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—Original Article—

Triploid human embryonic stem cells derived from tripronuclear zygotes displayed pluripotency and trophoblast differentiation ability similar to the diploid human embryonic stem cells

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Abstract. Because the diploid human embryonic stem cells (hESCs) can be successfully derived from tripronuclear zygotes thus, they can serve as an alternative source of derivation of normal karyotype hESC lines. The aim of the present study was to compare the pluripotency and trophoblast differentiation ability of hESCs derived from tripronuclear zygotes and diploid hESCs. In the present study, a total of 20 tripronuclear zygotes were cultured; 8 zygotes developed to the blastocyst stage and 1 hESC line was generated. Unlike the previous studies, chromosomal correction of tripronuclear zygotes during derivation of hESCs did not occur. The established line carries 3 sets of chromosomes and showed a numerical aberration. Although the cell line displayed an abnormal chromosome number, it was found the cell line has been shown to be pluripotent with the ability to differentiate into 3 embryonic germ layers both *in vitro* and *in vivo*. The expression of X inactive specific transcript (XIST) in mid-passage (passage 42) of undifferentiated triploid hESCs was detected, indicating X chromosome inactivation of the cell line. Moreover, when this cell line was induced to differentiate toward the trophoblast lineage, morphological and functional trophoblast cells were observed, similar to the diploid hESC line.

Key words: Differentiation, Embryo, Human pluripotent stem cell, Isolation, Karyotype

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Human embryonic stem cells (hESCs) can be established from the pluripotent cells of pre-implantation embryos. Under appropriate conditions, hESCs can be propagated unlimitedly *in vitro* and induced to differentiate into multiple cell lineages [1, 2]. The ability to differentiate has made hESCs is an important source of cells for replacement of degenerated cells and tissues. Besides generation of hESCs for medical purposes, hESCs can be generated for disease models or tools to access and discover the developmental stages of the human body. Chromosome-abnormal embryos, for example mono- or tripronuclear zygotes [3–6], and embryos carrying genetic abnormalities [7–9] have been used for hESC generation and have given rise to hESC lines. Although this type of hESC line cannot be used for cell therapies, it can be a useful tool for the investigation of cell physiology, early development and disease progression for the chromosomal abnormalities carried by a zygote [10]. Thus, chromosome-abnormal embryos can serve as the starting material for derivation of disease-specific or genetically abnormal cell lines.

In general, the major sources of human embryos used for derivation of hESCs, are discarded or donated embryos from the *in vitro* fertilization (IVF) units. Although these embryos usually display bad quality and are not suitable for transfer, they can still give rise to useful hESC lines. The morphology of diploid embryos likely has no impact on the successful derivation of hESC lines [11]. Embryos produced through the IVF process sometimes exhibit poor morphology, such as abnormal pronuclear formation and fragmented blastomeres, or they can progress to cell degeneration before being transferred. Clinically, embryos that display abnormal pronuclear after conventional fertilization or intracytoplasmic sperm injection (ICSI) are not transferred to recipients. However, some reports have mentioned that the transfer of such abnormal pronuclear embryos has resulted in healthy births [12, 13]. Moreover, blastocysts developed from abnormal pronuclear embryos have also been used for derivation of hESC lines and normal karyotype hESC lines can also be derived from these abnormal embryos [3–5, 14, 15]. Unlike the diploid zygotes, the efficiency of successful derivation of hESC line from aneuploid zygotes has been reported to be poor [6]. Currently, it remains unclear how abnormal pronuclear embryos can give rise to normal diploid hESC lines. It is believed that these diploid hESC lines are probably derived from mosaic zygotes consisting of aneuploid and euploid cells, and that *in vitro* selection favors euploid cells [4]. Interestingly, the results from a recent study demonstrated that the pluripotency and molecular biology of triploid hESC lines generated via somatic cell nuclear transfer were similar to those of diploid and triploid cell lines derived from fertilized embryos [16]. Although triploid hESC lines exhibit pluripotency, and display biological characteristics similar to those of diploid hESC lines but the genetic and epigenetic abnormalities might limit the use of such lines for therapeutic purposes. However, triploid hESCs have been used for

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studying genetic and epigenetic phenomena; for instance, it has been reported that the presence of two active X-chromosomes in a triploid hESC line caused the skewed X chromosome inactivation (XCI) pattern and that the presence of the maternal or paternal genome in the extra set of chromosomes might alter the expression of imprinted genes [17]. In this report, we describe the derivation, characterization, and trophoblast differentiation of a triploid hESC line derived from a tripronuclear zygote. This triploid hESC line differentiated into three embryonic germ layers for both *in vitro* and in *vivo*. Moreover, the triploid hESC line was induced to differentiate to trophoblast cells as shown by morphology, gene expression and secretion of human chorionic gonadotropin (hCG), similar to a diploid hESC line.

Materials and Methods

Embryos and ethical approval

Abnormal human embryos used in the present study were donated with informed consent from a couple that underwent an IVF program to treat infertility, at the Reproductive Medicine Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital. Isolation of hESCs was performed after approval by the Institutional Review Board (IRB number 096/50), Faculty of Medicine, Chulalongkorn University. Fertilized embryos that showed three pronuclei after fertilization via ICSI, were cultured in droplets of Global medium (LifeGlobal, Guelph, ON, Canada) supplemented with 10% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA), covered with light oil (LifeGlobal), and cultured at 37ºC in 5% O_2, 6% CO_2 and 89% NO_2 [18]. Embryos that developed to the blastocyst stage were collected and subjected to isolation of hESCs.

Preparation of a feeder layer

Commercial human foreskin-derived fibroblasts (HFFs; CRL-2429, ATCC, Manassas, VA, USA) were cultured and maintained according to the manufacturer's protocol. To use HFFs as the feeder layer, confluent HFFs were inactivated with 10 µg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 2.5–3 h, dissociated with 0.05% trypsin-EDTA (Life Technologies, Carlsbad, CA, USA), and plated on 0.1% gelatin coated-dish (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 5×10^4 cells/cm^2.

Isolation of inner cell masses and propagation of hESCs

The zona pellucida of blastocysts was removed by incubation with 0.1% Acid Tyrode's solution (Sigma-Aldrich) followed by five washes in hESC culture medium and plating of whole blastocysts on a feeder layer. The formation of a dome-like shape indicating an inner cell mass (ICM) was monitored for daily. The outgrowth from the ICM was mechanically removed from the trophectoderm (TE) with a needle at 4 to 5 days after initial plating and then plated on a new feeder layer. hESC-like cells were propagated by mechanical splitting with a needle or fine-drawn glass pipette. For propagation, colonies of hESCs were mechanically cut with a 23G needle into small pieces every 5–7 days, detached from the culture dish and plated onto new feeders, and the culture medium was changed on a daily basis.

A diploid hESC line (Chula2.hES) derived from frozen-thawed embryos [19], was cultured and propagated as described above.

The hESC culture medium consisted of knockout DMEM supplemented with 20% Knockout serum replacement (KSR), 1% GlutaMax, 1% non-essential amino acids, 1% penicillin-streptomycin, and 0.1 mM mercaptoethanol (all from Life Technologies) and 8 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA).

Immunostaining

Cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) for 15 min at room temperature. They were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min, blocked with 5% goat serum (Sigma-Aldrich) in PBS and incubated overnight with primary antibodies at 4ºC. The primary antibodies were detected after incubation with secondary antibodies for 45 min at room temperature. The primary antibodies used in this study were against OCT-4 (Abcam, Cambridge, MA, USA; 1:200), SSEA-4 (Abcam; 1:200), TRA-1-60 (Chemicon, Temecula, CA, USA; 1:100), TRA-1-81 (Chemicon; 1:100), Nestin (Chemicon; 1:200), Brachyury (Abcam; 1:200), alpha-fetoprotein (AFP, Chemicon; 1:200), CDX2 (Abcam; 1:200) and EOMES (Abcam; 1:200). The secondary antibodies used in this study were FITC conjugated- goat anti-rabbit (Abcam; 1:200), FITC conjugated- rabbit anti-goat (Abcam; 1:200), Alexa Fluor 568 goat anti-rabbit (Life Technologies; 1:500) and Cy3 conjugated- goat anti-mouse (Chemicon; 1:200). Cells were counterstained for nuclei with the 4'-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich; 1:1000).

RT-PCR analysis

Total RNA was extracted with TRIzol Reagent (Life Technologies). One microgram of total RNA was reverse transcribed with a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer's instructions. PCR was performed with PCR Master Mix (2X) (Fermentas, Thermo Fisher Scientific). PCR conditions and primers were used as described in the previous reports [20, 21].

Real-time PCR analysis for X inactive specific transcript

The relative expression level of *X inactive specific transcript (*XIST) in mid-passage of triploid hESCs (P42) was analyzed in comparison with diploid hESCs (P60) using real-time PCR analysis. Total RNA of undifferentiated triploid and diploid hESC lines was extracted and used for cDNA synthesis as described above. cDNA was subjected to PCR amplification for real-time PCR that was carried out with the ABI sequence detection software (Applied Biosystems) with XIST- and GAPDH-specific primers as described previously [22]. The relative gene expression level of XIST was calculated after having been normalized with the expression of GAPDH.

Differentiation of hESCs

Embryoid body (EB) formation was induced *in vitro* by cutting the colonies into small clumps and placing them in a suspension culture with hESC culture medium lacking bFGF. EBs were cultured in suspension for 7 days and then plated on Matrigel-coated dishes for an additional 14 days. After total culture for 21 days, cells were fixed and immunostained for markers of the ectoderm (Nestin), mesoderm (Brachyury) and endoderm (AFP).

For *in vivo* differentiation, teratoma formation was examined. Approximately 100–300 clumps of hESCs were injected underneath the testicular capsule of 4- to 6-week-old nude mice. Ten to twelve weeks later, mice were euthanized and the teratomas were removed, fixed in 10% buffered formalin phosphate (Sigma-Aldrich), embedded in paraffin blocks; and 4 micron sections were stained by hematoxylin and eosin. Care of animals was in accordance with the institutional guidelines as approved by the committee for ethics of laboratory animal use (Approval No. 15/52).

Karyotyping

Undifferentiated cells of Chula3.hES were subjected to karyotyping. Cells were incubated with 10 ng/ml of KaryoMAX (colcemid; Life Technologies) for 3 h at 37ºC in 5% CO_2. The cells were then trypsinized, treated with 0.075% KCl solution and fixed with fixative (3:1 of methanol: acetic acid). Metaphase spreads were prepared on microscope slides and stained using the standard G banding technique. Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN). About 15–20 metaphase spreads were analyzed.

DNA fingerprinting

Total genomic DNA was extracted from undifferentiated hESCs using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Fifteen short tandem repeat (STR) loci and Amelogenin were then amplified using an AmpFlSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, and detected using the 3100 Genetic Analyzer (Applied Biosystems).

Trophoblast differentiation of triploid hESCs

In this study, the established triploid hESC line (Chula3.hES), and the diploid hESC line (Chula2.hES) were induced for trophoblast differentiation with a protocol based on adherent culture as described in a previous report [21], with a slight modification. In brief, confluent hESCs were dissociated into small clumps using Versene (Life Technologies). Cells were plated on Matrigel-coated dishes and cultured with ReproFF medium (ReproCeLL, Yokohama, Japan) for 24 h. The following day, the culture medium was changed to hESC culture medium without bFGF. The culture medium was supplemented with 100 ng/ml of human recombinant BMP4 (R&D Systems). Cells were maintained in culture for 14 days with the medium changed on a daily basis. Supernatants were collected at D1, D7, and D14 for measuring hCG secretion using an ELISA kit (DRG diagnostic, Instruments, Marburg, Germany). After 14 days of differentiation, cells were collected for RNA extraction and gene expression was analyzed by RT-PCR as mentioned above. In addition, cells were fixed for detection of expression of CDX2 and EOMES by immunostaining.

Statistical analyses

Statistically analysis for the level of hCG, secreted by the differentiated cells was performed using the GraphPad Prism 6 software (http://www.graphpad.com). One-way analysis of variance (ANOVA) along with Turkey's multiple comparison test was assessed for statistical analysis. Student's *t*-test was used to evaluate differences in the relative expression of XIST mRNA. Data are expression displayed the mean ± SEM of 3 independent cultures. A value of $P \leq 0.05$ was considered statistically significant.

Results

Derivation and propagation of an hESC line from a tripronuclear zygote

In the present study, a total of 20 tripronuclear zygotes (Fig. 1A) were cultured and 8 zygotes developed to the blastocyst stage. Due to the low quality of blastocysts obtained (Fig. 1B), zona-free blastocysts (Fig. 1C) were plated on the feeder layer. Twenty-four hours after plating, zona-free blastocysts attached to the feeder layer, and this was followed by outgrowth of the TE (Fig. 1D). Five days after culture, prominent ICM outgrowth from the TE could be distinguished (Fig. 1E), and the ICM outgrowth was removed out and plated on newly prepared feeders. The first mechanical splitting of hES-like cells was performed at Day 5 after replating (Fig. 1F). As shown in Fig. 1F, primary hES-like colonies were mixed with undifferentiated- and differentiated cells. We separated the undifferentiated area of the colony by cutting the area with a 23G needle, detached it from the feeder layer, and then plated it on a new feeder layer. The mechanical propagation of undifferentiated cells was repeated until a stable hESC line could be established, and we named this cell line as Chula3.hES. Chula3.hES cells at passage number (P) 3 (Fig. 1G) and P40 (Fig. 1H) exhibit typical hESC morphology, with a defined colony border.

Characterization and identification of triploid hESCs

In order to prove whether the Chula3.hES line, exhibited the characteristics of pluripotent cells, the expression of pluripotent markers, and the ability to differentiate into embryonic germ layers were examined. The expression of pluripotent markers of the Chula3.hES line was compared with the expression of those of diploid hESC line, Chula2.hES. Colonies of Chula3.hES were positively immunostained for SSEA-4, TRA-1-60, TRA-1-81 and OCT-4 (Fig. 2A). Moreover, the RT-PCR results showed that the cells expressed the candidate genes for pluripotency, OCT-4, NANOG, SOX2, REX1, and NODAL, similar to those Chula2.hES (Fig. 2B). To examine its ability to differentiate, Chula3.hES was induced to differentiate *in vitro* through an EB formation assay. After 7 days of culture in suspension, Chula3.hES formed EBs. Then Chula3.hES-derived EBs were plated onto the Matrigel-coated dishes and allowed to differentiate further for another 21 days. The results showed that cells in EBs differentiated to embryonic germ layers as demonstrated by the positive immunostaining of Nestin (ectoderm), AFP (endoderm) and Brachyury (mesoderm) (Fig. 2C). Additionally, when Chula3.hES cells were injected into the testicular capsule of nude mice, the resulting teratoma tissue consisted of three embryonic germ layers, confirming the pluripotency of the Chula3.hES line (Fig. 2D).

For identification of the cell line, karyotype and DNA fingerprint analyses were performed. The G-banding results demonstrated that Chula3.hES at P8 (the earliest passage that the karyotyping could be performed) and P40 displayed three sets of each chromosome. A numerical aberration also occurred, as the cell line displayed mosaic triploid karyotypes of 70, XXY, +12 (Fig. 3A) and 69, XXY, +12,

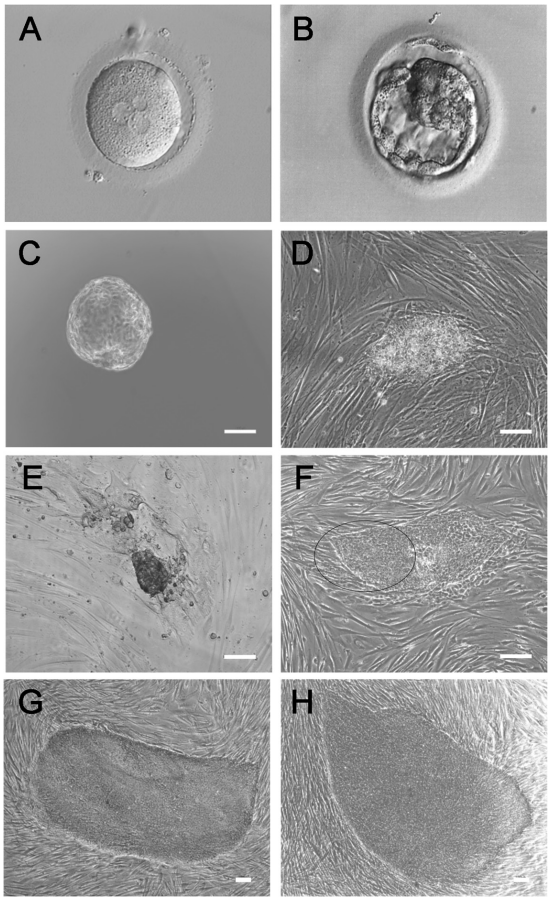

Fig. 1. Derivation of the hESC line, Chula3.hES, from a tripronuclear zygote. The images show the growth dynamics from a zygote to stable hESC line. The zygote presented 3 pronuclei after fertilization (A) and developed to the blastocyst stage (B). The zona-free blastocyst (C) was directly plated on the feeder layer. Twenty-four hours after initial plating, the blastocyst attached to the feeder, but the ICM could not be located (D). Prominent ICM outgrowth (E) was observed 5 days after the initial plating. The ICM outgrowth was removed from the surrounding TE and plated on a new feeder layer. Five days after replating, hES-like cells grew out from the ICM outgrowth (F). The primary colony consisted of undifferentiated hES-like cells which could be distinguished by the cells that had prominent nuclei; clear cytoplasm and a high nucleus and cytoplasm ratio (indicated by the oval); and differentiated cells (the rest of the colony). Passage number 3 (G) and passage number 40 (H) of Chula3.hES displayed typical hESC morphologies with a defined colony border. Scale bars = 100 μm, images in A and B were obtained using a 40 × objective phase-contrast microscope. hES, human embryonic stem; hESC, human embryonic stem cells; ICM, inner cell mass; TE, trophectoderm.

–15 (Fig. 3B). However, Chula3.hES at P53 displayed a mosaic triploid karyotype of 71, XXY, +9, +12 (Fig. 3C) In addition, the STR of Chula3.hES, showed triple peaks in some loci (arrows) which demonstrated the triploid characteristic of the Chula3.hES cell line (Fig. 3D).

Expression of X inactive specific transcript in triploid hESCs

The relative expression of XIST was assessed in Chula3.hES and Chula2.hES lines. The results demonstrated that the level of XIST expression in Chula3.hES (P42) was not significantly different ($P > 0.05$) from that in Chula2.hES (P60), as shown in Fig. 2E.

Trophoblast differentiation of triploid hESCs

After BMP-4 treatment morphological changes were observed in both Chula2.hES and Chula3.hES. Cells flattened after induced differentiation and enlarged prominent nuclei, which resembled early stage trophoblast differentiation, were observed (Fig. 4A). However, at day 14, some hES-like cells still remained in the culture (Fig. 4A). To confirm trophoblast differentiation, we analyzed the expression of pluripotent and trophoblast genes by RT-PCR as well as the expression of the trophoblast markers, CDX2 and EOMES, by immunocytochemistry in the Day14 differentiated cells. The RT-PCR results for the BMP-4 treated cells revealed a persistent expression of pluripotent genes including, OCT-4 and SOX2, while the expression of the trophoblast markers genes, CDX2 and EOMES was increased (Fig. 4B). In addition, CDX2 and EOMES markers were also detected by immunocytochemistry (Fig. 4C). To determine the link between morphological and functional changes of Chula3.hES and Chula2.hES during trophoblast differentiation, the secretion of hCG into the culture media by BMP-4-treated cells was determined. We found that cells differentiated from Chula3.hES and Chula2.hES secreted hCG hormone into the media as hCG hormone was readily detected in the culture media after 7 days of differentiation. The level of hCG secreted by Chula3.hES and Chula2.hES showed an increase consistent with the time of differentiation. However, the levels of hCG secreted by Chula3.hES and Chula2.hES were not significantly different ($P > 0.05$) when the levels of hCG were compared on the same day (day 1, 0.1 mIU/ml *vs*. 0.1 mIU/ml; day 7, 5.6 ± 1.4 mIU/ml *vs*. 2.6 ± 1.7 mIU/ml; and day 14, 339.4 ± 60.7 mIU/ml *vs*. 320.9 ± 88.5 mIU/ml, respectively).

Discussion

In general, two pronuclei form after fertilization of an oocyte by sperm, subsequently followed by fusion of the pronuclei, and cleavage to the diploid blastomere stage. However, aberrant pronuclear phenomena, such as no pronucleus, 1 pronucleus or 3 pronuclei (0PN, 1PN or 3PN respectively), have been observed in the IVF process. Zygotes carrying abnormal pronuclear are normally unable to develop to full term and correlate with early embryonic loss. Thus, chromosomally aberrant zygotes are not be routinely transferred to the uterus. Although some reports have claimed that transferring abnormal pronuclear zygotes can result in healthy births [12, 13], at the IVF unit of King Chulalongkorn Memorial Hospital zygotes of this type are routinely discarded or donated to research by the couples. Among those displaying abnormal pronuclear formation, tripronuclear zygotes are the abnormality that has been most commonly observed with a rate of 2–9% after conventional IVF or ICSI [23–25]. The presence of three pronuclei in the zygotes make them unsuitable for transfer; however, tripronuclei zygotes are able to develop to the blastocyst stage. Moreover, when tripronuclear zygotes are cultured on a feeder layer they can give rise to hESC lines [5, 6, 14].

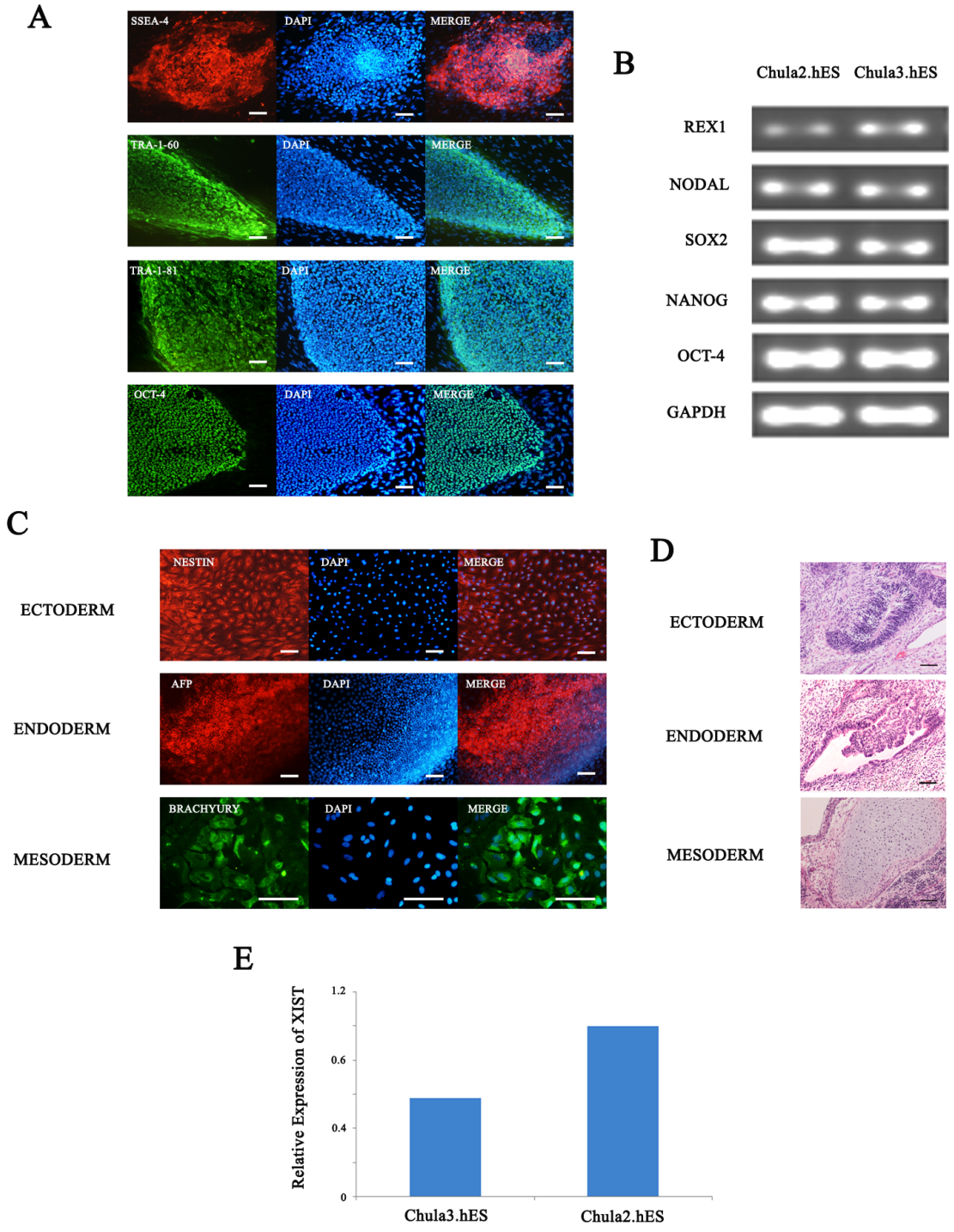

Fig. 2. Characterization of the triploid hESC line. The pluripotency of the triploid Chula3.hES line was characterized for pluripotent markers by immunostaining, gene expression by RT-PCR, *in vitro* differentiation by EB formation and *in vivo* differentiation by teratoma assay. The triploid Chula3.hES was positively immunostained for SSEA-4, TRA-1-60, TRA-1-81 and OCT-4 (A). The results of RT-PCR showed that Chula3.hES expressed OCT-4, NANOG, SOX2, NODAL and REX1, which was similar to the diploid hESC line, Chula2.hES (B). The *in vitro* differentiation of the Chula3.hES line was confirmed by the ability to form an EB and further differentiated to ectoderm (Nestin), mesoderm (Brachyury) and endoderm (AFP) (C). The hematoxylin and eosin staining of teratomas resulting from *in vivo* differentiation revealed a neuroepithelium (ectoderm), gut-like structure (endoderm), and cartilage (mesoderm) (D). The relative expression of XIST was assessed in the Chula3.hES and Chula2.hES lines. The level of XIST expression of Chula3.hES was not significantly different ($P > 0.05$) from that of Chula2.hES (E). Scale bars = 100 µm. AFP, alfa-fetoprotein; DAPI, 4'-6-diamidino-2-phenylindole; XIST, x inactive specific transcript.

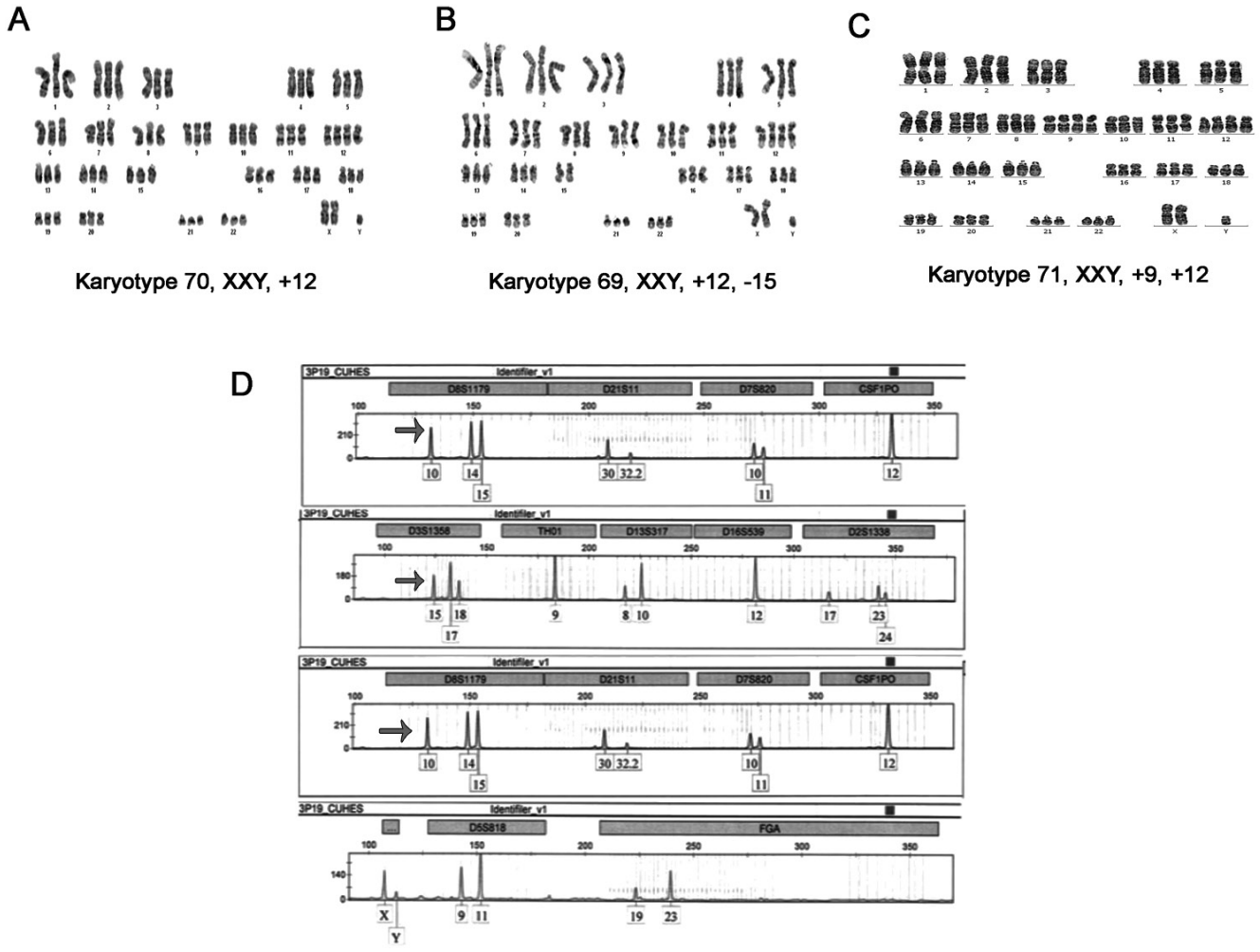

Fig. 3. Identification of the triploid hESC line. Identification of the triploid hESC line was performed by karyotype and DNA-fingerprint analyses. The results of karyotype analyses showed that the cell line displayed a mosaic triploid karyotype with numerical aberrations as demonstrated by 70, XXY, +12 (A) and 69, XXY, +12, –15 (B) at passage 8 and 40, while the cell line displayed 71, XXY, +9, +12 at passage 53 (C). The DNA fingerprint was analyzed by detection of short tandem repeats (STRs) of the cell line. The results demonstrated that the cell line showed triple peaks (as indicated by the arrows) in some loci (E), which confirmed their triploid characteristic.

Successful derivation of hESCs from blastocysts is influenced by several factors, including quality of embryos, timing and method of isolation of ICM, culture conditions, type of feeder cells, and the experience of the staff involved. There are no specific criteria for selecting the most suitable and effective conditions for deriving hESCs from blastocysts developed from triploid zygotes. Previous reports have shown that hESC lines from triploid zygotes have been generated by co-culture with mouse embryonic fibroblasts (MEFs) in the culture medium, supplemented with rhbFGF and insulin transferrin selenite [14] or rhbFGF alone [5]. However, the hESC derivation protocol that was used for deriving cell lines from normally developing embryos works properly with abnormally developing embryos [26]. Thus, for the derivation of hESCs from tripronuclear zygotes in the present study, we applied conditions similar to those for the isolation of the ICM, and applied culture conditions (including culture medium and feeder cells) that we reported previously [19]. The results regarding the growth dynamics of tripronuclear blastocysts to the hESC line were similar to those observed in diploid embryos, as shown by attachment of the blastocysts to the feeder layer within 24–48 h after initial plating, followed by collapse of the embryos, and emergence of a prominent ICM with a dome-like shape around day 4–5 [19, 27]. In our laboratory, the efficiency of derivation of triploid hESCs (1/20, 5%) was lower than that for the diploid hESCs (3/13, 23.1%) [19], when calculated from the blastocysts used. In addition, the efficiency of derivation of triploid hESCs in the present study was lower than that in the previous reports [3, 5, 6].

Previous reports have demonstrated that normal karyotype hESC lines can be generated from tripronuclear zygotes, and that the cell lines exhibited pluripotency, as well as differentiation abilities similar to those of diploid cell lines [5, 6, 14]. Although the mechanism of chromosome correction in these hESC lines remains unclear, it is hypothesized i) that zygotes correct their ploidy during cell division

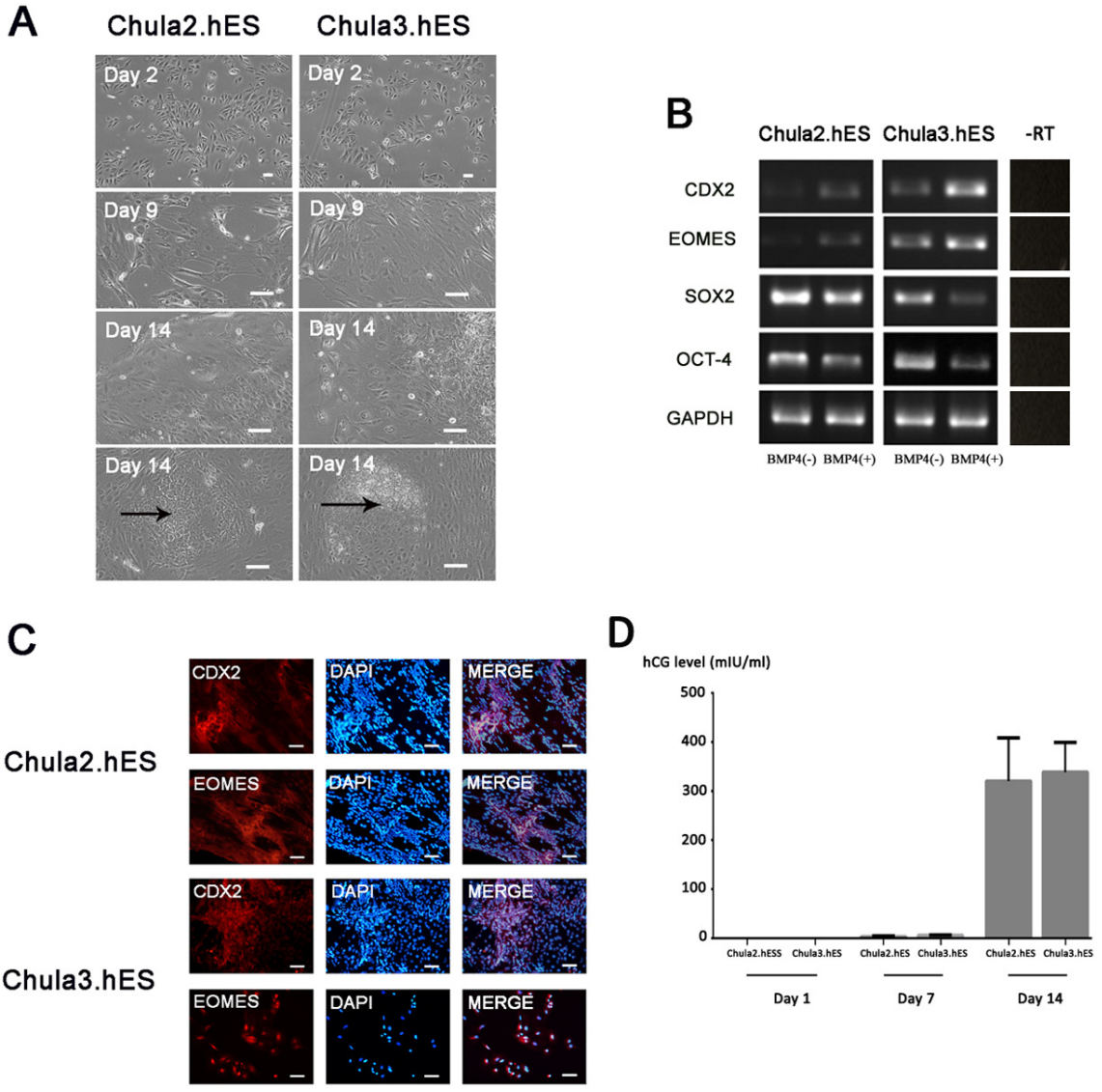

Fig. 4. Trophoblast differentiation of the triploid hESC line. Triploid and diploid hESC lines were induced for trophoblast differentiation by supplementation with BMP-4 into the culture media for 14 days. BMP-4 induced a change in the morphology of hESCs change and differentiated cells appeared flattened cells with enlarged prominent nuclei, which resembled the early stage of trophoblast differentiation as well as trophoblast-like cells. These morphological changes were observed, in both triploid and diploid hESC lines were observed (A). At day 14, some hES-like cells still remained in the culture (shown by arrows in A). Trophoblast differentiation was further confirmed by analysis of gene expression, immunocytochemistry and secretion of hCG into the culture media. Gene expression analysis showed that both undifferentiated and differentiated cells of triploid and diploid hESC lines expressed pluripotent genes, including OCT-4 and SOX2 (B). EOMES and CDX2, the candidate genes of trophoblast cells, were detected in the differentiated cells of triploid and diploid hESC lines (B). The differentiated cells of triploid and diploid hESC lines were positively immunostained for EOMES and CDX2 markers (C). Differentiated cells of triploid and diploid hESC lines secreted hCG into the culture media and the level of hCG increased in accordance with the duration of differentiation. The levels of hCG secreted by Chula3.hES and Chula2.hES was consistent with the time of differentiation. However, the levels of hCG secreted by Chula3.hES and Chula2.hES were not significantly different ($P > 0.05$) when they were compared on the same day (day 1, 0.1 mIU/ml *vs*. 0.1 mIU/ml; day 7, 5.6 ± 1.4 mIU/ml *vs*. 2.6 ± 1.7 mIU/ml; and day 14, 339.4 ± 60.7 mIU/ml *vs*. 320.9 ± 88.5 mIU/ml, respectively) (D). Data are expressed as the mean ± SEM of 3 independent cultures. Scale bar = 100 μm. BMP, bone morphogenetic protein; DAPI, 4'-6-diamidino-2-phenylindole; hCG, human chorionic gonadotropin; SEM, standard error of the mean.

by maintaining euploid cells and that while the triploid cells either enter apoptosis, resulting in the fragmentation of cells inside the embryos, or develop to mosaic zygotes consisting of triploid and euploid cells, or ii) that *in vitro* selection occurs to favor euploid cells during derivation or maintenance of hESCs [4, 10, 28]. Based on the results of karyotyping, our established Chula3.hES cell line, is a triploid cell line and the three sets of chromosomes in the cells are related to the three pronuclei observed in the zygote. It

is possible that after the first cycle of cell division of this embryo each blastomere contained three sets of chromosomes, resulting in triploidy of the ICM and ESCs. Furthermore, STR analysis showed triple peaks in some loci, confirming the triploid characteristic of the Chula3.hES cell line. Thus, our results correlate with previous reports that chromosome correction of triploid embryos does not always occur, as demonstrated by the generation of triploid hESC lines from triploid zygotes [5, 6, 14]. Alternatively, chromosomal abnormalities, such as diploidy and triploidy, are commonly observed during hESC culture [29–31]. Gains or losses of chromosomes such as chromosome 12, 17 or X in hESCs are the most common and have been reported in several studies. These chromosomal abnormalities could be the result of stress during adaptation of the cells to the culture environments, prolonged culture [32, 33] or enzymatic propagation of cells [34, 35]. In our laboratory, both triploid and diploid hESCs were similarly propagated by mechanical splitting using a needle and co-cultured with human foreskin fibroblast feeders. Interestingly, the karyotypic instability was found only in triploid hESCs but not in diploid hESCs. Besides the triploid characteristic of the Chula3.hES line, numerical aberrations of chromosome 12 and 15 were found in an early passage. The cells were propagated by mechanical splitting, thus the chromosomal abnormalities are not related to enzymatic dissociation but instead are probably related to the stress of the culture conditions, such as the culture medium or feeder cells during adaptation of the ICM to ESCs. Preimplantation genetic diagnosis (PGD) could be useful in proving the ploidy of embryos before generation of hESCs [36, 37]. However, in the present study, chromosomal analysis of the tripronuclear embryos was not performed prior to hESC isolation, thus numerical chromosome aberrations probably occurred in the blastomeres or the ICM of tripronuclear zygotes before hESCs were established. The observed chromosomal variants were probably artifacts of ES cell derivation and expansion. We conclude that analysis of the karyotype of an embryonic blastomere or early passage of an hESC line is not only useful for identification of newly established hESC lines, but also for proving chromosomal fidelity of hESCs intended for use in cell-based therapies, as atypical karyotypes in hESC lines have the potential to generate undesired effects after transplantation [38–40].

X chromosome inactivation (XCI) is a dosage compensation mechanism of X-linked genes that controls the embryonic development and the physiology of cells. The long non-coding RNA, XIST, controls XCI by mediating gene silencing on the inactive X chromosome [22]. Interestingly, the expression of XIST in Chula3.hES (XXY) indicated the inactivation of its X chromosome similar to a previous report [17]. Surprisingly, XIST expression was detected in Chula2.hES (XY), indicating that the X-linked genes should have been aberrantly repressed in this cell line. Although the XIST expression of Chula2.hES was unexpected, the XIST expression of XY-hESC line was also detected in an XY-hESC line in another study [22]. It has been suggested that the XIST expression in hESCs is not clear and highly variable even within the same cell line. The variability of the XIST expression may be correlated with the extended culture, the derivation conditions or the differentiation status of hESCs [17, 22].

Despite the chromosomal abnormalities, the Chula3.hES line showed multipotent differentiation capability, as confirmed by the results of spontaneous differentiation both *in vitro* and *in vivo*. However, little is known about their differentiation ability of this cell line toward specific lineages especially the trophoblast or TE lineage. Thus, the ability of the Chula3.hES line to differentiate to trophoblast cells was examined, in comparison with that of a diploid (Chula2.hES) line. The ability of a triploid hESC line to spontaneously differentiate to the trophoblast lineage has been previously reported by Baharvand and colleagues [14]. They demonstrated that hCG, which is the hormone secreted by trophoblast cells, was detected in the conditioned media at a level of more than 35 mIU/ml, after allowing their triploid hESC line to spontaneously differentiate *in vitro*. In the present study, instead of spontaneous differentiation, cell lines were induced toward trophoblast differentiation by a protocol based on adherent culture [21]. Cells were treated with 100 ng/ml of BMP-4, a member of the transforming growth factor-beta superfamily [41] for up to 14 days and morphological changes, gene expression, and the level of hCG in the culture media were observed. Our results confirmed that triploid hESCs were able to give rise to trophoblasts after induced differentiation, similar to diploid hESC lines [21, 42]. Supplementation of the culture media with only BMP-4 in the culture media during trophoblast differentiation was enough to induce hESCs toward trophoblast cells in a previous study [21], but we observed some differences during trophoblast differentiation in our study. We observed cellular heterogeneity after 14 days of differentiation in both triploid and diploid hESC lines, as shown by hESC-like cells remaining in the culture and the expression of pluripotent genes (OCT-4 and SOX2). Trophoblast differentiation of cells may have been delayed, due to the cell line having been induced to differentiation as small clumps instead of single cells. Merchand and colleagues [21] have suggested that in order to differentiate hESCs to trophoblast cells, the delay of differentiation and preservation of larger areas of undifferentiated cells may be avoided by plating cells as single cells. Supplementation with only BMP-4 may not have been sufficient to drive all the cells to differentiate toward the trophoblast lineage concurrently. It is also possible that supplementation with low molecular weight pharmaceutical agents that block activin A or FGF2 signaling [43] may improve the efficiency of trophoblast differentiation of hESCs. Nevertheless, the results regarding hCG detection in the culture media confirm that trophoblast cells differentiated from Chula3.hES and Chula2.hES cells were present in the culture. The results of the present study demonstrate that a triploid hESC line with chromosomal aberrations can be differentiated to trophoblast cells.

Although the triploid hESC line exhibited pluripotency, and differentiation ability similar to the diploid hESC line, the chromosomal aberration might limit the use of the Chula3.hES line for therapeutic purposes. However, Chula3.hES might be a good cell line for studying genetic and epigenetic phenomena; for instance, the X chromosome inactivation (XCI) pattern or the expression of imprinted genes due to the presence of the maternal genome in the extra set of X chromosomes. Furthermore, triploid hESCs might be a good model for studying the development of genetically abnormal human trophoblasts.

In summary, we successfully generated a triploid hESC line from a tripronuclear zygote. Not only did self-correction resulting in a diploid hESC line not occur, but numerical aberrations were also found in an early and late passage of the established cell line. Nonetheless,

this cell line showed the properties of pluripotency and the ability to differentiate into three embryonic germ layers both *in vitro* and *in vivo*. Expression of XIST by the triploid hESC line indicated X chromosome inactivation of the cell line. Lastly, the triploid hESC line can be induced to differentiate toward morphological and functional trophoblast cells.

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