

First clinical application of comparative genomic hybridization and polar body testing for preimplantation genetic diagnosis of aneuploidy

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Objective: To develop a preimplantation genetic diagnosis (PGD) protocol that allows any form of chromosome imbalance to be detected.

Design: Case report employing a method based on whole-genome amplification and comparative genomic hybridization (CGH).

Setting: Clinical IVF laboratory.

Patient(s): A 40-year-old IVF patient.

Intervention(s): Polar body and blastomere biopsy.

Main Outcome Measure(s): Detection of aneuploidy.

Result(s): Chromosome imbalance was detected in 9 of 10 polar bodies. A variety of chromosomes were aneuploid, but chromosomal size was found to be an important predisposing factor. In three cases, the resulting embryos could be tested using fluorescence in situ hybridization, and in each case the CGH diagnosis was confirmed. A single embryo could be recommended for transfer on the basis of the CGH data, but no pregnancy ensued.

Conclusion(s): Evidence suggests that preferential transfer of chromosomally normal embryos can improve IVF outcomes. However, current PGD protocols do not allow analysis of every chromosome, and therefore a proportion of abnormal embryos remains undetected. We describe a method that allows every chromosome to be assessed in polar bodies and oocytes. The technique was accurate and allowed identification of aneuploid embryos that would have been diagnosed as normal by standard PGD techniques. As well as comprehensive cytogenetic analysis, this protocol permits simultaneous testing for multiple single-gene disorders. (*Fertil Steril*® 2002;78:543–9. ©2002 by American Society for Reproductive Medicine.)

Key Words: Preconception diagnosis, preimplantation genetic diagnosis, comparative genomic hybridization, whole genome amplification, polar body, blastomere, aneuploidy, chromosome, FISH, embryo

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Data from natural cycles suggest that fewer than half of all human conceptions result in a live birth, most failing to survive beyond the first few days of life (1, 2). This is echoed in IVF, in which many embryos cease development before they can be transferred to the mother. Of those embryos that do survive transfer, only 5%–30% are thought to result in a live birth. There are many factors that negatively influence embryo survival, but one of the most important is chromosomal abnormality. Cytogenetic analyses have revealed that more than half of all human preimplantation embryos contain aneuploid cells (3–9). Most of

the chromosomal imbalances detected are not considered to be compatible with successful development.

With a few exceptions, aneuploid preimplantation embryos are morphologically normal (10), and consequently the usual assessments carried out in IVF clinics before embryo transfer do not allow them to be detected (11). Some infertility centers have now introduced preimplantation genetic diagnosis (PGD) in an effort to identify and preferentially transfer chromosomally normal embryos (for review, see Wells and Delhanty [(12)]). Usually, a single cell is biopsied from each embryo on day 3

after fertilization (8- to 10-cell stage) and is subjected to chromosomal analysis using fluorescence in situ hybridization (FISH). Selection of embryos on this basis has been shown to significantly reduce rates of spontaneous abortion, decrease incidence of aneuploid syndromes (such as Down's), and increase embryo implantation rates for several groups of patients (13–15). However, there are technical limitations to the number of chromosomes that can be analyzed by FISH. A human cell contains 23 pairs of chromosomes, and yet existing PGD protocols allow accurate assessment of only 5–9 chromosomes in each biopsied cell. Consequently it is likely that many abnormal embryos, incapable of forming a successful pregnancy, remain undetected and may be transferred.

We recently described a technique capable of detecting any chromosomal imbalance in a single cell. The method is based on amplification of the entire DNA content of the cell, followed by comparative genomic hybridization (CGH). This technique has provided useful research data (16–19), revealing the full extent of aneuploidy and mosaicism in human preimplantation embryos, but until recently, the time needed to perform the procedure had precluded clinical application. There is only a very narrow window of time available for preimplantation testing. Most PGD centers try to perform diagnosis and embryo transfer within 24 hours of receiving the biopsied cell, allowing a transfer on day 4 after biopsy on day 3. However, protocols for DNA amplification, labeling, and CGH require a total of 5–6 days to complete.

We have overcome this problem by developing a CGH protocol that allows comprehensive aneuploidy screening within a timeframe compatible with embryo transfer on day 4 after fertilization. To achieve this, it was necessary to develop an accelerated protocol capable of producing results with a hybridization time of just 30 hours, half the length usually employed for CGH. Additionally, we have chosen to apply CGH to polar bodies, available for biopsy on the day of fertilization, rather than to blastomeres, which cannot be biopsied until 3 days later. Chromosomal gain or loss in a polar body is accompanied by a reciprocal loss or gain in the oocyte. Polar-body analysis can therefore be used to infer whether an oocyte is normal or aneuploid. This is the first time that CGH has been successfully applied to polar bodies and the first report of clinical application of polar-body CGH.

Existing PGD protocols focus on one of two methods: FISH for chromosomal analysis or the polymerase chain reaction (PCR) for the diagnosis of single-gene disorders. Unfortunately, these two techniques cannot readily be applied to the same cell, and consequently few PGD tests that combine both aneuploidy screening and analysis of DNA sequence have been reported. We explored the possibility that whole-genome amplification could circumvent this limitation by providing sufficient amplified single-cell DNA to perform CGH and also multiple PCR-based single-gene

tests. This could be particularly useful for the significant numbers of couples seeking PGD for a single-gene disorder where the mother is of advanced reproductive age and therefore at increased risk for aneuploid pregnancy.

MATERIALS AND METHODS

Polar-body biopsy and aneuploidy screening were carried out with the approval of the institutional review board of St. Barnabas Medical Center.

Stimulation and Intracytoplasmic Sperm Injection

The patient was a 40-year-old woman, suffering from secondary infertility due to ovarian dysfunction. She had previously undergone six treatment cycles at a different fertility clinic (three involving transfer of frozen embryos). During these cycles, embryonic development and morphology were generally good; however, no pregnancies were achieved. After controlled ovarian hyperstimulation (20), oocytes were collected and their polar bodies removed (15, 21). The eggs were subsequently fertilized using intracytoplasmic sperm injection (ICSI), and the resulting embryos were cultured (22).

Polar Body Preparation

Polar bodies were washed in four 10- μ L droplets of phosphate-buffered saline–0.1% polyvinyl alcohol, transferred to a microfuge tube containing 2 μ L of proteinase K (125 μ g/mL) and 1 μ L of sodium dodecyl sulfate (17 μ M), and overlaid with oil. Incubation at 37°C for 1 hour, followed by 15 minutes at 95°C, was done to release DNA.

Whole-Genome Amplification Using Degenerate Oligonucleotide Primer PCR

Polar-body DNA was amplified using a modification of previously reported methods (16). Amplification took place in a 50- μ L reaction volume containing the following: 0.2 mM dNTPs; 2.0 μ M degenerate oligonucleotide primer, CCGACTCGAGNNNNNNATGTGG (23); 1 \times SuperTaq Plus buffer, and 2.5 U of SuperTaq Plus polymerase (Ambion, Austin, TX). Thermal cycling conditions were as follows: 94°C for 4.5 minutes; 8 cycles of 95°C for 30 seconds, 30°C for 1 minute, a 1°C/s ramp to 72°C, and 72°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 1.5 minutes; and finally, 72°C for 8 minutes. After amplification was complete, a 5- μ L aliquot of amplified DNA was transferred to a new PCR tube and retained for single-gene testing.

Stringent precautions against contamination, as discussed previously (24), were observed throughout polar-body isolation, lysis, and amplification procedures. The incidence of contamination was assessed by taking 2 μ L of phosphate-buffered saline from the final droplet used for washing each polar body and subjecting it to the entire degenerate oligonucleotide primed PCR and CGH procedure. No DNA

TABLE 1

Data from CGH analysis of polar bodies and FISH analysis of corresponding embryos.

Polar body No.	CGH interpretation of first polar body	Assessment of embryos produced	FISH diagnosis of embryo ^a
1	22, X, -22	At risk—trisomy 22	Poor development; not biopsied
2	22, -X	At risk—trisomy X or XXY	Oocyte failed to fertilize
3	23, X, -21, +22	At risk—trisomy 21 and monosomy 22	Oocyte failed to fertilize
4	22, X, -5	At risk—trisomy 5	FISH confirms trisomy 5
5	24, X, +16	At risk—monosomy 16	Oocyte failed to fertilize
6	24, X, +20	At risk—monosomy 20	FISH confirms monosomy 20
7	22, X, -14	At risk—trisomy 14	No confirmation by FISH ^b
8	22, X, -16	At risk—trisomy 16	Oocyte failed to fertilize
9	24, X, +2	At risk—monosomy 2	Oocyte failed to fertilize
10	CGH considered unreliable because of weak hybridization		FISH indicates trisomy 21
11	Polar body lost during biopsy, no CGH performed		FISH indicates trisomy 22
12	23, X	Normal	FISH indicates normal

^a A single embryo cell (blastomere) was biopsied from each embryo and tested by FISH for the following chromosomes: 13, 15, 16, 17, 18, 21, 22, X, and Y. Additionally, any chromosomes highlighted by CGH were also tested in this way. Only embryo 12 could be recommended for transfer on the basis of CGH analysis.

^b The FISH probe for chromosome 14 failed to give any result in this case.

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should be present in such negative controls, and consequently there should be no detectable fluorescence if they are amplified and used for CGH. Preclinical testing of this strategy involved analysis of >100 single cells (blastomeres, polar bodies, and other cell types) and revealed contamination affecting ~1% of negative controls.

Labeling of DNA and Probe Preparation

Amplified DNA samples (whole-genome amplification products) were precipitated and fluorescently labeled by nick translation. Polar-body DNA was labeled with Spectrum Green-dUTP (Vysis, Downers Grove, IL), whereas 46, XX (normal female) DNA was labeled with Spectrum Red-dUTP (Vysis). Both labeled DNAs were precipitated with 30 μ g of Cot1 DNA. Precipitated DNA was resuspended in a hybridization mixture composed of 50% formamide; 2 \times saline sodium citrate [SSC; 20 \times SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7]; and 10% dextran sulfate). Labeled DNA samples dissolved in hybridization mixture were denatured at 75°C for 10 minutes, then allowed to cool at room temperature for 2 minutes, before being applied to denatured normal chromosome spreads as described below.

Comparative Genomic Hybridization

Metaphase spreads from a normal male (46, XY; Vysis) were dehydrated through an alcohol series (70%, 85%, and 100% ethanol for 3 minutes each) and air dried. The slides were then denatured in 70% formamide, 2 \times SSC at 75°C for 5 minutes. After this incubation, the slides were put through an alcohol series at -20°C and then dried. The labeled DNA probe was added to the slides, and a coverslip was placed over the hybridization area and sealed with rubber cement.

Slides were then incubated in a humidified chamber at 37°C for 25–30 hours. After hybridization, the slides were washed sequentially in 2 \times SSC (73°C), 4 \times SSC (37°C), 4 \times SSC + 0.1% Triton-X (37°C), 4 \times SSC (37°C), and 2 \times SSC (room temperature); each wash lasted 5 minutes (modification of the procedure of Levy et al. [(25)]). The slides were then dipped in distilled water, passed through another alcohol series, dried, and finally mounted in anti-fade medium (DAPI II, Vysis) containing diamidophenylindole to counterstain the chromosomes and nuclei.

Microscopy and Image Analysis

Fluorescent microscopic analysis allowed the amount of hybridized polar body (green) DNA to be compared with the amount of normal female (red) DNA along the length of each chromosome. Computer software (Applied Imaging, Santa Clara, CA) converted these data into a simple red–green ratio for each chromosome; deviations from a 1:1 ratio were indicative of loss or gain of chromosomal material.

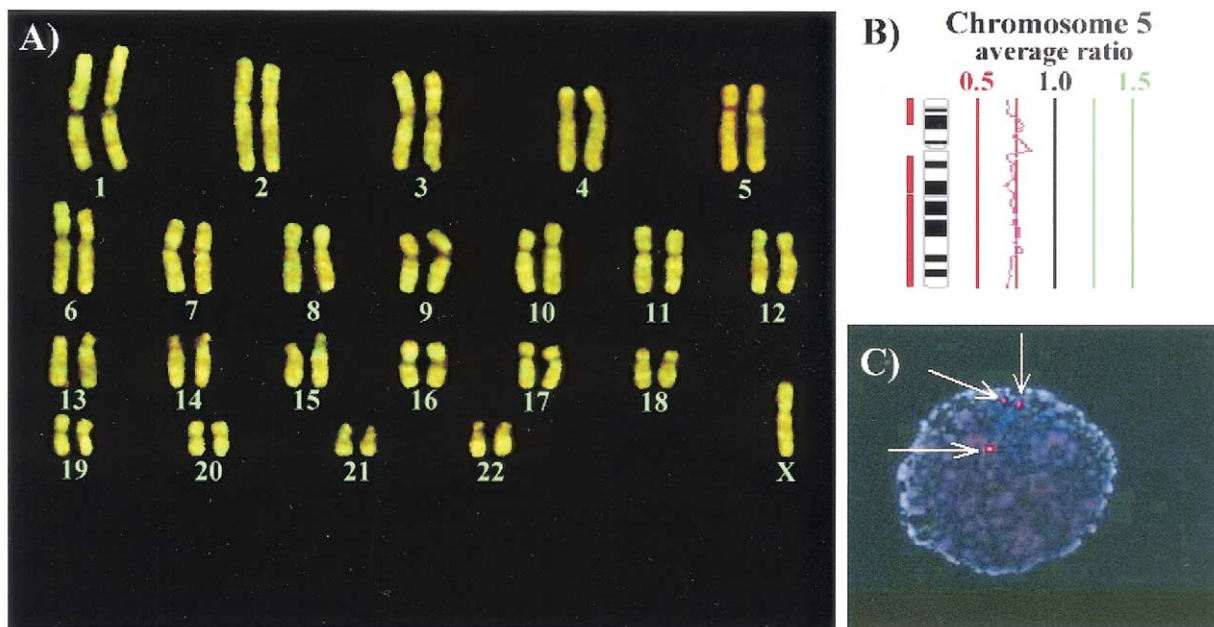
Blastomere Biopsy and FISH

During day 3 of development, one cell per embryo was biopsied by zona drilling using acidified Tyrode's solution, and the embryos were returned to culture as described elsewhere (26). All of the embryos were at the 4- to 12-cell stage of development at the time of biopsy. All blastomeres were fixed individually according to our protocol (27).

In addition to the nine chromosomes routinely assessed during PGD at St. Barnabas Medical Center (XY, 13, 15, 16, 17, 18, 21, 22), chromosomes that had been highlighted as potentially abnormal on the basis of the CGH were also tested in a third hybridization step.

FIGURE 1

Comparative genomic hybridization results from a first polar body. Normal metaphase chromosomes (46, XY) are hybridized with polar body DNA (green) and normal female DNA (red). (A) metaphase, (B) red–green ratio for chromosome 5. A deficiency of green fluorescence on chromosome 5 reveals this polar body to have a 22, X, –5 karyotype. The corresponding oocyte was considered likely to produce an embryo with an extra copy of chromosome 5. (C) Fluorescence in situ hybridization analysis of a cell from the resulting embryo; three red signals confirm trisomy 5.



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Oocytes and embryos were analyzed in accordance with guidelines approved by the internal review board of St. Barnabas Medical Center, including written consent from the patients. The patients were informed that the procedure was experimental.

Analysis of the Cystic Fibrosis Gene

As well as being subjected to chromosome testing using CGH, polar bodies were tested for cystic fibrosis mutation. A 5- μ L aliquot of degenerate oligonucleotide primer PCR-amplified polar-body DNA was taken before precipitation and CGH analysis. A fragment of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, encompassing the site of the common cystic fibrosis mutation, Δ F508, was then amplified from this aliquot. The CFTR fragments produced were analyzed by electrophoresis to determine whether the Δ F508 mutation was present. Amplification and analysis of CFTR were performed as described elsewhere (28).

RESULTS

We attempted biopsy of first polar bodies from 12 oocytes, retrieved after controlled ovarian hyperstimulation.

Eleven polar bodies were successfully biopsied, and 10 yielded CGH results. Only 1 of the 10 polar bodies was found to contain a normal number of chromosomes. A range of abnormalities, including six incidences of loss of chromosomal material and four examples of gain, were detected in the 9 aneuploid polar bodies (complete summary given in Table 1). Smaller chromosomes were more often involved in an imbalance than were their larger counterparts, with chromosomes 16 and 22 both affected twice.

No FISH data were available from one embryo that had arrested and five oocytes that had failed to fertilize or had not fertilized normally. Of the remaining four embryos for which CGH results had been obtained, FISH confirmation was possible in three (embryo 12, normal; embryo 4, trisomy 5; and embryo 6, monosomy 20) (Fig. 1). Confirmation was not possible for the fourth embryo, identified as at risk of trisomy 14 by CGH, because of failure of the chromosome 14 FISH probe. Embryo 10 (for which the CGH analysis had not worked) and embryo 11 (for which polar body biopsy had failed) were successfully analyzed by FISH and were shown to be trisomy 21 and trisomy 22, respectively.

No discordance between CGH and FISH results was seen on this occasion, although it is likely that a larger series of

polar bodies would reveal some discrepancies. This is anticipated because aneuploidy caused by precocious sister chromatid segregation, present in the oocyte at metaphase I, may be corrected at anaphase II in some cases.

On the basis of CGH results, only one embryo could be recommended for transfer. This embryo was developmentally normal and had compacted before transfer. Despite the success of the diagnostic strategy and an uneventful transfer, no pregnancy was obtained on this occasion.

To test whether chromosome analysis using CGH can be used in conjunction with mutation testing of specific genes, we retrospectively amplified surplus whole-genome amplification products, not used for CGH. Amplification of the CFTR gene was successful from 10 of 11 polar bodies. The only sample to fail amplification was embryo 10, which also failed to produce any CGH results. This suggests that the polar body was lost during transfer to the PCR tube or that its DNA was too severely degraded for efficient amplification. Precise electrophoretic analysis of the size of the amplified CFTR fragments revealed that none of the polar bodies were carriers of the $\Delta F508$ mutation (a deletion of three base pairs). This result was not unexpected, as the amplification and analysis of CFTR was a purely academic exercise in this case, aimed at testing the feasibility of combined CGH and mutation analysis. The patient was not known to be a cystic fibrosis mutation carrier.

DISCUSSION

Preimplantation aneuploidy screening can be used to assist in the identification of the IVF embryos most likely to form a successful pregnancy. Preferential transfer of the embryos ascertained in this way has improved outcomes for certain groups of IVF patients. However, the full potential of this strategy remains to be realized, as current protocols only provide data on approximately one third of the chromosomes in each cell. The use of CGH could theoretically boost success rates further by allowing a complete analysis of all the chromosomes. Unfortunately the time required to complete the procedure has, until recently, precluded clinical application.

Some have advocated a PGD strategy in which embryos are biopsied and then frozen, allowing as much time as necessary for CGH analysis (29). However, it is uncertain that this will benefit the majority of our patients, whose primary motivation for using PGD is to improve their chances of becoming pregnant. Current cryopreservation techniques diminish embryo viability even in couples with good prognosis (30). For embryos that have been biopsied before freezing, survival rates are lower still (31). A precise evaluation of CGH strategies involving embryo freezing has not yet been completed. However, it is possible that the decline in viability will offset any benefits gained by chromosomal screening.

We have opted for a CGH protocol that avoids cryopreservation and is compatible with embryo transfer on day 4 after fertilization. This is achieved by employing polar-body biopsy and an accelerated CGH protocol. Because the majority of human aneuploidies arise during the first meiotic division of the female, analysis of the first polar body allows identification of most chromosomal imbalances.

The principal causes of female meiosis I aneuploidy are nondisjunction and precocious sister chromatid segregation (PSCS). In the case of PSCS, a univalent may prematurely split into its component chromatids, which then segregate independently and at random. Whether the oocyte will ultimately be normal or unbalanced at the end of the second meiotic division depends on whether the unattached chromatid enters the second polar body at anaphase II or remains in the oocyte (32). A significant number of meiosis I errors caused by PSCS are expected to be corrected at anaphase II. Consequently, analysis of first polar bodies alone will overestimate the number of aneuploid oocytes. To avoid excluding potentially normal oocytes, it is necessary to confirm any abnormalities detected in the first polar body by subsequent testing of second polar bodies or embryos.

Our use of first-polar body CGH highlighted the chromosomes that were at risk of malsegregation; we then tailored our subsequent FISH analysis of biopsied blastomeres accordingly. Chromosomal screening with the nine FISH probes routinely used for PGD at our Center would have suggested that five embryos (embryos 4, 6, 7, 9, and 12) were chromosomally normal. However, CGH revealed that four of these were at risk of aneuploidy for chromosomes that are not usually tested by FISH. Previously, the chromosomes chosen for preimplantation screening were those that have a high frequency of aneuploidy in prenatal samples or spontaneous abortions. However, it is clear that a much wider variety of chromosome abnormalities can be found in oocytes and cleavage-stage embryos, and it has been suggested that screening for aneuploidies that are common later in pregnancy may not be the most appropriate strategy (33). Comparative genomic hybridization data such as those reported here may indicate which chromosomes are most relevant, allowing an optimal set of probes for PGD-FISH analysis to be selected.

Interestingly, 7 of the 10 unbalanced chromosomes in this set of polar bodies were equal to or smaller in size than chromosome 14. These data mirror recent findings achieved using spectral karyotyping (SKY) analysis of oocytes (34). Small chromosomes tend to form fewer chiasmata during meiosis, and it has been suggested that reduced recombination could predispose them to nondisjunction (35). The only large chromosomes to be affected by imbalance in this investigation were chromosomes 2 and 5. These chromosomes have been little studied in human embryos; however, there is some evidence to suggest that the aneuploidy rate of chromosome 2 may be unusually high for such a large

chromosome. Errors involving this chromosome have been reported in previous oocyte and preimplantation embryo studies employing CGH and SKY (18, 19, 34).

Nine of the oocytes from this case were considered to be at risk of producing an embryo with an unbalanced karyotype. Of the nine, only one (oocyte 2) had a predicted aneuploidy compatible with development to term. This high frequency of presumably lethal aneuploidy further illustrates the reasoning behind preimplantation testing. Five of the oocytes were predicted to have imbalance involving chromosomes 16, 22, or X, which are frequently aneuploid in spontaneous abortions.

Direct chromosomal analyses of oocytes and polar bodies, using techniques such as G-banding or SKY, have experienced problems related to chromosome morphology and spreading. These difficulties have compromised efforts to determine the rate of aneuploidy, particularly monosomy, in oocytes. Comparative genomic hybridization is a DNA-based technique and as such circumvents these problems, allowing an accurate estimation of the incidence of aneuploidy. Loss of chromosomal material was detected in six polar bodies (potentially leading to trisomy in the corresponding embryos), whereas a gain of material was detected in four (indicating a risk of monosomy in the embryo).

Preimplantation embryos can be screened for specific cytogenetic anomalies or single-gene defects by employing FISH or PCR, respectively. However, it is not possible to perform both of these methods on the same cell without an accompanying reduction in accuracy (36, 37). Consequently, diagnostic applications, for which accuracy is essential, must focus on chromosomes or DNA sequence, but not both. Our studies have revealed that certain whole-genome amplification methods provide sufficient DNA for the analysis of multiple genes as well as CGH (16). This has allowed us to perform tests for single-gene disorders as well as chromosomal imbalance in the same biopsied polar body or blastomere. During this study, the polar bodies were subjected to degenerate oligonucleotide primed PCR, and most of the resulting amplified DNA was used for CGH, whereas a smaller aliquot was used for the analysis of the cystic fibrosis gene. A significant proportion of couples seeking PGD for single-gene disorders are of advanced reproductive age. The ability to perform both cytogenetic and molecular genetic tests may be beneficial for such couples, allowing simultaneous screening for inherited disease and also for age-related aneuploidy.

Significant progress has been made since we first reported the application of CGH to blastomeres (16), with several groups working on the development of clinically applicable protocols (29). However, it is important to make clear that all CGH protocols reported to date are somewhat impractical and labor intensive. Protocols that use cryogenics to provide sufficient time for CGH analysis may be damaging the embryos and consequently confounding efforts to increase

pregnancy rates. The procedure that we report in this paper is also imperfect. Although much shorter than other CGH protocols, it is still labor intensive. Furthermore, although we believe that the impact of double biopsy (polar body followed by blastomere) on embryo viability is minor, this has not yet been confirmed. More advances will be required before CGH can be offered to a larger population of infertile patients.

Global aneuploidy screening by CGH in polar bodies will undoubtedly increase our understanding of errors in female meiosis and could help to extend the improvements in IVF success rates already achieved using the limited chromosomal tests currently available. The identification of the embryos most likely to produce a child could also allow pregnancy rates to be maintained while the number of embryos transferred per cycle is reduced, thus reducing the incidence of high-order multiple pregnancies. CGH requires further development before wider clinical application can be considered but holds great promise for the future.

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