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STEM CELL BIOLOGY

Vitrified blastocysts from Preimplantation Genetic Diagnosis (PGD) as a source for human Embryonic Stem Cell (hESC) derivation

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Abstract Embryos diagnosed as abnormal in Preimplantation Genetic Diagnosis (PGD) cycles are useful for the establishment of human Embryonic Stem Cells (hESC) lines with genetic disorders. These lines can be helpful for drug screening and for the development of new treatments. Vitrification has proved to be an efficient method to preserve human blastocysts. One hundred and three abnormal or undiagnosed vitrified blastocysts from the PGD programme at Institut Universitari Dexeus were donated for human embryonic stem cell derivation. The overall survival rate after warming was 70.6 %. Our results showed better survival rates when blastocysts have not started the hatching process (initial/expanded 87.8 %, hatching 68.3 % and hatched 27.3 %). Thirty-five blastocysts and 12 partially surviving embryos were seeded. One hESC line with the multiple exostoses type 2 paternal mutation was obtained.

Keywords Blastocyst · Vitrification · PGD · hESC derivation

Capsule Embryos diagnosis as abnormal in Preimplantation Genetic Diagnosis (PGD) cycles are useful as a source of human Embryonic Stem Cells (HESC) lines with genetic disorders for research and drug screening.

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Introduction

Embryonic stem cells are pluripotent cells than can be obtained from the blastocyst's inner cell mass [32]. They can be cultured in vitro and differentiated into different cell types [26]. These cells lines represent an ideal field of research to study cell differentiation and developmental biology, as well as other research areas focusing on their potential use for cell therapy for the treatment of degenerative human diseases. On the other hand, derivation of hESC lines from embryos with mutations from monogenic diseases or chromosomal abnormalities can offer the opportunity to generate in vitro disease models. These cell lines constitute an excellent source of cells for the study of human genetics, gene expression patterns and as a tool to understand the events that take place during human embryo development when genetic disorders are present. These lines can be also useful for drug screening and for the development of new possible treatments.

PGD is a procedure used to detect genetic defects in preimplantation embryos in order to avoid their transfer to the uterus and ensure a pregnancy from genetically normal embryos [12]. The establishment of hESC lines with genetic

disorders can be performed using embryos diagnosed as abnormal in PGD cycles, either PG Screening (PGS) for chromosomal abnormalities and monogenic disease diagnosis for the detection of gene mutations. These PGD-affected embryos are not used for transfer or cryopreservation but they can be a valuable source for the derivation of hESC lines. Around 60 hESC from abnormal PGD embryos have been described [3, 9, 11, 16, 19, 23, 28, 31, 33, 38, 39] and 83 lines have been registered in hESCreg (human Embryonic Stem Cell Registry) (www.hescreg.eu).

Surplus embryos generated at the PGD cycle (genetically abnormal and undiagnosed) need to be cryopreserved as the Spanish law (Act 14/2006) establishes that IVF embryos only can be generated for reproductive purposes. The couple has then different options for the use of the cryopreserved embryos including own use, donation to other couples, donation for research and to discard the embryos with no further use.e. Taking into account that PGD embryos have a hole in the zona pellucida and as such more fragile and prone to lysis, a safe and effective method for cryopreservation is needed.

Vitrification has demonstrated to be an effective method for the cryopreservation of blastocysts coming from biopsied PGD embryos [21]. However, there are few reports on data about the vitrification of blastocysts arising from biopsied embryos with a wide range of results in terms of survival and pregnancy rates [10, 14, 36, 37, 44].

We here describe the results obtained after warming blastocysts coming from two different vitrification methods from PGD cycles and their use for hESC derivation with genetic abnormalities.

Material and methods

Source of embryos

Human embryos diagnosed as abnormal (chromosomically abnormal or affected of monogenic diseases) or with a failed diagnosis and thus discarded for patient's use, were vitrified at blastocyst stage. Embryos were donated for research by couples submitted to PGD cycles at Institut Universitari Dexeus after informed consent. hESC Derivations were performed at the Stem Cell Bank of the Center for Regenerative Medicine in Barcelona (CMRB) after the approval of the Ethics Committee and of the *Comisión de Seguimiento y Control de la Donación de Células y Tejidos Humanos del Instituto de Salud Carlos III* (approved 19.02.2007).

Embryo culture and PGD

Gamete and embryo culture were performed according to standard protocols used in the *In Vitro* Fertilization (IVF) laboratory of the Reproductive Medicine Service of Institut Universitari Dexeus. Embryos were biopsied on day 3 as previously described [2]. One or two genetically normal blastocysts were replaced on day 5, when the diagnosis was established. All embryos that reached the blastocyst stage up to day 7 were vitrified for the couple or for research.

Three groups were established according to the blastocyst expansion degree: initial/expanded blastocysts, hatching blastocysts and hatched blastocysts.

Vitrification and warming

During the first period, from November 2004 to April 2005, blastocysts were frozen using a modified vitrification method, with home-made solutions [25], loaded in a open pulled straw [21, 35] and inserted into a high-security 0.3 ml straw (Cryo Bio System, France) before plunging them into liquid nitrogen avoiding direct contact with the sample.

Since 2005, the vitrification method used consists of a commercialized closed device (HSV; Cryobiosystem, France) and commercialized vitrification solutions (Irvine Scientific; USA).

In all cases, for warming, each straw was immersed into a 37 °C water bath for 5 seconds. Dilution of cryoprotectants was performed by six step incubations in decreasing sucrose solutions.

After warming, embryos were placed in culture medium (G2; Vitrolife, Sweden) and the evaluation of survival was performed after overnight culture. Re-expansion and morphological criteria such as the presence of structured ICM and trophoectoderm were considered for survival evaluation. Non structured and non re-expanded partially surviving blastocysts were also considered for use if viable cells were identified.

Derivation of hESC lines

After overnight culture, surviving blastocysts were classified as suggested by Stephenson et al. [29] taking into account the expansion degree (from 1 to 6; from initial to fully hatched blastocysts), the number of cells and appearance of the ICM (from A to E; from ICM with compacted cells to no visible ICM) and the number of cells and cohesion of the trophoectoderm cells (A to C; from many small identical cells to sparse cells).

The ICM of good quality blastocysts was isolated by exposing the trophoectoderm to cell lethal laser pulses (Octax EyeWare, Olympus).

All whole embryos and ICMs were cultured on irradiated human foreskin fibroblasts monolayers (HFF-1, CCD1112Sk ATCC) in derivation medium. This medium is composed of 50 % hES medium and 50 % hES conditioned medium (hES medium exposed to growing culture of hESC for one day with 2.5 % of hES cell tested FBS, Hyclone) supplemented with

2.5 % of ES cell tested FBS, following the methodology previously described [1, 6]. The derivation medium was gradually substituted for hES medium between first and third passages.

Poor quality as well as non structured and non re-expanded partially surviving blastocysts were cultured for 2–3 days, in hES conditioned medium, to stimulate hESC growth in the embryo. In this medium, ICM cells grow and fill in the blastocelic cavity and throphectoderm usually degenerates.

Characterization of hES cells

To determine if the line obtained was composed of hESC, phenotypic and genetic analysis of undifferentiated colonies were performed. Characterization included karyotype, analysis of human leukocyte antigen (HLA), assessment of expression of pluripotency markers and evaluation of pluripotency in vitro and in vivo as previously described [24].

Statistical analysis

Chi-square test was used considering $p<0.05$ to be statistically significant.

Results

In total, 103 non-transferable PGD blastocysts were donated for research. Blastocysts were warmed with a recovery rate (percentage of blastocysts recovered after warming) of 82.5 % (85/103). Eighteen embryos were lost during the process. From these 85 warmed blastocysts, 60 survived. The overall survival rate was 70.6 % (60/85).

Statistically significant differences have been observed in the survival rate according to the blastocyst expansion. The survival rates were 87.9 % (29/33) for initial and expanded, 68.3 % (28/41), for hatching blastocysts and 27.3 % (3/11) for hatched blastocysts. Differences were also observed in the survival rates according to the vitrification media used with lower results obtained when "homemade" medium when compared with commercial media were used (44.7 % versus 91.5 %). No differences were observed in the survival rate according the day of vitrification (day 5, 6 or 7). Survival rates results are shown in Table 1.

Sixty-six blastocysts came from PGD for aneuploidy screening (PGS) (77.6 %), while 19 blastocysts came from PGD cycles for monogenic diseases (22.3 %). No differences were observed in the survival rates (66.7 % and 84.2 % respectively) (Table 2).

Thirteen embryos degenerated during the culture process prior to seeding. Thirty-five blastocysts and 12 partially surviving embryos were seeded. One stem cell line was obtained from the monogenic disease group from a partially surviving embryo (Fig. 1). This line came from an embryo of a couple where the man was affected from multiple exostoses type 2. Hereditary Multiple Exostoses (MHE) is an inherited disorder of bone growth. Hereditary Multiple Exostoses is a condition in which people develop multiple benign (noncancerous) bone tumors called exostoses. The number of exostoses and the bones on which they are located vary greatly among affected individuals. This condition is inherited in an autosomal dominant pattern through the mutations of the *EXT1* and *EXT2* genes. This embryo was undiagnosed at the time of PGD and was discarded for transfer and was preserved for vitrification for possible use in research. DNA from the obtained hESC was analyzed by amplification of the exon 2 of the EXT2 gen by PCR amplification. The cell line established proved to have the paternal mutation. The line was named ES [11-EM].

ES[11-EM] was fully characterized. It expresses alkaline phosphatase, OCT4, NANOG , SOX2, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 2). Embryoid bodies (EBs)

Table 1 Survival rates according blastocyst stage and vitrification method and day

	N	Survival rate	
Blastocyst stage			
Blastocyst init/exp	33	87.9	
Blastocyst hatching	41	68.3	
Blastocyst hatched	11	27.3	
			$p<0.05$
Vitrification method			
Home-made	38	44.7	
Commercial	47	91.5	
			$p<0.05$
Day of vitrification			
D+5	54	77.8	
D+6	26	69.2	
D+7	5	0	
			n.s

Table 2 Survival rate and derivation efficiency according PGD method

	PGS	Monogenic diseases	N	%
Warmed blastocysts	66	19	85	
Survival blastocysts (%)	44 (66.7)	16 (84.2)	60	70.6
Blastocysts, ICM or partial survival embryos seeded	38	9	47	78.3
Initial growth rate	8	2	10	21.2
Lines obtained (%)	0	1 (11.1)	1 (ES[11-EM])	2.1

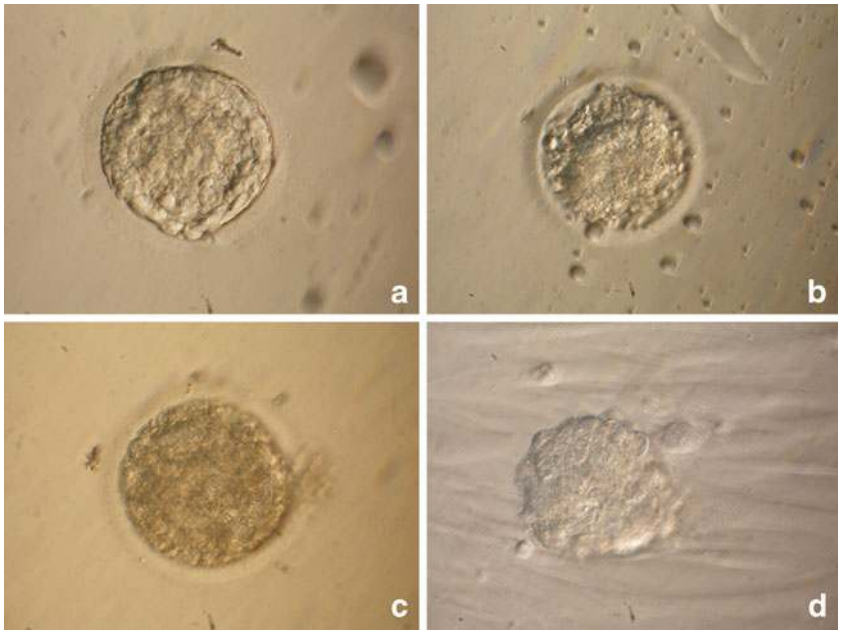

Fig. 1 Blastocyst used for ES[11-EM] derivation. **a** Blastocyst post-warming (day 6). **b** Blastocysts 4 h post-warming cultured in hESC conditioned medium. **c** Blastocyst on day 7. **d** Blastocyst seeded

were generated expressing markers of ectoderm (Tuj1), endoderm (α-fetoprotein and FoxA2) and mesoderm (α-actinin, GATA4 and SMA) after in vitro culture (Fig. 3). The supplementation of the differentiation medium with ascorbic acid resulted in approximately 20 % of the EBS generated displaying rhythmically beating areas. The ectoderm differentiation with PA6 coculture protocol give rise to mature neurons that expressed high levels of Tuj1 This line also had potential to form teratomas in SCID mice with presence of tissues from the three germ layers as cartilage, respiratory epithelium and neuronal tissue (Fig. 4). ES [11-EM] showed normal karyotype (46, XY) (Fig. 5). HLA and fingerprinting analysis were done (data not shown). This line was registered in the Spanish Stem Cell Bank and the hESCreg. Adequate frozen stocks are available and this line is provided at scientist's request.

Discussion

Eighty-two point 5 % of blastocysts were recovered after warming. This figure is not very high and probably affected

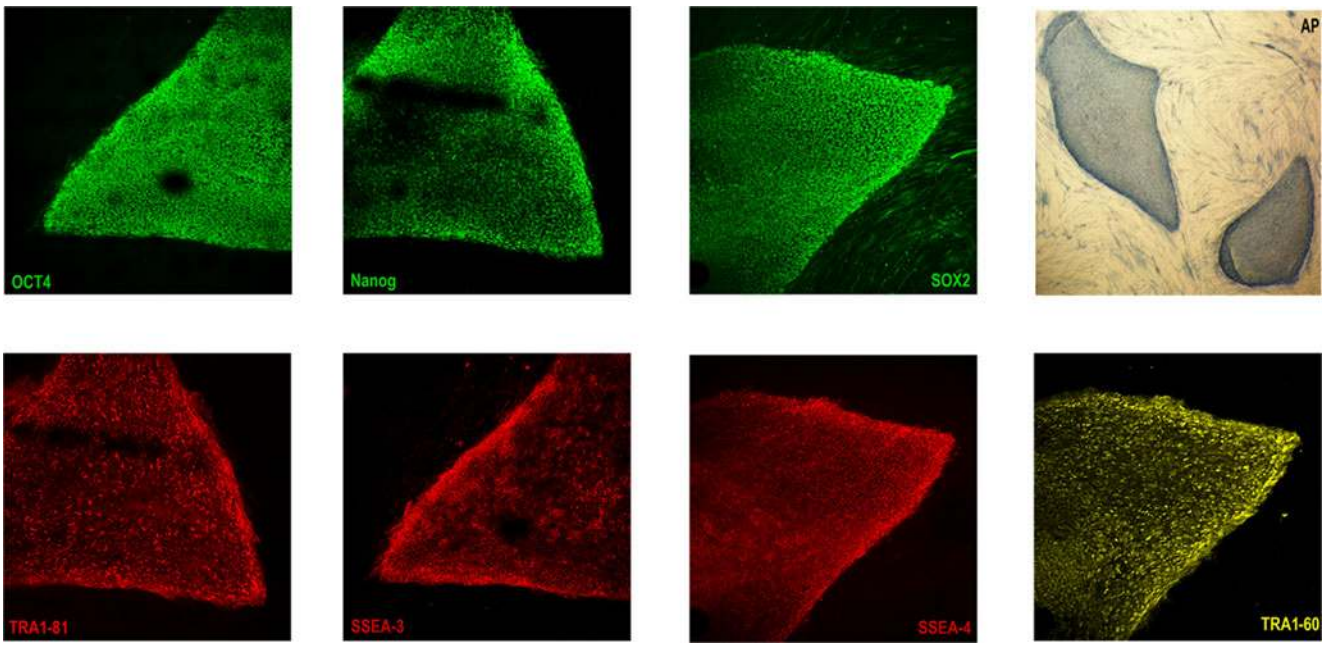

Fig. 2 Expression of pluripotency markers in ES[11-EM]. Immunofluorescence localization of Oct4, Nanog, Sox2, TRA-1-81, SSEA-3, SSEA-4 and TRA-1-60 and alkaline phosphatase activity (AP)

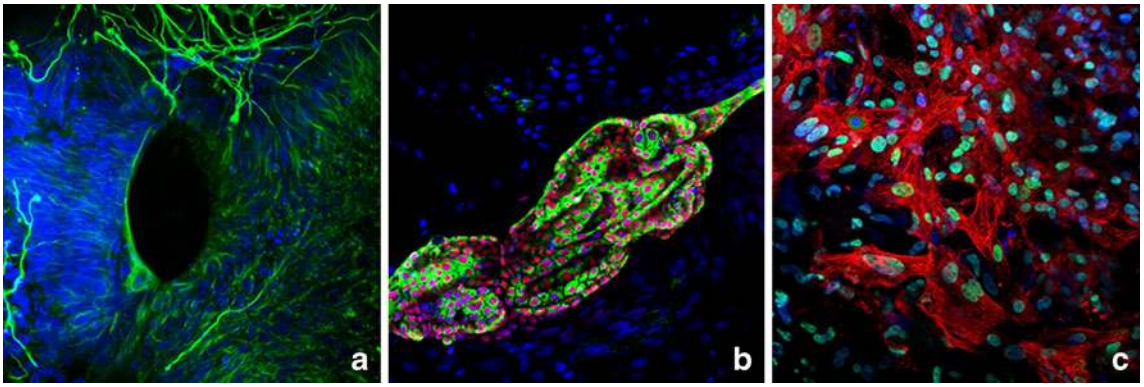

Fig. 3 In vitro differentiation of ES[11-EM] of ectoderm, endoderm and mesoderm. Cells immunoreactive for **a** Tuj1, **b** Alfa-fetoprotein (*green channel*) and FOXA2 (*red channel*) **c** GATA4 (*red channel*) and a-actinin (*green channel*)

by the learning curve of the vitrification technique that was partially put in place with non-transferable PGD blastocysts. Other reports describe similar results (84.5 %, [10])

Our overall survival rate was 70.6 %.

Differences in the survival rate were observed with the use of different vitrification media (home-made: 44.7 %; commercial: 91.5 %). It has to be taken into account that home-made media composition may be less homogeneous and show variability and that these media were used at the time of the learning curve of the technique.

Our results show similar survival rates for initial and expanded blastocysts (18/20; 90 % and 84.6 % respectively). It has been described that the degree of expansion of the blastocelic cavity is related to the survival of blastocysts after cryopreservation, with lower survival rates in intact expanded blastocysts [8, 18, 34, 37]. A possible explanation for the discordance of our results with these publications is the fact that our blastocysts arise from biopsied embryos. It has been hypothesized that the hole in the ZP may allow a better exposure to the cryoprotectant solution and result in a better dehidratation of the blastocoele [4, 44]. The possible beneficial effect of an artificial opening in the ZP before vitrification has also been reported, showing higher survival rates in expanded blastocysts after ZP opening. [42].

Decreased results in terms of survival rates were achieved for hatching and much lower for hatched blastocysts (68.3 % and 27.3 % respectively). No such differences were observed by Escribá et al. [10] and hatching and hatched blastocysts were not analyzed separately from expanded blastocysts in other similar studies [14, 37, 44]. Biopsied blastocysts that are inside the zona pellucida are exposed to the cryoprotectant but are also protected from mechanical damage. Hatching and hatched blastocysts have also a good exposure to the cryoprotectant but, as they are less protected by the zona pellucida, they can be more susceptible to mechanical damage during manipulation. This can be an explanation to the low survival rate in hatching and hatched blastocysts.

From 47 blastocysts and partially surviving embryos that were seeded only one cell line was derived. It is not possible from our data to establish a correlation between derivation efficiency and ICM quality, but as shown by our own results in previous reports [1, 24], low quality embryos can be successfully used in hESC derivation, with derivation

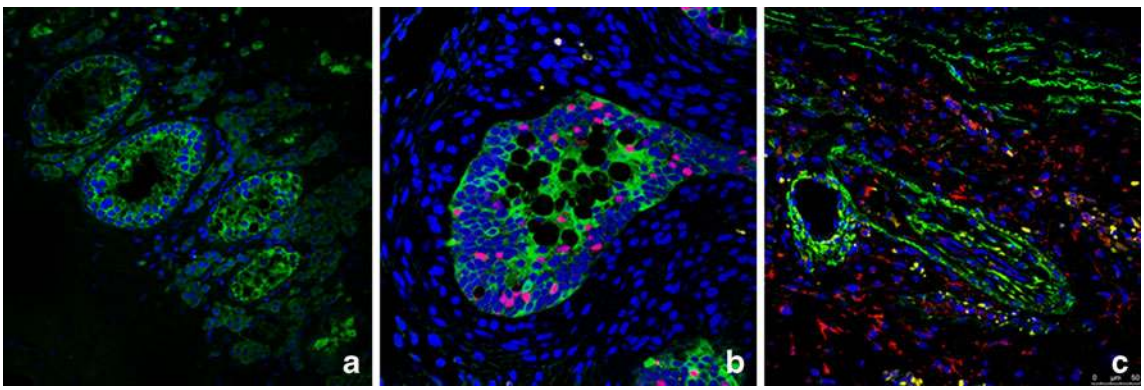

Fig. 4 Pluripotency of ES[11-EM] in vivo. **a** Ectoderm derivatives were detected by immunoreactivity for Tuj1; **b** Endoderm derivatives detected by immunoreactivity for alfa-fetoprotein (*green channel*) and FOXA2 (*red channel*); **c** Mesoderm derivatives for smooth muscle actin (*green channel*) and alfa-sarcomeric actinin (*red channel*)

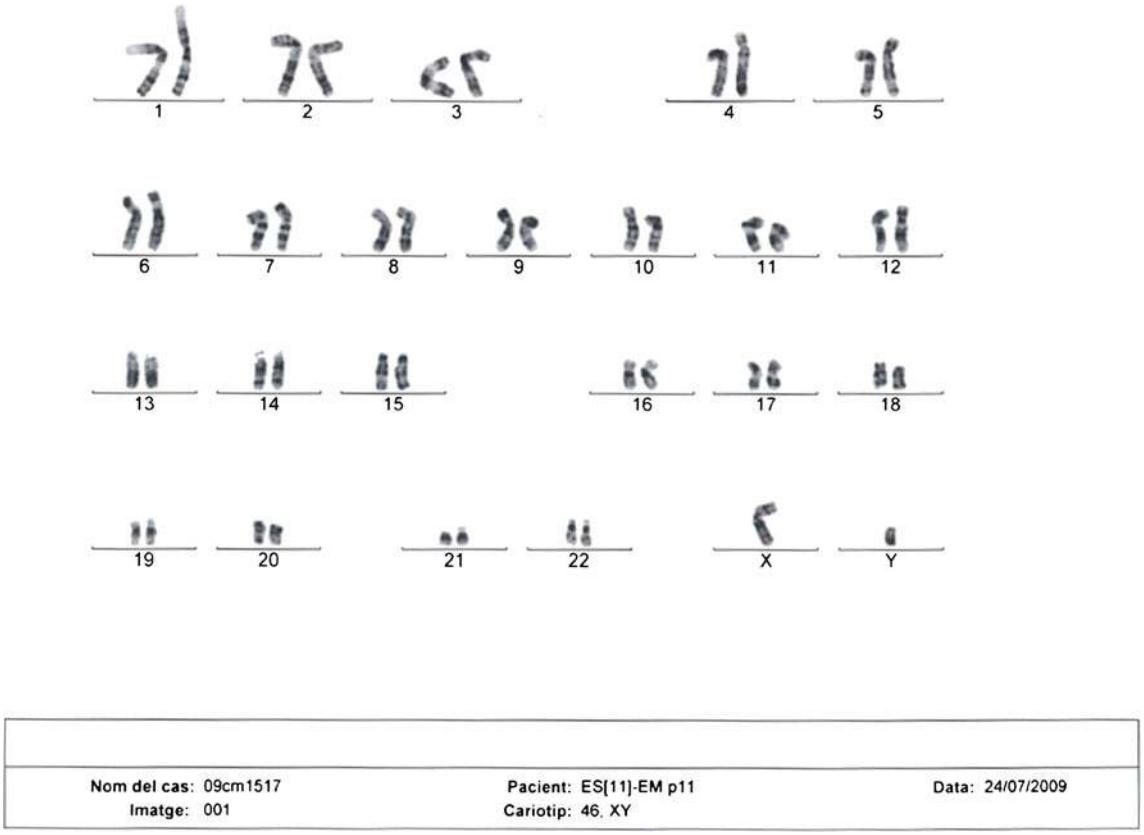

Fig. 5 Karyotype of ES[11-EM]

efficiency rates similar to the ones obtained with good quality embryos. Although many authors report the number of embryos and derivation methodology used, few reports provide information about the quality of the embryos used for derivation. Mitalipova et al. [17] reported a 21 % derivation efficiency using discarded embryos. The derivation efficiency presented by Chen et al. [5] with embryos with low morphological scores was 10.5 %. Cowan et al. [7] derived three lines from blastocysts that would have been discarded because of their poor morphologic characteristics and Zhang et al. [43] achieved a derivation efficiency of 5 % from arrested embryos. Ström et al. [30] found no correlation between embryo morphology and successful derivation of hESC lines, with an overall derivation rate of 12.8 % (30/234). However, O'Leary et al. [20] found a correlation between the number of poor-quality traits and blastocyst development and ICM quality, showing that good-quality ICMs from embryos with multiple poor-quality traits are unable to generate hESC lines.

The derivation efficiency of hESC with genetic abnormalities may vary according to the type of abnormality and how this abnormality affects embryo and cell viability. Our results show that no cell line was obtained from 31 chromosomally abnormal embryos and only one cell line was established from 14 abnormal monogenic diseased embryos. Several authors have reported hESC lines coming from embryos with chromosomal abnormalities. Frydman et al. [11] reported a low derivation rate (4.5 %) from PGD-blastocysts with chromosomal abnormalities whereas Peura et al. [22] and Taei et al. [31] presented derivation rates of 26.8 % and 18.2 % respectively. Verlinsky et al. [40, 41] found a correlation between chromosomal aneuploidies and the efficiency of establishing hESC lines; they observed that embryos with aneuploidies originated from meiotic non-disjunction have a lower derivation rate than those resulting from mitotic non-disjunction. Ilic et al., [13] referred no hESC lines established from 12 karyotypically abnormal embryos versus 2 lines from 4 normal embryos. Thirty three lines carrying chromosomal abnormalities have been registered in the hESCreg (www.hescreg.eu).

The derivation efficiency of hESC from blastocysts with monogenic defects can be similar to the efficiency obtained from normal embryos. Verlinsky et al. [38] obtained 18 hESC from 48 embryos affected or carriers of single gene mutations (37.5 %). Mateizel et al. [16] and Tropel et al. [33] reported similar derivation rates (21.4 % and 20 % respectively) from PGD-embryos with monogenic diseases.

Some of these PGD-derived cell lines are already being used in projects aimed to study the molecular mechanisms of diseases such as Fragil-X Syndrome, [9] Myotonic

dystrophy or Huntington disease [27, 28]. Recently, Marteyn et al. [15] have identified an early developmental defect in genes involved in neurite formation and neuromuscular connections using neuronal progeny derived from hESC carrying the DM1 mutation.

hESC lines derived from PGD-affected embryos represent an unlimited source of cells for the study of human genetics, drug screening, and the development of new therapeutics protocols and possible gene and cellular therapies for some diseases.

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Conflict of interest The authors declare that they have not conflict of interest.

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