

## —Mini Review—

## Preimplantation Genetic Diagnosis

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**Abstract:** Preimplantation genetic diagnosis (PGD) is a technology that allows for the selection and transfer of embryos unaffected by genetic disease. The limited number of cells available for genetic testing is a weakness of PGD and has been solved by means of the development of various strategies such as polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) and cell recycling. A confounding factor in PGD is the existence of preimplantation embryos with severe chromosomal abnormalities. Therefore, genetic analysis should be performed with the assumption that embryos have severe chromosomal abnormalities. The visualization of metaphase plates allows screening for numerical chromosomal abnormality and several kinds of structural chromosomal abnormality. In addition, in vitro culture of single isolated blastomeres makes it possible to reexamine samples to ensure accuracy of the results and to obtain additional genetic information.

**Key words:** Preimplantation genetic diagnosis, polymerase chain reaction, fluorescence in situ hybridization, cell recycling, aneuploid screening

Preimplantation genetic diagnosis (PGD) is a technology that avoids the transmission of inherited genetic disease by screening embryos for genetic abnormalities and allowing for the selection of only those embryos unaffected by such abnormalities. The advantage of PGD over classical prenatal diagnosis (PND), such as chorionic villi sampling (CVS) and amniocentesis, is that genetic information is obtained before implantation. When an affected embryo is diagnosed by classical PND, most patients choose artificial abortion. Some patients who are carriers of a genetic disease have more than one abortion and this may cause psychological/emotional and physical

damage. The ability to select unaffected embryos, and therefore avoid artificial abortion, is an expected benefit of PGD.

The initial development of PGD was as a tool to avoid transfer of embryos affected by genetic diseases such as X-linked recessive disorders [1]. The applications of PGD have increased and now include diagnosis for single gene disorders and for numerical and structural chromosomal abnormalities. Chromosomal abnormality, which is reported to be a frequent cause of implantation failure and abortion, could be detected by means of PGD-aneuploid screening (AS), thereby reducing embryo loss [2]. In the future, the technique has the potential to be routinely applied to genetic screening for cancer predisposition, late onset disorders with genetic predisposition, and blood type incompatibility.

In this mini-review, diagnostic strategies, pitfalls, and possible future developments of PGD are discussed.

### Diagnostic Strategies

#### Biopsy methods

Biopsy was required to obtain samples for genetic testing and this was performed at several developmental stages. The 1<sup>st</sup> and/or 2<sup>nd</sup> polar bodies could be obtained at the unfertilized oocyte and/or zygotes stages. These samples were used for PGD of defects of maternal origin. In most cases, the biopsy was performed on cleavage stage embryos. The one or two blastomeres obtained at each biopsy were later subjected to genetic testing. The use of blastocysts for PGD allowed us to obtain a large number of cells from trophectoderms, but methodological improvements were required to allow for routine clinical use of this technique.

#### Genetic tests

In the typical PGD protocol, one or two blastomeres

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are biopsied from cleavage stage embryos and analyzed by fluorescence *in situ* hybridization (FISH) and/or polymerase chain reaction (PCR) without affecting the viability of the embryo. The limited number of cells available for genetic testing is a weakness of PGD. In the case of classical PND, many cells are available for genetic testing and for reexamination to ensure the accuracy of results and to obtain additional genetic information. Many researchers are developing improved PGD strategies to remove the limitations of PGD.

A primary solution to the cell number limitation was the application of PCR, a core technique in the technology of molecular biology. The sensitivity of PCR is extremely high and this allows the analysis of DNA from a single genome. When PGD was first applied, gender was detected by amplification of a region specific to the Y chromosome in order to select male embryos and to avoid inheritance of X-linked genetic disease [1]. Although PCR is able to detect the existence of the target sequence, it does not allow quantification of the number of target chromosomes. Therefore, it is recommended that FISH be used for gender determination as FISH has advantages over PCR. Recently PCR has been mainly used to diagnose single gene disorders. Furthermore, multiplex nested PCR, which is able to analyze multiple regions simultaneously, was applied to obtain an increased amount of genetic information from single blastomeres.

A second way of overcoming the cell number limitation was the application of FISH. As mentioned above, the first application of FISH was as a substitute for PCR in gender determination [3]. Next, FISH was used to diagnose numerical chromosomal abnormalities such as aneuploidy. Recently, FISH has been applied to the detection of structural chromosomal abnormalities, such as reciprocal translocation and Robertsonian translocation. Furthermore, multiple-probe FISH makes it possible to analyze two or more chromosomes simultaneously, even at interphase.

PCR requires the preparation of primer sets to amplify target sequences and FISH requires the preparation of probes to hybridize to defined target regions. In other words, both PCR and FISH are only able to detect specific target genes and chromosomes.

Thornhill *et al.* [4] described a powerful technique called 'cell recycling', which combines both PCR and FISH on the same blastomeres for PGD. This technique made it possible to obtain information at both genetic and chromosomal levels from the same cells. The biopsied blastomeres were fixed on the tips of

miniature slides designed to be inserted into PCR tubes. The first step in this technique was PCR. Then cells on the miniature slides were used for FISH, and PCR products were used for genetic diagnosis. When there was insufficient PCR product for analysis, nested PCR was applied. The genomic DNA fixed on the miniature slides can be a template for amplification and then the same genomic DNA can provide a target for hybridization studies. We developed this technique for PGD of X-linked diseases such as Duchenne muscular dystrophy (DMD). Specifically, mutation of the dystrophin gene is detected by PCR, and gender and/or chromosomal abnormality, by FISH. When this numerical strategy is applied to PGD cases, allele dropout (ADO) is a possibility and this should be considered when analyzing results [5].

Benadiva CA *et al.* [6] described a 'cell recycling' method that performs sequential FISH on the same blastomeres. The biopsied blastomeres were fixed on regular slides and two-step FISH was applied on the same blastomeres with two different sets of probes. This technique made it possible to increase the information obtained from chromosomes. We developed this technique for the determination of numerical abnormality of seven chromosomes. FISH probes for seven chromosomes were divided into two groups; the first step was performed for chromosomes 13, 18, 21, X and Y and the second step for chromosomes 16 and 22 [7]. Recently, simultaneous analysis of numerous chromosomes has been made possible by improvement of the equipment and sequential FISH might already have become a classical method in PGD.

These two cell recycling methods increase the amount of genetic information that can be obtained from the same single cells. The limitation of cell recycling is the same as that of PCR and FISH, i.e., cell recycling methods are only able to detect specifically targeted genes and chromosomes.

### **Pitfalls of PGD**

It has been reported that preimplantation embryos include a large proportion of embryos with severe chromosomal abnormalities. Munne *et al.* [8] reported chromosomal abnormalities in 71.4% of arrested embryos, 50.8% of slow developing and fragmented embryos and 41.3% of morphologically normal embryos. Severe chromosomal abnormalities are a common cause of developmental arrest, implantation failure, and spontaneous abortion. Most fetuses with

severe chromosomal abnormalities are eliminated by natural selection in the first trimester. Consequently, the genetic characteristics of PGD targets are completely different from those of classical PND.

The fact that preimplantation embryos include a large proportion of embryos with severe chromosomal abnormalities is an important warning. It implies that the number of X-chromosome is not always one and two in blastomeres from male and female embryos, respectively. Our analysis of day three embryos, by FISH with probes for chromosome 13, 16, 18, 21, 22, X and Y [7], showed that undesirable embryos presumed to be extensively mosaic, were detectable. When genes responsible for X-linked disorders are analyzed by PGD, it is important that numerical analysis of sex chromosomes be performed, as this allows us to sex the embryo. This is important as the number of X-chromosomes present affects the interpretation of the results of gene analysis. For example, in PGD for Duchenne muscular dystrophy (DMD), deletion of exons in the dystrophin gene could be detected by multiple-nested PCR from single blastomeres. When a particular DNA fragment is amplified, the PCR only indicates the presence of the exon in the sample. The number of X-chromosomes could be three or four due to polyploidy or aneuploidy, or the product may be due to DNA contamination. PGD for X-linked disorders by gene analysis alone, without the numerical analysis of sex chromosomes, may lead to misdiagnosis and is not acceptable as a diagnostic strategy. In PGD, it should be confirmed that the results of gene analysis are consistent with the number of sex chromosomes present.

But only a few cells are available for PGD genetic tests. Then a way to obtain the results of both gene and chromosome analysis will be required. The primary method involves using PCR primer sets to amplify regions on the X-chromosome and target regions together. This could detect whether X-chromosomes are present within the blastomeres, but it does not detect the number of X-chromosomes. The second method is biopsy of two blastomeres from cleavage embryos followed by gene analysis with one blastomere and chromosome analysis with the remaining blastomere. When this method is used for PGD, mosaicism could be a possible cause of inconsistency between results from gene analysis and sex chromosome analysis. An acceptable solution for PGD is the method called 'cell recycling' which combines PCR and FISH on the same blastomeres [4].

Munne *et al.* [2] reported that PGD-AS reduced

embryo loss after implantation. The number of embryos that can be transferred to the uterus is limited. Therefore, simultaneous PGD-AS is recommended. Viable and unaffected embryos thus selected can then be used for transfer.

## Future Developments

### *Visualization of metaphase plates*

Chromosome analysis on good-quality metaphase plates makes it possible to diagnose both inherited and *de novo* chromosomal abnormalities, but a large proportion of blastomeres isolated from cleavage stage embryos are in the interphase and very few are in the metaphase. Even after overnight culture of isolated blastomeres in the presence of colcemid, metaphase plates suitable for karyotyping could be obtained from only one-third of the cells.

Willadsen, S. *et al.* [9] reported a procedure for visualization of metaphase chromosomes in single blastomeres by fusion with bovine oocytes. Similar studies on human, hamster and mouse zygotes or oocytes have been reported by Verlinsky Y *et al.* [10, 11]. The oocytes or zygotes were used as recipient cytoplasm and the blastomeres were electrically fused with the oocyte to transform them into metaphase cells [12]. Although this technique was a novel approach by which to obtain metaphase cells, it required highly skilled manipulation for successful nuclear transplantation. Improvements in this technique, for example, simplification of the procedures, increases success rates, and appropriate preparation of recipient cells is expected in the future. Furthermore, it is difficult to obtain good-quality metaphase plates suitable for G-banding from single metaphase cells. Instead of G-banding, advanced FISH techniques, such as whole chromosome painting or spectrum karyotyping (SKY) [13], could be applied. Those techniques allow screening for numerical chromosomal abnormalities and several kinds of structural chromosomal abnormalities on metaphase plates.

### *In vitro culture of single blastomeres*

The strategies described previously increase either the sensitivity of diagnostic tools or the amount of genetic information that can be obtained with these tools. Nevertheless, they are not fundamental solutions to the cell number limitation. The ideal solution to this limitation is likely to be in vitro culture of single isolated blastomeres. This solution makes it possible to reexamine samples to ensure accuracy of genetic test

results and to obtain additional genetic information. Wilton *et al.* [14] reported successful culture of blastomeres isolated from mouse four-cell embryos for a six-day period in wells coated with four kinds of extracellular matrix. A plaque consisting of approximately twenty cells was obtained from all fibronectin-coated wells.

Meanwhile, embryonic stem (ES) cell lines were established from inner cell masses of blastocyst embryos. They were able to be passaged continuously without differentiation and to transmit genetic characteristics accurately. It was presumed that the culture system for ES cells includes many factors that allow for cell proliferation. Delhaise *et al.* [16] described the establishment of ES cell lines from mouse single blastomeres at the 8-cell stage. We applied this culture system to isolated mouse blastomeres and observed explosive proliferation. It is expected that a culture system for ES cells inhibits differentiation and the accumulation of genetic abnormalities. Further investigations are required to confirm the genetic normality of proliferated cells. Although the proliferated cells in our trial were not confirmed to be ES cells yet, we expect that they have the potential to be established as ES cell lines.

### Conclusion

The limited number of cells available for genetic testing is a weakness of PGD and has been solved by means of the development of various strategies such as PCR, FISH and cell recycling. A confounding factor in PGD is the existence of preimplantation embryos with severe chromosomal abnormalities. The genetic characteristics of PGD targets are completely different from those of classical PND targets. Therefore, genetic analysis should be performed with the assumption that embryos have severe chromosomal abnormalities.

The visualization of metaphase plates allows screening for numerical chromosomal abnormality and several kinds of structural chromosomal abnormality. In addition, in vitro culture of single isolated blastomeres makes it possible to reexamine samples to ensure accuracy of the results and to obtain additional genetic information. With this technique it will be easier to obtain metaphase plates suitable for G-banding without cell fusion. In the future, a certain amount of DNA extracted from proliferated blastomeres would be applied to DNA chips for genetic screening. Furthermore, babies implanted after PGD might have their own ES cell lines available at birth.

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