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Derivation of the first Swiss human embryonic stem cell line from a single blastomere of an arrested four-cell-stage embryo

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Summary

Principles: Human embryonic stem cells (hESC) hold enormous potential for regenerative medicine. So far, the majority of hESC lines have been derived from the isolated inner cell mass (ICM) of blastocysts of variable quality, and several of them from low-grade embryos. Moreover, most of the lines have been obtained in media containing animal components such as foetal bovine serum. We aimed to derive hESC lines in xeno-free conditions using spare embryos frozen in Switzerland before 2001.

Methods: In cooperation with Swiss IVF centres we collected up to 199 donated embryos frozen between 1988 and 2000 at different stages of development.

Results: Embryo quality at thawing showed wide variability, reduced quality and low survival upon culture. Using early arrested embryos (n = 46), we report here the first Swiss hESC line, called CH-ES1, derived from a single blastomere of an arrested four-cell-stage embryo. Despite its polyploidy, already present at the third passage, CH-ES1 expressed ESC markers of pluripotency and differentiated into all three germ layers in embryoid bodies *in vitro* and in teratomas *in vivo*.

Conclusions: As the destruction of viable developing embryos, even spare ones, raises serious ethical concerns, deriving hESC lines from arrested embryos may be an alternative approach to avoid embryo destruction. However, given the reduced derivation efficiency they should not be considered a unique and/or selective source of hESC lines.

Key words: human embryonic stem cells; arrested embryo; blastomere; pluripotency; differentiation

Introduction

Human embryonic stem cells (hESCs) are known to be pluripotent since they have the ability to differentiate into virtually all cell types of the body. They hold great promise for regenerative medicine, and are a powerful tool for basic research [1, 2]. At present most of the existing pluripotent hESC lines have been derived from the inner cell mass (ICM) of five- to eight-day surplus human blastocysts. Only a few of them have been isolated from variable- or low-grade blastocysts [3–7] or morulae [8].

Recent studies have addressed the possibility of obtaining hESC from early cleavage embryos, ie with less than ten cells [9–11]. As previously reported, only small numbers of *in vitro* fertilised zygotes can successfully develop to the morula and blastocyst stage, since most of them arrest growth and are considered to be no longer viable [12–14]. Blastomeres of non-viable embryos do not normally continue to divide, even after 24 hours of observation. It was recently shown that late arrested embryos may be another source of human ESC derivation, with a derivation success rate of 0.8%. Moreover, arrested embryos were shown to express pluripotent genes, to adhere to feeders or extracellular matrices and to form primary outgrowths [9].

As the derivation process involves the destruction of viable developing human embryos, their use for hESC derivation, research and eventual medical applications has polarised ethical debates, despite the recent successful hESC deriva-

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tion from a single cell extracted from a still viable 8–10-cell embryo [11].

It is well known that with assisted reproductive technologies (ART), the number of resulting embryos produced after a cycle may exceed the number of embryos that can reasonably be transferred, thus producing embryos that are supernumerary to clinical requirements. Depending on each country's specific legislation, the number of surplus embryos can vary from one country to another. Switzerland has a conservative ethical framework regarding ART and constitutes a peculiar situation. On the one hand, the law on medically assisted procreation (LMAP), which entered into force in 2001, forbids the freezing of human embryos at different stages and allows 1) only the freezing of fertilised eggs or zygotes, defined as 'impregnated eggs' [15], and 2) culture of up to only three embryos for procreation purposes. On the other hand, the new law on hESC accepted by popular vote in 2004 allows the donation of surplus embryos to research [16]. This situation is however unlikely to occur, since cultured embryos that are not transferable at the last moment for medical or personal reasons are, and will continue to be, a rare event. Moreover, frozen fertilised eggs that become spare cannot be donated to research, since their culture beyond the zygote stage would correspond to the creation of an embryo 'for research purposes' and is thus forbidden by law. Compellingly, in 2005 we were confronted with the existence of a certain number of spare embryos frozen between 1988 and 2000 which had to be discarded to comply with the LMAP.

Here we report the state of the donated spare embryos frozen in Switzerland before 2001, as well as their quality at thawing and upon culture. We show the successful derivation of an hESC line, CH-ES1, from a single blastomere obtained from a poor-quality, four-cell-stage arrested embryo.

Materials and methods

Spare embryo collection

Surplus human embryos, frozen in Swiss IVF centres between 1988 and 2000 either at early cleavage or at the blastocyst stages, were collected from the various centres after being donated by patients through an informed consent process and transferred to our laboratory. No spare zygotes were used as Swiss law forbids their use for research purposes. The study was approved by the relevant institutional human ethics research committee of Geneva University Hospitals and by the Federal Office of Public Health (license # 6-001 [17]).

At the time of freezing, the quality of the embryos was quoted as being from good to heavily fragmented, ie defined as being of poor quality. Embryos that appeared to have stopped developing were processed only when it was clear that their development was irreversibly arrested [14, 18, 19], ie when none of the blastomeres had undergone cleavage within the last 24–48 hours of culture. Embryos were cultured in G-1/G-2 embryo culture media (Vitrolife) in 4-well plates (Nunclon, Roskilde, Denmark) in an atmosphere containing 6% CO_2, 5% O_2 and 89% N_2 at 37°C.

Feeder cells and culture media

All chemicals were obtained from Gibco BRL, Grand Island, NJ, USA, unless otherwise indicated. Feeder cells were commercially available human foreskin fibroblasts (CCL-110; ATCC1, Manassas, VA, USA). Fibroblast cultures (between passages four and ten) were mitotically inactivated using irradiation (35 Gy), and plated on 0.1% gelatin coated (Sigma-Aldrich, Switzerland) culture dishes (Nunclon, Roskilde, Denmark) at a density of $7x10^4$ cells/cm^2 [21]. The feeder cell culture medium was high glucose DMEM with the addition of 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin, 1x MEM-amino acids (ICN Biomedicals, USA), 1 mM sodium pyruvate (Sigma-Aldrich) and 10% FCS. Feeder cell dishes were used within one week from plating, changing the original culture medium to stem cell medium immediately prior to plating the embryos. Stem cell medium consisted of KO-DMEM with 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin, 1x MEM amino acids (ICN Biomedicals, USA), 0.1 mM β-mercaptoethanol (Sigma), 20% KSR and, initially, 50 ng/mL bFGF (R&D Systems, Minneapolis, USA), which was then reduced to 4 ng/mL upon the establishment of a stable hESC line.

Embryo plating and outgrowth culture

Embryo manipulation consisted of removal of the zona pellucida by mechanical cutting with an ultra-sharp splitting blade (Bioniche Animal Health, USA). If and when the blastocyst stage was reached, bisection into two parts was performed to separate the inner cell mass (ICM) and the polar trophectoderm from mural trophectoderm, with only the ICM-containing part plated onto feeder dishes, as published elsewhere [20]. Poor quality early-cleavage embryos (2–10 cells), ie with few intact blastomeres suitable for bisection, were plated whole and cultured as described above. Complete media change was carried out every second day of culture. The first passage of the outgrowth was performed 4–8 days after plating, and henceforth every 4–14 days until stable ESC-like growth was observed (fig. 2). Passaging was carried out manually by cutting outgrowths with an ultra-sharp splitting blade into 1–3 fragments and transferring them with a flame-pulled glass pipette to fresh feeder cell-coated dishes.

In vitro characterisation of the newly derived stem cell line 'CH-ES1'

Cell-surface markers characteristic of undifferentiated hESC were detected by immunocytochemistry. The cell colonies were fixed with 4% paraformaldehyde and incubated with antibodies against stage-specific embryonic antigen SSEA-1, and SSEA-4, Tra-1-85, nanog, alkaline phosphatase and oct-4 (Santa Cruz, CA, USA). Negative controls were performed by omission of the first antibody. The presence of markers was visualised using a Nikon fluorescence microscope.

The expression of genes characteristic of undifferentiated CH-ES1 was assessed using reverse transcriptase-PCR (RT-PCR). Total RNA was extracted using a Total RNA Extraction Kit (Qiagen, Switzerland) according to

Table 1

Embryo staging and development.

Code of embryos	Embryo stage at freezing	Number of intact blastomeres after thawing	Development	Outgrowth	Derived lines
BAA 1a	4	3	degenerated		
BAA 2a	4	0	degenerated		
BAA 3a	2	0	degenerated		
BAB 3a	5	3	blastocyst	No	
BAB 4a	6	1	degenerated		
BAC 2a	8	6	blastocyst	Yes	
BAD 1a	4	4	cut & plated		
BEB 1	4	4	cut & plated		
BEB 2	4	4	cut & plated		
BEG 1	2	2	cut & plated		
BEG 2	2	2	cut & plated		
BEG 3	4	1	cut & plated	Yes	CH-ES1
LOO 1	4	2	cut & plated		
LOO 2	4	4	cut & plated		
LOO 3	4	4	cut & plated		
LOO 4	5	3	cut & plated		
LOO 5	2	1	cut & plated		
LOP 1	4	0	degenerated		
LOP 2	4	0	degenerated		
LOP 3	2	0	degenerated		
LOG 1	7	7	cut & plated		
LOG 2	7	7	cut & plated		
LOG 3	4	3	cut & plated		
GER 1	8	8	cut & plated		
GER 2	6	3	degenerated		
GER 3	6	4	degenerated		
GEB 11	6	1	degenerated		
GEB 13	6	1	degenerated		
GEB 3	8	1	degenerated		
GEB 2	4	0	degenerated		
GEC 8	6	0	degenerated		
GEC 9	6	1	degenerated		
GEC11	4	2	degenerated		
GEC 5	4	1	degenerated		
GEC 3	4	4	degenerated		
GEG 1	2	0	degenerated		
GEM 1	5	5	Blastocyst	Yes	
GEP 1	2	0	degenerated		
GEP 2	4	0	degenerated		
LAHo1	3	1	cut & plated		
LAHa1	4	4	cut & plated		
LAHa2	4	4	cut & plated		
LAHa3	3	2	cut & plated		
LAHa4	7	1	cut & plated		
LAHa5	4	4	cut & plated		
LAHa6	4	3	cut & plated		
LAHa7	3	1	cut & plated		
LAHa8	6	3	cut & plated		
LAK1	4	4	blastocyst	Yes	
LAS1	2	2	blastocyst	No	
LACh1	3	3	cut & plated		
LACh2	2	2	cut & plated		

Table 1
(continued).

Code of embryos	Embryo stage at freezing	Number of intact blastomeres after thawing	Development	Outgrowth	Derived lines
LACh3	3	1	cut & plated		
LACa1	3	0	degenerated		
LACa2	6	0	degenerated		
LACa3	4	3	cut & plated		
LASe1	2	0	degenerated		
LAH9	4	4	cut & plated		
LAH10	4	4	cut & plated		
LAH11	4	4	cut & plated		
LAH12	4	4	cut & plated		
BEF 1	4	3	blastocyst	No	
BEF 2	7	7	cut & plated		
BEF 3	4	4	cut & plated		
BEF 4	4	4	cut & plated		
BEF 5	4	4	cut & plated		
BEF 6	4	4	cut & plated		
BEF 7	6	6	blastocyst	No	
BEP1	5	5	cut & plated		
BEP2	6	4	cut & plated		
BEP3	4	1	cut & plated		
BEP4	4	4	cut & plated		
BEFo 1	4	4	blastocyst	No	
BEFo 2	4	4	cut & plated		
BEFo 3	4	4	cut & plated		
BES 1	2	2	cut & plated		
BES 2	4	1	cut & plated		
BES 3	2	2	cut & plated		

the manufacturer's instructions for small quantities of cells. The concentration of RNA was assessed using a spectrophotometer (Biowave II, Biochrom, UK). Total RNA (1 µg) from each sample was used for first strand cDNA synthesis by using the Advantage RT-for-PCR Kit (Clontech, BD Biosciences, Switzerland) and following the manufacturer's protocols. The cDNA from each sample was used as a PCR template to detect expression of the genes listed in table 1. PCR reaction mixtures were prepared using 1 µg total cDNA as template, and were then denatured at 94ºC for four minutes, and cycled 32 times at 94ºC for 30 s, 60ºC for 30 s and 72ºC for one minute. A final extension at 72ºC for ten minutes was performed after cycling. PCR primers were designed using freely available Vector NTI software (Invitrogen, Switzerland) to show possible genomic contamination. Negative control samples, including characteristic differentiation genes from ectoderm, mesoderm and endoderm, were included in each reaction. PCR products were resolved on 2% agarose gels, stained with ethidium bromide and visualised in a trans-illuminator (Bio-Vision, USA).

The pluripotency of CH-ES1 was tested both *in vitro* and *in vivo*. For the *in vitro* differentiation study, embryoid bodies (EB) were generated as previously described [22]. Briefly, colonies were dissociated with collagenase IV treatment for thirty minutes at 37°C and then cultured in suspension on low-attachment 6-well culture plates. The differentiation medium consisted of KO-DMEM with 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin, 1x MEM amino acids (ICN Biomedicals, USA), 0.1 mM β-mercaptoethanol (Sigma), 20% FBS. Cells were cultured for 4–7 days in suspension, and then EBs were transferred onto gelatin-coated dishes and cultured for an additional 10–14 days. Total RNA was extracted as described previously for RT-PCR. To assess the expression of markers associated with differentiation of the three germ layers, the primers shown in table 2

Table 2
List of RT-PCR primers used to screen H1- and CH-ES1-ESC and EBs.

Gene	Sense primer (5'-3')	Anti-sense primer (3'-5')
Oct3/4	GACAACAATGAAAATCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA
Nanog	AAGACAAGGTCCCGGTCAAG	CCTAGTGGTCTGCTGTATTAC
REX1	GCGTACGCAAATTAAAGTCCAGA	CAGCATCCTAAACAGCTCGCAGAAT
hTERT	CGGAAGAGTGTCTGGAGCAAGT	GAACAGTGCCTTCACCCTCGA
PAX6	GAATCAGAGAAGACAGGCCA	GAGTAGGTATCATAACTCCG
NESTIN	GGCAGCGTTGGAACAGAGGTTGGA	CTCTAAACTGGAGTGGTCAGGGCT
T(Brachyury)	GCAAAAGCTTTCCTTGATGC	ATGAGGATTTGCAGGTGGAC
AFP	GGGAGCGGCTGACATTATTA	CCCTCTTCAGCAAAGCAGAC
GAPDH	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCAGCATCG

Table 3

List of the markers tested by immunohistochemistry on CH-ES1-derived teratomas.

Antibodies used	Specificity of immunoreactivity	Results in CH-ES1
AFP (alpha-foetoprotein)	Tumour marker of yolk sac component, but also in other germ cell tumours	Negative
β HCG (human chorionic gonadotrophin)	Tumour marker of choriocarcinoma	Negative
CD 30	Expression of nuclear factor KB; tumour marker of embryonal carcinoma	Negative
CD 34	Marker of dendritic interstitial cells and adult haematopoietic stem cells	Negative
CD 45	Marker of leucocytes	Negative
CD 56 (N-CAM)	Receptor involved in the regulation of nerve/muscle interactions and cell motility. Induction of neurite outgrowth	Positive in 70% of cells
CD 99 (O13)	Differentiation marker of primitive neuro-ectodermal cells and marker of Ewing sarcoma/PNET (primitive neuroectodermal tumours)	Negative
CD 117	C kit stem cell receptor; marker of gastrointestinal stromal tumours	Negative
Desmin	Muscle-type intermediate filament; marker of smooth/striated muscle cells and hormone dependent stromal cells.	Negative
KALL: pool of keratins (AE1=CK10, 13, 14, 15, 16, 19) (AE3 = CK1-8) (CK18/8)	Intermediate filament; marker of epithelial differentiation, regardless of endodermal, neuroectodermal, mesenchymal or germ cell derivation	Positive in 10% of cells
Myogenin	Myogenic nuclear protein; marker of skeletal muscle differentiation	Negative
Nanog	Pluripotent marker	Positive in 70% of cells
Nestin	Intermediate neurofilament; particularly abundant in neuroepithelial stem cells	Positive in 80% of cells
Neurofilaments	Intermediate filament; marker of neurons and some endocrine tumours	Negative
PLAP (placental-like alkaline phosphatase)	Reactivity observed in germ cell tumours and others	Negative
α smooth muscle actin	Contractile protein; isoform α is a marker of smooth muscle differentiation	Negative
Vimentin	Intermediate filament; expressed in mesenchymal cells	Positive in 80% of cells

were used, and PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and visualised in a trans-illuminator.

In vivo differentiation

In vivo differentiation studies were carried out by the generation of teratomas via subcutaneous flank injection of 5×10^4 undifferentiated hESC into SCID (severe combined immunodeficient) mice (n = 5). The resulting tumours were removed four weeks later, fixed in 4% formaldehyde, and embedded in paraffin. Four µm-thick sections were stained with hematoxylin-eosin (HE) for control, or immunostained with the streptavidin peroxidase system. To better characterise these tumours and detect the mesoderm, endoderm, and ectoderm derivatives respectively, immunohistochemistry was performed with different commercially available antibodies (table 3).

Freezing and thawing of CH-ES1

Cryopreservation of hESC colonies was successfully performed using the open pulled straw vitrification method developed by Reubinoff et al. [23]. The thawing protocol undertaken depended on the method used. Surviving colonies were plated on new irradiated feeder cell layers in hESC medium, and the growth curve was measured over three passages.

Karyotype of the CH-ES1

Karyotyping was performed in at least twenty metaphase spreads, using the GTG-banding method, by an independent laboratory (Genetic Service, Geneva University Hospitals, Switzerland). Briefly, hESC were incubated in hESC medium, supplemented with 0.2 µg/mL colcemid (Roche, Basel, Switzerland) at 37ºC for twenty minutes, and subsequently washed three times with 2 mL phosphate buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. A minimum of fifteen colonies were mechanically dissected in PBS from the feeder layer, collected in 2 mL 1× trypsin-EDTA (Invitrogen) and incubated at 37ºC for five minutes. The final mixture of cells was pipetted several times to disaggregate the cells. The trypsin activity was stopped with 4 mL hESC medium and spun at 300 g for 10 minutes. Subsequently the pellet was resuspended and incubated in 1 ml of pre-warmed potassium chloride solution (KCl, 0.075 mol / l) for ten minutes at 37ºC. Cells were then pre-fixed with 1 mL Carnoy fixative solution [methanol/acetic acid = 3 / 1] at –20ºC, and immediately spun at 1800 rpm for 10 minutes. Finally, the supernatant was discarded, the pellet resuspended again in Carnoy and the cells were prepared for analysis. CH-ES1 line was routinely karyotyped, the first time between passages 4 and 10, and then approximately every 20 passages, to monitor further changes in karyotype.

Results

State of spare embryos donated to research

To comply with the new 2001 Act on medically assisted procreation, which forbids the freezing of embryos beyond the zygote stage, left-over embryos frozen before 2001 had to be destroyed by the end of 2005, unless donated to research. With the assistance of FIV-NAT (the Swiss Society of Reproduction Medicine) and the active cooperation of seven out of 25 Swiss IVF centres, we collected 203 donated embryos frozen between 1988 and 2000. Donor anonymisation was performed by the IVF Centre of Geneva University Hospitals. Figure 1A shows the number and the stage of the donated embryos collected by each of the seven IVF centres. Out of 203 frozen embryos, 167 were at the 2–8-cell stage, while 36 were blastocysts.

Embryo culture for the establishment of hESC lines

As our first authorisation provided by the Federal Office of Public Health (FOPH) in 2006 allowed us to use only 100 embryos, we first processed 98 embryos retrieved from 108 thawed embryos. We were subsequently authorised to use the rest of the embryos in 2007. Figure 1 summarises the entire embryo culture procedure, namely the number of embryos thawed, retrieved and cultured, as well as the number of outgrowths obtained. At thawing we found, from a total of 203 donated embryos, 34 out of 36 blastocyst-stage embryos and 144 out of 167 2–8-cell-stage embryos. Only eight out of 34 blastocysts expanded and upon dissection and culture we obtained three outgrowths that did not grow further. In turn, of 144 retrieved 2–8-cell embryos, only 19 developed to blastocysts and further turned into seven outgrowths, but these ceased growing after five passages. The remaining 125 were scored as arrested embryos, in 79 of which all blastomeres were degenerated. To determine whether these early-cleavage stage arrested embryos could be suitable for hESC derivation, we cultured the remaining 46 embryos on inactivated human foreskin fibroblasts. After removal of the zona pellucida, only intact blastomeres were plated over feeder cells and cultured in stem cell medium. Overall, one outgrowth was observed coming from a four-cell-stage arrested embryo, of which only one out of four blastomeres survived thawing and the *in vitro* culture procedure. This blastomere gave rise to an embryonic stem cell line named CH-ES1 (fig. 2). The success rate of derivation from a single blastomere corresponded to 0.6% (1/158 blastomeres), while the success rate of derivation from arrested embryos was estimated at 2.2% (1/46). Table 1 summarises the data for each processed embryo.

Characterisation of CH-ES1

Morphologically undifferentiated colonies of CH-ES1 were selected for molecular and biochemical characterisation at passage level 3, 5, 10 and 15 (fig. 2). An immunocytochemical study confirmed that, like the control hESC line H1, CH-ES1 stained positive for the cell-surface markers SSEA4, Tra-1-85, and alkaline phosphatase as well as for the transcription factors oct-4 and nanog in the nucleus, with all of them being characteristic of the undifferentiated hESC (fig. 3).

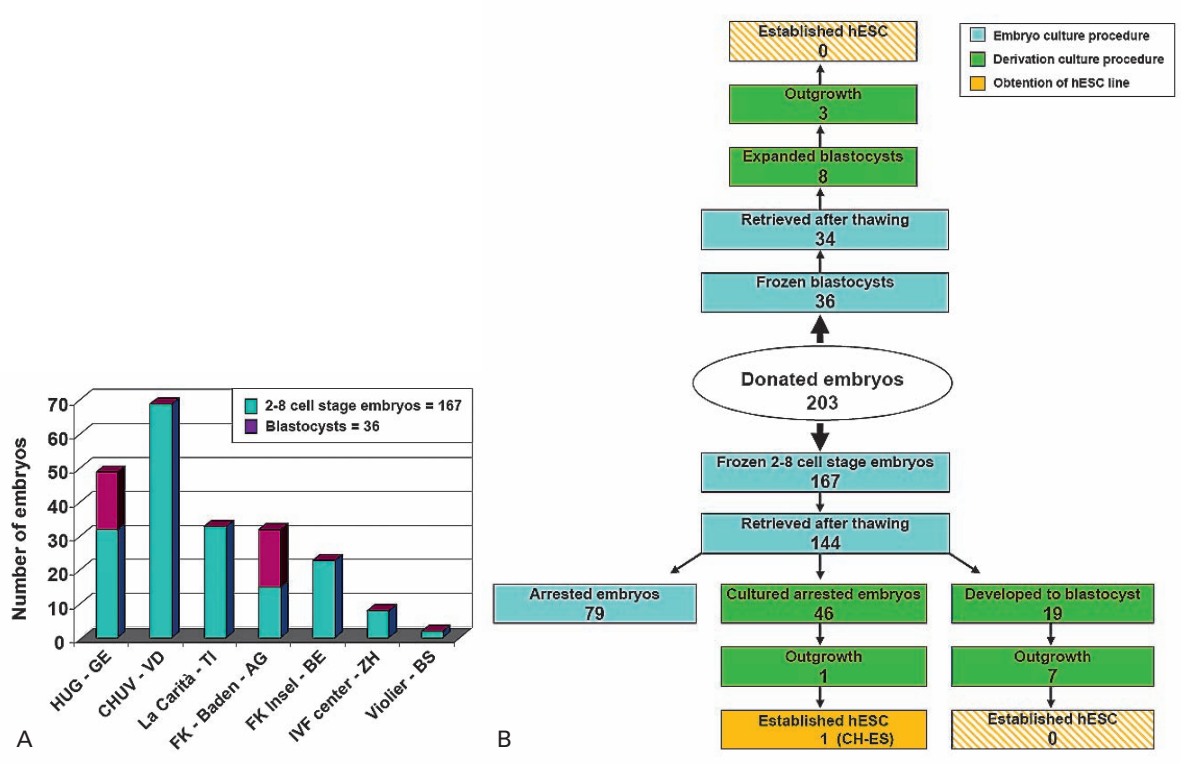


Figure 1

State of the spare embryos frozen in the 1990s in Switzerland and donated to research in 2005.
A) Number of embryos and conservation stages obtained by the 7 IVF centres that could contribute to the embryo collection. B) Organisation chart summarising the embryo stages, the recovery at thawing and the culture results.

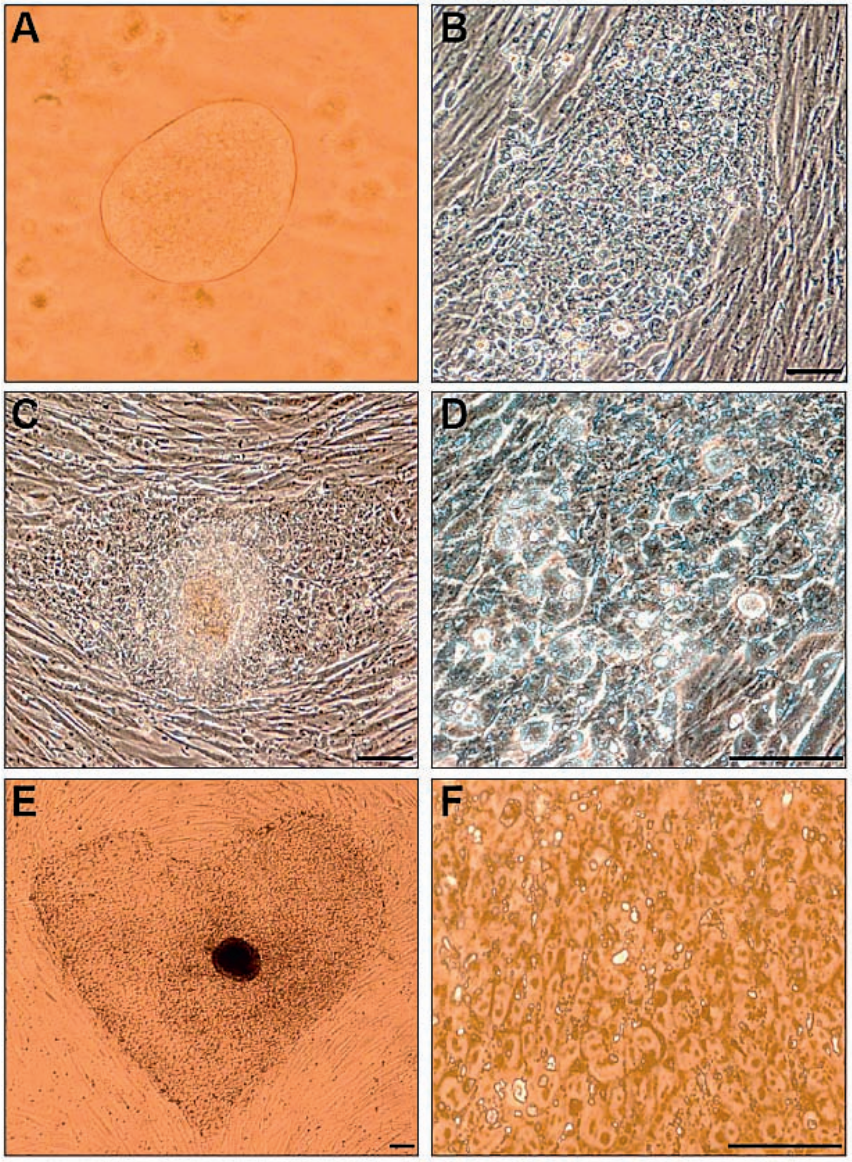


Figure 2

Culture steps leading to derivation of the hESC line CH-ES1 from a single blastomere obtained from an arrested 4-cell-stage embryo. A) A single blastomere after zona pellucida removal, placed on inactivated human foreskin fibroblast-derived feeders. B) Morphology of the first outgrowth obtained five days after blastomere plating. C, D) Human ESC colony formation after the first mechanical passage. E, F) Typical morphology of a CH-ES1 colony on human foreskin feeder cells. Bar: 20 µm.

The RT-PCR results further demonstrated that this hESC expressed the genes encoding for oct-4, sox2, nanog, rex-1 and hTERT when compared to the H1 human embryonic stem cell line (fig. 4A).

After freezing and thawing, the CH-ES1 line kept dividing and maintained its undifferentiated properties, with a short doubling time (15 hr). Karyotyping revealed that this new hESC line had an abnormal female karyotype (fig. 4B), with 61 chromosomes. Amongst these genetic abnormalities we detected trisomy 1, 4, 5, 6, 10, 13, 15, 16, 18, tetrasomy 3, 7, 12 and multiple rearrangements, thus explaining why this hESC had a short doubling time. Like H1, CH-ES1 was not able to adhere in the absence of feeder cells or extracellular matrices.

The pluripotency of CH-ES1 was then examined *in vitro* and *in vivo*. When grown in suspension, CH-ES1 colonies possessed the ability to form embryoid bodies (EB, fig. 5A) containing characteristic markers of all three germ layers (fig. 5B).

Injection of CH-ES1 cells into SCID mice resulted in consistent formation within four weeks of a locally highly invasive tumour that was primarily restricted to the site of injection (fig. 6A), whereas H1 induced teratomas only within 10–12 weeks after injection (not shown). Histologically, CH-ES1 tumours appeared malignant and rather undifferentiated, containing sheets of low cohesive round or oval-shaped cells (fig. 6B). The cytoplasm was scant and the nuclei were large with prominent atypical nucleoli. Mitotic figures were numerous (40 mitoses/10 high power fields) (not shown). Focal necrosis was observed, and invasion of adjacent adipose tissue and striated muscle was obvious. 70% of cells were positive for Nanog and CD 56 by immunohistochemistry, while 80% of cells stained positive for nestin and vimentin (fig. 6B, panel b). A pool of cytokeratins, described as KALL, was shown to be positive in less than 10% of the tumour cells (fig. 6B, panel d). However, cells were negative for all other immunostaining performed (see table 3).

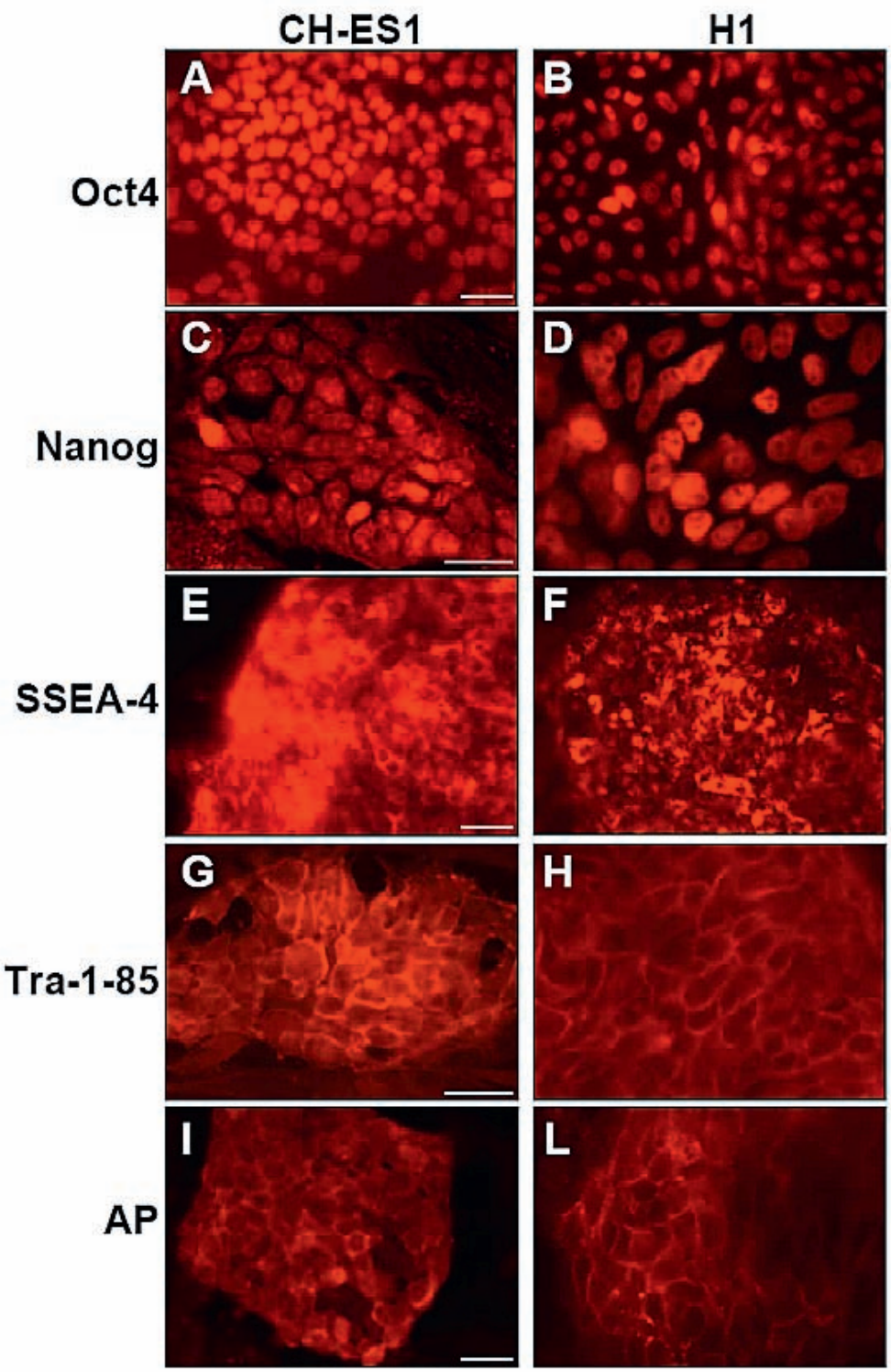


Figure 3

Immunocytochemical comparative analysis of CH-ES1 and H1 hESC lines for pluripotency markers. Colonies were analysed five days after mechanical passaging; (A, B) oct4, (C, D) nanog, (E, F) stage specific embryonic antigen SSEA-4, (G, H) Tra-1-85 and (I, L) alkaline phosphatase (AP). Bar: 20 μm.

Discussion

This is the first time the derivation and the characterisation of one hESC line, which originated from a single blastomere of an arrested four-cell stage embryo, have been reported. Only 35% of zygotes obtained from *in vitro* fertilisation successfully develop to the morula and blastocyst stage [13, 14, 18, 19, 24]. Hence, approximately 75% of embryos obtained from *in vitro* fertilisation exhibit different degrees of abnormalities leading to embryo arrest [12, 13, 19]. In our study, 87% of 2–8-cell-stage embryos arrested their development, while 13% reached the blastocyst stage. We expected such highly variable embryo quality at thawing, knowing that until 1998 in Switzerland culture and cryopreservation protocols varied greatly from one laboratory to another, and that all cultured embryos were frozen regardless of their quality. The main known causes of embryo arrest are inadequate oocyte maturation, chromosome abnormalities during embryo cleavage, cellular asymmetry, cytoplasmic and nuclear fragmentation, and inadequate *in vitro* embryo

Figure 4

Gene expression analysis and karyotyping of the newly derived CH-ES1 line.
A) Expression of pluripotency markers rex-1, oct-3/4, nanog, and hTERT by RT-PCR.
B) Karyotype analysis shows abnormalities in chromosomal number (61 XX) and the presence of iso-chromosomes.

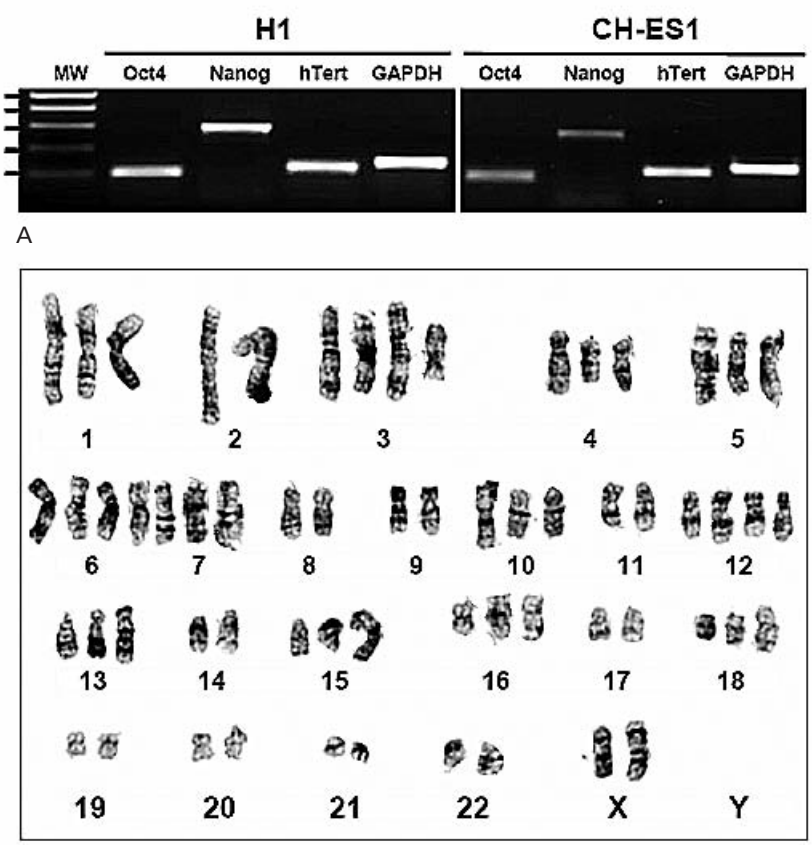
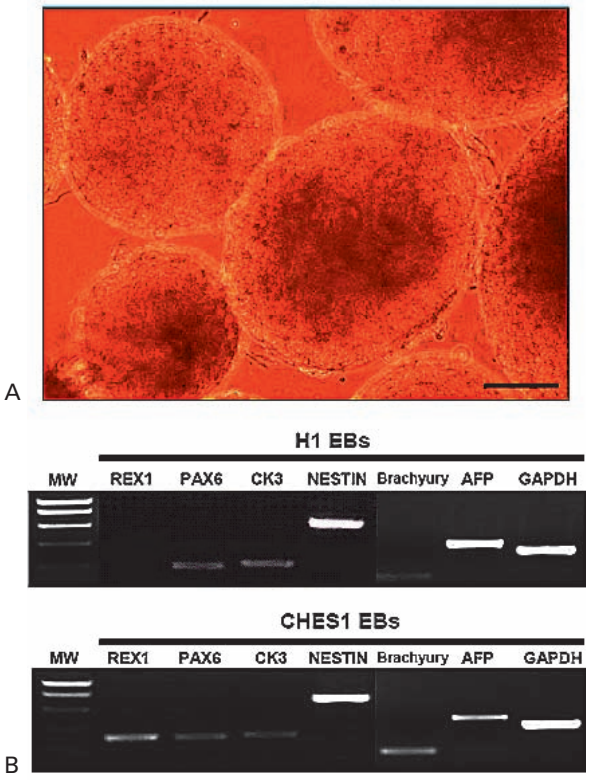


Figure 5

Induction of differentiation by embryoid body (EB) formation and expression of differentiation markers. A) EBs formed by fragments of hESC colonies removed from their feeders and grown in suspension. B) Expression of differentiation markers typical of endoderm and mesoderm, haematopoietic and cardiac mesoderm. The pluripotency marker oct4 remains elevated.
Bar: 100 μm.



culture conditions [13, 14]. There is a clear scientific interest in identifying the nature of the aberrations that cause either the embryo to arrest developmentally or cause pathological pregnancies. The derivation of hESC from arrested embryos and those carrying genetic anomalies would provide researchers with a large number of cells with which to elucidate some of the mechanisms behind developmental defects.

To date, the establishment rate of the majority of frozen blastocyst-derived hESCs has ranged between 3% and 10% [25]. Hardarson and coworkers found that only 42% of blastocysts originating from surplus embryos were chromosomally normal [26]. Of the abnormal embryos, most were mosaic. Hypothetically this would result in more chromosomally aberrant hESC lines than seems to be the case, since fewer than 10% of all stem cell lines worldwide and approximately 19% of those established in Gothenburg, Sweden, contain an innate aneuploid genome [26]. A low establishment rate in combination with a low frequency of chromosomally aberrant cell lines indicates that selection occurs during the development of an embryo to the blastocyst stage, as well as during establishment of hESC lines, causing cleavage arrest and degradation of embryos in which aberrations are too severe [25].

While the derivation of hESC lines is more efficient when using day six embryos, it has recently been shown that arrested embryos also have the potential to proliferate and form primary outgrowths and hESC-like colonies once freed from the zona pellucida [9]. Lanza et al. derived hESC lines from dissociated eight-cell stage embryos by co-culturing them with existing hESC lines, although with low efficiency (less than 4%) [11, 27]. They recently improved their derivation protocol and raised the efficiency above 20% using laminin and avoiding co-culture with hESCs [10]. These data suggest that viable blastomeres would have the potential to proliferate and may constitute an additional source for hESC derivation. However, *in vitro* culture conditions need to be improved to increase the derivation success rate.

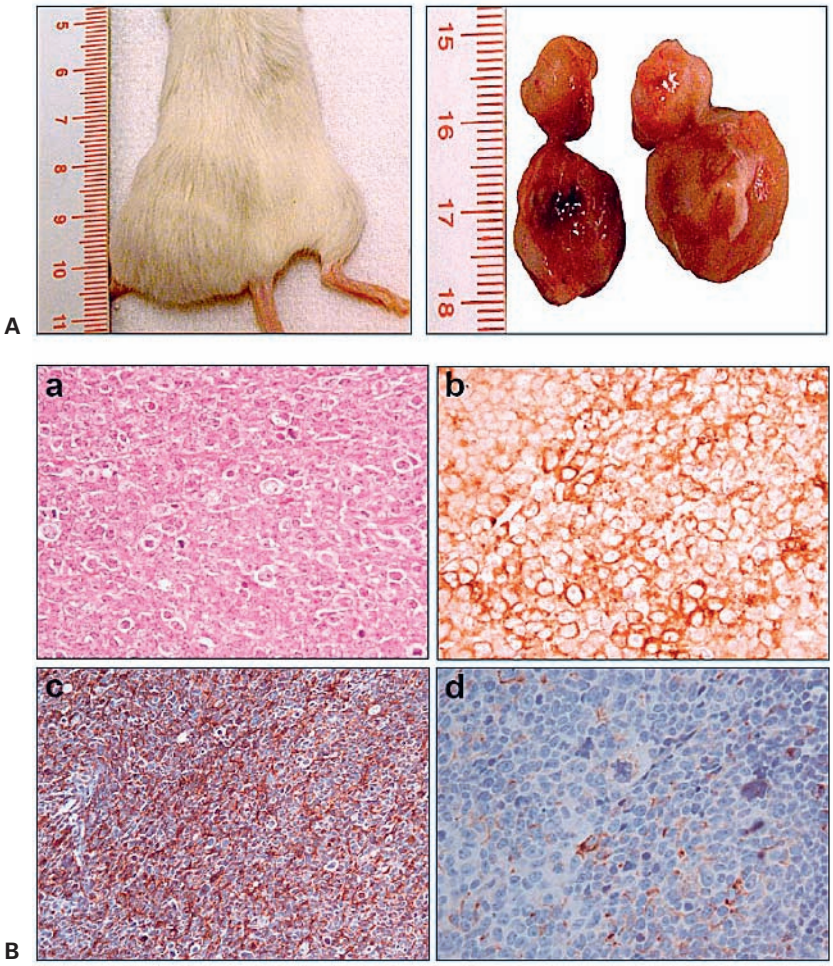
In our study, CH-ES1 line was derived in the same conditions as many other lines have been derived [20, 21, 28], ie without the use of laminin or any other components. Using the embryos which were available, our derivation rate was 2.2% (vs 4%).

Because the first karyotype was performed at passage 3, we hypothesise that polyploidy was acquired very early, possibly within the surviving blastomere. Despite these abnormalities, the CH-ES1 showed all hESC-like characteristics. They express all pluripotency markers (i.e. Nanog, Oct-3/4, and Rex-1) and can form EBs containing all three germ layer representatives; however, they fail to differentiate further under appropriate conditions. For instance, neither neurons nor beating cardiomyocytes were observed within differentiating EBs, despite the detection of tissue-specific genes by RT-PCR.

Little is known about abnormal hESC lines and their differentiation potential [30, 38]. In contrast to all derived lines which down-regulate

Figure 6

Developmental potential of CH-ES1 *in vivo*. A) Macroscopic view of tumour formation four weeks after CH-ES1 injection into SCID mice. B) Histological and immunohistochemical features of the CH-ES1-induced tumours. (a) The tumour cells are undifferentiated, with scant cytoplasm and large nucleus (H&E, magnification 400X). (b) Cells were strongly positive for nestin which is expressed in most of the tumour cells. (c) Vimentin-positive cells are widely expressed in the tumour. (d) KALL staining was present in a minority of cells.



pluripotency markers upon differentiation into EBs and produce teratomas upon injection into immunosuppressed mice [2, 3, 21, 28, 35-37], our newly derived hESC revealed the coexistence of pluripotency and differentiation markers during *in vitro* differentiation. These observations may be explained by the presence of genetic anomalies carried by the CH-ES1 line. Moreover, when injected into SCID mice CH-ES1 cells developed a highly invasive and rapidly growing tumour. According to histological criteria, this tumour should be considered a dysgerminoma since typical teratoma, epithelial or stromal structures were absent. The negative immunostaining for PLAP (placental-like alkaline phosphatase) suggested that the tumour was even less differentiated. Moreover, the negative immunostaining pattern for CD30, CD99, and b-hCG (human chorionic gonadotrophin) discards the histological diagnosis of embryonal carcinoma, primitive neurectodermal tumours, and choriocarcinoma. Instead, the expression of Nestin, Vimentin, Nanog and CD56 were consistent with a stem cell phenotype. The CH-ES1-derived tumours showed no morphological differentiation, but expressed cytoplasmic intermediate filaments involved in motility and mesenchymal/epithelial transformation.

Human ESC may be prone to acquire chromosomal anomalies while being cultured continuously *in vitro* [5, 29–33]. These genetic abnormalities could be due to the *in vitro* environment itself and to the culture pressure selection or conditions. The spontaneous occurrence of chromosomal aberrations reported in hESC lines, in combination with the fact that trisomic cells proliferated as fast as the chromosomally normal diploid stem cells, indicate that trisomy does not necessarily lead to a delayed cell cycle and hence a slower proliferation rate in hESC grown *in vitro* [5, 29-33]. In our case, the CH-ES1 line was passaged mechanically and colonies grew more rapidly when compared to the H1 line. However, it is important to stress that the CH-ES1 karyotype assessed at early passage 3 was maintained at passage 10. This would suggest that such anomalies may affect the known self-normalisation mechanisms [19, 34], and would thus lead to a high proliferation rate in this newly derived stem cell line.

All derived lines differentiated spontaneously by forming EBs and produced teratomas when injected into immunosuppressed mice [2, 3, 21, 28, 35–37]. Little is known about abnormal hESC lines and their potential for differentiation [30, 38]. In this study, the newly derived hESC line expressed all markers of pluripotency, such as Nanog, Oct-3/4, and Rex-1. When differentiation was induced *in vitro*, markers of the three germ layers coexisted with pluripotency markers typical of undifferentiated cells. These observations may be explained by the presence of genetic anomalies carried by the CH-ES1 line.

When CH-ES1 cells were injected into SCID mice, the latter developed a highly invasive and rapidly growing tumour. According to histological criteria this tumour should be considered a dysgerminoma, since typical teratoma, epithelial or stromal structures were absent. The negative immunostaining for PLAP (placental-like alkaline phosphatase) suggested that the tumour was even less differentiated. Moreover, the negative immunostaining pattern for CD30, CD99, and β-hCG (human chorionic gonadotrophin) discards the histological diagnosis of embryonal carcinoma, primitive neurectodermal tumours, and choriocarcinoma. Instead, the expressions of Nestin, Vimentin, Nanog and CD56 were consistent with a stem cell phenotype. The CH-ES1-derived tumours showed no morphological differentiation, but expressed cytoplasmic intermediate filaments involved in motility and mesenchymal/epithelial transformation.

In conclusion, this type of study, particularly in the case of mosaic embryos, may provide insight into the success rate of deriving abnormal hESC lines versus normal lines, thereby improving scientific knowledge of genetic anomalies and their possible involvement in causing embryo arrest. Although it is possible to obtain hESC lines

from highly abnormal arrested human embryos, the likelihood of abnormalities appears to be high and such embryos cannot be regarded as the only source of hESC lines.

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