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Original Article

Establishment of human-embryonic-stem-cell line from mosaic trisomy 9 embryo

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ABSTRACT

Objective: Human-embryonic-stem-cell (hESC) lines derived from chromosomally or genetically abnormal embryos obtained following preimplantation genetic diagnosis are valuable in investigating genetic disorders.
Materials and methods: In this study, a new hESC line, Center of Clinical Reproductive Medicine 8 (CCRM8) was established by isolation, culture, and passaging of the inner cell mass of mosaic trisomy 9 embryos.
Results: A karyotype analysis showed that the hESC line possessed a euploid (46 chromosomes). The undifferentiated hESCs exhibited long-term proliferation capacity and expressed typical markers of OCT4, TRA-1-60, and TRA-1-81. *In vitro* embryoid-body (EB) formation, differentiation, and *in vivo* teratoma production confirmed the pluripotency of the hESC line. The data represented here are the first detailed report on the characterization and differentiation of one Chinese hESC line generated from mosaic trisomy 9 embryos.
Conclusion: Our study showed that chromosomally aberrant embryos could generate a normal hESC line, which would be useful in investigating gene function and embryo development.

Introduction

Human embryonic stem cells (hESCs) possess pluripotent potentials for multidirectional differentiation *in vivo* and *in vitro*. These cells remain as undifferentiated features and keep the proliferative capacity during long-term *in vitro* culture, while maintaining the normal diploid karyotype [1]. For these reasons, hESCs are considered a superior model for studying embryogenesis, human development, control of gene expression, and etiopathogenesis of birth defects. In addition, these cells have been applied for drug screening and developmental toxicity test, and used as a source of cells for tissue replacement therapy in clinical settings [2–4]. Today, hundreds of hESC lines are generated following the establishment of the first hESC line by Thomson et al in 1998 [1].

Normal hESCs are generally isolated from surplus, frozen embryos donated by couples who have accepted *in vitro* fertilization (IVF) treatments and have fulfilled their family plan [1,4]. Such normal hESCs can satisfy the needs of most investigations when the application of human embryos for research is restricted by ethics concerns in many countries [5]. It is noteworthy that hESCs carrying genetic diseases have been successfully applied in studying the relevant etiopathogenesis. Such hESCs are more attractive study models that may face less ethics scrutiny. This is due to the fact that they are derived from an abundant source of discarded embryos that were readily diagnosed by preimplantation genetic screening (PGS) and preimplantation genetic diagnosis (PGD) for having chromosomal aberrations or carrying of genetic disorders [6]. PGD

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presents a viable alternative for couples at high genetic risk to avoid implantation of a fertilized egg carrying a serious genetic disease, thus increasing the rate of successful IVF and advanced single-cell-based technologies [7]. PGS was introduced into clinical practice for screening and discarding aneuploid embryos, thus improving the chance of healthy conceptions after an infertility treatment in patients with concerns of poor prognoses, such as advanced maternal age, repeated implantation failure, and recurrent miscarriage [8,9].

Normal hESC lines have been generated from discarded embryos (after PGS or PGD) obtained from IVF-embryo transfer or intracytoplasmic sperm injection by conventional inner-cell-mass isolation, culture, and passage [6,10–15]. Some of them were derived from the embryos with chromosome inversion through long- or short-arm repeat. To date, December 26, 2013, 243 hESC lines were eligibly registered in the National Institutes of Health Registration, including 69 hESC lines with disease-specific mutations, with 18 out of 69 hESC lines going through PGD. It should be pointed out that human stem cell lines from trisomy embryos, including trisomy 5, trisomy 16 [16], trisomy 1 [17], and trisomy 13 [18], have been established. However, no hESC line derived from trisomy 9 is currently available. Human chromosome 9 is highly polymorphic, and pericentric inversions occur in more than 1% of the pregnant population [19]. The frequent occurrence of inversions on human chromosome 9 can induce long arm and short arm to duplicate repeats [19–21], resulting in mosaic trisomy 9. This abnormal karyotype was reported as being related with many types of diseases, such as increased abortion rate [22], male sterility [23], and neonatal congenital malformation [24]. Thus, hESCs representing mosaic trisomy 9 embryos would be useful for *in vitro* studies of embryogenesis and gene function on chromosome 9.

Here, we established an hESC line, CCRM8, from a mosaic trisomy 9 embryo. We observed that CCRM8 grew in colony, expressed stem cell markers, were capable of long-term proliferation, and possessed pluripotent differentiation potentials. It is interesting that CCRM8 carried a normal karyotype of 46, XX. The hESC line would be useful in investigating gene function on chromosome 9 and the specific mechanism for euploid recovery.

Materials and methods

Human embryos and ethics approval

Human mosaic trisomy 9 embryos employed in this study for establishing hESCs were donated from couples that participated in the IVF program for infertility treatment. The study was approved (date of approval: October 13, 2008) by the local Institutional Review Board of the First Affiliated Hospital, Nanjing Medical University. All voluntary couples enrolled into this study signed an informed consent form.

Fluorescence in situ hybridization

An embryo was fixed at the positions of 2 and 4 o'clock with the holding pipette of a micromanipulator system (Narishige, Tokyo, Japan). The zona pellucida around the blastomere biopsy was fired, and one or two blastomeres were pumped out by negative pressure using a blastomere biopsy needle. The blastomeres were washed with phosphate-buffered saline (PBS) and placed in a hypotonic solution (1% sodium acetate + 6 mg/mL bovine serum albumin) for 5–10 minutes. Then, the blastomeres were transferred into the fixed liquid (0.01 mol/L hydrochloric acid + 0.1% TWEEN 20) before being moved to the hybrid zone on the glass slides coated with polylysine. Small drops of the fixed liquid were added onto the glass slides until the cytomembrane ruptured, and then rinsed with PBS. The blastomeres on the slides were dried in the air at room temperature.

The blastomeres were put into 2× standard saline citrate (SSC) for 10 minutes; dehydrated in turn by 70% alcohol, 85% alcohol, and absolute ethyl alcohol; and dried at room temperature. Probe mixture (chromosome 9 centromeric probes; Vysis, Illinois, USA) was denatured in 73°C water bath for 5 minutes, then added onto the blastomeres on the slides. After mounting, the glass slides were put into a humidified box overnight. The glass slides were washed by eluent (0.4× SSC/0.3% Nonidet P40; Sigma, St. Louis, USA) at 70°C for 2 minutes, then dried in the air. Afterward, the blastomeres were stained with 4′,6-diamidino-2-phenylindole, mounted, and photographed.

Preparation of the feeder layer

Mitomycin C-treated (MCT) mouse embryonic fibroblasts (MEFs) were used as a feeder layer to coculture hESCs. Pregnant Institute of Cancer Research mice were purchased from the Model Animal Research Center of Nanjing University, and embryos of 12.5 days old were isolated from the mice. Briefly, the heads and all viscera of the embryos were removed after the embryos were separated from the uterus. The remaining embryos were minced into pieces, digested with 0.25% trypsin/EDTA (Invitrogen, Waltham, USA), and incubated in Dulbecco's modified Eagle's medium (Invitrogen, Waltham, USA) plus 10% fetal bovine serum (Invitrogen, Waltham, USA) at 37°C with 5% CO_2. MEFs at passages 2–5 were inactivated with 10 μg/mL mitomycin C (Roche, Basel, USA) for 2.5–3 hours. The cells were collected following digestion with 0.05% trypsin/EDTA, counted, and plated onto 0.1% gelatin-coated (Invitrogen) dish or plate with a density of $2.5 \times 10^4/cm^2$.

Generation of hESCs

The zona pellucida of the blastocyst was removed by 0.1% Tyrode's solution (SAGE, New York, USA) on Day 5 or 6 of embryogenesis. Then, the whole zona-free blastocyst was plated onto MCT–MEFs and cultured in hESC medium, which was composed of Dulbecco's modified Eagle's medium/F12, 20% Knockout Serum, 1% glutamine, 1% nonessential amino acids, 0.1mM 2-mercaptoethanol, 1% penicillin–streptomycin (all purchased from Invitrogen), and 10 ng/mL basic fibroblast growth factor (bFGF; PeproTech, USA) at 37°C with 5% CO_2 and 20% O_2. The medium was changed daily. After 5–7 days, flat, colony-like cell masses appeared. They were plated onto fresh MCT–MEFs after mechanically dissociated into small pieces. For later passages, hESCs were expanded mechanically (before passage 5), or digested (passage 6 afterward) for 10 minutes with 1 mg/mL collagenase IV (Invitrogen), and then plated onto fresh MCT–MEFs every 5–7 days.

Karyotype analysis

The standard G-band chromosome analysis and the array-based comparative genomic hybridization were performed according to the manufacturer's protocols.

Alkaline-phosphatase staining

The Alkaline Phosphatase Detection Kit (Vector Laboratories, Burlingame, USA) was selected to carry out the alkaline phosphatase (AKP) staining of hESCs according to the manufacturer's protocol.

Immunostaining of hESCs

The primary antibodies of anti-Oct-3/4, anti-SSEA4, anti-Tra-1-60, and anti-Tra-1-81 (all from Chemicon, Salem, USA) were used