Cultures of hESM Human Embryonic Stem Cells: Chromosomal Aberrations and Karyotype Stability

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Cytogenetic analysis of karyotypes of hESM01-hESM04 human embryonic stem cells and substrains derived from these strains showed that all these strains retained normal karyotype during long-term culturing. Two substrains of embryonic stem cells with chromosome aberrations indicating clonal origin of these strains were detected. The potentialities of using analysis of chromosome variability of embryonic stem cells for evaluation of predisposition of the corresponding genotypes to the formation of chromosome abnormalities are discussed.

Key Words: *human embryonic stem cells; chromosomes*

The use of cells cultured *in vitro* for practical purposes should meet certain requirements. The possibility of using stem cells of different origin in cell therapy is now widely discussed. A criterium determining the possibility of *in vitro* utilization of cultured cells is preservation of their normal karyotype. The appearance of changes in karyotype of human embryonic stem cells (ESC) during long-term culturing was described. These changes included chromosome aberrations, amplification of small DNA loci, and epigenetic modifications of regulatory sequences [2-4,6,7].

We studied chromosomes in cells of four independent human ESC cells and in their substrains after long-term culturing.

MATERIALS AND METHODS

Four original human ESC strains were used: hESM01, hESM02, hESM03, and hESM04 [5]. Human ESC colonies were cultured in 35-mm gelatin-coated Petri dishes (Costar) in KODMEM (Invitrogen) with 20% serum substitute (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 1 mM glutamine (HyClone), a mixture of non-essential amino acids (Gibco), 4 ng/ml bFGF (Chemicon), and antibiotics; culturing was carried out in the presence of 6.5% CO_2 with a feeder from mitotically inactivated mouse embryonic fibroblasts.

After passage 15 of hESM01 culturing, a hESM01r18 substrain was isolated, characterized by the presence of abnormal chromosome 18 in the karyotype [1]. Two substrains (hESM03del4 and hESM03der9) were isolated after passage 10 from hESM03 strain.

ESC were fixed and metaphase chromosome preparations were obtained according to a previously described protocol [1] with minor modifications. Colcemide (0.05 μg/ml; Demecolcine, Sigma D1925) was added into the medium 1.5 h before fixation. The cells were then treated with trypsin (0.05%; 3 min at 37°C and 5% CO_2), and trypsin was inactivated with medium for cell culturing. After trypsin solution was discarded, 1.5 ml hypotonic solution (0.38 M KCl; 37°C) was added for 10-15 min, after which 2-3 drops of Carnoy fixative

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were added. The cells were mechanically removed from the dish, single-cell suspension was transferred into a 1.5-ml tube and centrifuged for 6 min at 1300*g* and 4°C. The supernatant was discarded and 1.5 ml cold fixative was added to the precipitate. The cell precipitate was carefully suspended and left on ice for 20 min. The cells were washed twice with cold fixative, pipetted onto slides, and dried at ambient temperature. The preparations were stained with DAPI (Sigma; 1 μg/ml, 10 sec), washed in distilled water, and dried at ambient temperature. Fibroblast-like cells were fixed by routine method [9].

The preparations were examined under an AXIOPlan 2 Imaging fluorescent microscope (Zeiss) with a Paco CCD camera and a CHROMA filter system. Microscopy was carried out at the Center for Microscopic Analysis of Biological Objects, Institute of Cytology and Genetics. The images of metaphase plates were recorded and processed using ISIS4 software (METASystems GmbH). Chromosomes were described using ISCN2005 standard nomenclature of human chromosomes.

RESULTS

Karyotypes of ESC strains hESM01-hESM04 (Fig. 1, *a*) and their derivatives isolated after differentiation into fibroblast-like cells (Fig. 1, *b*) were analyzed. hESM01 cells were analyzed during passages 11-49, hESM03 cells during passages 8 and 36-56, hESM04 cells during passages 11, 22, and 36-49, and hESM02 cells were analyzed during passages 16, 17, and 21.

Young (2-3-day-old) 1-2-layer colonies consisting of 100-200 cells were the most suitable for preparation of metaphase chromosomes. However, preparation of suspension of fixed mitotic cells even in this case is associated with some problems. In the majority of monolayer cultures, mitotic cells acquire a round shape and can be easily isolated, while human ESC remain tightly connected to the adjacent cells even during mitosis (Fig. 1, *a*).

Difficulties associated with preparation of metaphase chromosomes of human ESC are also determined by their high sensitivity to trypsin. The duration of trypsin treatment is a critical factors in the prefixation manipulations. An optimal result (destruction of cell conglomerations without impairing the integrity of individual cells) is attained by modifying parameters of trypsin treatment with consideration for cell density and age and feeder density and age.

If cell conglomerations were not destroyed, metaphase chromosomes poorly spread on the slide during the preparation. Mechanical destruction of cells in hypotonic solution or fixed suspension violated the integrity of individual cells: numerous metaphases with chromosomes collected in compact distorted groups appeared, which made cytogenetic analysis impossible. Excessive trypsin treatment did not lead to fatal cell injuries on condition of their subsequent culturing in nutrient medium, but cell injuries became critical during subsequent hypotonic treatment and eventuated in their destruction. In cytological preparations this manifested in changed chromosome morphology, poor spreading of metaphase cells, and decreased number of intact metaphase plates.

It is noteworthy that small volume of the cytoplasm in human ESC and, presumably, specific features of its organization, also impede isolation of high-quality chromosome preparations. This determines narrow range of hypotonic exposure providing satisfactory separation of individual metaphase chromosomes in a cytological preparation and not damaging metaphase plates. In addition, high elasticity of fixed cell material can lead to their partial shrinkage during chromosome spreading on slides.

Low yield of satisfactory metaphases from colonies of typical human ESC impeded the cytogenetic analysis, and therefore we realized an alternative method for material isolation for the cytogenetic study. Five cultures of differentiated fibroblast-like

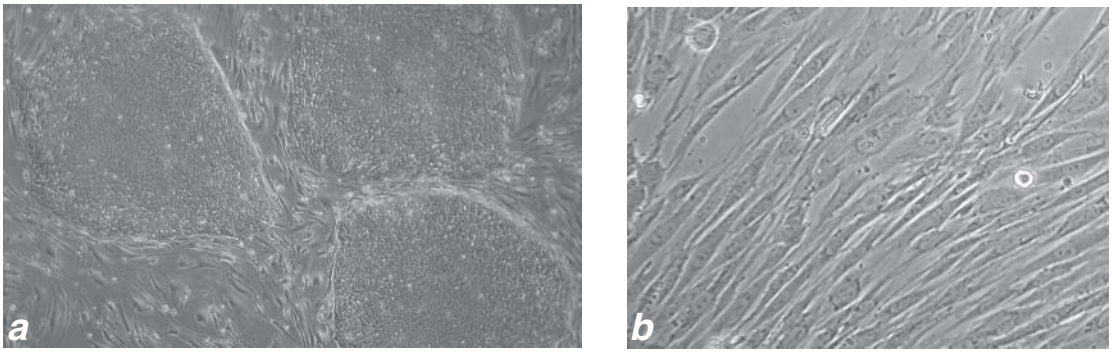

Fig. 1. hESM01 cells in culture. *a*) colonies (general view), phase contrast microscopy (×100); *b*) hESM01 fibroblast-like derivatives (×200).

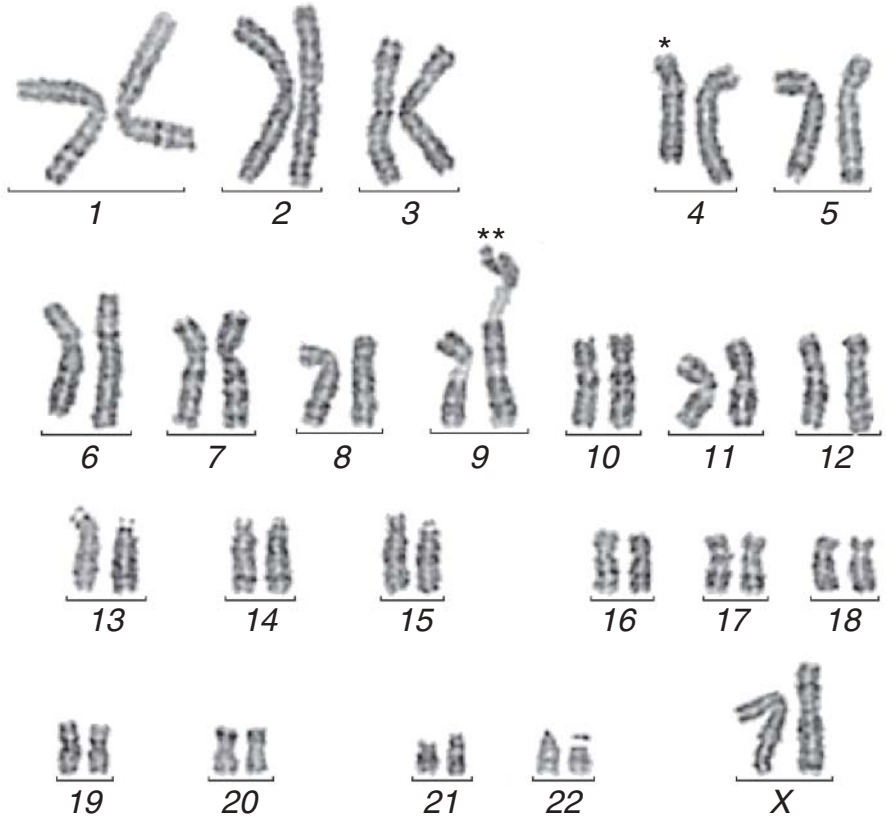

Fig. 2. Differentially stained chromosomes of a cell of hESM03der9 (46,XXdel(4), der(9)) strain. DAPI staining (×1000). *del(4)(pter->q2::q3->qter), **der(9).

cells were derived from hESM01, hESM03, and hESM04, which were used for additional chromosome analysis. In order to obtain fibroblast-like cells from hESM01, hESM03, and hESM04 strains, we cultured ESC colonies without feeder in DMEM with high glucose (HyClone) content and 10% fetal calf serum (Invitrogen), 1 mM glutamine (HyClone), and antibiotics at 6.5% CO_2. The resultant cultures consisted of cells similar to fibroblasts by morphological, immunohistochemical, and cultural characteristics (Fig. 1, *b*). These fibroblast-like derivatives were used as an extra source of material for cytogenetic analysis. A total of 3-5 additional passages were needed for obtaining differentiated derivatives. It cannot be excluded that only cells with a certain karyotype undergo differentiation, which could theoretically enrich the population with this or that chromosome aberration or lead to selection of cells with normal karyotype.

No chromosome aberrations were detected in the majority of analyzed hESM01-hESM04 cells and their derivatives. A total of 23 and 47 metaphase plates were analyzed for hESM02 and hESM04 ESC, respectively. Aberrant chromosomes were seen in some metaphase plates; some of them seemed to be artifacts of cell fixation and cytological preparation. During passage 36, a del(4)(pter->q2::q3->qter) chromosome was detected in all studied 15 cells of hESM03del4 substrain. This chromosome was then detected in all 50 cells of hESM03der9 substrain analyzed during passages 43-56. All analyzed cells (passages 43-56 of hESM03der9 substrain) also carried der(9) chromosome (Fig. 2). No abnormal chromosomes were detected in the initial hESM03 cells. The results of direct cytogenetic analysis of hESM03 substrain (passage 39) and its fibroblast-like derivatives were identical, and hence, hESM03 cell karyotype can be regarded as 46,XX.

Cells of substrain hESM01r18 were analyzed during passages 25, 26, 27, 32, 36, and 43. More than 100 cells were analyzed and only 2 cells (passage 32) did not carry r(18). However, even in these two cases one of chromosome 18 homologs was restructured, which could be a result of rupture of annular chromosome. No other repeating chromosome aberrations were detected.

Comparison of the characteristics of hESM03der9 and hESM01r18 cells with cells of parental strains showed more rapid cell proliferation in hESM01r18

and hESM03der9 substrains. Higher cell proliferation rates were detected by passage 25 in hESM01r18 substrain, and clonal origin of hESM03der9 substrain was detected by passage 43. Nonetheless, the cells of these substrains retained the main characteristics of human ESC, including expression of Oct-4, SSEA-4, alkaline phosphatase, and some other specific markers. On the other hand, the spectrum of resultant tissues was changed in teratomas from hESM01r18 cells, the capacity to spontaneous differentiation of hESM01r18 and hESM03der9 cells was reduced, the formation of embryoid bodies by hESM01r18 cells was delayed and these cells did not express some tissue-specific markers characteristic of embryoid bodies of the parental hESM01 strain.

Hence, permanent cytogenetic control makes it possible to maintain ESC in culture for a long time with preservation of the normal karyotype. Substrains with karyotypes containing stable aberrant chromosomes should be eliminated. On the other hand, a detailed description of aberrant chromosomes in these substrains can make them an important instrument in studies of the role of chromosome regions in the maintenance of ESC pluripotency and evaluation of the spectrum of their prospective differentiation.

However, initial predisposition of ESC strains giving rise to substrains with chromosome aberrations to generation of chromosome abnormalities cannot be excluded. Presumably, analysis of stability of ESC strains derived from certain couples liable to manifestation of chromosome instability can serve as a prognostic indicator for these patients. Further studies will show the possibility of predicting genotype instability by the analysis of chromosome variability of ESC. The possibility of using ESC for studies of the mechanisms of developmental abnormalities caused by chromosome aberrations, e.g. r(18), is also an unsolved problem [9]. Further studies of hESM01r18 substrain will promote creation of an experimental model for the study of mechanisms of development of these abnormalities and methods for their prevention.

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