Current Methods for Preimplantation Genetic Diagnosis

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Introduction

Couples undergoing in vitro fertilization (IVF) (1) have the unique opportunity to use pre-implantation genetic diagnosis (PGD) to select their best embryos for transfer, maximizing their chances of a successful pregnancy and the birth of a healthy child. In the 20 years since the first PGD babies were born (2), the technologies that have been available to fertility practitioners have advanced considerably.

PGD was originally applied to couples with a family history of known genetic disease that wished to avoid conceiving an affected child. Such application of PGD typically screens for X-linked disorders such as Hemophilia A, monogenic diseases such as cystic fibrosis or specific chromosomal abnormalities such as reciprocal or Robertsonian translocations.

Over time, the scope of PGD increased to include ploidy determination. Initially, only a small number of chromosomes were screened, typically 13, 18, 21, X and Y, allowing parents to avoid having a child afflicted with aneuploidy disorders such as Down Syndrome (Trisomy 21) or Edwards Syndrome (Trisomy 13). Currently, PGD is also used for couples that hope to improve the chances of a successful pregnancy based on the hypothesis that life-incompatible aneuploidies on other chromosomes are likely to result in embryo implantation failure or miscarriage. The following sections review the different technologies that have become available to fertility practitioners.

Disease-Linked Genes

The occurrence of genetic disease in both IVF and natural pregnancies can be devastating to families. Many genetic disorders are caused by rare single nucleotide mutations, whereas others are caused by insertions or deletions of stretches of nucleotides. Online Mendelian Inheritance in Man (OMIM), the largest public database of known genetic disorders, currently contains sequence information for more than 500 monogenic genetic diseases, and knowledge of genetic disease continues to grow. Parental carrier testing is currently available for over 1,000 heritable disease variants.

This information, combined with genotypic knowledge of specific embryos, empowers parents to select those embryos with the greatest likelihood of developing into a healthy baby by deselecting embryos afflicted with genetic disease variants. Some of the most frequently diagnosed autosomal recessive disorders include cystic fibrosis, beta-thalassemia, and sickle cell disease. For dominant diseases myotonic dystrophy and Huntington's disease are among the most common. In the case of X-linked diseases, fragile X syndrome, haemophilia A and Duchenne muscular dystrophy account for most of the tests. All of these diseases have been successfully screened against in IVF embryos.

Polymerase Chain Reaction (PCR) based methods

Currently, most PGD labs test for single gene disorders using techniques based on the polymerase chain reaction (PCR), wherein the disease-linked locus is amplified from blastomere DNA using targeted
primers designed specifically for the mutation of interest (2-4). Typically, the blastomere DNA is amplified using PCR, and is then sequenced using dye end terminator sequencing or sized by electrophoresis (5). Some investigators have used multiplex PCR to measure multiple loci of interest in a single reaction (4,6). PCR methods are generally labor intensive and difficult to scale.

One of the biggest drawbacks to the use of PCR-based techniques for gene detection in single cells is allele drop out (ADO), where the genetic variant of interest fails to amplify (5, 7-8). This may be caused by a number of factors, including cell loss upon handling, enucleated cells, failed lysis, and incomplete or differential amplification of one of the two alleles. ADO rates can vary widely given the conditions, anywhere from 10%, under ideal conditions, to more than 50% (5, 7-8).

Increasing the number of cells analyzed significantly lowers the ADO rate, however, only 1-2 cells can be reasonably biopsied from an 8-10 cell Day 3 embryo. Trophectoderm biopsy, typically performed on Day 5 embryos, allows the biopsy of a larger quantity of cells. An additional benefit is that waiting for Day 5 to perform the biopsy allows for some self-correction of mosaic embryos that may have tested as aneuploid at day 3, but will ultimately develop properly (9-10). A drawback to Day 5 trophectoderm biopsy is that embryos typically need to be cryopreserved and then thawed, a technique that is costly and carries the risk of damage to the embryos. However, newer preservation and thawing techniques have been demonstrated to significantly reduce the harm to embryos (11).

To address the problem of ADO, many PGD labs have begun using a method called preimplantation genetic haplotyping (PGH) (12-14). In PGH, known parental haplotypes at a set of alleles linked to the disease variant are amplified in parallel with the disease variant. If the disease variant allele drops out, then the linked alleles are used to determine whether the disease haplotype is present in the embryo. The method decreases the probability that ADO will cause a “no result” blastomere measurement.

Aneuploidy

PGD testing for numerical chromosome abnormalities (15-16) has four potential benefits: (a) to prevent the birth of a trisomic offspring by analyzing chromosomes X, Y, 13, 18 and 21, (b) to improve implantation rates, (c) to reduce pregnancy loss, and (d) to decrease multiple pregnancies by minimizing the number of embryos transferred per cycle.

Current estimates suggest that at least 50% of human embryos are affected by aneuploidy and other chromosomal abnormalities (17-18). Data collected by comparative genomic hybridization and fluorescent in situ hybridization (FISH) suggest that as many as 70% of embryos from the most common age group of women (35–37 years old) undergoing IVF have chromosomal abnormalities, and as many as 82% for women ≥40 years old (19-21). The high prevalence of aneuploidy in human oocytes has been repeatedly documented (22-29) and is likely a major contributor. Oocyte karyotyping has shown that while women younger than 35 typically have rates of aneuploidy in oocytes of between 5% and 10%, by the time a woman is over 40 that rate has risen to above 50% (19, 26-29).

Though current PGD methods significantly decrease the likelihood of a trisomic birth, it still remains the case that the majority of IVF cycles are unsuccessful, either due to a lack of embryo implantation or successful implantation followed by pregnancy loss. Regardless, we know that more than half of first trimester spontaneous miscarriages are chromosomally abnormal (30-32) and prenatal samples indicate a dramatic increase in aneuploidy rates in pregnancies with advancing maternal age (32), aneuploid fetuses that survive to term are often afflicted with developmental abnormalities such as Down's Syndrome and Edwards Syndrome.

Thus, given that aneuploidy is a common and universally negative result, the ability to exclude aneuploid embryos from transfer is expected to increase the likelihood of delivering a healthy baby. The most common method of embryo selection, based on embryo morphology, ignores the underlying genetic state of the embryo. A number of groups have attempted to identify aneuploidy in a non-invasive way, such as detecting cell surface proteins or cellular excretions that are correlated with aneuploidy (33-34), however, the most reliable methods for aneuploidy detection require blastomere biopsy of an embryo. Traditional karyotyping is not widely practiced given the difficulty of obtaining metaphase blastomeres (35). The next section describes the most common and promising new techniques for ploidy determination.
Fluorescent in situ Hybridization

Fluorescent in situ Hybridization (FISH) is a cytogenetic technique that uses a set of four molecular probes designed to anneal to specific regions of DNA on individual chromosomes (15-16). Each of the probes is labeled with a different dye, so that under a microscope the number of targeted chromosomes can be counted. FISH is able to detect aneuploidy in single blastomeres, and is currently the most common method for aneuploidy detection.

A number of studies have shown that FISH screening for aneuploidy has resulted in a decrease in spontaneous miscarriage (36-37), and in other cases an increase in implantation rate, and live birth rate (36, 38-41). Other recent clinical studies have suggested that PGS with FISH does not improve implantation rates or live birth rates (42-47). However there is debate as to the validity of the methodology used in these studies (48-51).

Despite its great promise, traditional FISH is limited in the number of chromosomes that can be tested: since there are only four dyes, only four chromosomes can be targeted at a time. To get around this limitation, multiple rounds of FISH on the same sample of DNA may be used, though the accuracy decreases with each round, and typically no more than eight chromosomes are screened. One study calculated that more than 20% of the embryos determined to be euploid using a typical eight chromosome FISH panel were likely aneuploid at untested chromosomes (18). This figure does not take into account the fact that the error rate with FISH for single cell screening is estimated to be between 3 and 15% (52-54).

Comparative Genomic Hybridization

Comparative Genomic Hybridization (CGH), a technique originally designed for evaluating chromosomal abnormalities in tumors (55), can be applied to embryos (17, 56-57). CGH involves amplifying DNA from both a blastomere and from a euploid control cell, under identical conditions, using fluorescently labeled nucleotides; green for the embryonic DNA, and red for the euploid control. The amplified, dye-labeled DNA is then hybridized to a slide spread with normal metaphase chromosomes. An excess of either red or green dye on a given chromosome, viewed manually under a microscope, indicates a chromosomal imbalance in the embryonic cell. Unlike FISH, CGH has no limitation as to the number of chromosomes that can be probed (17). However, accuracy rates of CGH have not yet been published. Additionally, a significant drawback to CGH is the length of time required for the procedure, typically four days, which precludes its use for PGD with fresh transfer, and necessitates embryo cryopreservation. Recent preliminary reports of shortened protocols indicate that this may not be a limitation in the future (58).

Array CGH is a recent modification to this technology wherein the amplified, labeled DNA is hybridized to DNA probes affixed to a microarray (59-60). Because analysis is automated, array CGH allows for higher throughput evaluation of the dye ratios on the different chromosomes. Additionally, the time needed for the entire protocol can be as little as 12 hours, well within the short window between blastomere biopsy and transfer of the embryo. However, per-blastomere accuracy rates of array CGH for detecting aneuploidy might be as low as 71% (59).

Methods That Test Concurrently for Both Aneuploidy and Single Gene Disorders.

In the last few years, the use of single nucleotide polymorphism (SNP) microarrays for PGD has been introduced (18, 60-61). Typically, SNP microarrays can interrogate millions of individual SNPs located at numerous positions along each chromosome. The vast amount of information gleaned from SNP microarrays can be used for ploidy calling as well as genetic disease variant diagnosis.

The microarray platforms typically involve oligonucleotide probes that are attached directly to the surface of solid supports, for example on slides or on beads, such that each probe has a specific, known location. In the Illumina system, the amplified and fluorescently labeled blastomere DNA is hybridized to the probes, which range from 25 to 85 nucleotides in length. Automated fluorescent readers then analyze the probes rapidly to determine hundreds of thousands to millions of genotypes in parallel (62).

A major advantage of SNP microarrays is that a vast amount of quantitative data is generated. This necessitates modern bioinformatics-based techniques, which can be advantageous. For example, data are processed and analyzed automatically and in parallel. They also allow other information, such as confidences, to be calculated. Moreover, some informatics techniques
can be used to improve the accuracy of individual allele calls beyond the accuracy of the SNP microarray data itself (18, 63). This is especially important when the measurements are made on single cells, such as blastomeres, where the ADO rate is significant.

**SNP microarrays in single gene detection**

Single gene detection using SNP microarrays is relatively straightforward if the gene of interest corresponds to one of the probes on the microarray. One drawback to using SNP microarrays to detect single disease-linked SNPs is the significant potential for ADO upon amplification, and consequently a lack of diagnostic accuracy at the SNP of interest. However, a linkage approach, similar in concept to PGH, can overcome this drawback by using the identity of neighboring SNPs that correlate strongly with the mutation site to infer the correct identity of the target SNP (63, 65-66). When combined with parental information, SNP microarrays can be used to follow the inheritance of each parental chromosome, yielding data on the inheritance of genes associated with disease (63, 65-66). However, all methods that combine single gene diagnosis with aneuploidy screening await clinical validation studies before they become more commonplace at IVF clinics.

One proprietary approach (18, 63) utilizes the microarray data from the embryonic DNA, the more accurate genetic data of the parents, and knowledge of the mechanism of meiosis to determine which segments of which parental chromosomes were inherited by the embryo. This allows the ploidy of the embryo to be determined with very high accuracy (18, 63).

**SNP microarrays in aneuploidy screening**

A number of strategies have leveraged the information available from SNP microarrays for ploidy determination. Average signal intensities of the probes corresponding to SNPs found on a given chromosome, or section of a chromosome, can be compared, and stronger or weaker responses are correlated with various types of aneuploidy (59-61, 64, 67).

Another strategy is to enhance measurements on embryonic DNA by supplementing parental genetic data (18, 63, 65-66). Because all embryonic chromosomes are derived from parental chromosomes, parental data can be used to "clean" noisy single cell data. Whereas certain methods detect only some meiotic errors (65-66), a recently reported method detects both mitotic and meiotic errors (18).

A major advantage to SNP microarrays is the potential for high accuracy of ploidy determinations. When SNP microarray data is processed using informatics-based techniques that incorporate parental information, error rates in ploidy determination can be up to an order of magnitude lower than with either FISH or CGH. In one study, where ploidy determinations were made using both embryonic and parental SNP...
microarray data, reported average error rates were less than 1% per chromosome (18, 63). More importantly, this technique allows confidences to be calculated for each ploidy state determination so that clinical decisions can be made with maximal accuracy.

In addition, some informatics-based methods are able to determine certain aneuploidy states that are otherwise not detectable using some other methods, such as uniparental disomy, maternal vs. paternal trisomy, and meiotic vs. mitotic trisomy (18, 63, 67). Given the high prevalence of mosaicism in embryos, and the ability for some mosaic embryos to self-correct (9-10, 68-73) the determination of the specific ploidy states can be helpful in predicting future outcomes. For example, it has been demonstrated that blastomeres with meiotic errors are less likely to belong to embryos with any euploid blastomeres, as compared to embryos with mitotic errors (18).

Summary

Over the last 20 years, the scope of PGD has expanded to include screening for a wide range of disease-linked genes, as well as screening for aneuploidy at all chromosomes. The vast amount of data available from SNP microarrays allows for simultaneous determination of most genetic conditions of interest using only the DNA from a single blastomere. When used in combination with informatics based data analysis, these determinations can be made rapidly, in parallel, and with accuracies significantly higher than possible with other techniques. The new PGD techniques will empower patients and clinicians to screen for almost any kind of genetic problem in embryos, with the potential to completely change the manner in which parents approach and manage genetic disease.

References


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