

Live birth after polar body array comparative genomic hybridization prediction of embryo ploidy—the future of IVF?

Simon Fishel, Ph.D.,^a Anthony Gordon, Ph.D.,^b Colleen Lynch, M.Sc.,^a Ken Dowell, D.M.,^a George Ndukwe, M.B.B.S.,^a Ehab Kelada, M.B., B.Ch.,^a Simon Thornton, M.D.,^a Lucy Jenner, Ph.D.,^a Ellen Cater, B.Sc.,^a Anthony Brown, Ph.D.,^a and Jose Garcia-Bernardo, Ph.D.^a

^a CARE Fertility, Nottingham; and ^b BlueGnome Limited, Cambridge, United Kingdom

Objective: To ascertain meiotic aneuploidy of the human egg using array comparative genomic hybridization to evaluate the 23-paired chromosome copy number of first polar body as an objective prognosticator of embryo viability for embryo transfer in the same cycle.

Design: Case report.

Setting: Independent-sector IVF program.

Patient(s): A 41-year-old woman with a history of 13 failed cycles of IVF.

Intervention(s): Polar body biopsy of metaphase II eggs.

Main Outcome Measure(s): Birth.

Result(s): Two of the nine eggs were euploid, and the resulting embryos, although morphologically inferior to sibling embryos, were selected for transfer to the uterus, resulting in the birth of a normal healthy baby.

Conclusion(s): Selection of euploid eggs, as an objective parameter of subsequent embryo viability and with the opportunity to transfer embryos in the same cycle could maximise the opportunity for live birth after IVF even in cases with poor prognosis. (Fertil Steril® 2010;93:1006.e7–e10. ©2010 by American Society for Reproductive Medicine.)

Key Words: IVF, polar body biopsy, PGS, PGD, array CGH, egg chromosomes, birth

More than 70% of all IVF embryos fail to implant, and only 35%–40% of all cycles succeed after an IVF procedure. It is believed that the single major cause of IVF failure is the quality of harvested eggs, and up to 75% of such eggs are aneuploid (1, 2). For >15 years, aneuploidy in IVF has been studied using polar body, cleavage-stage blastomere, or blastocyst trophectoderm tissue, with variable and conflicting success (3). Because ploidy and the conventional assessment of embryo viability, such as morphology, cleavage rates, and a raft of complex observational scores, are poorly correlated, the focus on chromosome copy number has become ever more vital. Most technology has focused on the analysis of a limited number of chromosomes, usually up to nine, with the majority of clinics using cleavage-stage blastomeres with fluorescent in situ hybridisation (FISH) technology (4, 5). Recent controversy has illuminated the deficiencies in both the use of the blastomere approach,

with the embryo's propensity for chromosome mosaicism, and FISH as a valuable prognosticator of embryo viability (6).

The introduction of comparative genomic hybridization (CGH) has realized the essential opportunity to analyze all 23 pairs of chromosomes in a single polar body or all 46 chromosomes of an embryonic cell (2, 7–9). However, until now, the use of CGH—also known as metaphase CGH—has required the use of embryo freezing, because results cannot be garnered for the technology to be of use in real time. Therefore, any uplift in delivery rates had to mitigate the loss of embryos due to the freeze-thaw process. More recently, a robust and reliable array CGH method has been pioneered and used to assess full 23 or 46 chromosome copy number of single cells in real time, permitting the transfer of fresh embryos. We report on our first case, resulting in the birth of the first baby using this technology on polar bodies, an intriguing insight into subjective microscopically assessed embryo quality, and argue the case for the use of this technology using polar body, blastomere, or trophectoderm cells in the future practice of IVF.

MATERIALS AND METHODS

Participants

The female partner was aged 41 years, and the couple had undergone 13 previous cycles of IVF at several clinics. During the previous cycles, FISH had been undertaken once; immune issues had been identified and the woman was treated with IV immunoglobulin, prednisolone, clexane, aspirin, and

Received July 10, 2009; revised September 25, 2009; accepted September 27, 2009; published online November 25, 2009.

S.F. has nothing to disclose. A.G. has nothing to disclose. C.L. has nothing to disclose. K.D. has nothing to disclose. G.N. has nothing to disclose. E.K. has nothing to disclose. S.T. has nothing to disclose. L.J. has nothing to disclose. E.C. has nothing to disclose. A.B. has nothing to disclose. J.G.-B. has nothing to disclose.

Reprint requests: Simon Fishel, Ph.D., CARE Fertility, John Webster House, 6 Lawrence Drive, Nottingham Business Park, Nottingham NG8 6PZ, United Kingdom (FAX: 44 115 967 0381; E-mail: simon.fishel@carefertility.com).

metformin; reproductive outcome was one ectopic pregnancy and two miscarriages before attending CARE Fertility in 2008. During this cycle pituitary desensitization was induced with daily 0.5 mL SC busserelin (Sanofi Aventis, Guildford U.K.), 500 mg metformin three times a day (Bristol-Myers Squibb, Uxbridge, U.K.), and, after confirmation of “down-regulation,” ovarian stimulation was induced using Menopur (Ferring, Slough, U.K.), 150 IU daily. When five follicles >17 mm were observed on day 12 of stimulation, 250 µg SC hCG (Ovitrelle; Merck-Serono, Feltham, U.K.) was administered. Twelve eggs, nine at metaphase II, were collected under sedation using ultrasound transvaginal control.

Procedures

The eggs were cultured in Fercult IVF medium (Microm UK, Bicester, U.K.) supplemented with 0–4% human serum albumin for 3 h before the nine metaphase 2 underwent ICSI (intracytoplasmic sperm injection) using a Research Instruments (Falmouth, U.K.) Integra Ti micromanipulator system. Immediately after ICSI, polar body biopsy was undertaken using a Saturn laser making a 7-µm opening in the zona pellucida along the horizontal plane of the polar body. Using a 13–15-µm inner diameter biopsy pipette (Humagen, Charlottesville, NC), each polar body was carefully extracted and placed into 2–5 µL phosphate-buffered saline in a sterile 0.2 mL polymerase chain reaction tube lysis buffer. Each was carefully labeled with the number of each egg, and each inseminated egg was cultured individually after ICSI in Quinn cleavage medium (Rochford Medical, Yarnton, U.K.) at 6% CO₂ in air at 37°C until day 2 after egg retrieval; on day 3, the embryos were transferred to Global IVF medium (IVFonline).

Polar bodies and blanks were amplified using the Genome Plex WGA4 Single Cell Whole Genome Amplification Kit (Sigma Aldrich, Poole, U.K.) modified according to the protocol of Fiegler et al. (10). Resulting amplified DNA products were assessed for DNA quantity by gel electrophoresis and 260 nm Nanodrop (ThermoFisher, Waltham, MA) spectrometry. Only samples which showed a high DNA quantity were labelled. Four hundred nanograms of sample DNA and 400 ng Promega Male DNA (G1471) were labelled with Cy3 and Cy5, respectively, using the BlueGnome fluorescent labeling system (BlueGnome, Cambridge, U.K.) according to the manufacturer's instructions. Labeled sample and Promega DNA were cohybridized onto BlueGnome 24sure BAC microarrays. Resulting 24sure microarrays were hybridized, washed, and scanned according to the BlueGnome CytoChip protocol.

Scanned images were analyzed and quantified, and whole chromosomal copy number ratios were reported using the CytoChip algorithm fixed settings in BlueFuse Software (BlueGnome). Determination of specific Genomeplex amplification on the resulting array CGH plot in BlueFuse was by visualization of Y nullisomy in the resulting profiles. Once specific amplification was observed, autosomal profiles were analyzed for gain or loss of whole chromosomal ratio using a 3 × SD assessment. Sample profiles were then reported as either euploid or aneuploid.

RESULTS

At 18.5 hours after insemination, seven eggs displayed two pronuclei and each of these zygotes cleaved to embryos. One polar body (egg no. 9) was highly fragmented, its corresponding egg degenerated after ICSI, and the polar body was not analyzed. As examples, the profiles from polar bodies corresponding to egg nos. 1 and 2 are represented in Figure 1. These data were available on day 3 after egg retrieval; seven of the eight polar bodies yielded data, one egg failed to fertilize, and only two of the seven embryos were scored as euploid. One of the two euploid embryos had developed to 6-cell grade 3 and the other to 4-cell grade 2 (grade 1 being the most uniform in cell size and cell membranes and devoid of intercellular fragments). These two embryos were transferred. Utrogestan 200 mg twice daily was prescribed for luteal support, and prednisolone 200 mg daily, clexane 40 mg daily, estradiol valerate 2 mg three times daily, and immunoglobulin 25 g IV were also prescribed in the luteal phase and early pregnancy.

Home pregnancy test 18 days after egg retrieval gave a positive result. The first pregnancy scan at 40 days after fertilization showed a single intrauterine gestation sac with a fetal pole, a crown rump length of 5.7 mm, yolk sac, and beating heart, and at 268 days after fertilization the couple were delivered of a healthy baby weighing 3.18 kg.

DISCUSSION

To our knowledge, this is the first reported birth resulting from full 23-paired chromosome copy number assessment of polar bodies in real time. Conventional approaches to a case with this background would predict a live birth rate no higher than 10% at best. It is axiomatic that, even with this couple's reproductive history, a live birth could result from IVF treatment at some time, however unlikely. However, at the time of writing, our ongoing trial with this technology has resulted in 20 embryo transfers with four confirmed pregnancies (20%), one after cryopreservation of zygotes, in a cohort of women with a mean age of 40.8 years and all having a history of repeated failure of IVF and miscarriage, and all had multiple indications for high-risk failure. This is an encouraging start for a group of patients with an expected live birth rate of ≤5%.

The intriguing question in the present case is whether this birth heralds a novel approach that will statistically and importantly enhance the chances of live birth for all IVF patients, and reduce the amount of unnecessary cryopreservation of aneuploid embryos by reliance upon objective chromosome information instead of conventional subjective microscopic morphologic observation. Had array CGH not been used, other embryos that were more advanced in cell number and/or possessing superior morphologic grade would have been transferred—as in this couple's previous 13 attempts at IVF—and these would have been aneuploid. Chromosome aberrations are known to be dominant in failed IVF, causing cleavage arrest, failed implantation, and miscarriage, irrespective of the advantage of preimplantation screening for viable aneuploidies such as Down, Klinefelter, Edwards, Turner, and Patau Syndromes. Earlier studies on polar bodies indicated that aneuploid eggs always propagate aneuploid zygotes, but if the first polar body indicates a euploid egg, only 9% of these will be scored as an euploid zygotes by second polar body screening (2). At this time, when the technology is expensive, it is important to get a balance on numbers of cells tested.

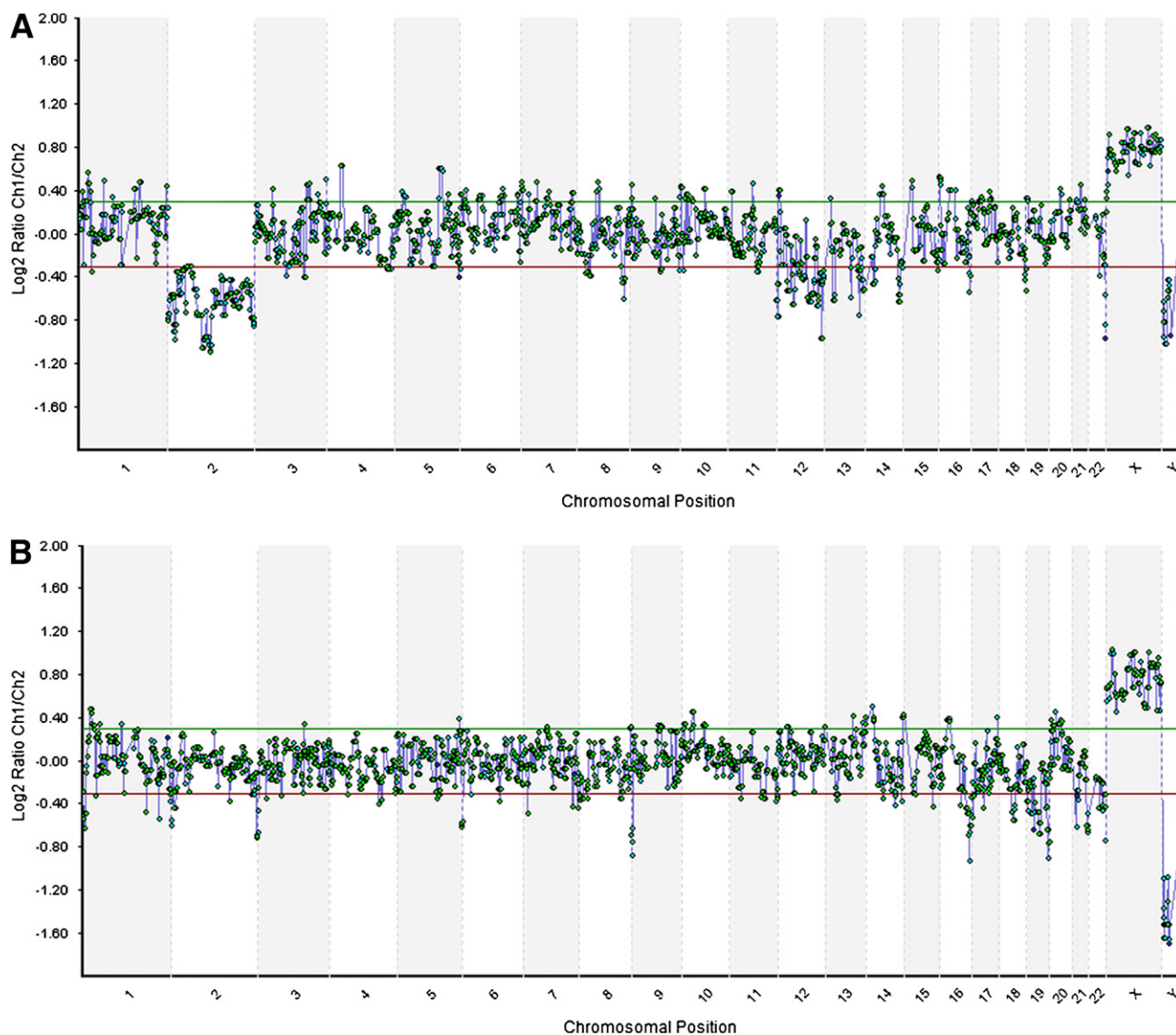
But this technology, or assessment of chromosome copy number, is not just for the most difficult of cases presenting for IVF. Indeed, given that younger and less complex cases still have an egg aneuploidy rate of up to 50% of harvested eggs, and that such cases statistically produce more embryos, a vital prognosticator should ultimately enhance live birth rates. Furthermore, it is evident that a high degree of aneuploidy also occurs *in vivo*, with up to 70% of human conceptions resulting in failure and >50% of spontaneous abortions having chromosome imbalances (11, 12), and in young fertile couples, as observed in those undergoing prenatal diagnosis for single-gene disorders (13).

The molecular genetics behind this case was made possible by advances in genome-wide assessment of copy number using array-based technologies, amplification procedures, and improved statistical methods for comparing copy number between test and reference samples. The technology, however, is changing at a rapid pace, with a variety of single-nucleotide polymorphism, whole-genome oligonucleotide, and bead-based chip formats becoming available (14, 15).

Research before this first clinical case confirmed that this technology is suitable for polar body, blastomere, or trophectoderm cells.

FIGURE 1

(A) First polar body result showing aneuploid egg as a result of loss of chromosomes 2 and 12. (B) Second polar body correlating euploid pattern in the corresponding egg.



Fishel. Birth after polar body array CGH. Fertil Steril 2010.

However, the best use of this technology needs to be determined and will depend on establishing definitive information on meiosis 1 and 2 errors compared with cleavage mitotic aneuploidy, spermatozoon contribution to aneuploidy, and, ultimately, conformity between trophectodermal and inner cell mass cells. Using the polar body does not require manipulation of the embryo, which is an ethical advantage for many patients and a few government regulators. Earlier studies have suggested that knowing the status of aneuploidy of the egg from the first polar body provides >85% concordance to embryo aneuploidy (2), which is supported by our knowledge of all human aneuploidy (1). However, for IVF it is essential that this information is established accurately and beyond doubt if the first, or first and second, polar bodies are to be used in preference to the embryo.

Conventionally, the most common assessment of aneuploidy has been a biopsy of a single blastomere from an embryo 3 days after fertilization, at the 6–8-cells stage. Clearly this approach will assess postzygotic aneuploidy, such as contribution from a spermatozoon or mitotic nondisjunction or more complex rearrangements (13). However, for chromosome copy number, embryo mosaicism leads to false positive and negative information, resulting in the transfer of affected embryos or the disposal of potentially viable embryos (16, 17). Understanding the level of mosaicism in early cleavage-stage embryos is vital, but the derivation of sophisticated algorithms from parental DNA and reference DNA for ranking such embryos for chromosome copy number may improve the probability of accurate selection (18, 19).

An alternative approach is the use of trophectodermal cells from blastocysts, but a significant number of patients will be at risk of losing viable embryos to extended in vitro culture, exposing some patients to having no embryos to transfer, and owing to the time it takes to obtain data, many aneuploid embryos will be unnecessarily cryopreserved. Analysis of trophectodermal cells, however, avoids direct biopsy of embryo tissue per se, which is ethically advantageous as well as a potential benefit to embryo viability. But this approach relies upon a strong concordance between trophectoderm and inner cell mass, which as yet is unproven. It will also require developing a more rapid analysis time to avoid cryopreservation, but will provide a critical advantage if mitotic nondisjunction turns out to be significantly more common than meiotic nondisjunction (13).

The present outcome, in addition to earlier research, has encouraged us to introduce a wider study on the use of this technology for improving IVF. An understanding of human aneuploidy and much research informs us that the logic of knowing chromosome copy number of all chromosomes is vital to embryo viability and healthy pregnancies. Having this technology, we shall now be able to evaluate if that knowledge alone, at a particular stage during ovulation to blastulation, enhances IVF outcome or simply informs us that preimplantation blastomeres have effective mechanisms for corrective or remedial action. One theoretic possibility is the early correction of mitotic aneuploidy errors limiting the number of affected blastomeres; another is the selective distri-

bution of euploid and aneuploid cells between the inner cell mass and trophectoderm, respectively.

There are several potential approaches on the horizon for establishing accurate copy number, but we decided that at this time first polar body chromosome analysis is less open to interpretation, a view recently supported by the European Society of Human Reproduction Task Force on Preimplantation Genetic Screening (20). However, we predict that trophoblast biopsy for full chromosome copy number and blastocyst cryopreservation could become the future of IVF. Indeed, if cost were not the issue, first polar body assessment of egg aneuploidy, followed by trophoblast biopsy and blastocyst cryopreservation, would prevent many aneuploid embryos from being frozen. This option would then maximize live birth rates with a single embryo while eliminating the number-one enemy of IVF, i.e., ovarian hyperstimulation syndrome. This would require a marked shift in patient acceptance to elect to have no fresh embryos transferred.

Until then, we have demonstrated that predicting egg karyotype by polar body array CGH may be a valuable objective prognosticator of embryo viability and could become an important tool in countries where regulation encourages elective single embryo transfer and where policy permits the transfer of only one or two embryos, such as in the U.K. It is essential that independent analyses using, where possible, randomized controlled trials, in addition to other clinical studies with patients of different age ranges and treatment history, are undertaken to validate this success.

REFERENCES

- Hassold T, Hall H, Hunt P. The original of human aneuploidy: where have we been, where are we going. *Hum Mol Genet* 2007;16:203–8.
- Sher G, Keskinetepe L, Keskinetepe M, Ginsburg M, Maassarani G, Yakut T, et al. Oocyte karyotyping by comparative genomic hybridization provides a highly reliable method for selecting competent embryos, markedly improving in-vitro fertilisation outcome: a multiphase study. *Fertil Steril* 2007;87:1033–40.
- Mastenbroek S, Twisk M, van Echten-Arends J, Sik-kema-Raddatz B, Korevaar JC, Verhoeve HR, et al. In-vitro fertilization with pre implantation genetic screening. *N Engl J Med* 2007;357:9–17.
- Wilton L. Preimplantation genetic diagnosis for aneuploidy screening in early human embryos: a review. *Prenat Diagn* 2002;22:512–8.
- Verlinsky Y, Cohen J, Munne S, Gianaroli L, Simpson JL, Ferraretti AP, et al. Over a decade of experience with preimplantation genetic diagnosis: a multicenter report. *Fertil Steril* 2004;82:292–4.
- Mastenbroek S, Scriven P, Twisk M, Viville S, Van der Veen F, Repping S. What next for preimplantation genetic screening? More randomised control trials needed? *Hum Reprod* 2008;23:2626–8.
- Wells D, Levy B. Cytogenetics in reproductive medicine: the contribution of comparative genomic hybridization (CGH). *BioEssays* 2003;25:289–300.
- Wilton L. Preimplantation genetic diagnosis and chromosome analysis of blastomeres using comparative genomic hybridization. *Hum Reprod Update* 2005;11:33–41.
- Le Caignec C, Spits C, Sermon K, De Rycke M, Thienpont B, Debrock S, et al. Single-cell chromosomal imbalances detection by array CGH. *Nucleic Acids Res* 2006;34: e68.
- Fiegler H, Geigl JB, Langer S, Rigler D, Porter K, Unger K, et al. High resolution array-CGH analysis of single cells. *Nucleic Acids Res* 2007;35: e15.
- Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy the “black box” of early pregnancy loss. *Hum Reprod Update* 2002;8:333–43.
- Fritz B, Hallermann C, Olert J, Fuchs B, Bruns M, Aslan M, et al. Cytogenetic analysis of culture failures by comparative genomic hybridization (CGH)—re-evaluation of chromosome aberration rates in early spontaneous abortions. *Eur J Hum Genet* 2001;9:539–47.
- Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, et al. Chromosome instability is common in human cleavage-stage embryos. *Nat Med* 2009;15:577–83.
- Carter NP. Methods and strategies for analysing copy number variation using DNA micro arrays. *Nat Genet* 2007;39:s16–21.
- Baldwin EL, Lee JY, Blake DN, Bunke BP, Alexander CR, Kogan AL, et al. Enhanced detection of clinically relevant genomic imbalances using a targeted plus whole genome oligonucleotide microarray. *Genet Med* 2008;10:415–29.
- Staessen C, Platteau P, Van Assche E, Michiels A, Tournaye H, Camus M, et al. Comparison of blastocyst transfer with or without pre implantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: prospective randomised control trial. *Hum Reprod* 2004;19:2849–58.
- Li M, DeUgarte CM, Surrey M, Danzer H, DeCherney A, Hill DL. Fluorescence in situ hybridization reanalysis of day-6 human blastocysts diagnosed with aneuploidy on day 3. *Fertil Steril* 2005;84:1395–400.
- Johnson DS, Rabinowitz M, Cinnioglu C, Keller J, Banjevic M, Singer J, et al. Novel technology for simultaneous reliable measurement of multiple alleles and copy number across 24 chromosomes in single human blastomeres. *Fertil Steril* 2008;89(Suppl 4): S5.
- Rabinowitz M, Johnson DS, Salzman J, Banjevic M, Cinnioglu C, Behr B. Reliable concurrent calling of multiple genetic alleles and 24-chromosome ploidy without embryo freezing using parental support technology (PS). *Fertil Steril* 2008;90(Suppl. 1):S23.
- Geraedts J. Training in polar body biopsy in advance of the ESHRE trial. *Focus Rep*. May 25, 2009.