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Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts *in vitro*

M.C.Magli[1], G.M.Jones[2], L.Gras[3], L.Gianaroli[1], I.Korman[4] and A.O.Trounson[2,5]

[1]SISMER, Reproductive Medicine Unit, Via Mazzini, 12, 40138, Bologna, Italy, [2]Centre for Early Human Development, Monash Institute of Reproduction and Development, Monash University, 27–31 Wright Street, Clayton, Victoria, [3]Monash IVF, Monash Private Surgical Hospital, Clayton Road, Clayton, Victoria and [4]Monash IVF Gold Coast, Allamanda Medical Centre, Southport, Queensland, Australia

[5]To whom correspondence should be addressed at: Centre for Early Human Development, Monash Institute of Reproduction and Development, Monash University, 27–31 Wright Street, Clayton, Victoria, Australia 3168.
E-mail: alan.trounson@med.monash.edu.au

In all, 143 human embryos obtained 3 days (day 3) after insemination or intracytoplasmic sperm injection (ICSI) were biopsied and a single nucleated cell removed for identification of aneuploidy by fluorescent in-situ hybridization (FISH) for chromosomes X, Y, 13, 16, 18 and 21. Fifty-one per cent of embryos were aneuploid and significantly more aneuploid embryos blocked in further development to morulae and blastocysts than euploid embryos (59 versus 34%; $P < 0.001$). Chromosomal analysis of the generated blastocysts revealed 40% were aneuploid (16 of 40 generated blastocysts). Re-examination of cells by FISH for the same chromosome probes of the inner cell mass (ICM) of expanded and hatching blastocysts derived from the aneuploid embryos revealed a high incidence of mosaicism of ICM cell lineages that were usually predictable from observations of day 3 single-cell biopsies. These data would not support the hypothesis of a preferential allocation of euploid cells to the ICM and aneuploid cells to the trophectoderm. A high concordance between day 3 aneuploidy diagnosis and ICM cell lineages was observed with trisomies (97%), and multiple complex chromosome numerical abnormalities (100%). A reduced concordance was observed with monosomies (65%) and haploidy (18%). Concomitantly, the proportion of ICM cell lineages was increased in blastocysts whose chromosomal condition was diagnosed as haploid (21%) or with complex numerical abnormalities (50%).

Key words: aneuploidy/blastocyst/chromosomal mosaicism/embryo biopsy/inner cell mass cells

Introduction

Variable morphology associated with different rates of cleavage and degrees of fragmentation are characteristic of human preimplantation embryos (Trounson, 1983; Bolton *et al*., 1989; Dorkras *et al*, 1993; Hardy, 1994; Alikani *et al*., 1999). However, it is not clear whether this is due to fertilization and culture *in vitro*, to follicular stimulation with high doses of gonadotrophins, or if it is a specific human embryo characteristic. In any case, the detrimental effects associated with fragmentation, and delayed or arrested cleavage, could substantially contribute to the reduced viability that characterizes the human embryo. This hypothesis has been confirmed by recent studies which report a strong correlation between slow cleavage rate of embryos and their chromosomal normality (Munné *et al*., 1995; Magli *et al*., 1998). Therefore, it has been recommended that culturing human embryos to the blastocyst stage instead of early cleavage stages will enable the selection and identification of healthy, chromosomally normal embryos endowed with a high potential for implantation (Janny and Ménézo, 1996; Jones *et al*., 1998a). If this is true, a mechanism of natural selection may operate during preimplantation development which eliminates abnormal embryos or selectively allocates aneuploid cells to the trophectoderm (TE) and euploid cells to the inner cell mass (ICM) (Hardy *et al*., 1989). This mechanism could become active at the time when a clear polarity arises in the human embryo. It has recently been reported (Evsikov and Verlinsky 1998) that, after fluorescent in-situ hybridization (FISH) analysis for the chromosomes 13, 18 and 21 of human blastocysts, mosaicism is present in the ICM. This observation would not support the hypothesis of a selective or preferential allocation of euploid cells in the ICM.

The present study was carried out with the aim of verifying the chromosomal status of the cells forming the ICM in blastocysts resulting from embryos identified as aneuploid at the 6–9-cell stage on the third day (day 3) after insemination. Blastocysts were obtained from patients, the majority of whom had a poor prognosis for pregnancy which has been demonstrated to yield 50–75% chromosomally abnormal embryos (Gianaroli *et al*., 1997). Following preimplantation genetic diagnosis (PGD) of aneuploidy on day 3 for chromosomes X, Y, 13, 16, 18 and 21, the resulting euploid, morphologically normal blastocysts, were either transferred or cryopreserved, whereas those blastocysts classified as chromosomally abnormal were reanalysed by FISH after immunosurgical isolation of the ICM. Data expected from the chromosomal analysis using a six chromosome panel of FISH probes were: (i) to assess the rate of mosaicism in the ICM; and (ii) to define the correspondence of ICM genotype with single cell diagnosis of aneuploid 6–9-cell embryos.

Materials and methods

Patients

In August 1998, 25 patients underwent induction of multiple follicular growth for infertility with PGD for aneuploidy at the Allamanda

Medical Center (Southport, Queensland, Australia). Indications for the study were; maternal age ⩾36 years (n = 12; mean 39, range 36–45 years), repeated IVF failures (n = 1): polycystic ovarian syndrome (PCOS) (n = 5) and idiopathic infertility (n =7; mean age 31, range 25–34 years). Ovarian stimulation was accomplished by exogenous gonadotrophin administration following a desensitization protocol with long-acting GnRH analogues (Jones *et al*., 1998b). At ~36 h after injection of human chorionic gonadotrophin (HCG), oocytes were retrieved transvaginally by ultrasound guidance and incubated in IVF medium overlaid with mineral oil (Scandinavian IVF Science AB, Göteborg, Sweden) in a 5% CO_2, 5% O_2, 90% N_2 humidified atmosphere at 37°C. Culture media and conditions were those previously described (Jones *et al*., 1998b). Oocytes were fertilized by ICSI or by brief exposure to spermatozoa for IVF (Gianaroli *et al*., 1996), depending on sperm sample parameters.

Fertilization and embryo development assessment

Oocytes were checked at 14–18 h after insemination for the presence of pronuclei and polar bodies. Fertilized oocytes with two pronuclei were cultured individually and scored 48 h later for the number and appearance of nuclei and blastomeres, and the degree of fragmentation was recorded (Alikani *et al*., 1999). Day 3 embryos with >5 cells and ⩽40% of fragmented cells were selected for embryo biopsy. After blastomere removal, embryos were transferred to G2 medium (Barnes *et al*., 1995) and cultured for two additional days. On the morning of day 5, embryos were scored and transferred to freshly prepared G2 medium. Further observations were performed the following day to evaluate blastocyst formation and morphology (Jones *et al*., 1998a).

Blastomere biopsy and FISH

Day 3 embryos selected for FISH analysis were manipulated individually in HEPES-buffered medium (Scandinavian IVF Science) overlaid with pre-equilibrated mineral oil. A breach of 20–22 μm was opened in the zona pellucida with acidic Tyrode's solution and a nucleated blastomere was gently aspirated by a polished glass needle (40 μm diameter). The biopsied embryo was carefully washed and returned to culture. The removed cell was left in the micromanipulation dish at room temperature. When the biopsy procedure was completed for all the embryos, the removed blastomeres were transferred to a hypotonic solution (1% sodium citrate), the nuclei were fixed on a glass slide using methanol–acetic acid 3:1, and dehydrated sequentially in 70%, 85% and 100% ethanol. Six DNA probes were used for the simultaneous detection of chromosomes X, Y, 13, 16, 18 and 21 which were labelled as follows: chromosome X with Spectrum Aqua, chromosome Y with Spectrum Aqua and Spectrum Green, chromosome 13 with Spectrum Orange and Spectrum Green, chromosome 16 with Spectrum Green, chromosome 18 with Spectrum Aqua and Spectrum Orange, and chromosome 21 with Spectrum Orange. Ten microlitres of the hybridization solution was applied to the fixed nuclei, denatured for 5 min at 73°C and left to hybridize for 4 h at 37°C in a moist chamber. After washing in 0.4×saline sodium citrate at 71°C for 2 min, DAPI (4′6-diamidino-2-phenylindole) in antifade solution was added and fluorescence was evaluated with a Leica microscope (Leitz, Wetzlar, Germany) at ×60 magnification, equipped with a triple band pass filter for the simultaneous observation of the Spectum Aqua, Spectrum Orange, and Spectrum Green signals. The X chromosome-specific signal appeared as blue, Y as white–yellow, 13 as orange, 16 as green, 18 as pink, and 21 as red.

Blastocyst immunosurgery

All the embryos which were diagnosed as chromosomally abnormal were cultured to day 5 or 6 to form blastocysts. Following the removal of the zona pellucida with 0.2% pronase (Sigma, St Louis, MO, USA) the ICM was isolated from fully expanded and hatching blastocysts by lysing the TE cells using a complement mediated reaction. Briefly, blastocysts were individually exposed to integrin β3 (N-20) affinity-purified goat polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. After extensive washings, the blastocysts were transferred to 10 μl droplets of baby rabbit complement-(Serotec Ltd, Oxford, UK) for 60–120 min at 4°C. The ICM were separated from the lysed TE cells and transferred to Ca^{2+}–Mg^{2+}-free medium (Scandinavian IVF Science AB). Cell spreading was accomplished by gentle pipetting with a polished glass capillary in hypotonic solution. The separated cells were fixed on a glass slide with methanol–acetic acid (3:1). Dehydration and FISH were performed as previously described.

Results

A total of 143 6–9-cell embryos derived from two-pronucleate zygotes was analysed by FISH on day 3. Seventy embryos (49%) had the correct complement of chromosomes and 73 embryos were identified as aneuploid (51%). Of the 70 euploid embryos 21 developed to morulae and 24 to blastocysts on days 5–6. Of the 73 aneuploid embryos 14 developed to morulae and 16 to blastocysts on days 5–6. In all, 40% of the resulting blastocysts were chromosomally abnormal (16 of 40 generated blastocysts). Aneuploid embryos had a significantly reduced capacity to develop to morulae and blastocysts on days 5–6 (59% of aneuploid day 3 embryos blocked in development compared to 36% of euploid day 3 embryos; $\chi^2 = 9.52$, $P < 0.001$).

Of the 16 aneuploid embryos that developed to expanded and hatching blastocysts on day 6, 15 were treated by immunosurgery. Another two blastocysts that developed from non-biopsied embryos (and were <6-cell and fragmented on day 3) and one euploid embryo were treated by immunosurgery and the ICM hybridized to FISH probes. On day 3, four embryos were trisomic, five were monosomic, one was haploid, five were complex (numerous abnormalities) and one was euploid. Two other embryos were not biopsied on day 3. The total number of cells analysed in the 18 blastocysts was 303 (16.8 ± 13.5 for each blastocyst). Fourteen blastocysts had five or more cells analysed (20.9 ± 12.5 for each blastocyst).

Table I summarizes the data obtained from FISH analysis of ICM cells of each blastocyst in relation to the diagnosis on day 3 and morphological evaluation on day 6. In all, 303 cells were examined by FISH with an average of 16.8 cells per embryo and a range of 1–46. In 14 embryos, the number of cells with a chromosomal diagnosis was at ⩾5. In the remaining four embryos, the low number of cells analysed reflected either the presence of a reduced ICM or failure to screen clearly the fluorescent signals, mainly due to cell clumping. No correlation was established between embryo morphology, chromosomal status, and number of cells that constituted the ICM.

Only in one case did all the analysed cells showed the same chromosomal complement (embryo no. 9), whereas in the remaining embryos mosaicism was represented by two to 16 different cell lineages. As summarized in Table II, euploid cells were present in the ICM of seven blastocysts (nos. 4, 6, 7, 10, 13, 14 and 17) in different proportions varying from

Table I. Fluorescent in-situ hybridization (FISH) results for inner cell mass (ICM) cells of human blastocysts

Embryo code	Blastocyst morphology	PGD on day 3	No. of ICM cells	No. of analysed cells	No. of cells with same genotype[a] (%)	No. of cell lines in ICM	No. of normal cells (%)	No. of monosomic cells	No. of trisomic cells	No. of complex abnormal	No. of haploid/ polyploid
Aneuploid day 3											
1	Hatched	Trisomy 21	40	32	31 (97)	3	0	1	31	0	0
2	Hatched	Trisomy 13	28	18	17 (94)	2	0	1	17	0	0
3	Expanding	Complex abn.	14	14	1 (7)	6	0	0	1	13	0
4	Hatching	Complex abn.	22	12	4 (33)	3	1 (8)	7[b]	0	4	0
5	Hatching	Monosomy 13	36	22	18 (91)	3	3	19[d]	0	0	0
6	Hatching	Monosomy 18	62	46	22 (48)	2	24 (52)	22	0	0	0
7	Hatching	Monosomy X,18	24	9	5 (56)	4	2 (40)	6	0	1	0
8	With discarded	Mosaic/ multinucleated cells	5	5	0	5	0	0	0	5	0
9	Hatching	Trisomy 13	24	10	10 (100)	1	0	0	10	0	0
10	Expanded	Complex abn.	23	3	1 (33)	3	1 (33)	0	0	1	1
11	Hatched	Haploid	44	33	6 (18)	7	0	12	0	13	8
12	Hatching	Monosomy 18	13	5	3 (60)	3	0	4	0	1	0
15	Hatching	Complex abn.	31	25	4 (16)	14	0	4	8	13	0
17	Collapsed	Nullisomy 13	39	29	23 (79)	5	1 (3)	27[c]	0	0	1
18	Expanded	Trisomy 13	16	2	0	2	0	0	0	1	1
Euploid or not examined day 3											
13	Expanding	Not biopsied	47	33	–	16	14 (42)	7	1	7	4
14	Expanding	Normal	10	4	1 (25)	4	1 (25)	0	1	2	0
16	Expanded	Not biopsied	5	1	–	–	–	1	0	0	0

[a]As assessed by PGD on day 3 embryos.
[b]Seven cells with nullisomy XY.
[c]23 cells with nullisomy 13.
[d]1 cell with nullisomy 13.
PGD = preimplantation diagnosis; abn. = abnormality.

Table II. Mosaicism in inner cell mass cells; presence of euploid cell lineages

Embryo code	PGD on day 3	No. of diagnosed cells	No. of cell lineages	No. of euploid cells	No. of aneuploid cells	Chromosomal abnormality
4	XO 2(13) 2(16) 1(18) 3(21)	12	3	1	4	Complex
					7	Nullisomy X,Y
6	XX 2(13) 2(16) 1(18) 2(21)	46	2	24	22	Monosomy 18
7	XO 2(13) 2(16) 1(18) 2(21)	9	4	2	5	Monosomy X,18
					1	Monosomy 18
					1	Complex
10	X,Y 1(13) 1(16) 1(18) 1(21)	3	3	1	1	Complex
					1	Triploid
13	Not biopsied	33	16	14	3	Monosomy Y
					2	Monosomy X
					1	Monosomy 13
					1	Monosomy Y,21
					1	XX, Y
					1	Haploid
					1	Triploid
					2	Tetraploid
					7	Complex
14	XX 2(13) 2(16) 2(18) 2(21)	4	4	1	1	XXX
					2	Complex
17	X, Y 0(13) 2(16) 2(18) 2(21)	29	3	1	23	Nullisomy 13
					2	Monosomy 13
					1	Haploid
					2	Complex

PGD = preimplantation genetic diagnosis.

8% to 52%. The chromosomal constitution of the abnormal cells is also shown in Table II.

Finally, the results obtained from the chromosomal screening of the ICM cells have been related to the diagnosis of the single cell that was biopsied from the embryos on day 3. Blastocysts with <5 cells diagnosed after immunosurgery and FISH were arbitrarily excluded from this analysis (nos. 10, 14, 16 and 18), as well as embryo 13 which was not selected

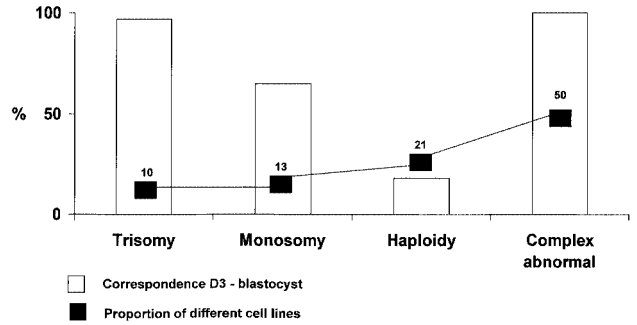

Figure 1. Correspondence between fluorescent in-situ hybridization (FISH) analysis of blastocyst inner cell mass (ICM) and FISH diagnosis performed on a day 3 (D3) biopsied cell. The bars indicate the percentage concordance between the day 3 diagnosis and the abnormality present in the ICM. The line joins the percentage variation in cell lineage in the ICM of blastocysts from that expected from day 3 diagnosis.

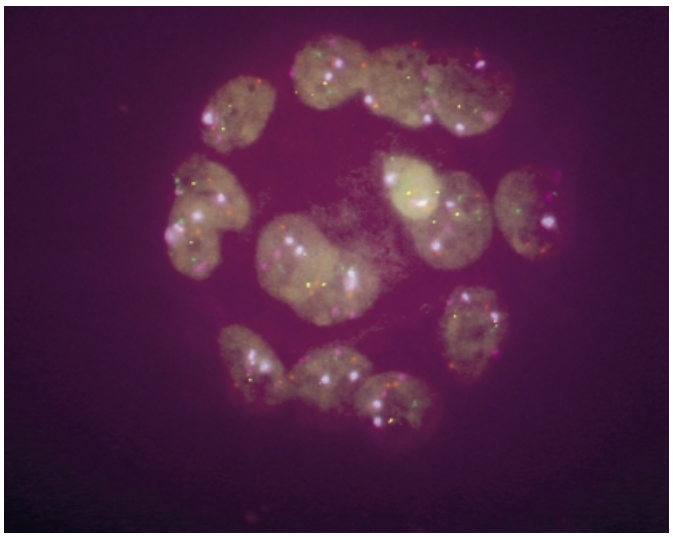

Figure 2. Fluorescence in-situ hybridization (FISH) analysis of the inner cell mass for the chromosomes X (light blue), 13 (yellow), 16 (green), 18 (pink) and 21 (red). The inner cell mass was isolated from an expanded, hatching blastocyst that had been diagnosed as monosomic for chromosome 13 after analysis on single blastomere performed on day 3. All the cells demonstrated to be diploid for the chromosomes X, 16, 18, and 21, and monosomic for chromosome 13 with the exception of 4 cells which were disomic ($n = 3$) and nullisomic ($n = 1$) for chromosome 13. This could suggest an altered mitosis in a few monosomic cells leading to unbalanced chromosomal division. The progression of this phenomenon in the following cleavage events, could be one of the causes of failed implantation associated to monosomic embryos.

for blastomere biopsy on day 3 because of its slow cleavage rate. The results, presented in Figure 1, show that the correspondence with day 3 diagnosis was 97% for trisomies (range 94–100%), 65% for monosomies (range 48–91%), 18% for haploidy (only one embryo analysed), and 100% for embryos classified as complex abnormal aneuploidies (multiple monosomies, trisomies and nullisomies). Concomitantly, an increase in the proportion of different cell lineages in the ICM, from 10% for trisomies, to 13% for monosomies, 21% for haploidy, and 50% for complex abnormalities was observed.

Figure 2 shows the FISH results of the inner cell mass from a blastocyst diagnosed as monosomic for chromosome 13 (embryo no. 5). All the analysed cells exhibited one signal for chromosome 13, with the exception of 3 cells which were disomic and 1 that was nullisomic for chromosome 13.

Discussion

Chromosomal abnormalities are responsible for a high proportion of embryonic loss, as already postulated by data from early abortions and animal studies (Magnuson *et al.*, 1985; Plachot *et al.*, 1988; Burgoyne *et al.*, 1991). According to FISH analyses of preimplantation human embryos, an abnormal chromosomal complement is more frequent in slow-cleaving embryos (Munné *et al.*, 1995; Magli *et al.*, 1998). As shown in the present study, aneuploid embryos are more likely to block (59%) in development between day 3 and days 5–6 than those diagnosed as euploid (34%). Although 41% of aneuploid day 3 embryos are still capable of developing further, most of them are destined to experience a further blockage of development and implantation failure. Consequently, culture to the blastocyst stage does identify more developmentally competent embryos than culture to day 2 or 3. This implies that a mechanism of natural selection operates in favour of normal concepti, possibly at two levels. (i) Chromosomally abnormal embryos are eliminated before blastocyst formation or later, prior to or shortly after implantation. This condition would be associated with the presence of an abnormal chromosomal complement in the vast majority of the cells constituting the embryo. (ii) If the number of aneuploid cells does not exceed a certain limit, they are diverted to TE, whereas normal cells are allocated to the ICM (Kaufmann *et al.*, 1995; Janny and Ménézo, 1996). This would imply that the major abnormalities are mostly excluded from the cell lineage that gives rise to the fetus. However, trisomies that originate from post-zygotic mitotic errors support the evidence that aneuploid cells are not strictly confined to TE (Delhanty and Handyside, 1995).

FISH analysis of interphase nuclei obtained from morphologically normal blastocysts have demonstrated that the transition from the morula to the blastocyst stage is critical in terms of starting a negative selection against aneuploid cells (Evsikov and Verlinsky, 1998). According to these authors, the degree of mosaicism observed up until the morula stage is dramatically increased in comparison to that for blastocysts. At this point, a mechanism of self-destruction is postulated to be triggered in concomitance with the first sign of clear functional polarity in the human embryo, when an evident dorsoventral symmetry arises in the blastocyst between TE and ICM cells. On the other hand, studies on 2-cell embryos have reported that TE and ICM lineages diverge very early in cleavage and mosaicism could reflect this polarity (Edwards and Beard, 1997).

The detection of major chromosomal abnormalities, including mosaicism, in in-vitro produced blastocysts has shown that an aberrant genome is not incompatible with blastocyst development. The results reported in the present study showed 34% of euploid embryos developed to blastocysts compared

to 22% for aneuploid embryos. It is apparent from these observations that long-term cultures do not exclusively select embryos with a normal chromosomal complement.

Hence in an unselected population of human blastocysts it could be expected that ~40% (as in the present study) could be chromosomally abnormal. Some of these embryos may develop to term (e.g. trisomy 21) but the majority will be lost prior to, or after implantation. The presence of blastocysts with severe chromosomal abnormalities will limit implantation and pregnancy rate even with blastocyst transfers. Claims of very high implantation and pregnancy rates after blastocyst transfer, without PGD for aneuploidy, need to be treated with caution because they might represent very highly selected patient subgroups where aneuploidy is probably very low (e.g. young women with male factor partners).

After immunosurgery and FISH on ICM cells, the numerical status of chromosomes X, Y, 13, 16, 18 and 21 was assessed in 303 ICM cells from 18 blastocysts. Multiple cell lines were demonstrated in 17 out of the 18 screened ICM. In blastocysts nos. 1, 2, and 5, the degree of mosaicism was very low (3, 6 and 9% respectively) and are probably of minor implication; monosomy was detected in two cells out of 48 (embryos nos. 1 and 2, one cell each), and monosomy and haploidy in two cells out of 22 (embryo no. 5). Similarly, in embryo no. 17, six cells out of 29 exhibited four different types of chromosomal numerical constitution. In four cases, it was due to an extra missing signal, one cell was euploid and the remaining cell was haploid. If we consider that the monosomies detected could be due to loss of micronuclei, the rate of mosaicism is possibly lower.

In the other 11 blastocysts (nos. 16 and 18 are not considered because of the small number of cells analysed by FISH: one and two respectively) the incidence of mosaicism was substantially increased. The highest values were observed in embryos nos. 8, 10 and 14, where all the screened cells appeared to be part of chaotic mosaicism. It must be considered, however, that only five, three and four cells could be diagnosed for each ICM respectively. Therefore, although present, the rate of mosaicism in these embryos could actually be similar to that detected in the remaining blastocysts. More reliable diagnoses were obtained from embryos 11, 13 and 15 which exhibited seven, 16 and 14 different cell lineages in each, out of 33, 33 and 25 screened cells respectively. This clearly demonstrates that an aberrant genome is not detrimental for blastocyst expansion and hatching.

Especially interesting is the observation of the presence of euploid cells in mosaic ICM (Table II). If mosaicism is a natural event that occurs at a low frequency in physiologically normal embryos, the reverse could also be true and result in the development of a normal fetus from the lineage of chromosomally normal cells in a mosaic embryo. Although speculative, this could possibly be related to several factors. Among them, the ratio of euploid to aneuploid cells may need to be below a hypothetical threshold. In this respect, embryo no. 6 represents an intriguing example not only in consideration of its potential development if transferred but also in relation to the reliability inherent to PGD performed on a single cell. This embryo was classified by PGD as monosomic for chromosome 18. The analysis of the ICM cells revealed 24 euploid and 22 monosomic cells. The two cell lines may originate at the first mitosis, and the chances of biopsying on day 3 a normal cell or an abnormal cell would be the same. If two cells had been removed for PGD and the result had been discrepant, the diagnosis of monosomy would probably be considered an error related to the whole procedure (loss of micronuclei during fixation or partially failed hybridization). Similarly embryo no. 13 had 14 normal out of the 33 FISH cells diagnosed. However, in this case the presence of multiple cell lineages was associated with slow cleavage rate to day 3 and probably identified a limited developmental potentiality.

According to the results of the present study, chromosomal analysis of the ICM cells of aneuploid embryos suggests that there is no limit imposed in preimplantation development by the chromosomal constitution for blastocyst formation, expansion and hatching, even in cases where a high degree of mosaicism is present. More data on ICM and trophectoderm cells are necessary to validate this hypothesis. This study has also shown a high concordance of PGD for day 3 embryos and blastocyst genotype. This affirms that the error rate due to the procedure itself (calculated to be ~8%; Gianaroli *et al*., 1999) is indeed low and the type of chromosomal pathology diagnosed at day 3 usually represents the onset of different cell lineages that can be identified in the ICM. In general, the correspondence between the result obtained by PGD at day 3 decreases with an increase of mosaicism in the ICM. It is also important to recognize that multiple FISH probes for the simultaneous screening of six chromosomes enables a more accurate definition of the embryo genotype. As a result, the embryos classified as carriers of complex abnormalities were all confirmed to be mosaic. Trisomies and monosomies were also confirmed with the exception of two monosomies (embryos nos. 6 and 7) where two clearly defined cell lineages, equivalent in size, developed in each embryo (one euploid and the other monosomic). There is no doubt that an increase in the number of chromosomes screened for PGD will more accurately identify the embryonic genotype in human blastocysts.

In conclusion, the chromosomal condition of the cells forming the ICM has been shown to be more heterogeneous than may have been anticipated. Further research is needed for understanding the regulatory mechanisms which determine the allocation of cell lineages within the embryo and trigger self-selection towards embryonic death or survival.

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