Am. J. Hum. Genet. 55:150–159, 1994

Chromosome Abnormalities in Human Arrested Preimplantation Embryos: A Multiple-Probe FISH Study

S. Munné,[1] J. Grifo,[1] J. Cohen,[1] and H.-U. G. Weier[2]

[1]Center for Reproductive Medicine and Infertility, Department of Obstetrics and Gynecology, New York Hospital—Cornell University Medical Center, New York; and [2]Division of Molecular Cytometry, Department of Laboratory Medicine, School of Medicine, University of California, San Francisco, and Division of Life Sciences, University of California, Lawrence Berkeley Laboratory, Berkeley

Summary

Numerical chromosome abnormalities were studied in single blastomeres from arrested or otherwise morphologically abnormal human preimplantation embryos. A 6-h FISH procedure with fluorochrome-labeled DNA probes was developed to determine numerical abnormalities of chromosomes X, Y, and 18. The three chromosomes were stained and detected simultaneously in 571 blastomeres from 131 embryos. Successful analysis including biopsy, fixation, and FISH analysis was achieved in 86.5% of all blastomeres. The procedure described here offers a reliable alternative to sexing of embryos by PCR and allows simultaneous ploidy assessment. For the three chromosomes tested, numerical aberrations were found in 56.5% of the embryos. Most abnormal embryos were polyploid or mosaics, and 6.1% were aneuploid for gonosomes or chromosome 18. Extrapolation of these results to all human chromosomes suggests that the majority of abnormally developing and arrested human embryos carry numerical chromosome abnormalities.

Introduction

Spontaneous developmental arrest during in vitro embryo culture in the human occurs very frequently at the four- to eight-cell stage, with the majority of embryos failing to form blastocysts (Bolton et al. 1988). Embryonic arrest and genome activation occur at approximately the same developmental stage, leading to the hypothesis that cleavage arrest occurs because of failure to begin transcription appropriately (Braude et al. 1988; reviewed by Telford et al. 1990). A recent study, however, reported transcriptional activity in half of the arrested embryos, most of which had an abnormal number of nuclei per cell (Artley et al. 1992). According to Artley and Braude (1993), embryonic arrest is most likely caused by cytoplasmic impairment produced by unsuitable in vitro conditions. For example, improvement of culture conditions or coculture could overcome cleavage arrest in some cases (Wiemer et al. 1989; Menezo et al. 1990; Lawitts and Biggers 1991). Although karyotype analysis cannot be performed in nondividing arrested embryos, it has been applied to abnormally developing nonarrested embryos, which show a high proportion of numerically abnormal karyotypes (Plachot et al. 1988; Bongso et al. 1991).

The goal of our study was to determine the frequency and extent of numerical chromosome aberrations in cleavage-arrested human embryos, as well as in other abnormally developing embryos. For interphase chromosome enumeration, FISH is the method of choice (Hopman et al. 1988). Aneuploidy studies on human blastomeres have been performed using FISH with probes that bind specifically to the X or Y chromosome or with a probe binding to chromosome 18 (Griffin et al. 1992; Munné et al. 1993*b;* Schrurs et al. 1993). The differentiation between cells that are either aneusomic for one particular chromosome or truly haploid or triploid can be done by simultaneous analysis of two or more chromosome pairs. Such studies are facilitated by selective staining and detection of individual chromosome pairs with different-color fluorescent probes. Multicolor FISH analysis has already been applied extensively on tissue cultures and lymphocytes (Dauwerse et al. 1992; Nederlof et al. 1992). Here we demonstrate a triple probe with a quadruple color scheme for individual interphase blastomere analysis. We applied the technique to determine the ploidy status and sex of arrested or abnormally developing human embryos during the fourth day of in vitro development.

Material and Methods

Embryos

One hundred thirty-one cleavage-arrested or abnormally developing embryos were obtained from patients undergoing in vitro fertilization (IVF) treatment for infertility at The Center for Reproductive Medicine and Infertility, Cornell University Medical Center. Patients' ages were 25–47 years, with an average age of 36 years and an

Received June 10, 1993; accepted for publication March 9, 1994.

Address for correspondence and reprints: Dr. S. Munné, The Gamete and Embryo Research Laboratory, Center for Reproductive Medicine and Infertility, Department of Obstetrics and Gynecology, New York Hospital—Cornell University Medical Center, P.O. Box 30, 1300 York Avenue, New York, NY 10021.

© 1994 by The American Society of Human Genetics. All rights reserved.

0002-9297/94/5501-0021$02.00

SD of 4.2 years. The number of pronuclei per zygote was scored after insemination, and those with two pronuclei (monospermic) were allowed to cleave for 3 d. Embryos that had developed to the 5–12-cell stage by day 3 were replaced into patients (Cohen et al. 1992). Monospermic embryos that had not yet reached the eight-cell stage on their fourth day of development and that had not cleaved during a 24-h period (i.e., were "arrested") were used for this study. Between the zygote and eight-cell stage, the majority of these embryos exhibited morphological abnormalities that included embryonic fragmentation, granular cytoplasm, multinucleation, and uneven cells. These embryos are normally discarded. Arrested embryos may be investigated by FISH, according to a protocol approved by the Human Investigation Committee of the New York Hospital—Cornell University Medical College (protocol 0890-701). Patients from whom the embryos were obtained consented to these procedures.

Embryo Biopsy and Blastomere Preparation

Embryos were biopsied on day 4. A hole was drilled (Gordon and Talansky 1986) through the zona pellucida with acidified Tyrode's solution (pH 2.4), and several blastomeres were removed from each embryo by micromanipulation (Grifo et al. 1992). Each blastomere biopsy took ~10 min/embryo.

In most cases, fixation of all the blastomeres from the same embryo was not possible, because some cells were used for other studies. Blastomeres selected for FISH were fixed individually on glass slides by using a slight modification of Tarkowski's technique (Tarkowski 1966; Munné et al. 1993*b*). Each blastomere was placed in a culture dish containing hypotonic solution (1% sodium citrate in water, 6 mg BSA/ml; Sigma), for 5 min at 25°C and then was transferred into a small volume of hypotonic solution on the slide. Ten milliliters of fixative (acetic acid: methanol, 1:3) were dropped on top, while the cell was observed under a stereo microscope (Wild M3Z). The fixative was spread by continuous and gentle blowing until the cytoplasm dissolved. The position of the nucleus was marked with a tungsten-carbide–tip pencil. The whole process lasted 10 min/blastomere. Slides were dehydrated (70%, 85%, and 95% ethanol, 2 min each) and were kept at −20°C before their analysis.

FISH Technique

DNA probes for chromosomes X, Y, and 18 labeled with fluorescent haptens (Imagenetics) allow FISH analysis with hybridization times 6 h (Munné et al. 1993*b*). Probes used in this study were labeled with either red (Spectrum Orange) or green (Spectrum Green) fluorochromes. The hybridization targets for chromosomes 18 and X were alpha- satellite repeat-clusters in the centromeric region, and that for chromosome Y was satellite-III DNA at Yqh.

Two chromosomes were chosen for hybridization with either a red or a green probe, while a third chromosome was stained with a mixture of both colors. The three targets on chromosomes X, Y, and 18 were hybridized with either a mixture containing probes CEP SpectrumOrange X, CEP SpectrumGreen Y, CEP SpectrumOrange Y, and CEP SpectrumGreen 18 (4–5 mg of each probe/ml; Y-mix) or a mixture containing CEP SpectrumOrange X, CEP SpectrumGreen X, CEP SpectrumOrange Y, and CEP SpectrumGreen 18 (4–5 mg of each probe/ml; X-mix) in 55% formamide (FA), 2 × SSC, 10% dextran sulfate (Pinkel et al. 1986), 0.1 mg human COT1 DNA/ml (Gibco/Life Technologies). All CEP probes were from Imagenetics. When double-labeled chromosomes were observed through a specialized dual-wavelength filter (ChromaTechnology), their intermediate color between red and green (from pale-yellow to pale-pink appearance) allowed differentiation from chromosomes hybridized with only a single probe.

Preliminary results with lymphocytes when a previously published protocol (Munné et al. 1993*b*) was used showed cross-hybridization of the probes to nontarget chromosomes. We determined optimal conditions by varying probe preannealing and hybridization temperatures (H.-U. G. Weier and S. Munné, unpublished data). In general, all hybridization protocols using lymphocytes from a healthy male donor showed 90% of nuclei with the expected number of hybridization domains—that is, one domain each—specifically labeling the X- and Y-chromosomal targets and two chromosome 18 domains (table 1). In the experiments described here, the hybridization solution was denatured at 76°C for 5 min and then incubated at 25°C or 37°C for 10 or 30 min, respectively. Blastomeres were denatured for 3 min at 75°C in 70% FA, 2 × SSC (pH 7.0) prior to dehydration in an ethanol series (75%, 85%, and 100%). The hybridization mixture was applied, and slides were covered with coverslips and sealed with rubber cement. Hybridization was performed at 37°C or 42°C for 6 h in a humidified chamber. The coverslips were then removed, and slides were immersed in 50% FA, 2 × SSC, pH 7.0 at 42°C for 15 min, followed by two washes of 15 min each in PN buffer (0.1 M sodium phosphate buffer[pH 8], 1% Nonidet P-40) at room temperature. The DNA was counterstained with 4′,6-diamino-2-phenylindole (DAPI; 0.5 mg/ml; Calbiochem) in antifade solution (Johnson and Nogueira-de Araujo 1981). Fluorescence microscopy was performed on a Nikon Optiphot microscope, using a dual filter set for simultaneous observation of FITC and Texas Red (ChromaTechnology). This allowed viewing of SpectrumOrange signals in combination with SpectrumGreen signals. A Nikon filter was used for DAPI observation. In rehybridization experiments, we used a chromosome 8–specific probe (CEP8 SpectrumOrange; Imagenetics) (Jenkins et al. 1992) under comparable conditions.

Table 1

FISH Analysis of Chromosomes X, Y, and 18 in Lymphocytes from a Male

Probe Mixture (preannealing conditions; hybridization temperature)	XY1818	YO1818	XO1818	XY18O	Others	N
X-mix (30 min, 37°C; 42°C)	95%	0%	2%	3%	0%	200
Y-mix (30 min, 37°C; 42°C)	95%	1%	0.5%	2.5%	1%[a]	200
X-mix (30 min, 37°C; 37°C)	93%	1%	1%	3%	2%[b]	200
Y-mix (30 min, 37°C; 37°C)	91.5%	0%	1.5%	5.5%	1.5%[c]	200
X-mix (10 min, 25°C; 37°C)	94%	1%	0%	4%	1%[c]	200
Y-mix (10 min, 25°C; 37°C)	94%	0%	1%	4%	1%[c]	200

[a] 0.5% XXYY1818 and 0.5% YO18O.
[b] 1% XXY1818, 0.5% XYY1818, and 0.5% XY181818.
[c] XY181818.

Analytical Criteria

Minor hybridization spots that had much lower fluorescence intensity were not scored, since they most likely represented cross-hybridization signals to nontarget chromosomes (Hopman et al. 1988). Spots found in close vicinity to one another or in paired arrangements were counted as one, as they most probably represented sister chromatids or split signals.

The specific FISH signals detected in a given blastomere were considered to reflect a true chromosome constitution in the following instances: (*a*) when there were blastomeres with two gonosome and two chromosome 18–specific signals; these were considered diploid blastomeres; (*b*) when there were embryos in which all the blastomeres had the same abnormality, such as aneuploid, haploid, or polyploid embryos; (*c*) when there were individual blastomeres that have only one signal per chromosome pair; these were considered haploid cells; (*d*) when there were individual blastomeres that had three or more signals per chromosome pair; these were considered polyploid cells; (*e*) when there were individual blastomeres that had extra or missing signals compensated respectively for the missing or extra signals in sibling blastomeres; we considered these blastomeres as belonging to an embryo with mosaicism generated by mitotic nondisjunction; (*f*) when there were blastomeres showing fewer signals than their sibling blastomeres and belonging to mosaic embryos resulting from the uneven cleavage of a blastomere without previous DNA synthesis; an example would be an embryo with mostly XX1818 cells, plus XO1818 and XO cells; and (*g*) when the same criteria (*a–f*) were also used for polynucleated blastomeres. Blastomeres with fewer or more than two gonosomes or chromosome 18–specific signals were considered, respectively, FISH false-negative or false-positive errors, unless one of the prior criteria (*a–g*) applied.

Results

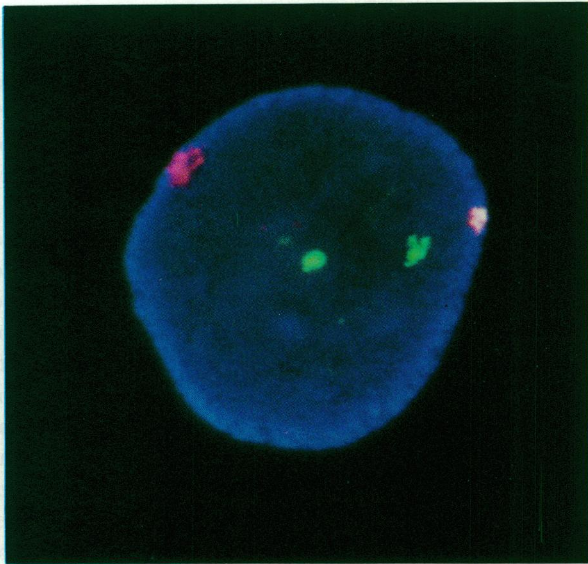
Analyzable Blastomeres

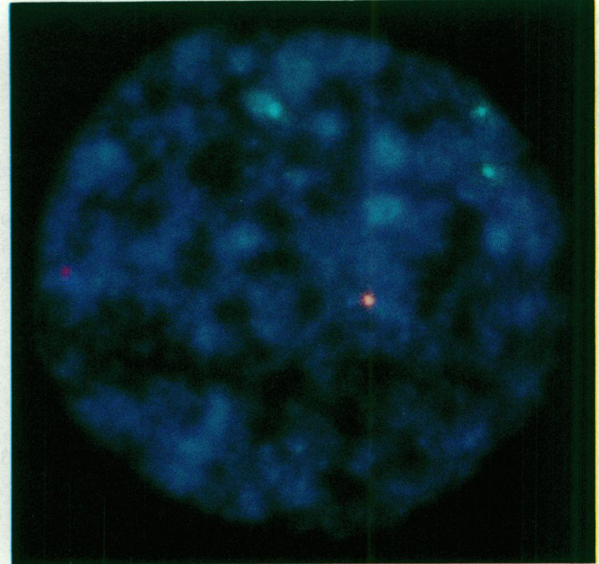
A total of 719 blastomeres from 131 human embryos were biopsied. The percentages of blastomeres lost after biopsy and fixation were 2.4% (17/719) and 3.5% (25/719), respectively. Nuclei were found in 571 of the fixed blastomeres, while the rest (106) were anucleated blastomeres or big cytoplasmic fragments (Appendix). Blastomeres hybridized with the X-mix and Y-mix combination of probes are shown in figure 1. After FISH analysis of 571 blastomeres, 554 cells (97%) showed hybridization signals. Seventeen cells were either damaged ($n=4$), covered with debris or cytoplasm ($n=3$), without clear signal ($n=5$), or not found ($n=5$). In addition, 26 cells (4.6%) with hybridization signals were FISH errors; 20 of them were false negatives, 5 were false positives, and one was simultaneously false positive for chromosome X and false negative for chromosome 18 (Appendix). The failure of the technique can be assessed as the number of blastomeres lost prior to FISH (5.9%), not including anucleated blastomeres, plus the number of blastomeres either not analyzable or showing false results after FISH (7.6%); this estimation is 13.5%. Anucleated blastomeres are a frequent event in embryonic development and are not caused by technique failure.

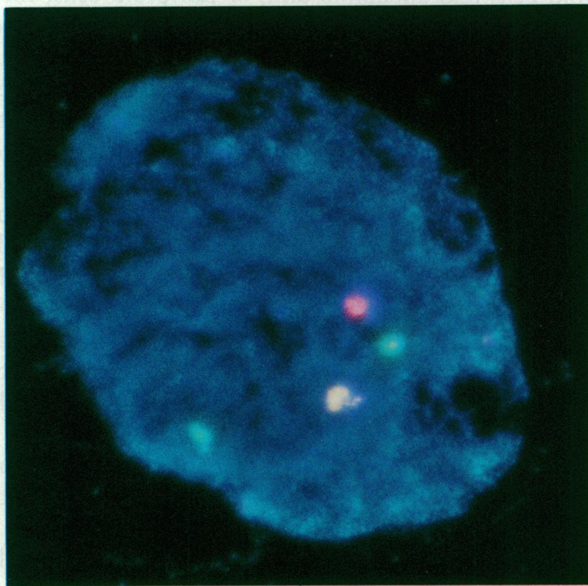
An incubation at 37°C after denaturation of the hybridization solution (preannealing), combined with hybridization at 42°C, was found to be optimal to reduce cross-hybridization without affecting the ability to detect specific signals. The nontarget signals were easily distinguishable from specific signals, which were substantially larger and brighter. Of the blastomeres, 36% and 18% showed cross-hybridization signals when hybridized at 37°C and 42°C, respectively.

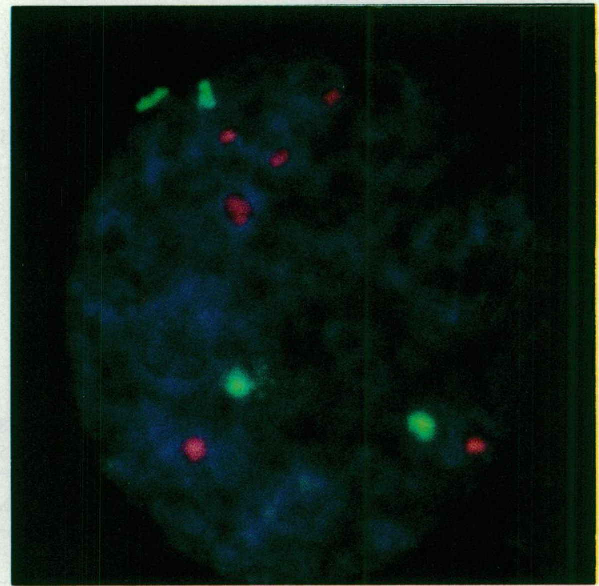
Embryos with Chromosome Abnormalities

Table 2 shows the results obtained after the FISH analysis of all or most of the cells of 131 embryos. Embryos









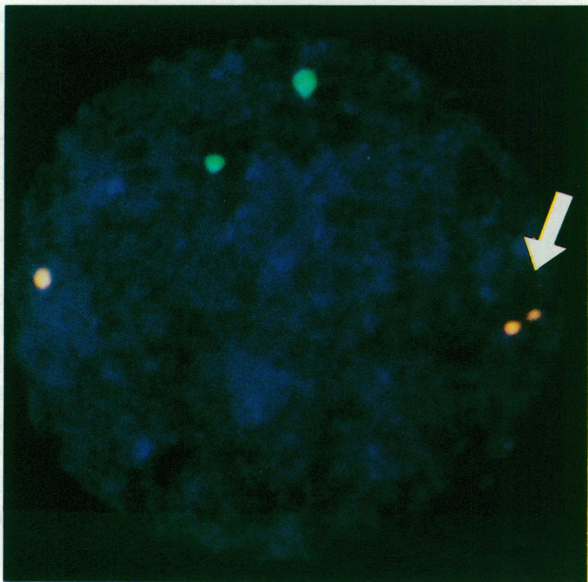


Figure 1 In situ hybridization of fluorochrome-labeled chromosome X–, chromosome Y–, and chromosome 18–specific DNA. *Left, top,* Hybridization of probe combination X-mix (chromosome X in mix, chromosome Y in red, and chromosome 18 in green) with male human lymphocytes. Minor cross-hybridization spots can be observed. *Left, middle,* Hybridization results obtained with the probe combination Y-mix (chromosome X in red, chromosome Y in mix, and chromosome 18 in green) with a male blastomere (XY1818). *Left, bottom,* Hybridization of probe combination X-mix with a female blastomere. The arrows point to a chromosome X–specific domain split in two. *Above, top,* Male blastomere with trisomy 18, hybridized with the Y-mix combination. *Above, bottom,* One nucleus of an MNB, hybridized with the Y-mix combination and showing six chromosome X–specific signals (in red) and four chromosome 18–specific signals (in green).

Table 2

Summary of 131 Chromosome X, Chromosome Y, and Chromosome 18 Numerical Abnormalities Found in Arrested or Abnormally Developing Human Embryos

	No. (%)
Diploid:	89 (67.9)
Normal	57 (43.5)
Aneuploid:	8 (6.1)
Monosomy XO1818	1
Monosomy YO1818	1
Double monosomy XO18O (embyro 22; table 3)	1
Trisomy 18	4
Trisomy 18 and mosaic (embryo 12; table 3)	1
Mosaics:	24 (18.3)
$2N/N$[a]	4
$2N/3N$	1
$2N/4N$	7
$2N/4N/8N$	1
$2N$/aneuploid/variable ploidy (embryos 1–11; table 3)	11 (8.4)
Haploid mosaics:	2 (1.5)
Mosaic $N/2N$[a] (embryo 21; table 3)	1
Mosaic haploid/aneuploid (embryo 13; table 3)	1
Polyploid:	40 (30.5)
All cells same ploidy:	24
$3N$	4
$4N$	7
$7N$	2
$8N$	4
Other ploidies	7
Mosaic polyploids:	16
Two ploidies (xN/yN[b])	9
Complex polyploid mosaic (embryos 14–20; table 3)	7
Total mosaics (any ploidy)	43 (32.8)
Total abnormal	74 (56.5)

[a] $2N/N$ diploid mosaics consist of $2N$ embryos with a single cell N, while $N/2N$ haploid mosaics consist of mostly N cells with one or more $2N$ cells.

[b] Mosaic embryos with two different polyploid cell lines: $4N/8N$ embryos, $3N/6N$ embryos, $8N/16N$ embryos, etc.

(n=89) with an average of two gonosomes and two chromosomes 18 were considered diploid. A total of 32 diploid embryos had an abnormal number of gonosomes and/or chromosomes 18. Eight (6.1%) of these 131 were aneuploid; that is, all of the cells from the same embryo had the same number of specific signals. The exceptions were an embryo with a false-negative FISH error in one blastomere and another embryo (number 12 in table 3) that was mosaic as well as being trisomic. This embryo is described later. Aneuploid embryos included five trisomics for chromosome 18, a monosomic for chromosome X, a monosomic for chromosome 18, a monosomic for chromosome Y, and a double monosomic for chromosomes X and 18 (embryo 22) (table 4). In order to be sure that embryo 22 (XO18O) was double monosomic instead of haploid, the embryo was rehybridized with a chromosome 8-specific probe showing an average of two chromosome 8 signals per cell (table 4). The other 24 (18.3%) abnormal diploid embryos were mosaics, that is, embryos with two or more cell lines differing in the number of gonosomes and or chromosomes 18. Thirteen diploid mosaic embryos were combinations of normal diploid cells and cells with a different number of chromosome complements: four had normal diploid cells, with the exception of a single haploid cell in each embryo; one had diploid and triploid cells; and eight contained only diploid cells, with the exception of one or two tetraploid cells. In the female embryos the tetraploid cells were XXXX18181818, while in the male embryos the tetraploid cells were XXYY181818, except that one embryo had a single tetraploid cell that was XXXY18181818. One of these embryos, in addition to being tetraploid, also contained an octoploid blastomere. The remaining 11 diploid mosaics were combinations of diploid cells and aneuploid and/or polyploid cells; they are listed in table 3. These embryos seem to be produced by three basic mechanisms: Embryos 1–3 from table 3 were probably produced by mitotic nondisjunction, because they contained monosomic as well as trisomic blastomeres in addition to normal cells. Embryos 4–9 seem to be produced by an abnormal DNA replication followed by uneven karyokinesis; and embryo 10 was probably produced by mitotic anaphase lag, since 44% (4/9) of its cells were monosomic; this percentage is much higher than the overall FISH error for this study. Embryo 11 could have been generated by a combination of the first two mechanisms. Finally, embryo 12, classified as trisomic for chromosome 18, was also a mosaic embryo and was probably produced by the second mechanism.

Two embryos (1.5%) were haploid mosaics. One (embryo 21; table 4) had all but one of its cells haploid; this cell was diploid. This embryo was rehybridized with a chromosome 8-specific probe to ensure that it was truly haploid and not double monosomic. As shown on table 4, embryo 21 showed also only one chromosome 8-specific signal per cell and was classified as haploid. However, it also showed a mitotic nondisjunction event involving chromosome 8 (cells 6 and 2 of embryo 21; table 4). The other haploid embryo (embryo 13; table 3) was a complex mosaic probably originating with a combination of nondisjunction and abnormal DNA replication followed by uneven karyokinesis. Embryo 13 could not be rehybridized with a chromosome 8 probe.

Embryos with an average of three or more gonosomes and chromosomes 18 per cell were considered polyploid. Approximately 30.5% (40/131) of the embryos were polyploid. Of these, 21 had the same ploidy in each cell: 4 were triploid; 7 were tetraploid; 1 was pentaploid; 1 was hexaploid; 2 were heptaploid; 4 were octoploid; 1 was $14N$; and 1 was $20N$. In addition, a four-cell embryo had four XXX18181818 cells, and two embryos with a single nucle-

Table 3

Mosaic Embryos for Chromosome X, Chromosome Y, and/or Chromosome 18

Mosaic Type and Embryo Number	Karyotype
Diploidy/variable ploidy/aneuploidy:	
1	XO1818, XXX1818, XX18O[a,b]
2	XX1818, XXX1818, XO1818
3	XX18O, XX1818, XX181818
4	XY1818 (3),[c] XX181818,[a] XY181818,[a] YO[a] (2)
5	XX1818,[a] XX1818[d] (2), XXXX181818, 18O
6	XY1818 (5), XY181818, XXY1818
7	XX1818,[a] XX181818,[a] XXX1818[a]
8	XX1818 (3), XX1818 (2),[d] XXXX1818,[a] 8X 10[e][18][a]
9	XX1818, XO1818, XO
10	XX1818 (2),[d] XXXX18181818, XXXX1818,[b] XXX1818, XO1818
11	XX1818 (5), XO1818 (4)
12	XY181818 (2), XXY1818181818,[a] XY1818[a]
Haploid:	
13	XO18O, XX18O (2), XX18O,[a] XO1818 (2), XO1818,[a] O18O[a]
Complex polyploid:	
14	NS,[f] XO 5[18],[a] 5XYYY14[18][a]
15	6X181818, XO181818, 6X5[18][a]
16	13[18],[a] XYY, 18O, XX1818, XYY181818
17	8X 9[18],[a] 5X181818[a]
18	XXYYYY 6[18],[a] XX18181818, YO[a]
19	7X 7[18] (2), 4X 8[18], 8X 16[18]
20	XXX181818, XX18O, 18O

NOTE.—Embryos with only a cell line of a different ploidy are not included in this table.
[a] Total no. of chromosomes in polynucleated blastomeres.
[b] Blastomere considered a FISH error.
[c] Blastomere number.
[d] Dinucleated blastomere with two signals per chromosome pair per nucleus.
[e] Total no. of chromosomes 18.
[f] NS = no signal.

ated cell in each were XXXX18181818181818 and XXXXYY13[18]. The rest of the polyploid embryos were mosaics. Of these, nine were combinations of two cell lines, with each line having a different number of chromosome complements: three were $4N/8N$; two were $4N$ with a haploid cell; two were $3N/6N$; one was $5N/6N$; and one was $8N/16N$. The remaining mosaics (embryos 14–20) were of complex origin and are listed in table 3.

Sixty (45.8%) of the 131 embryos had at least one multinucleated blastomere (MNB). However, the number of normal diploid embryos with MNBs was 26%, compared with 50% of the number of chromosomally abnormal embryos. Similarly, the percentage MNBs among the cells of a specific group of embryos ranged from 12.4% of the cells of normal diploid embryos to 43% of the cells of polyploid embryos.

FISH Errors, Polynucleation, and Polyploidy

The 4.6% (26/528) of cells with FISH errors were distributed unevenly among the different types of blastomeres. They were more common in MNBs (11.5% [13/113]) than in mononucleated cells (3.1% [13/415) and more frequent in cells from polyploid embryos (15% [12/79]) than in cells from from diploid embryos (3.2% [14/434]). The differences were highly significant ($P < .001$).

Discussion

Although the technique presented here can be applied with any other combination of three probes, chromosome X–, chromosome Y–, and chromosome 18–specific probes were chosen for their relevance in newborn chromosome abnormalities and genetic disease (Jacobs 1977). Previous FISH studies applied either a single probe for chromosome 18 or simultaneously detected chromosomes X and Y (Griffin et al. 1992; Munné et al. 1993*b;* Schrurs et al. 1993). These investigations could not differentiate between true trisomy or triploidy and monosomy or haploidy. Such abnormalities can now be differentiated by specific labeling of three chromosomes in blastomeres by using multiple-color FISH with fluorochrome-labeled probes. However, a single chromosome X signal and single

Table 4

Rehybridization with a Probe for Chromosome 8 of the Embyros 21 and 22, Previously Hybridized with Probes for Chromosomes X, Y, and 18

Blastomeres	No. of Nuclei/Cell	Results with Probes for Chromosomes X, Y, and 18	Rehybridization with a Probe for 8
Embryo 21:			
1	1	XO18O	8O
2	1	XO18O	88
3	2	XO18O[a]	8O[a]
4	1	XO18O	8O
5	2	XO18O[b]	8O[b]
6	1	XO18O	O
7	2	XX1818[b]	88[b]
Embryo 22:			
1	1	XO18O	88
2	3	XO18O[b]	88[b]
3	1	XO18O	88
4	1	XO18O	88

[a] Dinucleated blastomere with 1 signal/chromosome pair/nucleus.
[b] Total no. of chromosomes in polynucleated blastomeres.

chromosome 18 signal may be caused either by simultaneous loss of a gonosome and one homologue of chromosome 18 or by haploidy. We addressed this problem by rehybridization of the same embryos (21 and 22) with a probe specific for chromosome 8. This approach allowed differentiation between haploidy (embryo 21) and double monosomy (embryo 22).

Approximately 30% of the embryos were found to be polyploid. Two-thirds had all their cells with the same ploidy, which ranged from $3N$ to $20N$, and the rest were mosaic polyploids. Many of these embryos had only one nucleated cell, and the average number of cells per polyploid embryo was two, compared with five in diploid embryos. Arrested embryos with both a dominant blastomere and few or no other nucleated cells have been recently reported to be invariably polyploid (Munné et al., in press). It is unlikely that these embryos were produced by polyspermic fertilization, since only two pronuclei were observed after insemination. It may be that for these embryos their DNA synthesis continued although cellular division had stopped. In some instances they also continued karyokinesis, producing multinucleation in almost half of their cells. That DNA synthesis is not prevented by cleavage arrest has been demonstrated by Artley et al. (1992). According to Winston et al. (1991), even if karyokinesis and gene activation do not fail, impaired cytokinesis may arrest the embryo because there are insufficient cells to produce a functional inner-cell mass. Since most polyploid embryos arrested before the onset of genome activation, which occurs around the four- to eight-cell stage (Braude et al. 1988), oocyte quality or embryo culture conditions may be the cause of their arrest as well as the cause of their origin.

Mosaicism was also found to be very common. It was present in all the haploid embryos, in 40% of polyploid embryos, and in nearly 30% of diploid embryos. High rates of mosaicism in abnormally developing human embryos have already been described elsewhere (Plachot et al. 1987; Papadopoulus et al. 1988; Pieters et al. 1992). Mosaicism involving two cell lines, one with twice the ploidy of the other, was very common in both polyploid and diploid mosaics and most probably was caused by karyokinesis failure. However, the occurrence of tetraploid cells in diploid embryos seems to be a normal feature in the trophectoderm of human blastocysts (Benkhalifa et al. 1993). Other mosaics, such as diploid embryos with a single haploid cell, may have been the mistaking of a polar body for a blastomere, as some polar bodies can be as large as an eight-cell blastomere (Veek 1991). Diploid mosaics could be divided into two groups. One group consisted of mosaics that involved only a single chromosome pair (embryos 1–3, 10, and 11) and probably was produced by mitotic nondisjunction or by anaphase lag. Test of the diploid mosaics could arise through abnormal DNA replication sometimes followed by uneven karyokinesis. Such events are also very common in some MNBs; Munné and Cohen (1993) noted that in these MNBs cytokinesis also failed and that the uneven karyokinesis could be observed within the same cell. In the second type of mosaicism, most chromosome pairs in the affected cells would have an abnormal count, and consequently the system used here may have detected most of these mosaics. However, for all kinds of

mosaics, the rates described are tentative, since many of the embryos had only a fraction of their blastomeres analyzed, and some nonanalyzed blastomeres could have been abnormal. Little is known about the developmental viability of mosaic embryos—for example, how many normal cells are needed in a preimplantation embryo if it is to develop normally? In this study, some of the mosaic embryos contained a majority of blastomeres with normal karyotypes, as far as could be determined by testing with a limited number of chromosome-specific probes.

In the past, aneuploidy studies of human preimplantation embryos were limited by the inability to obtain metaphase spreads from arrested embryos, and only cleaving human embryos were karyotyped. In the present study, the rate of numerical abnormalities of gonosomes and chromosomes 18 from arrested or abnormally developing human embryos was assessed by FISH. For example, both a diploid embryo with an XO monosomy and another embryo with a YO monosomy were detected. Our results indicate that 4.6% (6/131) of the embryos were aneuploid for chromosome 18 and that 2.3% (3/131) were aneuploid for gonosomes. These rates appear to be very high, if it is assumed that all chromosome pairs had the same chance of being involved in aneuploidy. Under that assumption, 53%–100% of these embryos may be aneuploid. However, aneuploidy involved gonosomes more often than it involved autosomes. For instance, high rates of X-Y univalents (unpaired chromosomes) have been described in studies of meiosis I human male germ cells, which may predispose to nondisjunction of sex chromosomes (Laurie and Hulten 1985) and which explains the higher rate of gonosome hyperhaploidy found in sperm cells, compared with other chromosome pairs (Martin and Rademaker 1990). In addition, the aneuploidy rate for chromosome 18 has been shown to increase with maternal age (Hassold and Chiu 1985; Warburton et al. 1986). In our study, the mean maternal age was 36 years.

The interpretation of the extent of chromosome abnormalities by analyzing only two chromosome pairs and only a fraction of the cells in some embryos has clear limitations. It is possible to detect all polyploid, haploid, and aneuploid embryos for one of these chromosome pairs and for most mosaics. For instance, mosaics involving two cell lines with different numbers of haploid chromosome complements can be detected readily. Similarly, mosaics generated by uneven karyokinesis will also be detected in most cases, because they affect most chromosomes. However, mosaics would not be detected when produced by mitotic nondisjunction or when aneuploid embryos do not involve gonosomes or chromosomes 18. In addition, the analysis of only a fraction of the cells in a given embryo would result in underestimation of some additional mosaic embryos. A full "molecular" karyotype could only be achieved by the multiprobe-multicolor approach described by Dauwerse et al. (1992), but this would not apply to arrested embryos, as it would require metaphase cells. An alternative would be the use of "comparative genomic hybridization" (Kallioniemi et al. 1992), which has not yet been shown to work on single-cell templates. Nevertheless, our results suggest that most arrested or abnormally developing embryos are chromosomally abnormal.

Overall, most mosaics and polyploid embryos seem to be produced by cytokinesis failure and/or karyokinesis failures and are probably generated either by the same factors that determine cleavage arrest or by the arrest itself. However, on the basis of these results we cannot definitely determine whether an external factor, such as unsuitable embryo culture, was simultaneously the cause of embryonic arrest and chromosome abnormalities or whether chromosome abnormalities were the direct cause of developmental arrest. We are currently comparing arrested human embryos with normally developing embryos, to search for specific chromosome abnormalities found only in arrested ones.

Causes of reduced hybridization efficiency have been attributed to loss of DNA during denaturation, to poor probe penetration, or to insufficient binding of detection reagents (West et al. 1988; Pieters et al. 1990). However, the fact that FISH errors occurred three and five times more often in multinucleated and polyploid cells, respectively, compared with the error rate in other cells, suggests two different mechanisms. MNBs very often contain micronuclei, and during fixation some of them could get lost more easily than full nuclei, thus producing false-negative FISH errors. In contrast, polyploid cells contain more specific signals than do diploid cells, increasing the chances for two determinate signals to overlap or touch each other, thereby suggesting a single signal; this would also produce a false-negative FISH error.

The procedure described here may find widespread application in the preimplantation diagnosis of numerical chromosome abnormalities. The incidence of trisomic offspring increases exponentially with age (Warburton et al. 1986). IVF procedures (Medical Research International and Society for Assisted Reproductive Technology of the American Fertility Society 1992) are often performed in women 40 years of age, and routine screening of IVF embryos for chromosomes X, Y, 13, 18, and 21 might reduce the frequency of chromosomal abnormal pregnancies and births. In addition, because oocyte age, more than uterus age, seems to be the cause of reduced pregnancy rates in older women (Cohen et al. 1992; Drews et al. 1992), the transfer of chromosomally normal embryos may increase their chance of arriving to term. The FISH preimplantation diagnosis of aneuploidy, simultaneously using probes for chromosomes X, Y, 13, 18, and 21, recently has been described by Munné et al. (1993*a*), and it also can be used for the simultaneous aneuploidy-and-gender diagnosis of embryos from carriers of X-linked diseases, improving prior techniques (Handyside et al. 1989; Strom et al. 1991;

Grifo 1992). The preimplantation analysis of aneuploidy in IVF would have great impact in the way in which IVF is performed. In the transferring of embryos found to be normal for these chromosomes, the implantation and delivery rates per oocyte retrieved would not change; but these rates would increase significantly per embryo transferred. However, the high frequency of mosaicism that we observed suggests that single-cell preimplantation diagnosis at the eight-cell stage may lead to the possibility of misdiagnosis. If the cell being analyzed is abnormal, then the embryo would also be considered fully abnormal and would be discarded. However, if a cell is normal for the chromosomes analyzed, the embryo will usually be transferred, even though it might be mosaic. The importance of such misdiagnoses would depend on the extent of mosaicism in normally developing embryos, which to date is unknown, and also would depend on the developmental fitness of the abnormal cells. However, the present study does show that aneuploid embryos can be correctly diagnosed by single-cell preimplantation diagnosis.

Acknowledgments

Embryologists Mina Alikani, Alexis Adler, Adrienne Reing, Toni Ferrara, and Elena Kissin are acknowledged for technical assistance. We are grateful to Drs. Rosenwaks, Bedford, Estop, and Ledger for their support of this work. Giles Tomkin is acknowledged for assistance during preparation of the manuscript. We wish to thank the Laboratory for Cell Analysis, UCSF, for providing access to the microscope equipment. H.-U.G.W. was supported by the Office of Health and Environmental Research, Department of Energy, under contract DE-AC-03-76SF00098, with additional support from Amoco Technology Corp., Naperville, IL.

Appendix

FISH Efficiency in Blastomeres

- 719 cells biopsied from 131 embryos:
  - 17 (2.4%) broken during biopsy
  - 25 (3.5%) lost during fixation
  - 677 cells fixed:
    - 106 without nucleus
    - 571 with nucleus
- 571 cells analyzed by FISH:
  - 17 (3.0%) not analyzable
    - 3 covered by debris
    - 5 without clear signal
    - 4 damaged
    - 5 not found
  - 26 (4.6%) FISH failures
    - 20 false negative
    - 5 false positive
    - 1 false-positive and false-negative
  - 528 with correct number of signals

References

Artley JK, Braude PR (1993) Biochemistry of the preimplantation embryo. Assisted Reprod Rev 3:13–18

Artley JK, Braude PR, Johnson MH (1992) Gene activity and cleavage arrest in human pre-embryos. Hum Reprod 7:1014–1021

Benkhalifa M, Janny L, Vye P, Malet P, Boucher D, Menezo Y (1993) Assessment of ploidy in human morulae and blastocysts using co-culture and fluorescent in-situ hybridization. Hum Reprod 8:895–902

Bolton VN, Hawes SM, Taylor CT, Parsons JH (1988) Development of spare human preimplantation embryos: an analysis of the correlations among gross morphology, cleavage rates and development to the blastocyst. J In Vitro Fertil Embryo Transfer 6:30–35

Bongso A, Fong CH, Ng SC, Ratman S, Lim J (1991) Preimplantation genetics: chromosomes of fragmented human embryos. Fertil Steril 56:66–70

Braude P, Bolton V, Moore S (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 333:459–461

Cohen J, Alikani M, Trowbridge J, Rosenwaks Z (1992) Implantation enhancement by selective assisted hatching using zona drilling of embryos with poor prognosis. Hum Reprod 7:685–691

Dauwerse JG, Wiegant J, Raap AK, Breuning MH, Van Ommen GJB (1992) Multiple colors by fluorescence in-situ hybridization using ratio-labelled DNA probes create a molecular karyotype. Hum Mol Genet 1:593–598

Drews M, Bergh P, Williams M, Grunfeld L, Garrisi G, Navot D (1992) Age related decline in female fertility is not due to diminished capacity of the uterus to sustain embryo implantation. Fertil Steril Suppl 58:S39

Gordon JW, Talansky BE (1986) Assisted fertilization by zona drilling: a mouse model for correction of oligospermia. J Exp Zool 239:347–354

Griffin DK, Wilton LJ, Handyside AH, Winston RML, Delhanty JDA (1992) Dual fluorescent in-situ hybridization for simultaneous detection of X and Y chromosome-specific probes for the sexing of human preimplantation embryonic nuclei. Hum Genet 89:18–22

Grifo JA, Tang YX, Cohen J, Gilbert F, Sanyal MK, Rosenwaks Z (1992) Ongoing pregnancy in a hemophilia carrier by embryo biopsy and simultaneous amplification of X and Y chromosome specific DNA from single blastomeres. JAMA 6:727–729

Handyside AH, Pattinson JK, Penketh RJA, Delhanty JDA, Winston RML, Tuddenhanam EGD (1989) Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet 1:347–349

Hassold T, Chiu D (1985) Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. Hum Genet 70:11–17

Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, van der Ploeg M, Vooijs GP (1988) In-situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. Histochemistry 89:307–316

Jacobs P (1977) Epidemiology of chromosomal abnormalities in man. Am J Epidemiol 105:180–191

Jenkins RB, Le Beau MM, Kraker WJ, Borell TJ, Stalboerger PG,

Davis EM, Penland L, et al (1992) Fluorescence in-situ hybridization: a sensitive method for trisomy 8 detection in bone marrow specimens. Blood 79:3307–3315

Johnson GD, de Nogueira Araujo GM (1981) A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 43:349–351

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818–821

Laurie DA, Hulten MA (1985) Further studies on bivalent chiasma frequency in human males with normal karyotypes. Ann Hum Genet 49:189–201

Lawitts JA, Biggers JD (1991) Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol Reprod 45:245–251

Martin RH, Rademaker A (1990) The frequency of aneuploidy among individual chromosomes in 6821 human sperm chromosome complements. Cytogenet Cell Genet 53:103–107

Medical Research International and Society for Assisted Reproductive Technology of the American Fertility Society (1992) In-vitro fertilization-embryo transfer (IVF-ET) in the United States: 1990 results from the IVF-ET Registry. Fertil Steril 57: 15–24

Menezo YJ, Guerin JF, Czyba JC (1990) Improvement of human early embryo development in-vitro by coculture on monolayers of Vero cells. Biol Reprod 42:301–306

Munné S, Alikani M, Grifo J, Cohen J. Monospermic polyploidy and atypical embryo morphology. Human Reprod (in press)

Munné S, Cohen J (1993) Unsuitability of multinucleated human blastomeres for preimplantation genetic diagnosis. Hum Reprod 8:1120–1125

Munné S, Lee A, Rosenwaks Z, Grifo J, Cohen J (1993*a*) Diagnosis of major chromosome aneuploidies in human preimplantation embryos. Hum Reprod 8:2185–2191

Munné S, Weier HUG, Stein J, Grifo J, Cohen J (1993*b*) A fast and efficient method for simultaneous X and Y in-situ hybridization of human blastomeres. J Assisted Reprod Genet 10:82–90

Nederlof PM, van der Flier S, Vrolijk J, Tanke HJ, Raap AK (1992) Fluorescence ratio measurements of double-labeled probes for multiple in-situ hybridization by digital imaging microscopy. Cytometry 13:839–845

Papadopoulus G, Templeton AA, Fisk N, Randall J (1988) The frequency of chromosome abnormalities in human preimplantation embryos after in-vitro fertilization. Hum Reprod 4:91–98

Pieters MHEC, Dumoulin JCM, Ignoul-Vanvuchelen RCM, Bras M, Evers JLH, Geraedts JPM (1992) Triploidy after in-vitro fertilization: cytogenetic analysis of human zygotes and embryos. J Assisted Reprod Genet 9:68–76

Pieters MHEC, Geraedts JPM, Meyer H, Dumoulin JCM, Evers JLH, Jongbloed RJE, Nederlof PM, et al (1990) Human gametes and zygotes studied by nonradioactive in-situ hybridization. Cytogenet Cell Genet 53:15–19

Pinkel D, Gray JW, Trask B, van den Engh G, Fuscoe J, van Dekken H (1986) Cytogenetic analysis by in-situ hybridization with fluorescently labeled nucleic acid probes. Cold Spring Harb Symp Quant Biol 51:151–157

Plachot M, Junca AM, Mandelbaum J, De Grouchi J, Salat-Baroux J, Cohen J (1987) Chromosome investigations in early life. II. Human preimplantation embryos. Hum Reprod 2:29–35

Plachot M, Veiga A, Montagut J, De Grouchy, Calderon G, Lepretre JS, Junca AM, et al (1988) Are clinical and biological IVF parameters correlated with hromosomal disorders in early life: a multicentric study. Hum Reprod 5:627–635

Schrurs BM, Winston RML, Handyside AH (1993) Preimplantation diagnosis of aneuploidy using fluorescent in-situ hybridization: evaluation using a chromosome 18-specific probe. Hum Reprod 8:296–301

Strom CM, Rechitsky S, Verlinsky Y (1991) Reliability of gender determination using polymerase chain reaction for single cells. J In Vitro Fertil Embryo Transfer 8:225–229

Tarkowski AK (1966) An air drying method for chromosome preparations from mouse eggs. Cytogenetics 5:394–400

Telford NA, Watson AJ, Schultz GA (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 26:90–100

Veek LL (1991) Atlas of the human oocyte and early conceptus. Vol. 2. Williams & Wilkins, Baltimore, pp 167–178

Warburton D, Kline J, Stein Z, Strobino B (1986) Cytogenetic abnormalities in spontaneous abortions of recognized conceptions. In: Porter IH, Willey A (eds) Perinatal genetics: diagnosis and treatment. Academic Press, New York, pp 133–148

West JD, Gosden JR, Angell RR, West KM, Glasier AF, Thatcher SS, Baird DT (1988) Sexing whole human pre-embryos by in-situ hybridization with a Y-chromo some specific DNA probe. Hum Reprod 3:1010–1019

Wiemer KE, Cohen J, Amborski GF, Wiker S, Wright G, Munyakazi L, Godke RA (1989) in-vitro development and implantation of human embryos following culture on fetal bovine uterine fibroblast cells. Hum Reprod 4:595–600

Winston NJ, Braude PR, Pickering SJ, George MA, Cant A, Currie J, Johnson MH (1991) The incidence of abnormal morphology and nucleocytoplasmic ratios in 2-, 3- and 5-day human pre-embryos. Hum Reprod 6:17–24