

## Brief Report

**BIRTH OF A HEALTHY INFANT  
AFTER PREIMPLANTATION  
CONFIRMATION OF EUPLOIDY  
BY COMPARATIVE GENOMIC  
HYBRIDIZATION**

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**D**URING *in vitro* fertilization, morphologically normal embryos often fail to implant. The implantation rate, defined as the ratio of the number of fetal hearts detected to the number of cleavage-stage embryos transferred, is at best approximately 15 to 20 percent, even in experienced centers.<sup>1</sup> Early human embryos have a high frequency of aneuploidy, as determined by fluorescence *in situ* hybridization. Analysis by this method of three to six chromosomes from every cell in early human embryos generated by *in vitro* fertilization shows that 50 to 70 percent of these embryos have chromosomal errors, including aneuploidy, polyploidy, and mosaicism.<sup>2-4</sup> This rate is higher than the frequency of chromosomal abnormalities found in spontaneously aborted fetuses, indicating that many of the abnormal conceptuses die early in development. In particular, autosomal monosomy, with the rare exception of monosomy 21, has not been observed in fetuses in clinical pregnancies, making it likely that chromosomal abnormalities are responsible for a substantial proportion of implantation failures.

Because genetic abnormalities may lead to implantation failure, preimplantation diagnosis of aneuploidy is being carried out through testing of a single blastomere obtained by biopsy from an eight-cell embryo. Despite the inherent risk of misdiagnosis due to the high level of mosaicism observed in early human embryos, a number of centers now perform preimplantation genetic analysis for aneuploidy, using fluorescence *in situ* hybridization with probes for five to nine chromosomes, on single cells obtained by biopsy from embryos.<sup>5-7</sup> This procedure permits the selection of embryos that are normal, at least with respect to the chromosomes analyzed, for transfer to the pa-

tient and may lead to an increased rate of implantation in older women<sup>5</sup> and a decreased rate of embryo loss after implantation.<sup>6</sup> However, embryos identified by fluorescence *in situ* hybridization as normal with respect to five or nine chromosomes may be aneuploid for chromosomes that have not been examined. Other methods of testing blastomeres from preimplantation embryos are associated with particular problems; for instance, in spectral imaging with the use of probes labeled with a combination of fluorochromes, only 56 percent of nuclei provide interpretable results.<sup>8</sup>

Comparative genomic hybridization is a molecular cytogenetic technique that can be used with single cells in interphase to allow simultaneous enumeration of every chromosome.<sup>9,10</sup> We report the birth of a healthy infant to a woman with a history of implantation failure after the use of comparative genomic hybridization to determine that the karyotype of a single embryo was normal.

#### METHODS

All patients considering *in vitro* fertilization undergo mandatory counseling in the state of Victoria, Australia. At our center, patients considering preimplantation genetic diagnosis attend an additional session with a qualified genetics counselor before signing consent forms approved by our institutional review board; the permission encompasses approval to perform embryo biopsy and screening for aneuploidy by fluorescence *in situ* hybridization or comparative genomic hybridization.

A 38-year-old woman with a 7-year history of unexplained primary infertility underwent a cycle of ovarian stimulation, oocyte collection, *in vitro* fertilization, and embryo transfer with cryopreservation of excess embryos. A total of eight embryos were transferred in four separate procedures (regulatory restrictions limit the transfer of embryos to three per cycle), but no clinical pregnancy resulted. During a second cycle of ovarian stimulation, 15 oocytes were collected. After *in vitro* fertilization, because of the increased risk of aneuploidy associated with older maternal age, nine preimplantation embryos were tested with the use of fluorescence *in situ* hybridization for chromosomes 13, 16, 18, 21, and 22. Two embryos identified as normal with respect to these chromosomes were transferred, but no pregnancy resulted.

The patient underwent a third cycle of ovarian stimulation. Fourteen oocytes were collected, and each was inseminated with a single sperm with the use of intracytoplasmic sperm injection. The 11 embryos obtained were cultured for three days in human tubal-fluid medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10 percent maternal serum in 5 percent carbon dioxide in air, after which each contained between 6 and 12 cells. A single blastomere was obtained by biopsy from each embryo.

Blastomeres from six of the embryos were spread onto microscope slides with the use of 0.1 percent Tween 20 in 0.01 *N* hydrochloric acid<sup>11</sup> and analyzed by fluorescence *in situ* hybridization for chromosomes 13, 16, 18, 21, and 22 with a commercially available cocktail of chromosome-specific DNA probes according to the manufacturer's specifications (Vysis, Downer's Grove, Ill.). Two embryos with blastomeres that were identified as normal (with respect to chromosomes 13, 16, 18, 21, and 22) with the use of fluorescence *in situ* hybridization and chromosome-specific probes were transferred in human tubal-fluid medium with 10 percent maternal serum to the patient's uterus.

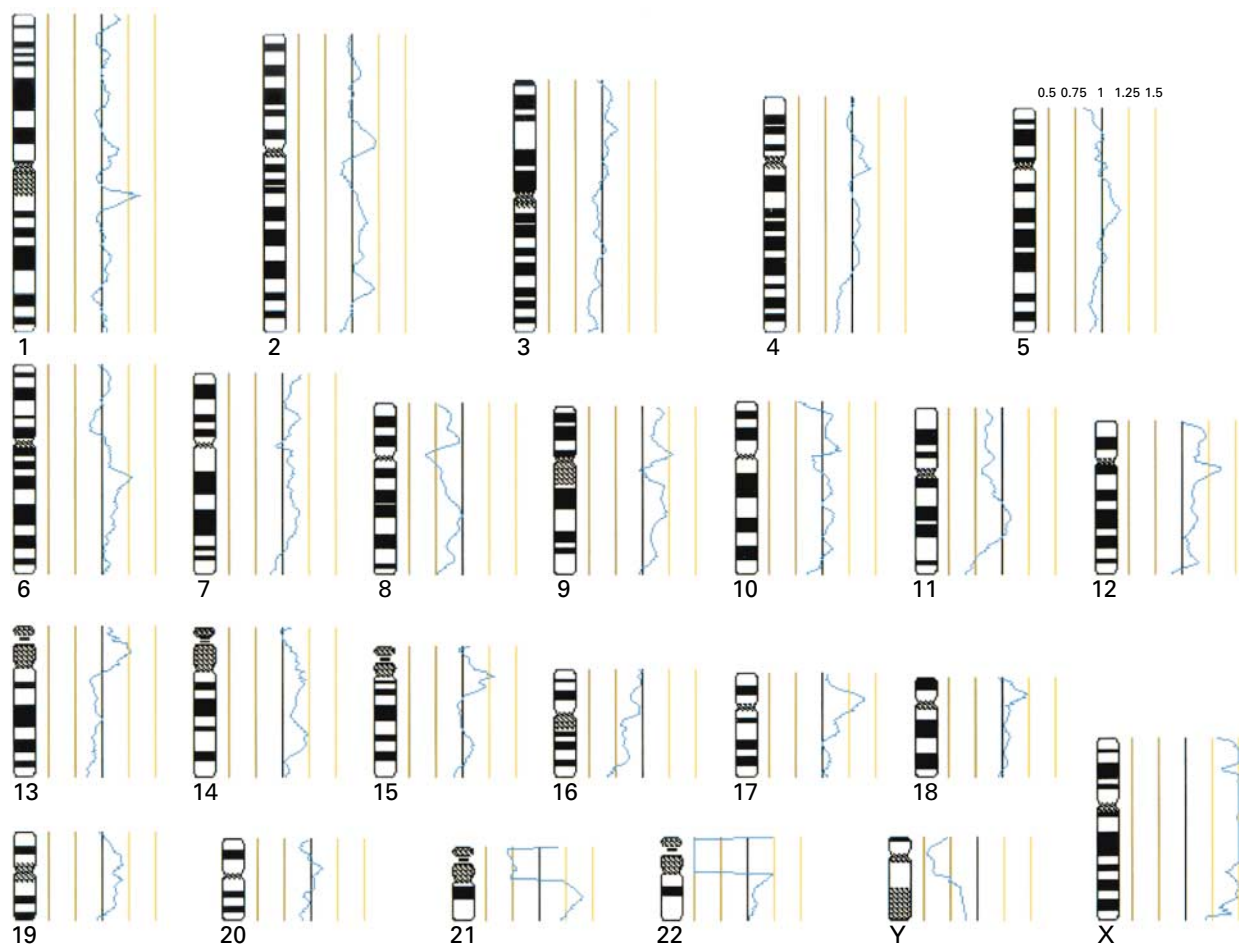
Blastomeres from the five remaining embryos were analyzed with the use of comparative genomic hybridization as previously described.<sup>9,12</sup> Individual blastomeres underwent alkaline lysis followed by whole-genome amplification by the polymerase chain reaction with random primers. A single cell from an amniocyte cul-

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ture shown by conventional chromosomal analysis to have a female karyotype with trisomy 21 was included as a control. The DNA amplification product (test DNA) was labeled by enzymatic incorporation (nick translation) of a nucleotide conjugated to a fluorochrome (Spectrum Green, Vysis). Genomic DNA extracted from lymphocytes from a normal male was also amplified with the use of the same procedure, and the amplified DNA was labeled with Spectrum Red (Vysis) by nick translation to provide a normal reference DNA sample.

Test and reference DNA were simultaneously hybridized to metaphase template slides from the normal male, and the fluorescence images were captured and analyzed with the use of Cytovision comparative-genomic-hybridization software (Applied Imaging, Santa Clara, Calif.) to determine the average ratio of green to red

fluorescence for each chromosome. When the number of copies of DNA in the test and reference DNA is the same, the ratio of green to red fluorescence is 1.0. When the DNA copy number is lower in the test DNA than in the reference DNA, the ratio is less than 1.0, and when it is more, the ratio is greater than 1.0. Thresholds for deviation indicative of chromosomal abnormality were set at 0.75 and 1.25. Comparative-genomic-hybridization profiles obtained after universal amplification of limited template by the polymerase chain reaction tend to show more random deviation than those obtained with the use of extracted genomic DNA. We have previously estimated that the limit of resolution with this technique is approximately 40 megabases.<sup>9</sup> Deviations of the profile at the telomeres and the heterochromatic (h) regions — including the centromeres, 1qh, 9qh, 16qh, Yqh, the short arm of the acrocentric



**Figure 1.** Profile Obtained after Comparative Genomic Hybridization with the Use of the Amplified DNA from a Single Cultured Amniocyte Known to Have a Female Karyotype with Trisomy 21.

When the number of copies of DNA is the same in test DNA and reference DNA, the profile is normalized to 1.0; deviations to the right indicate that the number of copies of DNA is higher for the test DNA than the reference DNA, and deviations to the left indicate that the number of copies of DNA is lower for the test DNA than the reference DNA. The deviation is marked with lines at intervals of 0.25: 0.5 and 0.75 (brown lines), 1.0 (black line), and 1.25 and 1.5 (yellow lines; these are identified on the profile for chromosome 5). Deviations greater than 1.25 or less than 0.75 were interpreted as indicative of chromosomal abnormality. Deviations of the profile at the telomeres and the heterochromatic regions — including the centromere, 1qh, 9qh, 16qh, Yqh, the short arm of the acrocentric chromosomes (i.e., chromosomes 13, 14, 15, 21, and 22), and the distal short arm of chromosome 1 — are regarded as normal artifacts and are not interpreted as indicating imbalance. The profile shows a deviation to more than 1.25 for chromosome 21, less than 0.75 for the Y chromosome, and more than 1.5 for the X chromosome, confirming the karyotype found by conventional cytogenetic analysis.

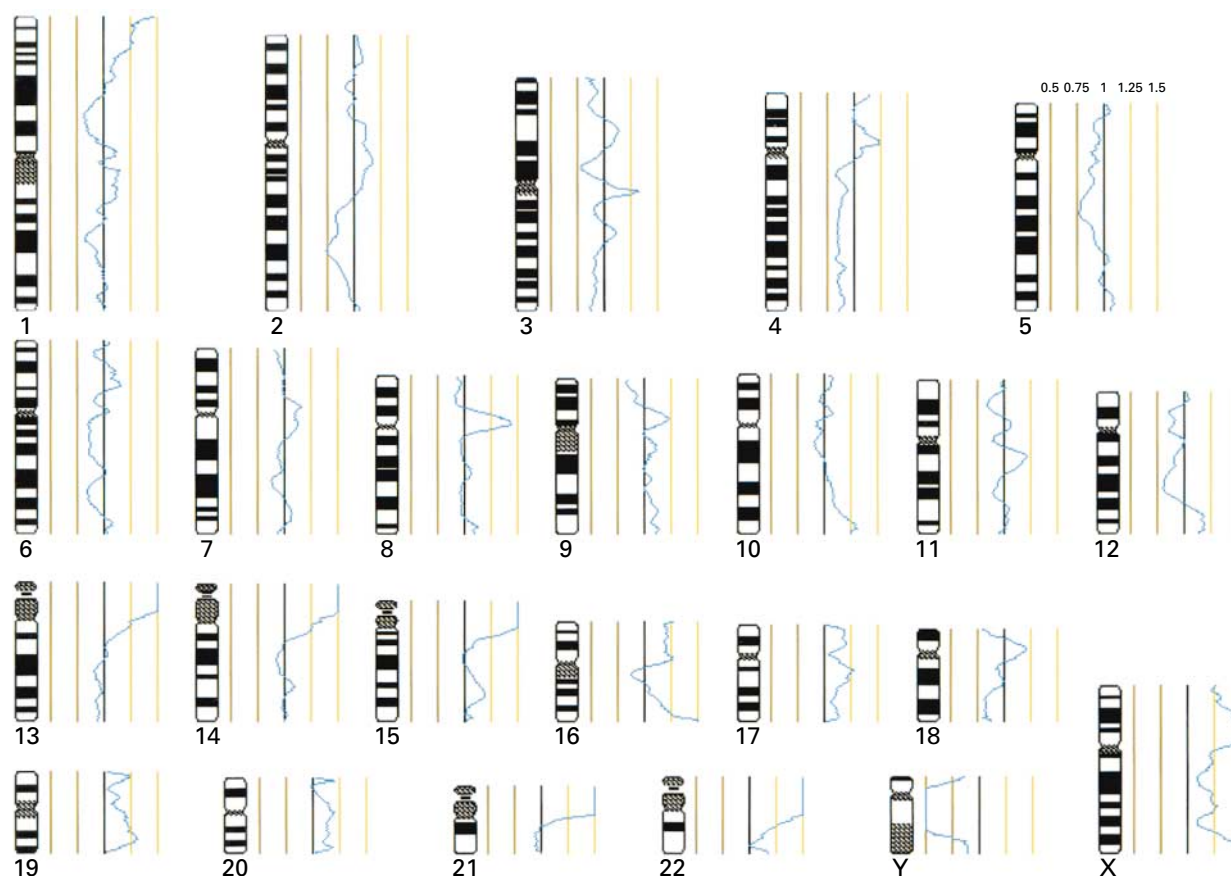
tric chromosomes, and the distal short arm of chromosome 1 — are regarded as normal artifacts and are not interpreted as indicating imbalance.<sup>12</sup> Comparative genomic hybridization takes approximately five days to complete, which is longer than three-day-old embryos can be maintained in culture, so embryos are cryopreserved until the results are available.

Embryos that underwent biopsy were cryopreserved as previously described<sup>13</sup> with the following modifications: HEPES-buffered human tubal-fluid medium (Irvine Scientific) was substituted for phosphate-buffered saline in all solutions, maternal serum (20 percent vol/vol) was substituted for human serum albumin, and the concentration of sucrose was increased from 0.1 M to 0.2 M. Thawing of embryos was carried out in HEPES-buffered human tubal-fluid medium supplemented with human serum albumin (20 mg per milliliter). 1,2-Propanediol was removed in two 5-minute steps in the presence of 0.3 M sucrose, and embryos were then transferred to 0.2 M sucrose for 10 minutes. Rehydration was completed by transfer of the embryos to HEPES-buffered human tubal-fluid medium for a further 10 minutes before incubation of the embryos in equilibrated culture medium at 37°C. Human tubal-fluid medium supplemented with 10 percent maternal serum was used for embryo transfer.

## RESULTS

From the patient's third cycle of in vitro fertilization, three of the six embryos were identified as normal with respect to chromosomes 13, 16, 18, 21, and 22 after investigation of a single blastomere by fluorescence in situ hybridization. One embryo was identified as monosomic for chromosome 18, and no results were obtained for two embryos. Two embryos identified as normal with respect to these five chromosomes were transferred to the patient, but no pregnancy resulted. The third normal embryo has been cryopreserved.

Single blastomeres were investigated from five additional embryos obtained during the same cycle of in vitro fertilization with the use of comparative genomic hybridization. Analysis of the control sample confirmed the female karyotype with trisomy 21 obtained by conventional chromosomal analysis (Fig. 1).



**Figure 2.** Profile Obtained after Comparative Genomic Hybridization with the Use of the Amplified DNA from a Single Blastomere from an Embryo.

The same format as in Figure 1 is used. The profile is interpreted as indicating a normal female karyotype in the test DNA. The X chromosome shows significant deviation to the right along most of the length, indicating two copies of the X chromosome in the test sample as compared with one copy in the reference DNA from a normal male. The profile of the Y chromosome in the euchromatic region shows significant deviation to the left, which is interpreted as the absence of Y-chromosome material in the test sample, as compared with the presence of a single Y chromosome in the reference DNA.

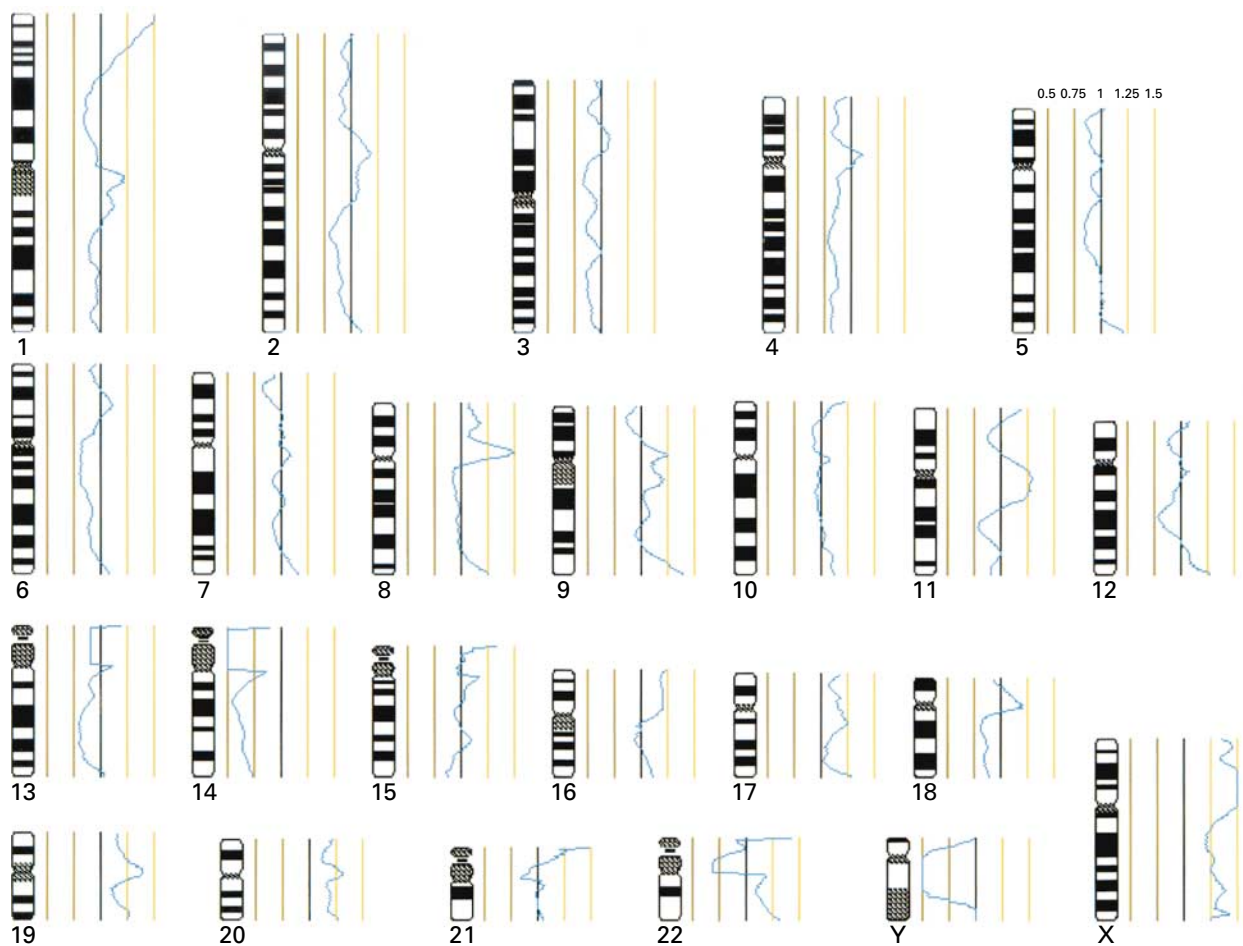
Analysis of the five embryos from the patient showed that the blastomere from only one embryo (with a female karyotype) appeared karyotypically normal for every chromosome (Fig. 2). A single blastomere from one embryo was identified as having monosomy 14 (Fig. 3), and a blastomere from another embryo was identified as having monosomy 4 and trisomy 16, suggesting possible meiotic aneuploidies. Blastomeres from two embryos showed more extensive chromosomal abnormalities: one was monosomic for chromosomes 8 and 9 and trisomic for chromosome 1, and the other was monosomic for chromosomes 4, 11, and Xq and trisomic for chromosomes 15, 16, and 6p. Such extensive abnormalities found in single blastomeres are associated with chromosomally disorganized embryos.<sup>12,14</sup>

The embryo identified as normal by comparative genomic hybridization was thawed and transferred and

resulted in a pregnancy. The patient declined invasive prenatal diagnostic testing, but a scan for nuchal translucency at 12 weeks' gestation, screening of maternal serum at 16 weeks' gestation, and fetal ultrasound at 20 weeks' gestation revealed no abnormalities. A healthy female infant was delivered by Cesarean section at 38 weeks' gestation. The infant had normal weight, length, and head circumference at birth; Apgar scores were 8 at one minute and 10 at five minutes.

## DISCUSSION

We report the clinical application of comparative genomic hybridization to obtain a complete embryonic karyotype before transfer of the embryo through in vitro fertilization. The transfer of a single embryo identified as normal by this technique resulted in the birth of a normal, full-term infant in a patient with



**Figure 3.** Profile Obtained after Comparative Genomic Hybridization with the Use of the Amplified DNA from a Single Blastomere from an Abnormal Embryo from the Patient.

The same format as in Figure 1 is used. The profile shows a deviation to less than 0.5 for chromosome 14 and the Y chromosome and more than 1.25 for the X chromosome. This was interpreted as indicating monosomy for chromosome 14 in the embryo.

a seven-year history of unexplained infertility. This patient previously had 12 embryos, including 4 identified as normal with respect to chromosomes 13, 16, 18, 21, and 22 by fluorescence in situ hybridization, transferred without any implantations. We attribute our success to comparative genomic hybridization, which is more effective than fluorescence in situ hybridization in detecting chromosomal abnormalities in single blastomeres, since it permits testing of all chromosomes rather than only five to nine. Analysis based on fluorescence in situ hybridization and probes for nine chromosomes (X, Y, 13, 14, 15, 16, 18, 21, and 22) as described by Munné et al.<sup>6</sup> would have detected only 4 of a total of 12 aneuploidies in the cells analyzed by comparative genomic hybridization. One of the four embryos found to have aneuploidy on comparative genomic hybridization (which was monosomic for chromosomes 8 and 9 and trisomic for chromosome 1) would have been identified as normal. With the use of fluorescence in situ hybridization with a five-probe set for chromosomes 13, 16, 18, 21, and 22, only 2 of 12 aneuploidies would have been detected, and two of four embryos with aneuploidy would have been identified as normal.

The benefit of being able to identify chromosomally normal embryos with the use of comparative genomic hybridization must be offset against the current necessity for embryo cryopreservation until the diagnosis can be completed. It has been estimated that cryopreservation reduces the implantation potential of human preimplantation embryos by 30 percent.<sup>13</sup> However, embryo cryopreservation is a routine part of in vitro fertilization treatment at many centers.

Analysis of nine chromosomes by fluorescence in situ hybridization increases the rate of implantation<sup>5</sup> and decreases the rate of embryo loss after implantation.<sup>6</sup> The use of comparative genomic hybridization to obtain a complete karyotype of all chromosomes can make it possible to identify karyotypically

normal embryos with greater certainty and may increase the number of live births per embryo transfer.

## REFERENCES

1. Gardner DK, Lane M. Embryo culture systems. In: Trounson AO, Gardner DK, eds. *Handbook of in vitro fertilization*. 2nd ed. Boca Raton, Fla.: CRC Press, 2000:205-64.
2. Delhanty JDA, Harper JC, Ao A, Handyside AH, Winston RML. Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. *Hum Genet* 1997;99:755-60.
3. Munné S, Marquez C, Reing A, Garrisi J, Alikani M. Chromosome abnormalities in embryos obtained after conventional in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril* 1998;69:904-8.
4. Iwarsson E, Lundqvist M, Inzunza J, et al. A high degree of aneuploidy in frozen-thawed human preimplantation embryos. *Hum Genet* 1999;104:376-82.
5. Gianaroli L, Magli MC, Ferraretti AP, Munné S. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with a poor prognosis: identification of the categories for which it should be proposed. *Fertil Steril* 1999;72:837-44.
6. Munné S, Magli C, Cohen J, et al. Positive outcome after preimplantation diagnosis of aneuploidy in human embryos. *Hum Reprod* 1999;14:2191-9.
7. Kahraman S, Bahce M, Samli H, et al. Healthy births and ongoing pregnancies obtained by preimplantation genetic diagnosis in patients with advanced maternal age and recurrent implantation failure. *Hum Reprod* 2000;15:2003-7.
8. Fung J, Weier H-UG, Goldberg JD, Pedersen RA. Multilocus genetic analysis of single interphase cells by spectral imaging. *Hum Genet* 2000;107:615-22.
9. Voullaire L, Wilton L, Slater H, Williamson R. Detection of aneuploidy in single cells using comparative genomic hybridization. *Prenat Diagn* 1999;19:846-51.
10. Wells D, Sherlock JK, Handyside AH, Delhanty JDA. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. *Nucleic Acids Res* 1999;27:1214-8.
11. Coonen E, Dumoulin JCM, Ramaekers FCS, Hopman AHN. Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence in-situ hybridization. *Hum Reprod* 1994;9:533-7.
12. Voullaire L, Slater H, Williamson R, Wilton L. Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. *Hum Genet* 2000;106:210-7.
13. Edgar DH, Bourne H, Speirs AL, McBain JC. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod* 2000;15:175-9.
14. Wells D, Delhanty JDA. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol Hum Reprod* 2000;6:1055-62.