

Advances in preimplantation genetic diagnosis/screening

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Preimplantation genetic diagnosis (PGD) gives couples who have a high risk of transmitting genetic disorders to their baby the chance to have a healthy offspring through embryo genetic analysis and selection. Preimplantation genetic screening (PGS) is an effective method to select euploid embryos that may prevent repeated implantation failure or miscarriage. However, how and to whom PGS should be provided is a controversial topic. The first successful case of PGD of a human being was reported in 1990, and there have been tremendous improvements in this technology since then. Both embryo biopsy and genetic technologies have been improved dramatically, which increase the accuracy and expand the indications of PGD/PGS.

preimplantation genetic diagnosis, preimplantation genetic screening, indications, biopsy methods, array comparative genomic hybridization, next-generation sequencing

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Preimplantation genetic diagnosis (PGD) is an established reproductive option for couples with a risk of conceiving a baby that is affected with some known genetic disease to have an unaffected child, avoiding the termination of pregnancy or recurrent miscarriages. PGD is useful in the diagnosis of a variety of genetic disorders that are caused by a known single gene mutation and chromosome number or structure abnormalities, which can be detected in a single cell.

Since 1990, when the first live birth following PGD for a recessive X chromosome-linked disease was reported [1], PGD has been routinely applied in a variety of genetic disorders, including Tay-Sachs disease, sickle cell anemia, and thalassemia, as well as predicting cancer predisposition

[2–4]. The most frequent single gene disorders detected by PGD are beta-thalassemia, cystic fibrosis, myotonic dystrophy, Huntington disease, fragile X syndrome [5], Comel-Netherton syndrome [6], and endocrine diseases (including persistent hyperinsulinemic hypoglycemia of infancy, congenital adrenal hyperplasia, and multiple endocrine neoplasia type 2A) [7]. Currently, over 10000 babies have been born by PGD and preimplantation genetic screening (PGS) worldwide [8].

In the last 20 years, PGD technology has made a series of steps that have improved its accuracy and specificity and allowed broader applications for a variety of genetic diseases. There are now multiple biopsy methods for PGD at different embryonic development stages with unique advantages. Strategies such as whole-genome amplification (WGA) have overcome the limitations of minimal template DNA from a single biopsied cell. Multiple genes can now

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be assessed simultaneously, and the accuracy of the method has been improved.

Herein, we reviewed the indications of PGD/PGS recent changes in genetic analysis, biopsy methods, WGA methods, array platforms and the application of next-generation sequencing (NGS) to PGD/PGS programs (Figure 1).

1 Indications for the application of PGD/PGS

1.1 PGD for single gene mutations and chromosomal disorders

PGD allows patients who are carriers or who are affected by genetic diseases to select unaffected embryos for transfer before pregnancy [9]. Single-gene diseases were originally the first indication for PGD and aimed to perform genetic testing and establish only unaffected pregnancies [1]. Today, PGD remains a valid alternative for couples at high risk of having a child with monogenetic diseases, i.e., cystic fibrosis, beta-thalassemia, Huntington disease, and myotonic dystrophy [1,10]. In Europe, more than 7000 cycles were performed for single-gene disorders in a total of 60 centers until 2010 [11]. Additionally, PGD can help carriers with structural chromosome abnormalities, such as reciprocal and Robertsonian translocations, inversions, deletions, and insertions, have a baby with a normal chromosome balance [12].

1.2 PGD for cancer patients with a susceptibility gene

Individuals carrying gene mutations predisposing them to hereditary breast/ovarian cancer have concerns about passing these health problems to their future children [13]. It is now feasible to use PGD during the process of *in vitro* ferti-

zation (IVF) to screen genes associated with high cancer risk. Once the mutation site is identified, patients can use PGD to select an embryo without familial cancer-predisposing mutations. This procedure has already been performed for several cancers, including the common syndromes of genetic predisposition to breast cancer [14] and neurofibromatosis type 1 [15].

Drusedau et al. [16] described a universal single-cell PGD test for carriers with BRCA1/2 (breast cancer 1/2) mutations based on haplotyping 6 (BRCA1) and 8 (BRCA2) microsatellite markers in a multiplex polymerase chain reaction (PCR), which have a clinical pregnancy rate of 23.5% per embryo transfer. Malcov et al. [17] reported that 48 PGD cycles in 14 couples resulted in eight pregnancies with the cancer predisposition mutation and flanking polymorphic markers according to single-cell multiplex nested PCR, which confirmed that PGD of cancer predisposition genes is a feasible and reliable procedure that is especially suitable for infertile carrier couples. More discoveries of cancer predisposition mutations will increase the demand for PGD to prevent the transmission of disease-related mutations to the next generation.

1.3 PGD for mitochondrial diseases

About 15% of mitochondrial or oxidative phosphorylation diseases are caused by maternally inherited mitochondrial DNA (mtDNA) mutations. Approximately one in 4000 children are born with an inherited mitochondrial disease in the United States [18]. Because of the severe phenotype, the limited effective treatments, and the high recurrence risk in the offspring of carrier females, couples with this high risk often wish to prevent the transmission of these mtDNA disorders to their babies. PGD provides an option to prevent

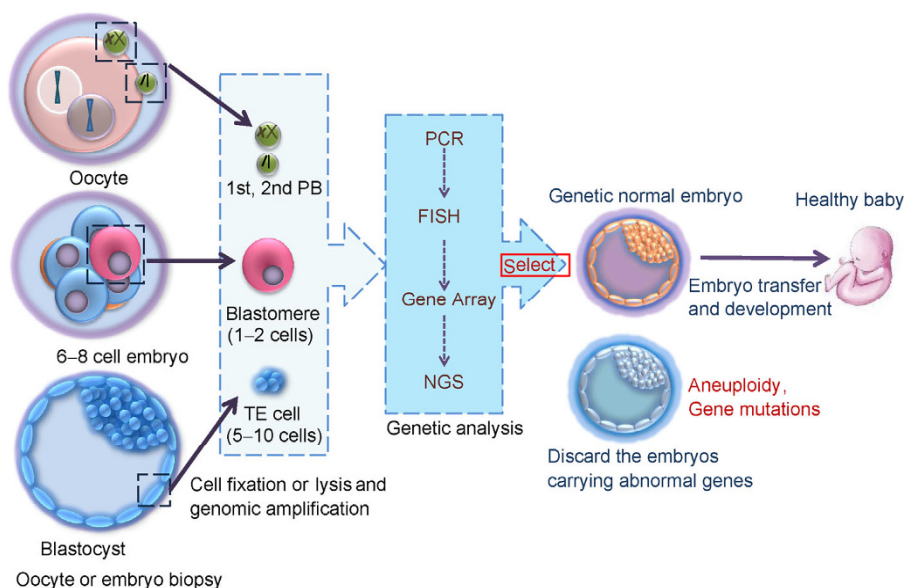


Figure 1 PGD procedures for patients at high risk of transmitting genetic disorders to their baby.

the transmission of mtDNA disorders by transferring an embryo below the threshold of clinical expression [19].

The successful clinical application of PGD of mtDNA diseases has been reported in some studies. Steffann et al. [20] found that the variation of mitochondrial heteroplasmy was fairly small among blastomeres from each embryo but the mutation load widely varied among embryos (from zero to 100%). They carried out PGD of an mtDNA mutation (8993T>G) carrier mother who already had an affected child; PGD resulted in a singleton pregnancy with delivery of a healthy child. Treff et al. [21] found that, for a carrier with 35% 3243A>G mtDNA mutation load, the variation in the mutation load within and among embryos varied from 9% to 90% in oocytes and 7% to 91% in embryos, and the mutation loads in the trophectoderm could be predictive of the inner cell mass. These researchers selected a male embryo with a 12% mutation load in trophectoderm cells for transfer, resulting in the delivery of a boy with tissue-specific mutation loads ranging from undetectable to 15%. Suzanne et al. [22] showed that PGD of mtDNA disorders was useful when there were oocytes below the threshold of the carriers and that the blastomere could be representative of the whole embryo in most cases. In this study, a healthy son was born from an m.8993T>G carrier, which confirmed the possibility of conceiving healthy offspring from those couples with mtDNA diseases. Despite the above successful case reports, PGD has limited efficacy in predicting mtDNA disease because of heteroplasmy and genetic bottleneck. PGD may only reduce, but not eliminate, the risk of transmitting abnormal mtDNA in subsequent generations, and couples should be informed of this during the counseling.

For women with homoplasmic or high levels of heteroplasmic mtDNA mutations, oocyte donation is currently the only option to have a healthy child. However, oocyte donation is limited in that there is no genetic link to the mother with mtDNA mutations.

Two other promising approaches, pronuclear and spindle transfer, have emerged recently that could be used to avoid mitochondrial disease. No successes have been reported in human until now. Only one group has reported embryonic stem cells derived from a blastocyst embryo by spindle transfer [23]. The safety of these technologies requires further evaluation.

1.4 PGD for human leukocyte antigen (HLA) matching

The use of PGD for combined analysis of mutations and HLA matching was reported for the first time in 2001 [24]. It enables the birth of an unaffected child with the closest HLA match to his (her) affected sibling. As a donor, the newborn baby could provide stem cells or some human tissue to the sibling in the transplant [25]. Numerous families have successfully used PGD for HLA matching to treat the affected child. In 2004, a group from Israel reported the

successful cord blood transplantation to a Fanconi anemia patient from his HLA-matched sibling, who was born after PGD that included mutation analysis for Fanconi anemia and HLA typing [26].

Based on the data from the European Society of Human Reproduction and Embryology, more than 500 PGD-HLA cycles have been performed until October 2010 [11]. The most common indication of HLA-matching is PGD combined with the exclusion of β -thalassemia and/or sickle-cell syndromes (~66% of cycles performed) [11].

1.5 PGS for couples with a special history

Because the first deliveries after embryo transfer following PGS were reported in 1995 [27], the procedure and technologies for aneuploidy detection have become readily available for clinical practice. Most of the early pregnancy losses are believed to be attributed to aneuploidy [28]. At present, PGS can be offered to couples with one or more of the following indications: (i) the woman with advanced maternal age (>35 and <40 years), (ii) a history of recurrent miscarriage (two occurrences or more), (iii) repeated implantation failure (more than three occurrences), and (iv) severe male factor infertility [29]. With the development of chromosome screening methods, blastocyst freezing, and trophectoderm biopsy techniques, women with advanced maternal age (>35 years of age) can use single-embryo screening for transfer to increase the birth rate [30]. Despite the above advances, problems still remain, such as how to optimize the procedure to safely offer the technique to patients and which group of patients would benefit more from routine biopsy [31].

2 Genetic analysis methods in PGD

2.1 PGD using PCR

Single-cell PCR was the first application used in PGD for two couples with a risk of transmitting adrenoleukodystrophy and X-linked mental retardation. Through DNA amplification of a Y chromosome-specific sequence, the female patients were confirmed to carry normal female twins [1]. Since then, the PCR method has been used to diagnose many single gene diseases during assisted reproductive technology processes.

However, using PCR for PGD also has difficulties such as contamination, amplification failure because of low-quantity DNA template, and allele drop-out (ADO) in heterozygous loci [32]. To obtain a more accurate diagnosis and simultaneously exclude ADO and contamination, the co-amplification of several loci by multiplex PCR is recommended [33,34]. Two upstream and two downstream markers are preferably applied within 1 Mb of the disease mutation to reduce the risk of recombination events. If there are no linked markers available and the multiplex PCR is

difficult, an alternative method to identify ADO is to biopsy two cells on day 3 or two cell clusters on day 5 with independent analysis [35–37].

2.2 PGD/PGS using fluorescence *in situ* hybridization (FISH)

The applications of FISH made the analysis of aneuploidies in embryos a reality. Screening a large number of chromosomes significantly increases the live-birth rate for mothers with an advanced maternal age [38]. The major limitations of FISH are the limited number of tested chromosomes that can be analyzed simultaneously and the technical difficulty of nuclear fixation and staining. Poor spreading could result in low-quality nuclei staining or even the loss of chromatin, decreasing the accuracy of the diagnosis [39,40]. Indeed, misdiagnosis can also result from overlapping signals, split signals, cross-hybridization, and polymorphisms [41]. However, some of these limitations might be overcome by using different strategies. To improve FISH accuracy, additional probes in the same chromosome could be applied to double-check ambiguous signals [42].

2.3 Array comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) applications in PGD or PGS

In recent years, several approaches for 24-chromosome analysis coupled with WGA have been developed to improve clinical outcomes. The first of these approaches to be applied in PGS was CGH [43], in which the genome of the interested cell is hybridized with a reference genome on a slide. More recently, array CGH has emerged, which provides high resolution and a rapid and simple procedure. There are two types of array platforms: CGH and SNP [44]. The advances of the array platforms could increase the reliability and stability of diagnosis by PGS, but the approaches need to be optimized for future applications.

The first successful application of array CGH using single cells, such as a single lymphoblast, fibroblast, and blastomere [45], suggested that it was the most appropriate method because of its high efficiency, chromosome detection, and time of analysis. Simpson et al. also pointed out that array CGH was the preferred diagnostic approach to assess 24 chromosomes for genetic screening in IVF treatment [46].

2.4 Next-generation sequencing (NGS) in PGD or PGS

Current approaches to evaluate the genomic state in PGD have significant limitations, such as providing limited resolution or restricted information (PCR for some loci, FISH for a few chromosomes, array for aneuploidy screening), which prevent the detection of aneuploidy and SNPs associated with Mendelian diseases and *de novo* mutations [47].

Therefore, it is necessary to establish a straightforward and reliable approach that can simultaneously analyze all mentioned PGD/PGS indications. Advances in WGA and DNA sequencing have made it practical to generate huge amounts of data by high-throughput NGS technologies.

The first study reported the extensive preclinical validation and assessed the accuracy of NGS-based comprehensive aneuploidy screening with single cells. In this study, NGS was demonstrated as a robust high-throughput methodology ready for clinical application in reproductive medicine, with the potential advantages of reduced costs and enhanced precision [48].

Genomics analysis in PGD/PGS using NGS, microarrays, or real-time PCR is often limited by the small amount of template available, which could rely on WGA from a single cell to generate enough DNA template for sequencing. Multiple displacement amplification could provide improvements over PCR-based methods [49], but it still has considerable bias.

A group from Harvard University developed a new WGA method, multiple annealing and looping-based amplification cycles (MALBAC), which showed high uniformity across the genome and achieved 93% genome coverage for a single human cell at 25× mean sequencing. MALBAC has been used to sequence a single circulating tumor cell [50], single sperm cells [51], and single oocyte cells [52].

Hou et al. [52] estimated the sensitivity and specificity of aneuploidy detection with different resolutions and showed that, for sub-megabase resolution (0.5 Mb), single-cell sequencing based on MALBAC achieves 94.8% sensitivity and 97.5% specificity, which is superior to most of the available CGH and SNP array technologies and allows accurate and cost-effective embryo selection for IVF. With MALBAC's significantly improved coverage uniformity [53], maternal point mutations can be detected with high sensitivity and accuracy. This approach can be applied to both polar bodies and blastocyst stage embryos, and it can detect all aneuploidies and gene mutations. Further improvements in the technology robustness and workflow, as well as finding new ways to reduce costs, will promote the use of NGS in clinical diagnosis. Eventually, the advantages of NGS will benefit PGD/PGS patients [54].

Perhaps many people want to use PGD to select embryos with a desired phenotype. In 2007, scientists in Europe identified the SNPs that are associated with a host of physical traits such as eye color, hair color, and freckles [55]. Currently, most clinics do not offer PGD for these features; however, from a technological point of view, it is a possible application in the future [25].

The authors believe that PGD should only be provided to couples with genetic diseases or a known serious disease, but not to anyone who expects a “designer baby.” PGD technologies require development to a high level to achieve

this goal. First, currently we cannot determine the relationship between most genotypes and phenotypes and the role of certain genotypes in human evolution. Second, we cannot distinguish the bad from the good genes in human beings. Third, PGD methods are performed on embryos *in vitro*; even using the so-called non-invasive technology, the long-term safety of this technology is still a concern. Therefore, PGD techniques cannot be used for “designer babies.” Governments should develop strict laws and regulations to prevent the abuse of this technology.

3 Cell samples provided for PGD

The first step in preimplantation genetic testing is to obtain DNA from the oocyte or embryo. Currently, there are three potential methods according to the source of DNA collection: polar body biopsy, blastomere biopsy (aspiration) from the 6- to 8-cell cleaving embryos, and trophoctoderm biopsy from the blastocyst. The optimal biopsy stage should be carefully considered.

The oocyte genotype can be deduced by analyzing the first and second polar body [52]. Polar body biopsy is less invasive and requires a longer time for the analysis than other methods, but it cannot be used to detect aneuploidies and mutations or aneuploidies inherited from the father and *de novo* mutations arising from mitosis.

Embryo biopsy is a more widely used approach because both paternal and maternal alleles can be detected. Embryo biopsy at the cleavage stage (6- to 8-cell stage) with the removal of one or two cells is the most common approach for PGD, which provides adequate samples but is detrimental to the embryo. About two of every five reproductively competent embryos will lose their ability to develop [56].

Blastomeric analysis is prone to error due to high ADO and mosaicism [57,58]. Blastocyst biopsy has been widely applied in PGD clinics in recent years because it is highly effective for the PGD of monogenic diseases [59]. Current data suggest that trophoctoderm biopsy may not adversely impact embryos. In addition, blastocyst biopsy provides primary DNA templates, which would improve the sensitivity and specificity of PGD. However, the time for molecular diagnosis is limited. Thus, blastocyst freezing would be necessary. Fortunately, clinical outcomes indicated that fresh IVF and cryopreserved embryo transfer cycles were equivalent [59]. Currently, the blastocyst stage is the optimal stage to perform biopsies for preimplantation genetic testing [56,60].

As for PGD with sperm, a group from France recently reported the first birth originating from couples with a male translocation carrier. By discontinuous gradient centrifugation, the proportion of unbalanced spermatozoa decreased from 63.6% to 52.3% in this patient [61].

4 Misdiagnosis by PGD

Notably, it is important to note that PGD is not as accurate as prenatal testing with chorionic villi sampling, amniocentesis, or testing of umbilical cord blood. Therefore, each patient must receive follow-up care, and are recommended prenatal diagnosis.

The causes of misdiagnosis include confusions of embryo and sample number (marking error, unclear, erased), the wrong embryo for transfer or thawing, maternal (cumulus cells) or paternal (sperm) contamination, ADO (one study reported 11.9% [62]), using incorrect and inappropriate probes or primers, probe or primer failure, and chromosomal mosaicism in cleavage-stage and blastocyst embryos. Unprotected sexual intercourse has been mentioned as a cause of adverse outcomes not related to PGD analysis errors [63]. The majority of these causes could be prevented by improved procedures, such as careful operation, double-check, sufficient pre-experimentation, and establishing a standard operation procedure. However, an exact diagnosis from a single cell is still a great challenge, and the risk of misdiagnosis cannot be completely eliminated.

The data collected by the European Society of Human Reproduction and Embryology (ESHRE) PGD Consortium indicated that the misdiagnosis was 0.15% for PCR-based cycles and 0.06% for FISH-based PGD cycles. However, these numbers may not reflect the reality. For example, some embryo transfer cycles have no follow-up records (no pregnancy or birth), and the majority of untransferred surplus embryos were not re-analyzed [11].

5 Pregnancy rate with PGD

The average pregnancy rate per oocyte retrieval cycle was about 23.04% (range, 0 to 100%) according to the 12th annual data from the ESHRE PGD Consortium [11]. Research about the PGD of monogenic diseases showed that the pregnancy rate per oocyte retrieval cycle reached 60% with an implantation rate of 50% [59]. Another group obtained a clinical pregnancy rate of 60.3% per transfer cycle with an implantation rate of 53.5% [64].

6 Summary

Reproductive medicine, a quickly developing field of medicine, is the discipline that can directly apply most of the advances from genomics to clinical treatment by providing effective strategies for personalized fertility and sterility treatment. PGD represents a valuable option for patients with the high risk of transmitting an inherited disorder to their offspring. PGD can be applied for any Mendelian disorder with known locations. Moreover, combined with linkage marker (short tandem repeats, SNPs, and haplotypes)

analysis, PGD can also be used if the mutation site is unknown. So far, more than 300 different conditions have been tested with high accuracy. As more genetic markers associated with diseases are identified, our ability to predict genetic disease will significantly increase. The era of PGD with deep sequencing is coming and will benefit more couples with genetic disorders or infertility problems. Additionally, PGD with deep sequencing also has the advantages of high sensitivity and specificity to detect both point mutations and aneuploidy, and it is cost efficient.

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