Germ-Line Contribution of Embryonic Stem Cells in Chimeric Mice: Influence of Karyotype and In Vitro Differentiation Ability

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Abstract: The effect of the karyotype and the ability to differentiate in vitro upon germ-line transmission by A3-1 embryonic stem (ES) cells in chimeric mice were examined. Germ-line transmission was confirmed in ES cells exhibiting 38% and more of the normal karyotype, but no chimeric mice and/or germ-line transmitters were observed regardless of the karyotype when the cystic embryoid body (CEB) was formed on day 8 and later in the suspension culture. Germ-line transmission of the ES cells was not significantly influenced by formation of the simple embryoid body (SEB). Germ-line transmitters were preferentially observed in chimeras when the ES cell contribution to coat color was markedly increased, but this contribution to coat color varied regardless of the karyotype or in vitro differentiation ability. These results suggest that A3-1 ES cells which exhibit CEB at 7 days after suspension culture and approximately 40% of normal karyotype are capable of germ-line transmission in chimeric mice.

Key words: chimera, ES cells, germline transmission, mouse

Introduction

ES cells are established as a cell line from the inner cell mass cells of a mouse blastocyst [5, 9] and universally exhibit a high differentiation ability. Their potential to differentiate *in vivo* makes them useful for

the preparation of gene disrupted mice by homologous recombination. When injected into a host blastocyst or aggregated with a host embryo, ES cells are capable of contributing to a wide variety of somatic tissues and germ cells in resultant chimeras [3].

Differentiation of ES cells will occur readily in the

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absence of chemical inducers. It can also be stimulated experimentally either *in vivo*, by their inoculation into syngenic host animals, or *in vitro*, by detachment from their substrate into a suspension culture to form endoderm cells [13]. To maintain their pluripotency, the ES cells are routinely cultured in a medium supplemented with leukemia inhibitory factor (LIF) [14, 23] on mouse fetal fibroblasts as feeder layer cells [13]. In general, ES cell lines at early cell culture passages exhibit pluripotency, but this and/or euploid chromosome constitution are generally lost after sustained periods under these conditions [11, 12]. Nevertheless a few exceptions do exist, namely, TT2 ES cell line which can maintain pluripotency after multiple passages [21].

It is widely accepted that the extent to which ES contribute to coat color indicates their ability to evoke germ-line transmission in chimeric mice. Germ-line contribution of the ES cells can not be conclusively identified without progeny testing of resulting chimeras, a process requiring culture of ES cells, collection of embryos, production and subsequent mating of chimeric mice, testing in each respective ES subline, and much time and effort. This process would be clearly facilitated if a direct relationship between the germ-line contribution of ES cells in chimeras, and respective karyotype and differentiation ability was established *in vitro*.

In this study, we examined ES cells with regard to the influence of karyotype and the ability to differentiate *in vitro* on their germ-line contribution in chimeric mice.

Materials and Methods

Cell culture: ES cells used in this study were A3-1 [2] derived sublines [8, 18 and our unpublished data] which were genetically manipulated and/or cultured for several passages. Identification numbers with E-, S- or G- in Tables 1 and 2 indicate the manipulated ES sublines. ES cells were cultured in SCM [13] supplemented with 10^3 units of LIF (Amrad, Australia) [14, 23] on mitomycin C-treated feeder layers derived from mouse fetal fibroblasts at 37°C in 5% CO₂ in air. The culture medium was changed every day. ES cells were frozen at every passage and stored as described previously [16, 20].

Karyotype analysis: Chromosome spreads of the ES cells were performed as described below. ES cells at 2 days after passage were arrested in the metaphase by adding colcemid (0.1 µg/ml at a final concentration) to

the culture medium for 1.5 hr at 37°C in 5% CO₂ 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 220 G for 5 min. The pellet was exposed to hypotonic shock by 0.56% KCl solution for 30 min at room temperature. After centrifugation at 90 G for 5 min, the hypotonic solution was removed, and the pellet fixed with methanol/acetic acid, 3:1 (vol/vol) via gently pipetting. After 10 min the suspension was centrifuged at 90 G for 5 min. Fixation was performed twice prior to spreading the cells on slides. The slides were air-dried overnight, stained in freshly made 5% Giemsa for 30 min and rinsed with distilled water.

Differentiation ability *in vitro*: The differentiation ability of the ES cells was examined by a suspension culture as described below. After trypsin-EDTA treatment of the confluent ES cells, the resultant single cell suspension was cultured at a density of 6×10^6 cells per 10 cm bacteriological dish (9015; Nissui, Japan) in DMEM (430-2100EB; Gibco, USA) supplemented with 10% fetal calf serum (FCS; JRH Bioscience, USA). Cells treated in this way did not attach to the bacteriological dish; instead, they aggregated together and formed small clusters within 24 hr. These cellular aggregates quickly delineate a layer of endoderm cells on their surface forming structures termed 'simple embryoid bodies' (SEB). When suspension culture was continued, the SEB differentiated further to form 'cystic embryoid bodies' (CEB). These typically contained an inner layer of columnar ectoderm-like cells and accumulated fluid in the interior of the structure [13]. The designated day for the appearance of SEB or CEB was determined by observing 10 or more embryoid bodies per dish.

Generation of chimeric mice: The chimeric embryos consisted of C57BL/6J embryos (CLEA, Japan) and ES cells were produced by both the injection [4] and coculture [16, 20, 24] methods.

In the injection method, host blastocysts were recovered from the uterus of natural mated C57BL/6J females at 3.5 days post-coitum. After trypsinization and collection into the injection pipette, from 10 to 15 ES cells were introduced into the blastocoel cavity of an expanded blastocyst previously immobilized by suction on the end of the holding pipette. Manipulated embryos were then cultured in Whitten's medium [22] supplemented with 100 µM EDTA [1, 7, 15] at 37°C in

5% CO₂ in air for 1–2 hr, and were then surgically transferred into the uterine horns of ICR recipients (CLEA, Japan) on day 2.5 of pseudopregnancy [17].

In the coculture method, zona-pellucida of *in vitro* or *in vivo* fertilized C57BL/6J embryos at between the 8-cell and morula stage were removed by treatment with acidified Tyrode's solution [6] or 0.5% pronase (Actinase E; Kakenseiyaku, Japan) [10]. Between 8 and 20 zona-free embryos were transferred into 15 μ l of frozen-thawed ES cell suspension covered with paraffin oil (Art 7162; Merck, USA) and then cocultured at 37°C in 5% CO₂ in air for between 3 and 3.5 hr [16, 20]. The ES cell suspension was prepared at a density ranging from 5–50 $\times 10^5$ cells/ml. After cocultivation, embryos were transferred into Whitten's medium supplemented with 100 μ M EDTA and cultured overnight. The blastocyst and morula stage embryos were subsequently transferred into the recipients as described above. The results of both injection and coculture methods were combined.

Newborns were delivered by cesarean section or natural parturition. Chimeras were identified by coat color and eye pigmentation; host embryos were black, whereas ES cells were derived from albino embryos. To confirm a germ-line contribution of the ES cells, mature male chimeric mice were mated with MCH:ICR (CLEA, Japan) mice. When no vaginal plug could be detected during several weeks of mating, *in vitro* fertilization was performed by the method of Toyoda *et al.* [19]. Phenotypic female chimeras were not tested as to the germline contribution of ES cells. Germ-line transmission of the ES cells in chimeras was confirmed by the coat color of their respective progeny.

The relationship between the ES cell contribution to coat color and germline transmission of ES cells in chimeras was analyzed by the Tukey-type test for non-parametric multiple comparisons. The correlation between the ES cell contribution to the coat color of germ-line chimeras and the percentages of newborns carrying genetic materials of ES cells was established by the least squares method.

Results

Twenty-four ES cell lines examined for karyotype and differentiation *in vitro* exhibited between 11–72% of the normal karyotype (2n=40); SEB and CEB were formed between 3–6 days and 5–11 days in suspension

culture, respectively (Table 1). Furthermore, chimeric mice and germ-line transmitters were obtained from ES cells exhibiting over 28% and 38% of normal karyotype, respectively. When the CEB was formed in the suspension culture on day 8 and after, chimeric mice and/or germline transmitters were not observed regardless of their karyotypes (G-22, G-20, S-164-2, S-164-1, S-7). No correlation was observed between SEB formation and germline transmission of ES cells. Germline transmission was observed when the ratio of phenotypic males with chimeras was 73% and over.

The relationship between *in vivo* differentiation of ES cells and, karyotype and CEB formation are shown in Fig. 1. With regard to A3-1 ES cells, 40% of normal karyotype and appearance in CEB on day 7 of culture were necessary to obtain a germline transmitter.

The production efficiency of chimeric mice and their ES cell contribution are shown in Table 2. Rates of development into chimeric mice ranged between 0 and 31% of transferred embryos. Unexpectedly, S-164-1 and G-22 exhibited 53–72% of normal karyotype (Table 1), but none of the transferred embryos developed to chimera. Formation of CEB in both S-164-1 and G-22 occurred between 8–10 days after the suspension culture (Table 1). The contribution of ES cells to coat color varied in chimeras regardless of the particular ES cell lines used, but a high contribution (over 90%) of ES cells to coat color was not observed in ES cell lines exhibiting 35% or less of normal karyotype (Tables 1 and 2).

Of 78 chimeras tested, 26 failed to reproduce after natural mating. Of these, 12 of 23 reproductively deficient chimeras produced offspring as a consequence of *in vitro* fertilization. One of these chimeras was a germline transmitter.

We analyzed the relationship between the ES cell contribution to coat color and germ-line transmission in male chimeras which were tested both by natural mating and *in vitro* fertilization, as shown in Table 3. Out of 22 chimeras, 12 (55%) in which ES cells contributed 80% and more to coat color, were germline transmitters. This frequency was significantly higher than that observed in chimeras where ES cell contribution to coat color was below 80% ($P<0.05$). Among germ-line transmitters, the lowest contribution of ES cells to coat color was 30%. The frequency of infertility in chimeras increased with the increasing

Table 1. Karyotype analysis and *in vivo* and *in vitro* differentiation of embryonic stem cells

Line	% of Chromosome no.			Appearance of embryoid body in culture (days)		Chimera (% of M)	Germ-line transmission
	<40	40	>40	SEB ¹⁾	CEB ²⁾		
A3-1-1	28	45	27	3	6-8	+(75)	+
A3-1-2		24		3	6-8	+(52)	-
G-4	18	72	10	4	7	+(71)	-
G-22	22	72	6	3	8	-	
G-20	27	69	4	5	11	+(50)	-
S-164-2	26	68	6	4	8	+(25)	-
G-7	30	64	6	3	7	+(73)	+
S-327	29	64	7	3	5	+(75)	+
S-307	29	61	10	3	7	+(100)	+
G-3	30	60	10	4	7	+(75)	+
S-164-1	20	53	27	4	8-10	-	
E-83	28	48	24	4	7	+(75)	-
S-237	17	41	42	3	7	N.T.	
E-6	49	40	11	4	7	+(100)	+
E-31	38	38	24	4	7-8	+(90)	+
S-97	32	35	33	4	7	+(67)	-
E-33	40	34	26	4	7	-	
S-23	12	30	58	3	5	+(56)	-
S-296	6	28	66	3-4	6-7	+(63)	-
E-87	28	24	48	4	10	N.T.	
G-95	19	24	57	6	11	N.T.	
S-7	41	22	37	4	8-9	-	
S-155	22	16	62	3	8	N.T.	
S-287	87	11	1	3	7	N.T.	

¹⁾Simple embryoid body. ²⁾Cystic embryoid body.

contribution of the ES cells ($P<0.05$). The germ-line transmitters passed on the ES cell derived material to between 3 and 100% of their progeny. These percentages did not correlate with the ES cell contribution to coat color ($R^2<0.5$ Fig. 2).

Discussion

Robertson first described a method to evaluate differentiation ability of ES cells *in vitro* [13]. With this method, cellular aggregates were not broken up by pipetting after trypsin treatment [13]. It is difficult to apply to quantitative analysis, since the numbers of cells in a clump can not be controlled before the suspension culture. We therefore modified her method in which the differentiation ability was evaluated by the formation of embryoid bodies from single cell suspension. When the single ES cells were suspended in culture

medium and cultured further, it was observed that cells did not only divide but they also adhered together and formed small clusters within 24 hr. Furthermore, we found that the appearance of 10 or more CEBs indicate that ES cells were able to form chimeras.

Except for a few sublines, germ-line transmission was confirmed in ES cell lines exhibiting approximately 40% of normal karyotype and showing a CEB formation after 7 days of suspension culture (Fig. 1). No chimeras were obtained from S-164-1 or G-22 subclones. These subclones, which exhibited 53-72% of normal karyotype, formed CEB on day 8-10 after the suspension culture. These results indicate that prolonged CEB formation reflects the failure of differentiation *in vivo* in ES cells regardless of their karyotypes (Table 1).

Germ-line transmission was not observed in A3-1 sublines when the ratio of phenotypic males with chimeras

Fig. 1. Relationship between karyotype and days for cystic embryoid body formation on the chimerism of ES cells. Double-circles indicate that at least one germ-line transmitter was obtained. Circles indicate that chimeric mice were obtained, but no germ-line transmission of the ES cells was observed. Crosses indicate that no chimeras were obtained.

Table 2. Results of ES cell contribution in chimeric mice

Line	No. of embryos transferred	No. of chimeras obtained (%)	Contribution of ES cells in coat color (%)	
			Male ¹⁾	Female
A3-1-1	100	20 (20)	10, 10, 40, 50, <u>70</u> , <u>70</u> , 80, 80, 80, 80, <u>90</u> , 90, <u>100</u> , 100, ?	10, 20, 60, 80, ?
A3-1-2	83	25 (30)	10, 10, 20, 20, 20, 20, 20, 40, 50, 60, ?, ?, ?	5, 5, 5, 5, 10, 10, 20, 50, 60, 60, ?, ?
G-4	88	7 (8)	5, 20, 40, 75, ?	5, 5
G-22	20	0 (0)		
G-20	34	2 (6)	10	5
S-164-2	137	4 (3)	10	10, 30, 70
G-7	119	11 (9)	60, 70, <u>85</u> , 85, <u>100</u> , 100, 100, 100	95, 100, 100
S-327	273	8 (3)	5, 5, <u>30</u> , <u>30</u> , 80, ?	10, 80
S-307	24	7 (29)	10, 20, 40, 50, 75, <u>95</u> , 95	
G-3	196	4 (2)	30, 70, <u>100</u>	95
S-164-1	100	0 (0)		
E-83	42	4 (10)	5, 5, 10	50
E-6	136	3 (2)	<u>95</u> , <u>100</u> , 100	
E-31	32	10 (31)	10, 40, <u>50</u> , 70, 80, 90, <u>95</u> , 95, 95	60
S-97	429	9 (2)	10, 50, 65, 70, ?, ?	20, 65, ?
E-33	111	0 (0)		
S-23	75	9 (12)	10, 20, 50, 70, 80	10, 30, 50, 70
S-296	89	8 (9)	10, 20, 40, 60, 70	30, 50, 60
S-7	74	0 (0)		

¹⁾Germline transmitters are underlined. ?: unknown. These newborns were classified as chimera by confirmation of eye pigmentation, but they died soon after birth.

was below 71% (Table 1). In explanation, the sex chromosome of A3-1 ES cells is XY [2], thus it is suggested that the appearance of phenotypic female chimeras at high frequency may reflect the low contribution to the germ line of chimeras. Thirty-three percent of male chimeras were not fertile in natural mating. Furthermore,

no progeny were obtained from 48% of these reproductively deficient chimeras even by *in vitro* fertilization. A major cause of the failure of *in vitro* fertilization was that the sperm were not collectable from the cauda epididymis in these chimeras. The high frequency of reproductive inefficacy where ES cells contributed profoundly to coat color may be an indication of functional damage to ES cells due to extended culture *in vitro* regardless of any genetical manipulations (Table 3).

The degree to which ES cells affected coat color was reflected by their contribution to the germ-line of chimeric mice. When ES cell derived coat color accounted for between 80 and 100% in chimeras, germ-line transmission occurred in over half of these mice (Table 3). Unexpectedly, as observed in the progeny tests, the contribution of ES cells to coat color did not correlate with that to germ cells (Fig. 3). Unfortunately, it was not possible, from this data alone, to interpret the factors which affect newborns obtained from ova fertilized with ES derived spermatozoa. Further studies of spermatogenesis would be necessary that account for the effects

Table 3. Relationship between ES cell contribution to coat color and germline transmission in male chimeras

% of ES cell contribution	No. of male chimeras examined	No. (%) of infertile ¹⁾	No. (%) of germline transmitters
5-29	24	0 (0) ^a	0 (0) ^a
30-79	22	1 (5) ^{a,b}	5 (23) ^a
80-100	22	5 (23) ^b	12 (55) ^b
Total	68	6 (9)	17 (25)

¹⁾When newborns were not obtained by both natural mating and *in vitro* fertilization, the chimeras were classified as infertile. Values with different superscripts are significantly different in the same column at $P < 0.05$.

Fig. 2. Relationship between ES cell contribution to coat color of germ-line chimeras and percentage of newborns carrying ES cell derived genetic materials.

of differentiation and self replication of spermatogonia which ultimately differentiate into spermatocytes.

These results suggest that the karyotype and *in vitro* differentiating ability of the A3-1 ES cells influence their germ-line contribution in chimeric mice. Both exhibiting approximately 40% of normal karyotype and showing CEB formation within 7 days after suspension culture are possible indication of their germ-line transmitting ability in chimeric mice.

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