Chapter 6
Embryology and PGD

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The Role of the Clinical Embryologist in PGD

Preimplantation genetic diagnosis (PGD) was first postulated as having potential for clinical application in 1965. However, the technical difficulties of sampling the preimplantation embryo, providing sufficient material and sufficient time for genetic analysis before embryo transfer, meant that successful realisation of this vision was not achieved for a further 22 years.

The pivotal role of the clinical embryologist in PGD is in minimising damage to embryos throughout the procedures that are necessary for the technique: in providing an environment for the routine fertilisation of oocytes and culture of resulting human preimplantation embryos; in the technical expertise enabling biopsy of embryos at whichever stage is deemed appropriate; in performing the biopsy procedure in an environment where DNA or adventitious agent contamination is minimised; in collecting biopsied cells using methods that minimise the risk of loss, contamination or mismatch

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T. El-Toukhy, P. Braude (eds.), *Preimplantation Genetic Diagnosis in Clinical Practice*, DOI 10.1007/978-1-4471-2948-6_6,
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between each embryo and its diagnosis; and in the successful cryopreservation of embryos, whether whilst awaiting results of the genetic diagnosis or for storage of unaffected embryos surplus to those transferred in a fresh cycle of treatment.

Fertilisation Methods

Where male fertility is not in doubt, and where genetic analysis of chromosomal disorders is to be undertaken using fluorescence in situ hybridisation (FISH), fertilisation may be achieved using conventional in vitro fertilisation (IVF), with insemination and overnight incubation of oocytes with prepared spermatozoa. In such cases, any residual cumulus cells that may remain adhered to and additional spermatozoa that may be bound to the *zona pellucida* (ZP, see Chap. 8) of the developing embryo will not impact upon the diagnostic test that is undertaken. However, if a molecular diagnostic technique is required, such as in the diagnosis of a single-gene disorder, additional steps must be taken to remove the risk of contamination with maternal or paternal DNA. Thus, the use of intracytoplasmic sperm injection (ICSI), where maternal cumulus cells are removed from the ZP before injection of a single spermatozoon to achieve fertilisation, is essential for all cases where PGD of single-gene disorders is to be undertaken; furthermore, any cumulus cells still adhering to the zona pellucida must be removed before biopsy.

Biopsy Procedures

Breaching the Zona Pellucida

Techniques for breaching the zona pellucida (ZP) were developed for the biopsy of blastomeres from cleavage stage embryos and include chemical drilling using acid Tyrode's or pronase solution, partial zona dissection (PZD) and laser ablation. Each has the potential to damage the embryo,

whether by exposure to the chemical agent used or through mechanical damage, and none has been shown unequivocally to be the method of choice. Indeed, no difference has been demonstrated between any of the techniques, in terms of the effect on implantation rates of manipulated embryos, when used for assisted hatching during cleavage. However, it has been recommended that mechanical, rather than chemical or laser, drilling should be used for polar body biopsy of oocytes because of the sensitivity of the meiotic spindle to damage, where care must be taken to minimise exposure of the embryo to reduced pH.

Adaptations of the original mechanical PZD method include the use of a piezo-micromanipulator, in which a piezoelectric pulse induces a vibratory motion in the dissecting needle; 3-dimensional PZD (3D-PZD), where the microneedle is used to make a cross-shaped breach in the ZP; and long zona dissection, where a long slit is made in the ZP using a modified holding pipette (LZD).

Whilst zona drilling using a laser is technically more straightforward than chemical techniques, and there are reports that laser drilling does not impair embryonic development to the blastocyst stage and implantation, its use is not without the potential to harm embryos.

Selection of a given method may be based on practical and economic considerations, including whether it is to be used for polar body biopsy, cleavage stage blastomere biopsy (Fig. 6.1) or trophectoderm biopsy of blastocyst stage embryos.

With all methods for zona breaching, the embryo is placed in a droplet of medium, usually buffered for use in air (HEPES or MOPS buffer) under oil on a warmed microscope stage. For cleavage stage biopsy, the medium used is free of Ca^{2+} and Mg^{+}, in order to reduce blastomere adhesion and facilitate removal of a single cell. The embryo is secured using a gentle vacuum applied to a holding pipette; once an opening has been generated in the ZP, cell(s) is aspirated through the breach in the ZP using the biopsy pipette (outer diameter 50 μm; inner diameter 35 μm).

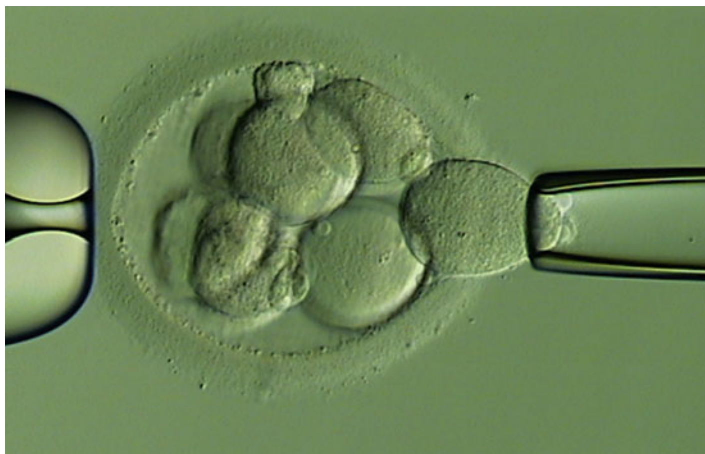

FIGURE 6.1 Stereophotomicrograph of a day 3 cleavage stage human embryo on a glass holding pipette, with a single blastomere being extracted through the hole breached in the zona pellucida. Note the intact nucleus in the cell being removed

The apparatus and micropipettes used for chemical drilling are shown in Figs. 6.1a and 6.2b. With this technique, a small hole is created in the ZP by controlled application of a stream of acid Tyrode's (or pronase) solution, expelled from the drilling pipette (inner diameter 5–6 μm).

For PZD, the embryo is secured with a holding pipette as with chemical drilling, and a slit is made in the ZP by rubbing a PZD pipette against the holding pipette with a sawing motion. The immobilised embryo may be squeezed with a biopsy pipette until a blastomere of a cleavage stage embryo is extruded, followed by aspiration with a biopsy pipette, or cell(s) may be aspirated as described for chemical drilling.

For laser ablation, the apparatus for which is shown in Fig. 6.3, the ZP of the secured embryo is breached, taking care with blastocyst stage embryos to orientate the embryo so that the breach is made in a region away from the inner cell mass (ICM), with aspirated trophectoderm cells excised using the laser.

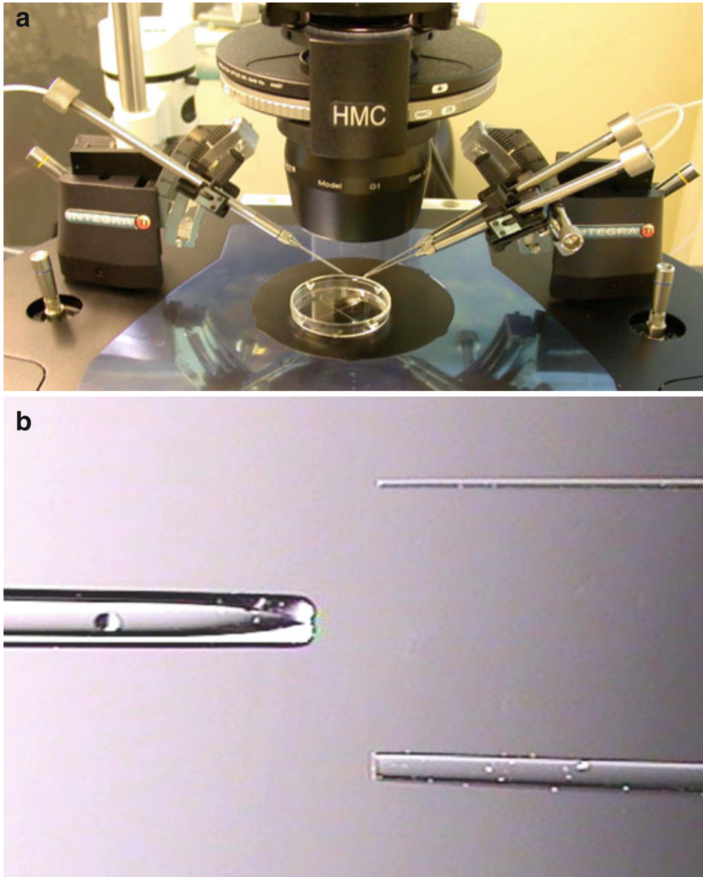

FIGURE 6.2 Micromanipulation apparatus for chemical zona drilling. (**a**) Micromanipulators attached to an inverted phase microscope, showing the attachments for the holding pipette (*left*) and drilling and biopsy pipettes (*right*). (**b**) Micropipettes used for holding (*left*), drilling (*upper right*) and biopsy (*lower right*)

Handling Biopsied Material

When required for processing for molecular analysis, precautions must be taken to prevent DNA contamination; when biopsied cells are to be processed for chromosome

analysis, such precautions may be less stringent. Biopsies should be carried out using sterile equipment in a minimum Class I flow hood, which provides material with protection from airborne contamination throughout the procedure, and the practitioner processing the biopsied cells should wear protective clothing (hairnet, facemask, theatre gown and sterile gloves) that must be changed if any contact is made with potential sources of contamination.

Biopsied cells must be rinsed thoroughly to ensure removal of all potential contaminants, but with care to avoid causing cell lysis, which may compromise the chance of yielding results. In practice, this entails rinsing in at least 3 sequential drops of wash buffer, consisting of a simple solution such as phosphate-buffered saline (PBS) supplemented with polyvinylpyrrolidone (PVP) to prevent adhesion of biopsied cells to the pipette and dish. The pipette is rinsed in fresh wash buffer between drops, before transfer of the cells into microcentrifuge tubes containing PBS, in no more than 1–2 μl wash buffer to avoid dilution of the reagents used for lysis and DNA amplification. Micropipettes used for rinsing and transfer of biopsied cells should be changed between samples to minimise cross-contamination. A sample of the stock of wash buffer used for each series of biopsy procedures should be taken for the preparation of a negative control.

Embryo Development

Biopsied embryos are rinsed through fresh drops of warmed culture medium before being returned to culture. This is particularly important when chemical drilling has been used and for cleavage stage biopsies where Ca^{2+}- and Mg^{+}-free medium will have been used, in order to remove traces of the chemical agent, biopsy medium and HEPES or MOPS buffer from the culture medium.

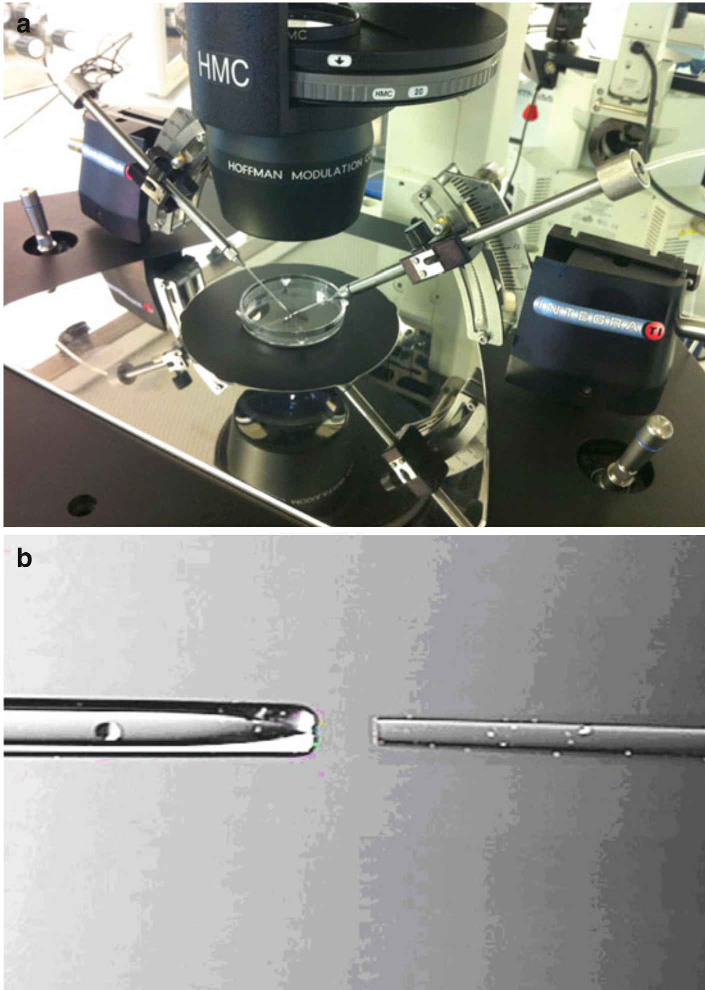

FIGURE 6.3 Micromanipulation apparatus for laser ablation. (**a**) Micromanipulators attached to an inverted phase microscope, showing the attachments for the holding pipette (*left*) and biopsy pipette (*right*). (**b**) Micropipettes used for holding (*left*) and biopsy (*right*)

Cryopreservation of Biopsied Embryos

Successful cryopreservation is an essential component of any PGD service, for several reasons. First, it may be necessary, on occasion, for biopsied embryos to be cryopreserved whilst allowing sufficient time for the results of genetic diagnosis of the biopsied material to become available; second, embryos found to be free of the genetic disorder under investigation, surplus to the fresh embryo(s) transferred in the initial treatment cycle and that appear morphologically to be developing normally, should be cryopreserved in order to increase the chance, and the number, of successful pregnancies following a single IVF/ICSI cycle; third, cryopreservation increases the cost-effectiveness of a single cycle of IVF/ICSI and PGD, both in terms of financial and emotional investment; and, finally, successful cryopreservation supports the incentive to transfer a single unaffected embryo in each cycle of treatment, in order to reduce the risk of multiple pregnancy.

Biopsied embryos may be cryopreserved using either long-established slow-freezing techniques or the more recently adopted technique of vitrification. It has been reported that implantation and successful pregnancy following transfer of slow-frozen and thawed embryos following blastomere biopsy at the cleavage stage is lower than following the same procedures carried out with intact embryos, possibly because of increased vulnerability to the toxic effects of the cryoprotectant reagents used in slow freezing when the zona pellucida has been breached. In contrast, vitrification and warming of both biopsied and intact embryos have been reported to yield equivalent survival, implantation and pregnancy rates. For this reason, vitrification may be the preferred method for cryopreservation following embryo biopsy.

With advances in the understanding of the culture requirements of human embryos, and the availability of media that support the development of embryos to the blastocyst stage in vitro, successful cryopreservation of embryos

surplus to those transferred fresh may be carried out routinely on days 5 and 6 of development, whether biopsy takes place during cleavage or at the blastocyst stage, once the results of genetic diagnosis are available. An alternative approach that allows genetic analysis to be undertaken with less time constraints is to vitrify all biopsied blastocysts immediately post-biopsy whilst awaiting the results of diagnosis and discarding those found to be affected once the results are known.

Documentation and Safety

It is essential that the result of genetic diagnosis specific for each embryo is reliable and specific to that embryo, and rigorous measures must be implemented to remove any risk of ambiguity. Embryos must be cultured after biopsy using a method that will ensure their accurate identification; culture in separate dishes, or culture in dishes with moulded, numbered wells that ensure embryo identification and segregation is mandatory. The cells biopsied from each embryo must be processed using corresponding labelling between cells and embryo, with clear documentation and witnessed processing steps to ensure that the genetic diagnosis for each embryo is matched unambiguously to the biopsied material from which the diagnosis is made.

Key Points

- The success of any PGD programme relies on there being a successful embryology laboratory for assisted conception.
- The method for breaching the zona pellucida may be selected according to practical and financial considerations.

- Rigorous measures must be implemented to eliminate all possible sources of contamination of biopsied material, including the use of ICSI for molecular diagnostic cases.
- Rigorous measures must be implemented to ensure segregation and accurate identification of individual embryos and the cells biopsied from them.
- A successful cryopreservation programme is essential to provide the maximum possible success rate for a PGD service.

Further Reading

Harton GL, Magli MC, Lundin K, Montag M, Lemmen J, Harper JC, et al. ESHRE PGD Consortium/Embryology Special Interest Group: best practice guidelines for polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS). Hum Reprod. 2011;26:41–6.