Derivation of Mouse Embryonic Stem Cells in Serum-Free and Feeder-Free Conditions

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The availability of murine embryonic stem (ES) cells has revolutionized the study of mammalian development and disease. A culture medium has been developed from the work of Ying *et al.*[1] that has enabled the identification of the essential signaling pathways that are required for maintenance of pluripotency *in vitro*. We have successfully derived and propagated mouse ES cells in the absence of feeder cells and serum using this medium. Here we describe some observations during derivation of new ES cell lines from mouse embryos in these serum and feeder free conditions. This work was used to develop the ESGRO Complete™ Derivation Kit by Stem Cell Sciences and is available from Millipore.

Introduction

ES cells are invaluable as an *in vitro* model for the study of development and as a means to create genetic modifications in mice for the study of gene function and disease. The derivation of ES cell lines is predominately achieved using the inbred 129 strain, as it is the most permissive to derivation. In this system, derivation of ES cells involves subjecting the embryos to delayed implantation or diapause; allowing the embryos to attach to the substrate for a few days, and then disrupting the cell contacts by disaggregation and replating. A major limiting factor in determining the efficiency of ES cell derivation is the quality of the serum used in the derivation process. The composition of serum is ill defined and variable between batches and must be prescreened before use. Also, reliance upon its inclusion in media for derivation and propagation of ES cells does not facilitate study of the minimal requirements and molecular responses involved in self-renewal and differentiation. There is, therefore, a requirement for a system that is reliable and free from batch to batch variation in order to obtain the most efficient procedures for mouse ES cell derivation. Here we describe ES derivation using a serum and feeder-free system developed from the work of Ying *et al.*[1].

DELAYED IMPLANTATION

Isolation of mouse ES cells is most efficient if the embryos are subjected to delayed implantation[2]. Delayed implantation can be induced in mice either by ovariectomy or by intraperitoneal injection of tamoxifen. 129 mice at 2.5 d post coitum (dpc) were subcutaneously injected with Depo-Provera (1-3 mg/mouse -as a source of progesterone) and tamoxifen (10 mg per mouse). The delayed embryos were flushed from the uterus of these mice 4 or 5 days after ovariectomy/tamoxifen injection using ESGRO Complete Basal Medium. Embryos were then transferred into ESGRO Complete Delayed Blastocyst Incubation Medium, a serum-free media containing LIF only, in gelatinized 4-well plates pre-equilibrated to 6% CO_2 and 37°C.

DISAGGREGATION

After 3 to 5 days in culture, outgrowths were disaggregated by gently detaching each cell clump. This was achieved by using a mouth controlled finely drawn plugged Pasteur pipette whose tip diameter was just bigger than the outgrowth. The clumps were transferred to a small (approximately 5 µL) drop of trypsin at 37°C for a few minutes until they began to dissociate. Taking care to minimize the amount of trypsin, the outgrowths were separated individually in a small amount of culture medium using a finely drawn plugged Pasteur pipette. The dissagregated cells were transferred to a new well of a 4-well plate containing ESGRO Complete Clonal Grade Medium as small clumps of about 1-5 cells. ES cell colonies should subsequently become identifiable after about 5 d (Figure 1). ES cell colonies were expanded into 96-well plates by trypsinization of the well, centrifugation (5 min at 1200 rpm) and resuspended in 50 µL of ESGRO Complete Clonal Grade Medium.

ROUTINE PASSAGING OF SERUM-FREE MEDIA DERIVED ES CELLS

To passage the cells from a gelatinized small flask (T25), we added ESGRO Complete Trypsin to cover the cells (about 1 mL) and incubated them at 37°C for a few minutes. We then added 19 mL of ESGRO Complete Basal Medium (without cytokines), to dilute the trypsin. centrifuged, aspirated off the medium completely, and resuspended in 0.5 mL ESGRO Complete Clonal Grade Medium (containing LIF and BMP4) and replated 1-2 x 106 cells into a new gelatinized T25 flask pre-equilibrated to 7% CO2 and 37°C. ES cells were routinely passaged every 2-4 days.

INJECTION OF SERUM-FREE MEDIA DERIVED ES CELLS INTO BLASTOCYSTS TO CONFIRM GERMLINE COMPETENCY

To confirm that ES cells derived in this serum free media are pluripotent, chimeras were made and checked for germline transmission. ES cell lines were trypsinized and the cells resuspended in a small volume (0.5 mL for a slightly sub-confluent T25 flask) of serum-containing medium (the addition of serum to the medium for cells for blastocyst injection reduces stickiness). 10-20 cells were injected into each C57BL/6 host blastocyst. Chimeras were monitored by coat color chimerism and were mated to outbred mice. Successful germline transmission was monitored by the presence of gray mice in subsequent litters (Figure 2).

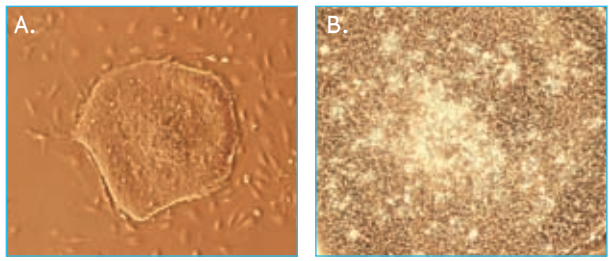

Figure 1. ES cell colonies form 5 days after dissaggregation of outgrowths (A). Undifferentiated ES cells have a distinct nuclear morphology that is easy to identify (B).

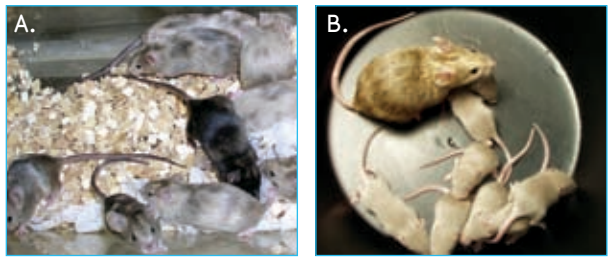

Figure 2. Chimeras produced after microjection of ES cells derived in serum-free conditions (A). Confirmation of germline transmission was seen as gray pups born to outbred mice (B).

Conclusion

The ESGRO Complete serum free media conditions offer an improved system for the efficient derivation, growth and passage of mouse ES cell lines, which avoids problems associated with the use of serum. These ES cell lines maintain their germline competency making this system suitable for gene targeting experiments. In addition, the fully defined conditions allow for a detailed and profound understanding of the conditions and factors needed for derivation of mouse ES cells which may affect the ability to generate ES cells from other strains of mice or other animals.Cells plated in 10% FCS containing medium tend to form colonies with a more irregular morphology and have more colonies that are clearly differentiated. When the plates are stained with alkaline phosphatase, the colonies grown in 10% FCS, in general, show less intense staining with many colonies displaying different levels of alkaline phosphatase staining (data not shown).

References

1. Ying *et al.* (2003). *Cell.* **115(3)**:281-92.
2. Nichols *et al.* (2001). *Development.* **128(12)**:2333-9.

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