Human Reproduction vol.13 no.11 pp.3151–3155, 1998

Mosaicism in the inner cell mass of human blastocysts

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Although mosaicism was shown to be a normal feature in cleaving embryos, its consequences for the late preimplantation stages are unknown. We performed blastocyst immunosurgery, followed by fluorescent in-situ hybridization (FISH), to determine the number of cells and degree of mosaicism in the inner cell mass (ICM) of human blastocysts. Of 47 ICM samples analysed, 20 had aneuploid cells, and two also had a few tetraploid cells. The average degree of aneuploidy in the ICM was similar to the overall blastocyst mosaicism, suggesting that there is probably no selection for euploid ICM. The lower degree of blastocyst mosaicism, compared with the cleavage-stage embryos, may be due to a mechanism of selection against the embryos with high frequency of mosaicism, leading to elimination of these embryos prior to blastocyst formation.

Key words: aneuploidy/FISH/human blastocyst/inner cell mass/mosaicism

Introduction

The development of fluorescent in-situ hybridization (FISH) techniques has provided the means for chromosomal analysis of practically every blastomere in preimplantation human embryos. The results obtained to date have revealed a high degree of mosaicism in human pre-embryos, especially those with abnormal morphology. Techniques of polymerase chain reaction (PCR) and FISH analysis have shown that up to 50% of 4- to 8-cell embryos are mosaics (Munné *et al.*, 1993, 1997; Ishikawa and Endo, 1995; Katagiri and Katayama, 1996; Kligman *et al.*, 1996; Delhanty, 1997; Laverge *et al.*, 1997). Compared with 2% of genetic defects at birth, these numbers suggest a presence of a strong selection against genetically abnormal embryos.

Two distinct cell lineages, inner cell mass (ICM) and trophectoderm (TE), separate at the blastocyst stage. The ICM, although contributing cells to the TE (Fleming *et al.*, 1984), is predetermined to give rise to the embryo proper. Since each blastomere of the early cleavage-stage human embryo can participate in both TE and ICM formation (Mottla *et al.*, 1995), genetically abnormal blastomeres should have the same chances of contributing to the ICM as euploid blastomeres.

It was suggested that only the 'fittest' embryos can reach the blastocyst stage *in vitro*, and therefore would have an increased viability (Edwards and Hollands, 1988; Kaufmann *et al.*, 1995; Janny and Ménézo, 1996). This implies that embryos with chromosomal abnormalities may be capable of cleavage but most of them will eventually be eliminated by the mechanisms of natural selection for normal concepti (Plachot *et al.*, 1987). If a mechanism of self-selection does exist at the preimplantation stage, it is important to determine whether this self-selection operates at the level of the whole embryo by elimination of abnormal embryos, or if selection acts by allocating euploid cells to the ICM in the human pre-embryo.

Using FISH analysis for chromosomes 13, 18 and 21 to determine a degree of mosaicism in human blastocysts, and specifically in the blastocyst ICM, we have been able to demonstrate that during human blastocyst formation there is no selection for a euploid ICM.

Materials and methods

Human preimplantation embryos

The research protocol was approved by IRB of Illinois Masonic Medical Center. We studied the following groups of embryos that would otherwise have been discarded in our in-vitro fertilization (IVF) programme. First, these were genetically abnormal embryos detected in our clinical trial for preimplantation genetic diagnosis. Secondly, there were spare embryos, unsuitable for cryopreservation because of poor morphology or low grading at the cleavage stage. The third group included embryos resulting from zygotes in which an abnormal number of pronuclei had been observed, but which had been proven to be diploid. In relation to patients from whom these embryos were obtained, only those cycles were included for analysis from which at least one embryo had developed into a blastocyst.

Embryos were cultured at 37°C in droplets of human tubal fluid (HTF) medium (prepared in-house using reagents from Sigma, St Louis, MO, USA) with 10% Plasmanate (Bayer Biological, New Haven, CT, USA) under equilibrated mineral oil (Squibb Pharmaceuticals, Princeton, NJ, USA) in 5% CO_2 in air.

Embryo grading in this study reflected both the embryo cleavage stage and the degree of fragmentation. The grade was calculated by multiplying the number of blastomeres by the embryo degree of fragmentation. Degrees of fragmentation used were: 4, no fragmentation; 3, 25% of total embryo volume occupied by cytoplasmic fragments; 2, 50% fragmented; 1, >75% fragmented. For example, a 6-cell embryo with slight (around 10%) fragmentation received a grading of $6 \times 3.5 = 21$.

Immunosurgery and differential labelling of ICM/TE nuclei of human blastocysts

The total number of cells in the blastocysts and overall degree of mosaicism were determined by embryo fixation followed by Giemsa staining or FISH. To isolate the ICM from human blastocysts, the

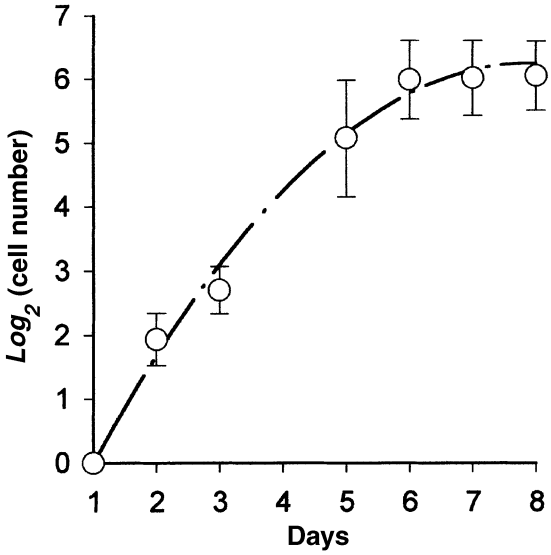

Figure 1. Human embryo cleavage rate *in vitro* expressed as a logarithm of the embryo cell number.

zonae pellucidae were first removed by treating the embryos with 0.5% Pronase (Sigma). In order to remove cytoplasmic fragments and blastomeres, which were excluded from the process of blastocyst formation, zona-free blastocysts were pipetted through a 120–180 μm (depending on the blastocyst diameter) flame-polished pipette. About 1 h later, blastocysts were subjected to immunosurgery according to the technique proposed by Hardy *et al.* (1989). Embryos were first incubated on ice for 10 min in 10 mM picrylsulphonic acid (TNBS) in HTF medium. After a brief wash in three droplets of HTF with 4 mg/ml polyvinlpyrrolidone (PVP) they were incubated for 30 min at 37°C in 0.1 mg/ml anti-DNP BSA (Sigma). After further washing in HTF/PVP, blastocysts were transferred in a 1:10 dilution of guinea pig complement serum (Sigma), containing two specific fluorochromes, propidium iodine (Sigma) and Hoechst 33342 (Sigma) at concentrations of 10 μg/ml each. After 1 h, ICM and lysed TE cells were transferred into a drop of HTF/BSA medium (see Figure 2A). At this stage, a Nikon Diaphot microscope equipped with epifluorescence for Hoechst stain (excitation filter 365 nm, barrier filter 400 nm) was used to count TE nuclei, 'inner cell' nuclei and to estimate the number of cells in the ICM. ICM nuclei and 'inner cells' nuclei appeared blue; nuclei from lysed TE cells were pink (Figure 2B). The separated ICM was transferred to a droplet of 1% Tween 20 in 0.01 N HCl on a poly-L-lysine-coated slide (Sigma). Cell spreading was achieved by gentle blowing. When the droplet

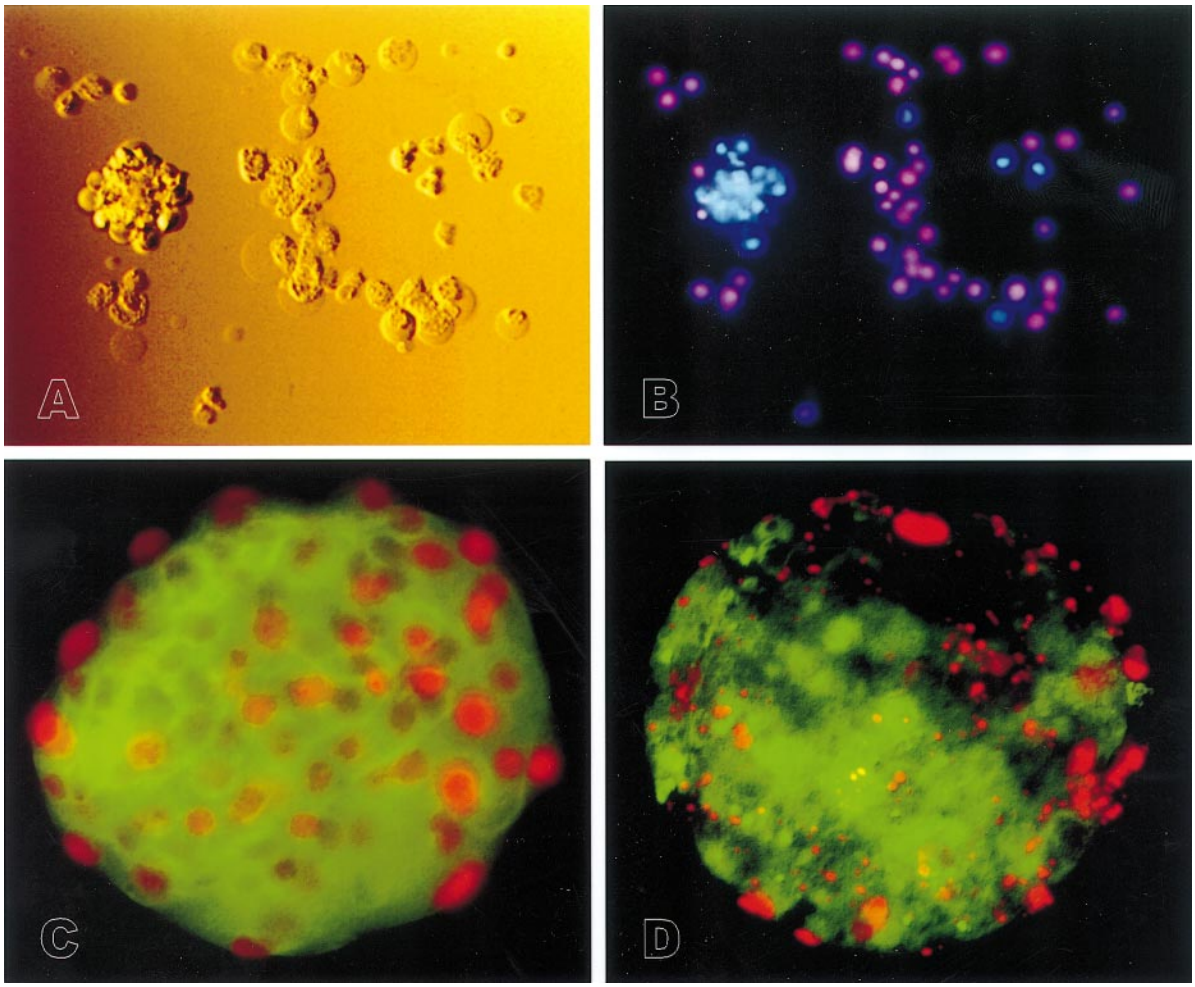

Figure 2. (**A**) Differential interference contrast view of the blastocyst after immunosurgery. (**B**) The same blastocyst, under epifluorescence. Inner cell mass and few 'inner cells' are blue, trophectoderm nuclei are pink. (**C**) Human blastocyst. Nuclei are red, tubulin microfilaments stained green. (**D**) Morphologically normal human blastocyst with abnormal nuclei and cytoskeleton.

had dried out, the slide was flooded a few times with methanol:acetic acid (3:1).

FISH

Commercially available (Vysis, Inc., Downers Grove, IL, USA) directly labelled probes for human chromosomes 13, 18 and 21 were used simultaneously, according to standard procedures for their hybridization and detection (Verlinsky *et al.*, 1996). The signals were registered using Nikon Microphot-MFA microscope and Quips Genetic Imaging Software (Vysis). During signal analysis we followed the scoring criteria set by Munné *et al.* (1994). Because of propidium iodine staining, TE nuclei had an additional fluorescence; thus contamination of ICM by TE cells was easily detected during FISH analysis by the reddish chromatin appearance (see Figure 3A).

Immunocytochemical analysis

The fixation and immunostaining method described by Emerson (1988) was used for tubulin visualization. The only modification of the

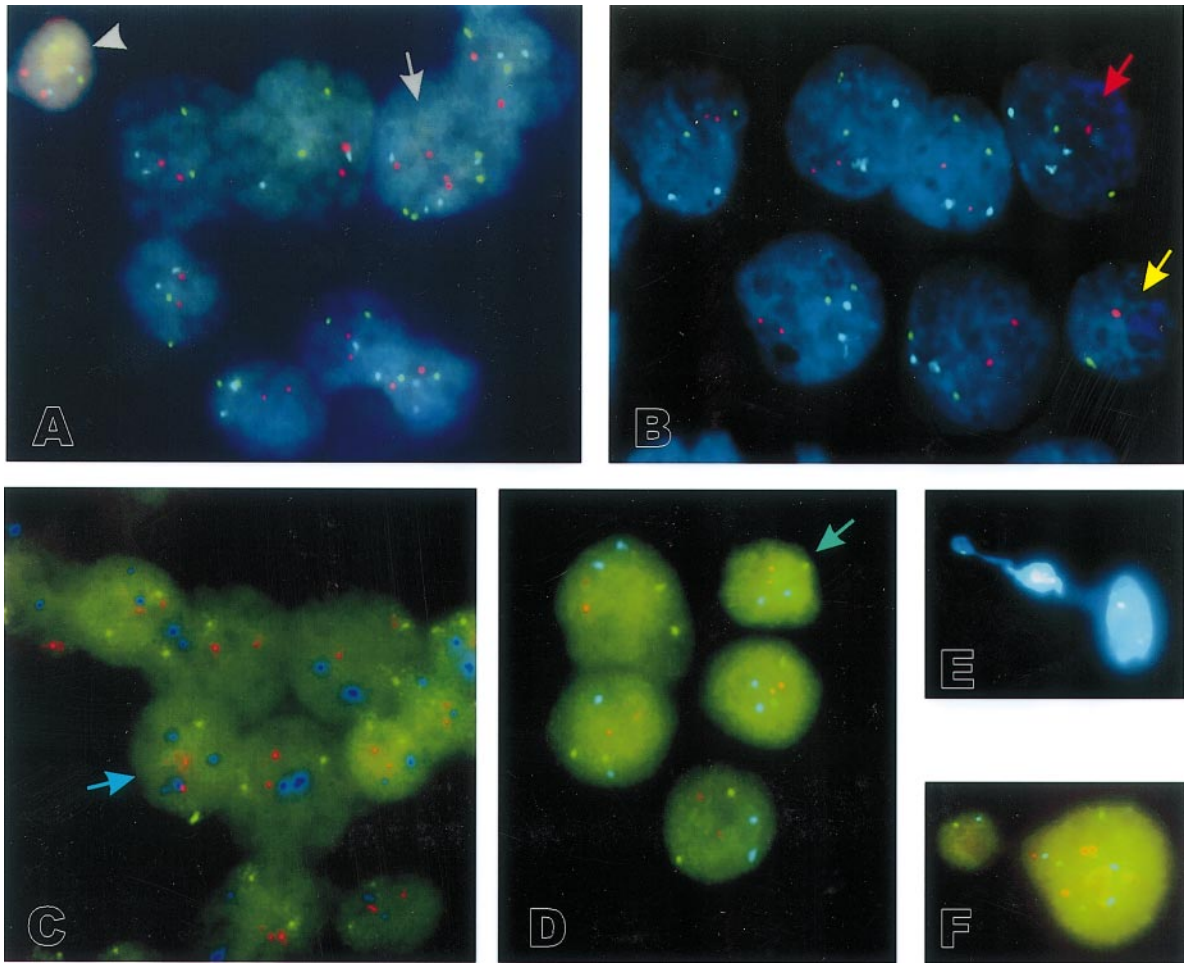

Figure 3. Fluorescent in-situ hybridization (FISH) analysis of the inner cell mass (ICM) (**A**–**E**) and trophoectoderm (TE) (**F**) cells for chromosomes 13 (green), 18 (aqua) and 21 (red). Slides (**A**), (**B**) and (**E**) were counterstained with DAPI for better chromatin visualization. (**A**) Contamination by the trophectoderm cell is easily detected by its reddish appearance (arrowhead). Note one tetraploid cell in the inner cell mass (arrow). (**B**) Monosomy 21 and trisomy 18 (red arrow). Nullisomy 18, monosomies 13 and 21 (yellow arrow). (**C**) Trisomy 18 (blue arrow). (**D**) Monosomy 13 (green arrow). (**E**, **F**) Origin of aneuploidy due to cell constriction or premature cytokinesis in ICM (E) or tetraploid TE (F) cells.

Table I. Mosaicism in overall diploid human blastocysts

Fixation technique	Blastocyst ploidy	Grading on day 3	Total cell number	No. of ICM cells (%)	Aneuploid ICM cells (%)	TE cells analysed[a]	Polyploid TE cells[a] (%)	Aneuploid TE cells[a] (%)
Blastocyst immunosurgery	Euploid ICM (n = 27)	22.9 ± 1.4	64.7 ± 4.4	13.0 ± 1.5 (20.2)	0	715	29 (4.1)	56 (7.8)
	Mosaic ICM (n = 20)	20.2 ± 1.6	61.3 ± 5.2	15.8 ± 1.9 (25.7)	3.3 ± 0.6 (20.1)	457	30 (6.6)	29 (6.4)
	No ICM (n = 2)	11.3 ± 1.3	35.5 ± 0.5	0	–	12	2	1
Whole blastocyst fixation	Euploid (n = 5)	22.1 ± 3.7	55.0 ± 5.8	ND	–	227	11 (4.9)	0
	Mosaic (n = 20)	20.3 ± 1.9	63.9 ± 7.8	ND	–	970	77 (7.9)	127 (13.1)

[a]In the case of the whole blastocyst fixation, TE + ICM cells.
ICM = inner cell mass; ND = not determined; TE = trophoectoderm.

Table II. Cell numbers and mosaicism in human blastocysts (aneuploid and polyploid cells are those deviating from the general type of ploidy)

General type of ploidy	No. of PN	Grading on day3	Stage at fixation	Total cell number	ICM cell number	Aneuploid ICM cells	TE cells analysed[a]	Polyploid TE cells[a]	Aneuploid TE cells[a]
Euploid (n = 74)	0/2	21.1 ± 0.9	Blastocysts	62.1 ± 3.0	13.6 ± 1.2	1.4 ± 0.3	2381	149	213
Chaotic	0	ND	Blastocyst	65	7	7	30	0	30
Haploid	1	ND	Blastocyst	130	ND	–	100	10	0
Triploid	2	8	Blastocyst	44	2	0	15	3	2
Triploid	2	12.5	Blastocyst	64	ND	–	60	0	5
Tetraploid	2	7.5	Hatching blastocyst	34	ND	–	34	2	0
Chaotic	2	12	Blastocyst	105	ND	–	105	0	105
Chaotic	2	14	Expanded blastocyst	53	10	10	40	0	40
Monosomy 18	2	14	Blastocyst	32	ND	–	32	4	5
Trisomy 18	2	14	Blastocyst	72	4	0	40	0	0
Monosomy 21	2	17.5	Blastocyst	50	8	0	42	0	0
Trisomy 21	2	24	Expanded blastocyst	83	ND	–	83	0	9
Tetrasomy 21	2	28	Blastocyst	45	8	0	29	0	0

[a]In the case of the whole blastocyst fixation, TE + ICM cells.
ICM = inner cell mass; ND = not determined; PN = pronuclei; TE = trophoectoderm.

technique was the use of monoclonal anti-α-tubulin FITC conjugate antibodies (Sigma). Embryos were mounted in Vectashield antifade (Vector Labs, Burlingame, CA, USA) with 0.5 μg/ml diamine propidium iodine (DAPI) counterstain, on glass slides with Teflon-bordered wells (12-well; Erie Scientific), under cut coverslips.

Statistical analysis

All results reported are expressed as mean ± standard error. Statistical comparisons of the data were done using Student's *t*-test. The correlation coefficient, *r*, was calculated using Microsoft Excel software.

Results

Figure 1 shows that embryo cleavage rate is almost constant until the sixth round of cleavage. On day 5, blastocysts had on average 49.1 ± 4.8 cells (n = 15). Although at least one day retarded, day 5 morula-stage embryos were considered viable, they had 15.5 ± 1.8 (n = 6) cells. Day 6 blastocysts had 64.3 ± 2.0 cells (n = 187). Seven- to nine-day-old blastocysts had the same number of cells; thus, when combined (n = 35) they gave an average of 64.5 ± 5.1 cells per blastocyst. Since we were interested in the developmental characteristics of the embryos at the most advanced stage, which could be reached *in vitro*, blastocysts were fixed on day 6.

Only morphologically normal blastocysts were fixed for cell counts or FISH analysis. After fixation, the majority of blastocysts had round nuclei of approximately equal size. Immunocytochemical staining of such a blastocyst is shown in Figure 2C. An insignificant proportion of blastocysts, although being morphologically normal, had abnormal-sized and irregular-shaped nuclei. Staining of such blastocysts for tubulin and chromatin also revealed gross abnormalities in the tubulin cytoskeleton (Figure 2D). These blastocysts were completely excluded from further analysis. It should be noted that a significant percentage of blastocysts presented in this study had a small percentage of picnotic or abnormal nuclei. Following immunosurgery, these abnormal cells stained blue and were clearly separated from each other and from the ICM (Figure 2B). We considered these cells to be excluded from the process of blastocyst formation and, wherever possible, they were not counted. Most of these nuclei were picnotic or degenerating and failed to hybridize during FISH analysis.

Blastocyst immunosurgery, followed by FISH analysis for chromosomes 13, 18 and 21, was used to determine the degree of aneuploidy in the ICM. ICM constituted on average 20% of the total blastocyst cell number. The number of cells in the ICM ranged from zero (no ICM, few 'inner cells' in the blastocele cavity) to 35 (65 TE cells). Cell numbers and the degree of mosaicism in overall diploid blastocysts are presented in Table I. As seen in Figure 3, ICM presents all types of aneuploid cells: tetraploidy (Figure 3A), complex aneuploidies in two (sister?) cells (Figure 3B), trisomy 18 (Figure 3C) and monosomy 13 (Figure 3D), as well as nuclei of abnormal shapes (Figure 3E).

Among 74 overall euploid blastocysts, 33 had no aneuploid cells in their ICM, and 15 blastocysts had no aneuploid cells at all. The average percentage of aneuploid cells was 9.1% (278 cells of 3048 analysed), ranging from 0 to 18 cells per blastocyst. Since polyploidization of TE cells is a normal process in blastocyst development, polyploid cells were counted separately. Polyploid (mostly tetraploid) cells constituted 4.9% of the total number of cells (149 of 3048 cells). Out of 47 ICM analysed, 20 had aneuploid cells, and two had a few tetraploid cells. The percentage of mosaicism in ICM containing aneuploid cells constituted 20.1% (65 of 315 cells). The average degree of aneuploidy in the ICM was 9.8% (65 of 667 cells), not different from the overall blastocyst aneuploidy (P = 0.57).

Among the embryos that reached blastocyst stage, there was no correlation between the degree of mosaicism (either total or mosaicism in the ICM) and number of cells in the blastocyst. Day 3 embryo quality also did not correlate with the degree of blastocyst aneuploidy (r = –0.09).

Out of 91 blastocysts analysed, four had turned out to be haploid, triploid and tetraploid, five had mostly aneuploid cells, and three had no predominant type of nuclei and were

marked as chaotic. The results on aneuploid blastocysts, as compared with overall diploid blastocysts, are presented in Table II. Note that 'tetraploid' and 'aneuploid' nuclei in these cases are those, deviating from the general type of aneuploidy, characteristic for that specific blastocyst.

Discussion

Our results showed that there is no selection for euploid ICM: the mosaicism rate in the ICM was similar to the overall blastocyst mosaicism, 10.5%. A similar conclusion, that aneuploid cells are not necessarily diverted to TE, was made by Delhanty and Handyside (1995), based on the fact that a substantial proportion of trisomic fetuses are due to postzygotic mitotic error.

Considering that, until the morula stage, a very high percentage of aneuploid cells is observed in the embryos of poor quality (Bongso *et al.*, 1991; Pellestor *et al.*, 1994; our observations), it may be concluded that cavitation initiates a negative selection against aneuploid cells. If the number of aneuploid cells at the morula stage reaches some threshold level, this leads to the self-elimination of the whole embryo. However, the presence of haploid, polyploid and mostly aneuploid blastocysts (Figure 2D; Table II) indicates that in some cases even the major abnormalities in embryo genome are not detrimental for preimplantation development. It might be speculated that at least two types of developmental anomalies lead to the embryo self-destruction. The first one is based upon some abnormalities in the spindle formation and chromosome segregation. Most probably originating in oogenesis, these abnormalities can be detected by analysis of polar bodies (Verlinsky and Kuliev, 1993; Verlinsky *et al.*, 1996). The other type of developmental arrest seems to originate during embryo cleavage and is based upon mitotic errors and faults in nucleocytoplasmic interactions. This latter case seems to be especially characteristic for human pre-embryos. During first-cleavage divisions, an asynchrony between karyokinesis and cytokinesis leads to embryo fragmentation and origination of multinucleate blastomeres. Premature cytokinesis may 'divide' an interphase nucleus, even at the blastocyst stage. A case of the ICM 'pulled' nucleus and trophectoderm tetraploid nucleus 'division' is depicted in Figure 2E and F.

In general, our results show that, compared with the early cleavage-stage embryos, human blastocysts have a significantly lower degree of mosaicism. This indicates that, at least under suboptimal in-vitro conditions, there is a selection against aneuploid cells. According to our results on cleavage-stage and morula-stage embryos, selection starts at the morula–blastocyst transition. If the number of abnormal cells at the morula stage reaches some threshold level, this leads to whole-embryo degeneration. Taking into account that an average number of aneuploid cells per blastocyst was five, and even presuming that at the morula–blastocyst transition aneuploid cells do not cleave, this renders 5–10 cells to be the maximum number of abnormal cells in a 30-cell morula. It should be noted, however, that the presence of blastocysts with mostly abnormal and aneuploid nuclei shows that even major genetic abnormalities do not necessarily affect preimplantation development. At present, we are investigating the possibility that autonomous cytoplasmic activity, found in mouse (Waksmundzka *et al.*, 1984; Evsikov and Solomko, 1994) and in lower species, may affect the process of human blastocyst development.

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Received on February 23, 1998; accepted on August 6, 1998