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Chromosome number variation in three mouse embryonic stem cell lines during culture

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Abstract Although mouse embryonic stem cell lines (mESCs) have been established since 1981, systematic studies about chromosomal changes during culture are lacking. In this study, we report the results of a cytogenetic analysis performed on three mESC lines (named UPV02, UPV06 and UPV08) cultured for a period of 3 months. At time intervals, the variation of the chromosome number together with the expression of markers of the undifferentiated status, i.e., OCT-4, SSEA-1, FOM-1 and alkaline phosphatase activity, were determined. The three mESC lines showed a progressive loss of euploid metaphases during the 3 months period of culture. Chromosome abnormalities were accumulated at the latest passages analysed. Metacentric chromosomes were the most frequent chromosome abnormality observed throughout the period of culture. Interestingly, in coincidence with, or few passages after, the drop of euploidy, the alkaline phosphatase activity was partially or totally lost, whereas the OCT-4, SSEA-1 and FOM-1 stem markers were always positive throughout the period of culture. Our results remark the necessity to perform the karyotype analysis during culture in order to develop new culture conditions to maintain the correct chromosome complement in long-term culture of mESC lines.

Keywords Mouse embryonic stem cell · Cell culture · Chromosome number variation · Chromosome abnormalities

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Introduction

Embryonic stem cells (ESCs) are pluripotent, self-renewing, undifferentiated cells obtained from the inner cell mass of a blastocyst (Evans and Kaufman 1981; Martin 1981; Thomson et al. 1998). ESCs provide an in vitro assay to study mammalian development and an invaluable source of differentiated cells for their potential use in regeneration medicine and tissue engineering (Segev et al. 2004; Joseph and Morrison 2005; Yuasa et al. 2005).

Although mouse ESCs (mESCs) represent the most widely studied model system and it has been shown that they are cells prone to accumulate chromosome abnormalities, very few studies have dealt with the systematic cytogenetic analysis of the

karyotype and the chromosome changes occurring during culture (Robertson et al. 1986; Nichols et al. 1990; Longo et al. 1997; Sugawara et al. 2006; Rebuzzini et al. 2008). A recent analysis of the karyotype variation of a mESC line at different time points during a period of 3 month culture showed a heterogeneous spectrum of abnormalities indicating a high frequency of chromosome mutations that are continuously arising during culture (Rebuzzini et al. 2008).

In this paper, we report a cytogenetic analysis of three new mESC lines, passaged by enzymatic dissociation to single cells and maintained in culture on a feeder layer for 31–35 passages with the aim to determine the chromosome number variation and the presence of specific chromosome abnormalities.

Materials and methods

Maintenance of mouse embryonic stem cell lines

The three mouse embryonic stem cell lines were derived in our laboratory as described in Neri et al. 2007. All mESC lines were expanded in Knockout-Dulbecco's modified Eagle's medium (GIBCO, Milano, Italy) supplemented with 0.5% penicillin–streptomycin solution (Sigma, Milano, Italy), 0.1 mM beta-mercaptoethanol (Sigma, Milano, Italy), 2 mM L-glutamine (Invitrogen, Milano, Italy), 1× non essential amino acids solution (Invitrogen, Milano, Italy), 20% ESC Qualified Fetal Bovine Serum (Invitrogen, Milano, Italy) and 500 units/ml of ESGRO-leukaemia inhibitory factor (LIF) (Chemicon International, Milano, Italy). Cells were subcultured by enzymatic passaging, alternating a passage on STO feeder cells, previously treated with mitomycin C (2 mg/ml final concentration), with two passages on gelatin coated p55 plate.

Chromosome preparation and staining

ES cells (5×10^5) were seeded onto a 24 × 50 slide and, after adhesion, incubated with 10 ml of fresh medium for 24 h. Chromosomes were prepared according to standard procedure and stained with DAPI (0.2 μg/ml in PBS 1× for 5 min). Chromosomes of UPV02 cell line at passage 25 were also C-banded as described in Sumner (1972).

ESCs cytochemistry

In order to detect the alkaline phosphatase (AP) activity, ESCs were fixed and stained with Sigma kit 85L-2 according to the manufacturer instructions. For immunocytochemistry, mESCs were seeded onto a coverslip in 1.9 cm^2 multiwell plates and incubated at 37°C for 24 h. Cells were washed with 1× PBS and fixed for 15 min with 4% cold paraformaldehyde in 1× PBS. Cells were incubated with anti-rabbit OCT-4 (Chemicon International, Milano, Italy; diluted 1:200), anti-mouse SSEA-1 (Chemicon International, Milano, Italy; diluted 1:200) and anti-rat FOM-1 (also named Forsmann antigen; BMA, Augst, Switzerland; diluted 1:100) antibodies for 1 h at 37°C; primary antibodies binding was revealed with anti-rat-TRITC (Jackson Laboratories; diluted 1:200), anti-rabbit-FITC (Sigma, Milano, Italy; diluted 1:500) and anti-mouse-FITC (Chemicon International, Milano, Italy; diluted 1:200) labelled secondary antibodies for 45 min at 37°C. Nuclei were finally counterstained with DAPI (0.2 μg/ml).

Results

The three mESC lines, named UPV02, UPV06 and UPV08, have a male chromosome complement, as determined by PCR amplification of *Sry* and *Zfy* gene sequences (data not shown) and chromosome analysis (Fig. 1a). Cells were subcultured by enzymatic dissociation and maintained in culture for 31–35 passages; in the three mESC lines, the chromosome number and the presence of chromosome abnormalities were evaluated, at time intervals, during the whole period of culture. UPV02 was analysed between passages 13 and 31, UPV06 between passages 9 and 29 and UPV08 between passages 7 and 22 (Table 1, Fig. 2). We analysed a total of 1,006 metaphases. On average, 77 metaphase spreads (with a minimum of 29 and a maximum of 103) were analysed for each mESC line and for each passage, for a total of 325, 354 and 327 metaphase for UPV02, UPV06 and UPV08 respectively. The frequency of metaphases with $2n = 40$ all acrocentric chromosomes was 58.8% in UPV02 at passage 13, 50% in UPV06 at passage 9 and 63% in UPV08 at passage 7 (Table 1, Fig. 2a). The frequency of metaphases with $2n = 40$ chromosomes decreased very rapidly to

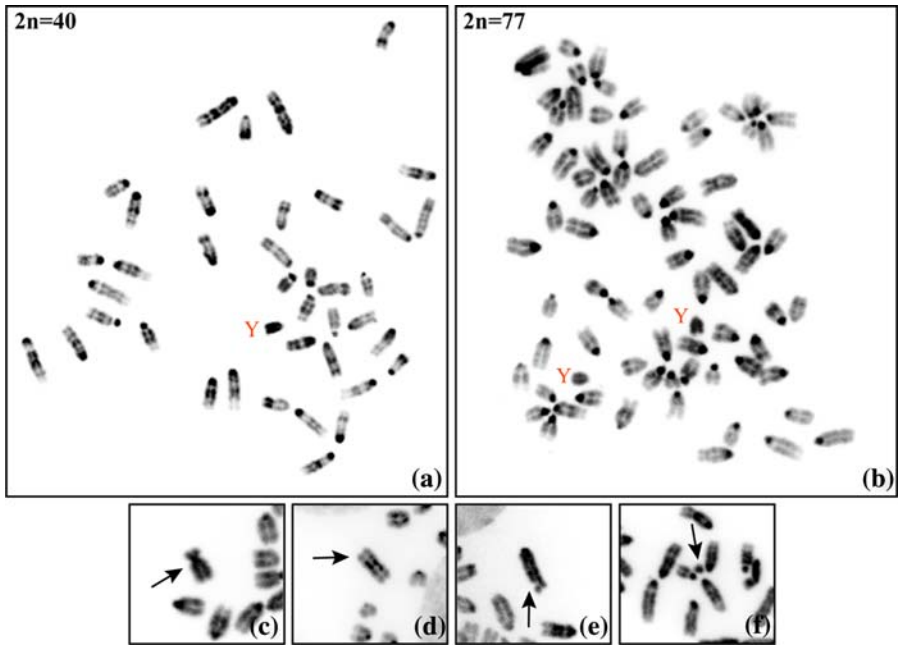

Fig. 1 Reverted images of DAPI banded karyotypes of a 2n = 40 (**a**) and a 2n = 77 (**b**) metaphase from UPV02 at passage 41. Chromosome abnormalities observed during the period of culture (*black arrows*): metacentric chromosomes (**c**), fragments (**d**), gaps (**e**) and spots (**f**)

Table 1 Total number of metaphases of the three mESC lines analysed at different time points and frequency of euploid metaphases and chromosome rearrangements

mESC line	Passage	No. of metaphases	% Frequency			
			Euploid metaphases	Metacentric	Dicentric	Other rearrangements[a]
UPV02	13	92	58.8	_	_	_
	25	71	43.8	2.8	_	1.4
	25 (C-banding)	60	38.3	3.3	1.7	8.3
	31	102	25.5	25.5	_	5.9
UPV06	9	80	50.0	_	_	_
	11	86	47.8	_	_	_
	17	29	62.2	_	_	_
	23	103	55.3	2.9	_	11.7
	29	56	32.1	28.6	_	_
UPV08	7	94	62.7	_	_	_
	8	86	63.9	_	_	_
	10	61	54.2	_	_	_
	22	86	20.8	17.4	_	3.5

Metaphases were stained with DAPI; metaphases of UPV02 cell line, at passage 25, were also C-banded

[a] Fragments, spots, gaps and other complex rearrangements

25.5% at passage 31 in UPV02, to 32% at passage 29 in UPV06 and to 21% at passage 22 in UPV08 cell lines (Table 1, Fig. 2a); at the latest passage analysed for each mESC line, the modal chromosome number was hypereuploid in UPV02 (Fig. 1b) and UPV06 mESC lines whereas it was both hyper- and

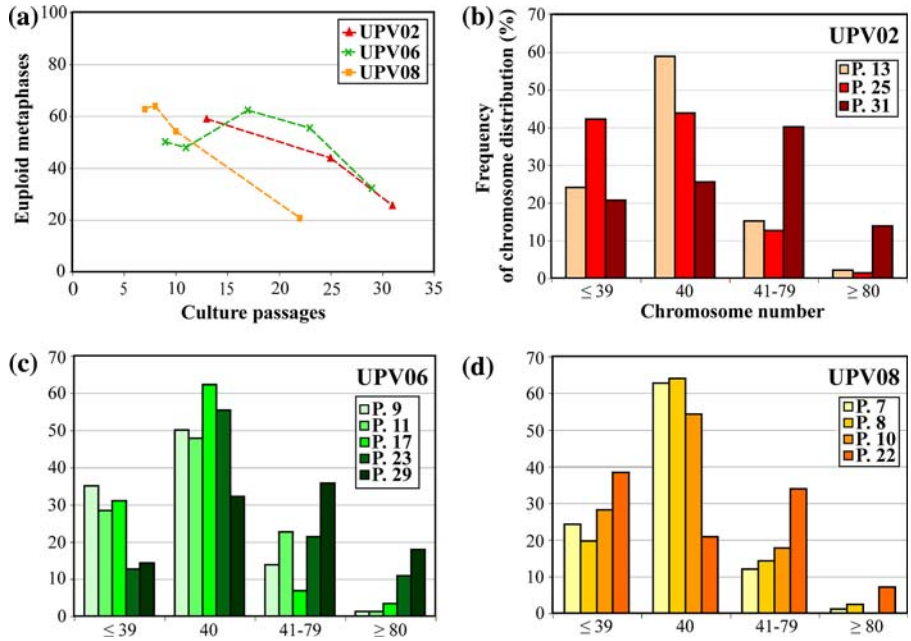

Fig. 2 In (**a**), frequency of euploid metaphases in UPV02, UPV06 and UPV08 mESC lines. In (**b**), (**c**) and (**d**), frequency of chromosome number distribution of UPV02, UPV06 and UPV08 mESC lines, respectively, during the period of culture

hypoeuploid in UPV08 mESC line (Fig. 2b–d). Hypoeuploid and hypereuploid metaphases were found in all three cell lines throughout the period of culture (Fig. 2). In UPV02, about 24% of metaphases presented a hypoeuploid chromosome number ($2n \leq 39$) at passage 13, that decreased to 20.6% at passage 31 (Fig. 2b); the opposite trend of variation was detected for hypereuploid metaphases being 15.1 and 40.1% at the first and last passage analysed (Fig. 2b). Tetraploid metaphases ($2n \geq 80$) increased from 2.1 to 13.8% during culture (Fig. 2b). In UPV06, at passage 9, 35% of metaphases presented a hypoeuploid chromosome complement ($2n \leq 39$), 13.75% of metaphases a chromosome number ranging from $2n = 41$ to $2n = 79$ and 1.25% had a tetraploid chromosome complement (Fig. 2c); at passage 29, 14.3, 35.7 and 17.9% of metaphases were hypoeuploid, hypereuploid and tetraploid, respectively (Fig. 2c). In UPV08, at passage 7, 24.2% of metaphases presented an hypoeuploid chromosome complement ($2n \leq 39$), in 12% of metaphases the chromosome number ranged between $2n = 41$ and $2n = 79$ and 1.1% were tetraploid (Fig. 2d); at passage 22 the frequency of metaphases with non-euploid chromosome number increased to 38.3, 33.8, and 7.1%, respectively (Fig. 2d).

The cytogenetic analysis revealed the presence of chromosome abnormalities that were classified into three classes (Table 1): metacentric chromosomes (Fig. 1c), dicentric chromosomes and other rearrangements, which included fragments (Fig. 1d), gaps (Fig. 1e), spots (Fig. 1f), and other complex rearrangements. At the early passages, none of the cell lines presented chromosome abnormalities, that began to appear at passage 25, 23 and 22 in UPV02, UPV06 and UPV08 cell lines, respectively, with an increasing frequency during the progression of culture (Table 1). In these three mESC lines, metacentric chromosomes represented the most frequent structural chromosome change at the last analysed passage (Table 1), rapidly accumulating in coincidence with the drop of the frequency of $2n = 40$ metaphases.

Immunocytochemical labelling showed positive immunoreactivity to OCT-4 protein, SSEA-1 and FOM-1 antigens throughout the period of culture (Fig. 3b, d, f, respectively). On the contrary, the activity of alkaline phosphatase, which was very high during the first passages of culture (Fig. 3g), was reduced or lost at the latest passages analysed in UPV02, UPV06 and UPV08 cell lines (Fig. 3h, i, j, respectively). In particular, in UPV02, alkaline

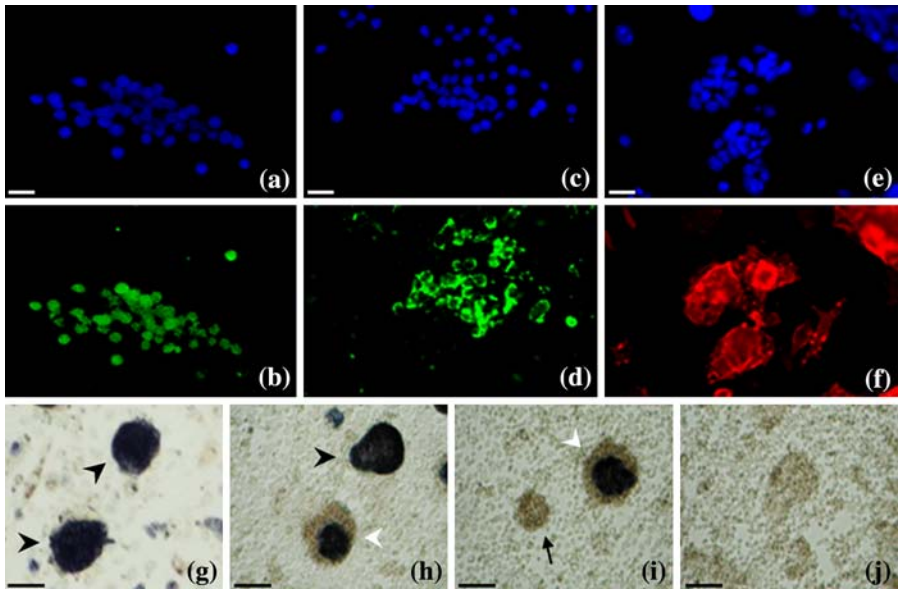

Fig. 3 The three mESC lines expressed OCT-4 protein (**b**), SSEA-1 (**d**) and FOM-1 (**f**) antigens throughout the period of culture. **a**, **c**, **e**, DAPI counterstaining. Cells from the UPV06 cell line, at passage 10, have been shown as an example. At early passages, the three mESC lines formed round-shaped colonies and had high alkaline phosphatase activity (**g**, colonies of UPV06 at passage 23 are shown as a representative example of all three mESC lines, *black arrowhead*). At passage 34, the alkaline phosphatase activity is maintained in some colonies (*black arrowhead*) whereas is lost at the edge of most of the others (*white arrowhead*) in UPV02 cell line (**h**); at passage 32 it is either present at the edge of some colonies (*white arrowhead*) or it has strongly decreased in others (*black arrow*) (**i**) in UPV06 cell line; at passage 28, it is completely lost in UPV08 cell line (**j**). Dark bar: 200 μm; white bar: 50 μm

phosphatase activity was present until passage 31, but at passage 34 and 35 it was mainly expressed in the centre of the colonies (Fig. 3h). In UPV06, the expression of alkaline phosphatase activity was revealed at passages 13 and 23, but at passages 29, 32, 34 it was present only in the central part of some colonies, whereas it was absent in others (Fig. 3i). A more dramatic situation was observed in UPV08, where at passages 28 and 31 the enzyme activity was completely absent (Fig. 3j) and cells had lost the capacity to form colonies.

Discussion

In this paper we report a cytogenetic analysis of three mESC lines, obtained from the inner cell masses of three different blastocysts and maintained in culture for a total of 3 months. The frequency of $2n = 40$ metaphases decreased with increasing number of passages. In UPV02 and UPV06 25.5% at passage 31 and 32.1% at passage 29 of metaphases were euploid, respectively. In UPV08, the decrease in the number of euploid metaphases was faster than in the other two cell lines, reaching 22% at passage 22. In a similar analysis Longo et al. (1997) found that the frequency of euploid metaphases of four different mESC clones dropped rapidly to 20% at passage 25 and Nichols et al. (1990) reported only one out of 15 mESC lines analysed showing a normal karyotype at very early passages, between 4 and 6, the remaining showing different degrees of chromosome abnormalities. A mESC line, UPV04, which we have derived with the same protocol used for the three mESC lines analysed in the present paper, maintained a higher frequency of $2n = 40$ metaphases during culture compared to UPV02, UPV06 and UPV08 (Rebuzzini et al. 2008). We cannot explain the differences in the maintenance of $2n = 40$ metaphases of the four mESC lines during culture, although it is likely that the first passages are crucial in keeping the proper chromosome complement, as also reported by other author (Nichols et al. 1990). However, the stability of the frequency of eu-, hypo- and hypereuploid populations of UPV04 compared to UPV02, UPV06 and UPV08 during culture might account for the

elimination of those cells that carry a high mutational burden in UPV04 mESC line, not active in the other three cell lines. It might be hypothesised that in UPV02, UPV06 and UPV08 mESC lines, a fast rate of occurrence of chromosome aberrations, likely not balanced by cell death, may lead to the rapid loss of euploid metaphases within 20–30 passages of culture. Chromosome abnormalities were accumulated at the latest passages analysed in coincidence with the drop of euploidy. Metacentric chromosomes were the most frequent chromosome abnormality observed throughout the period of culture. Proneness of the mouse genome to form metacentric has been described already in several feral house mouse populations (Capanna et al. 1976; Redi and Capanna 1988; Britton-Davidian et al. 2005) and in culture (Chakrabarti and Chakrabarti 1977).

Interestingly, in coincidence with, or few passages after, the drop of euploidy, the alkaline phosphatase activity was partially or totally lost in UPV02, UPV06 and UPV08 cell lines, whereas the OCT-4, SSEA-1 and FOM-1 stem markers were always positive throughout the period of culture in the three mESC lines. The loss or reduced activity of this stem cell marker accompanied the loss of euploidy, suggesting that alkaline phosphatase may be a sensitive marker, more precocious than the other pluripotency markers analysed, of those yet unidentified changes occurring in mESC during repeated passages in culture, including the loss of euploidy.

The rapid loss of euploidy and the accumulation of chromosome abnormalities within 22–35 passages of culture we have observed, may account for suboptimal culture conditions, which are known to cause chromosomal aberrations (Rice et al. 1986; Livingstone et al. 1992; Yin et al. 1992; Brown et al. 1983). Although mESC lines have been established many years ago (Evans and Kaufman 1981; Martin 1981) new culture conditions need to be developed to maintain the correct chromosome complement in long-term culture, for example using serum-replacement media (Wiles and Johansson 1999; Fletcher et al. 2006; Bryja et al. 2006; Amit and Itskovitz-Eldor 2006) and alternative methods for cell dissociation as applied for hESC lines (Suemori et al. 2006). The results of our study underline the importance of a cytogenetic analysis to further our understanding of the biology of mESCs and for the improvement of the culture conditions needed to maintain a stable chromosome complement.

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