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Karyotypically Normal and Abnormal Human Embryonic Stem Cell Lines Derived from PGD-Analyzed Embryos

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ABSTRACT

Although a normal karyotype is generally a requirement for stem cell lines, new applications are likely to emerge for stem cells with defined chromosomal aneuploidies. We therefore investigated the use of embryos found to be aneuploid on biopsy followed by preimplantation genetic diagnosis (PGD) with fluorescent in situ hybridization (FISH), and developmentally arrested embryos for stem cell derivation. Eleven stem cell lines were obtained from 41 embryos in 36 cultures, with higher success rate achieved from PGD-analyzed, developmentally advanced embryos (45%) than from clinically unsuitable non-PGD embryos (13%). The resulting stem cell lines were karyotyped, and surprisingly, six of the nine lines from aneuploid embryos as well as both lines from non-PGD embryos were karyotypically normal. Three lines from PGD embryos were aneuploid exhibiting trisomy 5, trisomy 16, and an isochromosome 13, respectively. None of the aneuploid lines presented the same anomally as the original PGD analysis. Our study has three important implications. First, we confirm the ability to produce stem cell lines from PGD-tested embryos as well as developmentally abnormal embryos, offering specialty stem cell lines for research into the clinically important aneuploidies. Second, we observe that stem cell derivation from apparently aneuploid embryos is often thwarted by underlying mosaicism and emerging dominance of the stem cell line by karyotypically normal cells. The corollary, however, is that regular production of normal stem cell lines from developmentally abnormal embryos ordinarity discarded opens a new source of embryos for stem cells, whether for research or for eventual therapeutic use within the donating families.

INTRODUCTION

THE FIRST HUMAN EMBRYONIC stem cell line to be derived was reported in 1998 (Thomson et al., 1998), and by 2006 the reported number of extant lines had increased to almost 300 (Wadman and Abbott, 2006). Nevertheless, it is becoming increasingly clear that there is a need for more lines, as increasingly specialized indications and opportunities for research involving stem cells unfold. Potentially, each line will have distinctive genetic characteristics of relevance to one or more particular research applications. For example, the promise of using stem cells for bioassays and laboratory-based drug discovery (Gorba and Allsopp, 2003) has coincided with a realization that

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drug actions can be particularly dependent on underlying genetic variations in target cells. These can include polymorphisms among genes for biochemical pathways responsible for drug metabolism and toxicity, indicating that platforms of stem cell lines with different genetic backgrounds will be important for predicting potentially idiosyncratic side effects or variability in efficacy in developing new drugs for common diseases. Second, stem cells carrying known mutations will be used to develop laboratory-based systems for screening drugs for efficacy for uncommon but serious monogenic diseases. Inevitably, however, given the increasing interest shown in exploring pathophysiological effects produced by gene–dosage anomalies and how these might in the future be addressed by modulation of gene expression to ameliorate the phenotype in conditions such as trisomy 21, standardized multipotent and immortal cell lines with known, stable karyotypic abnormalities are likely to become valuable tools for more fundamental research.

Besides research, new lines are needed for anticipated downstream clinical applications (Faden et al., 2003). In one reckoning it was estimated that for stem cell transplants, 150 stem cell lines would provide a relatively full match at HLA-A, HLA-B, and HLA-DR for just a minority of recipients (<20%), a beneficial match (i.e., one HLA-A or one HLA-B mismatch only) for 25–50% recipients and an acceptable match for 58–80% recipients (Taylor et al., 2005). For full matching across ethnic groups, however, orders of magnitude more lines will be needed (Faden et al., 2003).

In terms of opportunity, the last decade has seen preimplantation genetic diagnosis (PGD) become widely used in human assisted conception as an alternative to prenatal diagnosis and therapeutic abortion for couples and families with known genetic disorders. Besides identifying specific genetic mutations, PGD utilizing fluorescence *in situ* hybridisation (FISH) is also widely applied to screen for chromosomal aneuploidies prior to establishment of pregnancy, especially in cases of advanced maternal age, again reducing the need for therapeutic abortion. It has also been proposed that routine examination of embryos for aneuploidy could increase pregnancy rates and reduce miscarriages after IVF by preventing chromosomally abnormal but morphologically normal looking embryos from being transferred (Kuliev and Verlinsky, 2005). The reciprocal consequence is that PGD programs result in embryos with genetic abnormalities too severe for responsible establishment of pregnancy; to date, these embryos have been discarded, but they are now an obvious and valuable source of specialized stem cells

Several centers have published on the derivation of stem cell lines from PGD-analyzed embryos (Mateizel et al., 2006; Pickering et al., 2003, 2005; Verlinsky et al., 2005). The resulting cell lines have carried the expected mutations.

In this paper we describe our experience in the establishment of human embryonic stem cell lines from embryos with severely curtailed development considered to be at high risk of aneuploidy and from PGD-analyzed embryos with real-time diagnosis of aneuploidies based on limited screening with FISH. We show that high derivation rates can be achieved and that both normal and aneuploid stem cell lines can be obtained and maintained from these abnormal embryos.

MATERIALS AND METHODS

Embryo production and preimplantation genetic diagnosis

The research was approved by the human research ethics committees of Sydney IVF (Sydney, Australia) and of Sanatorium Helios (Brno, Czech Republic), where the derivations were performed. Embryos were donated by couples undergoing IVF treatment combined with preimplantation genetic testing through an informed consent process. The guiding principles of the process were (1) any decision to take or not to take part would not affect patients' current or future medical treatments, (2) all the patient information is kept strictly confidential, and (3) the patients were not offered payment. During the course of the IVF/PGD cycle, when the clinical embryologist and/or treating doctor discussed with the patients about possible options for outcomes (either no embryos developing or embryos developing and subsequently identified as aneuploid or euploid), patients were asked if they were interested in donating to research their affected or abnormally developing, clinically unsuitable embryos, which according to routine clinical practice would normally be discarded. If the patients expressed interest, they were informed about the research project both verbally

and in a written form. All discussions with the patients were carried out by the clinical staff and not the researchers directly involved with the project. If patients wished to proceed, they signed approved consent forms, after which the embryos could be used for the study.

The IVF and embryo culture procedures utilized a gas atmosphere of 6% CO_2, 5% O_2, and 89% N_2 in K-MINC-1000 mini-incubators (Cook Medical, Eight Mile Plains, QLD, Australia) together with two proprietary Sydney IVF stage-specific culture media which are described in detail elsewhere (Henman et al., 2005). Blastocyst biopsy and PGD analysis were performed as described by McArthur at al. (2005). Briefly, all embryos reaching the six- to eight-cell stage by day 3 were subjected to laser-assisted hatching using a Zilos-TK Infrared Laser (Hamilton Thorne Biosciences, Beverly, MA) and returned to culture. Partially hatched blastocysts were biopsied on day 5 or 6 by aspirating the herniated portion of the trophectoderm into a pipette and separating it from the rest of the blastocyst with short laser bursts (Fig. 1). The biopsy was then processed for FISH using fluorescence probes recognizing chromosomes 13, 18, 21, X, and Y following manufacturers' instructions (Vysis Inc, Downers Grove, IL) and the blastomeres examined by a fluorescence microscope with appropriate excitation and emission filters for the probes used. The results were obtained the same day. Karyotypically normal embryos were either transferred to the patient or frozen for later transfers, and karyotypically abnormal embryos on PGD were allocated to stem cell derivation. Some embryos not suitable for biopsy on days 5 or 6 (based on their delayed developmental stage and inferred very poor quality) were allocated to stem cell derivation on days 6, 7, or 8.

Embryonic stem cell derivation

Reagents used were obtained from Gibco BRL (Grand Island, NY) unless otherwise indicated. Feeder cells were human fetal fibroblasts either produced in-house or acquired commercially (ATCC, Manassas, VA). The feeder cell dishes were prepared by inactivating fibroblast cultures (between passages 4 to 10) by 10 μg/mL mitomycin for 2.5 h, followed by trypsinization and plating on 0.1% gelatin coated (Sigma Chemical Company, St. Louis, MO) culture dishes (Nunclon, Roskilde, Denmark) at a density of 7×10^4 cells/cm^2. The feeder cell culture medium was Dulbecco's minimum essential medium (DMEM) with the addition of 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin, 1× MEM-amino acids (ICN Biomedicals, Costa Mesa,

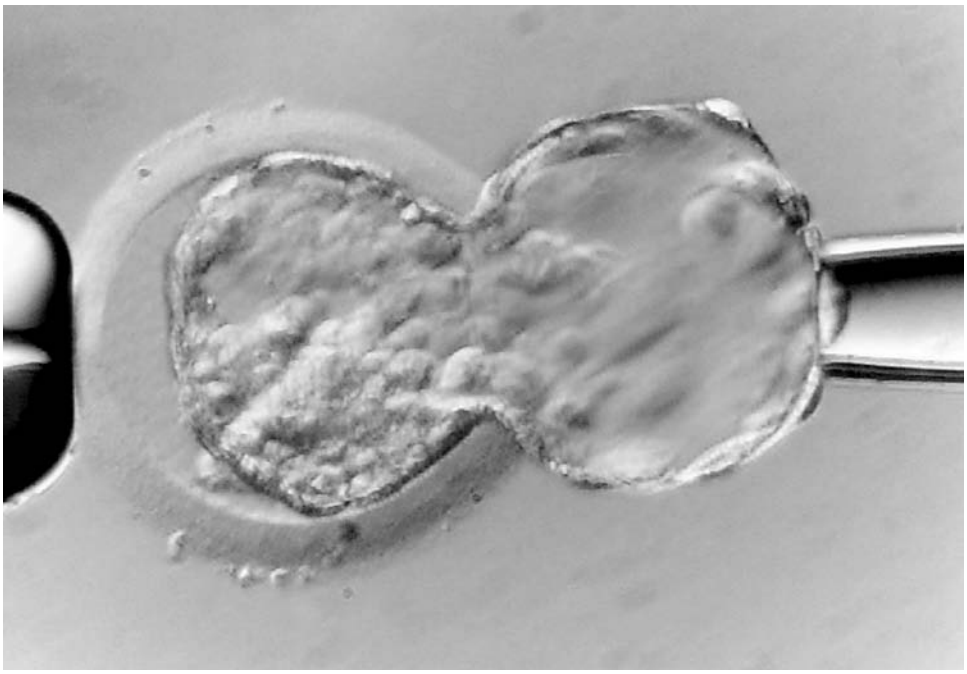

FIG. 1. Blastocyst biopsy of five day old human embryo; after laser-assisted hatching at day 3, the herniated portion of trophectoderm is separated from the rest of the embryo using short controlled laser bursts.

CA), 1 mM sodium pyruvate (Sigma) and 10% fetal calf serum (FCS). The stem cell medium for the first eight platings consisted of DMEM/F12 with 2 mM glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin, 1× MEM-amino acids, 1% ITS-G, 0.1 mM β-mercaptoethanol, and 20% FCS; for the remaining 28 platings KO-DMEM with the addition of 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin, 1× MEM-amino acids, 0.1 mM β-mercaptoethanol, and 20% knock-out serum replacement (KSR) was used. With each medium 4 ng/mL bFGF (Sigma) was added into the medium just before use. All cultures were performed in reduced oxygen atmosphere in K-MINC-1000 mini-incubators at +37°C.

Most of the embryos of good developmental quality were manually bisected using an ethanol-sterilized Ultra-Sharp Splitting Blade (AB Technologies, Pullman, WA) to remove the zona pellucida (if the blastocyst had not hatched) and to divide the embryo into two parts, plating only the ICM-containing part to the feeder dish. In some cases, poor quality embryos were plated whole after removal of the zona pellucida by 2-min incubation in 0.4% Pronase (Sigma) or by cutting with the splitting blade. Either a complete change of medium or an addition of fresh FGF was carried out every second day of culture. The first passage of ICM-outgrowth was performed 4 to 7 days after plating, and henceforth every 4 to 14 days until stable embryonic stem cell-like growth was observed. Passaging was done manually by cutting outgrowths into one to three fragments with the splitting blade and transferring the fragments with a flame-pulled glass pipette to a fresh feeder dish. Once colonies were established, passaging was done using a glass pipette both for cutting and transferring the colony fragments.

The resulting putative stem cell lines were expanded and passaged continuously, then cryopreserved at various passages using a modified open pulled straw vitrification method (Reubinoff et al., 2001; Vajta et al., 1997). The technique involves cutting undifferentiated colonies into pieces (approx. 0.8 × 0.8 mm), equilibration for a few minutes in warm (+37 °C) bench-medium of DMEM–HEPES + 20% FCS, then transfer for 20 to 30 sec to warm bench medium containing 10% ethylene glycol and 10% DMSO (v/v) (Sigma), then finally transfer for 20–30 sec to warm bench medium containing 20% ethylene glycol, 20% DMSO (v/v), and 0.5 M sucrose (Sigma) while being loaded to open-ended OPS-straws (LEC-Instruments, Scoresby, VIC, Australia) in approx. 2-μL volume and plunging into liquid nitrogen. The straws were immediately inserted into 0.5-mL straws that were sealed with BD Seal Ease (Becton Dickinson, Franklin Lakes, NJ), thus creating double-straw (Vajta et al., 1998) to avoid possible crosscontamination in liquid nitrogen storage.

Characterisation of stem cell lines

Karyotyping. Undifferentiated colonies of cells were incubated with 22.5 ng/mL colcemid (KaryoMAX) and 37.5 μg/mL BrdU for 17–19 h (Fisher et al., 1996), followed by PBS wash and dissociation into single cells with Cell Dissociation Buffer (Sigma). Later, this method was replaced with a shorter 2.5-h incubation in 5 ng/mL colcemid alone, followed by immediate harvesting. Harvested cells were fixed and stained as metaphase spreads for examination using standard Sydney IVF genetics laboratory G-banding techniques. Cell lines were routinely karyotyped between passages 4–10 and, if kept in the prolonged culture (>25 passages), were karyotyped again approximately every 20 passages.

Expression of markers by staining and immunocytochemistry. Undifferentiated colonies of cell lines were stained for alkaline phosphatase using an Alkaline Phosphatase Substrate Kit (Vector Laboratories Inc, Burlingame, CA) according to the manufacturer's instructions. For immunocytochemistry, the colonies were fixed in 4% paraformaldehyde for 10 min at room temperature, followed by overnight incubation at 4°C in blocking buffer (PBS + 0.5% BSA + 0.1% Triton X-100). The next day, primary antibodies were diluted with PBS + 0.5% BSA and applied to samples for 2 h in room temperature, followed by three washes in PBS. Similarly, the secondary antibodies were diluted with PBS + 0.5% BSA and applied for 2 h, followed by PBS washes, counterstaining with 1 μg/mL Hoechst 33342 (Sigma), and coverslip mounting. The primary antibodies were for SSEA-4 (Chemicon Temecula, CA; 1:200), Tra-1-60 (Chemicon, 1:200), OCT4 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) and SSEA-1 (Sigma; 1:100), and the secondary antibody was rabbit antimouse IgG FITC (Sigma; 1:1000). Immunocytochemistry studies were performed between passages 7 and 54, once or sev-

eral times per line, depending on the particular cell line.

Assessment of differentiation through embryoid body formation and teratoma formation. To characterize derived stem cell lines their ability to contribute to ectoderm, endoderm, and mesoderm *in vitro* was examined. Embryoid bodies (EBs) were formed by manually cutting undifferentiated colonies into approx. 0.5×0.5-mm fragments and culturing them in ES-medium without bFGF for 5 to 10 days. EBs were harvested and OCT4 (a marker of undifferentiated ES cells), AFP (an endoderm marker), VEGFR2 (KDR, FLK1 a mesoderm marker), and NCAM (an ectoderm marker) were assayed using RT-PCR. β-actin, a housekeeping gene, was assayed in the same reaction. Undifferentiated colonies of stem cell were analyzed for the same markers at the same time for control purposes. For PCR, total RNA from a single EB was extracted by three cycles of freezing and thawing in liquid nitrogen. Isolated RNA was reverse-transcribed at 42°C for 30 min with 1.5 units of reverse transcriptase primed with 0.25 mM oligo(dT) in 20 mL of reaction mix containing 3 mM $MgCl_2$, 60 mM KCl, 50 mM Tris-HCl, pH 8.3, 1 mM each dNTP, and 1 unit of RNAse inhibitor (all reagents, Perkin-Elmer Life Sciences, Norwalk, CT). The RT reaction was terminated by heating at 98°C for 5 min and cooling to 5°C. Each EB was then subjected to PCR for evaluation of the mRNA expression of OCT4, AFP, VEGFR2, and NCAM. An aliquot of cDNA was amplified using Promega PCR MasterMix protocol (Promega, Madison, WI), 2 ng OCT4, AFP, VEGFR2, and NCAM primers. At all times control samples of undifferentiated hES cells and genomic RNA were analyzed beside the RNA from the EBs. All RT-PCRs were performed on at least three different EBs and three passages of undifferentiated cells. Primers were as described by Abeyta et al. (2004). PCR products were visualized using an Agilent Technologies Bioanalyzer and analyzed using Agilent Technologies (Placerville, CA) 2100 expert program. PCR analyses were performed between passages 7 and 11 for all cell lines.

Teratoma formation in SCID mice was assessed by harvesting hESC cells from cultures that had been expanded over at least two to three passages with collagenase passaging, harvesting taking place between passages 7 to 13. Briefly, both for passaging and for cell harvesting the cells growing in T25 flasks were first washed with PBS, incubated with an overlay (1 mL) of collagenase IV solution (1 mg/mL) for 5–10 min, after which hESC medium was added and cells collected with a cell scraper to a centrifuge tube, broken into clumps by gentle pipetting, and centrifuged at $300 \times g$ for 4 min. For subsequent culture, the cell pellet was resuspended in hESC medium after removal of supernatant and transferred into T-flask(s). For teratoma induction studies no collagenase was used but cells were collected by scraping and resuspended in a maximum of 100 μL volume of hESC media. This cell clump solution was then injected into the upper thigh of 3–5 week-old SCID-mice. Once formation of a teratoma was observed, mice were killed and teratoma removed, processed for histochemistry by an experienced pathology laboratory and analyzed for the presence of differentiated cell types.

RESULTS

Cell line derivations

Thirty-six cultures were set up with 42 embryos from 21 patients (in five cases two non-PGD analyzed embryos were plated on one culture dish). In 30 cultures at least one embryo was plated. A total of 11 new stem cell lines were derived (Table 1).

We achieved higher derivation success rates with PGD-analyzed embryos than with non-PGD embryos (45% vs. 13%), probably because of the better than average quality of embryos able to be tested by PGD. At the time of plating, PGD embryos were assessed based on a clinical blastocyst grading system utilized at Sydney IVF where grade 1 represents embryos with uniformly well-formed inner cell mass and trophectoderm, grade 2 represents embryos with some cytological irregularities (arguably indicative of repair processes), and grade 3 embryos have a poor inner cell mass and/or defective blastulation. The distribution of embryos to quality grades 1, 2, and 3 was made at the time of biopsy and again at plating, when the distribution for PGD embryos was, respectively, 65:30:5. By methodologic design the non-PGD embryos were all of poor quality. Somewhat surprisingly, more cell lines were obtained from grade 2 (4 of 6, or 67%) than from grade 1 (5 of 13, or 38%) PGD embryos ($\chi^2 = 0.42$, not statistically significant).

Table 1. Embryonic Stem Cell Line Derivation Success from Preimplantation Genetic Diagnosis-Analyzed and Nonanalyzed Quality Grade 1 and 2 Embryos, Plated as Whole Embryos or After Bisection

PGD-status	*Embryo manipulation*	*Embryo grade*[a]	*Cultures established (No. of embryos)*	*Outgrowths observed (% of cultures)*	*Stem cell-lines obtained (% of cultures)*
PGD-analyzed	*Total*		*20 (20)*	*17 (85)*	*9 (45)*
	Whole	1	3 (3)	3 (100)	1 (23)
		2	3 (3)	3 (100)	2 (67)
		3	1 (1)	0 (0)	0 (0)
	Bisected	1	10 (10)	9 (90)	4 (40)
		2	3 (3)	2 (67)	2 (67)
		3	n.a.	n.a.	n.a.
Non-PGD	*Total*	*n.a.*	*16 (22)*	*13 (81)*	*2 (13)*
	Whole	n.a.	8 (12)	7 (88)	1 (13)
	Bisected	n.a.	8 (10)	6 (75)	1 (13)

[a]1 = good, 2 = fair, 3 = poor.

Cell line characterizations

Full karyotypes were performed at least once for each cell line (Table 2) and, depending on the accumulating number of passages, up to three times for some lines. In all repeat analyses the results were the same for each cell line, indicating overall genomic stability. Eight cell lines were found to be karyotypically normal (six female and two male). Three cell lines were aneuploid: one female trisomy 16 (46,XX,+16), one male trisomy 5 (46,XY,+5) and one mosaic line comprising isochromosome 13-carrying and normal female cells [46 XX,i(13)(q10)] and [46,XX] (Fig. 3). The trisomy 16 line came from an embryo that had been diagnosed as triploid in the original PGD analysis. The other two lines came from embryos categorized as "chaotic," that is, analyzed cells showed at least two different aneuploidies. The results of the original PGD analysis and the karyotypes of the resulting stem cell lines are shown in Table 2.

All 11 cell lines have been passaged at least 20 times since their derivation, most advanced cultures having reached over 70 passages at the time of writing. All lines at the undifferentiated stage stained positive for alkaline phosphatase. Immunocytochemical studies revealed that they

Table 2. Preimplantation Genetic Analysis (PGD) Results of FISH-Analyzed Embryos and Corresponding Karyotypes of Thereof Derived Stem Cell Lines

Stem cell line ID	*PGD-result of embryo biopsy*	*Karyotype of stem cell-line*	*Karyotyped at passage(s)*	*Latest passage cell line cultured before cryopreservation*
SIVF03	Triploid (3 × X, 3 × 13, 3 × 18, 3 × 21)	Female trisomy-16 [47,XX,+16]	P.6, 29, 55	P.74
SIVF04	"Chaotic" (No 21)	Normal female [46,XX]	P.7, 32, 65	P.74
SIVF05	"Chaotic" (e.g., 3 × 13, 3 × 18)	Normal male [46,XX]	P.8, 28	P.35
SIVF06	"Chaotic" (e.g., 3 × 13, 3 × 18)	Normal female [46,XX]	P.8, 28	P.35
SIVF07	"Chaotic" (e.g., 3 × 13, 3 × 18) 5 × 21)	Normal female [46,XX]	P.8, 26	P.22
SIVF08	"Chaotic" (e.g., 3 × X, 5 × 13) 3 × 18)	Normal female [46,XX]	P.8, 27	P.34
SIVF09	"Chaotic" (e.g., 1 × X, 1 × 21)	Mosaic [46,XX,i(13)q(10)/46,XX]	P.8	P.22
SIVF10	"Chaotic" (e.g., 3 × 13, 3 × 21)	Normal female [46,XX]	P.8	P.22
SIVF11	"Chaotic" (e.g., 3 × 13, 1 × 18)	Male trisomy-5 [47,XY,+5]	P.8	P.24
SIVF12	N/A	Normal female [46,XX]	P.9, 27	P.23
SIVF13	N/A	Normal male [46,XY]	P.8	P.21

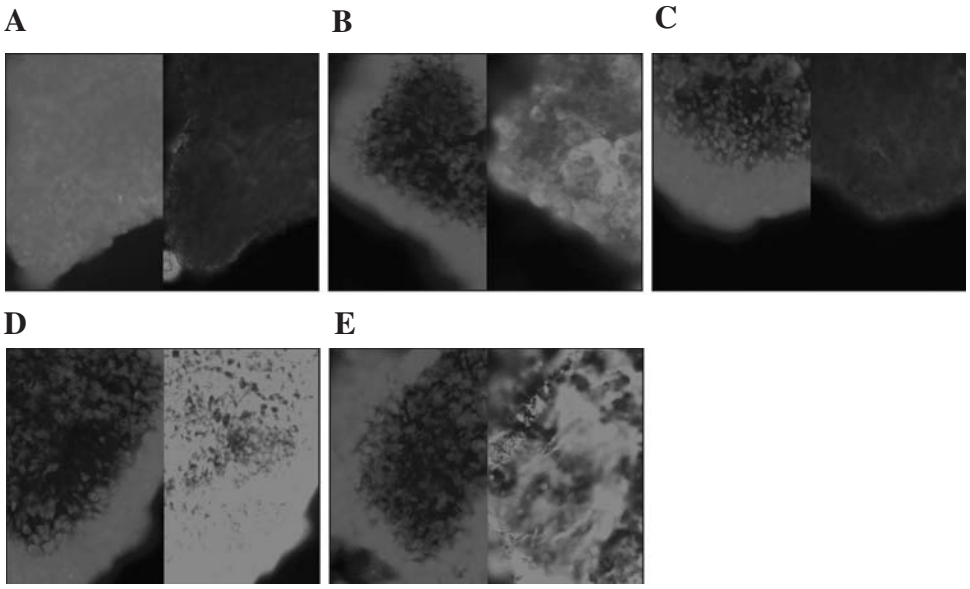

FIG. 2. Immunocytochemistry of undifferentiated human embryonic stem cell line SIVF12 (**A**) negative control (no antibody); (**B**) OCT4; (**C**) SSEA-1; (**D**) SSEA-4; (**E**) TRA-1-60 (left panel = Hoechst 33342 nuclear stain, right panel = FITC-conjugated secondary antibody).

also exhibited the common markers for undifferentiated human embryonic stem cells, namely OCT4, SSEA-4, and TRA-1-60, but none expressed SSEA-1, which is typically expressed in mouse but not human undifferentiated ES cells (Fig. 2). These analyses were performed at least twice for all but one line, and up to four times for two lines at different passages (Table 3). Two cell lines have not been analyzed for one marker each (Tra-1-81 for SIVF04 and SSEA-1 for SIVF05).

All cell lines produced well-formed embryoid bodies. In each case, subsequent PCR studies confirmed expression of all the analyzed germ layer-specific markers, namely AFP as an endoderm

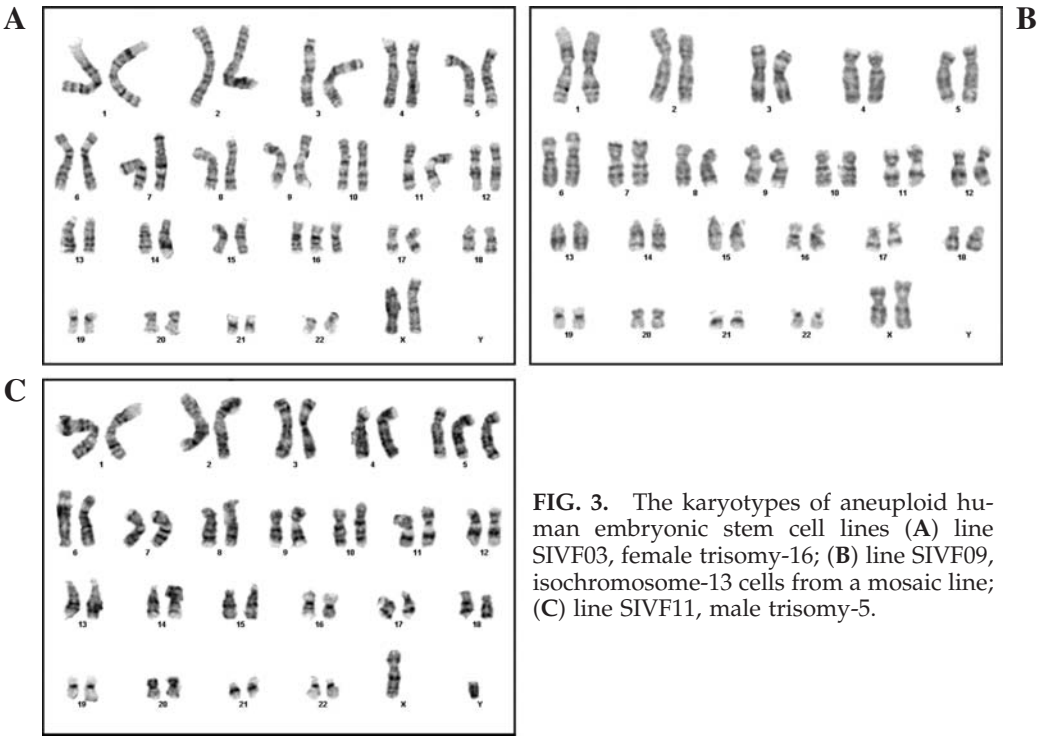

FIG. 3. The karyotypes of aneuploid human embryonic stem cell lines (**A**) line SIVF03, female trisomy-16; (**B**) line SIVF09, isochromosome-13 cells from a mosaic line; (**C**) line SIVF11, male trisomy-5.

Table 3. Immunocytochemistry Assay Passage Numbers and Their Results in hESC-Lines Derived from PGD-Analyzed Embryos

Stem cell line ID	*OCT4 passage no(s)*	*SSEA-4 passage no(s)*	*TRA 1-60 passage no(s)*	*TRA 1-81 passage no(s)*	*SSEA-1 passage no(s)*
SIVF03	34, 53	34, 53	34, 53	53	34
SIVF04	37	37	37	—	37
SIVF05	24, 26	24, 26	24, 26	24, 26	—
SIVF06	25, 26	26	26	25, 26	25
SIVF07	16, 24	16, 24	16, 24	24	16
SIVF08	7, 23, 24, 25	7, 23, 25	7, 23, 25	7, 23, 24, 25	7, 23, 24
SIVF09	10, 11	10, 11	10	10, 11	10, 11
SIVF10	10, 11	10, 11	10	10, 11	10, 11
SIVF11	10, 11	10, 11	10	10, 11	10, 11
SIVF12	7, 8, 24, 25	7, 8, 25	7, 8, 25	7, 24, 25	7, 8, 24
SIVF13	10, 11	10, 11	10, 11	10, 11	10

marker, VEGFR2 (KDR, FLK1) as a mesoderm marker, and NCAM as an ectoderm marker. β-Actin, a housekeeping gene, and OCT, the pluripotentiality marker, were also assayed in the same reaction. The Agilent Bioanalyzer 2100 program assess the concentration of DNA in a set of samples using a biophotometer, and thus allows for the semiquantitative analysis of the DNA. Each EB was analyzed by first normalizing against control β-actin, then the ratio of test DNA in the sample was assessed. All embryoid bodies had reduced OCT4 mRNA expression in combination with the presence of NCAM, AFP, and VEGFR2, indicating the presence of all three germ layers. For the undifferentiated cells, no expression markers for the three germ layers were detected, but OCT4 expression was high, as expected. Table 4 shows semiquantative gene expression data for all the cell lines, both for EBs and undifferentiated colonies.

Cell injections to SCID mice produced teratomas from 8 of the 11 lines, whereas three lines did not yield detectable growth (lines SIVF05, SIVF07, and SIVF11, with normal male, normal female, and trisomy 5 male karyotypes, respectively; see Fig. 3). Of the eight teratomas, seven clearly exhibited all three embryonic germ layers (Fig. 4), but in one line (SIVF03, with a trisomy 16 female karyotype) only mesoderm and endoderm could be seen. The teratoma inductions are being repeated with all four lines presently lacking a clear outcome in order to verify whether these results represent a random outcome of a single experimental procedure or a true and persistent compromise of differentiation ability.

Table 4. Gene Expression in Embryoid Bodies (EB) for Pluripotency and Differentiation Markers

	Embryoid bodies										
Gene	*SIVF03*	*SIVF04*	*SIVF05*	*SIVF06*	*SIVF07*	*SIVF08*	*SIVF09*	*SIVF10*	*SIVF11*	*SIVF12*	*SIVF13*
VEGF	++	++	+++	+++	++	++	++	+++	++	++	++
NCAM	++	++	++	+++	+++	++	++	++	+++	++	+++
AFP	++	+++	++	++	++	++	+++	+++	++	+++	++
Oct-4	N.D.	N.D.	N.D.	N.D.	N.D.	+	+	+	N.D.	+	+
	Undifferentiated cells										
Gene	*SIVF03*	*SIVF04*	*SIVF05*	*SIVF06*	*SIVF07*	*SIVF08*	*SIVF09*	*SIVF10*	*SIVF11*	*SIVF12*	*SIVF13*
VEGF	+	N.D.	N.D.	+	N.D.	N.D.	+	+	N.D.	N.D.	N.D.
NCAM	+	N.D.	+	N.D.	+	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
AFP	+	N.D.	N.D.	N.D.	+	N.D.	+	N.D.	N.D.	N.D.	N.D.
Oct-4	++	++	++	++	++	++	+++	+++	++	+++	++

N.D.: below detectable levels, + indicates 20–50% of actin; ++ 50–80% of actin, +++ 80% of actin.

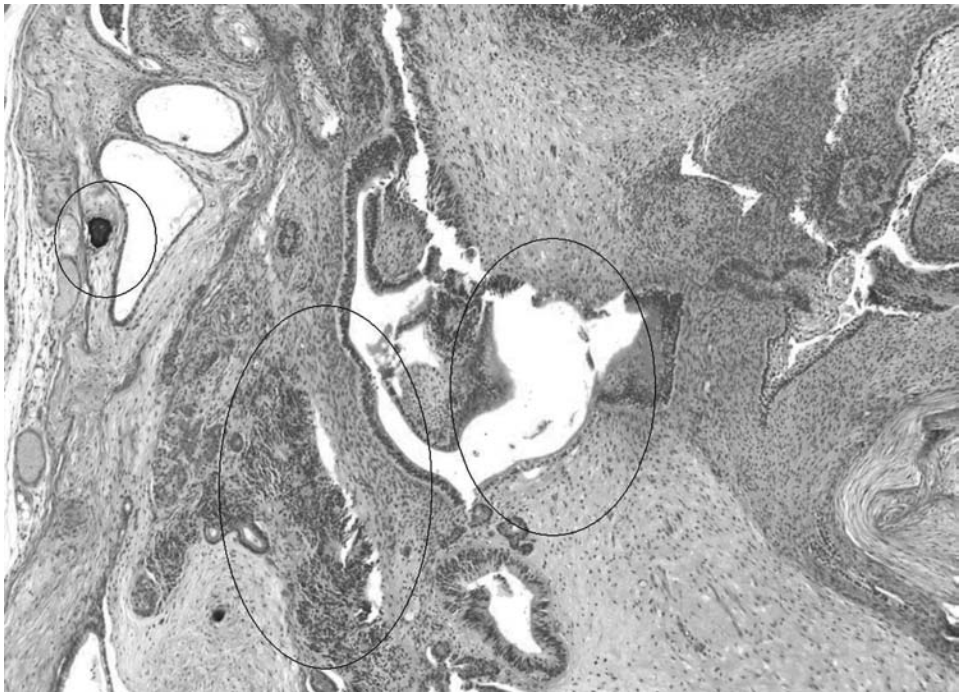

FIG. 4. Teratoma histology section from a tumor induced from SIVF12, indicating the presence of three embryonic germ layers; neuroepithelium (ectoderm), gut epithelium (endoderm), and bone (mesoderm).

DISCUSSION

The advent of preimplantation genetic diagnosis of embryos for aneuploidy screening as part of clinical *in vitro* fertilization (IVF) has resulted in defined abnormal embryos that are unsuitable for any conventional clinical purpose. Chromosomal analysis of cleavage stage embryos by comparative genomic hybridization, a method that reveals the number of all 24 chromosomes, has revealed that just 25% of day 3 embryos are composed only of euploid cells (Voullaire et al., 2000; Wells and Delhanty, 2000) and the incidence of chromosomal abnormalities in embryos of even relatively young women is high (Munne et al., 2006). The scene is therefore set to suppose that PGD might at once both improve the rate of normal pregnancy and birth (Kuliev and Verlinsky, 2005; Munne et al., 2005a) and, on the other hand, lead to reliable early recognition of abnormal embryos. If couples so wish, these embryos, instead of being discarded, can be used to produce stem cells suitable for research and, in time, for therapeutic use.

While the literature on the high prevalence of chromosomal abnormalities in preimplantation human embryos is considerable (Baart et al., 2004; Coonen et al., 2004; Munne et al., 1994; Sandalinas et al., 2001), the results are subject to a number of variables—whether embryos were growing normally or had arrested, the developmental stage of the embryos, the *in vitro* culture conditions, the analysis methods used (FISH, CGH, or full karyotyping), and the number of chromosomes defined—so that the final picture of the incidences and consequences of chromosomal abnormalities is still emerging.

We derived more karyotypically normal cell lines than would have been expected from the high reported incidence of chromosomal abnormalities in human embryos. Our study suggests that not all aneuploidies diagnosed among embryos *in vitro* are "permanent." The explanation no doubt lies in the fact that a relatively high proportion of embryos are mosaic (reviewed by Macklon et al., 2002; Wilton, 2005). Mosaicism can originate from nondisjunction during meiosis in which correction is taking place among daughter cells during cleavage. Mosaicism has also been shown in established pregnancies. For example, a prenatal sample in our clinic analyzed by qualitative fluorescence PCR analysis consisted of 70% trisomic and 30% normal chromosomes, the trisomic chromosome exhibiting a triallelic state as a proof of its meiotic origin (data not shown). However, more frequently mosaicism originates

from nondisjunction errors in mitosis during early cleavage stages (Coonen et al., 2004). Bielanska et al. (2002) reported an increased prevalence of mosaic embryos at advancing stages of preimplantation development, escalating from 15% of two to four cell embryos (one to three mitotic divisions, when persistence can result for example in confined placental mosaicism) to 91% in blastocysts, which have more than 100 cells (from many mitoses), indicative of the increasing impact of mitotic nondisjunction, albeit with a decreasing numeric contribution by the aneuploid lines the later the nondisjunction event occurs. For example, Gonzalez-Merino et al. (2003) reported almost 100% mosaicism in morulae (day 4), preexpanded (day 5), and expanded (day 6) blastocysts, but noted that the incidence and pattern of mosaicism differed between the developmental stages: the more advanced the embryos, the higher the proportion of diploid cells. This result is indicative of a combination of several distinct mechanisms producing a shifting effect. On the one hand, the probability of a nondisjunction event will increase cumulatively in step with the rising number of cells in the embryo subject to mitosis (but with a decreasing clonal share the later the first event); on the other hand, a probable tendency for "repair" through decreased survival of at least some chromosomally abnormal cells (typically a monosomic line of very short survival and a trisomic line that lasts for longer, depending on the chromosome involved and the genes whose expression is increased). Beyond these theoretical considerations and the limited empirical findings reported, the clinical impact of aneuploidy arising during cleavage remains poorly defined.

The observed difference in stem cell derivation success between PGD-analyzed embryos (relatively viable) and nonanalyzable embryos (nonviable and ordinarily discarded) reflects the difference in embryo quality. It should be pointed out that it is likely that the formation of an embryo capable of implanting successfully and developing into a normal fetus is a more demanding sequence of events than the origination of a uniform line of cells in culture. It should also be pointed out that the morphological grading system used is a graded categorical one, not a linearly scaled one. Although grade 2 embryos subjected to PGD produced stem cell lines with high efficiency, this apparently anomalous result is consistent with a relatively high implantation rate for grade 2 embryos clinically and, should the trend we found be confirmed with larger numbers, the explanation could reside in the likelihood that these embryos are under some degree of stress compared with grade 1 embryos and might plausibly be in a primed state to produce new cells by mitosis. The fact that the nonanalyzed grade 3 embryos had been deemed to be of too poor quality to undergo embryo biopsy at day 5 or 6 indicates that their development rate was indeed slower than that of good embryos that could be biopsied. In addition, more 7–8-day-old embryos, subjected to *in vitro* culture for 1–2 days longer than 6-day-old embryos, were plated in non-PGD group (6 of 16) than in PGD-analyzed group (3 of 20).

Two approaches for embryo manipulation before plating were used in this study, namely, whole embryo plating and mechanical separation of polar trophectoderm and ICM. In order to avoid introduction of animal-derived products to the derivation process, no immunosurgical methods were used. Also, the use of pronase was halted halfway through the project, and subsequently, the zona was removed by mechanical cutting. No differences in outcomes between the two embryo manipulation methods were obvious in the higher grades, whereas we had notably better results from whole embryo plating and culture with poorer quality embryos, where locating the ICM is made difficult because of its small size or the collapsed state of the embryo. Kim et al. (2005) compared stem cell derivation success rates after immunosurgical, partial, and whole embryo culture methods applied to embryos of different quality, where the partial embryo culture method was very similar as the embryo bisection method described here. We did not systematically compare partial and whole embryo culture in higher grade embryos, and our numbers are too low to draw definitive conclusions concerning the equivalence of the two procedures.

Because of mosaicism, the use of PGD for the detection of clinically important aneuploidy carries an inherent risk of misdiagnosis and can best be described as an imperfect screen. In almost all reported PGD series the blastomere biopsy is obtained at day 3, when one or sometimes two blastomeres are removed from a six- to eight-cell embryo for analysis (see McArthur et al., 2008, for a discussion in comparison with the novel practice of blastocyst biopsy used here). Several studies

have, however, compared the results of an initial day 3 embryo biopsy to the results obtained after reanalyzing the whole embryo at the blastocyst stage in an attempt to validate the role of PGD for aneuploidy screening (Baart et al., 2004a; Emiliani et al., 2004; Veiga et al., 1999), but suggesting also that the majority of apparent aneuploidies at this early cleavage stage are of meiotic origin.

Several studies have indicated that the incidence of chromosomal abnormalities and mosaicism is the same in trophectodermal and inner cell mass cells (Derhaag et al., 2003; Evsikov and Verlinsky, 1998; Magli et al., 2000), but this does not mean that a clinically meaningful aneuploid line present in the inner cell mass will always be reflected in the trophectoderm. Embryo biopsy at the stage of the blastocyst (de Boer et al., 2004) typically provides five or more trophectoderm cells for analysis, which while providing a secure means of detecting uncorrected meiotic errors could also increase the chance of detecting mosaicism, including aneuploidies confined to the placenta ("confined placental mosaicism"), that might or might not have a lasting clinical consequence (Kalousek and Vekemans, 1996; Leschot et al., 1996).

However, it remains a fact that detecting, say, an autosomal trisomy in a single cell removed from a six- to eight-cell embryo does not permit a distinction to be made between a dangerous meiotic nondisjunction from a now-innocuous mitotic trisomy–monosomy clone, the trisomic component of which has been excised and the monosomic component of which will not survive. Meanwhile, waiting for differentiation of the trophectoderm from the inner cell mass, and leaving the latter intact during PGD performed at the stage of blastocyst, is consistently producing higher embryo implantation rates and live births than is achievable from day 3, cleavage stage PGD (McArthur et al., 2008)

Several reports record the use of PGD-analyzed embryos with known heritable genetic disorders for the derivation of embryonic stem cells (Mateizel et al., 2006; Pickering et al., 2003; Verlinsky et al., 2005). Notwithstanding, a finding of outgrowths of OCT4-expressing outgrowth cells from embryos found to be aneuploid at the early cleavage stage (Munne et al., 2005b), the present report is, we believe, the first record of fully characterised aneuploid stem cell lines from aneuploid embryos. In related areas, Suss-Toby et al. (2004) derived a stem cell line from a zygote assumed to be aneuploid on the basis of a single pronucleus. Baharvand et al. (2000) described two triploid stem cell lines among five new lines derived from IVF without PGD analysis, one of the lines being derived from a zygote with three pronuclei.

We show that the use of PGD-determined aneuploid embryos can yield normal cell lines, cell lines with stable trisomies, or cell lines with abnormalities of chromosome number different from the aneuploidy diagnosed at embryo biopsy. Thus, we have shown that the results of the original PGD diagnosis and the final karyotyping of dominant cell lines cultured from the biopsied embryo are not always concordant.

In our study most of the cell lines were karyotypically normal, suggesting selective advantage for normal cells to establish the cell line. This is in accordance with the results reported by Munne and Alikani (Alikani and Munne, 2005; Munne et al., 2005b), where 24 PGD-analyzed chromosomally abnormal embryos were plated on feeder cells for 12 days and the initial outgrowths reanalyzed using limited FISH probes; seven outgrowths were karyotypically normal for the chromosomes tested, whereas 11 appeared to have experienced some degree of normalization, exhibiting 21–88% normal cells. In our study, six of the nine cell lines from PGD aneuploid embryos and both cell lines from non-PGD analyzed, abnormally developing embryos were normal on full, conventional, hig-resolution karyotyping. None of the karyotypically abnormal cases—trisomy 16, trisomy 5 and isochromosome 13—presented exactly the same anomaly as the original PGD analysis. However, most of the initial analyses deduced a chaotic status, based on analysis of one to eight cells. Complete trisomy 5 is a rare and lethal abnormality, whereas trisomy 5 mosaicism detected on chorionic villus sampling or on amniocentesis has been associated both with a normal outcome at birth and with children born with congenital anomalies (Hsu et al., 1997). Trisomy 16 is one of the most commonly occurring trisomy in humans, estimated to occur in more than 1% of clinically recognized pregnancies. Full trisomy 16 normally results in miscarriage in the first trimester, but, as with trisomy 5 mosaicism, trisomy 16 mosaicism is not rare and can lead to a normally developing fetus (Benn, 1998). Although it is impossible to say with certainty whether the original embryos in these cases had

any normal cells present, the emergence of a trisomic cell line over a karyotypically normal one suggests the same outcome might have happened in the developing fetus.

The production of karyotypically normal stem cell lines from abnormal IVF embryos is an important development, because these stem cells have between a one-in-four and one-in-five chance of HLA concordance with children born to the IVF couple, and thus could be reserved for future therapeutic treatments with compatible stem cells within the family. In terms of precedence, one of the stem cell lines derived from IVF embryos by Heins et al. (2004) initially showing a trisomy 13 karyotype was later demonstrated to be mosaic, with normal diploid cells comprising 5 to 15% of cells (Heins et al., 2006). By subcloning it was possible to obtain a normal diploid stem cell line, although with low efficiency: from 33 subclones (5% subcloning efficiency) just one line was found to be disomic for chromosome 13 (Heins et al., 2006). Typical initial stem cell line characterisation usually includes analysis of a limited number of metaphases, so it is possible that more cell lines could be mosaic than has so far been recognized and reported. The mosaic cell line SIVF09 described in this paper could be subjected to clonal propagation with a reasonable expectation of a karyotypically normal stem cell line.

It is possible that the continuing use of room-air oxygen concentrations in many IVF programs contributes to the apparent high incidences (Munne et al., 2006) of chromosomal nondisjunction in human IVF embryos. Likewise, the difficulty many authors have had in keeping karyotypes stable among human stem cells after high passage numbers (Draper et al., 2004; Mitalipova et al., 2005) could be a consequence of the almost universal use of 20% oxygen culture conditions for stem cell culture—a state greatly different to the virtual anoxia prevalent during the first 3 months of fetal development (Burton et al., 2003), when embryonic stem cells produce the primordial germ cells and over 200 "adult" stem cell populations in evolving tissue and organ niches. Both the success rate in producing stem cell lines and the stability of the karyotypes in our study are at the high end of reported results. Our application of substantially reduced oxygen level in culture, which we have shown to be beneficial for early human embryonic development (Catt and Henman, 2000), has led us now to conclude that an oxygen tension of approximately 5% appears also to be advantageous for culture and derivation of human embryonic stem cells (Peura et al., 2007). In an inbred mouse cell line prone to Y-nondisjunction, a reduced oxygen environment for cell culture has been shown to play a role in lowering the levels of mitotic nondisjunction to normal (*in vivo*) levels from the higher levels observed when mouse embryos are grown in atmospheric (20%) oxygen concentrations (Bean et al., 2002).

The characterization of the cell lines by immunocytochemistry, PCR, embryoid body, and teratoma formation suggests that at least most of these lines are true embryonic stem cell lines, although because of logistical and practical delays there are a few gaps in the complete characterization. Interestingly, of the three lines that did not produce teratomas in the first attempt, two were karyotypically normal (male and female), and one was a trisomy 5 line, and the line where only two embryonic germ layers were observed was trisomy 16. Whether this seeming lack of differentiation ability proves to be just a coincidence or is related to the chromosomal status of the trisomic lines remains to be determined by further studies. In any case, although these lines are chromosomally abnormal, and thus less appealing for most stem cell applications or studies, they could prove particularly useful for research into the genomic contribution of chromosomes 5 and 16 to normal development—or for research on trisomies more generally. We welcome inquiries from parties with such interests. But overall, these lines have all been cultured for more than 20 passages and have maintained the characteristics associated with true pluripotent, undifferentiated ES cells.

In summary, we report the production of chromosomally stable stem cell lines with both euploid and aneuploid karyotypes from demonstrably abnormal PGD-analyzed and ordinarily discarded IVF embryos. In some cases the aneuploidy of the embryo was corroborated in the resulting cell line, but in other cases the derived cell lines were karyotypically different from the original PGD-analysis result. Besides offering a further route to deriving clinically relevant aneuploid stem cell lines for further research or testing purposes, our approach invites the possibility of obtaining karyotypically normal stem cell lines from clinically unusable embryos, thus offering an alternative source of embryos for embryonic stem cell derivation within the families concerned

and for the wider community. The stem cell lines reported here are available for research to the scientific community for either academic or commercial application.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that no competing financial interests exist.

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