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## Preimplantation Genetic Testing: Current Status and Future Prospects

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### 1. Introduction

While *in vitro* fertilization (IVF) is most often employed as a remedy for infertility, a discussion of the field would not be complete if it did not address the application of IVF to avoid genetic disorders. IVF makes it possible to assess the genetic status of the embryo before establishing a pregnancy when couples are at risk for an affected child. Physicians in the field will benefit from being informed about the diverse set of molecular and cytogenetic technologies employed in preimplantation genetic diagnosis (PGD) and screening (PGS), and from understanding their relative power and limitations as tools for genetic counseling.

PGD and pre-implantation genetic screening (PGS) refer to two distinct types of clinical procedure that help a couple to have a healthy child: PGD determines the embryo's genotype, while PGS assesses an embryo's karyotype and has been used in screening chromosomal aneuploidy.

PGD and PGS require the use of the IVF technique. These technologies, initiated in the late 1980s as an alternative to prenatal diagnosis (PND), allows a couple at risk of a genetic disorder to give birth to an unaffected child by avoiding selective termination of an affected pregnancy. Genetic disorders could be due to either a single gene disorder, or an abnormal number or structure of chromosomes. The current agreed upon indications (Cooper & Jungheim, 2010) for PGD and PGS include:

1. Screening for embryo chromosomal aneuploidy in cases of advanced maternal age or known parental translocation
2. Family history indicating risk for known autosomal Mendelian genetic disorders
3. Sex selection with family history indicating risk for X-linked disorders
4. Sex selection for family balancing, e.g. parental preference for a male or female
5. Human leukocyte antigen (HLA) matching to achieve a child to provide hematopoietic progenitor cells from cord blood to an existing sibling who requires bone marrow transplantation

The medical need for these services is significant. The U.S. Centers for Disease Control and Prevention estimates that more than 6,000 single-gene disorders affect approximately 1 in 300 live-births (Benson and Haith, 2009), while cytogenetic abnormalities appear at about twice this rate in live births and cause approximately  $\frac{1}{4}$  of miscarriages and stillbirths (Thiesen & Shaffer, 2010). PGD makes it possible to assess the genetic status of at-risk embryos prior to implantation and initiation of a potentially affected pregnancy. There still remains considerable controversy regarding the need for and the ability of PGD to increase implantation rates for IVF.

### **1.1 The milestones of PGD and PGS development**

PGD began in the late 1980's with the pioneering work by Handyside and colleagues in selecting embryo gender by polymerase chain reaction (PCR) amplification (Handyside et al., 1990), and diagnosis for a recessive autosomal disorder (Handyside et al., 1992).

PGS was subsequently developed by Munne's team for gender determination (Munne et al., 1993), and by Handyside's team for aneuploidy screening (Schrurs et al., 1993) using fluorescent in-situ hybridization (FISH). Other PGD milestones include its application for chromosomal translocations (Munne et al., 1998), and for HLA matching that led to the first HLA matched baby unaffected with Fanconi anemia (Verlinsky et al., 2001; Grewal et al., 2004).

Between the birth of the first PGD baby in 1989 and the year 2000, about 500 babies were born worldwide using PGD. Since then, the PGD has been on a steep rise: about 1000 PGD babies were born worldwide between 2000 and 2002, and 1500 more PGD babies were born between 2002 and 2004 (Verlinsky et al., 2004; ESHRE PGD Consortium, 2002; Harper et al., 2010). In the past 21 years, while thousands of babies have been born following PGD, there are no confirmed reports of increased fetal abnormalities following PGD.

### **1.2 Assisted reproductive technologies that support preimplanation genetic testing**

Preimplantation genetic testing is supported by advances in assisted reproductive technology (ART). Methodologies of particular importance include intracytoplasmic sperm injection (ICSI), laser-assisted biopsies of embryos at the cleavage and blastocyst stages, sperm sorting, and cryopreservation of biopsied embryos.

#### **1.2.1 Intracytoplasmic sperm injection**

In conventional IVF each egg is combined with several thousand motile sperms on the day of egg retrieval to fertilize the egg. As a result, numerous sperms are present around the fertilized egg. At the next step, embryo biopsy, these excess sperms can contaminate the biopsy and contamination of the embryonic sample with sperm DNA can lead to incorrect PGD results. To guard against this risk a single sperm can be injected into each egg. This procedure is known as intracytoplasmic sperm injection (ICSI; Palermo et al., 1992; Harton et al., 2011a).

Through ICSI eggs are fertilized and embryos are formed. On day 3 of fertilization, a single blastomere is removed from each cleavage-stage embryo having 6-8 cells for PGD. When embryos develop into blastocysts on day 5 of fertilization, embryos selected by PGD are

transferred to the mother. Alternatively, a few cells can be removed from the trophectoderm of a blastocyst for PGD on day 5, but the embryo will need to be transferred on day 6. Otherwise, the blastocysts need to be stored frozen and transferred at the next fertilization cycle.

### 1.2.2 Laser-assisted biopsy of embryos

PGD is accomplished by evaluating genetic material in polar bodies from unfertilized and fertilized oocytes (not performed here), blastomeres from cleavage-stage embryos, or trophectoderm cells from blastocysts. Depending on the developmental stage (oocyte, embryo or blastocyst), the zona pellucida can be breached by one of three methods: mechanical zona drilling, acidified Tyrodes solution or laser.

At present, the cleavage stage is most widely used for embryo biopsy, and drilling a hole in the zona pellucida with a laser beam is the predominant method (Harton et al., 2011b). On day 3 of fertilization when normally developing embryos reach the 8-cell stage, one or two blastomeres are aspirated through the opening of a cleavage-stage embryo for PGD using a glass capillary pipette. This is also known as blastomere biopsy.

Biopsy of embryos at the blastocyst stage (on day 5 of fertilization) is more technically demanding (Fig. 1). An advantage of blastocyst biopsy is that this procedure is noninvasive

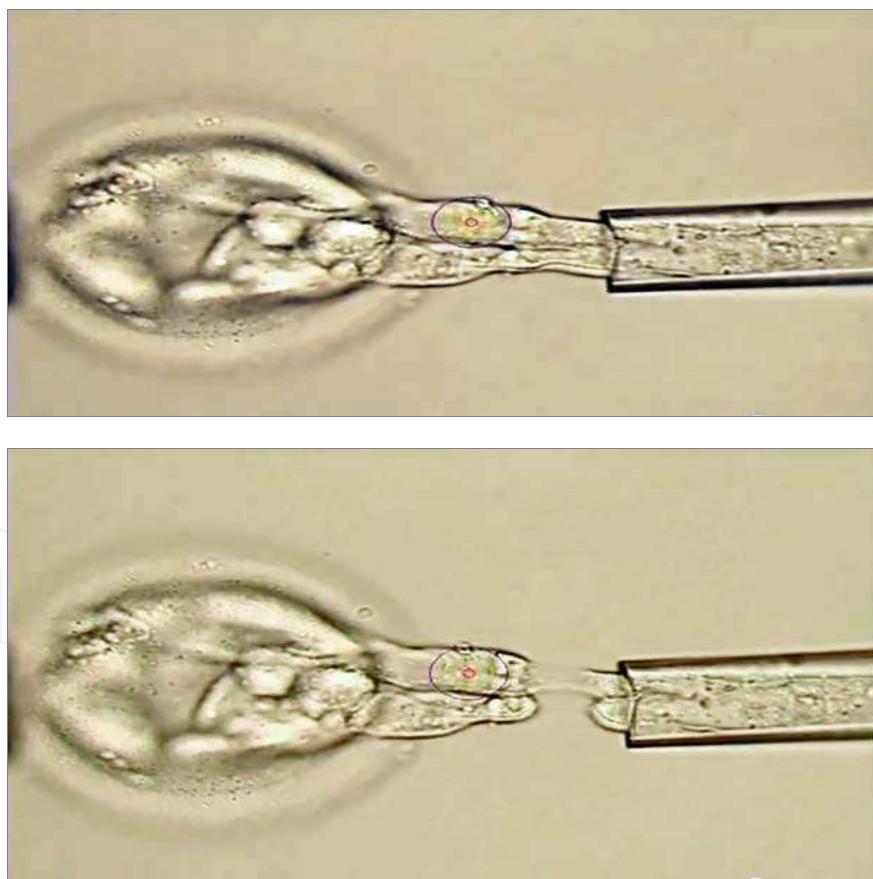


Fig. 1. Laser-assisted blastocyst biopsy. Shown in the *top* and *bottom* panels *before* and *after* the removal of a few trophectoderm cells from an embryo at the blastocyst stage for PGD, respectively. On day 5 post-fertilization, a few cells excised from the trophectoderm by laser beam are aspirated using a glass capillary pipette under stereo microscope.

to the inner cell mass of the embryo because cells are removed only from the trophectoderm that will become the placenta. A further advantage is that the removal of multiple cells for PGD will significantly lower the allele drop-out (ADO) rate and increase the accuracy of testing. A drawback of blasocyst testing is that it allows less time for PGD, and thus the embryo may need to be frozen for transfer in the next fertilization cycle if test results are not obtained within a day.

### 1.2.3 Sperm sorting

After binding non-intercalating fluorescent dye to their DNA, X- and Y-bearing sperms are separated by flow-activated cell sorting based on the difference in total DNA content. The purity is greater than 70% for Y-bearing sperms, but greater than 90% for X-bearing sperms. Sperm sorting can be used for preconception sex selection used in family balancing, or used in combination with IVF-PGD to prevent the transmission of X-linked recessive diseases.

### 1.2.4 Cryopreservation of biopsied embryos at the blastocyst stage

Using the standard freeze-thaw method, the survival rate is low when cleavage-stage embryos (on day 3 of fertilization) are biopsied and cryopreserved. The survival rate can, however, be improved by vitrification after incubating the cleavage-stage embryos for 6-8 h following biopsy (Zheng et al., 2005), or by cryopreservation in CJ3 medium (Stachecki et al., 2005). Recently higher survival rates of biopsied cleavage-stage embryos have been obtained by vitrification at the blastocyst stage (Magli et al., 2006; Zhang et al., 2009).

## 2. Current technologies for preimplantation genetic testing

At the Medical College of Wisconsin and Froedtert Hospital (Milwaukee, Wisconsin, U.S.A.) we provide PGD and PDS services for medically indicated conditions (Bick & Lau, 2006; Swanson et al., 2007). PGS for chromosomal aneuploidies (monosomy or trisomy) and chromosomal translocations is performed by FISH (Swanson et al., 2007). We also provide PGD for HLA matching (Bick et al., 2008), diagnosis of specific single-gene disorders (Swanson et al., 2007; Lau et al., 2010), and gender selection in cases of X-linked diseases (E.C. Lau, K. Wang & M.M. Janson, unpublished).

For a family with a child who needs bone marrow transplantation, PGD for HLA matching (tissue typing) can facilitate birth of an HLA-matched donor infant (Bick et al., 2008; Verlinsky et al., 2001). Hematopoietic progenitor cells are then transplanted from cord blood or bone marrow of the PGD infant to save the life of the affected sibling. In addition, we have developed PGD assays for a few common and/or severe childhood genetic diseases, such as spinal muscular atrophy, sickle cell anemia, autosomal recessive polycystic kidney disease (ARPKD), and cystic fibrosis.

### 2.1 PGD by multiplex PCR

Single-cell multiplex PCR has been used in the PGD assays for HLA matching, spinal muscular atrophy (SMA) and sickle cell anemia. In PGD for these disorders we perform multiplex PCR of single blastomeres followed by haplotyping analysis of embryos with several linked short tandem repeat (STR) markers (Bick et al., 2008). Multiple markers are used due to the high incidence of allele-drop-out for single-cell PCR.



In PGD for SMA, we perform multiplex nested PCR of single blastomeres followed by restriction cleavage analysis (Swanson et al., 2007). This technique detects the restriction fragment length polymorphisms (RFLPs) of the *SMN1* and *SMN2* genes at exons 7 and 8. We use Hinf I for cleaving exon 7 (Daniels et al., 2001), and Dde I for cleaving exon 8 of the *SMN* genes (Malcov et al., 2004). We use both fast PCR reaction mixture and rapid restriction enzymes to shorten the turn-around-time of PGD for SMA (E.C. Lau, unpublished). SMA, an autosomal recessive motor neuron disorder, is the most common fatal genetic disorder in childhood. It affects 1 in 6,000 to 1 in 10,000 live births, and has carrier frequency of 1 in 40 to 1 in 60 in the population. Deletion of both copies of *SMN1* gene accounts for 95% of SMA cases. *SMN2* gene is highly homologous to *SMN1*, but cannot substitute for *SMN1* gene functions (Prior & Russman, 2011).

In PGD for sickle cell anemia, we perform multiplex PCR of single blastomeres followed by detection of  $\beta$ -globin (*HBB*) gene mutations by mini-sequencing, which is also known as single-base primer extension (Kobayashi et al., 1995; Heinrich et al., 2009). Sickle cell anemia is an autosomal recessive disorder caused by single-base mutation in codon 6 of *HBB* gene, that substitutes a thymine for adenine. A conformational change in the hemoglobin S (Hb S) molecule reduces its ability to carry oxygen. Other types of sickle cell diseases result from co-inheritance of HbS with abnormal globin  $\beta$ -chain variants, such as sickle hemoglobin C disease (Hb SC), and sickle  $\beta$ -thalassemia. In PGD for sickle cell diseases, linkage analysis of *HBB* haplotypes is performed with linked STRs located within the *HBB* gene and its flanking regions (E.C. Lau, A.F. Licht & M.M. Janson, unpublished).

The major drawback of PGD by multiplex PCR is the interaction of PCR primers, which results in a long and tedious optimization process to work out a robust mixture for each patient family (see Section 3.1).

## 2.2 PGD by whole-genome amplification

A major challenge of PGD is to amplify DNA from single blastomeres and perform genotyping analysis within the time constraints of the IVF cycle. In order to meet the turn-around-time of 30 hours or less for PGD from receiving the biopsied cells to clinical report, we have developed a fast and reliable protocol for whole-genome amplification (WGA) from single cells using multiple displacement amplification (MDA; Lau et al., 2010). Amplification is necessary since a single cell does not contain enough DNA to fulfill these assays.

### 2.2.1 Techniques for single-cell whole-genome amplification

The earliest method for whole-genome amplification (WGA) from single cells was PCR-based primer-extension preamplification (PEP) of single sperms (Zhang et al. 1992), that was adapted for the amplification of single blastomeres in PGD (Sermon et al., 1996). Like other PCR-based methods for WGA, the drawbacks of PEP method were incomplete genome coverage, and amplification bias ( $10^3$  to  $10^6$  folds) between genomic loci in amplified products.

More recent technologies for WGA from single cells are multiple displacement amplification (MDA; Handyside et al., 2004; Lau et al., 2010; Dean et al., 2002) and PicoPlex® library methods. MDA is a non-PCR and isothermal method for DNA amplification. Handyside and colleagues used a commercial MDA kit to amplify single blastomeres for PGD (Handyside et

al., 2004; Hellani et al., 2004), but the reaction time was 16 hours, and the ADO rates for genotyping MDA products were as high as 34%. While commercial kits for MDA are optimized for greater than 10 ng input genomic DNA, the use of MDA for WGA of single cells is not a standard application supported by the kit manufacturers (Coskun & Alsmadi, 2007). We modified the reaction of a commercial rapid-MDA kit for single cells, and obtained genotyping results of less than 10% ADO after 4-h MDA reaction (Lau et al., 2010).

An alternative method for WGA from single cells is the PicoPlex® kit, which is adapted from the OmniPlex® technique for genomic DNA (Langmore 2002). The PicoPlex® library kit for WGA is a PCR-based technique that requires the fragmentation of large genomic DNA prior to the construction of a PCR-amplifiable library. Both MDA and PicoPlex® methods for single-cell WGA have been used in array-based preimplantation testing (Hellani et al., 2008; Johnson et al., 2010; see Section 3.1).

### 2.2.2 Linkage approach for embryo analysis

Besides direct mutation detection, we use a linkage approach for embryo and familial analysis in PGD. For PGD of single-gene disorders, genotyping analysis is performed for embryos using STR markers linked with the disease genes, followed by haplotyping analysis.

“Preimplantation genetic haplotyping” (PGH) is a test procedure for which the first round is whole genome amplification (WGA) of single blastomeres, and the second round is genotyping using whole-genome amplified products as template. A comparison of sizes and genotypes of STRs among family members allows inferences of the haplotypes for parents and an affected child. If the STR loci are closely linked to and flank the disease locus, unaffected embryos can be identified by comparing their haplotypes to those of the parents and affected sibling. The advantage of using single-cell WGA in the first round of PGD is that a single common protocol is used to make many copies of the entire genome for subsequent analysis.

We have successfully applied PGH to PGD for single-gene recessive disorders, such as autosomal recessive polycystic kidney disease (ARPKD; Lau et al. 2010) and cystic fibrosis (E.C. Lau, M.M. Janson & T. Boyle, unpublished). ARPKD is caused by mutations in the *PKHD1* gene, which is located at chromosome 6p12.2 and codes for fibrocystin protein. Although 2-5% of all cases of polycystic kidney diseases (PKD) are ARPKD, more than 75% of all PKD cases that present clinically in the first month of life are ARPKD, with a mortality rate of about 25% in the first month of life (Sweeney & Avner, 2006; Dell & Avner, 2008). ARPKD affects approximately 1 in 20,000 live-births, with a carrier frequency of 1 in 70 individuals. The first PGD case for a family at risk for autosomal recessive polycystic kidney disease (ARPKD) was successful using PGH (Lau et al., 2010). A PGD child, whose sibling died at birth from the disease and whose parents were both carriers of the gene, was born healthy with normal kidneys.

We have also applied the PGH approach to PGD for cystic fibrosis (CF). CF is the most common lethal autosomal recessive disorder among Caucasians of northern European ancestry, and is a common genetic cause of infant mortality. The carrier frequency among Caucasians in the United States is approximately 1 in 25, but is lower in other racial groups. Over 1,000 different mutations in the *CFTR* gene for CF have been described, but fewer than 25 occur with appreciable frequency (Amos et al. 2006).

PGD for single-gene disorders by PGH is a general approach based on linkage analysis. It does not require prior knowledge of the exact nucleotide sites of the mutations within a gene. PGH saves the time required to develop custom PGH assays for individual alleles, and thus is particularly useful for PGD of single-gene disorders involving many rare or private mutations. However, the haplotyping approach for PGD also has limitations. The method would be unable to determine the parental haplotypes when the affected child is deceased and a DNA specimen is unavailable. In addition, PGH will not infer the correct parental haplotypes if the affected inherited a chromosome that had undergone recombination at the disease locus (Altarescu et al., 2008).

### **2.3 PGD for gender selection**

The application of PGD for gender selection offers an alternative to single-gene assays for individual X-linked diseases. Instead of developing custom PGD assays for individual X-linked diseases, it is more practical to provide a single PGD assay of gender identification for families at risk of X-linked diseases. To prevent the transmission of X-linked Mendelian recessive diseases, PGD is used to select female embryos offspring. Because two copies of the mutant X allele are required for the diseases to occur in females, daughters of unaffected fathers will at worst be carriers for the trait. By contrast, in males only one copy of the mutant X allele is required for the disease to occur and so the male child of a carrier mother has a 50% risk of having the disease.

#### **2.3.1 Applications of PGD for gender selection**

The main medical use of PGD for gender selection is to prevent the transmission of X-linked Mendelian recessive disorders by giving birth to female offspring. Many X-linked Mendelian recessive disorders, such as Duchenne muscular dystrophy (DMD), and hemophilia A and B, are rarely seen in females because the child is unlikely to inherit two copies of the recessive allele. This may be because the condition is rare, or because affected males are reproductively disadvantaged.

PGD for gender selection may also be used for non-Mendelian disorders that are significantly more prevalent in one sex. For the prevention of these inherited disorders, the gender of offspring is selected based on the seriousness of inherited condition, the risk ratio in either sex, and the options for disease treatment (Amor & Cameron, 2008).

“Non-medical” applications of PGD for family balancing (also known as “social sexing” or “social sex selection”) are more controversial. There is no broad cultural preference for male or female offspring in the U.S., but there is a preference of males in some countries, such as China, India and the Middle East. This non-medical use of PGD for gender selection and family balancing is prohibited in many countries such as China, because it could disrupt the sex ratio of the population.

#### **2.3.2 Method of PGD for gender selection**

We have performed PGD for gender selection by WGA of single blastomeres by MDA, followed by multiplex PCR for detecting X- and Y-linked genetic loci (E.C. Lau, M.M. Janson & K. Wang, unpublished). The Medical College of Wisconsin and Froedtert Hospital do not



support non-medical use of PGD. We provide PGD for gender selection to help families with X-linked disorders give birth to unaffected children.

### **2.3.3 Sperm separation in combination with PGD for selecting female embryos**

Sperm sorting techniques prior to IVF can be combined with post-fertilization molecular diagnosis of the resulting embryos. To prevent the birth of affected children, female embryos can be selected by PGD after forming embryos through ICSI with X-bearing sperm. For families at risk for rare X-linked recessive disorders, such as Wiskott-Aldrich syndrome (WAS) and monocarboxylate transporter 8-specific thyroid hormone cell transporter (THCT) deficiency, sperm sorting can be combined with PGD for selecting female embryos instead of developing custom PGD assays for these disorders.

PGD for gender selection can also be used to increase the odds of conceiving an HLA-matched, unaffected sibling donor. For a family with an X-linked recessive disorder that has an affected child in need of a hematopoietic progenitor cell transplant from cord blood or bone marrow, the combined use of sperm sorting and PGD for gender selection increases the chance of finding an HLA-matched and unaffected sibling donor to approximately 1 in 5. Otherwise, the chance of finding an HLA-matched female embryo by PGD is only approximately 1 in 10.

## **2.4 PGS/PGD for cytogenetic anomalies**

Preimplantation screening for cytogenetic anomalies, in particular aneuploidies and translocations, has been performed using either FISH probes or array-comparative genomic hybridization. We shall discuss the challenges and limitations of both methods.

### **2.4.1 Aneuploidy for whole chromosomes**

The adoption of FISH in a preimplantation context was a natural adaptation of FISH techniques as used in prenatal diagnosis (PND), where its value in assessing aneuploidy is unassailable. In contrast to prenatal specimens, only single, or at most two cells, are available for preimplantation genetic testing (PGT), and thus single FISH probe cocktails must employ at least 5 fluorochromes to cover the most prevalent liveborn aneuploidies, namely, 13, 18, 21, the X and Y. The availability of commercially produced cocktails has alleviated the need to “home brew” probes, but has necessitated the acquisition of more comprehensive fluorescent filter sets than those used for most routine two- or three-color FISH.

Following biopsy of an embryo the single blastomeres are placed in a hypotonic solution, and then treated with acetic acid methanol to fix and spread the cells. All these are performed by an embryologist. Ideally this will result in a uniformly flat nucleus with little or no overlying cytoplasm. Larger swelled nuclei which have not ruptured and have therefore preserved the nuclear contents are desired in order to separate the hybridization signals, especially for the large centromere targets, 18, X and Y. Subsequent steps are performed in the cytogenetics laboratory. From this point forward a major departure from PND, where typically 50 nuclei are assessed, is the reliance on a single nucleus for aneuploidy diagnosis. Thus, there is a need for consistency in the fixation and spreading,

and freedom from artifacts such as spurious extra signals or weak signals, which are inconsequential in multi-cell analyses.

The partial coverage of a 5-probe set can be partly overcome by sequential hybridizations to the same nucleus. The quality and consistency of a second or subsequent hybridization are, however, generally progressively inferior due to the damaging hot treatments at 73 °C, which are necessary for denaturation and stringent washing. Preliminary indications of commercial development of a 6-step reduced-temperature sequential hybridization strategy to cover all 24 chromosomes have not to our knowledge been realized, and routine widespread use of such a strategy may not be technically realistic.

Nevertheless, a second hybridization cycle can detect the more common aneuploidies seen in early spontaneous pregnancy losses, namely, trisomies 15, 16 and 22. An example of a home-brew second cycle is shown in Fig. 2, and utilizes a contrasting color mixture of alpha satellite for 15, alpha satellite for 16 and the BCR locus for 22.

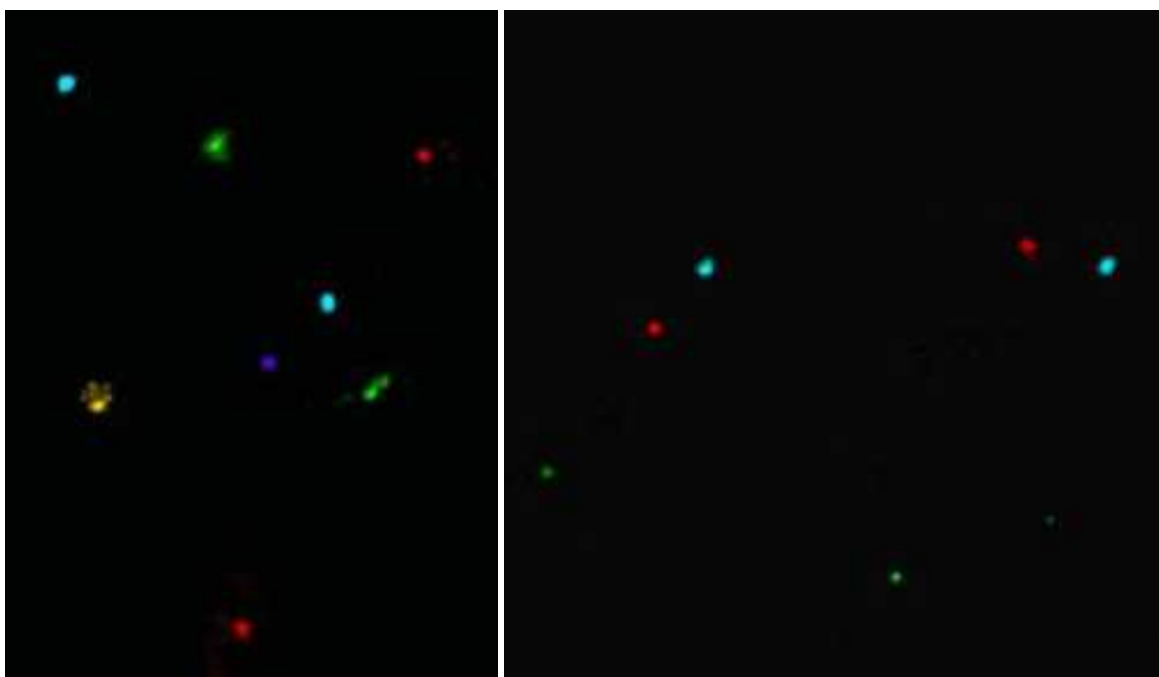


Fig. 2. Panel on left is a first cycle of hybridization using Abbott PGT cocktail. Fluorochrome scheme: red = 13, aqua = 18, green = 21, blue = X and Y = gold. This nucleus is euploid for these chromosomes and is male. Panel on right is cycle 2 of the same nucleus. Fluorochrome scheme: orange = 15, aqua = 16, green = 22, all obtained from Abbott Laboratories. The single copy unique sequence BCR is thus the smallest signal.

From the foregoing discussion one can deduce that several technical difficulties limit the usefulness of FISH in aneuploidy assessment. Due to artifactual variation in quality the clarity of results seen in Fig. 2 is not always achieved, resulting in ambiguous diagnosis or no diagnosis for an embryo. This limitation is inherent to the fact that a single data point (FISH signal) in a single nucleus is to be regarded potentially as representative of the entire embryo. Secondly, an obvious limitation is the extent of genome coverage, which often encompasses only the potentially liveborn trisomies, with a complete absence of information regarding other unsampled genomic regions.

Both these limitations are overcome by the use of genomic copy number microarrays (see Section 3 below). Firstly, each chromosome is represented by many data points by array hybridization, making 1, 2 or 3-copy number calls more statistically reliable. Secondly, all the chromosomes are represented on arrays, thus all aneuploidies leading to early pregnancy loss can be detected.

### 2.4.2 Aneuploidy for sub-chromosomal regions

Arrays using single-cell amplified DNA may not yet be necessarily sensitive enough to detect sub-chromosomal aneuploidy. Examples may include small extra marker chromosomes or unbalanced segregants of reciprocal translocation, both of which can lead to unfavorable pregnancy outcomes. FISH may continue to be of utility in assessing aneuploidy from translocation of small but genetically significant regions that have been associated with developmental delay or pregnancy loss. We have used a FISH strategy when the microarray service cannot provide assurance of reliable coverage of small regions.

An example in our experience is a couple which suffered two pregnancy losses due, in each case, to different unbalanced adjacent-1 meiotic segregation products. The small regions involved are shown in Fig. 3.

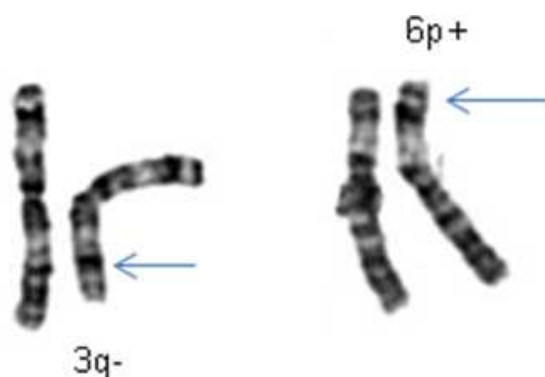


Fig. 3. Balanced  $t(3;6)(q25.3;p22.2)$  in a woman with repeated miscarriages and unbalanced pregnancy losses. Both unbalanced adjacent-1 products have been observed in karyotyped products of conception.

The probe scheme used to assess their embryos is illustrated in Fig. 4. It also illustrates the general rule that labeling three of the four arms of the quadrivalent with probes is necessary and sufficient to detect any of the 12 unbalanced products possible, including both adjacent-1, both adjacent-2 as well as all eight 3:1 segregants. An advantage of translocation FISH over aneuploid FISH is that often two data points will mark an unbalanced meiotic product, since all adjacent-2 and half of 3:1 products result in a duplication or deficiency for two probes. This strategy has been adopted in three pregnancy cycles in the aforementioned couple.

FISH patterns from an embryo clearly unbalanced with an extra 3p signal but a single 6 centromere are shown in Fig. 5a. This is interpreted as an adjacent-2 segregant, cartooned in Fig. 5b. This would result in 3 copies for most of 3 and monosomy for most of 6.

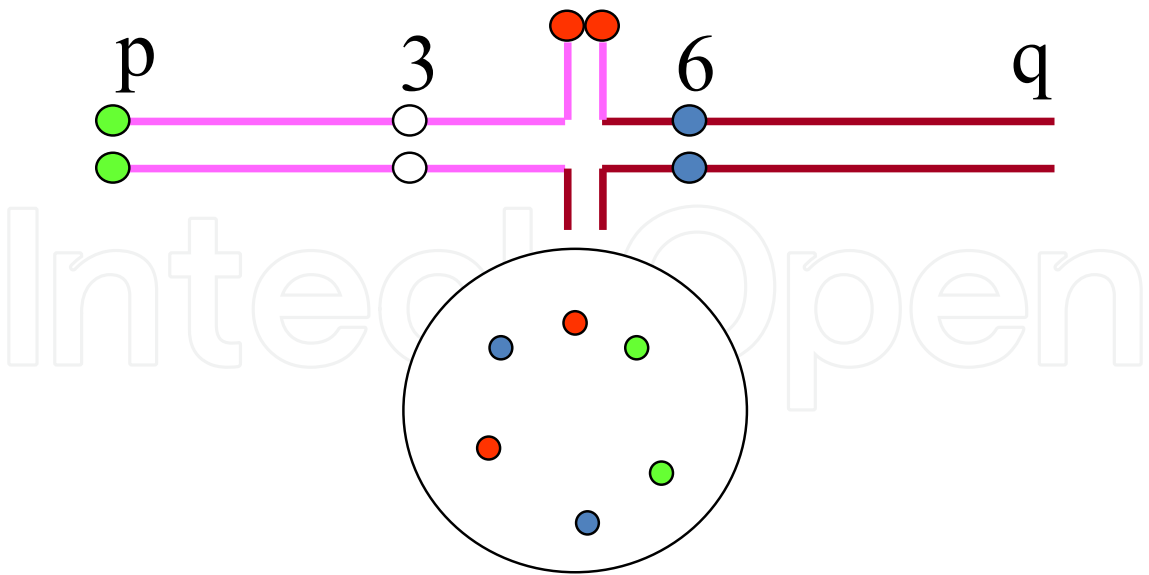


Fig. 4. *Top*, scheme to detect unbalanced t(3;6) meiotic products: subtelomere green (3p) and orange (3q) as well as 6 centromere (aqua). Three contrasting probes are sufficient to detect all unbalanced products. *Bottom*, this interphase pattern is balanced, any other pattern is unbalanced, following fertilization.

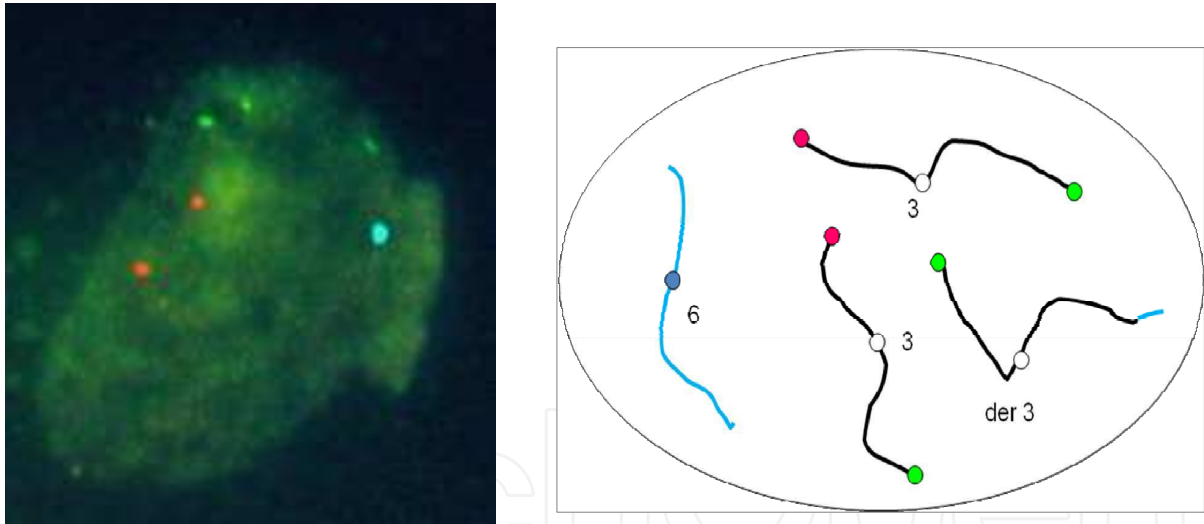


Fig. 5. *Left*, shows FISH patterns with an extra 3p signal and deficient for a 6 centromere signal. This is one of two cells from this embryo showing adjacent-2 segregation. *Right*, depicts this unbalanced complement.

To date adjacent-1 and adjacent-2 segregants have been detected in this couple, including those equivalent to both of the previous cytogenetically documented pregnancy losses.

It should be emphasized that both FISH and microarray approaches are limited in predictive value by strictly biological considerations. It is apparent by numerous studies that many early embryos are mosaic for aneuploidy. In addition, mosaic embryos often “correct”, leading to normal outcomes following an aneuploid finding based on a single cell (Barbash-Hazan et al, 2009). Thus, both false negative and false positive findings may be expected

from these single cell approaches. These considerations must be kept in mind when counseling couples to the value and accuracy of aneuploid PGD.

### 3. Technological advances in preimplantation genetic testing

Robust WGA protocols (see section 2.2 above) and high-throughput DNA technologies such as microarrays and next generation sequencing (NGS) make it reasonable to envision PGD by interrogating the complete genome of a single blastomere. Completing a whole-genome analysis within the time frame of an IVF cycle via either technology remains a technical challenge. Genomic strategies, however, offer the promise of universal diagnosis and screening methods with consequent advantages in cost efficiency and diagnostic power. Emerging technologies for whole genome analysis via DNA microarrays and DNA sequencing are rapidly becoming more powerful and more cost effective, and it will soon be feasible to apply these genomic tools widely in reproductive medicine.

#### 3.1 PGD/PGS by microarrays

DNA microarray technologies measure hybridization between the subject's DNA (the "target") and a matrix of known DNA sequences (the array "features") immobilized on a solid state matrix. Depending upon the array platform and hybridization protocol, microarrays can reveal gains or losses of genome segments or determine the subject's genotype for SNPs.

Array-comparative genomic hybridization (aCGH) has become an established alternative to FISH for PGS of aneuploidies, unbalanced translocations and complex karyotypes with multiple rearrangements (Hellani et al., 2008; Van den Veyver et al., 2009; Harper & Harton, 2010; Alfarawati et al., 2011; Fiorentino et al., 2011; Vanneste et al., 2011). In this procedure, target and control genomic DNAs are mixed and competitively hybridized to the same array. Changes in the hybridization ratio of target to control at a region indicate a gain or loss of material relative to the control genome. Since aCGH interrogates every chromosome and reveals events below the limits of microscopic detection, it is able to identify chromosome anomalies that a standard 8- or 12-chromosome FISH might fail to detect. However, aCGH does not detect balanced rearrangements or triploidy, where the target:control DNAs hybridized to the array features is constant along the genome (Thornhill et al., 2008; Harper & Harton, 2010; Fiorentino et al., 2011).

Single-gene disorders are amenable to whole-genome analysis using SNP microarrays. The SNP array features include alternative alleles for a large number of polymorphisms, and hybridization indicates which SNP allele(s) are present in the target genome. The SNP genotypes of two parents and a reference child define maternal and paternal haplotypes at a gene of interest, and linkage then establishes the genetic risk for a second child based on its combination of parental haplotypes. The analysis is similar to STR haplotyping discussed previously (see section 2.2.2). While custom PGD linkage assays have been developed for less than 10% of the known single gene disorders, a single microarray generates predictive SNP haplotypes for the entire genome.

Handyside et al. (2010) employed SNP genotype data to create "karyomaps" that represent the parental haplotypes and points of recombination along a child's chromosomes. In this



work, SNP haplotypes were shown to coincide with the inheritance of cystic fibrosis in families where both parents were carriers for CFTR mutations (Handyside et al., 2010).

Unpublished results from our laboratory (C.B. Ball & E.C. Lau) also serve as support the concept of SNP haplotyping for Mendelian disorders. SNP haplotypes linked to eleven loci of clinical interest were analyzed in a family of four individuals. At each locus, a region comprised of the gene of interest and 2 Mb flanking on either side contained sufficient informative SNPs to infer paternal and maternal haplotypes and predict the genetic status of a sibling to the reference child. We validated the SNP haplotypes analysis with PCR assays for STRs linked to three of the eleven loci (see section 2.2.2). Table 1 (a and b) summarizes the findings for these three genes:

(a) SNP haplotypes linked to loci of interest and diagnosis for proband child.

Locus	Linked SNPs		SNP Haplotypes				# of SNPs matching proband call
	Total	Informative	Father	Mother	Reference child	Proband child	
CFTR	903	255	P1 P2	M1 M2	P1 M1	P2 M2	245 / 253
HBB	1,747	528	P1 P2	M1 M2	P1 M1	P1 M2	499 / 528
PKHD1	1,492	488	P1 P2	M1 M2	P1 M1	P1 M2	472 / 488

(b) STR haplotypes linked to loci of interest and diagnosis for proband child.

Locus	STR location	STR haplotypes STR allele sizes in bp			
		Father	Mother	Reference child	Proband child
CFTR	3' flanking region	P1 P2 211, 204	M1 M2 204, 231	P1 M1 211, 204	P2 M2 204, 231
HBB	5' flanking region	P1 P2 137, 141	M1 M2 127, 119	P1 M1 137, 127	P1 M2 137, 119
PKHD1	5' flanking region	P1 P2 348, 363	M1 M2 352, 344	P1 M1 348, 352	P1 M2 348, 344
	intragenic	P1 P2 254, 260	M1 M2 260, 264	P1 M1 254, 260	P1 M2 254, 264
	3' flanking region	P1 P2 232, 228	M1 M2 230, 228	P1 M1 232, 230	P1 M2 232, 228

Table 1. Comparison of haplotyping for SNPs linked to three clinically significant loci (a) with haplotyping for STRs linked to the same loci (b).

It should be noted that of 21 custom primers tested, only five assays were informative in this pedigree (Table 1b). This illustrates the universality of SNP genotyping compared to STR methods and the value of avoiding custom assays.

SNP arrays are also of value in assessing aneuploidy and unbalanced chromosome rearrangements. Johnson et al. (2010) showed that integrating SNP genotype data with copy number improved the quality of analysis for noisy data such as those obtained from single-cell WGA. Similarly, Handyside et al. (2011) established that karyomapping across the whole genome would detect chromosome imbalances and identify the parent of origin for the imbalance based on the parental genotypes.

### 3.2 Future PGD/PGS by DNA sequencing

Besides high-density microarrays, SNPs can be detected genome-wide by DNA sequencing, which provides genotyping analysis at the highest resolution. For PGD of single-gene disorders, DNA sequencing would be the future method of choice. The assay can interrogate the entire genome, or target specific regions and genes of special interest. In principle, sequencing methods can search directly for a targeted base change, or generate SNP haplotypes for a linkage-based diagnosis. Second-generation sequencing systems, such as Roche 454 system, Illumina sequencers and ABI SOLiD system, have not been used for PGD due to the high cost and slow turn-around-time.

Even if the cost of whole genome DNA sequencing falls to \$1,000, it is still too costly for routine clinical PGD when the total cost of sequencing the parental samples, affected child and several embryos is considered. In contrast to accurately determining the genotypes of every SNP that requires sequencing at high genome coverage, it may lower the cost of PGD using a haplotyping approach that would require a lower genome coverage to identify the SNP haplotypes of embryos for transfer.

The recently released third-generation DNA sequencers, such as the Single-Molecular Real-Time System RS (Pacific Biosciences, CA), has enabled more rapid and cost effective genomic sequencing. Future sequencing technologies, such as DNA transistor nanopore sequencing system (Roche-IBM), and DNA tunneling silicon nanopore sequencer, will benefit PGD when the sequencing cost falls below \$250, and the turn-around-time to be less than 24 hours.

## 4. Genetic counseling for PGD/PGS

Genetic counseling is an essential step for patients contemplating PGD (Swanson et al., 2007). During the counseling session information is gathered including the patient's medical and reproductive history, the partner's medical and reproductive history, and their family histories. Both the patient and her partner should attend the counseling session. If an indication of risk is not covered, it is essential that the counselor obtains the physician and/or laboratory records supporting the information. Preimplantation genetic testing should not be undertaken without a firm diagnosis. With this information in hand the counselor can discuss disorder(s) in the family, the severity and variability of such condition(s), limitations of genotype/phenotype and the patient's probability of affected children. All of this information will help a patient decide whether PGD would be a reasonable option for her situation.

If PGD appears to be an option, then information is provided detailing the testing that can be carried out on single embryonic cells. Limitations of such testing are discussed including the patient's anticipated success rate for IVF with PGD and the possibility of both false positive and false negative PGD results. Prenatal testing through chorionic villus sampling (CVS) or amniocentesis is recommended to confirm the results of PGD. Patients should be reminded of alternative reproductive options including use of donor gametes, prenatal diagnosis (PND), accepting genetic risk without further testing, adoption, and having no children or no additional children (Harton et al., 2011c).

By combining the information from the reproductive medicine specialist and the genetic counselor, the patient can decide whether to pursue PGD. It is important that the counseling provided should be non-directive, to enable the patient to reach her own conclusion about the suitability of PGD (Shenfield et al., 2003).

## **5. Future prospects for preimplantation genetic diagnosis**

Severe genetic disorders are debilitating, expensive and incurable conditions. The patient, the family and society at large each have an interest in avoiding the birth of a child with such a disease. PGD provides that option in a cost-effective manner, without resort to termination of an affected pregnancy.

### **5.1 Current status of choice between PGD/PGS and PND in the U.S.**

Prenatal diagnosis (PND) of fetuses is performed by either amniocentesis or CVS performed at different stages of fetal development, but the procedures are invasive and parents may ultimately decide to terminate an affected fetus. Even though PGD has been available for 21 years, PND following natural conception still prevails in the U.S. For couples at risk of severe inherited genetic disorders, abortion of affected pregnancy is still the most common option, especially in those States which do not have mandatory insurance coverage for IVF and PGD.

### **5.2 Medical and economic considerations of universal access to PGD/PGS**

The medical community has recently begun to address the medical and economic implications of implementing a national PGD program to help couples who are carriers of severe single-gene disorders.

About 1000 children affected with cystic fibrosis (CF) are born annually in the U.S., in some part due to reluctance to terminate affected pregnancies. There is the potential to save 33 billion dollars in lifetime medical care for those affected with this disorder if carrier parents had the option of undergoing government-backed or insurance-mandated PGD and IVF (Tur-Kaspa et al., 2010). For couples who are carriers of severe inherited genetic disorders, prevention of affected pregnancy by PGD may be a preferred option to the termination of affected fetuses (Davis et al., 2009). Thus, economic and medical considerations favor a universal and affordable access to IVF, PGD or PGS services for carrier couples of severe single-gene disorders such as CF, or for individuals at risk for transmitting chromosomal translocations, but cannot afford it (Handyside, 2010).

## **6. Ethical concerns**

The non-medical use of PGD and PGS presents some ethical concerns. While PGD has mainly been used to select embryos unaffected with severe genetic diseases to avoid the transmission of some medical conditions, PGD may also have the potential to select an embryo affected with the same disability or disease that affects the parents, such as deafness. A deaf child born to a deaf couple would be better suited to the parents' shared culture.

There is resistance regarding the practice of reproductive technologies including IVF, PND, abortion, sperm sorting, PGD/PGS, and embryonic stem cells. In fact, many countries have

banned the use of PGD for gender selection, but it is permitted in the U.S. The opponents of “social sex selection” argue that the consequences of preimplantation procedures such as sperm sorting, PGD and PGS would artificially unequalize the ratio of females to males. The opponents of PGD and PGS fear that these technologies could be used for eugenic purposes, or increasing uses of PGD may open the door to other eugenic technologies.

PGD has successfully been used to prevent the transmission of single-gene disorders with Mendelian inheritance, but has not been used for complex genetic disorders because there is insufficient amount of amplified DNA from a single blastomere with high fidelity for testing a large number of genes and single-nucleotide polymorphisms (SNPs). Complex genetic disorders (e.g. diabetes mellitus), athletic ability and intelligence are controlled by many genes and environmental factors. Similarly, physical traits (e.g. body height), and cosmetic traits (e.g. hair color, eye color and skin pigmentation) are determined by multiple genes and SNPs (Sulem et al., 2007). With the recent advancement in blastocyst biopsy for removing multiple cells, cryopreservation of blastocysts, whole-genome amplification methods that generate amplified DNA with high fidelity, it has been technically feasible to provide preimplantation testing for complex genetic disorders and human traits. When PGD expands its scope to such a non-medical realm in potential attempts to make “designer babies”, it will spark more debates and controversy that might lead to legislation in this area.

## 7. Conclusion

Preimplantation genetic testing is an important application of IVF and has broad interest to reproductive medicine practitioners. Economic and medical considerations favor a universal and affordable access to IVF and PGD/PGS services for carrier couples of severe inherited genetic disorders. In addition to current methodologies, preimplantation testing is an expanding field poised to adopt cutting-edge genomic technologies for new advances in preventative medical care. Technologies for interrogating whole genomes via SNP microarrays and DNA sequencing are rapidly becoming more powerful and more cost-effective. It will soon be feasible to apply these genomic tools widely in reproductive medicine.

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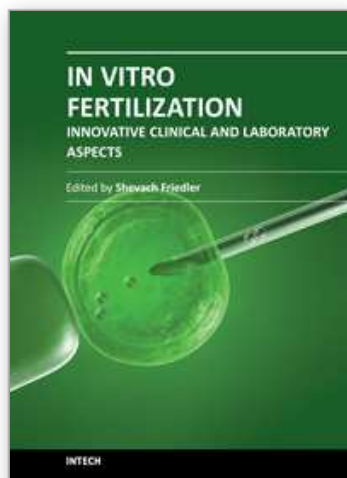
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## **In Vitro Fertilization - Innovative Clinical and Laboratory Aspects**

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The field of In Vitro Fertilization is a relatively new field in medicine, constantly on the move. This field is an exquisite example of the vast power in the complementary use of basic research with clinical practice and opened a new route of great basic and clinical research possibilities. The knowledge base that allowed the accomplishment of the idea of in vitro fertilization and embryo transfer has much developed since. The vast body of research pertaining to this field allowed deepening our understanding in the processes related to reproduction. In this book on in vitro fertilization we present new and interesting updated information in various aspects of this field. This work is a result of collaborative work of an international group of professionals dedicated to contribute to the advancement of our knowledge.

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