direct or one-step cloning) or to derive embryonic stem cells (also known as indirect or two-step cloning). Since it is much more efficient to generate ES cells, we choose the two-step procedure. In case the scientist is interested in direct cloning, the blastocysts can be transferred directly into pseudo-pregnant females as described in section 5.

There are many protocols describing the derivation of embryonic stem cells. The one described here is a standard technique, which utilizes feeder cells, and fetal calf serum.

1. The second day after nuclear transfer, prepare a 96-U-well plate for ES cell derivation.
2. Add 100 µL Gelatin into each well of a 96-U-well plate, incubate for a minimum of 10 min, and remove it afterwards.
3. Thaw feeder cells and resuspend in an according volume of ESD medium, e.g. feeder cells equivalent to an area of 25 cm^2 are resuspended in 10 mL ESD medium and 200 µL of the cell suspension is added into each of the gelatin-treated well.
4. Put the plate into an incubator and culture at 37°C, 5% CO_2.

5. After 3.5 days, the embryos should be at the blastocyst stage.
6. Prepare a sterile bacterial dish by plating a few drops ES cell medium and acidic thyrode.
7. The blastocysts are first transferred from the KSOM-AA drops into drops containing normal ES cell medium and washed twice.
8. Carefully transfer a group of blastocysts (5-10 blastocysts) into one drop of acidic thyrode and keep in there until the zona pellucida has dissolved.
9. The blastocysts are then transferred into a drop with ES medium, washed twice, and then placed into the feeder-coated 96-U-well plate (one blastocyst into one well).
10. The embryos are then cultured for 5-7 days in an incubator at 37°C, 5% CO_2 without disturbing them. This gives the embryo time to attach to the feeder layer, and to form an outgrowth.
11. Prepare 24-well plates, by plating feeders in ESD medium into each well, e.g. resuspend an equivalent of 50 cm^2 feeder in 12 mL ESD medium, and add 500 µL of the cell suspension into each well.
12. Carefully remove the ESD medium from the 96-U-well plate with the embryos, wash each well twice with 250 µL Hepes (or PBS), add 50 µL Trypsin and incubate for about 5 min at 37°C.
13. Pipette up and down multiple times, until outgrowth has fallen apart into a single-cell suspension, transfer into a 24-well plate and incubate at 37°C, 5% CO_2.
14. If ESC derivation was successful, ESC colonies should be visible after 7-10 days.

4.Blastocyst injection

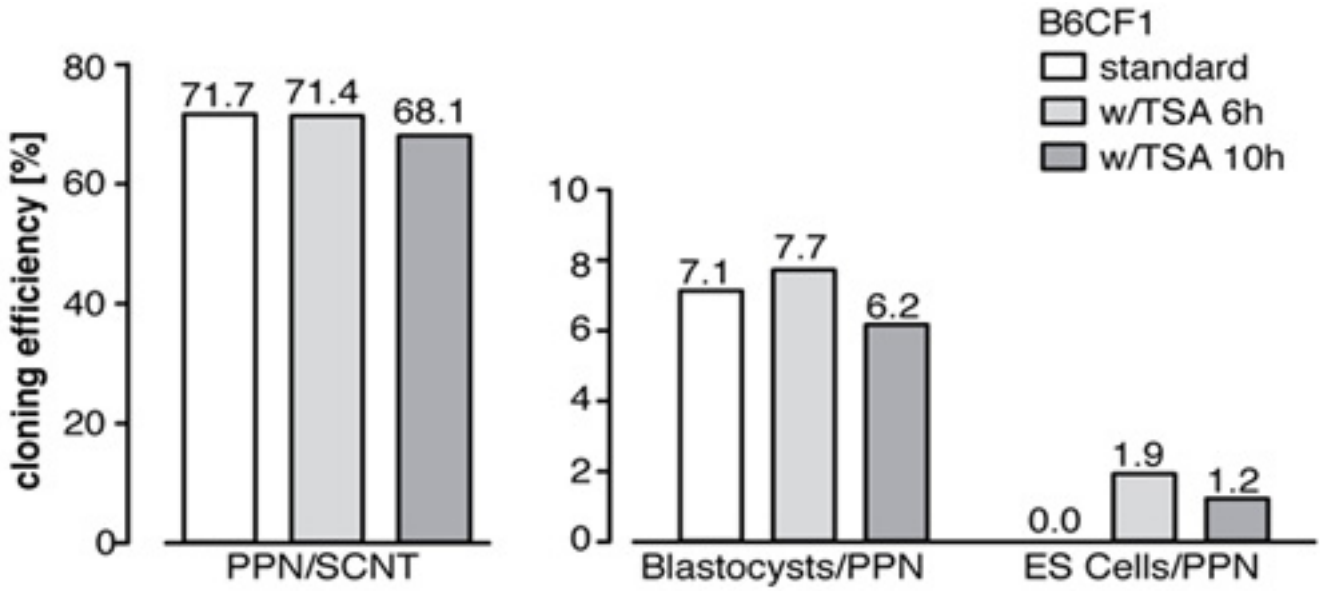
The injection of blastocysts is a routine technique to generate any kind of transgenic mouse model. It is important to mention that the injection of blastocysts and the subsequent transfer into pseudo-pregnant females needs to be timed well.

1. Prime female mice with PMS and HCG as described in section 2.1. After HCG injection put female mice together with male mice (one female and one male mouse per cage).
2. The next day, check females for plugs. This is counted as 0.5dpc (days post coitus).
3. Fertilized embryos are then isolated from the oviduct as described in section 2.1, and cultured in KSOM-AA for additional 3 days.
4. Prepare ES cells on the day of blastocyst injection (3.5dpc).
5. Remove medium from ES cells, wash twice with Hepes (or PBS), add Trypsin and incubate for 5 min at 37°C.
6. Resuspend trypsinized cells with ES medium, plate in a dish, and incubate for 45-60 min at 37°C, 5% CO_2 to reduce the amount of feeder cells (feeder cells adhere faster than ES cells).
7. Transfer the cell suspension through a cell strainer (40 or 70 µm) into a tube, and centrifuge for 5 min at 1000rpm.
8. Remove supernatant, resuspend cell pellet in 500 µL ES medium and store on ice until needed.
9. Prepare the lid of a sterile bacterial dish by plating drops containing PVP and HCZB.
10. Prepare a 15 µm needle by loading with mercury, placing it in position and washing it with PVP.
11. Place a group of blastocysts (10-30 blastocysts) into a drop with HCZB, and add 5-50 µL ES cell suspension (depends on the concentration) into another drop of HCZB.
12. Pick up ES cells (50-100 cells) with the injection needle.
13. Move to the drop with the blastocysts. Align the blastocysts horizontally, and fix one with the holding needle by applying vacuum.
14. Using the injection needle turn the blastocyst until the inner cell mass (ICM) is at the 9 o'clock position.
15. Place your injection needle at 3 o'clock, make sure that there are no ES cells at the tip of the needle, and apply piezo-pulse until the injection needle penetrates through the zona pellucida and the trophectoderm into the blastocoel.
16. Release a few ES cells (5-15 cells), so that the ES cells stick to the ICM. Continue until all blastocysts of a group have been injected with ES cells.
17. After one group of blastocysts is finished, wash them twice in KSOM-AA and keep them in a drop with KSOM-AA until all blastocysts are injected.

5. Embryo Transfer

The transfer of embryos into pseudo-pregnant females represents a surgical procedure, which needs to be carried out very carefully and according to the guidelines of the researcher's institute.

1. Anaesthetize female recipient mice, shave lower right quadrant, and disinfect.
2. Using scissors open the skin, and separate the peritoneum from the skin.
3. Locate the ovary through the peritoneum (red spot), and open the peritoneum in close proximity to the ovary.
4. Using a forceps, carefully grab the oviduct and pull out the uterus.
5. Pick up a group of about 10 blastocysts with a capillary connected to a mouth pipette.
6. Fix the uterus with one forceps, punch a small whole into it with a small needle, and insert the capillary with the blastocysts into the lumen of the uterus.
7. Carefully release the embryos into the uterus, and remove your capillary.
8. Push the uterus back into the abdominal cavity.
9. Locate the edges of the peritoneum and close it with a few stitches.
10. Close the skin of the mouse with a few staples.
11. For post-operative pain, administer 5 µg Carprofen per mg of body weight.



	SCNT	Pseudo-Pronuclei (PPN)	Blastocysts	ES Cells
standard	332	238	17	0
w/TSA 6h	290	207	16	4[a]
w/TSA 10h	119	81	5	1[b]

Resulting ESC lines: R7-I[a], R7-II[a], R7-III[a], T57[a], and G4[b]

Figure 1. Somatic cell nuclear transfer of pre-defined T cells in B6CF1 background. Absolute and relative numbers of embryos generated from CD8+ T cells and embryonic stem cells derived from SCNT blastocysts.

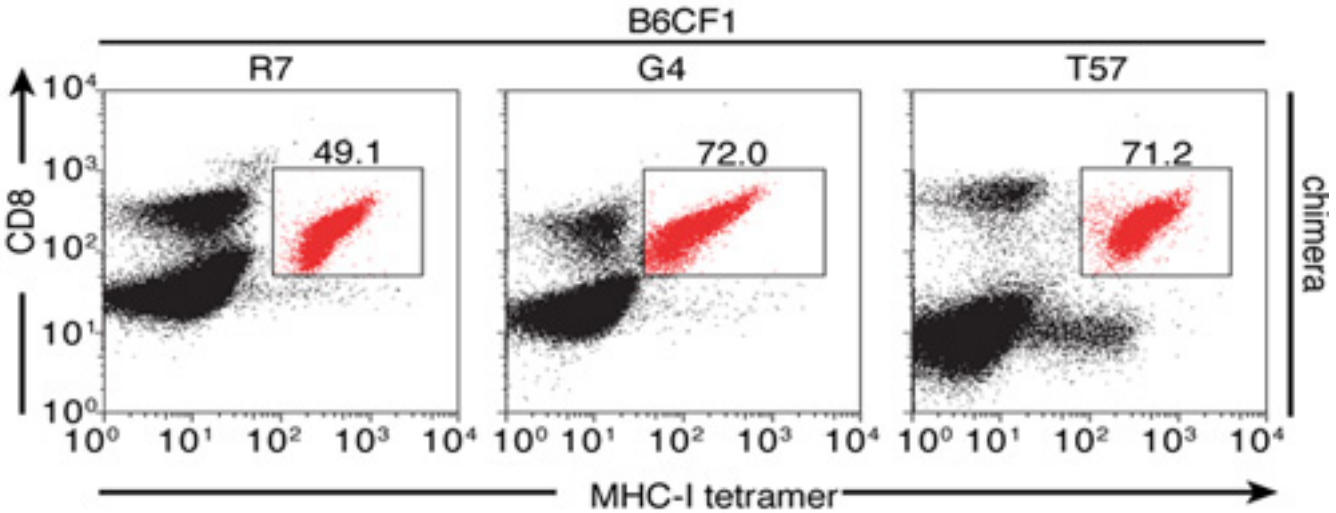


Figure 2. Representative flow cytometric analysis of chimeric mice injected with SCNT embryonic stem cells. Gate and number (percent per total CD8+ T cells) indicates the presence of specific CD8+ T cells in chimeric mice.

Discussion

We have shown here that SCNT can be used to generate transnuclear mice from T cells with pre-defined specificity. Although not shown here, the technique should also be usable to generate transnuclear mice from B cells with pre-defined specificity.

One important thing to consider is the strain and gender of mouse, which is infected or immunized and used to isolate T or B cells of interest. Since T and B cells have a very low reprogramming efficiency in general, we recommend using F1 hybrids of desired haplotype. Because the

donor cell also determines the sex, we recommend using T or B cells from male mice. This means that only male chimeric mice would transmit the TCR or BCR through the germline, with male mice being easier and faster to breed.

Taken together, we think that epigenetic reprogramming via SCNT represents a powerful tool to generate a novel type of mouse models, which will be of great advantage for the immunological field.

Disclosures

A patent has been filed.

Acknowledgements

The authors are thankful to H. Eisen, M. Gubbels, K. van Grinsven, E. Guillen, A. Drake, V. Mahajan, Q. Gao, and G. Yap for fruitful discussions and reagents. We are thankful to J. Dausman, R. Flannery, and J. Jackson for assistance with the management of the mouse colony. We are thankful to P. Wisniewski for FACSorting. EMF was supported by the Human Frontiers Science Program. R.J. was supported by National Institute of Health grants RO1-HD045022 and R37-CA084198. HLP was supported by grants from the National Institute of Health.

References

1. Schatz, D. G. V(D)J recombination. Immunol. Rev. 200, 5 (2004).
2. Barnden, M. J., Allison, J., Heath, W. R., & Carbone, F. R. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol. Cell Biol. 76, 34 (1998).
3. Eggan, K. *et al.*, Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci U S A* 98, 6209 (2001).
4. Kishigami, S. *et al.*, Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. *Biochem Biophys Res Commun* 340, 183 (2006).
5. Wakayama, T., & Yanagimachi, R. Mouse cloning with nucleus donor cells of different age and type. *Mol Reprod Dev* 58, 376 (2001).

