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Chromosome topology in normal and aneuploid blastomeres from human embryos

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Objectives To find whether chromosomes 13, 16, 18, 21, 22, X and Y in blastomeres of human embryos are nonrandomly localized, whether their aneuploidy affects their localization and if eventual early inactivation of chromosome X with peripheral localization is present.

Methods Relative distances from the nucleus center and edge of 1198 fluorescence *in situ* hybridization signals in 98 human blastomeres were measured in digital images for comparison with a mathematical model of random distribution in spherical nucleus.

Results Comparison with the mathematical model revealed that localization of chromosomes 13, 16, 21, 22, X and Y in normal and aneuploid blastomeres and that of chromosome 18 in normal blastomeres was not significantly different from random distribution. Similarly, chromosome X in blastomeres with more than one X did not appear to have a preferential localization. Only chromosome 18 in aneuploid blastomeres was differently distributed ($p < 0.0001$) with a shift to the nuclear periphery ($p = <0.0001$).

Conclusions Peripheral localization of chromosome 18 in aneuploid blastomeres is related to embryo aneuploidy. Conversely, a peripheral localization of the inactive X chromosome was not found in blastomeres from 3-4 day old embryos. These results open the possibility to improve embryo selection after pre-implantation diagnosis. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: chromosome topology; blastomeres; aneuploidy

INTRODUCTION

Chromosomes in nuclei of mammalian cells occupy discrete areas (chromosome territories—CT), reviewed by Cremer and Cremer (2001). The localization of CTs in the nucleus depends on chromosome size (Sun *et al*., 2000) and gene content (Boyle *et al*., 2001; Kreth *et al*., 2004). Smaller chromosomes are preferentially localized in the center of the nucleus while larger chromosomes are preferentially localized at the periphery. Chromosomes with a high gene density (e.g., chromosome 19) are localized in the interior of the nucleus, whereas chromosomes with a comparatively lower gene content (e.g., chromosome 18) are localized at the nuclear periphery. This arrangement is evolutionary conserved among birds and humans (Habermann *et al*., 2001). In tumour cells the correlation of gene density and radial chromosome arrangement is also present, but it is less pronounced (Cremer *et al*., 2003) suggesting a link between the chromosome localization and the malignancy.

The CT topology also depends on cell type, developmental stage and gene expression activity. The cell nucleus shape plays an important role (Cremer *et al*., 2001). In spherical nuclei of human lymphocytes from the postnatal period the chromosomes are arranged according to the gene content, with chromosome 18 at the periphery. In flat ellipsoid nuclei of human amniotic fluid cells and human fibroblasts from the postnatal period the arrangement according to the chromosome size prevails with the chromosome 18 located in the center of the nucleus. However in these flat nuclei, even the chromosomes in the center are in contact with the nuclear envelope (Cremer *et al*., 2001).

The localization of CT is dynamic during cell cycle. In quiescent cells from different human tissues, the centromeres are localized at the periphery, while in cycling cells a large fraction of centromeres is located in the interior in the early G1 phase and moves to the periphery in later stages (Solovei *et al*., 2004). In proliferating human fibroblasts the chromosome 18 is localized at the nuclear periphery, but it moves to the center upon cell cycle exit into G0 phase (Bridger *et al*., 2000).

An important example of functional significance of chromosome localization is the X inactivation in placental mammals. One of two X chromosomes in female cells is inactive and forms the late replicating and highly condensed sex chromatin. The inactive X differs from the active X also in histone methylation, histone acetylation and DNA methylation patterns (Chow and Brown, 2003) as well as in the localization and shape. The inactive X is typically localized at the nucleus periphery (Dyer *et al*.,

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1989). The exact time onset of X-inactivation characterized by peripheral localization in early human embryos is unknown.

A study of chromosomes 13, 16, 18, 21, 22, X and Y in human blastomeres (McKenzie *et al.*, 2004) found that in aneuploid nuclei more chromosomes are at the periphery than in normal nuclei and that chromosomes 13, 18, 21 and 22 are at the periphery, if monosomy or trisomy of these chromosomes is present. We have independently developed a method of fluorescence *in situ* hybridization (FISH) signal position measurement based on digital image analysis and comparisons with the mathematical model. Our previous study of localization of chromosomes 18 and X only in nuclei of human blastomeres (Diblik *et al.*, 2005) found that the chromosome 18 is shifted to the periphery of aneuploid blastomere nuclei.

The aim of this study was to improve the signal localization measurement by measuring both centers and peripheral edges of the signals. Furthermore, we have increased the number of analyzed chromosomes by adding chromosomes 13, 16, 21 and 22 in order to test whether the localization of these chromosomes is also influenced by aneuploidy.

MATERIALS AND METHODS

Patients and samples

Preimplantation genetic screening (PGS) for aneuploidy of chromosomes 13, 16, 18, 21, 22, X and Y was performed in 204 blastomeres from 32 IVF cycles of 28 patient couples with 4 couples having two cycles. Median female age at the time of oocyte pick up was 32, in the range 22–40 years.

As only blastomeres with clear FISH signals, without multinucleation, fragmentation of nuclei or FISH signals or damage were selected for the analysis, only 98 blastomeres with 1198 FISH signals from 22 IVF cycles from 20 couples were included in this study. Indications included advanced maternal age (6/22 cycles), repeated IVF failures (6/22), repeated miscarriages (3/22), previous pregnancy with aneuploid fetus (1/22), immunological infertility with presence of autoantibodies and repeated assisted reproduction failure (10/22), 45,X/46,XX mosaic karyotype (1/22), previous oncological therapy (3/22) and PGS with sex selection in hemophilia A carrier (1/22). The partner's oligozoospermia was present in 4/22 cycles.

The study was approved by the Ethics Commission of the University Hospital Motol in the frame of research project VZ FNM 00 000 064 203.

PGS

Blastomere biopsy was performed on day 3 or 4 according to the number of cells in embryos. Embryos with more than 5 blastomeres on day 3 were biopsied on day 3, the remaining embryos were biopsied on day 4, if they had more then 5 blastomeres. Blastomeres were washed in hypotonic solution (1% sodium citrate, 0.6% BSA) and fixed by the air-drying method with methanol and acetic acid (3 : 1) (Tarkowski, 1996).

PGS was performed by two-rounds of fluorescence *in situ* hybridization (Magli *et al.*, 2001). The Vysis Multivision PB (Abbott Laboratories, Illinois, USA) probe set consisting of centromeric probes for chromosomes 16 and 18 and locus specific probes for chromosomes 13, 21 and 22 was used in the first round. Slides were covered with glass cover slips and Parafilm M (Pechiney Plastic Packaging, Chicago, IL, USA), denaturation was performed for 5 min at 75 °C and hybridization either at 37 °C for 4–16 h or at 42 °C for 30 min followed by 90 min at 37 °C. Slides were washed by 0.7 × SSC with 0.3% NP-40 at 72 °C for 3 min 15 sec followed by 0.7 × SSC at room temperature for 1 min. Vectashield (Vector Laboratories, Burlingame, CA, USA) was used for mounting. After the first round FISH analysis, the probe mixture was washed away by immersing the slides in distilled water at 72 °C for 10 s. For the second hybridization, the Vysis CEP X + CEP Y Alpha (Abbott) combination of probes was used. Hybridization was performed at 42 °C for 30 min followed by washing for 30 s in 0.7 × σSSC with 0.3% NP-40 at 72 °C hybridization. Vectashield with DAPI (Vector) was used for mounting.

Axioplan 2 mot microscope (Carl Zeiss, Jena, Germany) with single (SpectrumRed, SpectrumAqua, SpectrumBlue, SpectrumGreen, SpectrumGold), double (Green/Red, Blue/Aqua) and quadruple (Blue/Aqua/Green/Red) band-pass filters and 63x Plan-Apochromat and 100x Plan-Neofluar objectives (Carl Zeiss) were used. Only single band pass filters were used for capturing the images in individual colour channels. Multiple band pass filters were used only for direct visual assessment of the FISH signals in microscope.

Image analysis

Images were captured using Axiocam MRm (Zeiss) camera and either Axiovision (Carl Zeiss) or ISIS (Metasystems, Altlussheim, Germany) software. Resulting bitmap files were analyzed by ImageJ 1.34s (National Institute of Health, USA, available on the Internet at http://rsb.info.nih.gov/ij/) software. The nucleus area was selected using the 'Freehand tool' with respect to the DAPI stained area. The co-ordinates of the center were measured using the 'Centroid' function that calculates the average of the co-ordinates of all pixels in the selection. The co-ordinates of the centers of signals and peripheral edges of signals were measured manually using the 'Crosshair' tool.

The following distances were calculated for each FISH signal:

R—nucleus radius. It was calculated for each signal separately to eliminate inaccuracies due to irregular nucleus shape.
Rc—signal center radius. Calculated as the distance between the signal center and the nucleus center.

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Re—signal edge radius. Calculated as distance between the peripheral signal edge and nucleus center.
Rs—inner signal radius. Calculated as distance between center of the signal and the peripheral edge of the signal.
RRc—relative radius of the signal center: RRc = Rc/R
RRe—relative radius of the edge of the signal: RRe = Re/R
CRR—corrected relative signal radius: CRR = Rc/(R-Rs). This value indicates the relative position of the signal in respect to the nucleus center and the nucleus edge irrespective of the signal size and allows a comparison with a model of random distribution of point signals.

The RRc, RRe and CRR calculations yielded values between 0 and 1 with 0 corresponding to the position of the signal in the center of the nucleus and 1 corresponding to the position at the extreme periphery of the nucleus. The diagram of the calculated distances is on Figure 1.

Statistical analysis

The signals of particular chromosomes were divided into categories according to the chromosomal status of the corresponding blastomeres. Blastomeres with two signals for each analyzed autosome and two signals for chromosome X or one signal for chromosome X and one for chromosome Y were designated as normal, all other as aneuploid.

The calculated RRc, RRe and CRR values were divided into 9 intervals (0–0.2, 0.2–0.3, 0.3–0.4, 0.4–0.5, 0.5–0.6, 0.6–0.7, 0.7–0.8, 0.8–0.9 and 0.9–1.0) corresponding to 9 concentric rings on top of a round nucleus and into two intervals ($\leq$0.75 and $>$0.75) corresponding to the central and peripheral zones. Comparison of signals distribution in 9 intervals reflects the differences in overall signal distribution from the center to the periphery of the nucleus, while the comparison of the proportion of signals in the 0.75–1.0 interval shows, whether there is a shift to the periphery. This value was chosen in order to compare the results with the study by McKenzie *et al.* (2004).

Comparisons of RRc and RRe distributions were performed by the independency test in contingency tables. Nine tests were performed in each group, therefore the statistical significance threshold was corrected according to the Bonferroni's principle to $p < 0.0056$.

To determine whether the signals are localized non-randomly, we compared the distribution of CRR values to expected frequencies. These were calculated using a mathematical model based on the presumption that the signals are randomly and uniformly distributed within the spherical nucleus and that the flat shape of a fixed nucleus is a result of the two-dimensional projection of this sphere. The expected frequency of signals in a given concentric ring is calculated as the relative portion of the sphere volume that would map on this ring under the projection. The relative volume V of each concentric ring with upper and lower borders ρ_1 and ρ_2 was calculated using following equations: $V = f(\rho_1) - f(\rho_2)$, where $f(\rho) = (3/2)(\rho^2 - \rho^2 v/2 + v^3/6)$, where $v = 1 - (1 - \rho^2)^{1/2}$.

Evaluation of differences between the observed and expected frequencies of FISH signals was performed by chi-square goodness-of-fit test. We have performed 26 comparisons for the distribution in the 9 rings and 18 comparisons for the proportion of peripheral signals. Therefore the cut-off p-value of statistical significance was corrected according to Bonferroni's principle to $p < 0.0019$ (for 9 rings) and $p < 0.0028$ (for peripheral zone).

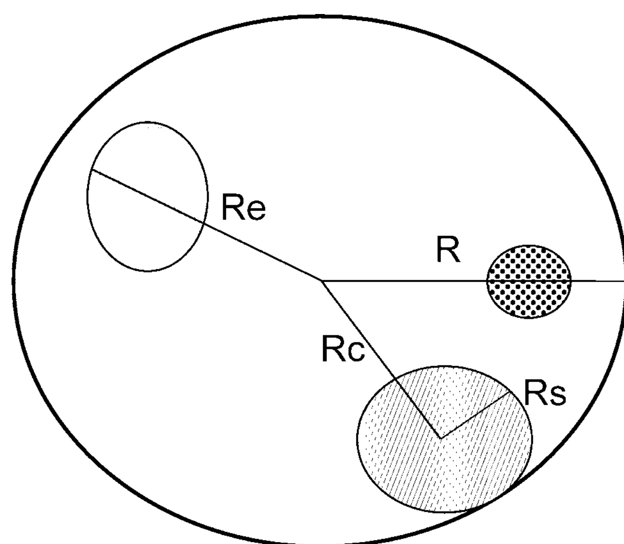

Figure 1—Scheme of measurements. R—nucleus radius, Rc—signal center radius—distance between the signal center and the nucleus center. Re—signal edge radius—distance between the peripheral signal edge and nucleus center. Rs—inner signal radius—distance between center of the signal and the peripheral edge of the signal

RESULTS

The normal PGS result was found in 49 (50%) of blastomeres, abnormalities included 16 trisomies, 11 monosomies and 22 complex aneuploidies (including 1 case of triploidy). An example of analyzed blastomere nucleus is in Figure 2.

Distribution in 9 intervals

The direct statistical comparisons (Tables 1 and 2) of the signals centers (RRc) and edges (RRe) distribution in 9 intervals did not reveal any statistically significant differences using the strict significance threshold $p < 0.0056$ corrected according to the Bonferroni's principle.

However, evaluation of signal centers and edges in several groups of normal and aneuploid blastomeres does not rule out possible different intranuclear distribution because of their p-values (chromosome 22 signal centers—$p = 0.0056$ and chromosome 18 signals edges—$p = 0.0221$).

The comparison of the distribution of the corrected relative radius (CRR) of all signals combined together

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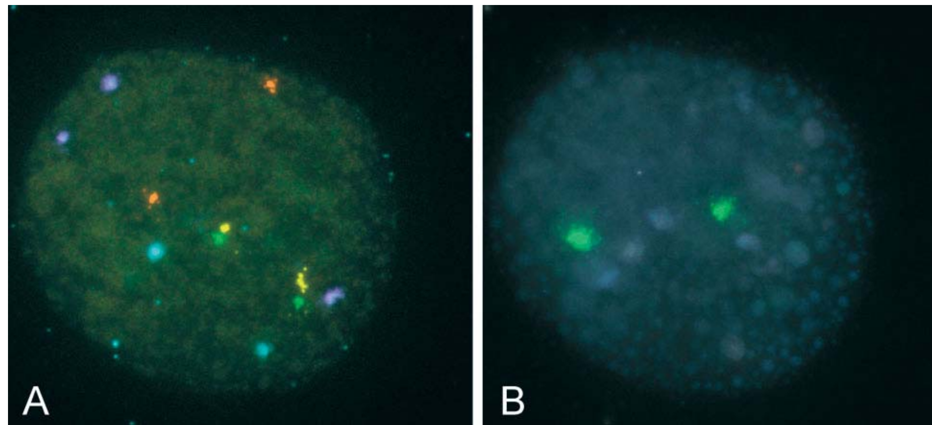

Figure 2—Example of analyzed blastomere nucleus. A—FISH with probes for chromosomes 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (yellow). Finding of trisomy 18 with peripheral localization of the chromosome 18 signals (CRR values: 0.82, 0.86, 0.84). B—FISH with probes for chromosomes X (green) and Y (red). Both chromosome X signals are in relatively central localization (CRR values: 0.26, 0.57)

Table 1—Direct comparisons of FISH signals centers (RRc) distributions in 9 intervals in normal *versus* aneuploid blastomeres and blastomeres with 1 *versus* more chromosomes X

Signals			Percentage of signal centers (RRc values) in intervals									
Chromosome	Blastomeres	Count	0–0.2	0.2–0.3	0.3–0.4	0.4–0.5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9	0.9–1.0	p_d
All	Normal	588	8.5	5.3	8.8	11.1	12.8	16.7	16.8	13.4	6.6	0.2558
	Aneuploid	610	5.6	7.5	8.5	11.0	16.4	14.1	17.0	13.1	6.7	
13	Normal	98	10.2	3.1	4.1	8.2	14.3	21.4	14.3	14.3	10.2	0.1527
	Aneuploid	101	5.0	6.9	7.9	14.9	17.8	9.9	17.8	11.9	7.9	
16	Normal	98	6.1	8.2	9.2	16.3	15.3	16.3	18.4	9.2	1.0	0.4126
	Aneuploid	92	7.6	9.8	16.3	9.8	9.8	14.1	16.3	10.9	5.4	
18	Normal	98	9.2	7.1	6.1	6.1	17.3	14.3	20.4	15.3	4.1	0.1863
	Aneuploid	108	3.7	5.6	4.6	10.2	15.7	5.6	23.1	23.1	8.3	
21	Normal	98	7.1	7.1	11.2	13.3	10.2	15.3	12.2	16.3	7.1	0.4863
	Aneuploid	104	5.8	6.7	9.6	6.7	16.3	17.3	19.2	9.6	8.7	
22	Normal	98	10.2	1.0	15.3	8.2	12.2	17.3	18.4	9.2	8.2	0.0056
	Aneuploid	105	4.8	11.4	7.6	11.4	16.2	21.0	14.3	12.4	1.0	
X	Normal	72	9.7	4.2	8.3	15.3	9.7	13.9	18.1	13.9	6.9	0.3810
	Aneuploid	75	6.7	6.7	6.7	13.3	24.0	12.0	13.3	6.7	10.7	
	1 X	53	11.3	5.7	5.7	18.9	17.0	9.4	17.0	9.4	5.7	0.8115
	>1 X	94	6.4	5.3	8.5	11.7	17.0	14.9	14.9	10.6	10.6	
Y	Normal	26	3.8	7.7	3.8	11.5	0.0	19.2	15.4	23.1	15.4	0.2192
	Aneuploid	25	8.0	0.0	4.0	12.0	16.0	32.0	4.0	20.0	4.0	

Notes: p_d—statistical significance of difference in distribution in 9 intervals and in central and peripheral zones when analyzed signal centers and edges distributions are compared between normal and aneuploid blastomeres and between blastomeres with 1 chromosome X and more than one copy of chromosome X.
Signals—categories of signals.
Chromosome—analyzed chromosome. All—all analyzed chromosomes merged together.
Count—number of signals in each category.
Blastomeres—analyzed blastomeres. Normal—with normal finding for chromosomes 13, 16, 18, 21, 22, X and Y. Aneuploid—blastomeres with abnormal finding for chromosomes 13, 16, 18, 21, 22, X and Y. 1 X—blastomeres with one copy of chromosome X. >1 X—blastomeres with more than one copy of chromosome X.
None of the differences in the distributions in all the 9 intervals has reached the significance threshold after the Bonferroni's correction ($p < 0.0056$). Therefore we cannot rule out that the signal centers in normal and aneuploid blastomeres are similarly distributed.

with the model describing random distribution of signals in the original sphere nucleus volume (Table 3) shows, that when all signals are grouped together, the distribution is significantly different ($p = 0.0001$) from the model and thus that they are not distributed randomly. The comparison of distribution of signals of each studied chromosome separately shows, that only distribution of signals of chromosome 18 is significantly different ($p = 0.0001$) from the model. This difference is even more significant when only chromosome 18 signals from aneuploid blastomeres were analyzed ($p < 0.0001$) but not from normal blastomeres ($p = 0.0539$). Some other categories of signals may also show a tendency to different distributions with p-values only slightly above the very strict significant threshold. These include all chromosomes in both normal ($p = 0.0120$) and aneuploid ($p = 0.0026$) blastomeres, chromosome 13 in all ($p = 0.0092$) and in normal ($p = 0.0020$) blastomeres,

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Table 2—Direct comparisons of FISH signals edges (RRe) distributions in 9 intervals in normal *versus* aneuploid blastomeres and blastomeres with 1 *versus* more chromosomes X

Signals			Percentage of signal edges (RRe values) in intervals									
Chromosome	Blastomeres	Count	0–0.2	0.2–0.3	0.3–0.4	0.4–0.5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9	0.9–1.0	p_d
All	Normal	588	5.6	7.7	7.1	8.0	12.9	17.3	15.6	15.0	10.7	0.1659
	Aneuploid	610	3.1	7.2	7.2	11.1	14.3	15.6	14.6	13.1	13.8	
13	Normal	98	8.2	5.1	2.0	6.1	13.3	19.4	16.3	12.2	17.3	0.3023
	Aneuploid	101	3.0	6.9	8.9	11.9	12.9	16.8	14.9	10.9	13.9	
16	Normal	98	4.1	9.2	8.2	9.2	18.4	19.4	16.3	11.2	4.1	0.4413
	Aneuploid	92	5.4	7.6	10.9	14.1	10.9	13.0	14.1	13.0	10.9	
18	Normal	98	7.1	8.2	6.1	4.1	10.2	21.4	13.3	20.4	9.2	0.0221
	Aneuploid	108	0.9	6.5	2.8	10.2	13.0	11.1	13.0	21.3	21.3	
21	Normal	98	3.1	10.2	10.2	9.2	13.3	15.3	11.2	17.3	10.2	0.9019
	Aneuploid	104	2.9	6.7	9.6	7.7	13.5	16.3	17.3	12.5	13.5	
22	Normal	98	5.1	7.1	10.2	9.2	12.2	13.3	20.4	11.2	11.2	0.7203
	Aneuploid	105	2.9	9.5	8.6	9.5	16.2	20.0	13.3	12.4	7.6	
X	Normal	72	6.9	5.6	6.9	12.5	11.1	15.3	16.7	15.3	9.7	0.2669
	Aneuploid	75	5.3	5.3	4.0	14.7	21.3	14.7	13.3	4.0	17.3	
	1 X	53	7.5	5.7	5.7	15.1	18.9	11.3	15.1	11.3	9.4	0.9446
	>1 X	94	5.3	5.3	5.3	12.8	14.9	17.0	14.9	8.5	16.0	
Y	Normal	26	3.8	7.7	3.8	3.8	7.7	15.4	15.4	23.1	19.2	0.7807
	Aneuploid	25	0.0	8.0	0.0	12.0	12.0	20.0	20.0	20.0	8.0	

Notes: p_d—statistical significance of difference in distribution in 9 intervals and in central and peripheral zone when analyzed signal centers and edges distributions are compared between normal and aneuploid blastomeres and between blastomeres with 1 chromosome X and more than one chromosomes X.
Signals—categories of signals.
Chromosome—analyzed chromosome. All—all analyzed chromosomes merged together.
Count—number of signals in each category.
Blastomeres—analyzed blastomeres. Normal—with normal finding for chromosomes 13, 16, 18, 21, 22, X and Y. Aneuploid—blastomeres with abnormal finding for chromosomes 13, 16, 18, 21, 22, X and Y. 1 X - blastomeres with one copy of chromosome X. >1 X—blastomeres with more than one copy of chromosome X.
None of the differences in the distributions in the all 9 intervals has reached the significance threshold after the Bonferroni's correction ($p < 0.0056$). Therefore we cannot rule out that the signal edges in normal and aneuploid blastomeres are similarly distributed.

chromosome 22 in normal blastomeres ($p = 0.0342$) and chromosome X in aneuploid ($p = 0.0141$) blastomeres.

Proportion of peripheral signals

Comparison of the ratio of peripheral localization defined by the relative radius above 0.75 (Table 4) does not show a significant shift to the periphery in any of the signal groups with regard to the strict significance threshold $p < 0.0056$. The difference in proportion of peripherally located signal centers ($p = 0.0095$) and edges ($p = 0.0293$) of chromosome 18 has not reached this strict significance threshold, but it indicates a trend to peripheral localization of these signal edges.

Comparisons with the model of random distribution (with significance threshold after Bonferroni's correction $p < 0.0028$) show that only chromosome 18 in aneuploid blastomeres has significantly ($p < 0.0001$) higher proportion of peripheral signals with 50.9% of signals in the peripheral zone. It also suggests a trend to increased proportion of peripheral signals localization in all chromosomes in all blastomeres (33.3%, $p = 0.0188$) and in all chromosomes in aneuploid blastomeres only (34.4%, $p = 0.0028$). A high proportion of peripheral signal localization was also found in chromosome Y in normal blastomeres (46.2%), but the difference is not significant ($p = 0.0529$).

DISCUSSION

Whole chromosome painting probes are ideal for chromosome topology studies, because they mark the overall position of the chromosome. However they are not suitable for PGS, due to large signal areas and high risk of signal overlap and splitting. We have used the commercially available set of probes consisting of centromeric probes for chromosomes 16, 18, X and Y and locus specific probes for loci at long arms of chromosomes 13, 21 and 22, that show only the position of the centromeres or the specific loci, not the position of the whole chromosome. It is also necessary to point out that our conclusions are based on FISH with probes for chromosomes 13, 16, 18, 21, 22, X and Y only. Some of the blastomeres in the normal group may thus be abnormal for one or more of the other chromosomes that we have not studied.

The developed method of FISH signal position evaluation in 9 intervals decreases the risk of errors, because it is based on measurement of distances in digital images. This method is reproducible, rather easy and feasible for possible future diagnostic application. We have calculated three parameters of the signal position: the relative radius of signal center (RRc), relative radius of the peripheral signal edge (RRe) and the corrected relative signal radius (CRR). The CRR indicates the relative position of the signal irrespective of its size and thus it

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Table 3—Corrected signal radius (CRR) values distributions compared with the model of random distribution

Signals			Percentage of signals (CRR values) in intervals									
Chromosome	Blastomeres	Count	0–0.2	0.2–0.3	0.3–0.4	0.4–0.5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9	0.9–1.0	p_m
All	All	1198	6.5	6.6	7.5	9.7	14.0	15.3	14.8	13.7	11.9	0.0001
	Normal	588	8.0	5.6	8.0	8.8	13.1	16.3	15.1	14.1	10.9	0.0120
	Aneuploid	610	5.1	7.5	7.0	10.5	14.9	14.3	14.4	13.3	13.0	0.0026
13	All	199	7.5	5.0	5.0	10.1	15.1	16.1	15.6	10.6	15.1	0.0092
	Normal	98	10.2	3.1	3.1	6.1	15.3	18.4	16.3	10.2	17.3	0.0020
	Aneuploid	101	5.0	6.9	6.9	13.9	14.9	13.9	14.9	10.9	12.9	0.8113
16	All	190	5.8	10.0	9.5	12.1	14.2	14.7	14.7	12.1	6.8	0.9504
	Normal	98	4.1	10.2	7.1	12.2	18.4	18.4	15.3	10.2	4.1	0.4678
	Aneuploid	92	7.6	9.8	12.0	12.0	9.8	10.9	14.1	14.1	9.8	0.8569
18	All	206	4.4	7.3	6.3	5.3	14.1	13.6	13.1	20.9	15.0	**0.0001**
	Normal	98	8.2	8.2	6.1	4.1	11.2	20.4	12.2	21.4	8.2	0.0539
	Aneuploid	108	0.9	6.5	6.5	6.5	16.7	7.4	13.9	20.4	21.3	**<0.0001**
21	All	202	6.4	6.4	8.9	8.9	13.9	15.3	13.4	14.4	12.4	0.5732
	Normal	98	7.1	6.1	11.2	10.2	13.3	14.3	10.2	16.3	11.2	0.8559
	Aneuploid	104	5.8	6.7	6.7	7.7	14.4	16.3	16.3	12.5	13.5	0.5940
22	All	203	7.4	6.4	10.8	8.4	13.8	16.7	16.3	12.3	7.9	0.8192
	Normal	98	10.2	1.0	15.3	7.1	12.2	13.3	19.4	10.2	11.2	0.0342
	Aneuploid	105	4.8	11.4	6.7	9.5	15.2	20.0	13.3	14.3	4.8	0.4054
X	All	147	8.2	4.8	5.4	13.6	16.3	13.6	15.6	8.8	13.6	0.0870
	Normal	72	9.7	4.2	5.6	13.9	11.1	12.5	18.1	15.3	9.7	0.6771
	Aneuploid	75	6.7	5.3	5.3	13.3	21.3	14.7	13.3	2.7	17.3	0.0141
	1 X	53	11.3	3.8	3.8	18.9	17.0	11.3	13.2	11.3	9.4	0.3713
	>1 X	94	6.4	5.3	6.4	10.6	16.0	14.9	17.0	7.4	16.0	0.1720
Y	All	51	5.9	3.9	2.0	13.7	3.9	19.6	15.7	19.6	15.7	0.0983
	Normal	26	3.8	7.7	3.8	11.5	0.0	15.4	15.4	19.2	23.1	0.1387
	Aneuploid	25	8.0	0.0	0.0	16.0	8.0	24.0	16.0	20.0	8.0	0.4656

Notes: p_m-statistical significance of difference in distribution in 9 intervals and in central and peripheral zone when analyzed signals are compared to the model based on presumption of random signal localization in the original spherical nucleus.
Model—distribution of signals according to a model of random localization.
Signals—categories of signals.
Chromosome—analyzed chromosome. All—all analyzed chromosomes merged together.
Count—number of signals in each category.
Blastomeres—analyzed blastomeres. Normal—with normal finding for chromosomes 13, 16, 18, 21, 22, X and Y. Aneuploid—blastomeres with abnormal finding for chromosomes 13, 16, 18, 21, 22, X and Y. 1 X—blastomeres with one copy of chromosome X.>1 X—blastomeres with more than one copy of chromosome X.
A distribution significantly different (below the corrected threshold $p < 0.0019$) from the model of random localization of signals in the spherical nucleus was found for the following categories: signals of all chromosomes from all blastomeres, signals of chromosome 18 in all blastomeres and signals of chromosome 18 in aneuploid blastomeres.

can be compared with a model of random distribution of point signals. Both RRc and RRe are influenced by the signal size and so they can only be used for direct comparisons between different groups of signals, but not for comparisons with the model.

Special cell preparation methods conserve the original three-dimensional structure of cells (Kozubek *et al.*, 2000). Such methods are not feasible for pre-implantation diagnosis, because of increased risk of cell loss or misdiagnosis. Nevertheless it has been proven, that even after standard cytogenetic fixation resulting in the nucleus spreading and flattening, the relative organization of the nucleus is not significantly perturbed (Croft *et al.*, 1999). However a portion of the peripheral signals originally positioned on top and bottom of the nucleus will end up in the center of the fixed nucleus.

Therefore we developed a mathematical model of random distribution of signals in the flat nucleus after fixation. This allowed us to find out whether the distributions that we have found are random or non-random. We had to adjust the statistical significance threshold value according to Bonferroni's principle to avoid false positive results due to the large number of simultaneous analyses of chromosomes 13, 16, 18, 21, 22, X and Y that were performed.

In our study, the most clear indication resulting from comparing the percentage of signals in the peripheral zone (>0.75 - see Table 4) is about the non-random preferential localization of chromosome 18 in aneuploid blastomeres with shifting to the periphery. These results confirm our previous finding in which different methods of signal localization measurement and analysis were used (Diblik *et al.*, 2005). In addition, the data point to a peripheral localization of chromosome Y in normal blastomeres and to a different localization of chromosomes 13 and 22 in normal and aneuploid blastomeres. Nevertheless, these findings must be confirmed by further studies on higher number of blastomeres.

The detection of the preferential localization of chromosome 18 in aneuploid blastomeres is in agreement with the observations in normal lymphocytes during postnatal development (Cremer *et al.*, 2001). This may

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Table 4—Comparison of proportions of central and peripheral signals

Signals			Percentage of signal centers (RRc values) in intervals			Percentage of signal edges (RRe values) in intervals			Percentage of signals (CRR values) in intervals		
Chromosome	Blastomeres	Count	< = 0.75	>0.75	p_d	< = 0.75	>0.75	p_d	< = 0.75	>0.75	p_m
All	Normal	588	71.8	28.2	0.7638	65.3	34.7	0.6619	66.7	33.3	0.0188
	Aneuploid	610	71.0	29.0		64.1	35.9		65.6	34.4	0.0028
13	Normal	98	68.4	31.6	0.7679	61.2	38.8	0.7553	65.3	34.7	0.2089
	Aneuploid	101	70.3	29.7		63.4	36.6		65.3	34.7	0.2053
16	Normal	98	81.6	18.4	0.3487	76.5	23.5	0.2135	76.5	23.5	0.2326
	Aneuploid	92	76.1	23.9		68.5	31.5		69.6	30.4	0.7515
18	Normal	98	70.4	29.6	0.0095	63.3	36.7	0.0293	64.3	35.7	0.1391
	Aneuploid	108	52.8	47.2		48.1	51.9		49.1	50.9	**<0.0001**
21	Normal	98	71.4	28.6	0.7937	66.3	33.7	0.9976	65.3	34.7	0.2089
	Aneuploid	104	73.1	26.9		66.3	33.7		67.3	32.7	0.3985
22	Normal	98	72.4	27.6	0.5417	66.3	33.7	0.3493	66.3	33.7	0.3012
	Aneuploid	105	76.2	23.8		72.4	27.6		73.3	26.7	0.6078
X	Normal	72	69.4	30.6	0.0938	61.1	38.9	0.3825	65.3	34.7	0.2791
	Aneuploid	75	81.3	18.7		68.0	32.0		69.3	30.7	0.7413
	1 X	53	79.2	20.8	0.4291	66.0	34.0	0.7881	67.9	32.1	0.6145
	>1 X	94	73.4	26.6		63.8	36.2		67.0	33.0	0.3876
Y	Normal	26	57.7	42.3	0.2851	50.0	50.0	0.3129	53.8	46.2	0.0529
	Aneuploid	25	72.0	28.0		64.0	36.0		72.0	28.0	0.9176

Notes: p_d—statistical significance of difference in distribution in 9 intervals and in central and peripheral zone when analyzed signal centers and edges distributions are compared between normal and aneuploid blastomeres and between blastomeres with 1 chromosome X and more than one chromosomes X.

p_m—statistical significance of difference in distribution in 9 intervals and in central and peripheral zone when analyzed signals are compared to the model based on presumption of random signal localization in the original spherical nucleus.

Model—distribution of signals according to a model of random localization.

Percentage of signal centers (RRc values) in intervals—percentages of relative signal center radius values < = 0.75 and >0.75.

Percentage of signal edges (RRe values) in intervals—percentages of relative signal edge radius values < = 0.75 and >0.75.

Percentage of signals (CRR values) in intervals—percentages corrected relative signal radius values < = 0.75 and >0.75.

Signals—categories of signals.

Chromosome—analyzed chromosome. All—all analyzed chromosomes merged together.

Count—number of signals in each category.

Blastomeres—analyzed blastomeres. Normal—with normal finding for chromosomes 13, 16, 18, 21, 22, X and Y. Aneuploid—blastomeres with abnormal finding for chromosomes 13, 16, 18, 21, 22, X and Y. 1 X—blastomeres with one copy of chromosome X. >1 X—blastomeres with more than one copy of chromosome X.

No significantly (below strict threshold corrected using the Bonferroni's principle $p < 0.0056$ for RRc and RRe) different proportion of peripherally located signal centers and edges was found.

A proportion of peripheral signals significantly increased (below the corrected threshold $p < 0.0028$ for CRR) compared to the model of random localization of signals in the spherical nucleus was found only for signals of chromosome 18 in aneuploid blastomeres.

contribute to elucidate the correlation among aneuploidy, peripheral localization of some chromosomes and the presumed changes in gene expression and silencing, that might be linked also to the processes of biological ageing.

Several studies show a special importance of the peripheral nuclear zone. Peripheral localization of chromatin in *Saccharomyces cerevisiae* promotes transcriptional silencing (Andrulis *et al*., 1998). In different human cell types the non-transcribed DNA sequences are found predominantly at the nuclear periphery or at the nucleoli, whereas genes tend to localize on the surfaces of the CT, that are exposed to the nuclear interior (Scheuermann *et al*., 2004). The interspersed Alu sequences and housekeeping genes are concentrated in the nuclear interior (Bolzer *et al*., 2005).

The detection of an altered chromosome localization in aneuploid embryos is in agreement with the study by McKenzie *et al*. (2004). However, in contrast with this study, we have found this behaviour only for chromosome 18. This might be due to the fact that we have used different and more precise methods of FISH signal position determination and statistical analysis supplemented by comparisons with a model of random distribution of signals in the spherical nucleus. The study by McKenzie *et al*. (2004) was based on assignment of the FISH signals to one of four concentric shells using transparency sheets, that were placed on the computer monitor. We have used exact measurement of FISH signals center and peripheral edge co-ordinates instead. These measurements allowed calculation of the corrected relative signal radius value that is not dependent on the signal size. Our definition of peripheral localization is more stringent with the limit 0.75, while the study by McKenzie *et al*. (2004) uses 0.5.

The peripheral shift of chromosome 18 can be a direct physiological result of the changes in the gene dosage resulting in activation of mechanisms of gene silencing in areas in contact with the nucleus periphery. It can also be a result of disruption of mechanisms

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responsible for the functional chromosome positioning due to the gene dosage imbalance. Another possibility is that the peripheral localization reflects the initial stages of fragmentation and cell death.

It can also be speculated that aneuploidy is secondary to the chromosome localization changes in embryos with abnormal development. Chromosomes in the nucleus periphery might be prone to non-disjunction or anaphase lagging due to incorrect kinetochore-microtubule attachments.

Defects in the mitotic apparatus might be responsible for both the difference in chromosome localization and aneuploidy, as chromosome localization is at least partially dependent on the position of chromosomes after mitosis. The centrosome of the zygote derives from the centriole inherited from the sperm (Palermo *et al.*, 1994). Aneuploidy is increased in embryos conceived with sperm from males with severe forms of infertility, especially when testicular sperm is used (Gianaroli *et al.*, 2000). The chromosomal imbalance in the embryo could be originated either by the aneuploidy condition in the fertilizing spermatozoon, or by the defective mitotic spindle in the zygote, that was organized by a defective centrosome of male origin. Defects in the centrosome organization might be a major cause of the post-meiotic aneuploidy and mosaicism that are often found in pre-implantation embryos (Silber *et al.*, 2003).

If the correlation between aneuploidy and the peripheral localization of chromosomes in pre-implantation embryos is confirmed by further studies, this approach might significantly contribute to a better understanding of both the pathogenesis of early development disorders and the inter-chromosomal interactions entering this process. It could also represent a new criterion for the selection of the most suitable embryos for transfer by the indirect detection of increased risk of aneuploidy involving other chromosomes.

Future studies should analyze larger numbers of blastomeres in relation to the type of aneuploidy, poor prognosis indications, parental age, fertilization and cultivation methods and media, as well as IVF outcome. Suitable methods of blastomere fixation with preservation of the original three-dimensional structure should be tested in order to optimize the technique.

According to our results, there is no peripheral localization of chromosome X in the earliest phase of human development in any of the studied categories of blastomeres. This is in agreement with the data reported by McKenzie *et al.* (2004) in which the proportion of chromosome X signals in the peripheral quadrants in normal embryos is not different. They did not analyze the possible correlation between aneuploidy and the number and localization of chromosome X. If our observations are confirmed by further studies, provided that the presumed relationship between peripheral localization of chromosome X and its gene inactivation exists in this phase, it would suggest that aneuploidy does not change gene transcription activity of chromosome X in day 3–day 4 embryos.

CONCLUSIONS

Normal and aneuploid blastomeres differ in the localization of chromosome 18 signals, being shifted to the nucleus periphery in aneuploid blastomeres. Conversely, localization of chromosome 13, 16, 21, 22, X and Y signals do not significantly differ from a random distribution model both in normal and aneuploid blastomeres.

No difference in the localization of chromosome X signals in blastomeres with one or more copies of chromosome X, or in blastomeres with aneuploidy of the studied autosomes was disclosed. The peripheral localization of the inactive chromosome X was not found in day 3–day 4 human pre-implantation embryos with more than one X chromosome.

On the other hand, the suggested peripheral localization of chromosomes 13 and Y in normal blastomeres and the difference in localization of chromosome 22 in normal and aneuploid blastomeres must be confirmed by further studies.

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