Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF

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BACKGROUND: In order to assess the frequency of aneuploidy and mosaicism in embryos obtained from IVF patients aged <38 years, preimplantation genetic screening (PGS) was performed after biopsy of two blastomeres. Furthermore, the reliability of this diagnosis was assessed by performing reanalysis of the embryo on day 5. METHOD: The copy numbers of 10 chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X and Y) were investigated by fluorescence *in situ* hybridization (FISH) analysis. Embryos that were found to be abnormal or of insufficient morphological quality were cultured until day 5 and reanalysed. Results obtained were compared to the day 3 blastomere analysis. RESULTS: After analysis of 196 embryos (one cell in 38% and two cells in 62%), only 36% of the embryos were found to be normal on day 3. After analysis of two blastomeres, 50% showed chromosomal mosaicism. Comparison of the FISH results from day 3 blastomeres and day 5 embryos yielded an overall cytogenetic confirmation rate of 54%. CONCLUSIONS: The rates of mosaicism and aneuploidy in these embryos from young IVF patients are similar to those published for older women. We found the best confirmation rate after a diagnosis based on two cells, where both blastomeres showed the same chromosomal abnormality. In contrast, after a mosaic diagnosis the confirmation rate was low. The present study provides the first detailed reanalysis data of embryos analysed by PGS and clearly demonstrates the impact of mosaicism on the reliability of the PGS diagnosis.

Key words: aneuploidy/chromosomal mosaicism/confirmation of diagnosis/human preimplantation embryos/preimplantation genetic screening

Introduction

The advent of IVF as a treatment for infertility has created the opportunity to study the chromosomal constitution of human preimplantation embryos. An increasing body of evidence suggests that the incidence of chromosomal abnormalities in embryos is extremely high (as reviewed by Macklon *et al.*, 2002; Wilton, 2002) and good embryo morphology does not necessarily exclude an abnormal chromosomal constitution (Magli *et al.*, 2000; Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Magli *et al.*, 2001; Sandalinas *et al.*, 2001). Since aneuploidies are considered the main cause of embryonic wastage and loss, this phenomenon may be primarily responsible for the relatively poor pregnancy rates reported after IVF, as well as the poor fertility performance of humans *in vivo* (Delhanty, 2001).

The introduction of fluorescence *in situ* hybridization (FISH) for preimplantation genetic diagnosis (PGD) has enabled screening of embryos for chromosomal aneuploidies before transfer. This preimplantation genetic screening (PGS)

would be of special interest for couples who are thought to have a higher risk of developing chromosomally abnormal embryos, with the aim of improving their chances for an ongoing pregnancy after IVF. Although PGS is offered in many IVF centres around the world, its clinical value remains uncertain. A positive effect on implantation and ongoing pregnancy rates in a group of patients with advanced maternal age has been observed in retrospective studies (Munné *et al.*, 1999, 2003). However, a recent prospective randomized study failed to show a positive effect of PGS on clinical outcome per initiated cycle in patients with advanced maternal age (Staessen *et al.*, 2004). Other indications for which PGS has been proposed include recurrent implantation failure and recurrent miscarriage. Again, clinical benefits have not yet been convincingly demonstrated (Gianaroli *et al.*, 1999; ESHRE PGD Consortium Steering Committee, 2002; Pehlivan *et al.*, 2003; Rubio *et al.*, 2003; Platteau *et al.*, 2005).

Studies testing the efficiency of PGS have so far used clinical parameters such as implantation rates and ongoing pregnancies

as outcome measures. However, it has not yet been demonstrated that the screening of one or two blastomeres obtained from an 8-cell embryo for the presence of aneuploidies will provide a reliable prediction of the chromosomal status of the remaining embryo. An important factor affecting the reliability of the diagnosis is the phenomenon of mosaicsm in embryos (Los *et al.*, 2004). Reanalysis of blastocysts can be a useful tool to investigate whether FISH results from blastomeres obtained from day 3 embryos are representative for the remaining embryo (Gianaroli *et al.*, 1999; Veiga *et al.*, 1999; Emiliani *et al.*, 2000; Magli *et al.*, 2000). In a previous study, we have biopsied frozen–thawed good quality embryos and performed FISH analysis for 10 chromosomes (Baart *et al.*, 2004a). After biopsy, the embryos were cultured until day 5 and subsequently reanalysed using the same probe panels. We observed a high percentage of mosaic embryos on day 3 (57%) and found that the chromosomal constitution of these embryos is subject to changes during development to the blastocyst stage. This yielded mostly false positive results and a low confirmation rate, confirming our suspicion that chromosomal mosaicism at the 8-cell stage poses a serious problem when performing PGS.

PGS has been mostly applied to women of advanced maternal age or with an indication, such as recurrent implantation failure or recurrent miscarriage. Moreover, in most of the studies mentioned above, the diagnosis has been based on the biopsy of only one blastomere. Therefore, only limited data are available concerning the incidence of chromosomal abnormalities and especially mosaicism in embryos of younger IVF patients (<38 years) with no specific indication for PGS. In order to assess the frequency of aneuploidy and mosaicism in embryos obtained from such a group of women, we performed PGS using FISH for 10 different chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X and Y) on day 3 embryos after biopsy of two blastomeres. Furthermore, the impact of chromosomal mosaicism on the accuracy of the day 3 diagnosis was studied. Embryos diagnosed as normal and of morphologically sufficient quality were either transferred or cryopreserved on day 4. Those embryos diagnosed as abnormal or normal embryos of insufficient quality were cultured further until day 5 to study the developmental capacity of the embryo. The embryo was subsequently completely analysed by FISH and the reliability of the diagnosis was evaluated by comparing the results obtained on day 3 with the chromosome constitution of the embryo on day 5.

Materials and methods

Patients and embryos

Between November 2002 and August 2004, preimplantation embryos were obtained from couples participating in an ongoing study on PGS. The present study was designed to investigate the incidence of chromosomal aneuploidies in embryos from young IVF patients with no specific indication for PGS. Prior to commencing the study, ethical approval was received from the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local institutional ethics committee. Only women aged <38 years and with a partner with normal semen characteristics were invited to participate in the study and written informed consent was obtained from each couple. Additional inclusion criteria included: (i) a history of regular menstrual cycles, ranging from 25 to 35 days; (ii) a body mass index of 19–29 kg/m^2;

(iii) no known karyotype abnormalities; and (iv) no history of recurrent abortions. Couples could participate in the study for one cycle only.

Ovarian stimulation, oocyte retrieval and IVF procedures were performed as described previously (Huisman *et al.*, 2000; Hohmann *et al.*, 2003). Before the biopsy procedure, the embryos were scored for quality and number of blastomeres. Embryo quality scores were assigned according to previously described criteria (Huisman *et al.*, 2000; Hohmann *et al.*, 2003). Biopsied embryos were cultured until day 4, by which time FISH analysis was completed. Only embryos that were diagnosed as normal and of sufficient morphological quality were transferred, with a maximum of two embryos per patient. Remaining good quality, normal embryos were cryopreserved on day 4. Embryos diagnosed as abnormal or of insufficient quality were cultured until day 5, scored for morphology and the entire embryo was fixed for FISH analysis.

Biopsy procedure and fixation of blastomeres and embryos

The biopsy procedure was performed on day 3 after fertilization as described previously (Baart *et al.*, 2004a). In short, embryos were washed and then incubated in EB-10 medium and later in the study, G-PGD medium (both Vitrolife, Göteborg, Sweden) for 5 min at 37°C. One blastomere was biopsied if the embryo consisted of five or six cells and two blastomeres if the embryo had at least seven cells. The biopsied embryos were returned to normal culture conditions. The removed blastomeres were fixed as described previously (Dozortsev and McGinnis, 2001) with some modifications (Baart *et al.*, 2004b). In short, the blastomere nucleus was isolated with spreading solution (0.01 N HCl/0.1% Tween 20; Coonen *et al.*, 1994; Harper *et al.*, 1994) and subsequently fixed with freshly made fixative (methanol:acetic acid, 3:1). Whole surplus embryos were fixed in the same way, only now a wash in spreading solution was also used to dissolve the zona pellucida, before transferring the embryo to the slide.

FISH procedure

A two-round FISH procedure was performed as described previously (Baart *et al.*, 2004b), allowing the detection of chromosomes X, Y, 1, 7, 13, 15, 16, 18, 21 and 22. The DNA probes used in the first round were centromere probes for chromosomes 1 (pUC 1.77; Cooke and Hindley, 1979), 7 (7t1; Waye *et al.*, 1987), 15 (pTRA-20; Choo *et al.*, 1990), X (pBamX5; Willard *et al.*, 1983) and a Y chromosome heterochromatin probe (RPN1305X; Lau, 1985). The probes for chromosomes 1, 7, 15 and Y were labelled with Pacific Blue, Alexa Fluor 350, Alexa Fluor 594 and Alexa Fluor 488 respectively. The probe for the X chromosome was labelled with both Alexa Fluor 488 and Alexa Fluor 555, resulting in a yellow fluorescence. The DNA probes used in the second round were centromere probes for chromosomes 16 and 18, labelled with Spectrum Aqua and Spectrum Blue, combined with LSI probes for chromosomes 13, 21 and 22 labelled with Spectrum Red, Green and Gold respectively (Multivysion PB kit; Vysis, Downers Grove, IL, USA). Signals from the second round were recorded and compared with the ones from the first round to ensure that they had not persisted.

As most of the probes used are repetitive DNA probes, signal size can vary due to individual variability in size of the heterochromatic region. To detect this, lymphocyte nuclei from both parents were used as controls. Slides were prepared from blood samples according to standard protocols and they were hybridized using the same two-round FISH protocol as for the embryonic cells. Signals were observed in 10 nuclei from each parent and images were obtained after each round to check for persisting signals. Probe hybridization effiency on lymphocyte nuclei was 86% for the first round of hybridization and 90% for the second round (Baart *et al.*, 2004b).

Microscopy and interpretation of FISH results

Slides were examined with a Zeiss Axioplan 2 imaging epifluorescence microscope, equipped with appropiate filters (Baart *et al.*, 2004b). Images were captured with the ISIS FISH Imaging System (MetaSystems, Altlussheim, Germany). For enumeration of the signals after both rounds, we used scoring criteria previously published (Munné *et al.*, 1998). Interpretation of the FISH results from single blastomeres and embryos was done according to the definitions published previously, with some modifications (Baart *et al.*, 2004a). Based on the analysis of two blastomeres per embryo, we classified day 3 embryos as normal (both nuclei showing the normal number of signals for the chromosomes investigated), aneuploid (both nuclei carrying the same chromosome abnormality), abnormal/normal mosaic (one normal nucleus and one abnormal) or abnormal/abnormal mosaic (each nucleus showing one or more different chromosome abnormalities). If only one blastomere was available for diagnosis, the embryo was classed as normal or aneuploid based on the FISH result from this nucleus. If a blastomere showed aneuploidy for two or more chromosomes, it was defined as double or multiple aneuploidy respectively.

After analysis of day 5 embryos, we classified them as normal (~80% normal nuclei and, more importantly, <10% of the nuclei with the same chromosome abnormality), aneuploid (≥90% of the nuclei showing the same abnormality) or mosaic (>10% and <90% of the cells showing the same chromosome abnormality). Embryos with ≥90% haploid, tetraploid or triploid nuclei were classed as such. However, we considered the occurrence of some tetraploid cells as a normal phenomenon of *in vitro* cultured embryos (Evsikov and Verlinsky, 1998; Bielanska *et al.*, 2002; Coonen *et al.*, 2004) and treated them as normal cells.

An abnormal diagnosis made on day 3 was considered cytogenetically confirmed, if at least one of the chromosomal abnormalities seen on day 3 was recovered in >10% of the cells analysed on day 5.

Results

Clinical results

A total of 60 couples started their IVF cycle within the study period and the clinical results are summarized in Table I. Five cycles were cancelled due to either poor response or ovarian hyperstimulation. After 55 oocyte retrievals, two cycles showed no fertilization and in seven cycles none of the embryos were suitable for biopsy on day 3. The 46 cycles where a biopsy could be performed yielded a total of 323 embryos, from which 224 embryos were suitable for biopsy. The average age of these 46 women was 33.1 years (range 25–37). This was their first (61%), second (11%), third (19%) or fourth IVF cycle (9%).

Biopsy and diagnosis on day 3

A total of 178 embryos consisted of at least seven blastomeres, enabling two cells to be biopsied (79%), and from the remaining 46 embryos, only one blastomere could be taken (21%; Table I). From 28 embryos, blastomere(s) were lost during the spreading procedure or the FISH results were inconclusive, so no diagnosis could be made. From the other 196 embryos, a diagnosis was obtained based on two cells in 121 embryos (62%) and on one cell in 75 embryos (38%). After analysis of two blastomeres, the diagnosis was normal for 43 embryos (36%), aneuploid for 17 embryos (14%) and mosaic for 61 embryos (50%), of which 34 (28%) embryos were abnormal/normal mosaic and 27 embryos (22%) abnormal/abnormal mosaic. After analysis of only one blastomere, the diagnosis was normal for 27 embryos (36%) and 48 embryos (64%) were found to be aneuploid.

Reanalysis of day 5 embryos and interpretation of FISH results

After transfer or cryopreservation on day 4, 108 embryos were left for further culture until day 5. On day 5, 49 embryos (45%) had developed to the blastocyst stage. Twenty embryos (19%) had arrested after (arrested day 4) and 14 embryos (13%) before compaction (arrested day 3). A further 25 embryos (23%) had degenerated and could not be analysed by FISH.

In total, detailed FISH analysis was performed on 83 embryos, the results of which are presented in detail in Appendix I. After interpretation of the FISH results from the blastocysts according to the criteria described, 16 (33%) were found to be normal, 11 (22%) aneuploid and 22 (45%) mosaic. In the group of arrested embryos, five (15%) were found to be normal, seven aneuploid (21%) and 22 mosaic (65%). The chromosomal constitution of the day 5 embryo (blastocyst or arrested) was compared to the original diagnosis on day 3 after analysis of two blastomeres (Table II) or one blastomere (Table III). A summary of the confirmation rates is presented in Table IV.

Cytogenetic confirmation of day 3 diagnosis

Normal day 3 diagnosis

For the embryos diagnosed as normal on either one or two cells, we found poor confirmation rates (20 and 43%, respectively; Table IV), leading to eight cases with a false negative diagnosis (Table II and III). It has to be kept in mind that these embryos, although diagnosed as normal, were found to be unsuitable for transfer or cryopreservation on the basis of development and morphology on day 4.

Aneuploid day 3 diagnosis based on two cells

The highest confirmation rate was established for embryos diagnosed as aneuploid based on two cells. Here, we found only two false positive cases out of 11. Case 10 demonstrated a monosomy

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Table I. Clinical IVF results and FISH diagnosis based on one or two blastomeres obtained from day 3 embryos

Started cycles	60
No. of cycles with:	
Oocyte retrieval	55
Fertilization failure	2
Insufficient development of embryos	7
Biopsy	46
No. of embryos obtained	323
No. of embryos biopsied	224 (69)
No. of embryos diagnosed	196 (88)
No. of embryos diagnosed based on 2 cells	121 (62)
Normal	43 (36)
Aneuploid	17 (14)
Abnormal/normal mosaic	34 (28)
Abnormal/abnormal mosaic	27 (22)
No. of embryos diagnosed based on 1 cell	75 (38)
Normal	27 (36)
Abnormal	48 (64)

Values in parentheses are percentages.

225

Table II. Embryo development, FISH results and interpretation from embryos after analysis of two blastomeres on day 3 (for a detailed presentation of the FISH results on day 5, see Appendix)

Case no.	Day 3		Day 5			
	Morphology score[a]/ no. of cells	FISH results	No. cells analysed	% of normal cells	Interpretation of FISH results	Confirmed
Embryos reaching the blastocyst stage						
		Normal				
1	3/10	2N/2N	51	73	Normal	+
2	2/7	2N/2N	15	80	Mos mon7/2N	–
3	2/9	2N/2N	58	78	Mos mon21/2N	–
		Aneuploid				
4	2/8	X0/X0	18	56	Mos monX/2N (XY)	+
5[e]	2/8	–22/–22	41	0	Mon 22	+
6	2/8	+15/+15	43	79	Mos tris15/2N	+
7[e]	3/8	+15/+15	57	16	Mos tris15/2N	+
8[e]	2/10	+22/+22	26	0	Tris 22	+
9	2/8	3N/3N	19	0	Triploid	+
10	2/8	X0/X0	38	71	Normal (XY)	–
		Abnormal/normal mosaic				
11	2/8	–16/2N	64	84	Mos mon16/2N	+
12	2/8	–16/2N	66	83	Mos mon16/2N	+
13	2/8	–**16**/2N	17	47	Mos mon15/**mon16**/mon13/X0/2N (XY)	+
14[e]	1/10	+22/2N	62	10	Tris 22	+
15	2/12	X0/2N (XY)	30	73	Mos mon7/2N (XY)	–
16	1/8	+7/2N	76	88	Normal	–
17	2/8	+15/2N	81	100	Normal	–
18	2/8	+21/2N	61	89	Normal	–
19	2/8	–15/2N	34	94	Normal	–
20	2/8	–7,–7/2N	48	92	Normal	–
		Abnormal/abnormal mosaic (single aneuploidy)				
21	2/8	–7/–21	38	82	Mos mon15/2N	–
22[d]	2/9	+7/–1	38	74	Mos mon7/2N	–
23	2/8	+13/–21	52	94	Normal	–
		Abnormal/abnormal mosaic (multiple aneuploidies)				
24[e]	2/8	+22,+22/+**22**	20	10	Mos **tris22**/tris1,22/tris1, mon7,tris 22	+
25[e]	2/7	–18,–21/–21	40	0	Mon 21	+
26	2/8	+16,+22/–**21**	27	52	Mos tris15 and **mon 21**/tris15/2N	+[b]
27[d,e]	2/7	XXXY/**XXY**	49	27	Mosaic XY/**XXY**	+[b]
28[d,e]	2/8	+**1**,–15,–21/**XXX**,+**16**,+16	23	0	Mos **trisX,16**/**tris16**/**tris1**,15,**16**	+[b]
29	1/8	X0,–**16**,–16,–22/–1, –7,–**16**,–16	50	76	Mos **mon16**/2N	+[b]
30	2/8	+15/–18,–21	27	100	Normal	–
31	2/8	XO,+15/+22,+22	52	94	Normal (XX)	–
32	1/8	XXX/3N,–1,–15	32	63	Normal (XX)	–
33	2/8	–7,–13,–15,–18,–22/–7, +13,+15,+18,+22	127	87	Normal	–
34	2/8	XXY,+15,–7,–13/XXY,+13	103	91	Normal (XY)	–
35	2/8	–7/N,–7,–21,–13	28	82	Normal	–
36	2/8	–13,–18,–18,–21/–7,–7,–18	32	94	Normal	–
Embryos arrested at day 3 or 4						
		Normal				
37	1/8	2N/2N	22	91	Normal	+
38	2/8	2N/2N	11	82	Normal	+
39	3/8	2N/2N	6	0	Mos Near 4N[c]/monX/mon15/mon1,7	–
40	2/8	2N/2N	10	0	Near tetraploid[c]	–
		Aneuploid				
41	3/7	–22/–22	8	38	Mos 4N/N/multiple aneuploidy (incl. **mon22** in 25%)/2N	+
42	1/8	3N/3N	6	0	Triploid	+
43[d,e]	4/8	–**7**,–15,–18,–21,–**21**/–**7**, –15,–18,–21,–**21**	3	0	**Mon7**,tris15,18,**mon21**	+[b]
44	2/8	+7,+13/+7,+13	3	100	Normal	–
		Abnormal/normal mosaic				
45	2/8	–**18**/2N	28	46	Mos mon21/**mon18**/mon7/2N	+
46	2/8	–**1**,–21/2N	9	22	Mos mon18/tris18/**mon1**,16/2N	+[b]
47	3/8	–16,–18/2N	10	0	Triploid	–
48	2/7	–13/2N	11	73	Normal	–

Table II. Continued

Case no.	Day 3		Day 5			
	Morphology score[a]/ no. of cells	FISH results	No. cells analysed	% of normal cells	Interpretation of FISH results	Confirmed
		Abnormal/abnormal mosaic (multiple aneuploidies)				
49[e]	2/8	+1/+1,+15	19	0	Tris 1	+
50[d,e]	2/8	−7,−22/−22	7	0	Mos Mon 22/tris7,mon22	+
51[e]	2/10	XO,−13,−18,−21,−21,−22/ XO,−7,−18,−21,−21,−22	10	0	Mos multiple aneuploidy (same chromosome abnormalities involved)	+
52	1/8	−**18**/−7,+21,−13,−13, −16,−22	14	79	Mos **mon18**/2N	+
53[d]	3/8	XXY,−**15**/XXY,−**15**,+22	7	86	Mos **mon 15**,YO/2N (XY)	+[b]
54	1/10	XXY,−1/**XO**,−21	20	70	Mos tris1/**monX**/2N (XY)	+[b]

[a]Morphology score: 1 = excellent; 2 = good; 3 = average; 4 = poor quality.
[b]Confirmation of at least one of the chromosome abnormalities observed on day 3 (in bold type).
[c]Near tetraploid = 92 ± chromosomes (ISCN, 1995).
[d]Cases with potential mitotic non-disjunction before the 8-cell stage, in which the reciprocal product was recovered on day 5.
[e]These are cases where the abnormality or at least one of the abnormalities most likely resulted from a meiotic error.
2N = normal copy number for the chromosomes investigated; Mos = mosaicism; mon = monosomy; tris = trisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 1995).

X on day 3 and was found to be normal on day 5 according to our definitions (Table II). However, there were four cells present in the embryo with either a monosomy or a trisomy X, so a low level of mosaicism at the 8-cell stage cannot be excluded, in which case we may have biopsied most of the abnormal cells.

Aneuploid day 3 diagnosis based on one cell

From the embryos diagnosed as aneuploid based on one cell, nine embryos showed a single abnormality on day 3, and the same abnormality was recovered in the day 5 embryo in three cases (56, 57 and 72, Table III). A further 15 embryos were diagnosed with double or multiple aneuploidy. Eleven cases could be confirmed on day 5, although in 10 cases only one of the abnormalities seen on day 3 was recovered. Only in case 62 was the exact double aneuploidy present in all cells of the blastocyst.

Day 3 diagnosis of mosaicism

After analysis of two cells on day 3, 36 embryos were found to be either abnormal/normal or abnormal/abnormal mosaics (Table II and IV). Of these, 18 (50%) were confirmed to be mosaic or aneuploid involving the same chromosomal abnormality in cases of single aneuploidy (e.g. cases 11 and 14) or at least one of the chromosomal abnormalities observed on day 3 in cases of double or multiple aneuploidy (e.g. cases 26 and 29). Of the 18 embryos without confirmation, 14 presented a normal chromosome constitution on day 5, from which 13 had developed into blastocysts. One embryo turned out to be triploid on day 5 (case 47) and three embryos were abnormal/normal mosaics, all involving another abnormal cell line (cases 15, 21, 22). The trisomy 7 observed on day 3 in case 22 was not confirmed on day 5, but interestingly, a cell line with a monosomy 7 was observed. It is not unlikely that the trisomy and monosomy 7 were the products of a non-disjunction event during the second or third cleavage division. Biopsy of the trisomic cell then left the embryo with the corresponding monosomic cell, next to normal cells.

If we look at the abnormal/abnormal mosaic cases in more detail (Table II), we find that in 19 out 22 cases, multiple aneuploidy was involved. From these mosaic embryos with multiple aneuploidies, in 12 cases the two blastomeres share the same chromosome abnormality, next to other abnormalities (cases 24, 25, 27, 29, 33, 34, 35, 36, 49, 50, 51, 53). In eight of these 12 cases (67%) we were able to confirm the common aneuploidy on day 5.

Identification of the origin of chromosome aberrations observed

By comparing the day 3 and day 5 diagnosis for each embryo, valuable information on the origin of the abnormalities observed can be obtained. As mentioned above for embryo 22, there are 11 further cases where a mitotic non-disjunction event is likely to have occurred before the 8-cell stage and where the reciprocal product of such an event is recovered on day 5 (Tables II and III). However, it can never be excluded that the monosomy and trisomy resulted from two separate events. With respect to embryos 46 and 71, a non-disjunction event was detected in the day 5 embryo, where both a monosomic and a trisomic cell line for the same chromosome were found. Another interesting example is embryo 33, where a cell with a monosomy 7 divided with non-disjunction for chromosomes 13, 15, 18 and 22. These daughter cells were then biopsied, probably leaving the embryo with only normal cells.

In 19 out of 83 cases (23%), a chromosome abnormality was involved that most likely originated during meiosis (Tables II and III). Besides the meiotic chromosome abnormality, in all of these cases the embryo was additionally hit by one or more mitotic events, such as anaphase lagging and non-disjunction (see Appendix). An interesting example is embryo 27, where the results on day 3 and on day 5 are consistent with a chromosome constitution of XXY from a meiotic event, followed by non-disjunction of one of the X chromosomes during the second cleavage division (Figure 1).

Table III. Embryo development, FISH results and interpretation from embryos after analysis of one blastomere on day 3 (for a detailed presentation of the FISH results on day 5, see Appendix)

Case no.	Day 3		Day 5			
	Morphology score[a]/ no. of cells	FISH results	No. cells analysed	% of normal cells	Interpretation of FISH results	Confirmed
Embryos reaching the blastocyst stage						
		Normal				
55	4/5	2N	37	0	Tetraploid	–
		Aneuploid				
56[e]	2/7	–21	22	0	Mon21	+
57[e]	2/8	+16	47	0	Tris16	+
58	3/6	+18	30	93	Normal	–
59	4/6	–16	36	64	Mos mon15/2N	–
60[d]	1/8	**+7**	30	60	Mos mon7/2N	–
		Double aneuploid				
61[e]	1/12	–18,**+22**	86	5	**Tris22**	+[b]
62[e]	2/8	–16,–22	35	0	Double mon16 and 22	+
63[e]	2/7	–7,**–22**	57	0	**Mon22**	+[b]
64[d]	2/8	–7,–21	30	23	Mos tris21/tris7/2N	–
65[d]	3/8	–15,+13	25	88	Mos mon13,21/2N	–
		Multiple aneuploid				
66	2/8	X0,–16,**–21**	57	82	Mos **mon21**/2N (XY)	+[b]
67	2/8	N,–1,+15,+15,–21	42	43	Mos mon18/2N	–
Embryos arrested at day 3 or 4						
		Normal				
68	4/5	2N	12	100	Normal	+
69	1/9	2N	18	77	Mos mon22/2N	–
70	2/7	2N	10	90	Mon21	–
71	4/6	2N	9	11	Mos mon1/tris1/monX/trisX/2N	–
		Aneuploid				
72	3/6	–15	13	23	Mos Multiple aneuploidy (incl. **mon15** in 38% of cells)/2N	+
73	2/6	+15	10	30	Mos triploidy/2N	–
74	3/6	+21	5	0	Mos null1/null1, monX, mon15	–
75	3/8	–13	6	83	Mos mon16/2N	–
		Double aneuploid				
76[d]	1/8	+22,**–16**	14	50	Mos **mon16**/mon22/2N	+[b]
77[e]	2/8	+15,**–22**	24	0	Mos **mon22**/mon7, **22**	+[b]
78	2/7	**–1**,–7	15	67	Mos **mon1**/2N	+[b]
79[d]	1/8	X0,+13	24	63	Mos mon13/2N (XX)	–
		Multiple aneuploid				
80	2/12	N,XX,+1,+15	5	0	Mos **haploidy**/tetraploidy	+[b]
81[d]	3/8	XXX,**+13**,+16,–21	10	50	Mos monX,**tris13**/mon7/2N	+[b]
82[d,e]	3/9	**X0**,+7,+13,+13,+18,+22	1	0	**X0**,–7,–15,–21,–22	+[b]
83	3/8	–1,–1,–13,**–16**,–21,–21,–22	3	33	Mos **mon16**/2N	+[b]

[a]Morphology score: 1 = excellent; 2 = good; 3 = average; 4 = poor quality.
[b]Confirmation of at least one of the chromosome abnormalities observed on day 3 (in bold type).
[d]Cases with potential mitotic non-disjunction before the 8-cell stage, in which the reciprocal product was recovered on day 5.
[e]These are cases where the abnormality or at least one of the abnormalities most likely resulted from a meiotic error.
2N = normal copy number for the chromosomes investigated; Mos = mosaicism; mon = monosomy; tris = trisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 1995).

Embryos 6 and 7 were both trisomic for chromosome 15 on day 3 and turned out to be mosaic for the same abnormality on day 5. This chromosome abnormality may, however, have originated from two different mechanisms. In case 6, trisomy 15 most likely originated post-zygotically through non-disjunction, followed by loss of the monosomy 15 cell line. In contrast, in case 7 the trisomy 15 most probably arose meiotically, with a post-zygotic loss of the extra chromosome 15 resulting in only 16% normal cells in the day 5 embryo.

Discussion

Here we present the data from good quality human preimplantation IVF embryos after screening for aneuploidies of 10 different chromosomes on day 3. In addition, for embryos not suitable for transfer on the basis of the PGS results or poor morphology, subsequent analysis of the entire embryo on day 5 is also presented. Although the embryos investigated came from a group of relatively young IVF patients (mean maternal age 33.1 years, range 25–37), we found only 36% of the embryos to be normal, after analysis of both one or two blastomeres. Interestingly, Staessen *et al.* (2004) reported the exact same percentage of normal embryos after PGS on one or two blastomeres in a group of older-aged patients. So although older women are thought to have a lower chance of producing chromosomally normal embryos, this could not be confirmed by our results. In fact, in 23% of the reanalysed embryos, we observed a chromosome

Table IV. Overview of the diagnosis made on day 3 and rate of cytogenetic confirmation after reanalysis on day 5.

Diagnosis on day 3	No. of embryos reanalysed on day 5	No. of cases confirmed (%)
Based on two cells		
Normal	7	3 (43)
Aneuploid	11	9 (82)
Mosaic	36	18 (50)
Abnormal/normal mosaic	14	6 (43)
Abnormal/abnormal mosaic	22	12 (55)
Total	54	30 (56)
Based on one cell		
Normal	5	1 (20)
Aneuploid	24	14 (58)
Total	29	16 (55)
Overall confirmation rate	83	45 (54)

Values in parentheses are percentages.
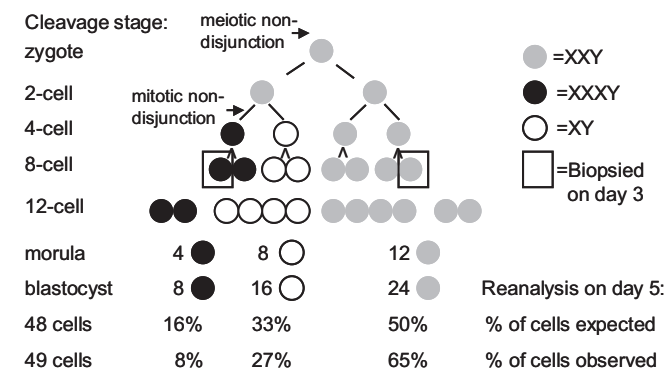
Figure 1. Schematic representation of the most likely origin of the chromosomal abnormalities observed in case 27, on day 3 and day 5.

abnormality that most likely originated during meiosis. Another study reported PGS analysis in a group of women aged <35 years (Munné *et al*., 2004). These patients were undergoing PGS either because they were carriers of X-linked diseases, because they had two or more previous IVF failures or because of a previous aneuploid conception. After analysis of a single cell for 6–9 chromosomes, the percentages of normal embryos were found to be 52, 47 and 29% respectively. Comparison with our data is difficult, since we analysed two cells and more chromosomes, and a lower percentage of normal embryos would therefore be expected. Furthermore, the first group probably consisted primarily of fertile patients with no indication for IVF. Most of the patients in our study (61%) had their first IVF cycle, but our group may be more heterogeneous.

We observed a high rate of mosaic embryos after both day 3 (50%) and day 5 analysis (45% for blastocysts and 65% for arrested embryos). Artefacts of the FISH procedure resulting in misdiagnosis are one possible explanation for the high rate of chromosomal mosaicism. However, most of it will represent true abnormalities in a mosaic embryo. This has been elegantly demonstrated in a recent publication, where they used probes for two different loci on the same chromosome (Daphnis *et al*., 2005) and found an error rate caused by artefacts of 5% per

nucleus. Furthermore, other studies, using techniques other than FISH, also described mosaicism in preimplantation embryos. This phenomenon was reported after performing conventional karyotyping on day 2 or 3 embryos (Jamieson *et al*., 1994). Comparative genomic hybridization (CGH) offers the advantage of allowing all the chromosomes to be analysed. Two groups using CGH on a small number of embryos confirmed the high rates of mosaicism observed by FISH (Wells and Delhanty, 2000; Voullaire *et al*., 2000, 2002). Using single cell multiplex fluorescent (FL)-PCR, mosaicism of trisomy 21 was confirmed in day 3 embryos diagnosed as aneuploid for chromosome 21 by FISH (Katz-Jaffe *et al*., 2004). This technique demonstrated a mitotic origin of trisomy 21 in half of the embryos investigated. Recently, a direct insight into the mechanisms leading to mosaicism has been provided in a study using confocal laser scanning microscopy in embryos immunolabelled with antibodies against tubulin (Chatzimeletiou *et al*., 2005). They observed various spindle abnormalities including abnormal shape and chromosome loss from the spindle, presumably leading to chromosome malsegregation to the daughter cells.

Although mosaicism is now becoming a well-accepted phenomenon in preimplantation embryos, its implications for PGS require more attention. The present study provides the first detailed reanalysis data of embryos analysed by PGS and clearly demonstrates the impact of mosaicism on the reliability of the PGS diagnosis. Our results show that the chromosomal constitution of the embryo on day 3 is by no means fixed. The first cell divisions may be successively hit by mitotic events leading to chromosome loss as well as chromosome gain, as hypothesized by Los *et al*. (2004). These abnormal cell divisions can persist as long as the embryonic genome is not fully active and cell cycle control is absent. So, mechanisms such as non-disjunction and anaphase lagging are responsible for the high percentage of mosaicism as observed in 8-cell embryos and in blastocysts (Coonen *et al*., 2004; Daphnis *et al*., 2005).

Reanalysis on day 5 can be used to investigate the reliability of the day 3 diagnosis. This has been used by several groups and very high confirmation rates have been reported (Magli *et al*., 2000; Gianaroli *et al*., 2001; Sandalinas *et al*., 2001; Emiliani *et al*., 2004; Staessen *et al*., 2004). However, very few details were given as to how the term 'confirmed' was defined. In the current study, we considered this from a cytogenetic point of view, so confirmation entails the chromosome constitution of the investigated blastomeres to be reflected in the embryo after analysis on day 5. In the current group of embryos, this was the case for only 54%. Confirmation rates could also be established from a clinical viewpoint, i.e. the embryo was correctly replaced or discarded after a normal or abnormal diagnosis. However, since it is not known how many diploid cells an embryo needs to be able to develop into a healthy child, this is impossible to determine for mosaic embryos.

We found the best confirmation rate after a diagnosis based on two cells, where both blastomeres showed the same chromosomal abnormality, either as a single aneuploidy or in combination with other abnormalities. In these embryos the aneuploidy most likely arose during meiosis or fertilization. In contrast, after a mosaic diagnosis the confirmation rate was

low. Especially from the 26 mosaic day 3 embryos that had developed into blastocysts on day 5, half of the embryos turned out to be chromosomally normal at that point. In line with these findings, a diagnosis based on one cell yielded poor confirmation rates, since a distinction between mosaicism and an abnormality from a meiotic origin cannot be made after analysis of one cell.

Another point for consideration is the impact of the biopsy procedure itself on the confirmation rate, since the removal of blastomeres changes the constitution of a mosaic embryo. When a biopsy of two cells is performed, two blastomeres lying next to each other are removed. The biopsy is therefore not random and the chance of removing the reciprocal daughter cells is ~25%. We found several examples in which the biopsy of two abnormal blastomeres may have 'cured' the embryo, yielding a grossly normal embryo on day 5.

Because of the biological phenomenon of mosaicism, PGS at the 8-cell stage will never be fully reliable. Even if the diagnosis is based on two cells, they are removed from the embryo and the chromosomal constitution of the remaining blastomeres is not known. Moreover, because of the compromised functioning of cell cycle checkpoints, the remaining embryo can continue to change cytogenetically until the embryonic genome becomes fully active, probably at the blastocyst stage (Wells *et al.*, 2005). The developmental potential of mosaic embryos will depend on the proportion of normal cells (Bielanska *et al.*, 2002). Although the general consensus is that embryos with <50% normal cells would be unlikely to survive beyond the implantation stage, this is impossible to assess. Therefore, no matter how many improvements are made to the technique of aneuploidy detection, it will be impossible to predict with 100% certainty the chromosomal status of the embryo at the time of transfer and beyond by performing genetic analysis at the 8-cell stage. A better understanding of the fate of mosaic embryos is needed before these embryos can be considered for transfer. Until this is resolved, PGS may result in good embryos being discarded or in chromosomally abnormal embryos being replaced.

In conclusion, reanalysis by means of FISH of the embryos on day 5 provides an improved understanding of the fate of abnormal blastomeres during embryo development and a valuable insight into the mechanisms of aneuploidy formation. We show that PGS after analysis of two blastomeres is effective in detecting abnormal embryos resulting from a meiotic non-disjunction event. Although current techniques of PGS result in limited accuracy, PGS may still offer an additional marker for embryo quality, and can thus contribute to an overall positive effect on ongoing pregnancy rates (Munné *et al.*, 1999, 2003).

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Appendix. Detailed FISH results from embryos cultured until day 5

Case no.	No. of cells	FISH results [no. of cells]
1	51	**2N [29] + 4N [8]**
		+1 [4]
		−18 [4]
		+22 [2]
		−21 [2]
		−1 [1]
		3N [1]
2	15	**2N [11] + 4N [1]**
		−7 [3]
3	58	**2N [42] + 4N [3]**
		−21 [8]
		+21 [2]
		−18 [1]
4	18	2N (XY) [9] + 4N [1]
		−X [7]
		+16 [1]
5	41	−22 [29]
		4N, −22, −22 [5]
		−18, −22 [1]
		−13, −22 [1]
		−1, −22 [1]
		+7, +15 [2]
		3N [2]
6	43	2N [34]
		+15 [5]
		+7 [2]
		−7 [2]
		+18 [1]
		−18 [1]
7	57	2N [9]
		+15 [45]
		−13, +15 [1]
		−15 [2]
8	26	2N [1]
		+22 [18]
		4N, +22, +22 [3]
		−15, +22 [2]
		+15, +22 [2]
9	19	3N [16] + 3N, +1 [1]
		3N, −13 [2]
10	36	2N (XY) [25] + 4N [2]
		3N [2]
		−7 [2]
		−X [2]
		+X [2]
		N [1]
11	64	**2N [53] + 4N [1]**
		−16 [7]
		−13 [2]
		+13 [1]
12	66	**2N [51] + 4N [4]**
		−16 [7]
		−21 [4]
		−13 [1]
13	17	2N, XY [8]
		−15 [4]
		−16 [1]
		−13, **−16**, −18 [1]
		−13 [1]
		−Y [1]
		−Y, −22 [1]
14	62	2N [6]
		+22 [55]
		+22, −15 [1]
15	30	2N (XY) [22]
		−7 [5]
		−1 [1]
		−1, −21 [1]
		XO [1]
16	76	**2N [64] + 4N [3]**
		−21 [5]
		+7 [1]

Appendix. Continued

Case no.	No. of cells	FISH results [no. of cells]
		–16 [1]
		–15 [1]
		–7, –7 [1]
17	81	**2N [81]**
18	61	**2N [54]**
		–X [3]
		–7 [2]
		–1 [1]
		3N [1]
19	34	2N [32]
		–18 [1]
		–15 [1]
20	48	**2N [44]**
		N [1]
		–21 [1]
		–13, –18 [1]
		–22 [1]
21	38	2N [29] + 4N [2]
		–15 [5]
		+7 [1]
		–7 [1]
22	38	2N [28]
		–7 [2]
		–7, –18 [1]
		–7, –22 [1]
		–1 [2]
		+1 [2]
		–18 [2]
23	52	2N [47] + 4N [2]
		–16 [1]
		–21 [1]
		–1 [1]
24	20	2N [2]
		4N +22, +22 [3]
		+1, +22 [5]
		+1, –7, +22 [5]
		+22 [1]
		+15, +22 [1]
		–7 [1]
		+1 [1]
		+1, +13, +22, +22 [1]
25	40	–21 [33]
		4N, –21, –21 [5]
		N –21 [1]
		N [1]
26	27	2N [14]
		+15, –**21** [6]
		+15 [4]
		–1 [1]
		–7, –1 [1]
		–13 [1]
27	49	2N (XY) [13]
		XXY [31]
		XXY, +1 [1]
		XXXY [4]
28	23	+**X**, +**16** [12]
		+X, –1, +15, +**16** [1]
		+**16** [5]
		+**1**, +15, +**16** [5]
29	50	2N [38]
		–**16** [7]
		+22 [3]
		–22 [1]
		–21[1]
30	27	2N [27]
31	52	2N [46] + 4N [3]
		+15 [1]
		–7 [1]
		–1 [1]
32	32	2N [20]
		3N [2]
		–15 [2]
		+22 [2]

Appendix. Continued

Case no.	No. of cells	FISH results [no. of cells]
		+15 [1]
		+7 [1]
		–7 [1]
		+13 [1]
		–13 [1]
		–16 [1]
33	127	2N [110]
		+1 [3]
		+18 [4]
		+21 [8]
		–21 [2]
		+22 [2]
34	103	2N [94]
		–22 [2]
		–13 [2]
		–18 [3]
		+15 [2]
35	28	2N [23]
		–13 [2]
		–21 [2]
		–22 [1]
36	32	2N [30]
		–X [1]
		–15 [1]
37	22	2N [20]
		+16 [1]
		+18 [1]
38	11	2N [9]
		–1 [1]
		–15, –X, –Y [1]
39	6	Near 4N [3]
		–15 [1]
		–X [1]
		–1, –7 [1]
40	10	Near 4N [8]
		–Y, –13, –21, –22 [1]
		–X, –Y [1]
41	8	2N [3]
		4N [2]
		N[1]
		–1, –13, +18, –16, –**22**, –**22** [1]
		–1, +13, –16, –18, –21, –**22** [1]
42	6	3N [6]
43	3	–**7**, +15, –**21** [3]
44	3	2N [3]
45	28	2N [13]
		–**18** [5]
		–21 [7]
		–7 [3]
46	9	2N [1]
		4N [1]
		–18 [4]
		+18 [2]
		–**1**, –16 [1]
47	10	3N [9]
		4N [1]
48	11	2N [8]
		+X [1]
		–X [1]
		–1 [1]
49	19	+1 [19]
50	7	–22 [6]
		+7, –22 [1]
51	10	–X, –18, –21, –21, –22 [2]
		–X, –18, –21, –21 [3]
		–X, –21, –21, –22 [1]
		–X, –1, –13, +16, –21, –21 [1]
		–X, –13, –16, –18, –21, –21, –22 [2]
		–X, +13, –18, –21, –21 [1]
52	14	2N [11]
		–**18** [5]
53	7	2N [6]
		–**15**, –X [1]

Appendix. Continued

Case no.	No. of cells	FISH results [no. of cells]
54	20	2N [14]
		+1 [2]
		−**Y** [2]
		−21 [1]
		−13 [1]
55	37	4N [33]
		3N [4]
56	22	−21 [18]
		−X, −21 [2]
		+15, −21 [1]
		4N, −21, −21 [1]
57	47	+16 [37]
		−7, +16 [4]
		+16, −22 [3]
		4N, +16, +16 [3]
58	30	2N [28]
		−1, −7 [1]
		+Y, +1 [1]
59	36	2N [22]
		4N [1]
		−15 [10]
		−21 [2]
		−18 [1]
60	30	2N [18]
		−7 [8]
		−21, −21 [2]
		−7, −15, −22 [1]
		−1 [1]
61	86	2N [4]
		+**22** [73]
		4N, +**22**, +**22** [4]
		3N, +**22** [2]
		−16, +**22** [1]
		−18, +**22** [1]
		−21, +**22** [1]
62	35	−16, −22 [33]
		−16, −18, −22 [2]
63	57	−**22** [51]
		4N, −**22** [5]
		−18, −21, −**22** [1]
64	30	2N [7]
		+21 [12]
		+7 [7]
		−21 [2]
		−7 [2]
65	25	2N [22]
		−13, −21 [3]
66	57	2N [45]
		N [2]
		−**21** [9]
		−1 [1]
67	42	2N [14]
		4N [4]
		−18 [20]
		−1 [3]
		+1 [1]
68	12	2N [11]
		4N [1]
69	18	2N [14]
		−22 [2]
		+1, −22 [1]
		−15 [1]
70	10	2N [9]
		−21 [1]
71	9	2N [1]
		−X, −1 [2]
		+1 [2]
		−X [2]
		+X [1]
72	13	2N [3]
		−7 [2]
		−**15** [1]
		−**15**, −16 [1]

Appendix. Continued

Case no.	No. of cells	FISH results [no. of cells]
		−1, −7, −**15** [1]
		−7, −**15**, −18 [1]
		−1, −15, −**15**, −21 [1]
		−7, −22, −18 [1]
		−7, −13, −18, −22 [1]
		−13, −16 [1]
73	10	2N [3]
		3N [4]
		+21 [1]
		+21, +22 [1]
		+13, +13, +21 [1]
74	5	2N [1]
		−1, −1 [2]
		−X, −1, −1, −15 [2]
75	6	2N [5]
		−16 [1]
76	14	2N [7]
		−**16** [5]
		−22 [2]
77	24	−**22** [19]
		−7, −**22** [4]
		+1, −**22** [1]
78	15	2N [10]
		−**1** [1]
		−**1**, −**7** [1]
		−**1**, +7 [1]
		−**1**, −21 [1]
		−**1**, +18, +18 [1]
79	24	2N [15]
		−13 [3]
		XXX [2]
		−1 [1]
		+1 [1]
		−18 [1]
		−22 [1]
80	5	**N** [4]
		4N [1]
81	10	2N [5]
		−X, +**13** [3]
		−7 [1]
		−7, −7 [1]
82	1	**XO**, −15, −7, −21, −22 [1]
83	3	2N [1]
		−**16** [2]
		−18 [1]