Article

Impact of meiotic and mitotic non-disjunction on generation of human embryonic stem cell lines

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Abstract

At least 50–60% of oocytes derived from IVF procedures are chromosomally abnormal due to meiotic I or II errors. Through the use of polar body and blastomere diagnosis, euploid embryos suitable for transfer can be identified. Those embryos that are aneuploid are usually discarded, or otherwise can be used to generate chromosomally abnormal human embryonic stem cell (hESC) lines. The authors' centre has one of the largest repositories of hESC lines with genetic and chromosomal disorders generated from preimplantation genetic diagnosis (PGD) abnormal embryos. The results, studying hESC lines derived from PGD abnormal zygotes, imply that aneuploidies resulting from meiotic non-disjunction have a greater impact on viability of cells of the human embryos than those originating from post-zygotic mitotic non-disjunction.

Keywords: *aneuploidy rescue, human embryonic stem cells, in-vitro fertilization, non-disjunction, polar body diagnosis, preimplantation genetic diagnosis*

Introduction

Preimplantation genetic diagnosis (PGD) is a method used to test for genetic abnormalities in oocytes [polar body (PB) diagnosis] or embryos (blastomere or blastocyst biopsy) after IVF procedures to improve reproductive outcome in certain IVF patients (Gianaroli *et al.,* 1999, 2001, 2004; Munné *et al.,* 1999, 2003, 2006; Verlinsky *et al.,* 2005a; Findikli *et al.,* 2006).

It is known that approximately 50% of preimplantation embryos are abnormal in women at 35 and older, rising to nearly 80% in patients ≥40 years of age (Magli *et al.,* 2007; Munné *et al.,* 2007). The majority of these chromosomally abnormal embryos seem to be eliminated before implantation, as only 1 in 10 of recognized pregnancies is chromosomally abnormal. Furthermore, it is now widely accepted that reduced fertility with age mostly arises from chromosome abnormalities deriving from meiotic errors (Abdalla *et al.,* 1997).

Knowing that over 90% of chromosomal errors originate from maternal meiosis, oocyte testing is the method of choice to detect these abnormalities and is performed by removing the first and second PB (PB1 and PB2) from oocytes, representing the by-products of meiosis I (MI) with PB1 and meiosis II (MII) with PB2 respectively. This approach makes it possible to infer the resulting maternal contribution to the embryo and is also of importance due to a relatively high rate of mosaicism at the cleavage stage, which is the major limitation of the blastomere-based PGD for chromosomal disorders.

Sequential testing of both polar bodies in the oocytes, followed by blastomere biopsy in day-3 embryos, is therefore useful for reliable diagnosis of chromosomal status of embryos. Sequential testing also helps to exclude both pre-zygotic meiotic and post-zygotic mitotic errors, as most mosaic embryos may originate from aneuploid oocytes through the process known as 'aneuploidy rescue' (Kuliev and Verlinsky, 2004), which is also reflected in the comparable overall prevalence of aneuploidies in oocytes and embryos (Gianaroli *et al.,* 2001; Munné, 2002; Kuliev *et al.,* 2003).

Taken together, PGD for chromosomal aneuploidy allows the avoidance of transfer of the overall 50–80% of abnormal embryos, thus making a potential contribution to the pregnancy outcome of IVF patients.

Human embryonic stem cells (hESC) are usually produced through the use of surplus embryos derived from IVF procedures and aneuploid zygotes obtained in PGD, which otherwise are discarded. Recently the early-arrested or highly fragmented embryos, which have achieved blastocyst stage, also became a robust source of normal hESC (Lerou *et al.,* 2008). As mentioned, another source for hESC lines is genetically abnormal tested embryos after PGD (Verlinsky *et al.* 2005b). These hESC lines with monogenetic and chromosomal abnormalities represent an extremely valuable source for investigation of primary mechanisms of monogenetic diseases or chromosomal abnormalities.

Materials and methods

The preimplantation embryos for the establishment of ESC lines with genetic disorders were obtained from PGD cycles, which were performed either by sequential biopsy of PB1 and PB2 in oocytes followed by removal of one blastomere in day-3 embryo biopsy or by single blastomere biopsy on day 3, as described elsewhere (Verlinsky and Kuliev, 2000). Following fluorescence in-situ hybridization analysis of the biopsied materials using probes specific for chromosomes 13, 16, 18, 21, 22 and re-hybridized with specific probes for 9, 15, 17, X and Y chromosomes in blastomere analysis, the unaffected embryos were transferred back to patients, while the chromosomally abnormal ones were used for derivation of hESC lines, according to informed consent approved by the Institutional Review Board of Reproductive Genetics Institute. The policy of the institute is not to transfer any embryos, where chromosomal errors cannot be completely ruled out because of false-positive or false-negative results (ambiguous result).

Four groups of aneuploidies were defined and studied. Group I comprised exclusively PB diagnosis where blastocysts originated from zygotes tested positive for monosomy, trisomy or multiple errors. Such chromosomal abnormalities originate comparably from meiosis I and II (Kuliev and Verlinsky, 2005). Usually, all cells in the resulting embryos are abnormal for the same chromosome(s).

Group II were exclusively PB diagnosis, where blastocysts originated from zygotes showing a balanced karyotype. It is known that approximately 43% of oocytes with meiosis I errors also have sequential meiosis II errors, resulting in 33% of cases in karyotypically balanced zygotes. The cause for the formation of such balanced zygotes is not yet understood. It is suggested that the underlying mechanism is an 'aneuploidy rescue' event in female meiosis. This event may be similar to the well-known phenomenon of 'trisomy rescue' in post-zygotic embryo development, which may result in uniparental disomy and imprinting disorders (Kuliev and Verlinsky, 2005). Such embryos either have a stable diploid karyotype, or may show a predisposition for further post-zygotic and thus mitotic non-disjunction events.

Group III comprised PB1 and/or PB2 karyotypically normal and blastomere biopsy showing chromosomal errors. Such chromosomal abnormalities may arise from mitotic errors in one or more blastomeres of an otherwise karyotypically normal embryo. These embryos can be classified as being mosaic, with a mix of karyotypically normal and abnormal cells.

Group IV included embryos testing positive for chromosome errors after only blastomere diagnosis.

It is assumed that the major sources of chromosomal abnormalities in embryos are meiosis I and II errors, with mitotic errors playing a tangential role (Kuliev and Verlinsky, 2005). However, because data on PB are missing in group IV, the underlying cause for the chromosomal abnormalities (meiotic error with predisposition to further mitotic errors, 'aneuploidy rescue' of an abnormal zygote during the first mitotic divisions or primarily post-zygotic mitotic errors of single cells) remains speculative.

Depending on the developmental stage of these aneuploid embryos, different techniques for the establishment of hESC lines were used, as described previously (Strelchenko *et al.,* 2004). The initial disaggregation of the cells (passage 0) was performed approximately 8–14 days after growth in the feeder layer, by treating the cells with EDTA and cutting and transferring the soft cell clumps into a new dish with feeder layer. Fast proliferating colonies with ES-like morphology were isolated and propagated further. Within the next two to five passages, the uniform proliferating cells were selected, and colonies of established ESC lines were passaged using EDTA, followed by the harvesting procedure with a cell lifter, as described previously (Strelchenko *et al.,* 2004).

The cell lines were tested for the following ES cell markers: alkaline phosphatase, stage-specific antigens SSEA-3 and SSEA-4, high molecular weight glycoproteins or tumour rejection antigens, TRA-1–60 and TRA-1–80, and Oct-4, detected with polyclonal antibodies, as well as by Gene Choice One Tube real time-polymerase chain reaction kit (Vector Laboratories Inc., Burlingame, CA, USA), as described previously (Strelchenko *et al.,* 2004).

Regular chromosomal analysis was performed using G-banding technique on 10–20 cells of the established hESC lines before freezing at passage 10–15.

Statistical analysis

To compare the different groups, a double-sided Fisher's exact test was conducted using Statistics Package for Social Sciences for Windows 11.0 (Chicago, IL, USA). Percentages were rounded up to the first decimal place. *P*-values <0.05 were considered as statistically significant.

Results

Four groups of aneuploidies were studied, as described under Materials and methods.

As shown in **Tables 1** and **2**, those hESC lines derived from group I with a proven trisomy and monosomy in the oocytes carried the same chromosomal error. On the other hand, two stem cell lines originating from ambiguous trisomy results had a normal karyotype on two different passages. As shown in

Figures 1 and **2**, most probably these two oocytes had a normal chromosome make-up, but were not classified as normal due to a large signal; no additional testing was done due to sufficient normal zygotes available for transfer (**Figure 1**: ambiguous result on first polar body for stem cell line SC-327; **Figure 2**: ambiguous result on first polar body for stem cell line SC-343). The same holds true for the tested zygote with an ambiguous result on monosomy, which produced a karyotypically normal hESC line, probably due to a hybridization failure. **Tables 3** and **4** show the outcome of hESC line derivation from the embryos resulting from a balanced chromosome abnormality (group II). All of these cell lines were karyotypically normal after karyotype analysis on 10–20 cells at the different passages.

Groups III and IV represent the hESC lines derived from mosaic embryos, detected as described above. As shown in **Tables 5–8**, all but three hESC lines were karyotypically

Table 1. Embryos abnormal based on polar body (PB) 1 and/or 2 analyses.

No.	*Stem cell line*	*Result of PGD*	*Karyotype*
1	SC-184	Trisomy13	47XX+13
2	SC-321	Trisomy 21	47XX+21
3	SC-327	Trisomy 13	46XX
4	SC-342	Monosomy 21 (only 2nd PB)	46XY
5	SC-343	Trisomy 21	46XY
6	SC-344	Monosomy 22 (only 2nd PB)	46XY
7	SC-267	Monosomy 22	45XX–22

PGD = preimplantation genetic diagnosis.
No. of blastocysts = 174; blastocysts with trisomy = 87; blastocysts with monosomy = 45;
blastocysts with multiple abnormalities = 42

Table 2. Success rate of generating human embryonic stem cell (hESC) lines from group I embryos.

	Polar body 1 and/or 2 errors			*Total*
	Trisomy	*Monosomy*	*Multiple errors*	
No. of karyotypically abnormal blastocysts	87	45	42	174
No. of embryos with ambiguous results	2	2	0	4
No. of hESC lines	4	3	0	7
No. of euploid hESC lines from ambiguous results	2	2	0	4
No. of euploid hESC lines from proven errors	0	0	0	0
Success rate per blastocyst				
No. of hESC lines (%)	4/87 (4.6)	3/45 (6.7)	0/42 (0.0)	7/174 (4.0)
No. of hESC lines from proven errors (%)	2/85 (2.4)	1/43 (2.3)	0/42 (0.0)	3/170 (1.8)
Euploid hESC lines from proven errors (%)	0	0	0	0

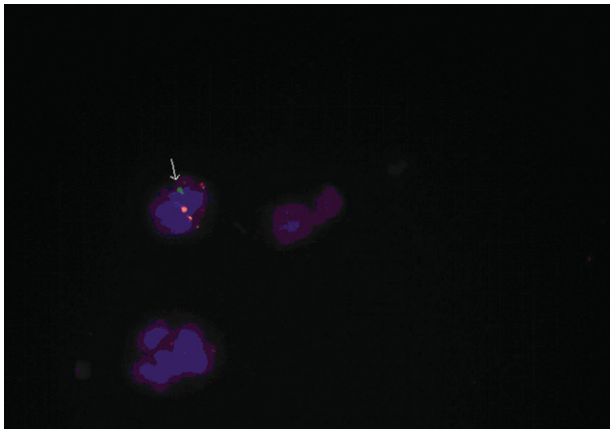

Figure 1. Ambiguous result on polar body 1, polar body 2, normal on zygote producing stem cell line SC-327: trisomy 13 could not be ruled out due to one large signal in 13q, even after rehybridization (white arrow in picture: one large signal in 13q). Colour of signals on re-hybridization: 13q = green, 16q = orange.

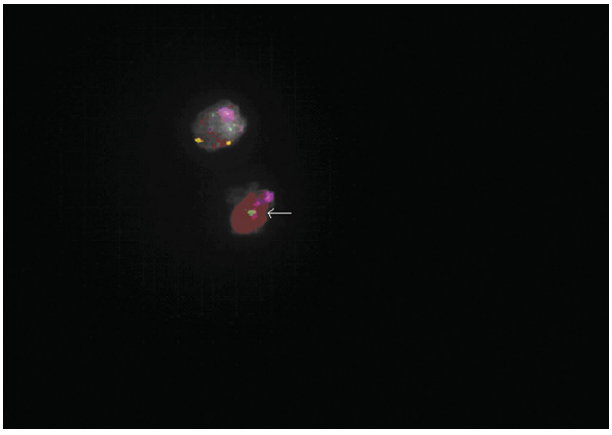

Figure 2. Ambiguous result on polar body 1, polar body 2 normal on zygote, which produced stem cell line SC-343: trisomy 21 could not be ruled out due to a large signal (white arrow: one large signal in chromosome 21). Colour of signals: 13 = red, 16 = aqua, 18 = blue/purple, 21 = green, 22 = gold.

Table 3. Embryos with balanced abnormalities after analysis of polar body 1 and 2.

No.	*Stem cell line*	*Result of PGD*	*Karyotype*
1	SC-160	–21+21	46XX
2	SC-217	+16–16	46XY
3	SC-221	+16–16	46XY
4	SC-294	+22–22	46XX
5	SC-295	–13+13	46XX
6	SC-338	–22+22	46XY
7	SC-340	+22–22	46XX
8	SC-345	–21+21	46XX

PGD = preimplantation genetic diagnosis.
No. of blastocysts = 37.

Table 4. Success rate for the generation of human embryonic stem cell (hESC) lines from group II embryos.

	Balanced by polar body analysis
No. of karyotypically abnormal blastocysts	37
No. of hESC lines	8
No. of euploid hESC lines	8
Success rate per blastocyst	
No. of hESC lines (%)	8/37 (21.6)
Euploid hESC lines (%)	100

Table 5. Embryos with normal polar body 1 and 2, blastomere abnormal.

No.	*Stem cell line*	*Result of PGD*	*Karyotype*
1	SC-155	+13	46XX
2	SC-159	polyploid	46XX
3	SC-206	XYY	46XY
4	SC-209	+21	46XY
5	SC-220	+22	46XX
6	SC-270	+21	46XY/49XXY+12+15
7	SC-297	+21	46XX
8	SC-325	–18–21–22	46XX
9	SC-336	Haploid	46XX

PGD = preimplantation genetic diagnosis.
No. of blastocysts = 99; trisomy = 35; monosomy = 47; multiple abnormalities = 17.

Table 6. Success rate for the generation of human embryonic stem cell (hESC) lines from group III embryos.

	Polar body 1+2 normal, blastomere errors			*Total*
	Trisomy	*Monosomy*	*Multiple errors*	
No. of blastocysts	35	47	17	99
No. of hESC lines	6	1	2	9
No. of euploid hESC	5	1	2	8
Success rate per blastocyst				
No. of hESC lines (%)	6/35 (17.1)	1/47 (2.1)	2/17 (11.8)	9/99 (9.1)
Euploid hESC lines (%)	83	100	100	89

Table 7. Embryos abnormal by blastomere analysis only.

No.	*Stem cell line*	*Result of PGD*	*Karyotype*
1	SC-183	+13	46XX
2	SC-185	XO	46XY
3	SC-199	+22	46XY
4	SC-240	+15	46XY
5	SC-243	–21	46XX
6	SC-245	XXY	47XXY
7	SC-253	–11	46XX
8	SC-269	+21	46XX
9	SC-284	XO	46XX
10	SC-285	-13	46XX
11	SC-302	+21	46XX
12	SC-300	XO	45XO
13	SC-331	+13+21	46XY
14	SC-334	XO	46XX
15	SC-335	+21	46XX
16	SC-337	XO	46XX
17	SC-354	+18	46XY

PGD = preimplantation genetic diagnosis.
No. of blastocysts = 139; trisomy = 44; monosomy = 55; multiple abnormalities = 40

Table 8. Success rate for the generation of human embryonic stem cell (hESC) lines from group IV embryos.

	Blastomere errors, no polar body diagnosis			*Total*
	Trisomy	*Monosomy*	*Multiple errors*	
No. of blastocysts	44	55	40	139
No. of hESC lines	8	8	1	17
No. of euploid hESC	7	7	1	15
Success rate per blastocyst				
No. of hESC lines (%)	8/44 (18.2)	8/55 (14.5)	1/40 (2.5)	17/139 (12.2)
Euploid hESC lines (%)	88	88	100	88

normal, two of which carried the same sex chromosomal error as diagnosed in the embryo (stem cell line SC-245 and SC-300) and one presenting with a different anomaly to the original PGD analysis (SC-270).

All in all, the plating efficiency for hESC originating from blastocysts carrying a proven meiotic error was 1.2% and differed statistically significantly ($P < 0.01$) from the plating efficiency for hESC originating from blastocysts with balanced meiotic errors (21.6%) or mitotic errors [after sequential testing [(9.1%]) and after only blastomere testing [(12.2%)]. Groups II–IV did not differ statistically significant in terms of plating efficiency.

Discussion

hESC self-renew perpetually in culture, maintaining an undifferentiated phenotype and normal karyotype when cultured in appropriate conditions and are capable of developing into all three primary germ layer derivatives (ectoderm, mesoderm and endoderm, both *in vitro* and *in vivo*) (Draper and Andrews, 2002).

To date, blastocysts used for hESC derivation have been obtained from donated normal embryos (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Lanzendorf *et al.*, 2001; Amit and Itskovitz-Eldor, 2002) or from poor quality discarded embryos (Mitalipova *et al.*, 2003). Furthermore, it was shown that aneuploid embryos can be used as a source for the derivation of aneuploid hESC lines for research purposes (Verlinsky *et al.*, 2005b).

This study demonstrates that, depending on the origin of aneuploidy (pre-zygotic and thus meiotic non-disjunction errors, balanced meiotic errors or post-zygotic and thus non-disjunction errors in mitosis), resulting hESC may either be stable euploid or maintain their initial chromosome defect with very low plating efficiency, such as those that are derived from the embryos originating from zygotes with MI or MII errors.

It was previously shown that a mononuclear zygote could produce an hESC line with normal diploid karyotype (46,XX) (Suss-Toby *et al.,* 2004). The authors speculate that a regular fertilization event formed asynchronous pronuclei resulting in a normal blastocyst and subsequently euploid hESC line. In addition, karyotypically normal hESC lines may be produced, in addition to the abnormal ones, from the embryos with chromosomal abnormalities detected by PGD based on blastomere biopsy (Peura *et al.,* 2008). None of the aneuploid lines presented the same anomaly as the original PGD analysis. The authors speculate that the underlying mosaicism with an emerging dominance of chromosomally normal cells lead to their findings of the production of normal stem cell lines from chromosomally abnormal embryos.

The data suggest that errors in MI or MII may not be corrected in the post-zygotic development of the embryo (Kuliev and Verlinsky, 2004), which points to a pre-zygotic origin of the majority of the embryo chromosome abnormalities. The low efficiency of establishing hESC lines from such embryos with MI and/or MII error (4.0%) compared with embryos from group III (9.1%) and IV (12.2%) (post-zygotic mitotic non-disjunction errors) further demonstrates that post-zygotic errors might not represent constitutional abnormalities or be more easily corrected in the preimplantation development, such as a result of 'aneuploidy rescue' (as the majority of the resulting hESC lines had a normal karyotype), seeming altogether more viable.

The one abnormal hESC line from group III (SC-270: 46XY/49XXY+12+15) had a different abnormality than the originally tested blastomere (trisomy 21). This is not surprising, as it has been shown recently that aneuploid lines do not have to present the same anomaly as the original PGD analysis (only blastomere diagnosis) (Peura *et al.,* 2008).

The two abnormal cell lines from group IV (SC-245: 47XXY and SC-300: 45XO) most probably originated in the male meiosis during spermatogenesis (with a trivalent or X univalent/YY bivalent configuration in pachytene) (Palmer *et al.,* 1990). Less likely is fertilization of an oocyte with X-chromosome non-disjunction in the first meiotic division with a normal spermatozoon. This is a rare event occurring in only 2.4% of cases, and MI/MII errors may be corrected only in about one-third of zygotes through 'aneuploidy rescue'.

Only those hESC lines that had an ambiguous trisomy result, which are usually not transferred, or possible monosomy due to technical artefacts such as signal overlap, loss of nuclear material during fixation, or poor hybridization, had normal karyotypes.

Furthermore, those embryos that had a balanced chromosome abnormality after sequential errors in the first and second meiotic division generated hESC lines efficiently (21.6%), comparable with the success rate of 20–25% using chromosomally normal embryos (Verlinsky *et al.,* 2006). However, as shown earlier (Kuliev *et al.* 2005), only approximately 32.5% of all types of sequential errors in MI and MII are rescued, leaving the majority of oocytes still aneuploid.

It is clear that classical karyotype analysis is not fully informative because cryptic disorders (microdeletion or microduplication syndromes) cannot be excluded and microarrays for genome investigations might be included in future studies.

Taken together, the results imply that aneuploidies originating from meiotic non-disjunction have a greater impact on cells of human embryos than those resulting from mitotic non-disjunction.

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