Preimplantation genetic diagnosis: present and future

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Abstract *Purpose:* Preimplantation genetic diagnosis (PGD) was developed more than a decade ago and aims to identify embryos free of genetic disease attributed either to gene mutations or chromosome errors. The purpose of this article is to provide an update on the current status and future prospects of PGD.

Methods: Review of studies employing different strategies for the detection of single gene defects, and chromosome abnormalities, both structural and numerical in the context of PGD.

Results: Amplification of several DNA fragments is feasible via multiplex PCR for the PGD of single gene disorders, whilst current FISH protocols employ up to 10 probes to identify embryos with a normal chromosome complement. New methods are being developed which will enable the assessment of the entire chromosome complement of embryonic blastomeres.

Conclusions: PGD has come a long way since its first application, and has become very accurate and reliable. Technical advances in the field of preimplantation genetics mean that PGD holds great promise for the future.

Keywords Preimplantation genetic diagnosis · Single gene defect · Chromosome abnormality · PCR · FISH

Introduction

The aim of Preimplantation genetic diagnosis (PGD) is the identification and preferential transfer to the mother's uterus

of embryos unaffected by a genetic disorder. As a result, a healthy pregnancy is achieved, a possible termination can be avoided, and in certain cases an inherited disease can be eradicated from a particular family. PGD involves the biopsy of blastomeres from IVF generated day 3 (cleavage stage) embryos, or first and/or second polar bodies (PBs) extruded from the oocyte during meiosis progression, and their subsequent testing with one of two methods, fluorescent in situ hybridization (FISH), or the polymerase chain reaction (PCR) [1].

PGD was first clinically applied in the early nineties, and was initially utilized in sexing cases for couples who were at risk of transmitting an X-linked recessive disorder [2]. Since that time, the number of diseases diagnosed has increased dramatically, as have the different patient groups who use PGD to achieve a healthy pregnancy. These patients fall into the following categories:

- (i) Carriers of single gene disorders, dominant and recessive, autosomal or X-linked.
- (ii) Carriers of structural chromosome abnormalities, reciprocal and Robertsonian translocations, inversions, deletions, insertions, etc.
- (iii) Women of advanced maternal age, to avoid having chromosomally abnormal offspring.
- (iv) Couples with repeated implantation failure following assisted reproduction treatments (ART).
- (v) Couples with repeated unexplained miscarriages.

At present, PGD is considered as an alternative to prenatal diagnosis [3], while the related method known as preimplantation genetic screening (PGS) is employed to increase success rates of ART. This review will focus on the current status of PGD, and its future prospects.

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Diagnosis of single gene defects

PCR enables the increase in quantity of a specific fragment in a DNA sample to the extent that it can undergo further genetic testing. The first methods to be applied for the PGD of X-linked disorders were PCR-based, involving amplification of a repeat sequence on the long arm of the Y chromosome. This allowed the determination of embryo sex, and the transfer of unaffected females [2]. Soon after these early PGD cases, PCR-based protocols were developed for inherited diseases such as cystic fibrosis and α -1-antitrypsin. These tests involved the amplification of the DNA fragment which contained the causative mutation and its detection using mutation analysis techniques [4, 5].

As time has progressed, PCR strategies have become more complex, leading both to an increase in the number of disorders for which PGD could be employed, and to increased accuracy rates. The number of diseases currently diagnosed via PGD-PCR are approximately 200 (D. Wells personal communication), and include some forms of inherited cancers such as retinoblastoma and the breast cancer susceptibility gene (BRCA2). Additionally, PGD has been applied to new indications that have not traditionally been the subject of prenatal testing, such as HLA-antigen matching [6–8]. Table 1 shows the different diseases for which PGD was carried out between January and December 2003, according to the ESHRE data [9]. Although the ESHRE data only presents a partial record of the PGD cases conducted worldwide, it is indicative of general trends in the field of PGD.

The development of PGD-PCR protocols can be technically very demanding, as the DNA content of single blas-

tomeres is small (5–10 pg). This fact necessitates a large number of amplification cycles, in order for the mutation to be visualized. The large number of PCR cycles leads to a high risk of contamination, either by extraneous or parental DNA. As all PCR-based PGD strategies analyse minute amounts of genetic material, any contaminating DNA will lead to an increase to the misdiagnosis risk for a particular case. A way around this setback is the amplification of additional hyper-variable DNA fragments along with the alleles used for the diagnosis. This approach is effectively similar to DNA fingerprinting, and enables the detection of contamination by an external DNA source, by identifying alleles which are non-embryonic in origin. If two alleles from the same parent are present, then this indicates either that the contaminating DNA is of parental origin [19] or that the specific embryo is trisomic, carrying two copies of one of the parental chromosomes. In both cases such embryos are eliminated from transfer. Additionally, the use of ICSI instead of IVF eliminates the risk of sperm or cumulus cell contamination, and is routinely used for all PGD-PCR cases. Denuding the oocyte of cumulus cells is also standard practice for PCR-based PGD.

An additional problem which is common to all single-cell based PCR tests, is a phenomenon known as allele dropout (ADO). ADO can be defined as amplification failure affecting only one of the parental alleles present in the single cell [10]. ADO's incidence varies, but in extreme cases has affected 20% of amplifications [11–14], and in the past has led to several misdiagnoses [15–17].

The simultaneous amplification of one or more polymorphic markers, located on the same chromosome and near the disease-causing gene is a way to ensure that a PCR-based PGD approach will be error-free, as far as ADO is concerned. This strategy is termed multiplex PCR, and effectively enables diagnosis to take place by scoring the mutation itself, or the polymorphic allele(s) which are inherited with it, as it is very unlikely that ADO will affect both amplified fragments in the same reaction [18].

Generally, the most reliable PCR PGD protocols employ multiplex PCR. In addition to amplification of a DNA fragment encompassing the mutation site, extra fragments containing linked polymorphisms are amplified to avoid misdiagnosis due to ADO, and at least one highly polymorphic marker is amplified to detect possible contamination [1].

It is very likely that as molecular genetics and associated technologies advance, PGD-PCR strategies will become simpler and more accurate. This will lead to a significant increase in the number of disorders diagnosed. Effectively PGD will find more widespread use, benefiting many more couples who are at risk of transmitting an inherited disease to their children.

Table 1 Clinical application of PGD for single gene disorders

Disease	Number of cycles
Cystic fibrosis	69
β -thalassaemia	53
β -thalassaemia + HLA matching	8
Spinal muscular atrophy	29
Sickle-cell anaemia	9
Huntington disease	90
Huntington disease exclusion	8
Myotonic dystrophy type 1	67
Adenomatous polyposis coli	9
Marfan syndrome	8
Duchenne muscular dystrophy	17
Becker muscular dystrophy	4
Haemophilia	14
Fragile-X syndrome	27
Others	104
Total	516

Cycles performed between January and December 2003 [adapted from Sermon et al., 2006 (current data reported by ESHRE)]

Diagnosis of structural chromosome abnormalities

Although the use of PGD for the diagnosis of monogenic disorders is growing rapidly, the most common indication for preimplantation embryo testing remains the risk of chromosomal imbalance (aneuploidy). Unlike PCR-PGD where embryonic blastomeres are placed in microcentrifuge tubes, PGD for chromosome abnormalities involves as an initial step the spreading and fixation of a single cell on a microscope slide with its subsequent cytogenetic analysis. Classical cytogenetic techniques (e.g. G-banding) are not applicable at the single cell level, as they require chromosomes at the metaphase stage of the cell cycle. Embryonic blastomeres, however, in their majority are found to be in interphase. To overcome this problem, PGD protocols commonly employ a molecular cytogenetic method termed fluorescent in situ hybridization (FISH). This technique involves the hybridization of chromosome-specific DNA probes, labelled with different colors, to nuclei or chromosomes spread on microscope slides. The method is rapid, and performs equally well regardless of whether applied to metaphase or interphase nuclei (reviewed in [20]).

One of the main groups of patients seeking PGD for aneuploidy in order to achieve a healthy pregnancy, are carriers of a structural chromosome abnormality. Table 2 shows the different types of structural chromosome anomalies and the mechanism by which they are generated. Patients, who are balanced carriers of such abnormalities, have a dramatically elevated risk of producing gametes with an incorrect number of chromosomes. They therefore frequently have complex reproductive histories, involving subfertility or complete infertility, multiple spontaneous miscarriages, or the birth of children with congenital abnormalities.

Reciprocal and Robertsonian translocations are the two types of structural chromosome anomalies for which PGD

is most frequently employed. Two different FISH strategies have been used for blastomere analysis during the PGD of these two types of structural anomalies. The first approach used probes which spanned the breakpoints of a translocation [21], whilst the second used probes which flanked the breakpoints [22]. In both schemes, a distinct combination of signals was seen in interphase nuclei, each corresponding to one of the four chromosomes involved in the rearrangement (two normal and two derivatives). Chromosome imbalance due to segregation of translocated chromosomes was detected by both of these schemes. However, the relative simplicity of the “flanking probe” approach, along with the commercial availability of sub-telomeric probes specific for each chromosome arm, has made it the most popular strategy for PGD of reciprocal translocations [23–28].

A similar FISH strategy was devised for the PGD of Robertsonian translocations. This involves the application of two probes, each one hybridizing to one of the two chromosomes that form this rearrangement. In cases where one of the chromosomes participating in this type of translocation could result in a viable trisomic pregnancy, such as chromosome 21, then it is advisable to use two probes for this chromosome, to ensure its visualization during diagnosis [29].

FISH strategies have been developed for other less common chromosome abnormalities, such as pericentric inversions [30, 31], deletion of part of chromosome 22 leading to Di George syndrome [32], and for patients who are gonadal mosaic for a trisomic cell line [22]. In all, the wider availability of commercial probes has increased the number of FISH PGD cases feasible, and hence the number of patients that could be treated.

A limitation associated with the PGD of chromosome abnormalities is the level of mosaicism- the presence of two or more cytogenetically distinct cell lines within the

Table 2 Different types of structural chromosome abnormalities and their causal mechanisms

Structural chromosome rearrangement	Mechanism
Reciprocal translocation	A break occurs on each of two chromosomes with the segments being exchanged to form two new derivative chromosomes.
Robertsonian translocation	Two acrocentric chromosomes (13, 14, 15, 21, and 22) break at a position on or close to their centromeres. Fusion of their long arms and formation of a single metacentric chromosome follow.
Paracentric inversion	Two break rearrangement involving one chromosome. Segment involving one of the chromosome is reversed in position
Pericentric inversion	As paracentric inversion, but participating segment involves the chromosome centromere
Deletion	Loss of part of a chromosome leading to monosomy for the particular segment
Insertion	Segment of one chromosome inserts into another chromosome
Ring chromosome	Break occurs on each arm of a chromosome. The generated sticky ends reunite as a ring
Isochromosome	Loss of one chromosome arm and duplication of the other

resulting embryos [22, 28, 29, 33]. Several published reports have identified highly abnormal chromosome complements in 70–100% of embryos generated from some patients with poor reproductive histories attributed to a structural rearrangement (reviewed in [34]). Such high levels of mosaicism are likely to adversely influence the success rates of PGD for chromosome rearrangements. However, as such couples may be unable to establish or maintain a pregnancy naturally, PGD is still the most attractive option.

Preimplantation genetic screening

In recent years PGD technology has been increasingly used by infertile patients undergoing IVF treatment, in order to screen their embryos for chromosomal abnormalities. Aneuploidy is extremely common in human embryos and leads to developmental arrest, implantation failure and spontaneous abortion. The inadvertent transfer of chromosomally abnormal embryos is believed to explain a significant proportion of failed IVF cycles. By screening for aneuploidy and ensuring the transfer of chromosomally normal embryos it has been suggested that a variety of IVF outcomes (including implantation and pregnancy rates) can be improved. This approach is known as preimplantation genetic screening (PGS) (reviewed in [35]).

Most PGD centers offer PGS to couples with one or more of the following indications: advanced maternal age (AMA) (cut off varies between 35 and 40 years of age, depending on the center), three or more previous unsuccessful embryo transfers with regular IVF procedures (repeated implantation failure RIF), or repeated spontaneous loss of pregnancies, when parents have a normal karyotype (recurrent miscarriage RM). As described previously, PGD tests for the diagnosis of single gene disorders or structural chromosome anomalies, are generally patient specific. However in the case of PGS, an identical probe combination and protocol is employed for all patients. PGD laboratories offering this service, examine 6 to 15 chromosomes per embryo [36–38]. This restricted number of chromosomes is attributed to a technical limitation of FISH. There are only five spectrally distinct fluorochromes available for probe labeling, and consequently the number of chromosomes that can be simultaneously assessed is limited to five.

Early PGS protocols examined chromosomes 13, 18, 21, X, and Y, aneuploidies for which are sometimes compatible with viable pregnancies, and aneuploid syndromes (e.g. Patau, Edwards, Down, Turner, and Klinefelter). This probe combination led to a reduction in the incidence of aneuploid syndromes, but did not result in any statistically significant improvement to implantation rates [39]. The latter suggested that other chromosomes which were not included in this initial combination could be participating in aneu-

ploidy events. Current protocols involve the combination of up to five probes in a single experiment and investigate up to 15 chromosomes in two sequential FISH rounds [38]. Preliminary data obtained with such protocols have demonstrated a doubling in implantation rates and a significant increase in pregnancies per retrieval for women of AMA and/or RM couples [37, 40, 41]. No notable benefits, however, were seen for younger women, and RIF couples so far, and there is an ongoing debate about whether one or two blastomeres should be biopsied from individual embryos and examined via PGS [42, 43]. Moreover it has been shown that aneuploidy could affect any chromosome during preimplantation development, and this along with embryo mosaicism could be reducing the efficiency of current PGS strategies.

While it is generally accepted that PGS succeeds in reducing miscarriage rates and the incidence of aneuploid syndromes, there is conflicting data concerning the efficacy of PGS in raising implantation and birth rates. Additional large randomized studies are necessary, prior to a more routine application of this diagnostic approach. Moreover, application of novel techniques, capable of screening the entire chromosome complement of embryonic cells is essential so as to improve the existing PGS methodology.

The future

Several researchers employed FISH to analyze non-transferred preimplantation embryos and have observed bizarre chromosomal compliments, which are not seen in later fetal development [44–49]. These findings suggest that aneuploidy screening would be more effective if it allowed analysis of all 46 chromosomes.

Classical and molecular cytogenetic methods capable for investigating all 23 chromosome pairs, such as G-banding and spectral karyotyping (SKY) require metaphases, whilst embryonic nuclei are generally found at interphase. However, methods such as SKY can be used for PGD if combined with nuclear conversion, a technique that involves injection of a biopsied blastomere into the perivitelline space of enucleated oocytes, or abnormally fertilized zygotes, followed by cell fusion with the aim of pushing the embryonic cell to enter metaphase. PGD cases using this strategy for the identification of unbalanced embryos generated by reciprocal translocation carriers have been reported [50, 51], but this approach is technically demanding and has yet to find wider application, due to technical problems.

Another method which permits the investigation of the entire chromosome complement, but does not require cells in metaphase, is comparative genomic hybridisation (CGH) [52]. CGH allows the copy number of every chromosome to be assessed in a single hybridisation by reference to a normal

DNA sample. In principle, a green fluorescent molecule is incorporated in the “test” DNA (unknown karyotype), while a DNA sample coming from a karyotypically normal individual (46, XY, or 46, XX) is labelled in red, and serves as reference. The two are then mixed and co-hybridized to normal male (46, XY) metaphase spreads on a microscope slide. Test and reference DNAs compete for hybridisation sites on each of the 23 chromosomes. In the case that the test DNA is karyotypically normal (i.e. has the same karyotype as the reference DNA) no difference in fluorescence intensities would be observed and the chromosomes would have a yellow/orange colouration. If however, the test DNA carried a trisomy for a specific chromosome, then this chromosome would appear to be greener. The opposite would happen if the test DNA was monosomic for a chromosome, which would then appear more red. Differences in fluorescent intensities are identified with the aid of specialized computer software, which is able to recognize chromosome areas that are either over- (gain) or under- (loss) represented in the test DNA sample. CGH sensitivity ranges between 3–5 Mb [53–55], and can therefore accurately detect both whole and partial chromosome aneuploidy.

The challenge for CGH analysis of embryonic blastomeres is that the method requires ~200 ng of DNA, whereas a single cell contains only 5–10 pg. For this reason it is necessary to perform a whole genome amplification (WGA) reaction prior to CGH analysis. The method of choice for this purpose is known as degenerate oligonucleotide primed (DOP)-PCR, an approach employing a heterogeneous mixture of semi-degenerate primers that anneal and initiate DNA synthesis at numerous sites throughout the genome [56, 57]. It has been shown that amplification of the genome generates sufficient DNA to allow both CGH and also multiple PCR tests to be carried out on the same cell. This can possibly overcome the incompatibility of FISH and PCR, allowing analysis of single gene mutations and aneuploidy to be conducted in the same cell [57].

WGA and CGH have been applied for the cytogenetic analysis of preimplantation embryos, metaphase II oocytes and their corresponding PBs, both in a research and clinical context [58–62]. Apart from confirming that aneuploidy could affect all chromosomes during human female meiosis and the early embryonic divisions, CGH identified other types of abnormality, including chromosome breakage, embryos which were highly mosaic, and others which had undergone a complete mitotic breakdown and were classified as chaotic [58–62].

Hence, CGH is the most promising method for the investigation of the full chromosome set of a single cell. As with FISH though, it has some limitations. The principal setback for a wider clinical application of CGH is that the method requires approximately 5 days to yield results. This length of

time is not compatible with the restricted timeframe available for preimplantation testing. One strategy of overcoming this problem is to cryopreserve the embryos after biopsy, with the transfer taking place in a subsequent cycle [60]. The main drawback in this case is that freezing and thawing can reduce the embryo implantation potential, a problem exacerbated by blastomere biopsy.

An alternative approach is the investigation of the 1st PBs, which are available for analysis three days earlier than the blastomeres, providing sufficient time to perform CGH, and avoiding embryo cryopreservation [61]. However, further FISH analysis of biopsied blastomeres should occur, as chromatid anomalies detected in meiosis I have only a 50% chance of leading to an aneuploid embryo. An additional misdiagnosis risk involves the presence of meiosis II or a paternally or post-zygotically derived error.

Other problems associated with CGH include the complexity of the technique. The protocol is labor intensive, and requires expertise in both molecular genetic and cytogenetic methods which are not generally available to fertility clinics. A less complex approach will be essential prior to CGH being applied in a wider clinical setting.

Currently, the best hope for a simplified, clinically applicable, CGH methodology is microarray CGH. As with conventional CGH, this method involves the competitive hybridization of differentially labeled test and reference DNA samples. However, in this case the labeled DNAs are hybridized to DNA probes affixed to a microscope slide rather than metaphase chromosomes. Each probe is specific to a different chromosomal region and occupies a discrete spot on the slide. Chromosomal loss or gain is revealed by the color adopted by each spot after hybridization (ratio of red: green fluorescence). The evaluation of red: green fluorescence is simple and easily automated.

Microarray CGH has been successfully applied for the detection of aneuploidies in single cells after whole genome amplification using DOP-PCR or an alternative method known as multiple displacement amplification (MDA) [63–65]. Comprehensive chromosome analysis was achieved in less than 48 hours, within the timeframe of a regular embryo transfer after PGD. Consequently the future application of microarray CGH for PGD appears to be extremely encouraging and its relative technical simplicity is bound to make it more amenable to routine application in the IVF laboratory.

Very recently, there has been a trend into towards developing new approaches for determining embryo viability. Different research groups have set out to investigate gene and protein expression of oocytes and embryos (Fragouli and Wells unpublished, [66]) in an attempt to develop new non-invasive methods of determining the embryo(s) with the highest implantation potential. It is therefore clear that PGD has come a long way since its early application with the technical innovations leading to increased accuracy and an

expanded of indications. Novel techniques currently under development will undoubtedly lead to further advances and hold great promise for the future.

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