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.
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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

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

to characterize hESCs. Briefly, cells cultured on coverslips were fixed with 4% paraformaldehyde (Sigma, USA) at room temperature for 10 minutes, permeated with 0.1% Triton X-100 (Sigma, St. Louis, USA)/PBS on ice for 10 minutes, and blocked with fresh 2% bovine serum albumin (Sigma)/PBS at room temperature for 30 minutes. The treated hESCs were washed with PBS for 5 minutes, and then incubated with primary antibodies overnight at 4°C. After the 5-minute rinse with PBS, the hESCs were stained with Cy2-conjugated or fluorescein-isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in the dark for 30 minutes. The stained hESCs were mounted with 4′,6-diamidino-2-phenylindole (Vector Laboratories) after being washed with PBS for 5 minutes, and photographed under a fluorescence microscope (Tokyo, Nikon, Japan).

Reverse transcription–polymerase chain reaction

The harvested cells were washed with PBS, and RNA was extracted with TRIzol reagents (Invitrogen, Waltham, USA). Complementary DNAs were synthesized by reverse transcription using a reverse-transcription kit (Takara, Shiga, Japan) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out in 50 μL volume reactions containing 5 μL of 10× PCR buffer, 4 μL of 2.5mM deoxyribonucleotide triphosphates, 1 μL of each primer (10μM), 0.5 μL of Taq enzyme (5 U/μL), 8 μL of complementary DNA template, and 31.5 μL of ultrapure water. The reverse transcription–PCR conditions were as follows: pre-denaturing at 94°C for 2 minutes, denaturing at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and elongation at 72°C for 45 seconds. Following 30 cycles of amplification, final elongation was performed at 72°C for 5 minutes. The primer sequences are shown in Table 1. The PCR products were resolved by agarose gel electrophoresis, and the DNA bands were visualized by ethidium-bromide staining and observed under an ultraviolet automatic image analyzer.

Embryoid body and teratoma formation

Following 5–7 days of culture, hESC colonies were suspended with collagenase IV and dispase. For the formation of EBs, cell clusters were plated onto bacteria-culture dishes and allowed to culture for 1–2 weeks in embryonic-stem media without bFGF and feeder. For teratoma formation, 5×10^6 to 10×10^6 hESCs were injected intramuscularly into mice with severe combined immunodeficiency disease. After 2–3 months, the mice were sacrificed and teratoma tissues were dissected into sections, and then fixed in 4% paraformaldehyde. The sections from the fixed teratoma tissues were stained with hematoxylin and eosin, and photographed.

Table 1
Designations, sequences, and the sizes of reverse transcription–polymerase chain reaction amplicons.

Name	Sequence from 5′-3′	Size (bp)
AFP forward	AGCTTGGTGGTGGATGAAAC	182
AFP reverse	TCTGCAATGACAGCCTCAAG	
BMP4 forward	AAGCGTAGCCCTAAGCATCA	197
BMP4 reverse	ATGGCATGGTTGGTTGAGTT	
Nestin forward	GCAGCACTCTTAACTTACGATC	176
Nestin reverse	CTGACTTAGCCTATGAGATGGA	
GAPDH forward	GAAGGTCGGAGTCAACGGATTT	223
GAPDH reverse	CTGGAAGATGGTGATGGGATTTC	

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; AFP = alpha fetoprotein; BMP4 = Bone Morphogenetic Protein 4.

Results

Characterization of mosaic trisomy 9 embryos

During the IVF treatment, the chromosome-abnormality couples go through a preimplantation diagnosis by fluorescence *in situ* hybridization (FISH) to choose a normal karyotype embryo for transplantation, and, generally, abnormal embryos are discarded. In some cases, infertile couples voluntarily donated embryos with abnormal signals for hESC research. In our study, the donation couple obtained 20 ovules after egg retrieval in a menstrual cycle, and 16 of them were fertilized after intracytoplasmic sperm injection. Eight embryos grew to the eight-cell stage. We found two normal and six abnormal embryos, of which one embryo showed mosaic trisomy 9 karyotype (Table 2). The short-arm repeat on trisomy 9 was confirmed by PGD, in which a single blastomere of the eight-cell stage of the embryo was sucked out (Figure 1A–D) and subjected to FISH assay (Figure 1E–H). The FISH results clearly showed that a single blastomere biopsy expressed two red spots (Figure 1F) and three green spots (Figure 1G), which meant chromosome 9 short-arm terminal repeat.

Derivation and characterization of hESC line

The whole zona-free blastocysts from chromosome 9 short-arm terminal repeat embryo were plated onto MCT–MEFs, and cultured in an hESC medium after being removed from the zona pellucida in Tyrode's solution. After 5–7 days, the appearing flat, colony-like masses were subcultured through mechanical scraping into small pieces. At last, one independent stable hESC line, CCRM8, was successfully established by mechanical (before the 5th generation) and enzymatical (after 6th generation) passage and expansion. During culture, the hESCs grew as flat colonies with clear cell boundaries (Figures 2E and 3B), and large nuclei and clear nucleoli presented in growing hESCs, giving a high nucleoplasmic ratio. We observed that the hESCs proliferated fast during Days 5–6, and should be subcultured on Days 6–7; otherwise, the hESCs would begin to spontaneously differentiate. After AKP staining, the hESC colonies turned into violet blue, indicating the strong expression of AKP (Figure 3C). Furthermore, expressions of the stem-cell markers TRA-1-60, TRA-1-81, and OCT4 were detected in the hESCs (Figure 3D–F). Interestingly, CCRM8 carried a normal diploid karyotype of 46, XX (Figure 3A), although it derived from the embryo with chromosome 9 short-arm terminal repeat. In our study, CCRM8 cells could maintain the hESCs' distinctions even if they were cultured to the 50th generation. Thus, CCRM8 was a typical hESC line, capable of self-renewal, expression of stem-cell markers, and long-term culture.

Differentiation of hESCs

The hESCs exhibited their pluripotency when they spontaneously differentiated into morphologically distinct cell types representing all the three embryonic germ layers *in vitro* and *in vivo*. We found that EBs were formed when CCRM8 cells were cultured in an ultralow-attachment dish without bFGF and feeder layer. The EBs grew and differentiated gradually, and cyst-shaped EBs were presented on Day 7 (Figure 4A). Moreover, the reverse transcription–PCR results showed expression of early differentiation markers, including nestin (ectoderm), Bone Morphogenetic Protein 4 (BMP4) (mesoderm), and alpha fetoprotein (AFP) (endoderm) in EBs on Day 7 (Figure 4B), indicating CCRM8 *in vitro* could differentiate into the three germ layers. Subsequently, the *in vivo* pluripotency of hESCs was accessed by teratoma-formation experiments. We observed a lump at the injection site after the

Table 2
Clinical data of the donor couple.

Female		Male		Embryos	
Age	Karyotype	Age	Karyotype	No. of embryos	FISH signals
28	46, XX, inv(9) (q11;q22)	29	46, XY	3	2 Red, 0 green
				5	2 Red, 0 green
				7	0 Red, 0 green
				8	0 Red, 3 green
				9	0 Red, 0 green
				10	2 Red, 2 green
				11	2 Red, 2 green
				15	2 Red, 0 green

"Red" represents long-arm terminal; "green" represents short-arm terminal. The "2 red and 2 green" means normal embryos; the "3 green" means mosaic trisomy 9 embryos (short-arm repeat).
FISH = fluorescence *in situ* hybridization.

hESCs were injected into the hind legs of mice with severe combined immunodeficiency disease at 8–12 weeks of postinjection (Figure 4C). The gross-anatomy analysis indicated that the lump indeed came from injected hESCs rather than intrinsic tumorigenesis based on the absence of peplos or the destruction in lump-surrounding tissues. Hematoxylin and eosin staining analysis confirmed that nerve cells (ectoderm lineage, Figure 4D), gland (endoderm lineage, Figure 4E), and cartilage (mesoderm lineage, Figure 4F) were present in the retina and choroid plexus. These data indicated that the injected hESCs could develop into all three germ layers in derived teratoma, confirming the hESC pluripotency *in vivo*. In summary, although established from a mosaic trisomy 9 embryo, CCRM8 was characterized as normal hESC, in that it not only maintained hESC-like morphology and expressed stem-cell
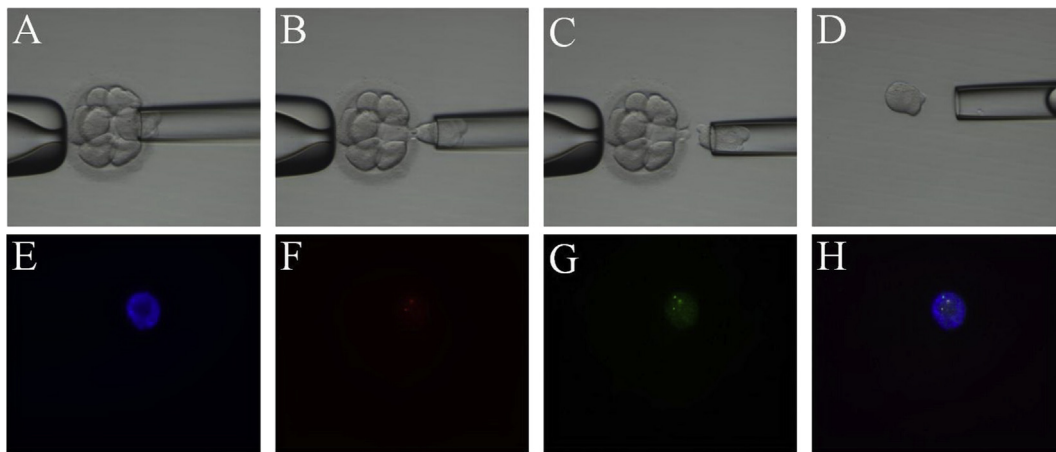
Figure 1. Identified chromosome 9 short-arm repeat by fluorescence *in situ* hybridization (FISH). (A–D) A single blastomere was sucked out after laser drilling on an eight-cell embryo. (E) The blastomere nucleus was indicated by 4′,6-diamidino-2-phenylindole staining. (F) FISH showed chromosome 9 long-arm terminal normal (two red dots). (G) FISH showed chromosome 9 short-arm repeat (three green dots). (H) Picture merged from (E–G).
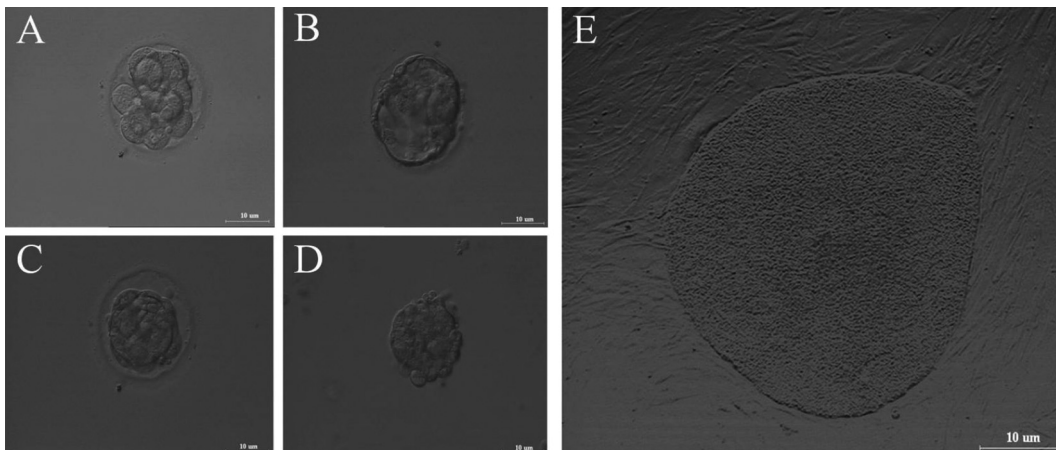
Figure 2. Morphology of zygotes and human-embryonic-stem-cell colony. (A) The embryo at eight-cell stage; (B) the embryo at morula stage; (C) the embryo at blastocyst stage; (D) the whole embryo after zona removal; and (E) human-embryonic-stem-cell colony at passage 2. (Scale bar: 10 μm).
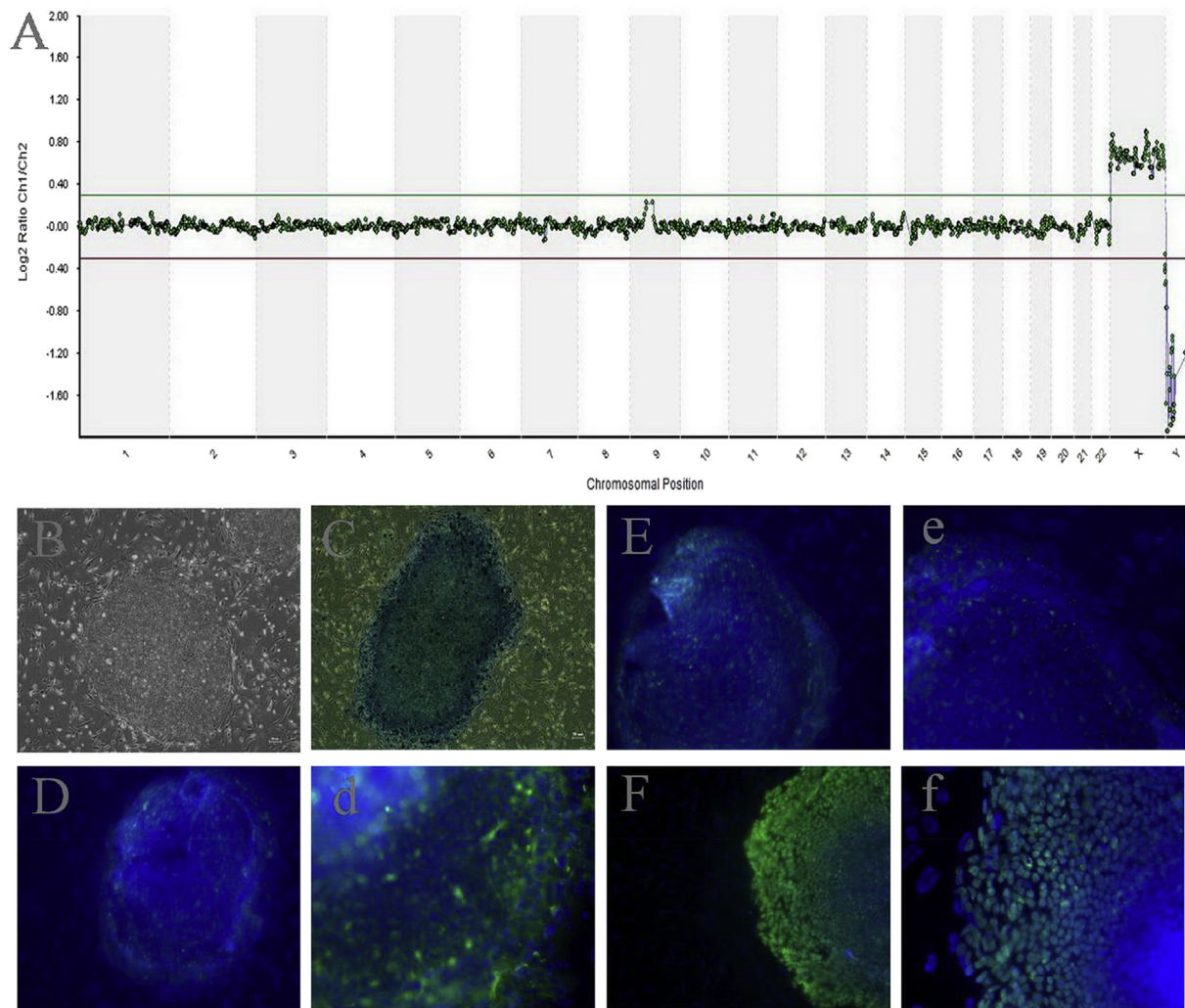
Figure 3. Characterization of CCRM8 by karyotype and immunohistochemistry analysis. (A) Karyotype analysis of CCRM8 by array-based comparative genomic hybridization; normal 46, XX was observed. (B) Human-embryonic-stem-cell (hESC) clones at passage 17 under a phase-contrast microscope. Scale bar: 10 μm. (C) The hESCs were determined by alkaline-phosphatase staining. Scale bar: 10 μm. (D—F) and (d—f) Detection of stem-cell markers expressed in hESCs. The nuclei were stained by 4′,6-diamidino-2-phenylindole staining. (D) and (d) TRA-1-60. (E) and (e) TRA-1-81. (F) and (f) OCT4. (D—F) 4× magnification. (d—f) 20× magnification. CCRM8 = Center of Clinical Reproductive Medicine 8.

markers, but also possessed the differentiation pluripotency *in vitro* and *in vivo*.

Discussion

In our study, one new hESC line, CCRM8, was generated from PGD-discarded embryo donated by a Chinese couple. Several studies have shown that hESC lines can be derived from abnormal embryos donated following PGD [25,26]. Such hESC lines can be useful models for studying genetic anomaly in relevant diseases. The CCRM8 cell line was established from an embryo diagnosed as mosaic trisomy 9. However, karyotypic analyses at an early stage (P17) showed the cell line was of normal karyotype (46, XX). Previous investigators have reported that both normal and abnormal hESC lines were successfully derived from PGD-diagnosed aneuploid embryos [16,27]. There are also studies that demonstrated the successful establishment of normal pluripotent hESC lines from both monosomic and trisomic embryos [28—30], indicating that, just like natural embryonic development *in vivo*, extensive genetic alterations and selection could occur under *in vitro* culture conditions. In our case, the normal karyotype, CCRM8, is derived from an embryo diagnosed as mosaic trisomy. However, we could not

completely exclude the possibility of new mutations, and the cells with normal karyotype most likely arose from the normal cells without trisomy defects. Presumably, the normal cells enjoyed a proliferation or survival advantage over the trisomic cells, leading to selective expansion in the culture. This transformation may contribute to the state of mosaicism and rapid growth of the stem cells with normal karyotype [31]. In spite of the mechanism, from a practical point of view, such possibility pointed to a feasible approach with which normal cell cultures can be obtained from mosaic (discarded) embryos that are relatively easy to obtain in a clinical setting.

Alternatively, the hESCs with normal karyotype may come from trisomic cells. In this case, we have to assume that the trisomic stem cells have a self-correction capability, which enables them to revert back to chromosomally normal cells either partially or completely. Indeed, it has been reported that aneuploidy mammalian hESCs can instinctively revert to diploid cells. The hESCs derived from tri-pronuclear embryos with or without extra-pronuclear removal spontaneously recover to diploid karyotype in a long-term culture [32]. Additionally, haploid ESCs can also spontaneously transfer to diploid karyotype during culture [33]. Thus, mosaic trisomy 9 hESCs have a chance to transform back to chromosomally normal cells
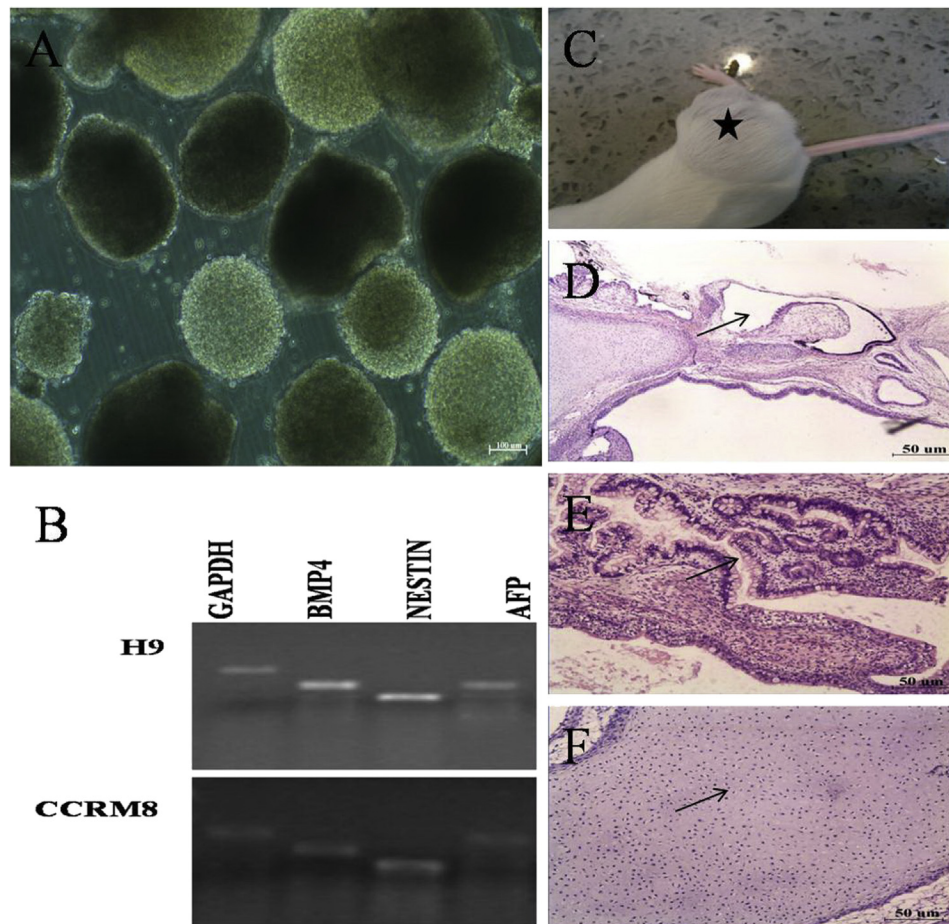
Figure 4. Differentiation of CCRM8 cells *in vitro* and *in vivo*. (A) and (B) Human-embryonic-stem-cell (hESC) differentiation *in vitro*. (A) Embryoid bodies at differentiation, day 7. Scale bar: 100 μm. (B) Three germ-layer markers expressed in embryoid bodies as detected by reverse transcription–polymerase chain reaction. Lane 1, glyceraldehyde-3-phosphate dehydrogenase as control. Lane 2, mesoderm marker BMP4. Lane 3, ectoderm marker, nestin. Lane 4, endoderm marker, AFP. (C–F) The hESCs differentiated *in vivo*. (C) Teratomas (asterisk) were formed after hESCs injected into mice with severe combined immunodeficiency disease for 2–3 months. Nerve cells (D, arrow), gland (E, arrow), and cartilage (F, arrow) were indicated by hematoxylin and eosin staining of teratoma sections. (D–F) Scale bar: 50 μm. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; AFP = alpha fetoprotein; BMP4 = Bone Morphogenetic Protein 4; CCRM8 = Center of Clinical Reproductive Medicine 8.

through a similar, unknown mechanism. Unfortunately, in our study, along with others [34], the karyotype analysis was not performed at the initial passages, leaving the possibilities of mosaicism and instinctive reversion unsolved.

It has been reported that mosaic blastomeres are frequently found in early-stage embryos [33,35], and the self-correction phenomenon may also happen in the embryo stage. The heterogeneity may result in false-positive or false-negative artifact of PGD test. Therefore, it can be speculated that the normal karyotype of CCRM8 was possibly due to one of the aforementioned possibilities.

Although the karyotypes of hESC lines derived from trisomy 9 embryos appeared normal, we could not eliminate the possibility of new mutations in the cell line. Once again, such mutations reversed the cell karyotype, and at the same time, might lead to increased survival capability, thus enabling their dominance under the *in vitro* culture conditions. This possibility would be cleared if more precise experiments, such as second-generation sequencing, were applied. Thus, further characterization is needed to obtain a complete picture of the genetic background of the new cell line.

Despite of the aforementioned uncertainty, here, we first report the successful derivation of an hESC line, CCRM8, from a mosaic trisomy 9 embryo donated by a Chinese couple. The hESC line exhibited all the key characteristics of hESCs, demonstrating that the PGD procedure does not affect the characteristics and differentiation potential of hESCs. Now, the cell line is available for distribution to the stem-cell research community, which will facilitate embryonic stem-cell research.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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