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Chromosomal Mosaicism in Cleavage-Stage Human Embryos and the Accuracy of Single-Cell Genetic Analysis

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Purpose: *Our purpose was to assess the effect of chromosomal mosaicism in cleavage-stage human embryos on the accuracy of single-cell analysis for preimplantation genetic diagnosis.*
Methods: *Multicolor fluorescence in situ hybridization with X, Y, and 7 or X, Y, 7, and 18 chromosome-specific probes was used to detect aneuploidy in cleavage-stage human embryos.*
Results: *Most nuclei were diploid for the chromosomes tested but there was extensive mosaicism including monosomic, double-monosomic, nullisomic, chaotic, and haploid nuclei.*
Conclusions: *Identification of sex by analysis of a single cleavage-stage nucleus is accurate but 7% of females are not identified. One or both parental chromosomes 7 were absent in at least 6.5% of the nuclei. With autosomal recessive conditions such as cystic fibrosis, carriers would be misdiagnosed as normal or affected. With autosomal dominant conditions, failure to analyze the affected parents allele (1.6–2.5%) would cause a serious misdiagnosis and analysis of at least two nuclei is necessary to reduce errors.*

KEY WORDS: human preimplantation embryos; preimplantation genetic diagnosis; chromosomal mosaicism; multicolor FISH; single-gene defects.

INTRODUCTION

The development of single-cell genetic analysis for preimplantation genetic diagnosis (PGD), over the last 10 years, has focused attention on the cleavage-stage human embryo (Handyside and Delhanty, 1997). Two main techniques have been used for single-cell analysis, nested polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH) for the detection of single-cell defects and chromosomal aneuploidy, respectively. Both of these techniques have been demonstrated to work efficiently and accurately at the single-cell level under optimal conditions. However, for a variety of technical reasons, false-positive and -negative results can arise with either of them. Additionally, it has become clear that analysis of single-cells biopsied from cleavage-stage human embryos may inherently be problematic since they have a high incidence of nuclear and chromosomal abnormalities, which could add to these diagnostic errors. These include binucleate, multinucleate (Hardy *et al.*, 1993; Winston *et al.*, 1991), and apopotic (Jurisicova *et al.*, 1996) nuclei, abnormal ploidy including haploid, triploid, and tetraploid nuclei, and aneuploidy both affecting the whole embryo and arising postzygotically, resulting in chromosomal mosaicism (Munné *et al.*, 1994; Delhanty *et al.*, 1997).

To assess the consequences of these nuclear abnormalities and chromosomal mosaicism at cleavage stages of human preimplantation development for the diagnostic accuracy of single-cell genetic analysis, a series of embryos was examined by three- or four-color FISH with probes specific for chromosomes X, Y, and 7 and X, Y, 18, and 7, respectively. These were selected for their relevance for identification of sex in X-linked disease and PGD of cystic fibrosis (CF), which is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene on chromosome 7.

MATERIALS AND METHODS

Human Preimplantation Embryos

As described previously (Muggleton Harris *et al.*, 1993), women were superovulated and oocytes col-

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lected by transvaginal ultrasound-guided aspiration and inseminated with prepared sperm or fertilized by intracytoplasmic sperm injection (ICSI) (day 0). Oocytes were examined the following morning, 16–20 hr postinsemination, and classified as normally fertilized or polyspermic, depending upon the presence of two or more pronuclei, respectively. Fertilized embryos were cultured in Universal IVF medium [Medi-Cult (UK) Ltd.] until day 2, when they were transferred to M3 medium [Medi-Cult (UK) Ltd.]. After selection of the most suitable embryos for transfer (normally on day 2) and cryopreservation of other good-quality embryos, if requested, surplus embryos were used with the couple's informed consent. The Assisted Conception Unit, St. Thomas' Hospital, is licensed for this research by the Human Fertilisation and Embryology Authority and it was approved by the ethics committee, St. Thomas' Hospital.

Cytogenetic Preparation

The nuclei of cleavage-stage embryos on day 2 or 3 were spread on silanized slides using the Tween–HCl method, as described previously (Harper *et al.*, 1994). Briefly, the embryos were washed in phosphate-buffered saline (PBS) for 2 min and the zona removed using acid Tyrode's solution (pH 2.4). The zona-free embryo was then transferred into a small drop of spreading solution (0.01 *N* HCl, 0.1% Tween 20) on a silanized slide. As the blastomeres lysed, extra spreading solution was added and removed to clear the nuclei of cytoplasm. Following air-drying the slides were washed in PBS for 5 min, treated with pepsin (100 μg/ml) in 0.01 *N* HCl for 10–20 min at 37°C, rinsed with distilled water followed by PBS, dehydrated through an ethanol series, and air-dried again. The location of nuclei was confirmed by phase-contrast microscopy and slides were either stored at 4°C until use or hybridized immediately.

Control Lymphocytes

The hybridization efficiency of the chromosome-specific probes was tested in both combinations with a cytogenetic preparation of lymphocytes from a normal male prepared by standard procedures.

Multicolor FISH

For three-color FISH, DNA probes specific for the centromeric alpha satellite repeats of chromosomes X, Y, and 7 (Oncor, Gaithersburg, MD) were used. The probes for the X and Y chromosomes were labeled with biotin and digoxigenin and detected using Texas Red-labeled avidin and FITC-labeled antidigoxigenin (Vector Laboratories; Burlingame, CA), respectively, resulting in red and green hybridization signals. Two probes were used simultaneously for chromosome 7, one labeled with digoxigenin and the other with biotin, resulting in yellow hybridization signals. For four-color FISH, centromeric probes for chromosomes X, Y, and 18 directly labeled with fluorescent haptens, CEP Spectrum green, CEP Spectrum orange, and CEP Spectrum aqua, respectively (Vysis, Naperville, IL) were used and combined with the same two indirectly labeled probes for chromosome 7 detected as for three-color FISH.

The FISH protocol was similar to those published previously (Munné *et al.*, 1993). Briefly, following spreading of the nuclei and slide preparation, probes and nuclear DNA were codenatured at 75°C for 6 min and hybridized for 30 min or 1 hr, for three- and four-color FISH, respectively. The slides were then washed and hybridization of the indirectly labeled probes detected by incubation first with the Texas Red-labeled avidin for 5 min at 37°C and, after further washes, in FITC-labeled antidigoxigenin, also for 5 min at 37°C. Finally, the slides were washed again, air-dried, counterstained, and mounted in Vectashield (Vector Laboratories) antifade medium containing 1 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei and examined under a Zeiss microscope fitted with a Photometrics cooled charge-coupled device camera with dedicated Software. The four probes were distinguished using a triple-wavelength filter, a dual-wavelength filter, and individual single-wavelength filters for green, red, and blue. Analysis of the FISH signals was carried out by direct microscopy and image analysis according to the criteria of Munné *et al.* (1996).

RESULTS

Control Lymphocytes

Five hundred control male lymphocytes were analyzed with both the three- and the four-color combination of probes. Hybridization efficiencies for the individual probes were in the range of 96.2–99.0%. In addition, 1.5 and 0.3% of the nuclei had greater than the expected number of diploid signals for the chromosomes analyzed with the three- and four-color combination, respectively.

Cleavage-Stage Human Embryos

Eighty normally fertilized, two pronucleate, human embryos at cleavage stages ranging between 2-cell and approximately 10-cell stages on day 2 or mainly day 3 postinsemination or ICSI were prepared for cytogenetic analysis of nuclei. The efficiency with which all of the nuclei from each embryo were successfully spread was not complete but improved with later preparations. Overall, a total of 400 nuclei was analyzed by three-color (embryos 1–38) or four-color (embryos 39–80) FISH ranging between two and nine nuclei per embryo (excluding three embryos which had uniformly triploid nuclei, presumably as a result of dispermic fertilization). Most nuclei (351/400; 88%) appeared normal and in interphase. Some nuclei, however, were paired or partly overlapping and assumed to be from binucleate cells or were fragmented, possibly apoptotic, or simply damaged in preparation. These nuclei were excluded from further analysis since they are known to be associated with high levels of aneuploidy (Kligman *et al.*, 1996).

The results are summarized in Tables II and III. In Table II, the results are categorized according to the sex of the embryo based on the signals of the majority of the nuclei, interpreted according to the criteria set out in Table I. In Table III, the results are categorized according to the ploidy or aneuploidy status of one or both of the autosomal chromosomes analyzed.

DISCUSSION

The efficiency of hybridization of the probes used in this study with control lymphoctyes was very high (in excess of 96.2%). Nevertheless, hybridization failure does occur occasionally, and because of differences in the way the nuclei are prepared, it cannot be excluded that hybridization efficiencies with embryo nuclei are lower. For the purposes of the current analysis, therefore, failure of a single probe to give the expected diploid number of signals cannot conclusively be attributed to aneuploidy and must be excluded from consideration of diagnostic errors. We have therefore taken into account only those nuclei in which abnormalities of two or more probes make it unlikely that the result was caused by FISH failure *per se,* for example, haploid nuclei, in which all three or four chromosome pairs are consistent with this interpretation, or double-monosomies, where two chromosome pairs are missing one signal. Our estimates of diagnostic errors caused by nuclear and chromosomal abnormalities are, therefore, only conservative estimates. On the other hand, the embryos examined here were surplus to therapeutic IVF cycles and, in many cases, for poorer quality, and this probably explains the high proportion of abnormal nuclei that were excluded from the analysis (12%) because of the established correlation with aneuploidies and other abnormalities as detected by multicolor FISH (Kligman *et al.*, 1996).

For identification of sex in X-linked disease, it is now well established that it is necessary to analyze for the presence of both X and Y chromsomes and also include an autosomal probe to control for aneuploidy (Delhanty *et al.*, 1993). Furthermore, because of the risks of sex chromosomal aneuploidy combined with an X-linked defect, other categories of abnormal FISH results exclude the embryo from consideration for transfer, for example, XO nuclei (Table I). On this basis, we analyzed the results of the three-and four-color FISH and categorized them according to whether the embryo was predominantly male or female (Table II). In a typical X-linked recessive condition, the aim of identifying the sex of the embryos is to avoid transferring males, of which 50% will be affected by the defect, and to select female embryos, of which 50% will carry the defect. Among 172 nuclei analyzed from female embryos and 163 cells analyzed from males, there were only 2 cases in which the basic rule that identification should be based essentially on the normal combination of the two sex chromosomes could have caused a misdiagnosis. One of 163 (0.006%) nuclei from 23 male embryos was apparently XX181877 but it was ambiguous whether the nucleus was binucleate. The only other discrepancy was in (triploid/diploid mosaic embryo in which the chromosomal constitution of three nuclei was as follows: XXY777181818, XX771818, XX771818. How this arose is not known but it is possible that the triploid nucleus with the Y

Table I. Criteria for Identification of Sex by Multicolor FISH Analysis of Single Cells with X, Y, and an Autosome (N)-Specific Probe

Male	Female
XYNN	XXNN
Possibly suitable for transfer:	
XXYNN, XYYNN	XXN, XXXNN, XXXXNNNN
Should not be transferred in X-linked disease	
Any combination including a Y signal	X0NN, 00NN and chaotic combinations (?)
No diagnosis possible	
X or "00" (?Hybridization failure/overlapping signals or aneuploidy)	

Table II. Three- and Four-Color FISH Analysis of Nuclei from Cleavage-Stage Human Embryos on Day 2–3 Postinsemination or ICSI with X, Y, and 7- or X, Y, 18, and 7-Specific Probes Categorized as Male or Female on the Basis of the Signals in the Majority of Nuclei

	Embryos 1–38: X, Y, and 7	Embryos 39–80 X, Y, 7, and 18	Both series (%)
Females (n = 172)			
XXNN	73	82	155 (90.1)
XXN	2	3	5 (2.9)
X or 00	7	5	12 (7.0)
Males (n = 163)			
XYNN	43	77	120 (74.0)
XYN	0	14	14 (8.6)
X or 00	6	14	20 (12.3)
Others		9	9 (5.5)

signal may have resulted from fusion of a diploid zygotic nucleus with an extra Y-bearing sperm nucleus.

If binucleate, fragmented, and damaged nuclei are excluded, therefore, successful analysis of a single interphase nucleus for identification of sex is highly accurate. However, 7.0% of female embryos will not be positively identified because only a single X chromosome or no X chromosomes are identified (Table II). Analysis of two nuclei from two biopsied cells would reduce this loss if the missing signal in one of the nuclei is discounted, and it may also improve detection of chromosomally mosaic embryos. In a few X-linked conditions, for example, incontinenti pigmenti, it may be necessary to select males for transfer since carrier females may be affected and affected males die *in utero*. Identification of male embryos was considerably less efficient in this series, with 12.3% of nuclei failing to hybridize with the Y probe and 5.5% of nuclei showing other combinations of increased signals that would probably eliminate the embryo from being considered for transfer. This reinforces the importance of not trusting a single X signal in any combination since it could be from a male embryo and, in typical X-linked cases, at risk of being affected. The reason for the apparently lower hybridization of the Y probe is not clear but may be a result of using indirectly labeled probes, which are associated with higher background levels of nonspecific hybridization, causing some weak red Y signals to be discounted as background.

For detection of single-gene defects, nuclear and chromosomal aneuploidy could also potentially cause misdiagnoses if they involve the chromosome to which the disease gene maps, especially in a situation where one of the parental chromosomes is missing. As the common ΔF508 mutation of the cystic fibrosis transmembrane regulator (CFTR) gene causing cystic fibrosis (CF) was the first to be identified by PGD (Handyside *et al.*, 1992) and remains one of the main applications of PGD clinically (Ao *et al.*, 1996), we specifically analyzed the incidence of aneuploidy for chromosome 7 to which CFTR maps as well as chromosome 18 for comparison (Table III).

Again excluding nuclei considered to be abnormal and nuclei with extra hydridization signals in unusual or apparently chaotic combinations, the combined results of the three- and four-color FISH indicated that 298 of 346 (87%) of nuclei had the normal two signals for chromosome 7 and 18 (where analyzed). Analysis of these nuclei by PCR should therefore accurately reflect the status of the embryo for CF and any other conditions mapping to chromosome 7 or 18, and any errors would be technical. Of the remaining nuclei, single chromosome 7 or 18 signals were absent in 3.7 and 3.3%, respectively, suggesting monosomy. In 3.3% of nuclei examined by four-color FISH both a single 7 and a single 18 signal were missing, indicating double-monosomy, and in 3.2% the nuclei were haploid for all of the chromosome pairs examined. (Additionally, 0.4 and 0.5% of nuclei were nullisomic for chromosome 7 or 18, respectively, but this would result in complete failure of PCR amplification and no diagnosis would be possible). As discussed previously, nuclei which are apparently monosomic could simply result from hybridization failure. In the other situations, totaling 6.5% of nuclei for chromosome 7, FISH failure is unlikely and hence one of the parental alleles would escape detection. For autosomal recessive conditions such as CF, this will only cause a misdiagnosis of a carrier as either homozygous normal or affected. For

Table III. Multicolor FISH analysis of Nuclei from Cleavage-Stage Human Embryos on Day 2–3 Post Insemination with X, Y, and 7- or X,Y, 18, and 7-Specific Probes Categorized According to Ploidy or the Aneuploid Status of the Autosomes Examined[a]

	Embryos 1–38: X, Y, and 7 (%) (n = 136)	Embryos 39–80: X, Y, 7, and 18 (%) (n = 210)	Both series (%) (n = 346)
77 or 771818	124 (91.2)	174 (82.8)	298 (87.0)
Monosomy 7	4 (2.9)	9 (4.2)	13 (3.7)
Monosomy 18		7 (3.3)	
Double-monosomy		7 (3.3)	
Nullisomy 7	0	2 (0.9)	2 (0.5)
Nullisomy 18		1 (0.4)	
Haploid nucleus	8 (5.8)	3 (1.4)	11 (3.2)

[a] Others: XYY71818 (2), XXY7771818, XY777181818, XXY71818, XXY777181818, XYY7718.

autosomal dominant conditions, a serious misdiagnosis could occur since it would result in failure to detect the affected chromosome from the affected carrier parent in 6.5 × 0.25, or about 1.6%, of single cells (2.5% for chromosome 18). It is clear therefore that at least two cells should be analyzed when the risk is of an autosomal dominant condition, if possible combined with analysis of both the specific mutation and closely linked markers as demonstrated recently for familial polyposis coli (Ao *et al.*, 1998).

The use of one analytical method, in this case, multicolor FISH, to analyze the effects of nuclear and chromosomal abnormalities for identifying sex or the consequences for other techniques such as PCR is complicated by the inherent limitations of FISH itself or of having any independent means to verify definitively the chromosomal status of individual embryo nuclei. Our aim was simply to assess the magnitude of possible errors that are inherent to the cleavage-stage human embryo itself and these will clearly vary depending on embryo quality and stage. However, some useful observations do arise from our analysis. Clearly, it is necessary in deciding how many cells to biopsy from cleavage-stage embryos to balance diagnostic accuracy with potential to implant and develop, which is progressively compromised as a greater proportion of the embryo is removed (Liu *et al.*, 1993). For selection of females in X-linked disease, even though some females will fail to be identified, analysis of single nuclei is sufficient for an accurate diagnosis. With analysis of recessive single-gene defects as exemplified by CF, again single-cell analysis should not in itself increase the risk of a serious misdiagnosis unless a haploid cell is sampled that is unrelated to the embryo genotype, for example, if an extra sperm nucleus is not incorporated at syngamy. Further research will be necessary to assess this risk. However, a significant number of carrier embryos will be misdiagnosed as affected, reducing those available for transfer. For autosomal dominant conditions, however, it is clear that even our conservative estimates strongly indicate that the risk of analysis of only a single cell is likely to be unacceptably high.

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