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Mitotic errors in chromosome 21 of human preimplantation embryos are associated with non-viability

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Fluorescent *in situ* hybridization (FISH) studies of human preimplantation embryos have demonstrated a high proportion of chromosomal mosaicism. To investigate the different timings and nature of chromosomal mosaicism, we developed single cell multiplex fluorescent (FL)-PCR to distinguish meiotic and mitotic cell division errors. Chromosome 21 was investigated as the model chromosome as trisomy 21 (Down's syndrome) represents the most common chromosomal aneuploidy that reaches live birth. Sister blastomeres from a total of 25 chromosome 21 aneuploid embryos were analysed. Of these, 13 (52%) comprised cells with concordant DNA fingerprints indicative of meiotic non-disjunction errors. The remaining 12 (48%) aneuploid embryos comprised discordant sister blastomere allelic profiles and thus were mosaic. Errors at all stages including metaphase (MI) (12%) and first (38%), second (31%) and third (19%) mitotic cleavage divisions were identified from the types and proportion of different allelic profiles. In addition, three embryos showed combined meiotic and mitotic cell division errors including non-disjunction and anaphase lag, suggesting that diploid cells had resulted from an aneuploid zygote. However, the majority of the mosaic aneuploid embryos showed mitotic gains and losses from a diploid zygote occurring prior to the activation of the embryonic genome. Allelic profiling of amniocytes from 15 prenatal diagnosis samples displayed only meiotic errors. There appears to be a large difference between the proportion of mosaic mitotic-derived trisomy 21 embryos and fetuses. These findings indicate that mosaic mitotic error of chromosome 21 is associated with non-viability.

Key words: aneuploidy/chromosome 21/early embryos/mosaicism

Introduction

Chromosomal mosaicism is defined as the presence of two or more different chromosomal complements in a cell and is believed to develop in preimplantation embryos as a result of abnormal mitotic cell division. Mitotic errors can produce either an aneuploid cell line in a diploid conception or a diploid cell line in an aneuploid conception. About 20% of human oocytes (depending on maternal age) and 2–4% of human sperm are aneuploid due to a meiotic error in gametogenesis (Hassold and Jacobs, 1984; Hook, 1985). Post-fertilization mitotic errors, such as mitotic non-disjunction and anaphase lag, have been associated with the high incidence of chromosomal mosaicism detected in human preimplantation embryos (Coonen *et al*., 1994; Munné *et al*., 1994; Harper *et al*., 1995; Delhanty *et al*., 1997; Kuo *et al*., 1998; Bielanska *et al*., 2002). Chromosomal mosaicism has been shown to increase with abnormal embryonic cleavage and morphology (Almeida and Bolton, 1996; Magli *et al*., 2000; Sandalinas *et al*., 2001), and both sub-optimal embryo culture and exogenous hormonal stimulation have been implicated (Munné *et al*., 1997). In a recent study of a large series of human embryos, a significant maternal age effect was observed for mosaics involving mitotic non-disjunction of a single chromosome including chromosomes 13, 18 and 21 (Munné *et al*., 2002).

Our current understanding of the origin, nature and regulation of mitotic chromosomal segregation during early human embryonic development remains inadequate. In addition, the impact of chromosomal mosaicism on potential implantation and further fetal development is not well understood. Cytogenetic studies have shown that most aneuploid fetuses result in spontaneous miscarriages during the first trimester (Hassold *et al*., 1980) and that only ~5% of those trisomies compatible with development are mosaic (Hook and Cross, 1983; Antonarakis *et al*., 1993; Mikkelsen *et al*., 1995; Yoon *et al*., 1996). There seems to be a large difference in the proportion of mosaic embryos detected on day 3 or 4 of development (Munné *et al*., 1994; Delhanty *et al*., 1997; Kuo *et al*., 1998) compared to the proportion of mosaic fetuses detected in the pre- and post-natal period (Hsu and Perlis, 1984; Boué *et al*., 1985), indicating that there is a strong selection against the latter. The frequent occurrence of chromosomal mosaicism among human preimplantation embryos may contribute to the relatively low success rate of human IVF. Based on mosaicism assessment after embryo cryopreservation (Tarin *et al*., 1992) and *in vitro* culture of embryos to blastocyst stage (Sandalinas *et al*., 2001), >3/8 abnormal blastomeres in a preimplantation embryo is considered detrimental for embryo survival. In reality, it is not known how many diploid cells in a preimplantation embryo are required to establish a viable pregnancy.

To precisely map the nature and origin of chromosomal aneuploidy that leads to embryonic mosaicism, we developed a multiplex fluorescent (FL)-PCR system to specifically assess the allelic status of chromosome 21. FL-PCR analysis of sister blastomeres from day 3 embryos diagnosed as aneuploid for chromosome 21 by FISH, identified both metaphase (M) I and MII errors and mapped the timing of mitotic errors that preceded the development of these embryos. FL-

Table I. The performance of the chromosome 21 microsatellite markers on single cells

Microsatellite markers[a]	Allelic size range	Label	Heterozygosity index (%)	Reliability (%)	Allele drop-out rate (%)
D21S11	172–264	6-FAM	0.90	95	4.5
D21S1411	269–313	HEX	0.93	89	11
D21S1413	152–184	NED	0.88	84	7
D21S1437	107–151	6-FAM	0.93	94	5
D21S1442	225–261	NED	0.80	94	6

[a]Primers for each marker were multiplexed in a single reaction.

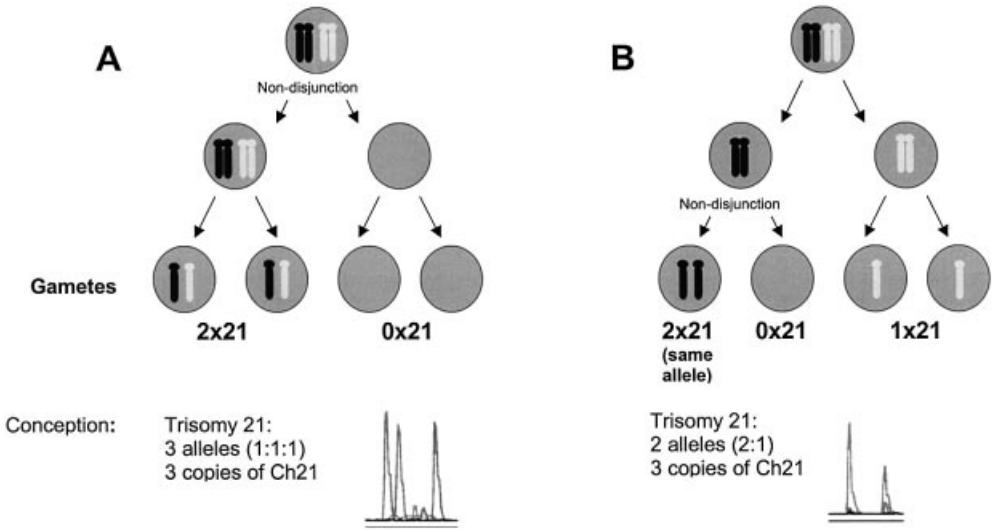

Figure 1. Meiotic non-disjunction: (**A**) metaphase (M) I error, (**B**) MII error (unable to dicriminate between MI and MII error for monosomy 21 embryos).

PCR analysis of amniocytes identified only meiotic errors, demonstrating a large difference between the proportion of mosaic mitotic-derived trisomy 21 embryos and fetuses.

Materials and methods

Embryos

Twenty-five chromosome 21 aneuploid cleavage stage embryos (average of six to eight blastomeres) diagnosed first by FISH (probing five chromosomes: 13, 18, 21, X and Y) as genetically abnormal were obtained from 21 PGD patients (mean age 39.3 years) undergoing IVF treatment for infertility. The majority (65%) of these aneuploid chromosome 21 embryos were euploid for the other chromosomes analysed by FISH. Under guidelines established by the Infertility Treatment Authority in Victoria, Australia, aneuploid embryos deemed unsuitable for transfer were left to 'succumb' on the bench for 24 h. The vast majority of these embryos were morphologically normal with varying degrees of fragmentation and contained on average between six and eight blastomeres. Succumbed aneuploid embryos were treated with pronase (2 mg/ml in HEPES-buffered human tubal fluid culture medium) for 1 min to dissolve the zona pellucida and transferred into Ca^{2+}/Mg^{2+}-free medium to dissociate the blastomeres. Single blastomeres were examined under an inverted microscope (Leica MS5) and collected in finely pulled 22.9 cm long glass Pasteur pipettes (Becton Dickinson, USA). Each blastomere was carefully washed through three 5 µl drops of phosphate-buffered saline (PBS) and transferred into sterile 0.2 ml PCR tubes for FL-PCR analysis.

Amniocytes

Clear amniotic fluids (n = 15) were collected from women between 14 and 20 weeks of gestation. The most frequent indication for fetal sampling was advanced reproductive age (ara) and abnormal biochemical or ultrasound screening. After cytogenetic analysis identified the amniotic cultures as aneuploid for chromosome 21, cells were made available for molecular diagnosis. An aliquot of cells was removed from the culture dishes and washed several times in PBS, centrifuged at 6000 g and re-suspended in 500 µl PBS. A 10 µl aliquot was examined for nucleated cells and only intact single cells were collected and washed through three 5 µl drops of PBS before transfer into sterile 0.2 ml PCR tubes. A total of 12 single amniocytes were collected and analysed for each sample obtained.

FL-PCR and Genescan analysis

The five chromosome 21 tetranucleotide microsatellite markers used in this multiplex FL-PCR system were chosen with high heterozygosity indices and broad allelic distributions (Table I). Primers were synthesized and fluorescently labelled (6-FAM, HEX or NED) by Applied Biosystems, Australia. All primer pairs were diluted in molecular biology grade H_2O (Sigma, Australia) to 200 pmol/µl stock solutions under sterile conditions and stored in aliquots of 100 pmol/µl at –20°C until use. Single blastomeres were subjected to the single cell multiplex FL-PCR system as previously described (Katz *et al*, 2002): 2.5 µl of 10×Taq PCR Buffer (500 mmol/l KCl, 100 mmol/l Tris–HCl, pH 9.0 and 15 mmol/l $MgCl_2$), 0.5 µl of 10 mmol/l dNTP (200 µmol/l), 0.3 µl of Taq polymerase (5 IU/µl) (Amersham Pharmacia Biotech, Australia), 12.7 µl MQ-H_2O and 9 µl of primer mix (5–25 pmol of each primer pair) making a final volume of 25 µl. A total of 36 thermal cycles using the 9700 Thermocycler PCR machine (Applied Biosystems) of denaturation for 45 s at 94°C, annealing for 45 s at 60°C, and extension for 1 min at 72°C were performed. Positive control tubes contained 10–20 cells in 1–2 µl of PBS buffer, whereas negative control tubes contained either 1–2 µl of PBS buffer from the last wash droplet or no cell. All FL-PCR products were analysed using the ABI Prism 3100 DNA sequenator coupled with Genescan software (Applied Biosystems).

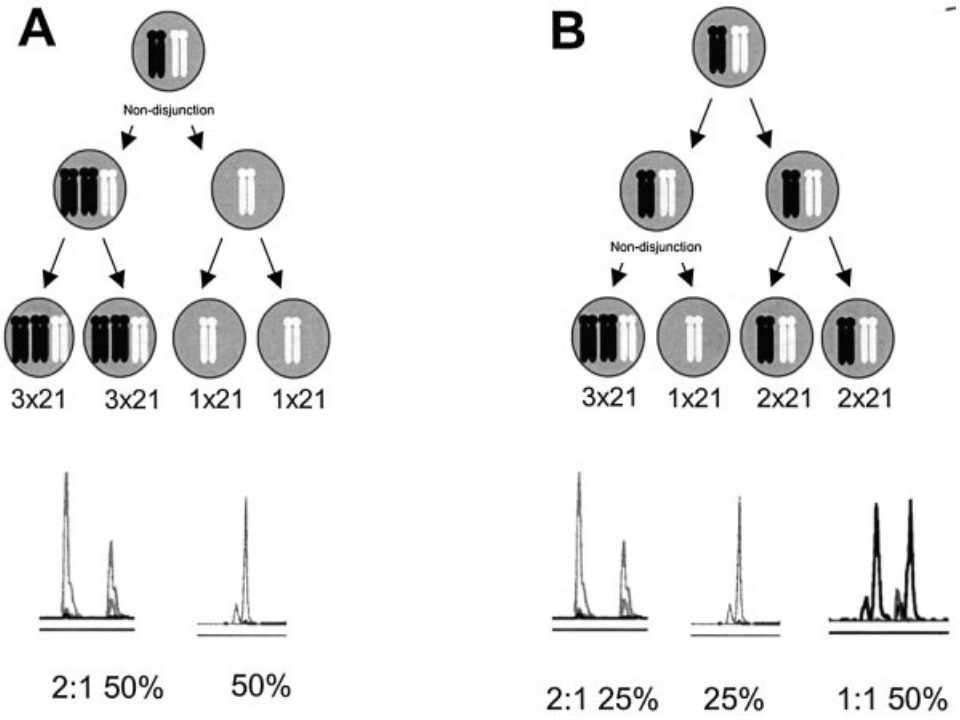

Figure 2. Mitotic non-disjunction post fertilization diploid conception (the proportion and type of allelic profiles identifies the timing and nature of the cell division error). (**A**) First cleavage division: 50% trisomic (2:1) and 50% monosomic blastomeres. (**B**) Second cleavage division: 25% trisomic (2:1), 25% monosomic and 50% diploid (1:1) blastomeres.

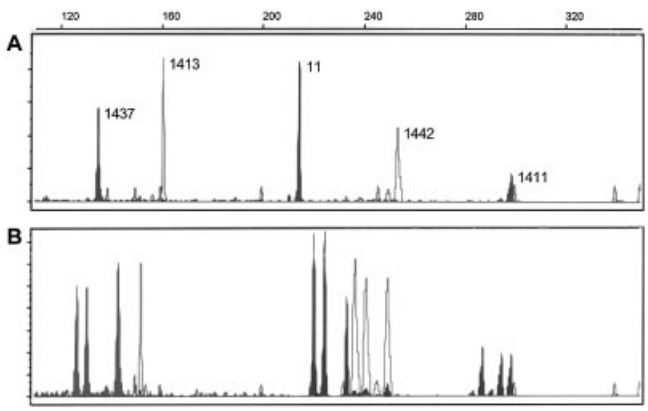

Figure 3. Allelic profiles of single blastomeres: (**A**) monosomy 21, (**B**) trisomy 21.

Allelic profile analysis

Profiles were analysed according to the allelic ratios at each individual microsatellite marker locus and the cumulative results used to identify the chromosome 21 status of each single cell and respective embryo. A diploidy status was determined by the presence of two alleles with an allelic ratio of 1:1, a monosomy status by a consistent mono-allelic pattern across all five microsatellite marker loci and a trisomy status by either the presence of a tri-allelic pattern or a double dosage di-allelic pattern across at least three microsatellite marker loci. Analysis of the respective allelic patterns in the blastomeres of preimplantation embryos allows the identification of the nature, origin and timing of the cell division error. The different chromosomal complements and respective allelic profiles expected for non-disjunction during meiosis (Figure 1) or from non-disjunction during mitosis (Figure 2) after a diploid conception are shown for reference. For example, an MI error during oogenesis results in an oocyte with both maternal copies of chromosome 21, thus two distinguishable alleles at a heterozygous locus (Figure 1A).

Results

Performance of the chromosome 21 FL-PCR multiplex system

The chromosome 21 FL-PCR multiplex system was firstly optimized on single cells to achieve strong and consistent amplification of alleles before assessment for reliability and accuracy on 50 single buccal cells diploid for chromosome 21 and 50 single buccal cells from a patient with Down's syndrome (Table I). Reliability was defined as the number of successful allelic amplifications, calculated at 91% for the diploid cells and 93% for the trisomy 21 cells. Accuracy was defined as the number of correct amplifications, calculated at 96% for the diploid cells and 94% for the trisomy cells. Allele drop-out rate (ADO) was observed at <8% for both cell types, while allelic preferential amplification was observed at 10–12% (Katz *et al*., 2002).

Analysis of chromosome 21 aneuploid human IVF embryos

A total of 172 blastomeres (average 6.9 per embryo) were analysed from the 25 chromosome 21 aneuploid embryos and the DNA fingerprint profiles for most blastomeres showed strong allelic amplification with virtually no non-specific background interference to confound interpretation. Figure 3 shows an example of an allelic profile of a blastomere with only a single copy of chromosome 21 and a blastomere with three copies of chromosome 21. Both of these diagnoses were concordant with the original FISH result. Reliability was calculated at 85%, with an informative allelic profile identified when three or more microsatellite markers amplified from a single cell. The overall chromosome 21 status of each embryo was established when the allelic profiles of the majority (>50%) of the blastomeres were acceptable. The 85% reliability observed on blastomeres was significantly lower than the >91% obtained from buccal cells and amniocytes (χ^2-test, $P < 0.05$). Aneuploid embryos

Table II. Nature and timing of chromosomal aneuploidy in 25 chromosome 21 aneuploid human preimplantation embryos

Embryo class	Cell division error	No. with error
Homogeneous aneuploid embryos (n = 13)	Trisomy 21	n = 6 (MI = 5, MII = 1)
	Monosomy 21	n = 7 (MI or MII)
Mosaic aneuploid embryos (n = 12)	1st cleavage division	n = 6 (38%)
	2nd cleavage division	n = 5 (31%)
	3rd cleavage division	n = 3 (19%)
	Meiotic and mitotic combined errors	n = 2 (12%)

MI = metaphase I; MII = metaphase II.

were left to 'succumb' on the bench for 24 h before dissociation whereas buccal cells were freshly isolated and analysed. It has been widely reported that blastomeres from arrested and fragmented embryos yield much lower amplification efficiencies (Ray *et al*., 1998; Findlay *et al*., 1999), possibly due to partial or total nuclear DNA degeneration (Cui and Matthews, 1996).

Of the 25 chromosome 21 aneuploid embryos diagnosed by FISH, 13 (52%) comprised sister blastomeres with identical chromosome 21 results. All the blastomeres from each of these embryos showed the same chromosomal error. The respective allelic profiles were indicative of meiotic non-disjunction errors displaying either only monoallelic (n = 7), tri-allelic or double dosage di-allelic (n = 6) (MI and MII error) profiles (Table II). The remaining 12 (48%) aneuploid embryos comprised discordant sister blastomeres reflecting chromosome 21 chromosomal mosaicism. From the proportion of blastomeres within an embryo showing different allelic profiles at the five test loci, we were able to estimate the timing and nature of the cell division error (refer to Figures 1 and 2). Errors at all stages including MI (12%) and first (38%), second (31%) and third (19%) mitotic cleavage divisions were identified in these mosaic aneuploid embryos. Two of these embryos showed combined meiotic and mitotic cell division errors suggesting that diploid cells had resulted from an aneuploid zygote after anaphase lag. However, the majority of the mosaic aneuploid embryos showed mitotic gains and losses from a diploid zygote with different proportions of diploid, monosomy and trisomy cells (Figure 2).

Analysis of chromosome 21 aneuploid amniocytes

A total of 180 single amniocytes from 15 trisomy 21 fetal amniocyte samples were analsyed. The allelic profiles showed strong amplification of all five microsatellite markers, with reliability calculated at 92%. All allelic profiles displayed a combination of both tri-allelic and double-dosage di-allelic marker patterns indicative of meiosis non-disjunction errors. The majority of samples (13 out of 15) analysed exhibited profiles indicative of a MI non-disjunction error, while the remaining two amniocyte samples showed a reduction to homozygosity with two copies of the same chromosome 21 in a single gamete indicating a MII non-disjunction error.

Discussion

This study demonstrates the power of single cell allelic profiling to distinguish between and identify the timing and nature of chromosome 21 meiotic and mitotic cell division errors in human preimplantation embryos. Of the 25 aneuploid embryos studied, over half displayed allelic profiles indicative of meiotic non-disjunction errors. From the total number of meiotic errors in this study, there were close to equal numbers of nullisomy versus disomy gametes and >85% of the trisomic embryos with meiotic errors were of MI origin. These results are comparable with previous population studies investigating the origin of chromosome 21 non-disjunction (Antonarakis *et al*., 1992; Lamb *et al*., 1996; Petersen and Mikkelsen, 2000). Unfortunately it was impossible to determine the origin and nature of nullisomy gametes that produce homogeneous monosomy embryos.

Mitotic errors were observed to occur during each cleavage stage with the majority identified during the first and second cell divisions prior to the activation of the embryonic genome. More than 80% of the mosaic aneuploid embryos showed mitotic gains and losses with different proportions of diploid, monosomy and trisomy cells, suggesting that the original zygote was diploid (Figure 2) and that both gametes contained only one copy each of chromosome 21. Two of the mosaic aneuploid embryos showed combined meiotic and mitotic cell division errors, suggesting that the observed diploid cells were a result of anaphase lag of chromosome 21 in an aneuploid zygote and that one of the gametes had two copies of chromosome 21 resulting in the aneuploid conception. However, the majority of these mosaic embryos were originally diploid conceptions.

From the analysis of trisomy 21 amniocytes, all chromosome 21 cell division errors originated during meiosis, predominantly MI. On comparison with the results from the preimplantation embryos, mosaic embryos appear to be lost either before or shortly after implantation. In a recent FISH study of human blastocysts, there appeared to be a strong selection at the morula–blastocyst transition against some types of mosaics, specifically monosomies (Sandalinas *et al*., 2001). Several mechanisms have been proposed for the incidence of chromosomal mosaicism including the deregulation of the mitotic process caused by cytoplasmic impairment (Wells and Delhanty, 2000; Voullaire *et al*., 2002), temporary relaxation of the centromere function (Choo, 1997) and/or low expression of the normal cell cycle checkpoints (Delhanty and Handyside, 1995; Delhanty *et al*., 1997). However, the cause and consequence of chromosomal mosaicism in human preimplantation embryos remains elusive. It has been hypothesized that mosaic embryos with different chromosomal complements are non-viable, contributing to the large number of IVF failures. Our data lend support to this hypothesis. Through allelic profiling of sister blastomeres from 25 chromosome 21 aneuploid embryos, we demonstrated that 12 (~50%) had mitotic cell division errors. In the majority of these embryos, the prior FISH analysis had excluded errors in four other chromosomes (13, 18, X and Y), indicating that these embryos were predominantly euploid. Analysis of amniocytes from trisomy 21 fetuses identified only meiotic errors. Taken together, the difference in the nature of chromosome 21 errors between preimplantation embryos and first and second trimester fetuses suggests that mitotic non-disjunction of chromosome 21 is non-viable. Even though there are clinical reports of chromosome 21 mosaic pregnancies and confined placental mosaicism (Kalousek and Vekemans, 1996; Nicolaidis and Petersen, 1998) these are considerably less in number than the frequency of chromosome 21 mosaicism from mitotic cell division error observed in the early embryo. In addition, the vast majority of trisomy 21 fetuses during pregnancy and at live birth originate from a meiotic cell division error.

One possible explanation for the non-viability of embryos with chromosome 21 mitotic error to successfully implant and maintain a pregnancy could be due to imprinting errors. Imprinted genes function from just one allele, either maternal or paternal, while methylation silences the other allele during early embryo development (Vastag, 2001; Butler, 2002; Miozzo and Simoni, 2002). It is conceivable that mitotic cell division error of chromosome 21 causes aberrant expression of imprinted genes in the embryo that is lethal at or post-implantation. Given that all the other chromosomes (excluding Y) contain a larger number of imprinted genes (Butler, 2002), it is likely

that mitotic cell division error in any chromosome of the preimplantation embryo is also associated with non-viability.

It is probable that different IVF parameters may influence the occurrence of embryonic mosaicism. With the availability of single cell allelic profiling in conjunction with human as well as non-human primate models for aneuploidy, it will now be possible to use allelic profiling as a tool to define factors and parameters that cause meiotic and mitotic errors, by directly comparing these errors with IVF variables such as hormonal stimulation protocols and culture conditions. Such knowledge may enable revision of IVF protocols to produce a lower proportion of embryos with mosaic aneuploid chromosome constitutions. This could then translate into a higher number of euploid non-mosaic embryos available for transfer and improved viable pregnancy outcomes, i.e. take-home babies, for future IVF patients.

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