Short Specialist Review

21. Preimplantation genetic diagnosis for chromosome abnormalities

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1. Introduction

Preimplantation genetic diagnosis (PGD) is the earliest form of prenatal diagnosis, and usually involves the analysis of a single cell after biopsy from a three-day old embryo created through assisted reproductive techniques (ART), or sometimes the first and second polar bodies of the eggs prior fertilization, also through ART. Because the embryo should be replaced in the future mother in no more than one or two days after biopsy and as there are only one or two cells for analysis, the diagnostic tests must be fast and highly sensitive. However, obtaining analyzable metaphases of karyotyping quality from a single cell is not possible even with cell-conversion methods (Willadsen *et al*., 1999; Verlinsky and Evsikov, 1999); so the analysis for cytogenetic purposes is performed using FISH, which allows chromosome enumeration of interphase cell nuclei, that is, without the need for culturing cells or preparing metaphase spreads. Since 1993, FISH has been used for PGD of common human aneuploidies with either blastomeres (cells from 2- to 16-cell stage embryos) or oocyte polar bodies (Munné *et al*., 1993; Munné *et al*., 1995a,b; Munné *et al*., 1998b; Munné *et al*., 1999; Munné *et al*., 2003; Verlinsky *et al*., 1995; Verlinsky *et al*., 1996; Verlinsky *et al*., 1998; Verlinsky *et al*., 2001; Verlinsky and Kuliev, 1996; Gianaroli *et al*., 1997; Gianaroli *et al*., 1999; Gianaroli *et al*., 2001b; Pehlivan *et al*., 2002; Kahraman *et al*., 2000; Rubio *et al*., 2003). Currently, probes for at least chromosomes X, Y, 13, 15, 16, 18, 21, and 22 are being used simultaneously (Munné *et al*., 2003), with the potential of detecting 83% of aneuploidies found in spontaneous abortions (Jobanputra *et al*., 2002).

2. PGD to improve pregnancy outcome in ART

PGD was first thought of as a tool for selecting against genetically abnormal embryos from couples carrying genetic diseases; but about 90% of the PGD cycles performed so far have been for aneuploidy to improve the pregnancy outcome of ART patients, with over 5000 cases performed to date for that purpose (Munné *et al*., 1999; Munné *et al*., 2003; Gianaroli *et al*., 1999; Gianaroli *et al*., 2001b;

ESHRE PGD Consortium Steering Committee, 2002; Verlinsky and Kuliev, 2003) and close to a thousand babies born thereafter (Verlinsky *et al*., 2004).

The rationale for using PGD to increase pregnancy rates and reduce miscarriage rates is as follows. Oocyte quality is the major cause of reduced implantation with advancing maternal age (Navot *et al*., 1994), and one of the clearest links so far between maternal age and embryo competence is aneuploidy. The increase in aneuploidy with maternal age in spontaneous abortuses and live offspring (Hassold *et al*., 1980; Warburton *et al*., 1980; Warburton *et al*., 1986; Simpson, 1990) has also been observed in embryos and oocytes (Munné *et al*., 1995a; Márquez *et al*., 2000; Dailey *et al*., 1996) but with much higher rates of chromosome abnormalities than in spontaneous abortions, which indicates that a sizable part of chromosomally abnormal embryos are eliminated before clinical recognition. This embryo loss, rather than endometrial factors, largely accounts for the decline in implantation with maternal age.

To compensate for the low implantation potential of human embryos created in vitro, fertility centers normally generate a larger cohort of embryos (average >10); those with the highest potential to implant are then selected on the basis of morphology and developmental characteristics. Unfortunately, trisomy is not correlated with embryo morphology or development (Munné *et al*., 1995a; Márquez *et al*., 2000), and only some monosomies can be selected against by culturing the embryos to blastocyst stage (Sandalinas *et al*., 2001).

Because of the correlation between aneuploidy and declining implantation with maternal age, we hypothesized that negative selection of chromosomally abnormal embryos could reverse this trend (Munné *et al*., 1993). While the probes currently used in PGD check only a limited number of chromosomes, the results so far indicate that PGD of aneuploidy actually does increase implantation while reducing trisomic offspring and spontaneous abortions (Munné *et al*., 1999; Munné *et al*., 2003; Gianaroli *et al*., 1999; Gianaroli *et al*., 2001a,b; Werlin *et al*., 2003).

As determined in a recent study, the chromosomes most involved in aneuploidy at the cleavage stage (first 3 days of embryo development) are different than those found in prenatal diagnosis (Munné *et al*., 2004). So when the most common chromosomes are analyzed by PGD with eight or more probes, including probes for chromosomes 13, 15, 16, 18, 21, and 22, the implantation rate (embryos implanting/embryos replaced) doubles.

In one study, we observed a significant twofold increase of implantation, from 10.2 to 22.5% ($p < 0.001$) (Gianaroli *et al*., 1999); in another more recent study with an older population (average age 40), and with two or less previously failed IVF attempts, we found a 20% implantation rate after PGD compared to 10% in the control group ($p = 0.002$) (Munné *et al*., 2003).

It is clear that not only does implantation reduce with advancing maternal age but also those embryos that do implant have a higher risk of chromosomal abnormality and miscarriage. Since the objective of ART is to ensure a healthy baby for couples seeking to conceive, both factors are extremely important.

FISH with probes for 13, 15, 16, 18, 21, 22, X, and Y can detect 83% of all chromosomally abnormal fetuses detected by karyotyping (Jobanputra *et al*., 2002). Since this combination of probes is the current standard (Munné *et al*., 1999; Gianaroli *et al*., 1999), PGD is able to eliminate close to 80% of all chromosomally

abnormal embryos at risk of causing a miscarriage. Among many examples, two studies reported abortion rates of only 9% after PGD in women >36 years (Munné *et al*., 1999; Gianaroli *et al*., 2001a,b) compared to the 24% spontaneous abortion expected for such populations of infertile patients (SART-ASRM, 2000).

Increased implantation and decreased spontaneous abortion result in a higher chance of patients achieving viable pregnancies (Munné *et al*., 1999). However, PGD works properly only when a larger group embryos is available for testing in a given procedure. If patients have less than five embryos available, then replacing all the embryos will in general give the same result as if PGD had been performed.

3. PGD to reduce the risk of aneuploid conceptions

The current PGD technique works by analysis of a single cell with an error rate around 10%. This error rate is mostly due to mosaicism, which is very common in human cleavage-stage embryos (see review Munné *et al*., 2002). Thus, when diagnosing trisomic offspring, PGD can significantly reduce the occurrence but not completely prevent it. Indeed, four misdiagnoses have already occurred after PGD (Munné *et al*., 1998b; Gianaroli *et al*., 2001a). Nevertheless, the rate of trisomic offspring detected after PGD is significantly lower than expected ($p < 0.001$). For instance, 2 of 666 (0.3%) fetuses were found with aneuploidies for chromosomes XY, 13, 15, 16, 18, 21, and 22 (Munné *et al*., 2003 and unpublished results) compared to a 2.6% rate expected in a population of the same age range (Eiben *et al*., 1994). Interestingly, the reduction from 2.6% to 0.3% is a 90% reduction, which is as expected if the error rate is indeed 10%. Similarly, Verlinsky *et al*. (2001) reported 140 healthy children born after PGD of aneuploidy using polar body analysis, with no misdiagnoses.

4. PGD for translocations

Balanced translocations occur in 0.2% of the neonatal population. However, they are identified in 0.6% of infertile couples, 2–3.2% of infertile males requiring intracytoplasmic sperm injection, and 9.2% of fertile couples experiencing three or more consecutive first-trimester abortions (Testart *et al*., 1996; Meschede *et al*., 1998; Van der Ven *et al*., 1998; Stern *et al*., 1996; Stern *et al*., 1999). PGD for translocations can reduce spontaneous abortion and minimize the risk of conceiving an unbalanced baby, thus being a realistic alternative to prenatal diagnosis and pregnancy termination of unbalanced fetuses. So far there have been close to 500 cycles of PGD of translocations performed worldwide (Munné *et al*., 2002; Verlinsky *et al*., 2002; Cieslak *et al*., 2003; Gianaroli *et al*., 2003).

4.1. Methods

Several approaches to PGD of translocations have been developed. The first involved the analysis of first polar bodies, after the observation that more than 90%

of first polar bodies fixed for 6 or fewer hours after retrieval are in metaphase stages (Munné *et al*., 1998a). The translocation can then be identified using chromosome-painting probes for the two chromosomes involved in the translocation (Munné *et al*., 1998c,d; Durban *et al*., 2001).

Cell-conversion methods have also been used to transform blastomere nuclei (usually in interphase to metaphase) by fusing them to oocytes or zygotes (Willadsen *et al*., 1999; Verlinsky and Evsikov, 1999; Evsikov *et al*., 2000; Verlinsky *et al*., 2002), achieving close to 80% rates of analyzable metaphases. Alternatively, Tanaka *et al*. (2004) observed 4–6-cell stage embryos every hour, and when the nuclear envelope of a blastomere disappeared, the blastomere was biopsied within an hour. Disappearance of nuclear envelope was observed in 89% of embryos, and all produced analyzable metaphases.

Two different interphase approaches have also been developed for PGD of translocations. The first developed specific probes spanning the breakpoints of each translocation (Munné *et al*., 1998c; Weier *et al*., 1999) or inversion (Cassel *et al*., 1997). The second used probes distal to the breakpoints or telomeric probes in combination with proximal or centromeric probes, either for translocations (Munné *et al*., 1998c; Munné *et al*., 2000; Pierce *et al*., 1998; Van Assche *et al*., 1999) or inversions (Iwarsson *et al*., 1998). The exception is a Robertsonian translocation (RT), for which chromosome enumerator probes are used to detect aneuploid embryos (Conn *et al*., 1998; Munné *et al*., 1998c; Munné *et al*., 2000). Only the first approach (spanning probes) can differentiate between balanced and normal embryos.

4.2. Results

For most translocation patients, the risk of consecutive pregnancy loss is their major incentive in enrolling in a PGD program. The unbalanced products of a translocation are usually lethal and therefore the true risk is that of pregnancy loss. We have demonstrated that PGD of translocations substantially increases a couple's chances of sustaining a pregnancy to full term (Munné *et al*., 1998e; Munné *et al*., 2000). So far, 115 patients undergoing PGD for translocations have lost 84% (233/278) of their prior conceptions, but after PGD only 5% (4/78) was lost (Munné *et al*., 1998e; Munné *et al*., 2000, and unpublished data). Data from Verlinsky's group also indicates a significant reduction in spontaneous abortions to 20% (7/34) (Verlinsky *et al*., 2002; Cieslak *et al*., 2003), when 88% of pregnancies in these patients prior to undertaking PGD procedure resulted in spontaneous abortions.

However, some translocation patients produce 80% or more unbalanced gametes, and with the 50% baseline of chromosome abnormalities in cleavage-stage human embryos, it is nearly impossible to find normal ones for replacement. Previous studies have used clinically recognized pregnancies to formulate rules to predict unbalanced offspring (Jalbert *et al*., 1988). However, these specimens were probably the most viable segregation types because selective processes had already occurred. Thus, when analyzing zygotes and preimplantation embryos, it is not surprising that different translocations involving the same chromosomes show very different meiotic behavior (Escudero *et al*., 2000; Van Assche *et al*.,

1999). Escudero *et al*. (2003) determined the level of chromosome abnormalities in spermatozoa that would preclude a chromosomally normal conception, and found that the percentages of abnormal gametes and of abnormal embryos were correlated, thereby establishing that patients with 65% or fewer chromosomally abnormal spermatozoa have a good chance of conceiving.

5. Molecular methods for PGD of chromosome abnormalities

Ultimately, speedy and efficient analysis of all 24 chromosomes is PGD's true goal, as some embryos diagnosed as normal are undoubtedly still abnormal for other aneuploidies not analyzed in current protocols. One approach still needing improvement is comparative genome hybridization (CGH) (Kallioniemi *et al*., 1992). For CGH of single cells, the whole genome of the cell must be amplified (Wells *et al*., 1999). Trials applied to human blastomeres from discarded embryos have promising results (Wells and Delhanty, 2000; Voullaire *et al*., 1999; Voullaire *et al*., 2000), but so far, the process takes too long. To gain enough time for analysis, Wilton *et al*., (2001, 2003) applied this method to blastomeres from embryos that were frozen after biopsy, and the first babies have recently been borne following this procedure. However, cryopreservation and thaw destroys some embryos and ultimately outweighs the benefits of CGH.

Recently, we have been able to obviate cryopreservation by applying CGH to polar bodies and get results prior to embryo replacement on day four of development (Wells *et al*., 2002). But as with any polar body analysis, postzygotic abnormalities, which account for more than half of all abnormalities, as well as paternally derived aneuploidies, are not detectable.

DNA microarrays are being developed for aneuploidy and translocation analysis (Weier *et al*., 2001), but as presented in the last International symposium of PGD, current methods still need improvement to differentiate in single cells ratio changes of 0.5 in order to be used for PGD (Leight *et al*., 2003).

Once optimized, microarrays will have the advantage over CGH of being more robust and probably faster, and possibly not requiring embryo freezing. Also, they will supply redundancy and more accurate diagnosis of subchromosomal regions useful for translocation analysis.

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