



Embryo Biopsy for PGD: Current Perspective

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Learning Objectives

- To describe the rationale for blastocyst-stage biopsy in comparison to biopsies performed at earlier embryo developmental stage.
- To review the settings where blastocyst-stage embryo biopsy can be performed.
- To provide a detailed description on how blastocyst-stage embryo biopsy is performed.
- To present the applications of blastocyst-stage biopsy.
- To review the objectives and means of single gene testings using embryonic cells.

This chapter sets out to examine the major recent advances in embryo biopsy, specifically blastocyst-stage biopsy and preimplantation genetic testing and diagnosis (PGD), which are transforming singleton live birth rates in families at risk of passing on monogenic diseases or chromosomal translocations.

50.1 The Development of Embryo Biopsy

Embryo biopsies for clinical PGD generally were performed on day 3, when the embryo typically was at the six- to eight-cell stage, and involved the removal of one or two blastomeres. The zona was breached by dissolving the protein with acid Tyrode's solution. The embryo was incubated in a calcium-/magnesium-free medium to reduce cell–cell interactions and make the removal of cells easier. There has been debate about the impact of removing multiple cells, and in general, it was considered that two-cell removal was more detrimental than removing just a single cell and should not be performed—although the reliability of amplification of two cells was considered less prone to allele dropout. An alternative biopsy approach involved the removal of either the first or second polar bodies—either sequentially on day 0 and day 1 or both on day 1. PCR analysis of blastomeres or polar bodies involves amplification of a single allelic copy of the target. Similarly, analysis by FISH is a single-cell test. Biopsy at the blastocyst stage, when embryos typically comprise upward of 100 cells, enables the removal of 3–5 trophoctoderm cells without significant cell mass depletion. Putting several cells into a PCR reaction should decrease the

likelihood of amplification failure (allele dropout, or ADO) and, with FISH analyses, should provide an opportunity to confirm signal patterns.

During the early 2000s, Genea (formerly Sydney IVF) moved comprehensively to blastocyst culture and blastocyst-stage transfers and cryostorage and experienced a corresponding increase in take-home baby rates, substantial reductions in multiple pregnancies, and reduced rates of miscarriage [1–7]. In 2004, we described the first clinical application of blastocyst biopsy to routine PGD practice [1]. Embryos were “hatched” on day 3 using a Hamilton Thorne ZILOS-tk near-infrared laser and were then incubated for another 2 days to enable blastocoel expansion and herniation of trophoctoderm cells through the opened zona for biopsy [1, 6]. Suitable embryos were placed in 5 μ L drops of standard medium under oil. A holding pipette, the same as used in ICSI practice, was employed to immobilize the embryo, while a 30- μ L biopsy pipette was used to collapse the blastocyst cavity and hold the tissue sample. Several pulses with the laser set at low level loosened cell–cell interactions, permitting a small piece of tissue to be teased off the exposed trophoctoderm. The embryo was then removed and placed into fresh medium for further incubation (until the results were known and the embryo was transferred, cryostored, or disposed of). The tissue piece was washed and placed into PCR tubes or fixed to glass slides for analysis using FISH.

The advantage of moving from cleavage-stage to blastocyst-stage PGD was demonstrated by comparison of embryo biopsies performed (a) at the day 3 cleavage stage and followed by the transfer of embryos that went on to blastulate successfully, with (b) embryo biopsies taken *at* the blastocyst stage (day 5–6) and followed by almost immediate transfer [7]. This study, in other words, *examined the efficacy of day 3 biopsy vs. day 5 or 6 on the embryo while controlling for the embryo's ability to blastulate*; patients in this trial had PGD not for infertility or miscarriages but to prevent further propagation of a serious monogenic family disease. The outcome (Table 50.1) implies that, in comparison with the biopsy of blastocysts, day 3 cleavage-stage PGD reduces the implantation potential of at least some embryos. (The same could still also be true of blastocyst biopsy but appears to be to a much lesser extent.)

Table 50.1 shows the technical outcome data for the embryos biopsied (595 for day 3; 656 for days 5–6), with an

Table 50.1 Embryos available for testing for monogenic disease mutations by biopsy on day 3^a and on day 5–6

	Egg retrievals	Embryos biopsied	Inconclusive test result	Conclusive, favorable test	Embryos transferred fresh	Tested embryos cryostored
Day 3 biopsy + day 5–6 transfer ^a	91	595 av. 6.5 embryos	61 (10.3%)	261 (43.8%)	103	158 (60.5%)
Day 5–6 biopsy + day 5–6 transfer	177	655 av. 3.7 embryos	46 (7.0%)	305 (46.5%)	121	184 (60.3%)

See McArthur et al. [7] for more detailed interpretation of the data

^aAll embryos were developed to blastocysts before transfer

Table 50.2 Clinical outcomes following biopsy at the cleavage stage vs. biopsy at the stage of blastocyst, each with transfer of embryos fresh on day 5 or 6

	Embryos transferred <i>n</i>	Transfer procedures <i>n</i>	Pregnancy per retrieval	Implantation per embryo ^a	Miscarriage	Live birth or ongoing pregnancy	Multiple at confinement		
							Single	Twin	Triple
Day 3 biopsy + day 5–6 transferred <i>n</i> = 91 retrievals	1	38	11	11	4	7	7		
	2	28	12	15	1	10	7	2	1
	3	3	1	1	0	1	1		
	All av. 1.5	69 (75.8%)	24/91 (26.4%)	27/103 (26.2%)	5/91 (20.1%)	18/91 (19.8%)	15	3 multiples (16.7%)	
Day 5–6 biopsy + day 5–6 transferred <i>n</i> = 177 retrievals	1	105	54	54	8	46	46		
	2	8	4	5	1	3	2	1	
	3	0							
	All av. 1.1	113 (63.8%)	58/177 (32.8%)	59/121 (48.8%)	9/58 (15.5%)	49/177 (27.7%)	48	1 multiple (2%)	

Data from McArthur et al. [7]

^aThe implantation rate for blastocysts biopsied from the trophectoderm as blastocysts (48.8%) was highly significantly better than the implantation rate for blastocysts biopsied using a single cell removed at the day 3 cleavage stage (26.2%, $P < 0.01$)

average of 6.5 embryos biopsied and tested per retrieval at cleavage, compared with an average of 3.7 embryos biopsied and tested per retrieval when the blastocyst stage was awaited before performing PGD. The proportion of embryos with a conclusive test and with a normal result, thus suitable for transfer, was still approximately 50% in each series, which means that taking the biopsy later in embryo development conferred appreciable laboratory and clinical efficiency through not having to test embryos whose development was compromised. The late-biopsied blastocysts had almost twice the chance of implanting than did the blastocysts that had been biopsied on day 3.

Table 50.2 shows the outcomes of the embryo transfer procedures. In spite of a lower average number of embryos transferred (1.1 vs. 1.5 per transfer procedure), and without taking into account later further pregnancies from cryostored, biopsied embryos, the day 5–6 biopsy transfers resulted in fewer miscarriages and a higher absolute ongoing pregnancy rate, as well as the expected lower rate of multiple pregnancy. There was one obvious monozygotic twinning event, involving an embryo biopsied on day 3. In about 60% of cases in each series, additional embryos that had tested normally were cryostored for further attempts at pregnancy.

50.2 Preimplantation Screening for Aneuploidy

It has been known for more than 15 years that IVF embryos show a high rate of chromosome aneuploidy [8]. It has also been understood for many more years that a chance acquisition of an abnormal number of chromosomes is a frequent

event in human conception and, in particular, is the commonest cause for pregnancies to miscarry. It might therefore be expected that screening IVF embryos for aneuploidies before selecting an embryo to transfer should materially improve the chance of pregnancy, reduce the risk of miscarriage, and (by enabling embryos to be transferred efficiently and efficaciously one at a time) greatly reduce the multiple pregnancy rate, thus lessening perinatal morbidity and mortality. The target—improved live birth rates from IVF and less costs for community—is worthy and logical.

50.3 Aneuploidy Risk

Several authors have reported that the age of the woman undergoing IVF has a significant bearing on the extent of aneuploidy in the resulting embryos. Studies on the origin of nondisjunction chromosome anomalies have suggested that most of the abnormalities originate predominantly from female meiosis, especially meiosis I, although analysis of preimplantation embryo polar bodies with FISH has indicated that meiosis II errors could be similar in number [9]. Analysis of later-stage embryos would therefore be able to identify both meiosis I and meiosis II errors (as well as reveal aneuploidies brought by the fertilizing sperm). Generally, a predisposition to aneuploidy beyond maternal age effect has been hampered by the fact that few studies have looked for or been able to identify genetic causes; rare recessive genetic states that interfere with meiosis have been described [10]. While not extensive, there have been a number of reports suggesting that among women undergoing IVF and experiencing subsequent implantation failure, the chromosome

abnormality rate in their embryos is quite high compared to the other IVF cohorts [11–13]. Screening embryos for aneuploidy could reduce the number of embryos subsequently needed to initiate a successful and continuing pregnancy [14]. The efficacy of the screening process must obviously take into account any detrimental aspects of the biopsy and culture processes to be considered truly beneficial for the patient's progress.

50.4 Aneuploidy Screening in IVF Programs

Examination of a restricted number of chromosomes using FISH for aneuploidy screening as a routine may not be helpful in all cases and in fact can be harmful if biopsy procedures are not efficient. Mastenbroek et al. showed that biopsy of day 3 (cleavage-stage) embryos for limited PGS—screening for aneuploidy of chromosomes 13, 16, 17, 18, 21, X, and Y—can reduce the chance of an ongoing pregnancy in women aged 35–41 having in vitro fertilization (IVF) [15]. Our considerations above (■ Tables 50.1 and 50.2) suggest that interfering with an early embryo might lie behind this detrimental result, but other factors could also be important. For the Mastenbroek study, these included such straightforward concerns as the time the embryos spent being manipulated in potentially altered culture conditions across the variety of IVF clinics where the biopsies were performed. They also include more complex issues, such as the inadvertent exclusion from transfer of mosaic embryos in which the biopsied cell happened to be the only cell with trisomy (a situation that can follow a mitotic non-disjunction event) [9].

Between August 2004 and November 2006, we studied the impact of screening for aneuploidy in younger infertile women (<38 years, median 33.5 years), employing biopsies of blastocysts [5]. All women were in their first or second attempt at IVF. Agreement to have one embryo transferred (eSET) was a precondition for entry. Patients were withdrawn from the study if there were fewer than eight ovarian follicles over 1 cm diameter at 8–10 days of stimulation, fewer than four embryos with seven or more cells on day 3 of culture, or fewer than three blastocysts for biopsy on day 5 or 6; no women had cycles canceled because of a poor response. The biopsies consisted of 2–9 trophectoderm cells and were tested by at least five-color fluorescent in situ hybridization for, at minimum, chromosomes 13, 18, 21, X, and Y. We compared outcomes between the screened group (Group A, normal ³five-color pattern in all the removed trophectoderm cells for the transferred embryo) and the principal control group (Group B, with zona opening but no biopsy); we also made comparisons with the women who were withdrawn from the study before randomization because of suboptimal responses to stimulation (Group C) and with women who were eligible but elected not to take part in the study (Group D). ■ Table 50.3 gives the results up to the time the trial was suspended. Pregnancies are clinical pregnancies

■ **Table 50.3** Pregnancy rates after preimplantation genetic screening for aneuploidy from biopsy of blastocysts on day 5 or 6 of development using five- or seven-color FISH

Group A. Biopsy				Group B. No biopsy (control)			
N	P	NP	%P	n	P	NP	%P
56	25	30	45.5%	48	26	20	56.5%
Group C. Poor response, withdrawn				Group D. Eligible, nonparticipating			
N	P	NP	%P	n	P	NP	%P
107	36	71	33.6%	1194	564	630	47.2%

From Jansen et al. [5], with permission
P pregnant; NP not pregnant

with a normal fetal heart rate on ultrasound scanning in the first trimester. The clinical pregnancy rate (pregnancies with a normal fetal heart rate at 6 weeks' gestation) was high (46.4% of egg retrieval procedures overall), irrespective of whether PGS was performed or not, and is consistent with results we [2, 6] and others [16] have reported previously for elective single blastocyst transfers.

Among the women who underwent biopsy for aneuploidy screening (Group A), the pregnancy rate at 45.5% was insignificantly less than among women who were eligible for the trial but did not take part (Group D, 47.2%) and was trending to be higher than among women who were withdrawn from the trial prior to randomization because of a suboptimal response (Group C, 33.6%; $c^2 = 1.7$, $P < 0.1$, 1-tailed). We could thus find no evidence of clinically important detriment from blastocyst biopsy in women of normal reproductive age. The pregnancy rate compares favorably, with the 25% clinical pregnancy rate reported by Mastenbroek et al.

Unexpectedly, Group B, the embryos subjected to zona opening by near-infrared laser, a standard preparatory step for biopsy and performed on day 3 or 4 (see above), produced the highest clinical pregnancy rate of the groups (56.5%). While the results in Group B were not statistically significantly different from either the biopsied embryos (Group A, $c^2 = 0.8$) or the eligible but nonparticipant women's embryos (Group D, $c^2 = 1.2$), the trend was opposite to that required to disprove the null hypothesis, and the clinical trial was stopped.

The reason for the strong performance of the embryos in the principal control group, if it is true, is not clear. Assisted hatching by opening of the zona, while advocated from time to time for the embryos of older women to facilitate hatching and implantation, has not been shown to be beneficial among women under 40 or with good blastocyst development. More likely, a too strict set of criteria for assumed meiotic nondisjunction led to overinterpretation and rejection of some blastocysts that would, if left unscreened, have developed normally and contributed to the total number of embryos suitable for transfer.

50.5 Testing for Chromosomal Translocations

Reciprocal translocations occur in about 1 in 625 newborns and usually result from the exchange of two terminal segments from different chromosomes, ordinarily resulting in a genome that is balanced. Exchanges can also take place close to the centromeres of two acrocentric chromosomes; these Robertsonian translocations, which occur in about 1 in 900 newborns, also ordinarily provide a balanced genome and bring the overall prevalence of balanced translocations among newborns to about 1:380 [17]. When diploid germ cells with these karyotypes eventually undergo meiosis, however, the chromosomes involved segregate abnormally and yield a varying but significantly high level of unbalanced haploid states among oocytes and spermatozoa—an unbalanced state that is continued into the embryo and which results in implantation failure, miscarriage, stillbirth, or abnormalities at birth. Balanced translocations are ten times more common among couples presenting for treatment with IVF [18].

With *reciprocal translocations*, homologous pairing during meiosis I produces a tetravalent structure instead of the usual bivalent. Subsequent segregation to respective daughter cell spindles takes one of three modes: 2:2 *alternate segregation* (producing alternately a normal or a balanced abnormal complement, the latter perpetuating the familial condition but both with a balanced genome); *adjacent 1 and 2 segregations* (producing segmental monosomies and trisomies); and, comparatively rarely, 3:1 *segregations* (involving nondisjunction of a whole chromosome and producing more complete monosomies and trisomies) [19]. Overall, 75% of embryos from a parent with a balanced reciprocal translocation show partially or fully aneuploid chromosome complements (14 different unbalanced combinations compared to two balanced combinations), considerably reducing the number of otherwise healthy appearing embryos available for transfer after PGD.

In *Robertsonian translocations*, a trivalent structure is formed during meiosis I, with three main segregation modes possible (and nine different chromosome combinations), namely, *alternate* (which returns dosage to its balanced state), *2:1 segregations* (producing complementary monosomies and trisomies), and *3:0 segregation* (producing double trisomy or double monosomy).

Traditional PGD for translocations involves FISH, utilizing either breakpoint-spanning probes (which require access to extensive probe libraries and complicated workups) or (much more simply) combinations of commercially available, quality-controlled centromeric, locus-specific, and subtelomeric probes attached to standard fluorochromes. The use of PGD to screen balanced from unbalanced chromosome sets in the embryos then significantly reduces the failure rate for implantation and should result in fewer miscarriages among the embryos available for transfer [13, 20]. Again, to be truly beneficial, the process of biopsy must do the least amount of harm to the embryo's continued development and to its ability to implant. ■ Table 50.4 shows our

■ Table 50.4 Clinical outcomes of PGD for balanced translocation using FISH and from using STR-based PCR

	Reciprocal translocations		Robertsonian translocations	
	FISH	STR-PCR	FISH	STR-PCR
Patients	54	22	17	7
Mean age	35.2	33.1	36.0	33.3
Cycles with egg retrieval	112	22	51	6
Cycles with embryo biopsy	73	22	31	6
Biopsied embryos	320	61	142	17
Actionable PGD result	304	60	136	17
Embryos for transfer	74 (23%)	20 (33%)	44 (31%)	13 (76%)
Embryo transfer cycles	54	13	27	5
Embryos transferred	1.2	1	1.2	1
Total embryos transferred	62	13	32	5
Positive pregnancy test	20	7	14	3
Implantation rate/embryo (%)	32	54	44	60
FH-positive pregnancy rate per transfer	15 (24%)	6 (46%)	12 (38%)	2 (40%)
FH-positive pregnancy rate per egg retrieval (%)	13	27	24	33

Data from McArthur et al. [7] and Traversa et al. [22]

All embryos were biopsied and transferred at the blastocyst stage. Results do not include pregnancies from the embryos cryostored

experience with cleavage- and blastocyst-stage biopsies among couples with recurrent miscarriage attributable to a balanced reciprocal translocation in one of them. The live baby results have been lower compared to those we obtain after testing for monogenic disease (■ Table 50.2), possibly reflecting the large decrement in transferable embryos seen with reciprocal translocations following the demonstration of unbalanced cells by FISH-based PGD. These apparent unbalanced outcomes can be of biological origin but can also be false, due to inherent error rates observed with FISH-based protocols [21] or reflective of a benign mosaic state,

but in either case contributing to false-positive interpretation of FISH signals and leading to the exclusion of otherwise normal embryos.

In our published series for translocations using FISH [7], 95 egg retrievals were performed and led to biopsy and testing among couples with a *balanced reciprocal translocation*; there were 10 pregnancies among 26 patients who had day 3 biopsies, seven of which went to term—a miscarriage rate of 33%. Of the 12 pregnancies among 21 couples for whom biopsy was performed on day 5–6, eight miscarried (38%). Twenty-three egg retrievals among 15 couples with a *Robertsonian translocation* led to biopsies and testing; there were three pregnancies among seven couples with day 3 biopsies, each of which went to term, and eight pregnancies among seven couples with day 5–6 biopsies, one of which miscarried and one of which was an ongoing monozygotic twin pregnancy. Combining day 3 with day 5–6 biopsies, the miscarriage rate after PGD for Robertsonian translocation exclusion was 18%, whereas PGD for excluding unbalanced reciprocal translocations was followed by a miscarriage rate of 45%.

50.6 Monogenic Diseases

Monogenic diseases considered appropriate for PGD are those uncommon or rare, fatal, or chronically disabling familial conditions that occur as a result of mutations in a single gene. The location of the mutation can be in an exon, a splice point, or within the control regions and affects the functioning of the specific gene. Inheritance is Mendelian, and classically there are three major classes of phenotypic expression:

1. *Dominant inheritance*, where every individual who inherits the single gene change is likely to be affected by the disorder and will carry a 50% chance of passing on the affected gene to offspring. An example is Huntington's disease. PGD analysis for such mutations must be reliable in detecting a mutation change in a background of normal DNA sequence.
2. *Recessive inheritance*, where carriers of mutations themselves are not affected by the disorder but who partner with another carrier for a mutation in the same gene then produce a reproductive risk for their offspring of 25% for an affected child and 50% for a carrier child. An example is cystic fibrosis. Mutation analysis for these conditions needs to address the ability to analyze for a mutation in a homozygote state or often in a compound heterozygous state.
3. *X-linked inheritance* where, essentially, mutations on the X chromosome typically result in female carriers who have a 25% risk of producing affected male offspring and a 25% risk of reproducing the carrier state in female offspring. An example of a recessive X-linked gene disorder is hemophilia A. An example of an incompletely dominant X-linked disorder is fragile X syndrome, which causes severe mental retardation in males but

which also has a heterozygous female phenotype that includes premature ovarian failure. Analysis must be reliable but, unlike other recessive diseases or the dominant diseases, there is no normal background DNA sequence for males. Female carriers contribute a nonmutated X chromosome, so confidence with the analysis must be the same as for the autosome mutations.

The starting point for PCR in the case of a single cell from a day 3 biopsy is usually just a single copy of DNA (there is obviously more DNA available with multicellular trophectoderm biopsies). In principle—and regrettably sometimes also in practice—failure of the mutated DNA to amplify (ADO) produces a false-negative result, leading to an incorrect conclusion of a normal state. Any biopsy testing process must be as reliable as possible to avoid any miscalls.

50.7 The Near Future for Translocation Testing

50.7.1 STR-Based Molecular Strategies

Our experience, above, revealed no obvious advantage for blastocyst-stage biopsies compared with day 3 cleavage-stage biopsies when FISH is used to infer balanced chromosomal patterns for either reciprocal or Robertsonian translocations. In each case, miscarriage rates remain particularly high for apparently balanced reciprocal translations. We have since reported a molecular strategy utilizing PCR for PGD in translocation carriers that examines highly polymorphic short tandem repeat sequences (STRs), application of which has significantly improved outcomes after biopsies at the blastocyst stage [22].

Using STR profiling to identify chromosomal segments on either side of the known breakpoints, in conjunction with standard cytogenetic segregation tables to predict each unbalanced state, we directly identify the monoallelic and triallelic states that are the direct cause of the phenotypic abnormality and reproductive loss which results from these malsegregants and, in turn, is the immediate pathogenic mechanism behind the reason PGD is offered to translocation carriers. The method requires extensive screening of chromosome-specific STRs to define those markers for which the carrier is heterozygous and where alleles are not shared with the partner. To make this PCR-based test efficient, chosen markers are multiplexed to obtain results within primary or secondary amplifications. The method also lends itself to other PCR-based PGD objectives conducted simultaneously, such as monogenic disease exclusion. Verification of the method has come from the rebiopsy of embryos diagnosed as unbalanced: in each of six cases in which samples were assessed for segmental chromosomal gains and losses using conventional CGH (see below), the predicted malsegregations were confirmed.

Conclusive results in our hands rose to 99% using STR profiling, compared with 93% with blastocyst-based FISH.

Any apparent mosaicism seen in the trophectoderm sample has the potential to complicate the interpretation of the translocation state, especially when using FISH, where any visible abnormality tends to disqualify the embryo for transfer, on the subjective basis that failure of a chromosome to hybridize or to hybridize ambiguously is always possible. STR profiling, on the other hand, encompasses multiple loci on each side of the translocation point, reducing ADO-based errors (false monoallelic states from diploid alleles, false biallelic states for trisomic alleles).

■ Table 50.4 compares our FISH-based blastocyst biopsy experience with our STR-PCR experience. Patients with reciprocal translocations still show the expected predominantly unbalanced segregation patterns predicted by theory, but fewer embryos are falsely disqualified from transfer. Patients with Robertsonian translocations also fare better. Robertsonian translocation carriers can be prone to uniparental disomy, especially when chromosomes 14 and 15 are involved (see [22]); STR-PCR, unlike FISH, enables biparental inheritance to be looked for and to be confirmed or excluded. Finally, the time needed for actionable results with STR-PCR is just 4–5 h, compared with the 6–16 h required for FISH hybridization and interpretation.

50.8 The Near Future for Aneuploidy Screening

The majority of aneuploidies arise during female meiosis. The minority are brought to the embryonic genome by the fertilizing sperm and are equally pathogenic. A small number take origin in the first few cleavage divisions through mitotic nondisjunction. The latter lead to mosaic states in the embryo: clearly, the later this happens, the smaller the proportion of triploid cells and the more patchy the distribution among inner cell mass and trophectoderm derivatives. There is a large body of published knowledge on the recognized outcomes, such as confined placental mosaicism. In the embryo proper, trisomic cells will be at a disadvantage compared with their euploid neighbors as tissues and organs develop. Our experience with karyotyping 82 cell lines derived from inner cell masses of slow and stalled embryos, assumed to disproportionally display aneuploidies, provides an indication of this process (Bradley et al., manuscript under review). Sixty-nine (84%) displayed only a normal, diploid karyotype, indicating likely self-correction of mitotic nondisjunction-based mosaic states; a limited number tested showed no cases of loss of heterozygosity, which would indicate uniparental disomy as a consequence of self-correction of meiotic errors. The 13 cellular outgrowths that were cytogenetically abnormal included six single trisomies, a double trisomy, a monosomy, three triploidies, a triploidy with an additional chromosome 22, and a balanced reciprocal translocation. In each of the trisomies, meiotic nondisjunction was confirmed by demonstrating triallelic states for STRs on the affected chromosome. There were no mosaic cell lines.

Thus, for reasons of both relative numbers (mitotic trisomies are from the start mosaic states, whereas meiotic trisomies are pure) and, possibly, a qualitative difference between independent aneuploid states compared with diploid states, the key objective of screening for aneuploidy should be less to count chromosomes than it is to recognize dominant original parent of origin states for any of the 24 chromosomes.

50.9 Fluorescent In Situ Hybridization

Specific staining of embryo chromosomes with FISH has been the preferred method to identify the chromosome copy number in a fixed cell preparation. The probes bind to defined regions on the cell chromosomes immobilized on a standard microscope slide, usually at interphase. Generally, the probes are purified cloned regions of the specific chromosome, subtracted for repetitive sequences. These probes are labeled with a unique fluorophore, which can be visualized with fluorescence microscopy. The preparation and quality control of such material generally means that a commercial supply of the probes is the preferred choice for routine clinical use.

There are limitations to commercially available probe sets. The number of fluorophore colors falls far short of the minimum of 24 required. The fluor needs to be chemically active to attach to the DNA probe and also stable enough to remain attached during the hybridization process. Once hybridized, the color must be able to be visualized using, typically, UV excitation and filtered emission. High-energy wavelength excitation can result in rapid photo bleaching of the fluorophore and hence insufficient time to enumerate the hybridization pattern. The commercial suppliers have limited their probe labels to a very small set that meet manufacturing standards and the exacting requirements for clinical use. These fluors must be spectrally separable using specific but simple microscope filters. In practice, this means that only 5–7 or so chromosomes can be checked in one hybridization event. Consequently, the number of chromosomes that are there to be counted using FISH means that multiple cycles of hybridization, enumeration, probe stripping, and rehybridization are needed. Each cycle runs the risk of target loss and/or degraded target sites, either of which can result in incorrect chromosome enumeration and thus a misreading of chromosome number—technical considerations that preclude more than two or three rounds of hybridization. Temporally, adding more than a very few hybridizations would take too much time to permit the transfer of IVF embryos fresh.

Single blastomere biopsy from day 3 embryos gives a single, simple answer: a normal chromosome complement or an abnormal complement. The problems of mosaicism and technical difficulties discussed above, however, still lead to embryos being incorrectly classified and then being excluded from transfer. Biopsy at the blastocyst stage does not resolve these problems but does offer an opportunity to see multiple

hybridization signals for a set of cells. Nonetheless, a conservative reading of those signals has meant that observation of mosaic states in multicell biopsies has resulted in the exclusion of embryos that are likely to be substantially normal and suitable for transfer. The policy of disqualifying an embryo for transfer on the basis of one or two aberrant cells might need to be reexamined.

All other current karyotyping methods applicable to extremely low copy numbers of chromosomes, including analyses of single cells with day 3 embryo biopsies and of typically fewer than ten cells with blastocyst trophoctoderm biopsies, require preliminary amplification of DNA copy number.

The first way to satisfy this challenge is to greatly increase the number of chromosome targets to be amplified, enabling any genomic shortcomings in genome-wide amplifications to be overcome by averaging. Over the last few (very few) years, advances in whole genome amplification has advanced the place of the technique of *comparative genomic hybridization* (CGH) by increasing its resolving power within chromosomes as well as improving its quantitative reliability in estimating preamplification DNA copy number.

50.10 Comparative Genomic Hybridization

Developed as a chromosomal screen to analyze genomic changes in cancers almost 20 years ago [23], CGH reveals copy amounts of all 22 autosomes and the 2 sex chromosomes to a resolution of 10 million base pairs or so. The technique uses a combination of molecular and cytogenetic approaches to evaluate chromosome complements. Testing cancers with CGH is simpler than testing embryos, however, because generally with cancer samples there is no shortage of extracted DNA to be tested, whereas embryo biopsy specimens are much more limited.

Wells and Delhanty reported CGH analysis of individual cells from human day 3 embryos a decade ago [24]. Wilton and others reported the first successful clinical preimplantation use of CGH technique a year later [25]. While the use of CGH promised to deliver a total chromosome aneuploidy screen and the possibility of identifying any chromosome imbalance in an embryo, its labor intensity and its time-consuming nature (which required the embryos to be frozen while testing proceeded over periods of many days) precluded transfer of embryos during the biopsy cycle. There were only a few further reports over the ensuing 6 years [26–28]. Often, what was observed were relatively complex chromosome combinations; these then were given causal roles to explain implantation failure, but screening out aneuploid embryos did not improve embryo implantation rates. In spite of its promise, CGH has not been reported to be in routine use by any group. Recently, however, a report from Wells et al. has reported high implantation rates for blastocysts biopsied and analyzed with CGH after improved whole genome amplification [29]. The embryos

were transferred after vitrification and later thawing and produced an impressive thawed blastocyst implantation rate of 67%—which would warrant routine use, at least in selected patients.

The use of classical CGH on metaphase chromosomes demands high levels of skill, many days of analysis, and the freezing of biopsied embryos until the karyotype is known. One approach to minimizing labor requirements and shortening the testing time has been to employ DNA microarrays [30]. On the one hand, the timing suits polar body analyses and day 3 cleavage-stage biopsies, but both of these sample types offer only a single-cell genome for amplification and analysis, whereas blastocyst biopsy offers several cells to average out the amplification biases more effectively. On the other hand, the additional expense of CGH, however performed, is coming to be more generally appreciated as another reason for identifying embryos that can blastulate before biopsy and testing, in effect providing a self-screening process that reduces the costs of the testing for the individual patient. A pilot study looking at the analysis of polar bodies for aneuploid detection of female origin has been commenced by a consortium from the European Society of Human Reproduction and Embryology. Implantation rates and pregnancy outcome data are still to be collected. Recent advances combining blastocyst-stage biopsy, micro-array CGH, and vitrification have produced high embryo implantation rates and clinical pregnancy outcomes allowing a viable clinical service to be offered [31].

To date, therefore, it is blastocyst-stage biopsy that has given valuable improvement in implantation rates, and it waits to be seen whether the still prevalent day 3 biopsies and day 0–1 biopsies of polar bodies can achieve the same outcomes. With the use of a DNA amplification-based approach, the interpretation problems associated with low-level somatic mosaicism common in embryos and seen with FISH are partially overcome. The tissue sample, typically consisting of 3–5 cells, is analyzed as a whole (and is taken to represent the embryo as a whole), thus producing an averaging effect for the constitutional chromosomes under investigation.

Review Questions

1. Please define the rationale of blastocyst-stage embryo biopsy.
2. Please describe the required settings for blastocyst-stage embryo biopsy.
3. Please explain the procedure of blastocyst biopsy and the removal of trophoctoderm cells.
4. Please describe the general principles of single gene mutation testing (PGD or PGT-M) and the reasons for performing it.

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