New approaches for multifactor preimplantation genetic diagnosis of monogenic diseases and aneuploidies from a single biopsy

Preimplantation genetic diagnosis (PGD) is an alternative to spontaneous conception and chorionic villus sampling or amniocentesis for couples at risk of transmitting a defined genetic disorder. It involves the diagnosis of genetic disease on embryo biopsies during an IVF cycle before a clinical pregnancy has been established and represents a preferred reproductive option for many patients owing to medical, emotional, and ethical questions raised by the need to consider termination of pregnancy after prenatal testing. PGD is in its 25th year of clinical application since the first autosomal single gene disorder for cystic fibrosis was diagnosed at the preimplantation stage in 1992 (1). Since then, the strategies for PGD have increased in sophistication and the number of diseases to which PGD has been applied has grown steadily. Nowadays, PGD is a widely established procedure around the world that theoretically can be applied for any genetic disease with a definitive molecular diagnosis and/or defined marker linkage within a family.

The small amount of DNA found in a single diploid cell made the development of PGD protocols very challenging and introduced several problems not usually seen in routine genetic diagnostic laboratories. These problems included the risk for amplification failure (AF) affecting the reliability of the approach and especially the considerable risk of DNA contamination and allele dropout (ADO) frequently observed when working on single cells. These are very well documented phenomena that can lower the clinical effectiveness of a PGD cycle and potentially lead to misdiagnosis. To overcome these issues, the analysis of multiple markers linked to the mutation was introduced in the course of a multiplex polymerase chain reaction (PCR) by identifying family-specific informative polymorphisms that can increase the robustness and accuracy of the PGD. The use of closely linked short tandem repeat (STR) markers flanking the affected gene, combined with mutation detection, became the "criterion standard" approach for PGD of single-gene defects for decades, with few technologic improvements until recently.

Despite advances in DNA amplification technology, PGD remained limited by several aspects: the high workload and times needed to develop and validate a patient-tailored multiplex protocol, where it is not unusual for couples requesting PGD to have to wait for several months before they can start their IVF cycle; the relatively high AF and ADO, where >10% of the embryo testing procedures usually fail to produce a conclusive diagnosis; and the difficulty to integrate this approach with comprehensive chromosome screening (CCS) protocols to simultaneously analyze single-gene disorders (SGDs) and chromosome aneuploidies from a single biopsy.

In particular, because blastocyst biopsy and CCS have proved to significantly improve the efficacy and safety of IVF treatments, the development of reliable multifactor genetic testing technologies (SGD+CCS) from a single biopsy has become one of the most attractive goals for investigators in the PGD field in modern times.

Recently, an SGD+CCS method for PGD, known as karyomapping, was developed on a single-nucleotide polymorphism (SNP) array platform to conduct linkage analysis for the gene of interest in a given family and determine whether the embryos have inherited mutant or normal copies. One advantage of karyomapping is that it can provide a single protocol applicable to a wide range of patients, reducing the need for extensive work-up and times. Furthermore, karyomapping provides information on the copy number of all 24 types of chromosome, identifying monosomies as well as some trisomies from the same embryo biopsy, thus providing a multifactor PGD test. However, one major limitation of this approach is the absolute need for analysis of at least one additional family member, affected child, or relative, to identify which of the parental haplotypes are associated with the disease-causing mutation. Unavailability of these essential DNA samples may account for a significant proportion of the cases in clinical practice (about one-fourth) and necessitates the incorporation of direct mutation detection and STR analysis (2). This may also apply for genomic regions with less coverage, such us CFTR, SMN1, and telomeric genes, where there is a low concentration of informative SNPs, as well as for consanguineous cases, making the procedure not really universal but still case dependent. Importantly, in a recent publication by Kostantinidis et al. (3), the first clinical experience with karyomapping was reported on 55 IVF-PGD cycles. A high diagnostic consistency compared with conventional PCR approach was reported. However, the study highlighted a clinically important limitation in the reliability of the technology, where ~14% (49/300) of the embryos are expected to remain without a conclusive result. Because failure to produce a conclusive diagnosis may necessitate for those embryos a second additional round of thawing, biopsy, and cryopreservation, this can compromise the embryonic viability and in general the chance of a patient to achieve a healthy pregnancy.

In this issue of the *Fertility and Sterility*, Zimmerman et al. (4) report a new approach for simultaneous CCS and SGD from a single-trophectoderm biopsy with the use of quantitative real-time PCR (qPCR) without the need for whole-genome amplification (WGA). This method, unlike all other multifactor PGD protocols, where WGA is required, has the advantage of using targeted PCR for DNA enrichment, therefore benefiting from the well established improved locus-specific amplification reliability followed by Taqman based genotyping of the mutation and linked informative SNP markers.

The authors first examined the risk of ADO and AF on fibroblast cell lines of different cell numbers with known genotypes, assessing 40 previously described SNPs by means of Taqman qPCR. When assessing cell samples resembling a trophectoderm (TE) biopsy (≥2 cells), the observed ADO and AF frequencies were as low as 0.02% (1/4,426) and 1.09% (35/3,200), respectively. On rebiopsies of TE samples, ADO and AF rates were 0% with the use of qPCR, highlighting a major breakthrough in the reliability of this new method for PGD. The new qPCR-based SGD PGD technology was then applied in clinical practice in 44 cases to detect dominant,

recessive, and X-linked conditions as well as small duplications/deletions with the use of either a direct mutation analysis or a linkage-based approach. In the clinical setting, this new qPCR-based approach entails a primary SNP array analysis of relevant family members, usually the parents, to define informative SNPs. Then predesigned or custom qPCR assays for informative SNPs and the mutation are ordered and validated on purified genomic DNA and 5-cell lymphocyte samples from the family. The reported average time to complete the work-up was 4 weeks.

In this case series, a conclusive diagnosis was achieved with the use of qPCR-based PGD in 98.7% of embryos (300/304). Only four embryos received inconclusive results, owing to the detection of recombination events. Interestingly, ~30% of embryos negative for SGDs were aneuploid, highlighting the importance of performing CCS in PGD cycles. Encouraging clinical outcomes in this young female patient population after the transfer of euploid unaffected blastocysts also were reported, with delivery rates per embryo transfer of 69% and per patient of 82%.

From a technical point of view this new method is the first describing a multifactor PGD approach from a single TE biopsy without the use of WGA. Targeted PCR-based DNA enrichment and Taqman genotyping are universally used methods in molecular biology with known levels of accuracy, and this study showed a very high reliability for PGD application. In contrast, WGA introduces significant additional cost and time to the analysis. Furthermore, especially when used on single or few cells, a significant portion of the genome fails to amplify, leading to poor reliability on a locus-by-locus basis and, possibly, introducing chromosome-specific amplification bias due to the unique GC content of each chromosome (5). ADO rate was shown to at least double after WGA of embryo biopsies (2).

In addition, with this new approach, linked markers are generally nearer the mutation when using SNPs instead of STRs. In practical terms, this makes it possible to have better control over recombination events, thus minimizing the risk of discarding embryos because of a proximal crossover event that can cause misinterpretation. Indeed, the use of closely linked informative SNPs combined with mutation detection provides a more generic and universal method for PGD where it is possible to find out informative markers for almost all, including telomeric, conditions and to provide a PGD service in almost all SGD cases.

The rapid nature of the qPCR-based approach may also provide the first opportunity for TE biopsy, multifactor PGD, and fresh blastocyst transfer within the window of endometrial receptivity. No other methods at present are capable of giving accurate genotyping and CCS results within 4 hours from the biopsy.

By pushing back the technical boundaries of genetic analyses on single cells, PGD has opened up new possibilities for more effective multifactor genetic testing from a single TE biopsy and has offered hope to couples who wish to have healthy children but are unwilling to undergo termination of pregnancy. As PGD technologies have entered the genomic era, more accessible, effective, and lower-cost treatments with improved live birth rates have been made possible, as presented by Zimmerman et al.

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http://dx.doi.org/10.1016/j.fertnstert.2015.10.039

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