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Trisomy 8 does not affect differentiative potential in a murine parthenogenetic embryonic stem cell line

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Abstract

Murine parthenogenetic embryonic stem (ES) cell lines expressing *lac Z* reporter gene were isolated after co-transfection with *lac Z* reporter gene (pENL) and *neo^R* gene (pSTneo) to TMA-48P cell line of 129/Sv origin. Karyotype analyses showed that all of four transfected cell lines examined contained 41 chromosomes with trisomy 8. Bacterial *neo^R* transgene required for G418 selection were integrated into several chromosomes including chromosome 8. Histological studies of teratomas formed in syngenic mice and embryoid bodies grown *in vitro* showed that the differentiative potential remained almost identical in chromosomally normal parental cell line and its derivative cell lines trisomic for chromosome 8.

Key words: embryonic stem cells, parthenogenesis, transfection, trisomy 8

Introduction

The remarkable pluripotency of ES cell derived from inner cell mass of blastocysts^{6,17)} has made them convenient vectors for exogenous genes into mouse germ line, and for the functional loss of specific genes through homologous recombination or gene targeting^{4,27)}. Recently, ES cells with genomes from only one parent have been used for the investigation of genomic imprinting mainly through the assessment of the ability of their contribution to chimeras^{16,24)}. It has been shown that the androgenetic ES cells lacking the maternal genome contribute mainly to mesodermal tissues such as bones and muscles, whereas gynogenetic or parthenogenetic ES

(PGES) cells lacking the paternal genome contribute to various tissues as a minor component except tissues of mesodermal origin^{1,15)}. These findings are in agreement with the data obtained from studies on chimeras generated by aggregation of embryos^{7,19)}.

It has been broadly recognized that the cell marker is a convenient tools for tracing the fate of single cells and the distribution of their descendants in the embryos. Over the decades, various cell markers, such as malic enzyme, glucose phosphate isomerase and horseradish peroxidase, have been developed¹⁹⁾. For the ES cells in chimeric studies, it is desired that the marker should be localized, stable, cell autonomous, ubiquitous and easily detectable. The *lac Z*

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gene coding for *E. coli* β -galactosidase is compatible with ubiquitous expression and easily visualized in cells and tissues by simple histochemistry^{2,13}. We tried to introduce the bacterial *lac Z* reporter gene into a PGES cell line in preparation for studying behavior of PGES cells in chimeras *in vivo* and *in vitro*. This report deals with properties of four *lac Z*-positive cell lines isolated after transfection.

Materials and Methods

Cell lines and plasmids

TMA-48P cells, a PGES cell line which is derived from an ethanol-activated diploid 129/Sv blastocyst were used for transfection²³. NR2 cells (a kind gift from Dr. M. A. Surani, Wellcome/CRC Institute, UK) expressing *lac Z* reporter gene, are derived from a male transgenic mouse line¹. ES cells used in the present study were maintained in an undifferentiated state on the mitomycin C (Kyowa, Japan) treated STO cell feeder layer in Dulbecco's Modified Eagle's Medium (DMEM, Dainippon Pharmaceutical Co.) supplemented with 10% fetal bovine serum containing 1×10^3 unit/ml of recombinant leukemia inhibitory factor (LIF, Amrad) on collagen coated dishes at 37°C, in humidified air containing 5% CO₂²⁵. For electroporation, ES cells grown without feeder layer were trypsinized and resuspended in phosphate-buffered saline (0.1 M PBS, containing NaCl 8.0 g/l, KCl 0.2 g/l, Na₂HPO₄ 1.44 g/l and KH₂PO₄ 0.24g/l). The plasmid, pENL containing *lac Z* gene under the control of elongation factor 1 α promoter (a generous gift from Dr. Kazunori Hanaoka, Kitasato University) and pSTneo¹¹ (a kind gift from Drs. Kazuto Katoh and Hisato Kondoh, Osaka University) as a selection marker were used in this study.

Transfection and cell line isolation

Electroporation was carried out in PBS as previously described²⁷, using Shimadzu GTE-1 electroporator. TMA-48P cells (4×10^6) were submitted to an electric discharge (400 V and 500

V; 1 msec in pulse width; 7 in pulse number without interval) in the presence of linearized 25 μ g pENL and 2.5 μ g pSTneo plasmids. Selection was initiated 24 hr after electroporation with 250 μ g/ml of Geneticin (G418, Sigma). G418-resistant (*neo*^R) colonies appeared after 10 days of selection and they were transferred individually onto 24-well tissue-culture plates. Each well was subcultured into two wells and X-gal staining was performed on one of the wells as described by Hogan *et al.*¹⁰. Briefly, cells fixed with 4% paraformaldehyde were washed in PBS containing 0.02% NP40 and 0.01% sodium deoxycholate, and stained for the expression of β -galactosidase by incubation in 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.02% NP40 and 0.01% sodium dexoycholate, and 1 mg/ml X-gal in PBS, at 37°C overnight. β -galactosidase positive cell lines were subcultured for karyotyping and cryopreservation.

Chromosome analysis

ES cells were allowed to incorporate 5-bromo-2'-deoxyuridine (BrdU: 100 μ g/ml) for 7–8 hr at 37°C, in an atmosphere of 5% CO₂ in air. Colcemid (0.1 mg/ml) was present in the culture medium during the last 1 hr of incubation. The labeled ES cells were trypsinized and treated with hypotonic treatment in 0.075 M KCl solution for 10 min, and fixed in methanol-acetic acid (3 : 1). Chromosome slides were prepared by a routine air-drying method, stained with acridine orange and examined under a fluorescence microscope. Chromosome slides were also stained with Hoechst 33258 (50 μ g/ml) for Q-banding in McIlvaine buffer for 10 min, dried in air, mounted with 50% glycerol and examined under a fluorescence microscope.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was carried out on R-banded metaphase chromosomes prepared from one of 4 transfected cell lines, which incorporated BrdU. After denaturation of DNA *in situ* hybridization was

carried out with pSTneo probe labeled with biotin-16-dUTP (Boehringer Mannheim) by nick translation for 36 hr at 42°C. The biotinylated probes were detected after binding with anti-biotin antibody (Boehringer Mannheim) coupled to FITC (Biomed) under a fluorescence microscope.

Differentiation of ES cells

Embryoid bodies (EBs) were produced as described before^{18,23,25}. About 1×10^7 ES cells grown without the feeder layer in an undifferentiated state were trypsinized and seeded onto bacteriological petri dishes to which they did not adhere. EBs were cultured in DMEM supplemented with 10% fetal bovine serum without LIF. To produce tumors, six- to eight-week-old male 129/Sv mice were used for transplantation. Five to ten EBs produced by method described were selected by the morphology and size of consistence at given stage of culture days. These EBs were implanted under the kidney capsule using general surgical procedures⁵. Mice were sacrificed 4 weeks after the transplantation. Tumors thus obtained were fixed with 4% (w/v) paraformaldehyde in PBS, embedded in paraffin and sectioned for histology.

Histological examination of embryoid bodies and tumors

All EBs and tumors were fixed in PBS containing 4% (w/v) paraformaldehyde. For detection of β -galactosidase activity, EBs were stained in X-gal reaction mixture mentioned above. For a histological examination, EBs and tumors embedded in paraffin were sectioned at 3 mm in thickness, and stained with haematoxylin and eosin. β -galactosidase activity in tumors was detected after sectioning by X-gal histochemistry.

Results

Isolation of β -galactosidase positive cell lines

Selection with G418 was initiated 24 hr after electroporation. The cells which did not in-

corporate pSTneo began to be eliminated and the dead cells and cell debris were abundant 24 hr after addition of G418. After 10 days of selection, *neo*^R colonies were large enough for expansion. Among 139 *neo*^R cell lines picked up, 9 cell lines were *lac Z* positive. Four of nine cell lines showed patched staining pattern after several passages *in vitro* and the remaining five cell lines were stained stably. Four of these five cell lines (T48PZ4, 5, 6 and 8) were used in this study.

Chromosome and FISH analysis

The parental TMA-48P cells had 40 chromosomes including two synchronously replicating X chromosomes. However, the modal chromosome number shifted to 41 in some *neo*^R cell lines. In T48PZ4, out of 103 cells examined 75 (72.8%) had 41 chromosomes, and only 4 (5.6%) had 40 chromosomes at passage 5. Karyotype analysis of TMA-48P and T48PZ4 cells is summarized in Table 1. A total of 58 (80.5%) out of 72 cells karyotyped had trisomy 8 as shown in Figure 1. The same karyotype was also prevalent in remaining cell lines: 72.7% (8/11) in T48PZ5, 83.8% (15/18) in T48PZ6 and 76.2% (16/22) in T48PZ8. Sex chromosome constitution was relatively stable in these cell lines; loss of one X chromosome was found in 2.8% (2/72) of T48PZ4 cells and deletion of a single X chromosome was found in two T48PZ6 cells. FISH using pSTneo as a probe revealed that in T48PZ4 the plasmid was integrated mainly on chromosome 8B, but signals were also found on chromosomes 2E, 5E, 14D, and 16C (data not shown).

Differentiative potential of PGES cell lines expressing β -galactosidase

No apparent histological difference was found in 7–10 days old EBs formed by TMA-48P cells and those derived from T48PZ4 cells. Typical EBs consisted of the sparse outer endodermal layer and the poorly organized inner core with a plenty of dead cells at the center. EBs derived from fertilized ES cells, NR2, were usually cystic with a well formed columnar

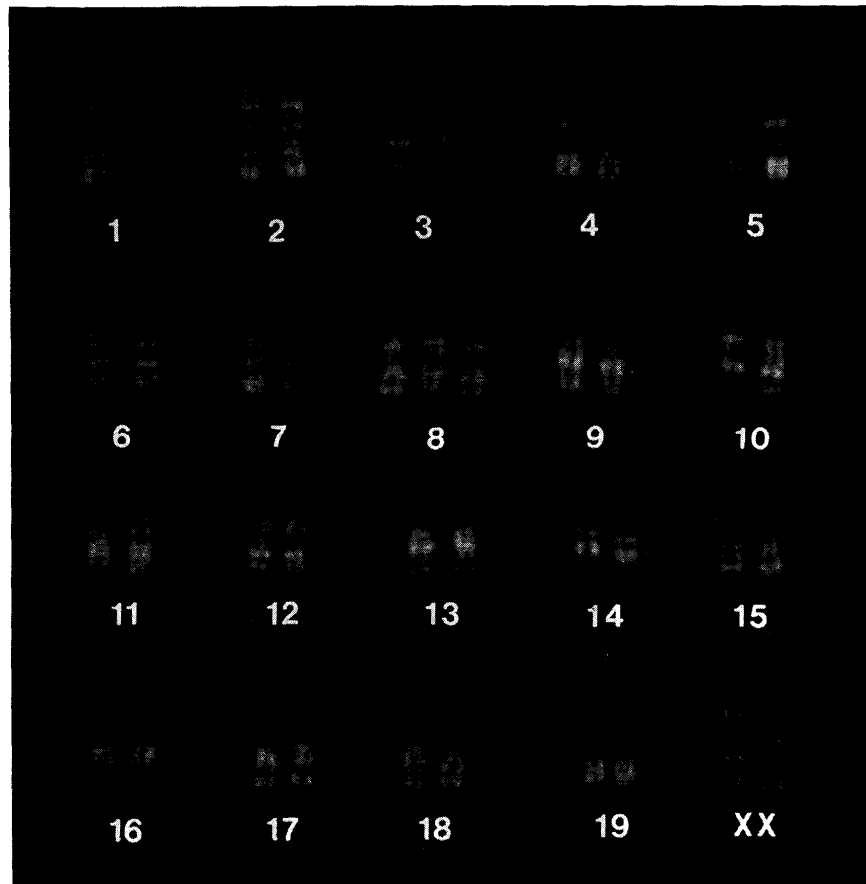


Figure 1. A replication banded karyogram from transfected cell line, T48PZ4 stained with acridine orange, showing trisomy 8.

Table 1. Karyotype analysis of TMA-48P and T48PZ4 cells

Cell lines	No. of cells examined	No. & (%) of cells with different chromosome numbers			
		39	40	41	42
TMA-48P	247	16(6.5)	183(74.1)	43(17.4)	5(2.0)
T48PZ4	103	10(9.7)	4(5.6)	75(72.8)	14(13.6)

epithelial tissues (Fig. 2; A, B, and C). Histological examination (Fig. 2; D, E, and F) showed that teratomas derived from TMA-48P and T48PZ4 cell lines contained various tissues derived from ectodermal and endodermal origin, with reduced contribution to mesodermal derivatives, compared to teratomas derived from fertilized ES cell lines (Table 2). T48PZ4 and NR2 cells and their derivatives in both EBs and teratomas were positively stained with X-gal (data not shown).

Table 2. Tissue composition in tumors from NR2, TMA-48P and T48PZ4 derived embryoid bodies transplanted under kidney capsule

Tissue types	Cell lines (No. of samples examined)		
	TMA-48P(2)	T48PZ4(2)	NR2(1)
Ectodermal derivatives			
Nerve tissue	+	+	+
Keratinized epithelium	+	+	+
Hair follicles	-	-	-
Mesodermal derivatives			
Connective tissue	+	+	+
Cartilage	-	-	+
Bone	-	-	+
Muscle fiber	-	-	+
Endodermal derivatives			
Ciliated epithelium	+	+	+
Gut epithelium	+	+	+

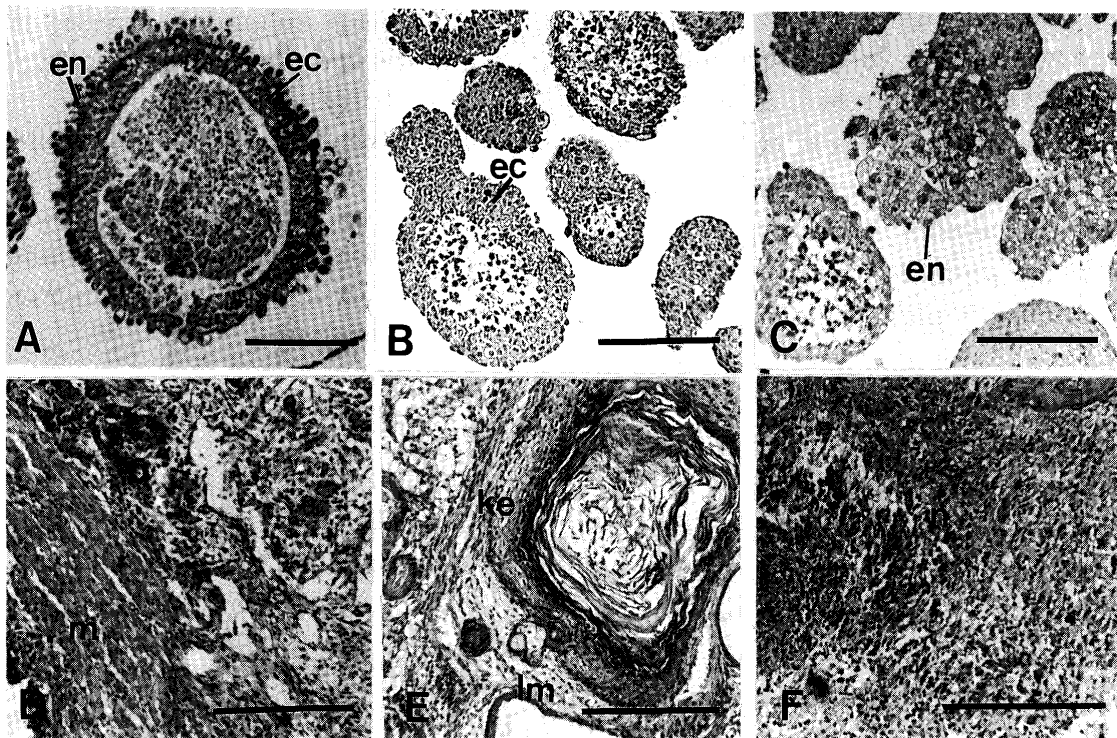


Figure 2. Sections of embryoid bodies (EBs) at 10 days (A-C) and representative regions of EBs derived-tumors (D-F). Note the formation of embryonic ectoderm-like cell layer (ec), endoderm (en), in NR2 (A), TMA-48P (B), and T48PZ4 (C) cell lines. Histological section of tumors showed the muscle fibers (m), neuroblast-like cells (n), keratinized epithelium (ke), and luminal structure (lm) in NR2 derived-tumor (D), TMA-48P (E), and T48PZ4 (F). Bar; 0.5mm. Haematoxylin and eosin staining.

Discussion

The remarkable characteristics of pluripotency have made it possible for ES cells to be used extensively as vectors for exogenous genes into mouse germ line, and for functional disruption of specific genes through homologous recombination or gene targeting. Recently, there has been numerous reports which document the application of ES cells for the study of developmental and physiological events using techniques mentioned above^{3,4,20,22}. In the present study, bacterial β -galactosidase gene was introduced into PGES cell line, as a first step to study the behavior of PGES cells *in vivo* and *in vitro*. The transfection efficiency of the present study was comparable to those of previous studies⁹.

Chromosomal aberration occurred in ES cell lines during tissue culture was seldom reported.

X chromosome instability was reported in PGES cell lines¹². Loss of Y chromosome was also reported in ES cells integrated with foreign genes for gene targeting²⁶. The present karyotype analysis revealed that the isolated cell lines had trisomy 8. However, it seems unlikely that the integration of the *neo*^R gene into chromosome 8 is responsible for the genesis of trisomy 8. Frequent occurrence of trisomy 8 has recently been reported in ES cells that are incapable of germ line integration¹⁴. However, the same paper suggested that trisomy might have a particular growth advantage because it arose independently and was selected for in three different ES cells. Chromosome 8 and the syntenic human chromosome 8 and 16 carry oncogenes such as *Jun* b and d, *Lyl*-1, and *Ras* 15-2, 6¹⁴. It has been also reported that trisomy 8 or 16 in humans has been associated with acute lymphoblastic leukemia¹⁴.

Probably trisomy 8 has given ES cells certain growth advantage in culture and outgrown karyotypically normal ES cells. Although there is no definitive evidence, it is likely that the four PGES cell lines were derived from a single cell spontaneously gained an extra copy of chromosome 8 in the parental culture.

In mice, most trisomies including chromosome 8 result in prenatal death in the midgestation period, whereas trisomies 16, 18, and 19 are usually survive until term⁸⁾. In morphological and histological features from EBs and tumors, both cell line with trisomy 8 and chromosomally normal cell line exhibited the capacity of differentiation to the same extent in the present work. After 7–10 days in suspension culture, EBs from both TMA-48P and T48PZ4 cells consisted of the sparse outer endodermal layer, and the inner tissue resembling embryonic ectoderm. These observation was similar to those seen in EBs reported previously^{18,23)}. Tissue sections showed that tumors derived from both cell lines contained various tissues derived from ectodermal and endodermal origin with reduced contribution of mesodermal derivatives. Tumors and EBs originated from T48PZ4 cells were homogeneously positive for β -galactosidase activity. It may suggest that T48PZ4 and TMA-48P cells had restricted developmental potential as previously reported for PGES cells¹⁾.

Thus, T48PZ4 cells have retained developmental potential characteristic to undifferentiated PGES cells¹⁾. Trisomy 8 may not be a fatal defect for experiments clarifying certain behavior of the PGES cells in development. It was also reported that ES cells with trisomy 8 can contribute and be allocated in somatic tissues of chimeric embryos¹⁴⁾.

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