

Spectral karyotyping of fresh, non-inseminated oocytes

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The object of this study was to determine the mechanisms producing aneuploidy in female meiosis I by analysing the whole chromosome complement of human non-inseminated and unfertilized fresh oocytes. For this purpose, 131 fresh oocytes were obtained from 16 oocyte donors (24–48 years old). These oocytes were fixed immediately after retrieval and 47 good quality metaphases from 13 donors were analysed by spectral karyotyping to identify all 23 chromosome types. The data was divided into two maternal age groups, 24–34 ($n = 31$) and ≥ 35 years ($n = 16$). More non-disjunction (13 and 25%), unbalanced predivision (10 and 44%, $P < 0.01$) and balanced predivision (6 and 62%, $P < 0.001$) events were found in the older group of oocytes. There was an increase in balanced predivision with decreasing chromosome size ($P < 0.001$). The present results are the first obtained in fresh oocytes where all chromosomes were specifically identified, and support previous theories that predivision of chromatids is a major factor causing aneuploidy. Previous reports with inseminated, non-fertilized oocytes fixed ≥ 24 h after retrieval suffered from artefactual predivision of chromatids triggered by in-vitro ageing of oocytes.

Key words: aneuploidy/balanced predivision/PGD/preimplantation genetic diagnosis/trisomy 21

Introduction

The favourable pregnancy rates in post-menopausal women along with low miscarriage rates obtained after oocyte donation (Abdalla *et al.*, 1993; Yaron *et al.*, 1993; Balmaceda *et al.*, 1994) suggest that uterine receptivity is not the cause of implantation decline with maternal age. Direct assessment of the endometrium has shown no impairment with age in women 25–60 years old (Sauer *et al.*, 1993). A prospective study of paired recipients aged older and younger than 40 years sharing randomized oocytes from the same donor and receiving on average the same number of embryos showed no difference in pregnancy rates (Navot *et al.*, 1994; Hull *et al.*, 1996). The problem could be in the oocytes. The most obvious link between maternal age and embryo competence is the increase of aneuploidy with maternal age described in oocytes, embryos and spontaneous abortions (Hassold *et al.*, 1980; Warburton *et al.*, 1980; Munné *et al.*, 1995a; Dailey *et al.*, 1996).

Assessment of the chromosome component of human meiosis II (MII) oocytes has been attempted using different methods. Karyotype analysis has been extensively used, giving a 30% average rate of chromosome abnormalities (Martin *et al.*, 1986; Djalali *et al.*, 1988; Pellestor and Sèle, 1988; Ma *et al.*, 1989, 1994; Papadopoulos *et al.*, 1989; Angell *et al.*, 1991, 1993, 1994; Selva *et al.*, 1991; Tarin *et al.*, 1991; Zenzes *et al.*, 1992; Kamiguchi *et al.*, 1993; Roberts and O'Neill, 1995; Angell, 1997; Boiso *et al.*, 1997; Lim and Tsakok, 1997). However, because of the difficulty of banding oocyte chromosomes, most of these studies have not identified specific missing or extra chromosomes, but merely carried out chromosome counts. Information on specific aneuploidy rates was first obtained with multicolour multi-probe fluorescence in-situ hybridization (FISH)

(Dailey *et al.*, 1996; Dyban *et al.*, 1996; Verlinsky *et al.*, 1996a,b; Mahmood *et al.*, 2000). This proved the expected correlation between non-disjunction and maternal age in oocytes (Dailey *et al.*, 1996), as observed previously in prenatal data (Hassold *et al.*, 1980; Hassold and Chiu, 1985).

Regardless of the method used, several mechanisms causing aneuploidy have been identified. One, non-disjunction (or premature disjunction) of bivalent chromosomes during meiosis I (MI), is observed as extra or missing monovalent chromosomes in MII oocytes. This is the mechanism most commonly reported. A second mechanism, premature division of chromatids during MI (Angell *et al.*, 1991; Angell, 1997), produces missing chromatids ($22+1/2$), extra chromatids ($23+1/2$) or a balanced karyotype with separation of chromatids for one univalent chromosome ($22+1/2+1/2$) (Kamiguchi *et al.*, 1993; Lim *et al.*, 1995; Dailey *et al.*, 1996; Verlinsky *et al.*, 1996a,b; Angell, 1997; Lim and Tsakok, 1997; Marquez *et al.*, 1998). A less commonly reported mechanism is the occurrence of gonadal mosaicism produced by mitotic non-disjunction in primordial cells (Vig, 1984; Cozzi *et al.*, 1999; Costa and Wilton, 2000; Mahmood *et al.*, 2000).

Angell proposed that predivision was the major mechanism of aneuploidy (Angell *et al.*, 1994; Angell, 1997), but other studies attributed most balanced predivision events to oocyte ageing *in vitro* (Munné *et al.*, 1995a; Dailey *et al.*, 1996) or oocyte degeneration (Lim and Tsakok, 1997). Because most studies had analysed oocytes aged 24–72 h *in vitro*, the importance of predivision of chromatids in human aneuploidy remained unclear. One of the main purposes of the present study was to determine the importance of predivision of chromatids in human aneuploidy by analysing non-inseminated, fresh oocytes, thus minimizing in-vitro ageing.

The second purpose was to ascertain which specific chromosomes are more susceptible to maternal MI errors resulting in aneuploidy. Because FISH can only identify a few chromosomes simultaneously, an alternative is the use of spectral karyotyping (SKY), which can identify each one of the 23 chromosome types present in the oocyte (Schröck *et al.*, 1996). This technique was first applied in human oocytes by Márquez *et al.* (Márquez *et al.*, 1998) and was also used in the present study.

Materials and methods

Oocytes

Oocytes were obtained from the IVF programme of The Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center in accordance with guidelines set by their internal review board. Written consent was obtained from the participants in each case. Oocytes were produced from fertile oocyte donors with the sole purpose of being donated for research. These donors underwent hormonal stimulation to produce superovulation. At no time were any of these oocytes fertilized, in accordance with the National Institute of Health (NIH) guidelines for NIH funded research on gametes and embryos.

Immediately after retrieval the oocytes were denuded from the corona cells; those that presented a first polar body were dipped in Tyrode's acid to remove the zona pellucida, and oocytes and polar bodies were fixed independently according to a previously described method (Tarkowski, 1966). The position of the oocytes and polar bodies was circled with a diamond pencil and the slide was dehydrated in 70, 85 and 95% ethanol (2 min each) and then incubated at 65°C for 12 h prior to SKY. Fixed oocytes and polar bodies were hybridized with SKY probes as previously described (Márquez *et al.*, 1998) without modification, and the images were analysed using a SKYVision spectral imaging system (Applied Spectral Imaging, Carlsbad, CA, USA) mounted on an Olympus BX60 fluorescence microscope also as described previously by the same authors.

Scoring criteria and nomenclature

Only MII oocytes with all their chromosomes identified by SKY were included in this study. When the oocyte had 23 monovalent chromosomes, each with two associated chromatids, the oocyte was considered to be normal. However, when there were extra or missing monovalent chromosomes, the oocyte was considered to be aneuploid due to non-disjunction. In the event that single chromatids were present, the oocyte was considered to have suffered premature predivision of chromatids. Such predivision could be balanced, if there were 22 monovalent chromosomes plus two single chromatids for the monovalent missing, or unbalanced when there were 23 monovalent chromosomes plus a chromatid or 22 monovalents plus a chromatid. Both balanced and unbalanced predivision may produce normal or abnormal zygotes depending on the random distribution of single chromatids during MII.

Most studies on oocytes have found higher rates of monosomy than trisomy, usually attributing it to chromosome loss during fixation (Pellestor and Sele, 1988; review), and thereafter counting non-disjunction as double the frequency of trisomy. Here, we counted monosomies as real based on our previous studies of human embryos that show more monosomies than trisomies, accepting that this excess is not entirely caused by chromosome loss during fixation (Munné *et al.*, 1995b). Nevertheless, when cells with three or more nullisomies were found, they were not included in the study and were considered fixation chromosome losses.

Statistical analysis

The incidence of abnormalities from variable numbers of oocytes was subjected to a logistic regression analysis in order to investigate the association between maternal age and these abnormalities. The variable analysed was a transformation of the proportion of oocytes (for each donor) displaying the various abnormalities, and the maternal age was categorized into two maternal age groups. In order to summarize the findings from the analyses, the variable analysed was back-transformed onto the original scale of proportions.

Results

A total of 131 oocytes (MII oocyte plus polar body complexes) were obtained from 16 donors with maternal ages of 24–48 years. Of these 131 oocytes, 126 showed a metaphase after fixation, but 68/126 had at least two overlapping chromosomes and 11/126 had incomplete metaphases. Although oocytes with overlapping chromosomes produced a chromosome count, they were uninformative for aneuploidy because overlapping chromosomes could be of any type, thus masking aneuploidy. The remaining 47/126 oocytes could be analysed by spectral karyotyping. These 47 oocytes were obtained from 13 donors (six from patients aged 20–34 years and seven from patients aged 35–45 years). Only three first polar bodies produced analysable results and they were not included in the study. Representative spectral karyotyping of oocyte and polar body metaphases are shown in Figures 1–3.

Of the 47 analysable oocytes, eight had non-disjunction events, 10 had extra or missing chromatids, two were diploid and two had chromosome breaks (Table I). In addition, regardless of whether they were aneuploid or not, 12 oocytes had balanced predivision. The data was divided into two maternal age groups, 24–34 ($n = 31$) and ≥ 35 years ($n = 16$). More non-disjunction (13 and 25%, not significant), unbalanced predivision (10 and 44%, $P < 0.01$) and balanced predivision (6 and 62%, $P < 0.001$) were found in the older group of oocytes. When balanced predivision was analysed across three maternal age groups, it increased from 6.5% in women ≤ 34 years to 25% in women 35–39 years, and up to 75% in women ≥ 40 years ($P < 0.001$). Table II shows the specific abnormalities found in each oocyte.

Table III shows the aneuploidy rates per chromosome. There was a clear increase in balanced predivision with decreasing chromosome size, with most balanced predivision events accumulating in the smaller chromosomes, groups E–G ($P < 0.001$), rather than in larger chromosomes, groups A–D.

Discussion

This study had two major purposes. One was to determine the importance of predivision of chromatids in human aneuploidy by assessing fresh oocytes, which are supposedly not affected by artefactual predivision of chromatids produced by in-vitro ageing. The other purpose was to determine, using SKY, which chromosomes were more involved in human aneuploidy originating in maternal MI.

While SKY provided important data for each chromosome, the spreading technique was an obvious limitation. Metaphases with a few overlapping chromosomes can usually be analysed by dual chromosome painting to identify translocations (Munné *et al.*, 1998, 2000), but a whole SKY karyotype cannot be obtained because the overlapping chromosomes cannot be identified. To date, we have only been able to obtain SKY results in ~35% of the oocytes fixed (Márquez *et al.*, 1998; present study); results with first polar bodies are even worse, with only three of the polar bodies from this study producing analysable SKY results. Better fixation methods may help to increase the yield of analysable oocytes and polar bodies. Otherwise, an optimized probe set consisting of seven fluorochrome combinations instead of five may reduce ambiguity in certain overlap situations (Azofeifa *et al.*, 2000).

Balanced predivision and maternal age

The present results are the first obtained from fresh, non-inseminated MII human oocytes in which all chromosomes were individually identified. The results indicate that with increasing maternal age there is a very significant increase in balanced predivision, a moderate increase in unbalanced predivision and an as yet insignificant increase

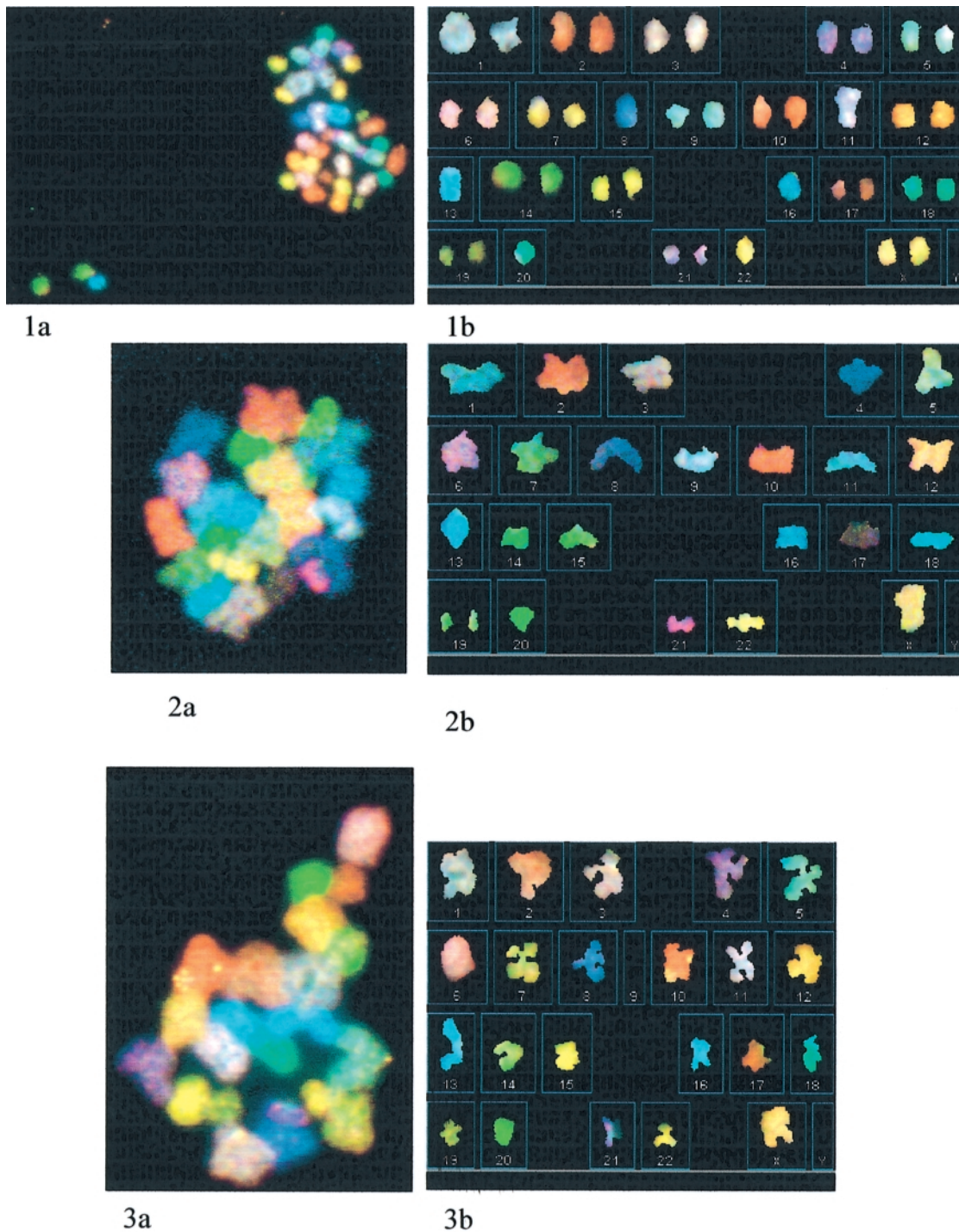


Figure 1. (a) Polar body metaphase. (b) Karyotype (23,X) of the same metaphase showing balanced predivision of chromatids for all chromosomes except for 8, 11, 13, 16, 20 and 22.

Figure 2. (a) Oocyte metaphase. (b) Karyotype of the same metaphase showing an extra chromatid 22 and balanced predivision for chromosome 19.

Figure 3. (a) Oocyte metaphase. (b) Karyotype of the same metaphase with a missing univalent (22,X,-9).

in non-disjunction. The results support previous reports by Angell and Lim and Tsakok, who detected an increase of predivision with maternal age (Angell, 1997; Lim and Tsakok, 1997). In the past we had questioned Angell's results because they were performed on in-vitro aged oocytes, which already contained balanced predivision caused by the ageing process (Munné *et al.*, 1995a; Dailey *et al.*, 1996). However, the present results demonstrate that Angell's theory is correct and that predivision of chromatids is an important cause of aneuploidy strongly linked with maternal age.

An increase in balanced predivision with maternal age has also been reported by Lim and Tsakok, but they found 20–25% more predivision of chromatids in each maternal age group than did the present study (Lim and Tsakok, 1997). This extra 20–25% predivision is the amount expected to be produced after 48 h of in-vitro ageing, as previously described (Dailey *et al.*, 1996). Thus, by analysing fresh oocytes, we obtained a more accurate assessment of predivision than previous studies.

Individual chromosome data from cleavage-stage human embryos

Table I. Number of abnormal karyotypes in non-inseminated fresh human oocytes

	20–34 years (%)	35–45 years (%)	Total	<i>P</i> -value
Donors	6	7	13	
Oocytes analysed	31	16	47	
Oocytes normal or haploid (no predivision)	20	4	24	
Oocytes with non-disjunction ^a				
Extra univalent	0	2	2	
Missing univalent	4	2	6	
Total non-disjunction	4 (13)	4 (25)	8	NS
Oocytes with unbalanced predivision ^a				
Extra chromatid	1	5	6	
Missing chromatid	2	2	4	
Total unbalanced predivision ^a	3 (10)	7 (44)	10	< 0.1
Oocytes with balanced predivision	2 (6)	10 (62)	12	< 0.001
Total aneuploidy excluding balanced predivision ^a	9 (29)	9 (56)	18	< 0.01
Oocytes with structural abnormalities ^a	2	0	2	
Diploid oocytes ^a	2	0	2	

^aOocytes with one or more types of abnormality are counted in each line that applies, but only once in the total of aneuploidy. NS = not significant.

(Munné *et al.*, 1995b; Márquez *et al.*, 2000) show aneuploidy rates similar to the rates found in this study for non-disjunction plus unbalanced predivision. If confirmed after a larger series of oocyte studies, it would mean that balanced predivision does not have a great impact on aneuploidy. More research on balanced predivision and its importance or not in producing aneuploid embryos is clearly needed.

Chromosome mosaicism in human cleavage-stage embryos does not seem to increase with maternal age (Munné *et al.*, 1995b; Márquez *et al.*, 2000), and therefore the observed complex chromosome imbalances in human oocytes may result not in mosaicism but mostly in multiple aneuploidy.

The fact that non-disjunction found with SKY did not increase with maternal age in this study nor in that of Márquez *et al.* is probably due to the small sample size of oocytes from women of ≥ 40 years old (Márquez *et al.*, 1998). When a sizeable group was analysed, as per Dailey *et al.*, non-disjunction did increase with maternal age (Dailey *et al.*, 1996).

Balanced predivision and chromosome size

There was a clear increase in balanced predivision with decreasing chromosome size, with most balanced predivision events involving chromosomes 16, 17, 18, 19, 20, 21 and 22, but no correlation was observed between chromosome size and unbalanced predivision, nor for non-disjunction. If predivision occurred solely at MI and the products of predivision were distributed randomly, a proportion of 2:1 balanced:unbalanced predivision would be expected (Angell, 1997). However, that proportion was 2.6:1 for all chromosomes and 3.4:1 for chromosomes 16–22. This suggests that some balanced predivision events were produced during MII, before anaphase II. This excess of balanced predivision could be a result of oocyte degeneration, as has been previously indicated (Lim and Tsakok, 1997). For example, four oocytes (5–19, 5–20, 5–23, 10–37 in Table II) concentrated 20/34 of balanced predivision events, indicating that they could have been degenerating. The embryos resulting from these

Table II. Results divided according to oocyte

Patient	Oocyte	Age (years)	Karyotype	Chromosome with balanced predivision
1	1	35	24,X,+14,+ctd 22,+ctd 18	
2	2	37	23,X,+ctd 14	22
2	3	37	23,X	
2	4	37	23,X	
3	5	32	23,X,csb 11	
3	6	32	23,X,-ctd 16	21
3	7	32	23,X,+ctd 6	
3	8	32	23,X,+ctd 22,+ctd 19	
3	9	32	46,XX	
3	10	32	23,X	
4	11	24	22,X,-18,-ctd 17	
4	12	24	22,X,-9	
4	13	24	23,X	
4	14	24	46,XX	
4	15	24	23,X	
4	16	24	23,X	
4	17	24	23,X	
4	18	24	23,X	
5	19	43	23,X,-ctd 14	16, 17, 18, 19, 20, 22
5	20	43	23,X,+ctd 8	2, 8, 11, 16, 21
5	21	43	22,X,-12	22
5	22	43	22,X,-11	
5	23	43	23,X	10, 19, 20, 22
6	24	32	23,X,csb 6	2
6	25	32	22,X,-22	
6	26	32	21,X,-9,-20	
6	27	32	23,X	
6	28	32	23,X	
6	29	32	23,X	
6	30	32	23,X	
6	31	32	23,X	
7	32	27	23,X	
7	33	27	23,X	
7	34	27	23,X	
8	35	48	23,X	
9	36	44	24,X,+21	18
10	37	40	23,X,-ctd 14	2, 7, 16, 17, 22
10	38	40	23,X	19, 20, 21
10	39	40	23,X	16, 21
11	40	40	23,X,+ctd 22	19
11	41	40	23,X	
13	42	26	23,X	
13	43	26	23,X	
14	44	29	23,X	
14	45	29	23,X	
14	46	29	23,X	
14	47	29	23,X	

Ctd = chromatid; csb = chromosome break.

four oocytes would probably never have implanted, explaining why most aneuploidy detected in first trimester pregnancies is classified as occurring at MI (Antonarakis *et al.*, 1991; Hassold *et al.*, 1995; Lamb *et al.*, 1996; Robinson *et al.*, 1996).

Oocyte degeneration in older women may imply deficient protein synthesis, thus affecting the proteins that maintain cohesion between sister chromatids up to anaphase II. Several mutations producing premature separation of chromatids have been described (Cohen-Fix, 2000; Van Heemst and Heyting, 2000). For instance, lack of Rec8 activity, a protein from the cohesin complex that confers cohesion to sister chromatids, results in MI in equatorial separation (of chromatids) instead of reductional separation (of homologues). Rec8 is removed in anaphase I from the arms of the chromatids, allowing the chiasmata to be terminated and the homologues to separate. This is mediated by a complex called separin, which is inhibited by securin up to anaphase I. Mutations in securin also produce predivision of chromatids. After anaphase I, Rec8 remains in the centromeric region

Table III. Aneuploidy rates for each specific chromosome

Chromosome number	Non-disjunction	Balanced predivision	Unbalanced predivision	Total per chromosome
1	0	0	0	0
2	0	3	0	3
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	1	1
7	0	1	0	1
8	0	1	1	2
9	2	0	0	2
10	0	1	0	1
11	1	1	0	2
12	1	0	0	1
X	0	0	0	0
13	0	0	0	0
14	1	0	3	4
15	0	0	0	0
16	0	4	1	5
17	0	2	1	3
18	1	2	1	4
19	0	4	1	5
20	1	3	0	4
21	1	4	0	5
22	1	5	3	9
Total	9	31	12	52

The smaller chromosomes (groups E–G) were involved in balanced predivision significantly more often ($P < 0.001$) than the larger chromosomes (groups A–D).

up to anaphase II. Separin cannot remove Rec8 from the centromere because it is presumably protected there by MEI-S322. Mutations of MEI-S322 cause Rec8 to disassociate from the centromere, resulting in equatorial (chromatid) separation.

Recently, Mam1 has been described to promote monopolar attachment of microtubules to sister chromatid kinetochores (Toth *et al.*, 2000), thus ensuring that sister chromatids segregate to the same pole during MI. In mutants of Mam1, the sister kinetochores are pulled to different poles by microtubules, against Rec8 cohesion forces, and a proportion of sister chromatids separate prematurely during anaphase I.

Any disturbance of the equilibrium of these meiosis-specific proteins could result in the premature predivision of chromatids. For instance, the longer the chromosome, the more Rec8 would be involved in their cohesion and therefore, if these proteins are synthesized in suboptimal quantities in compromised oocytes, the chromatids from small chromosomes may suffer more predivision during MII. Low levels of Mam1 transcripts may allow bipolar attachment of microtubules to sister chromatid kinetochores, and small chromosomes containing less cohesin to hold together sister chromatid arms, and fewer chiasmata between homologues, may cause more susceptibility to premature predivision of chromatids. For instance, it has been described that distal chiasma predispose to non-disjunction of chromosome 21 (Lamb *et al.*, 1996), probably because there is less cohesion distal to the chiasma, and when the arm cohesion is lost, homologues and/or chromatids disassociate and segregate at random. The first evidence that disturbances in the genetic expression of meiosis-specific genes may be related to aneuploidy comes from Steuerwald *et al.* who described that the transcripts of spindle attachment checkpoint genes, such *MAD2* and *BUB1*, are found in lower concentrations in oocytes of older women, who are usually more likely to produce aneuploid oocytes (Steuerwald *et al.*, 2001).

Future studies should study chromosome numerical abnormalities simultaneously with the expression and localization of the proteins involved in meiosis. An initial step in this direction was the elegant work of Hunt *et al.* who simultaneously studied chromosome X non-disjunction and spindle structure (Hunt *et al.*, 1995).

Note added in proof

Individual chromosome data from cleavage-stage human embryos (Munné *et al.*, 1995b; Márquez *et al.*, 2000) show aneuploidy rates similar to the rates found in this study for non-disjunction plus unbalanced pre-division. If confirmed after a larger series of oocyte studies, it would mean that balanced pre-division does not have a great impact on aneuploidy, but it may still have an effect on other chromosome abnormalities. Overall chromosome mosaicism in human cleavage-stage embryos did not seem to increase with maternal age (Munné *et al.*, 1995b; Márquez *et al.*, 2000). However, we have recently found that a subgroup of mosaics, those produced by mitotic non-disjunction, do increase significantly with advanced maternal age (Munné *et al.*, 2002). A compromise meiotic/mitotic apparatus may result in either aneuploidy or mitotic non-disjunction during the cleavage stage. It is therefore possible that this age-related increase in balanced pre-division could be the cause of mitotic non-disjunction.

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