Preface

As the major task of sequencing the human genome is near completion and full complement of human genes are catalogued, attention will be focused on the ultimate goal: to understand the normal biological functions of these genes, and how alterations lead to disease states. In this task there is a severe limitation in working with human material, but the mouse has been adopted as the favored animal model because of the available genetic resources and the highly conserved gene conservation linkage organization.

In just of ten years since the first gene-targeting experiments were preformed in embryonic stem (ES) cells and mutations transmitted through the mouse germline, more than a thousand mouse strains have been created. These achievements have been made possible by pioneering work that showed that ES cells derived from preimplantation mouse embryos could be cultured for prolonged periods without differentiation in culture, and that homologous recombination between targeting constructs and endogenous DNA occurred at a frequency sufficient for recombinants to be isolated. In the next few years the mouse genome will be systematically altered, and the techniques for achieving manipulations are constantly being streamlined and improved.

Recently new technologies have developed for inducible gene expression in transgenic mice that in combination with conventional gene targeting can give temporal and tissue-specific expression. These advances have been spurred on by the desire to study the function of genes that show an embryonic-lethal phenotype when deleted or "knocked out". Gene targeting in mice initially concentrated on making gene knockouts or null mutations, but increasingly the technology is being used to create subtle point mutations to simulate human disease states.

Gene Knockout Protocols brings together distinguished contributors with extensive experience in the gene targeting and mouse genetics fields. In line with the successful format of *Methods in Molecular Biology*, the volume contains step-by-step protocols for the design of targeting constructs to protocols for the analysis of the mouse phenotype. Emphasis has been paid to the inclusion of other techniques used in mouse genetics that are relevant to researchers performing gene targeting. These include embryo transplantation, In vitro

ES cell differentiation, creation of aggregation chimeras, mouse pathology, embryo cryopreservation, and transplantation. Issues such as the use of existing mouse mutation resources and the influence of genetic background and epigenetic effects upon phenotype are also covered. We hope that *Gene Knockout Protocols* will be an invaluable source of proven protocols for those just entering the field of gene targeting, but also a valuable reference for researchers in the process of describing the phenotype of mutant mice.

Martin J. Tymms
Ismail Kola

2

Isolation and Maintenance of Primate Embryonic Stem Cells

Vivienne S. Marshall, Michelle A. Waknitz, and James A. Thomson

1. Introduction

Primate embryonic stem (ES) cells are derived from preimplantation embryos and are capable of prolonged undifferentiated proliferation in culture. Under particular conditions, these cells differentiate into derivatives of endoderm, mesoderm, ectoderm, and trophoblast ***(1,2)***. In mammals, many developmental events are studied using mouse embryos or ES cells, but some aspects of development differ significantly between humans and mice, such as the timing of embryonic genome expression ***(3)***, fetal membrane and placental structure and development ***(4–6)***, and the formation of an embryonic disc instead of an egg cylinder ***(7,8)***. These and other features of human development are better studied using a primate model.

Recent embryological investigation in primates chiefly has addressed gamete interactions and preimplantation development. Primate ES cells provide an opportunity to use techniques that have never been developed in primates, such as lineage analysis, chimera formation, and transgenesis to study postimplantation events. Primate ES cell lines offer exciting possibilities for establishing a robust experimental primate embryology, and provide a powerful new model for understanding human development and disease.

Murine ES cells, unlike primate ES cells, have characteristics that make them relatively easy to culture. They can be readily passaged with a reasonable cloning efficiency, allowing large numbers of cells to be propagated for uses such as transfection or homologous recombination. Additionally, murine ES cells can be maintained in an undifferentiated state in the absence of feeder layers when culture medium is supplemented with leukemia inhibitory factor

From: *Methods in Molecular Biology, vol. 158: Gene Knockout Protocols*
Edited by: M. J. Tymms and I. Kola

(LIF) ***(9)***. In contrast, primate ES cells differentiate or die in the absence of fibroblast feeder layers, even in the presence of LIF ***(1,2)***. Primate ES cells require regular and meticulous attention to detail in all aspects of the culture process. Here we present a concise summary of the methods we use to isolate and maintain primate ES cells in vitro.

2. Materials

2.1. Immunosurgery

1. 0.5% pronase E (Sigma) in Milli-Q water.
2. Rabbit anti-rhesus or anti-marmoset spleen cell antiserum: Antiserum is raised as described previously ***(10)***, except primate spleen cells were used.
3. Guinea pig complement diluted 1:10 (Gibco-BRL; *see* **Note 1**).

2.2. Culturing Primate ES Cells

1. Irradiated mouse embryonic fibroblasts (MEF) plated on 0.1% gelatin ***(11)***.
2. Embryo culture-grade water (*see* **Note 2**).
3. Dulbecco's modified Eagle medium (DMEM) with D-glucose (4500mg/L) and L-glutamine but without sodium pyruvate or sodium bicarbonate (Gibco-BRL).
4. Primate ES cell culture medium: 79% DMEM, 20% FBS, 1% nonessential amino acid stock, 0.1 m*M* 2-mercaptoethanol, and 1 m*M* L-glutamine. Combine and filter (0.22 μm) before use.
5. Sodium bicarbonate (Sigma).
6. Fetal bovine serum (FBS; *see* **Note 3**).
7. 2-Mercaptoethanol (Sigma).
8. L-glutamine (Gibco-BRL).
9. 50X MEM nonessential amino acid stock without L-glutamine (Gibco-BRL).
10. Ethylenediamine tetraacetic acid (EDTA; Sigma).
11. Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; Gibco-BRL).
12. Dimethyl sulphoxide (DMSO; Sigma).

2.3. Handling Primate ES Cells

Primate cells should always be considered biohazardous because some viruses such as herpes B, which can be carried by rhesus macaques without noticeable clinical signs, are potentially fatal when transmitted to humans. Never use a mouth pipet for handling primate cells, and dispose of used equipment (pipets, test-tubes, and the like) according to local regulations.

1. 15 mL polystyrene tubes.
2. Glass pipets (1 mL, 5 mL, 10 mL, 25 mL).
3. Glass Pasteur pipets (9 in. borosilicate).

4. Micrometer syringe apparatus ***(12)***.
5. 4-well and 6-well tissue culture plates, 35mm tissue culture dishes (Nunc), T25 and T75 polystyrene tissue culture flasks (Becton Dickinson).
6. Cryogenic vials (Nalgene).

3. Methods

3.1. Preparation of Mouse Embryonic Fibroblasts

Instructions for the isolation, preparation, and plating of MEF can be found elsewhere ***(11)***. MEF should be isolated and frozen in quantity well before required.

1. Culture MEF to 70–80% confluence. To keep differentiation of ES cells to a minimum, passage MEF regularly, and do not allow MEF to reach confluence immediately before irradiation.
2. Mitotically inactivate by exposure to 3000 rads γ-radiation.
3. Plate at 5×10^4 cells/cm^2 at least 2 h (preferably 12 h) prior to immunosurgery or ES cell passage.

3.2. Isolation of the Inner Cell Mass

All solutions used for the immunosurgical procedure must be made fresh (from frozen stocks) on the day of the procedure, and allowed to equilibrate in an incubator at 37°C for at least 1 h.

1. Incubate blastocyst **(Fig. 1A)** briefly in 0.5% pronase until the zona pellucida disappears. This takes approximately 30 seconds, so constant attention is required.
2. Immediately remove the zona pellucida-free embryo and wash three times in DMEM + 20% FBS.
3. Incubate the blastocyst in antibody for 30 mins at 37°C in 5% CO_2 in air.
4. Wash three times in DMEM + 20% FBS.
5. Incubate in guinea pig complement for 30 mins at 37°C in 5% CO_2 in air.
6. Wash in DMEM + 20% FBS.
7. Attach to the micrometer syringe apparatus a finely drawn glass pipet that has an internal diameter slightly larger than the inner cell mass (ICM).
8. Draw the embryo into the pipet and expel. If the immunosurgery was successful, the trophoblast cells will lyse, leaving the ICM as a small clump of tightly bound intact cells **(Fig. 1B)**.
9. Using the pipet, transfer the ICM onto the prepared MEF feeder layer. The ICM will usually attach within 24 h and after approximately 72 h, the ICM will have flattened on the feeder layer **(Fig. 1C)**. Four to six days after immunosurgery a small colony will be evident **(Fig 1D)**, and the first passage should be performed (*see* **Note 4**).

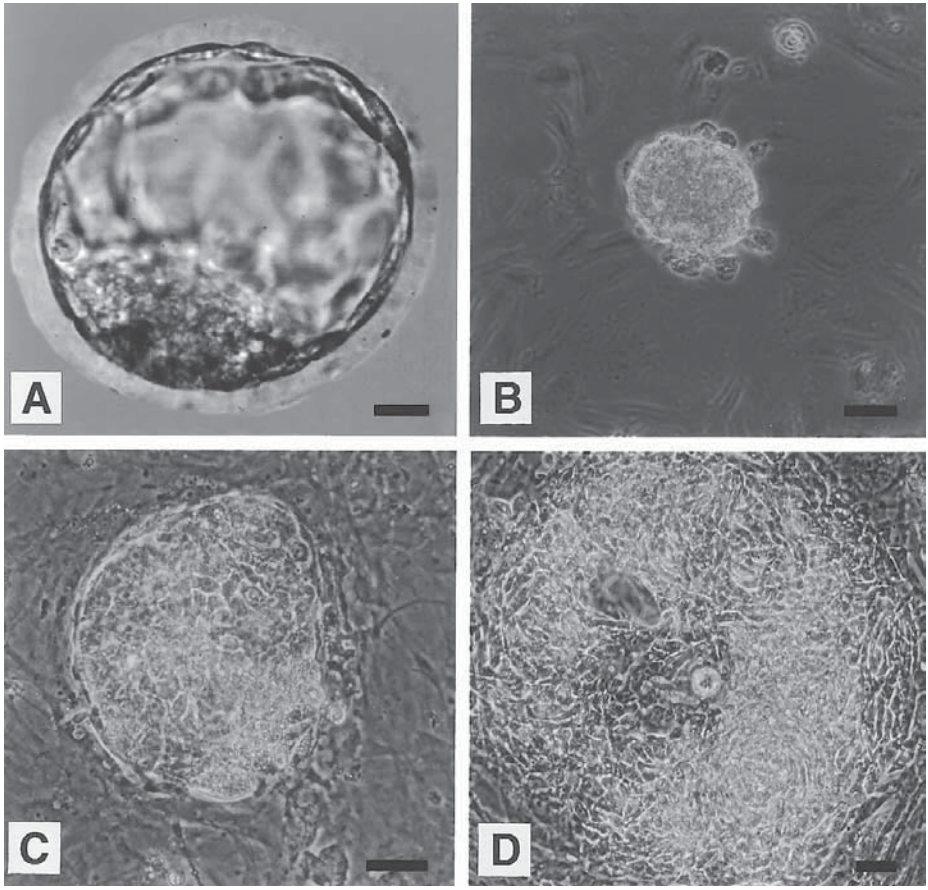

Fig. 1. Primate embryonic stem cell isolation. **(A)** Rhesus blastocyst. Bar = 25 μm. **(B)** Rhesus inner cell mass (ICM) immediately following immunosurgery. Bar = 50 μm. **(C)** ICM 3 d postimmunosurgery, attached to feeder layer. Bar = 50 μm. **(D)** ICM 7 d postimmunosurgery, immediately prior to initial dissociation for ES cell isolation. Bar = 50 μm.

3.3. Passaging Primate ES Cells

1. Remove culture medium.
2. Wash with Ca^{2+}/Mg^{2+}-free PBS with 0.5 m*M* EDTA and 1% FBS.
3. Reapply Ca^{2+}/Mg^{2+}-free EDTA/FBS and observe cells under phase contrast microscopy.
4. When cells show signs of individualization (3–5 min), immediately either:
   a. Aspirate ES cells with a small bore glass pipet attached to a micrometer syringe apparatus and expel onto fresh feeder layers (*see* **Note 5**), or

b. Scrape ES cells with the tip of a glass 5 mL pipet and aspirate. Expel cells into a 15 mL centrifuge tube. Centrifuge at 1000*g* for 5 min in a benchtop centrifuge. Resuspend ES cells in culture medium and plate onto prepared feeder layers.

3.4. Maintenance of Primate ES Cells in Culture

Primate ES cells are difficult to maintain in vitro **(Fig. 2)**. Differentiation of primate ES cells can be minimized by careful attention to detail in all aspects of the culture process:

1. Feed every 2 d and more often as colonies grow.
2. Eliminate differentiated cells from the continuing culture when passaging by selecting individual undifferentiated colonies, as described in **Subheading 3.3., step 4a.** Failure to remove most of the differentiated cells from the culture will result in rapid loss of the culture to complete differentiation.
3. Try to keep time in suspension minimized during all procedures. Primate ES cells fragment and die rapidly when removed from feeder layers.

3.5. Freezing Primate ES Cells

1. Remove cells from the culture plate as for passaging.
2. Spin in a 15 mL tube in a benchtop centrifuge at 1000*g* for 5 min.
3. Remove supernatant.
4. Resuspend in 0.25 mL 20% FBS: 80% DMEM. Add an equivalent volume of 20% DMSO: 20% FBS: 60% DMEM dropwise into the tube, mix and transfer to a 1.5 mL cryogenic vial.
5. Place the cryogenic vial between two polystyrene racks and freeze at –70°C overnight.
6. Transfer to liquid nitrogen for long-term storage.

3.6. Thawing Primate ES Cells

1. Remove cryogenic vial from liquid nitrogen.
2. Gently swirl vial in 37°C water bath until thawed and wash vial in ethanol.
3. Pipet contents of vial up and down once to mix.
4. Place contents of cryogenic vial in a 15mL centrifuge tube.
5. Add an equal volume of ES medium and mix.
6. Spin cells for 5 min at 1000*g* in a benchtop centrifuge.
7. Remove supernatant and resuspend cells in ES medium.
8. Place cell suspension on a culture plate previously plated with irradiated MEF.

3.7. Primate ES Cells for Tumor Formation (see Note 6)

ES cells can be injected into severe combined immunodeficient (SCID) mice for tumor formation. In this environment, undifferentiated ES cells can differenti-

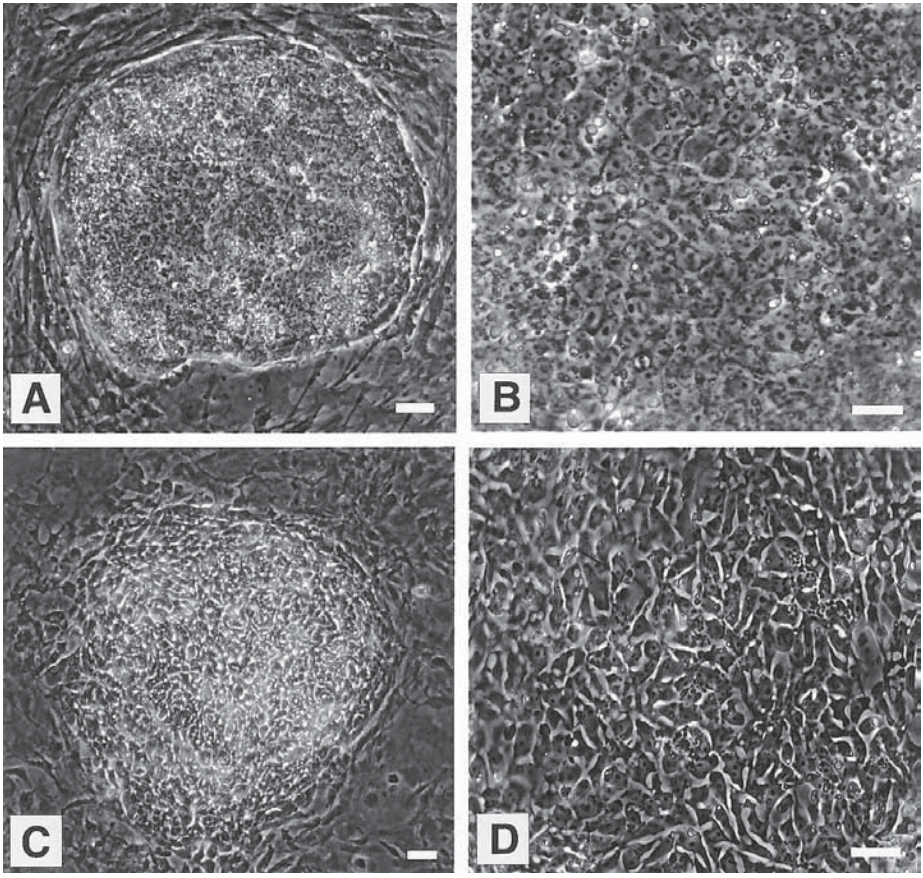

Fig. 2. Primate embryonic stem cells. **(A, B)** Marmoset ES cell colony. **(C, D)** Rhesus ES cell colony. Bar = 50 μm.

ate into cell types and complex structures that may not form if ES cells differentiate in vitro. This provides a way to assess the developmental potential of the ES cells and to study the development of specific cell types or tissues.

1. Culture at least 2×10^6 cells per injection site.
2. Remove cells from culture plate as described in **Subheading 3.3., step. 4a**.
3. Centrifuge gently for 5 min in benchtop centrifuge, resuspend in 0.1 mL culture medium, and place on ice.
4. Load cells into a 1 mL tuberculin syringe.
5. Using a 22 gage needle, inject the cell suspension into the hind leg muscle of a SCID mouse.
6. Observe mouse daily and palpate hind leg weekly. Palpable tumors are usually present within 4 wk.

4. Notes

1. Batches of guinea pig complement may give variable results and must be tested for toxicity.
2. Primate embryonic stem cells require extremely high-quality water for all culture media. We use a Milli-Q filtration system (Millipore), which is sanitized monthly. Batches of water are stored in multiple 2 L bottles and tested in culture medium before use.
3. FBS suitability for culture medium varies among lot numbers and needs to be tested before use. Primate ES cells are particularly sensitive to endotoxin. We test sera of different lot numbers directly on primate ES cells but sera can be tested also by assessing the cloning efficiency of mouse ES cells, grown in the presence of leukemia inhibitory factor (LIF) without feeder layers.
4. We usually perform the first passage within a week of immunosurgery. If this procedure is performed too soon after immunosurgery there will be too few cells, and the culture may be lost. If the first passage is left too long, there is a risk of losing the culture to differentiation.
5. This method is most appropriate when dealing with small numbers of cells, or when it is necessary to select undifferentiated colonies from a partially differentiated culture.
6. Always follow local animal care-and-use protocols.

Acknowledgments

The authors thank Robert Becker for the photograph shown in **Fig. 1A**. This research was supported by NIH grants RR00167 and RR11571-01 (to J.A.T.). This is publication number 40-007 of the WRPRC.

References

1. Thomson, J. A., Kalishman, J., Golos, T. G., et al. (1995) Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* **92,** 7844–7848.
2. Thomson, J. A., Kalishman, J., Golos, T. G., et al. (1996) Pluripotent cell lines derived from common marmoset *(Callithrix jacchus)* blastocysts. *Biol. Reprod.* **55,** 254–259.
3. Braude, P., Bolton, V., and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332,** 459–461.
4. Benirschke, K. and Kaufmann, P. (1990) *Pathology of the Human Placenta*, Springer-Verlag, New York.
5. Luckett, W. P. (1975) The development of primordial and definitive amniotic cavities in early rhesus monkey and human embryos. *Am. J. Anat.* **144,** 149–168.
6. Luckett, W. P. (1978) Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. *Am. J. Anat.* **152,** 59–98.

7. O'Rahilly, R. and Muller, F. (1987) *Developmental Stages in Human Embryos*, Carnegie Institution of Washington, Washington.
8. Kaufman, M. H. (1992) *The Atlas of Mouse Development*, Academic Press, London.
9. Solter, D. and Knowles, B. (1975) Immunosurgery of mouse blastocysts. *Proc. Natl. Acad. Sci. USA* **72,** 5099–5102.
10. Robertson, E. J. (1987) Embryo-derived stem cell lines, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, (Robertson, E. J., ed.), IRL Press, Washington, DC, pp. 71–112.
11. Hogan, B., Beddington, R., Costantini, F., and Lacey, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, N.Y.