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Normal Human Embryonic Stem Cell Lines Were Derived From Microsurgical Enucleated Tripronuclear Zygotes

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ABSTRACT

A normal fertilized human zygote contains two pronuclei, but zygotes may also display one, three, or even more pronuclei resulting from irregular insemination or meiotic division. Today diploid and triploid human embryonic stem cell (hESC) lines have been derived from tripronuclear (3PN) triploid zygotes, and an in-vitro fertilization (IVF) baby was born from a rescued diploid zygote by removing the extra male pronucleus of the 3PN zygote. However, whether hESCs can be derived from a rescued 3PN zygote is still unknown. Here, by microsurgical pronuclear removal, we restored 61 diploid zygotes from 3PN zygotes donated by 35 couples, and 11 blastocysts developed with a blastocyst rate of 18.0%, which seems higher than that of nonrescued 3PN zygotes according to previous reports. After the whole zona pellucida free embryos were plated onto feeder cells to grow and passage, 2 hESC lines (CCRM-hESC-22 and CCRM-hESC-23) were generated and both carried normal karyotype (46, XY). The hESC lines were then characterized by morphology, expansion in vitro, and expression of specific markers of alkaline phosphatase, OCT4, SSEA4, TRA-1-60 and TRA-1-81. Furthermore, the pluripotency of these 2 hESC lines was confirmed by in vitro embryoid body formation and in vivo teratoma production. Our study indicates that depronucleared 3PN zygotes can improve the blastocysts formation rate, and normal hESC lines can be derived from those corrected 2PN embryos. Based on their multi-directional differentiation potential in vitro, the established hESC lines could be applied to the developmental risk assessment for IVF babies born from restored zygotes. J. Cell. Biochem. 114: 2016–2023, 2013.

KEY WORDS: HUMAN EMBRYONIC STEM CELLS; MICROSURGICAL PRONUCLEAR REMOVAL; TRIPRONUCLEAR ZYGOTES

Since the first successful derivation of human embryonic stem cell (hESCs) lines by Thomson et al. [1998], many additional hESC lines have been established worldwide. Derived from blastula inner cell mass(ICM), hESCs possess properties of undifferentiated proliferative capacity during long-term in vitro culture, multi-directional differentiation potential in vivo and in vitro, as well as maintaining normal diploid karyotype [Thomson et al., 1998]. Thus, hESCs not only play an important role in basic research, such as in embryogenesis, etiopathogenesis and the investigation of human gene function, but also may play a valuable role in application studies, for example as the drug screening model, and giving cells, tissues or organs for clinical replacement therapy [Kaufman et al., 2001; Olson et al., 2011]. Till the end of 2012, 186 hESC lines have been eligibly registered in the National Institutes of healthy Registration (http://grants.nih.gov/stem_cells/registry).

Conventionally, hESCs are isolated from surplus, frozen embryos donated by couples who completed in vitro fertilization (IVF) treatments and have no further plans to utilize the embryos [Thomson et al., 1998; Cowan et al., 2004]. However, the application of such high-quality human embryos in research is restricted by ethics in many countries [Ehrich et al., 2011]. Discarded embryos, identified by preimplantation genetic diagnosis (PGD) and preimplantation genetic

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screening (PGS), carrying genetic disorders or having chromosomal aberrations [Taei et al., 2010], are an important source for producing hESCs. According to classic embryology, embryos with abnormal morphology are also defined as discarded embryos, which indicate decreased implantation potential, a high degree of fragmentation, multinucleated blastomeres, delayed development, and non-2PN at fertilization [Staessen and Van Steirteghem, 1997; Van Royen et al., 1999; Van Royen et al., 2003; Stone et al., 2005; Scott et al., 2007; O'Leary et al., 2011]. These embryos are generally not utilized in fertility treatment. Normal hESC lines have been established from discarded embryos gained from in vitro fertilization-embryo transfer (IVF-ET) or intracytoplasmic sperm injection (ICSI) [Scott et al., 2007; Niclis et al., 2009; Verlinsky et al., 2009a,b; Taei et al., 2010; Altarescu et al., 2011; Aran et al., 2012]. There are also hESC lines derived from 3PN zygotes by conventional ICM isolating, culture and passage and those cell lines show triploid or diploid karyotypes [Baharvand et al., 2006; Huan et al., 2010; Chen et al., 2012; Wang et al., 2012].

It has been reported that approximately 6–10% polypronuclei zygotes in conventional IVF cycles and 3PN zygotes account for 15–18% of cytogenetically abnormal cases among spontaneous abortions [McFadden and Robinson, 2006]. Although Matt et al. [2004] reported a normal birth after transferring a single-embryo with polypronuclear in zygote formation [Matt et al., 2004], ploynuclear zygotes were often abandoned in IVF treatment if there were better choices. However, some researchers have tried to rescue diploidy by removing the extra male pronucleus of the tripronuclear zygote. As early as in 1980s, the first removal of one of the two presumed male pronuclei from human 3PN zygotes was reported by Rawlins et al. [1988] and Gordon et al. [1989]. A study by Ivakhnenko et al. [2000] showed a high efficiency of removing the extra pronuclei from human 3PN zygotes and 100% of the corrected zygotes survived. Moreover, in 2003, Kattera and Chen [2003] were the first to report that a healthy boy was born after the microsurgical depronucleation of a single pronuclear from a three tripronuclear zygote. The rescued embryo was transferred to a 38-year-old woman on day 3 after fertilization. This is the valuable evidence supporting the totipotent potential of a restored dipronuclear zygote and the safety of polypronuclear correction. However, whether normal human embryonic stem cells can be derivated from the rescued 3PN zygotes is not well-known.

In the current study, we derived two hESC lines from corrected 2PN embryos by excluding one extra pronuclear. These hESC lines exhibited typical features of normal hESCs, such as growing as a colony, expressing undifferentiated markers, capable of long-term proliferation, and possessing pluripotent differentiation potential. Moreover, both hESC lines carried a normal 46, XY karyotype. Thus, our hESC lines would be useful in the in vitro developmental risk assessment for IVF babies born from restored zygotes.

MATERIALS AND METHODS

HUMAN EMBRYOS AND ETHICAL APPROVAL

Human 3PN embryos employed in this study for establishing human Embryonic Stem cells were donated from couples that participated in an IVF program for infertility treatment. All study methods were approved (Date approved: 2008-07-16) by the local Institutional Review Board of the First Affiliated Hospital, Nanjing Medical University. All couples enrolled voluntarily and signed formal consent.

IN-VITRO FERTILIZATION, PRONUCLEAR REMOVAL, AND EMBRYO CULTURE

The patients underwent IVF treatment using either the gonadotrophin-releasing hormone (GnRH)-agonist long protocol or the flare-up protocol were described in our previous report [Liu et al., 2003]. Briefly, ovarian stimulation was administered by human menopausal gonadotrophin (hMG) or recombinant follicle stimulation hormone (rFSH, Gonal-F; Serono). hCG (Pregnyl, Organon) was administered 36 h prior to oocyte collection, which was performed by ultrasound-guided transvaginal aspiration. Cumulus-oocyte-complexes (COC) were cultured for 3–4 h in IVF medium (Universal IVF medium, Origio) to induce full maturation of oocytes. Thereafter, in the conventional IVF program, COC were inseminated with prepared husband's spermatozoa and cultured in IVF medium. After 12–14 h post-insemination, all oocytes were denuded with pipettes and examined for the number, shape and distribution of pronuclear and nucleoli using the pronuclear scoring system, which was modified from Scott and Smith [1998] under an inverted microscope (TE-2000U, Nikon, Japan) equipped with Hoffman modulation optics. The normal zygotes were processed for the next steps of the IVF program. The 3PN zygotes with three distinct pronuclei and the first and second polar bodies were picked up separately. The abnormal zygotes would be studied for research purpose only after the patients were informed and after they consented to donate. All human oocytes and embryos were in vitro cultured in IVF culture system.

Pronuclear removal of donated 3PN zygotes under micro-manipulation system (Narishige, Japan) was performed within 1–2 h after pronuclear assessment on day 1 (Fig. 1). First, zygotes were transferred to M-HTF medium and were then held with Holding pipette (Humagen) in the orientation that the whole pronuclear was far away from the polar body. Second, the 3PN zygote was gently rotated with a holding pipette until the assumed male pronucleus was at a 3 O'clock position, furthest from the second polar body. Third, an ICSI needle (Humagen) was injected into the cytoplasm to aspirate the assumed male pronucleus, and small amounts of surround cytoplasm were removed. Finally, the integrity of the removed pronucleus, the remaining two pronuclei and the cytoplasmic membrane was examined 4–6 h later to confirm whether the pronuclear removal was successful.

Zygotes that survived and continued to cleavage were collected and cultured to developmental day 3 in embryo cleavage culture medium (SAGE) covered with mineral oil, at 37°C and 5% CO_2, 5%O_2, and 90%N_2. At this stage, the embryonic development was observed every 24 h. At day 3, the embryos were moved to a blastocyst culture medium (SAGE) and cultured for an additional 3 days. The blastocyst formation was recorded at day 6 and the blastocyst quality was assessed according to the criteria of Gardner and Schoolcraft [Schoolcraft et al., 1999].

PREPARATION OF THE FEEDER LAYER

Mitomycin C-treated (MCT) mouse embryonic fibroblasts (MEFs) were used to co-culture hESCs. ICR mice were purchased from Model

Fig. 1. A: Put the 3PN zygote into the correct position. B: Injected the microsurgical needle into the cytoplasma. C: Aspirated the assumed male pronucleus. 200× magnification.

Animal Research Center of Nanjing University and 12.5-day old embryos were employed. Briefly, embryos were isolated from uterus and heads and all viscera of embryos were removed. Minced embryo pieces were then digested with 0.25% trypsin/EDTA (ethylene diamine tetraacetic acid; Invitrigen), cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrigen) containing 10%FBS (Invitrigen) at 37°C with 5% CO_2, and performed cryopreservation in passage 1. MEFs at passage 2–5 were mitotically inactivated with 10 μg/ml mitomycin C (Roche) for 2.5–3 h, then dissociated with 0.05% trypsin/EDTA, counted, and plated onto 0.1% gelatin-coated (Invitrigen) dish or plate with the density of $2.5 \times 10^4/cm^2$.

GENERATION OF hESCs

On day 6, the zona pellucida of the blastocyst was removed by 0.1% Tyrode's solution (SAGE). Then the whole zona-free blastocyst was plated onto MCT-MEFs and cultured in hESCs medium which composed of DMEM/F12, 20% knockout serum replacement, 1% Glutamin, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1% penicillin streptomycin (all purchased from Invitrogen), and supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech) at 37°C with 5% CO_2 and20% O_2. Medium was changed daily. After 5 days, flat, colony like cell masses appeared plated onto flesh MCT-MEFs by mechanically dissociated into small pieces. For later passages, hESC cells were expanded mechanically (before passage 5) or digested (passage 6 afterwards) for 10 min with 1 mg/ml collagenase IV (Invitrogen) and then plated onto fresh MCT-MEFs every 5–7 days.

KARYOTYPE ANALYSIS

Standard G-band chromosome analysis was performed by the Medical Test Institute, Nanjing Medical University.

ALKALINE PHOSPHATASE STAINING

Alkaline phosphatase detection Kit (Vector Lab) was selected to carry out alkaline phosphatase (AKP) staining of hESCs according to the manufacturer's protocol.

IMMUNOSTAINING OF hESCs

The following primary antibodies were used for immunostaining hESCs: anti-Oct-3/4, anti-SSEA4, anti-Tra 1-60, and anti-Tra 1-81 antibodies (all from Chemicon). Briefly, cells cultured on coverslips were fixed with 4% paraformaldehyde (PFA; Sigma), permeated with 0.1% TritonX 100 (Sigma)/Phosphate Buffer Solution (PBS), and blocked in fresh 2% bovine serum albumin (BSA; Sigma)/PBS at room temperature for 30 min. The treated hESCs were washed with PBS for 5 min and then incubated with primary antibodies over night at 4°C. After the 5 min rinse with PBS, the hESCs were incubated with FITC-conjugated or Cy2-conjugated secondary antibodies (Jackson Immunoresearch, West Grove) for 30 min in the dark. After being washed with PBS for 5 min, the hESCs were mounted with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Lab) and photographed under a fluorescence microscope (Nikon, Japan).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

For reverse transcription-polymerase chain reaction (RT-PCR), cells were first washed with PBS and then RNA was extracted with Trizol (Invitrogen) to synthesize cDNA through reverse transcription, finally cDNAs underwent RT-PCR amplification. The PCR reaction mix was 50 μl volume, composed of 5 μl of 10× PCR buffer, 4 μl of 2.5 mM dNTPs, 1 μl of each primer (10 μM), 0.5 μl of Taq enzyme (5 U/μl), 8 μl of cDNA template, and 31.5 μl of ultra-pure water. RT-PCR reaction conditions were as follows: pre-denaturing at 94°C for 2 min, denaturing at 94°C for 45 s, reannealing at 55°C for 45 s, elongation at 72°C for 45 s, 30 cycles; finally elongation at 72°C for 5 min. The PCR products were performed agarose gel electrophoresis, and then the electrophoretic results were analyzed with an ultraviolet-automatic image analyzer. Detailed procedures were carried out strictly according to the kit instructions (Takara, Japan). Primer sequences were shown in Table I.

TABLE I. Primer Sequence for RT-PCR

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	

TABLE II. Survival and Development of Corrected 2PN Zygotes

Total manipulated 3PN zygotes	Development stage of corrected 2PN zygotes			
	2-Cell stage N (%)	8-Cell stage N (%)	Morula N (%)	Blastocyst N (%)
61	58(95.1%)	54(88.5%)	31(50.8%)	11(18.0%)

EMBRYOID BODY AND TERATOMA FORMATION

After 5–7 days in the culture, hESCs colonies were disassociated from feeder cells with collagenase IV and dispase, plated onto bacteria culture dishes to form embryoid bodies, which were allowed to culture for 1–2 weeks in ES media without bFGF and feeder. For teratoma formation, $5–10 \times 10^6$ hESCs were injected intramuscularly into Severe Combined Immunodeficiency Disease (SCID) mice. After 2–3 months, the mice were sacrificed and teratoma tissues were dissected and then fixed in 4% PFA. The sections from fixed teratoma tissues were then stained with hematoxylin and eosin and photographed.

RESULTS

DEVELOPMENT OF CORRECTED 3PN EMBRYOS

Our study employed sixty-one 3PN embryos donated by 35 couples, whom suffered from tubal factor infertility and underwent IVF treatment by either GnRHa long or flare-up protocol from March 2009 to May 2009. The maternal age of these couples ranged from 24 to 37, while paternal was 25–39. These sixty-one 3PN embryos occur in a total of 341 populations after normal ovulation induction, oocyte retrieval and insemination, and the 3PN rate in these couples varies from 8% to 40% (Supplementary Table I). After microsurgical

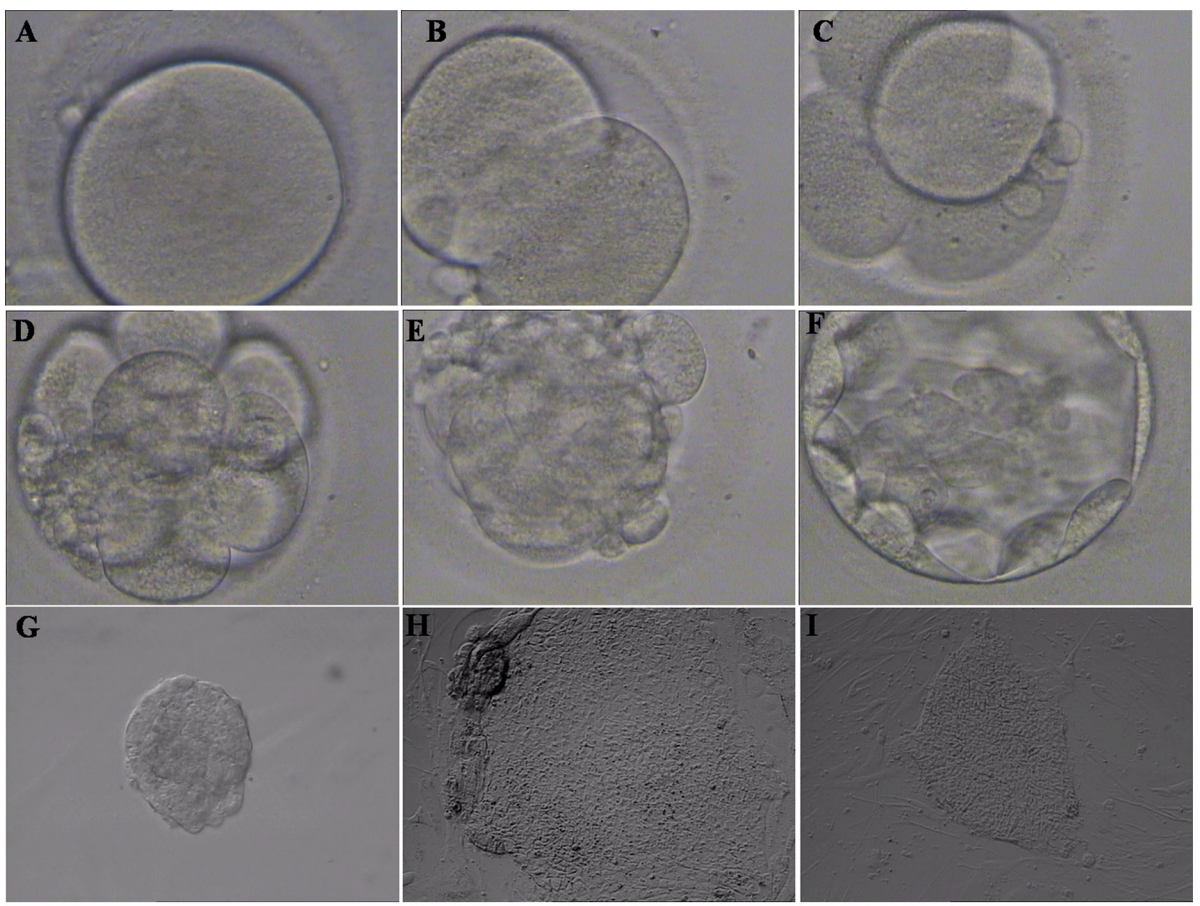

Fig. 2. A: Zygotes, 16–18 h after micromanipulation, 400× magnification. B–F: developmenting embryo, stage of 2 cell (B), 4 cell (C), 8 cell (D), morula (E), and blastocyst (F). Figures were all taken under 400× magnification. G: Whole embryos after zona removal, 200× magnification. H: Outgrowth of inner cell masses (ICMs) at 5 days post plating. 100× magnification. I: Morphology of hESC lines at passage 2. 100× magnification.

TABLE III. Quality of Blastocysts

No. of blastocyst	Quality	hESCs Derived
B1	4AA	Failed
B2	4CC	Failed
B3	4BB	hES-CCRM-22
B4	4CC	Failed
B5	4BB	Failed
B6	3AA	Failed
B7	4CC	Failed
B8	5CC	Failed
B9	4CC	Failed
B10	3CC	Failed
B11	4BB	hES-CCRM-23

manipulation, each of the sixty-one 3PN zygotes was aspirated one extra proneuclear and the corrected 2PN embryos were sequentially incubated in embryo cleavage culture medium and blastocyst culture medium. We observed that of the 61 embryos, 58 embryos survived and developed to 2-cell stage at 30 h postinsemination, 54 embryos reached 8-cell stage at day 3 or day 4, while 31 embryos went through morula stage at day 4 to day 5, and at last, 11 embryos showed blastocysts morphology (Table II and Fig. 2). Thus the rate of blastocyst formation of the corrected 2PN embryos was about 18.0% (11 of 61), which is a little high than previous reports [Balakier, 1993; Sathananthan et al., 1999; Reichman et al., 2010]. The quality of blastocysts developed from corrected 2PN zygotes were classified as 3–5CC (Table III).

DERIVATION AND CHARACTERIZATION OF hESC LINES

On day 6, the whole Zona-free blastocysts were plated onto MCT-MEFs after being removed from the zona pellucida by Tyrode′s solution, and were cultured in hESCs medium. After 5 days, the coming out flat, colony like cell masses were passaged by mechanically dissociated into small pieces. Finally, from the 11 blastocysts, two independent, stable hESC lines, CCRM-hESC-22 and CCRM-hESC-23, were successfully established through mechanically and enzymatically passaged and expanded. Both of the two hESC lines were proliferated to over 50th generations and they still maintained their distinctions as those of early passages.

During culture, the hESCs grew in flat colonies with clear cell boundaries (Fig. 3A). Among these cells, large nuclei with clear nucleolus and a high nucleoplasmic ratio were observed under inverted microscope. We noticed that the hESCs reached their growth peak during day 4–6, and should be passaged on day 5–7, otherwise the hESCs would begin to spontaneously differentiate. After AKP staining, the hESC colonies showed violet-blue (strongly positive) while MEFs failed to be stained (Fig. 3B). Furthermore, hESCs also positively expressed stem cell markers such as OCT4, SSEA4, TRA-1-81 and TRA-1-60 (Fig. 3D–G). Importantly, the karyotype analysis showed that both cell lines carry a normal 46, XY diploid karyotype (Fig. 3C). In summary, the cell lines derived from the corrected 2PN zygotes could undertake self-renewal, express stem cell markers and be long-term cultured, characterizing them as hESCs.

DIFFERENTIATION OF hESCs

Next we investigated the pluripotency of our established hESC lines through spontaneously differentiating the cells into morphologically distinct cell types representing all three embryonic germ layers in vitro. We observed that embryoid bodies (EBs) could be formed when hESCs were cultured in ultralow-attachment dish without bFGF and feeder layer after passaged. The size of EBs were gradually increased and cyst-shaped embryoid bodies would present at day 7 (Fig. 4 A). Furthermore, RT-PCR revealed expressions of early differentiation

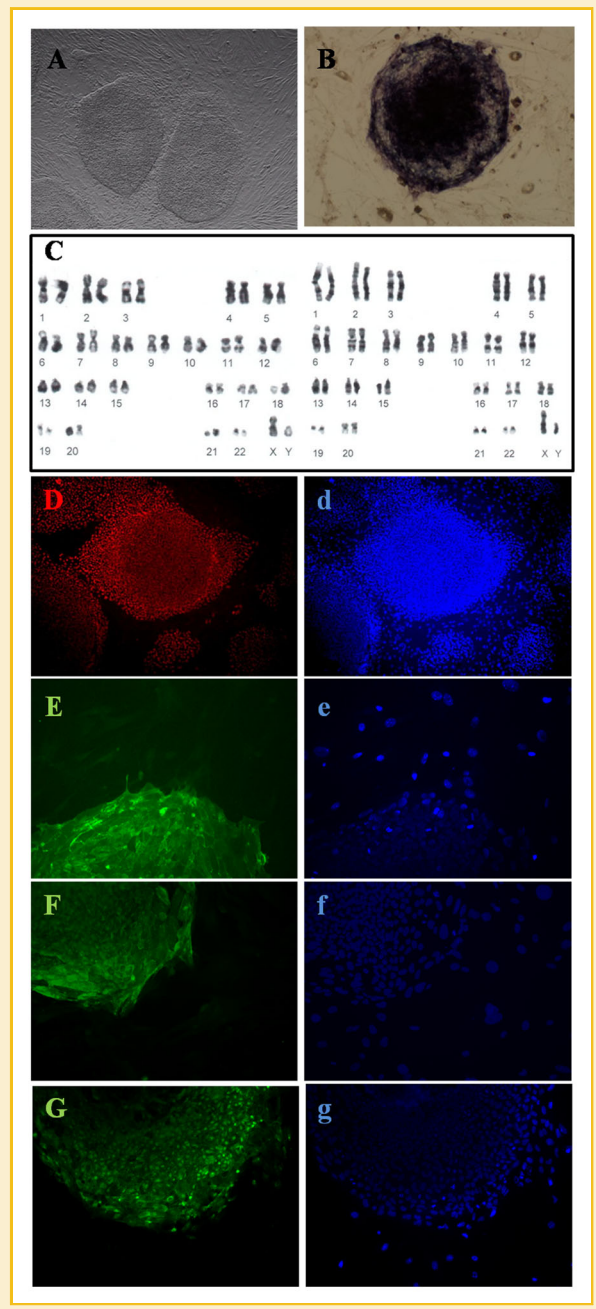

Fig. 3. A: hESC clones under inverted microscope (4× magnification, hES-CCRM-22, Passage 35). B: Alkaline phosphatase staining. C: Karyotype analysis, normal 46XY (left: hES-CCRM-22, right: hES-CCRM-23). (DG) hESCs Expressed stem cell markers. OCT4 (D), TRA-1-60 (E), TRA-1-81 (F), and SSEA-4 (G) were stained. D–G: Nuclear of hESC were stained by DAPI. All photos taken under 200× magnification.

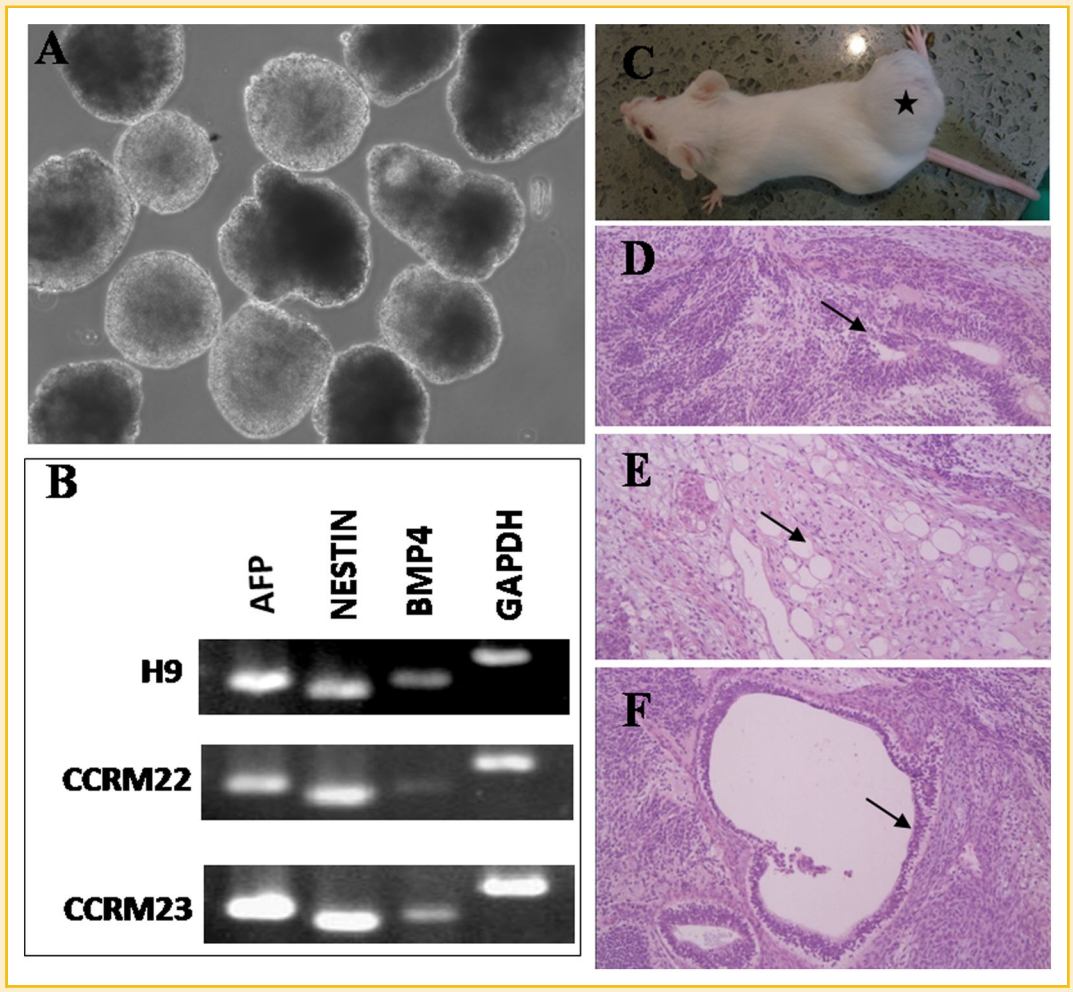

Fig. 4. Differentiation of established hESCs in vitro and in vivo (hES-CCRM-22). A–B: hESCs differentiation in vitro. A: Embryoid bodies at differentiation day 7.100× magnification. B: Three-germ layer markers expressing in embryoid bodies detected by RT-PCR. Lane 1, endoderm marker AFP; Lane 2, mesoderm marker BMP4; Lane 3, ectoderm marker Nestin; Lane 4, GAPDH, as control. C–F: hESCs differentiation in vivo. (C) Teratomas (asterisk) formation after hESCs injected into SCID mice. primary neural tube (D, arrows), fat tissue (E, arrows), Glandular tissue (F, arrows) were indicated by HE staining of teratomas sections. 200× magnification.

markers including Nestin (ectoderm), BMP4 (mesoderm), and AFP (endoderm) in EBs on day 7 (Fig. 4B).

Teratoma formation experiments were performed to access the in vivo pluripotency of hESCs. A lump was seen at the injection site after the hESC cells were injected into the hind legs of SCID mice for 8–12 weeks (Fig. 4C). The gross anatomy analysis showed that the lump mass neither marked peplos, nor marked destruction in lump-surrounding tissue, indicating that the lump indeed came from injected hESCs rather than intrinsic tumorigenesis. HE staining analysis confirmed the lump tissue contained nerve cells (ectoderm lineage, Fig. 4D), fat tissue (mesoderm lineage, Fig. 4E), and glands (endoderm lineage, Fig. 4F), showing that the injected hESCs could develop into three germ layers-derived teratoma and demonstrating the hESC lines are pluripotency in vivo. Our results showed that CCRM-hESC-22 and CCRM-hESC-23, derived from corrected 2PN zygotes, not only maintained hESC-like morphology, but also owned the differentiation pluripotency in vitro and in vivo, indicating those were the normal hESCs.

DISCUSSION

In general, zona reaction (and oolemma) of an oocyte prevents polyspermy during fertilizations, resulting in 2PN zygotes. However, the block to polyspermy may fail during IVF, and thus enable two or more sperms to fertilize an oocyte and produce a polyploid (≥3PN) zygote [Sachs et al., 2000], which occurs at a rate of 5% in traditional IVF cycles [Gu et al., 2009]. However, human tripronuclear zygotes may also result from diploid spermatozoa or oocytes blocked in the meiotic division or diploid oocytes [Tarin et al., 1999]. It was reported that 3PN zygotes show slower cleavage at day 3 and lower implantation rates [Reichman et al., 2010], and only 6–10% can reach the blastocyst stage [Balakier, 1993; Sathananthan et al., 1999]. Even after successful implantation and development, a fetus from a 3PN embryo will often spontaneously abort or have malformations [Sherard et al., 1986]. Therefore, 3PN zygotes have to be deselected in clinical IVF [Gu et al., 2009]. To correct triploidy, some strategies have

been attempted since the first successful removal of extra pronuclei in 1989 [Gordon et al., 1989; Malter and Cohen, 1989; Ivakhnenko et al., 2000; Gu et al., 2009]. However, the rate of blastocyst formation from corrected zygotes varies in different labs. Gu et al. [2009] and Escriba et al. [2006] reported that tripronuclear zygotes correction give a higher blastocyst formation rate than control groups, while Chen et al. [2010] found that the early embryo development is not improved by removal of a male pronucleus. Here we successfully rescued triploid zygotes at an early cleavage stage with a survival rate of 95%, and achieved a blastocyst formation rate of 18.0% (11 of 61) which is similar to the previously reported rate of 16% by Gu et al. [Chen et al., 2010]. Kattera and Chen [2003] previously reported that a healthy boy was born in an IVF program after the microsurgical depronucleation of a single pronucleus from the three tripronuclear zygote and implantation. This is valuable evidence for the totipotent potential of a restored dipronuclear zygote and the safety of ploidy correction. However, the development risks to babies born from the corrected zygote are still unclear. Normal hESCs derived from the corrected zygotes, which can differentiate into all three germ layers and their derivatives, may be useful to in vitro evaluate the developmental risk for those babies. However, such hESC lines have not been reported.

In the current study, two hESC lines were derived from diploid blastocysts which were corrected from 3PN zygotes. Both of the cell lines showed typical hESCs morphology, expression of undifferentiated hESCs markers, capability of long-term proliferation, and pluripotent differentiation potentials. Moreover, both of them carried a normal 46, XY diploid karyotype. Our results provide valuable evidence regarding the safety and importance of ploidy correction. First, the hESCs derived from corrected zygotes face less ethical problems, thus previously discarded 3PN zygotes may be an important resource for new hESC lines in the future. Second, these hESC lines can be applied as an embryo developmental model in vitro to analyze the health risks of IVF babies from corrected 2PN embryos. Third, our results indicate that patients looking for IVF treatment may have another choice if there is no diploid zygotes production.

It has been suggested that microsurgical removal of the pronucleus which located at the farthest position to the second polar body will generate heteroparental blastocyst [Escriba et al., 2006]. In the current study, the assumed male pronucleus was aspirated to produce heteroparental zygotes. However, due to the protection of parents′ information, here we cannot take genetic samples from parents to further confirm whether these hESC lines are parthenogenetic or androgentic. Thus, that the generations of hESC lines with normal 46, XY karyotype here may be indirect evidence for heteroparental pronuclei survival after microsurgical rescue. We believe this question will be answered in the future when more hESC lines are derived from the corrected 2PN zygotes, meanwhile genetic samples from parents are carefully analyzed simultaneously.

In conclusion, our study is the first one to report the successful derivation of normal hESC lines from corrected 3PN embryos, demonstrating that removal of extra pronuclear at early cleavage stage would not adversely affect the development of a human embryo, and the corrected embryo maintains its′ pluripotency similar to the normal embryos.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table SI.