Subcloning of Mouse A3-1 Embryonic Stem Cells

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Abstract: *Three methods for subcloning embryonic stem (ES) cells, namely the isolation, precipitation and colony pickup method, were examined as follows. Isolation method: ES cells were trypsinized and classified morphologically as spherical and unspherical cells. Each type of ES cell was isolated with a micropipette and micromanipulator and was cultured separately. Precipitation method: ES cells were trypsinized and incubated for 15–45 min. Both the adhesive and floating cells were cultured separately. Colony pickup method: morphologically undifferentiated ES cell colonies were picked up with a micropipette. The colony was trypsinized and replated on a new feeder layer. Before the subcloning, there were 30–48% of normal diploid sets of 40 chromosomes in ES cells. Both the isolation and precipitation methods have not shown the effect of subcloning on the karyotype. The most effective subcloning method in this study was the colony pickup method. All of three sublines derived by the colony pickup method showed 68–78% of normal karyotypes and simple embryoid and cystic embryoid bodies were formed 3–4 days and 7–8 days after suspension culture, respectively.*

Key words: *Mouse, Embryonic stem cell, Differentiation, Subcloning.*

Embryonic stem (ES) cells are widely used for making gene disrupted mice by homologous recombination. They are maintained in an undifferentiated pluripotent state by culturing on an embryonic fibroblast feeder layer [1] or in conditioned medium containing differentiation inhibitory activity (DIA) [2]. ES cells spontaneously differentiate in the absence of feeder layer cells or DIA [1, 3]. Although DIA was originally identified as a leukemia inhibiting factor (LIF) [4], factors affecting the maintenance of undifferentiated pluripotency and differentiation of ES cells have not been fully clarified. In general, ES cells are routinly cultured in a medium supplemented with LIF on feeder layer cells. In this culture condition, however, their pluripotency has been lost with extended culture [5, 6]. Therefore, maintenance of an undifferentiated state might be regulated by many unknown factors other than by the LIF. As ES cell lines are difficult to establish in the absence of specialized laboratory facilities, it is important to develop simple and reliable methods to reestablish ES cell lines by subcloning. To date suitable subcloning methods for ES cells have not been fully evaluated. In this paper we compare three different methods for the subcloning of ES cells.

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Materials and Methods

A3-1 ES cells [7], which were kindly supplied by Dr. Toyoda of The Institute of Medical Science, The University of Tokyo, were used in this study. A3-1 cells were cultured in SCM [1] supplemented with 10^3 units of LIF (Amrad, Australia) [2, 4] on mitomycin C-treated feeder layers derived from mouse fetal fibroblasts. The culture medium was changed every day. The ES cells were frozen at every passage and stored as described previously [8, 9]. Subcloning was performed by the three methods described below.

Isolation method: Confluent ES cells were trypsinized in trypsin-EDTA solution [1] and classified morphologically as either spherical or unspherical cells with an inverted microscope (Fig. 1-A). About one hundred ES cells from each morphological group were isolated with a micropipette and a micromanipulator and cultured in 24-well microplates (25820GEL, Corning, USA). When inducing proliferation, ES cells were transferred and cultured in 6-well microplates (25810GEL; Corning, USA) and/or culture dishes (2510GEL; Corning, USA). Spherical ES cells subjected to 2 h of trypsin-EDTA treatment were also isolated with a micropipette and then cultured

as described above. These cells were kept for 2 h at 4°C after the trypsin-EDTA treatment.

Precipitation method: Confluent ES cells were trypsinized and incubated for 15–45 min. Adhesive and floating ES cells were cultured separately. This separation was performed at each passage.

Colony pickup method: ES cells were cultured at a density of 5×10^4 cells/ml to a dish. Morphologically undifferentiated ES cell colonies were picked up with a micropipette at 72 h after culture (Fig. 1-B). The colonies were then trypsinized and cultured in a 24-well microplate. The ES cells before and after the subcloning were evaluated by karyotype analysis [1] and differentiation ability *in vitro* [10].

Chromosome spreads of the ES cells were performed as described in our previous report [11]. ES cells at 2 days after passage were arrested in the metaphase by adding colcemid (0.1 μg/ml at the final concentration) to the culture medium for 1.5 h at 37°C with 5% CO_2 in air. The cells were treated with trypsin-EDTA for 5 min at room temperature. After vigorous pipetting, the single cell suspension was centrifuged at 1,000 rpm for 5 min. The pellet was exposed to hypotonic shock with a 0.56% KCl solution for 30 min at room temperature. After centrifugation at 500 rpm for 5 min, the hypotonic solution was removed, and the pellet was fixed with methanol/acetic acid, 3 : 1 (vol/vol) by gently pipetting. After 10 min the suspension was centrifuged at 500 rpm for 5 min. Fixation treatment was performed twice prior to spreading the cells on slides. The cells were air-dried overnight, stained with fresh 5% Giemsa for 30 min and rinsed with tap water. Before the subcloning, there were 30–48% normal karyotypes (2n=40) in A3-1 ES cells, which had been cultured for several passages in our laboratory.

The differentiating ability of the ES cells was examined in a suspension culture as described below. After trypsin-EDTA treatment of the confluent ES cells, the single cell suspension of the ES cells was cultured at a density of 6×10^6 cells per 10 cm bacteriological dish (9015; Nissui, Japan) in DMEM (430-2100EB; Gibuco, USA) supplemented with 10% fetal calf serum (FCS; JRH Bioscience, USA). The cell aggregates formed in this way are unable to attach to the surface of the bacteriological dish. The cells adhere together and form small clusters within 24 h. The cell aggregates formed by this procedure will quickly delineate a layer of endoderm cells on their surface forming structures termed as 'simple embryoid bodies (SEB)'. When the suspension culture is continued the SEB differentiate further to form 'cystic embryoid bodies (CEB)'. They typically contain

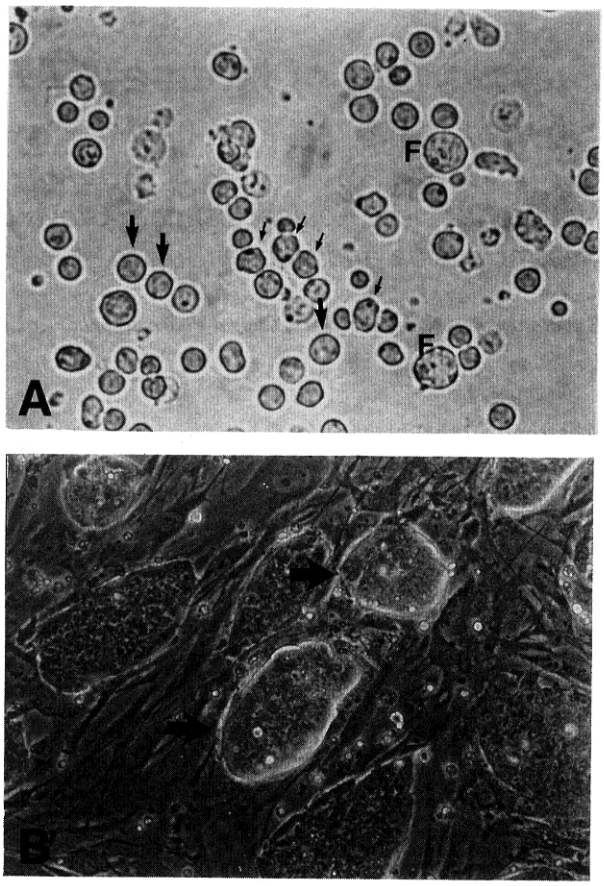

Fig. 1. A: A3-1 ES cell suspension after trypsin-EDTA treatment. Large and small arrows indicate spherical and unspherical ES cells, respectively. F: feeder cell. B: A3-1 ES cells at 2 days after subculture. Arrows indicate morphologically normal and undifferentiated ES cell colonies.

an inner layer of columnar ectoderm-like cells and accumulate fluid in the interior of the structure [1]. The day of the appearance of SEB or CEB was determined by observing 10 or more embryoid bodies per dish [10].

Results and Discussion

Results of subcloning by three different methods are shown in Table 1. In the isolation method, the proportion of the ES cells of the normal karyotype derived from spherical and unspherical cells was 16% in both cases, but spherical ES cells exhibited 51% of the normal karyotype at 2 h after trypsin-EDTA treatment. The proportions of unspherically shaped cells tended to increase with time. In the precipitation method, 46% and 42% of floating and adhesive cells derivatives exhibited the normal karyotype. There was no difference in the

Table 1. Karyotype and *in vitro* differentiation ability of A3-1 ES cells before and after subcloning

Method	Type of selection	After subcloning					Before subcloning				
		% of chromosome no.			Appearance of embryoid body in culture (days)		% of chromosome no.			Appearance of embryoid body in culture (days)	
		<40	40	>40	SEB**	CEB***	<40	40	>40	SEB**	CEB***
Isolation											
	Spherical cell	6	16	78	-	-	14	40	46	-	5
	Unspherical cell	16	16	68	-	-					
	Spherical cell*	0	51	49	-	-					
Precipitation											
	Floating cell	18	46	36	3	7	35	48	17	3	6
	Adhesive cell	18	42	40	3	7					
	Floating cell	38	24	38	4	6	12	30	58	3	5
	Adhesive cell	36	24	40	5	8					
Colony pickup		22	68	8	3	7	35	48	17	3	6
		16	72	12	4	8					
		18	78	4	3	7					

*: Showing at 2 h after Trypsin-EDTA treatment. **: Simple embryoid body. ***: Cystic embryoid body.

distribution of 40, less than 40 and more than 40 chromosomes in ES cells before and after the subcloning by the precipitation method. On the other hand, when morphologically undifferentiated ES cell colonies were picked up and cultured after trypin-EDTA treatment, the proportion of the normal karyotype in all of three ES cell types subcloned increased significantly. The derivatives of three colonies exhibited 68%, 72% and 78% normal karyotype, respectively.

There was a tendency to delayed appearance of both the SEB and CEB after the subcloning by these three methods.

One of the most important features of ES cell lines is that they universally exhibit a high differentiation ability. ES cells will differentiate readily in the absence of chemical inducers. The cells are induced to form endoderm after segregating them from their substrate into a suspension culture. Our previous datum has indicated that the karyotype and differentiation ability of ES cells *in vitro* influenced their germ-line contribution in chimeric mice [10].

In the isolation method, the selection of both the spherical and unspherical cells after trypsin-EDTA treatment did not increase the normal karyotype. But when the spherical cells at 2 h after trypsin-EDTA treatment were isolated and cultured, the proportion of normal karyotypes among ES cells was slightly increased and the cells having less than 40 chromosomes were decreased. This indicates that the karyotype abnormality was reflected in the cell shape. It appeared that the ES cells having less than 40 chromosomes were also not spherical. There were no significant differences in the proportion of normal karyotypes before and after the subcloning in the precipitation method. It appeared that the adhering ability and/or specific gravity of the cells do(es) not reflect the karyotype.

The most effective subcloning method in this study was the colony pickup method. The proportion of normal karyotypes among ES cells increased 20–30% with this method. In our previous data regarding A3-1 ES cells, it was indicated that the karyotype and *in vitro* differentiating ability of the ES cells influenced their germ-line contribution in chimeric mice. Both exhibiting approximately 40% of the normal karyotype and showing CEB formation at 7 days after suspension culture have been capable of indicating their germ-line transmission ability in chimeric mice [10]. After subcloning by means of the colony pickup method, two of three ES cell sublines colonies included 68 and 78% normal karyotypes, and the SEB and CEB were formed 3 days and 7 days after the suspension culture. These properties may be important for germ-line transmission in chimeric mice.

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