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BRIEF METHOD

Efficient Germline Transmission of Mouse Embryonic Stem Cells Grown in Synthetic Serum in the Absence of a Fibroblast Feeder Layer

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Colonization of the germ layer by mouse embryonic stem (ES) cells is essential for the production of knockout mice. The factors affecting germline transmission of ES cells are complex (Brown et al, 1992; Liu et al, 1997); therefore, cells are maintained under optimal conditions by growth on a fibroblast feeder layer and in suitable batch-tested serum (Akiyama et al, 2002; Elder et al, 1998; Harris et al, 2002). However, serum and feeder layer batch testing is time consuming and, because they differ between batches, fully defined culture of ES cells is not possible under these conditions. The culture of ES cells in synthetic serum in the absence of a feeder layer would provide fully defined growth conditions and greatly reduce the time required for ES cell culture. We report here for the first time that the mouse ES cell line E14TG2a (129/OLA derived) grown in synthetic serum (Knockout SR; Invitrogen Corporation) in the absence of a fibroblast feeder layer can result in efficient chimera production and germline transmission. The ES cells could also be efficiently resuscitated following cryopreservation in 10% DMSO/Knockout SR growth medium. The efficiency of germline transmission of E14TG2a in synthetic serum is consistent with that claimed by the serum manufacturer and was increased compared with in-house germline competent ES cell lines grown on a fibroblast feeder layer in batch-tested serum. Feeder-free growth of ES cells in synthetic serum therefore provides a more efficient method for the production of knockout mice, and the fully defined culture conditions will allow the investigation of factors involved in ES cell self-renewal, differentiation, and germline transmission.

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The E14TG2a ES cell line (a gift from Dr. A. Smith, Edinburgh, United Kingdom; also available from American Type Culture Collection [ATCC CRL-1821]) has been used for the production of knockout mice (Koller et al, 1991; Thompson et al, 1989). E14TG2a can be grown in the absence of a fibroblast feeder layer in the presence of leukemia inhibitory factor (LIF) and suitable batch-tested serum (Smith, 1991). The E14TG2a ES cell line was initially grown in medium supplemented with batch-tested fetal calf serum (FCS) and weaned onto medium containing the synthetic serum (SR; Table 3) by subsequent passages in 75/25%, 50/50%, 25/75%, and 0/100% FCS/SR medium on gelatin-treated tissue culture-grade plates (approximately 3×10^6 cells seeded per 9-cm dish; Nunc, United Kingdom; see legend to Table 3). Some differentiation of the cells was apparent upon the initial change of serum (assessed by morphological examination); however, this ceased after several passages. The growth medium was changed daily and the cells incubated at 37° C/5% CO_2 in a humidified atmosphere. The cells were passaged every other day and viewed daily by phase contrast microscopy to ensure undifferentiated growth.

Because synthetic serum does not contain trypsin inhibitors, there is no need to wash the cells with PBS prior to trypsinization. Instead, the medium was removed and 4 ml of trypsin/EDTA solution (Sigma, Dorset, United Kingdom) added to the cells for 10 seconds. All of the trypsin solution was removed and the plates incubated at 37° C/5% CO_2 for a further 30 seconds. At this point, the cells could be easily detached by gently tapping the culture plate. Cells were resuspended in 3 ml of prewarmed culture medium and agitated to ensure a single-cell suspension. The cell suspension was split 1:3 to 1:6 and 8 ml of fresh prewarmed medium added (approximately 3×10^6 cells seeded per 9-cm dish). The cells were expanded to provide adequate stock for future knockout studies and frozen in SR medium (5×10^6 cells/ml) supplemented with 10% v/v DMSO as a cryopreservent (prior to passaging, each 9-cm dish contains approximately

Table 1. Comparison of Germline Transmission Efficiencies of Four ES Cell Lines

	E14TG2a	MESC20	R1	C57/BL6
Blastocysts injected	62	112	50	100
Live pups	30 (48.4%)	39 (34.8%)	27 (54%)	44 (44%)
Chimeric pups	8 (26.7%)	10 (25.6%)	6 (22.2%)	4 (9.1%)
Male chimeras	7 (23.3%)	10 (25.6%)	6 (22.2%)	4 (9.1%)
Germline transmission	4 (13.3%)	2 (5.1%)	2 (7.4%)	1 (2.3%)

E14TG2a ES cells were grown as described in the text and MESC20, R1 (Nagy et al, 1993) and C57/BL6 ES cells were grown in batch tested FCS on primary embryonic fibroblast feeder layers (Elder et al, 1998). Cells were trypsinized, suspended in growth medium and 10–15 cells injected into 3.5 day old blastocysts and implanted into pseudo-pregnant BDF-1 female mice (Hogan et al, 1994; glass capillaries from Clark Electromedical Instruments, Kent, United Kingdom; Axiovert 10 microscope, Carl Zeiss, Herts, United Kingdom; MMO-202ND injection manipulation arm, Narishige Int. Ltd., London, United Kingdom; Kopf 750 pipette puller, Tunjunga, California). Chimeric pups and germline transmission efficiency was determined by mating to a mouse genotype that enabled donor coat color to be distinguished. Mice were housed according to Home Office guidelines (Home Office, 1986) and kept on a 12 hour-light/dark cycle in which the dark period was from 7 PM to 7 AM.

Table 2. Chimera and Germline Transmission Data for E14TG2a ES Cells

Chimera	Litter 1	Litter 2	Litter 3	Total % germline
1 male	0 /10	1 /7	Culled	1/17 (6%)
2 male	0 /4	0 /7	0 /9	0/20
3 male	2 /10	1 /9	7 /7	10/26 (38%)
4 male	No offspring			No offspring
5 male	0 /10	NFO	—	0/10
6 male	1 /15	5 /8	5 /10	11/33 (33.3%)
7 male	8 /8	Culled	—	8/8 (100%)
8 female	No offspring			No offspring

Chimeric mice were produced using E14TG2a ES cells and mated as described in the legend to Table 1. "Culled" refers to the stage at which the mice were killed; NFO = no further offspring.

1×10^7 cells). Frozen E14TG2a ES cell vials were resuscitated by rapid thawing at 37° C and the cells incubated as described above. If the cells were not ready for splitting within 2 days, they were trypsinized and replated in a fresh gelatin-treated dish. For use in gene-targeting methods, the ES cells could be selected by the addition of 350 μg/ml neomycin (G418; PAA, Linz, Austria) to the medium and resistant clones picked by Day 9.

Compared with in-house ES cells cultured using the conventional technique of fibroblast feeder layer and batch-tested FCS (MESC20, C57/BL6, and R1 ES cell lines), E14TG2a was between two and six times more efficient at germline transmission (Tables 1 and 2). Interestingly, the number of chimeras compared with live births was similar in MESC20, R1, and E14TG2a cell lines (25.6, 22.2, and 26.7%, respectively); thus, it is germline transmission rather than chimera formation that is more efficient in the latter cells. The germline efficiency of E14TG2a is also above average compared with independent studies of ES cells grown on a feeder layer in batch-tested serum (Wood et al, 1993, 3.8% germline efficiency; Fedorov et al, 1997, 3.2–8.2% germline efficiency). The results reported here also compare favorably with the data provided by the manufacturer of the synthetic serum (reagent notes to Knockout SR). For example, the efficiency of germline transmission compared with total chimeras (50% for both studies), the range of germline transmission (6–100% and 4–100%, respectively), and the percentage of male chimeras (87.5 and 70%, respectively) is similar between the two studies. Mating of the chimeric progeny (agouti C57/129) mice resulted in BL/6, agouti, and 129 coat colors (ratio 1:3:1 from 10 pups),

Table 3. Culture Conditions for Feeder-Free Growth of ES Cells in Synthetic Serum

Knockout DMEM (Invitrogen Corporation, Paisley, United Kingdom; 10829-018)		500 ml
Serum replacement (Knockout SR; Invitrogen; 10828-028)		80 ml
Nucleosides: Adenosine (Sigma, Dorset, United Kingdom; A4036)	80 mg	6.0 ml
Guanosine (Sigma; G6264)	85 mg	
Cytidine (Sigma; C4654)	73 mg	
Uridine (Sigma; U3003)	73 mg	
Thymidine (Sigma; T1895)	24 mg	
Dissolve in 100 ml ddH_2O at 37°C and filter sterilize		
Sodium bicarbonate (7.5% w/v; filter sterilized)		9.6 ml
L-Glutamine (Invitrogen; 25030-024)		6.0 ml
2-Mercaptoethanol (Invitrogen; 31350-010)		0.6 ml
Nonessential amino acids (Invitrogen; 11140-035)		6.0 ml
ESGRO (LIF; Chemicon Int., Middlesex, United Kingdom, ESG 1107)		1000 units/ml

Cells were incubated at 37°C/5% CO_2 in a humidified atmosphere and passaged as described in the text. Media was prepared fresh under sterile conditions and stored at 4°C for a maximum of 2 weeks. Gelatin-treated plates were made by the addition of 5 mls of 0.1% w/v gelatin (in ddH_2O) to tissue culture grade flasks/dishes and incubated overnight at 4°C. The excess solution was removed, the plates air dried and stored at 4°C for a maximum of 4 weeks.

showing that germline transmission is retained by subsequent progenies (data not shown).

We have successfully weaned four further ES cell lines from the fibroblast feeder layer and FCS growth into SR medium and gelatin-coated plates (MESC20) (Brown et al, 1992), 129 (a gift from Dr. Wolfgang Breitwieser, PICR), C57/BL6 (Kagi et al, 1994), and D3 (American Type Culture Collection). Attempts to simultaneously wean the cells onto SR medium and gelatin plates resulted in significant differentiation of the cells (data not shown). Therefore, ES cells should be weaned first onto the SR medium followed by removal of the fibroblast feeder layer. The mitotically inactive fibroblast feeder layer can be removed by passaging the ES cells without further addition of feeder cells, resulting in feeder-free cultures within two to three passages. When maintained in an undifferentiated state, these ES cell lines exhibited characteristic ES cell colony morphology (Smith, 1991) and expressed various ES cell-specific markers (Oct-3/4, Rathjen et al, 1999; Rex-1, Ben-Shushan et al, 1998; Forssman antigen and SSEA-1, Ling and Neben, 1997), although chimeric studies have not been carried out on these cell lines.

The ability to culture germline-competent ES cells under fully defined growth conditions represents a major advancement in the use and manipulation of these cells. Notwithstanding the decreased time required for ES cell culture, it enables a more systematic approach to the study of these cells. For example, variations between feeder layer and serum batches are eliminated, allowing comparative studies between independent ES cell lines to be carried out. This will allow analysis of factors involved in ES cell growth and differentiation to be investigated and may allow elucidation of the factors responsible for germline transmission in these cells. In summary, the culture of feeder-free ES cells in synthetic serum maintains germline transmission of the cells, provides fully defined growth conditions, and greatly reduces the time required for cell culture.

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