PREIMPLANTATION GENETIC DIAGNOSIS

Peter Braude, Susan Pickering, Frances Flinter and Caroline Mackie Ogilvie

Preimplantation genetic diagnosis (PGD) is an evolving technique that provides a practical alternative to prenatal diagnosis and termination of pregnancy for couples who are at substantial risk of transmitting a serious genetic disorder to their offspring. Samples for genetic testing are obtained from oocytes or cleaving embryos after *in vitro* fertilization. Only embryos that are shown to be free of the genetic disorders are made available for replacement in the uterus, in the hope of establishing a pregnancy. PGD has provided unique insights into aspects of reproductive genetics and early human development, but has also raised important new ethical issues about assisted human reproduction.

REPRODUCTIVE RISK
The risk of establishing a pregnancy in which a fetus miscarries or has a phenotypic abnormality as a consequence of the familial genetic condition.

ANEUPLOIDY
The presence of extra copies, or fewer copies, of some chromosomes.

BLASTOCYST
A preimplantation embryo that contains a fluid-filled cavity called a blastocoel.

Centre for Preimplantation Genetic Diagnosis, Thomas Guy House, Guy's Hospital, London SE1 9RT, UK.
Correspondence to P.B.
e-mail: obgyn@kcl.ac.uk
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Preimplantation genetic diagnosis (PGD) is a clinical diagnostic procedure that has evolved from the substantial advances in assisted reproductive technology that have occurred since the first birth resulting from *in vitro* fertilisation (IVF) nearly 25 years ago. PGD was originally developed as an alternative to prenatal diagnosis to reduce the transmission of severe genetic disease for fertile couples with a REPRODUCTIVE RISK[1]. In PGD, cellular material from oocytes or early human embryos that have been cultured *in vitro* (FIG. 1) is tested for a specific genetic abnormality. After diagnosis, only the unaffected embryos are selected for transfer to the uterus. In contrast to this specific and limited application, the same technology has recently been used more frequently to improve IVF success for infertile couples by screening embryos for common or age-related ANEUPLOIDIES (aneuploidy screening, PGD-AS).

The first successful clinical application of PGD for genetic disease involved the use of PCR to amplify a specific repeat on the Y chromosome to sex embryos in the presence of X-linked genetic conditions[1] — in this case, adrenoleukodystrophy (ALD) and X-linked mental retardation. Substantial groundwork for the clinical application of PGD to various conditions (see below for further discussion) was undertaken in the late 1980s and, in 1992, the first live birth was reported following PGD for cystic fibrosis (CF)[2].

Preimplantation testing of embryos is not new[3]. In 1968, Gardner and Edwards were able to sex rabbit embryos using a sex-specific chromatin pattern in BLASTOCYST biopsies, before their transfer to the uterus[4]. Preimplantation testing of embryos is also used routinely in animal husbandry to produce animals of the preferred sex[5]. However, the clinical application of this type of technology, in an attempt to prevent transmission of genetic disease in humans, is still evolving. Measuring cytoplasmic enzyme activity in individual embryonic cells was first investigated, as a method of PGD, for clinical conditions characterized by an absence or a reduction of specific enzyme activity. Among such conditions are severe combined immunodeficiency disorder[6,7] (SCID; adenosine deaminase deficiency), Lesch–Nyhan syndrome (LNS; hypoxanthinephosphoribosyl transferase deficiency) and Tay–Sachs disease[8] (TSD; hexosaminidase deficiency). However, this method turned out to be of limited use when it became clear that it was difficult to distinguish maternally inherited enzyme activity that was present in the oocyte, from the embryo's own enzyme activity. In the mid 1980s, the advent of PCR provided a far superior method for genetic testing, making it possible to carry out a diagnostic test on highly concentrated and relatively pure amplified PCR fragments that spanned the appropriate genetic mutation[9]. The ability to extract DNA and genetically characterize single sperm and diploid cells

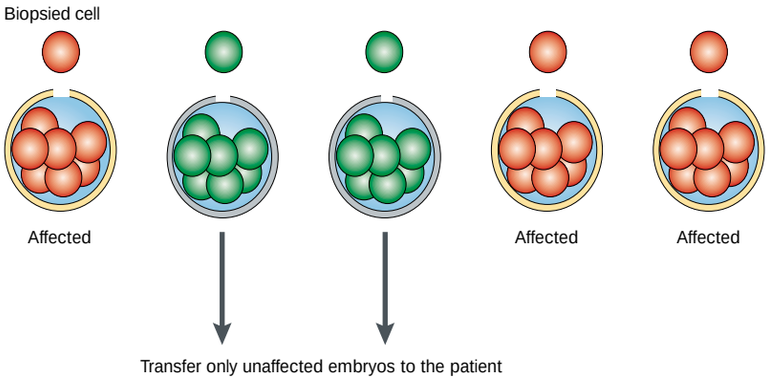

Figure 1 | **Principle of preimplantation genetic diagnosis.** A single cell (or cells) is removed from each embryo of an *in vitro*-developing cohort, on which a diagnostic genetic test is carried out. Up to three of the embryos that are unaffected are transferred to the patient in the hope of establishing a pregnancy.

AMNIOCENTESIS
A procedure in which a small sample of amniotic fluid is drawn out of the uterus through a needle that is inserted into the abdomen. The fluid is then analysed to detect genetic abnormalities in the fetus or to determine the sex of the fetus.

CHORIONIC VILLUS SAMPLING
(CUS). Sampling of the placental tissue of the conceptus for laboratory analysis.

TRIMESTER
One of the ~12-week stages into which pregnancy is divided for clinical purposes.

FLUORESCENCE ACTIVATED CELL SORTING
(FACS). A method whereby dissociated and individual living cells are sorted, in a liquid stream, according to the intensity of fluorescence that they emit as they pass through a laser beam.

provided a powerful impetus to pursue this technology clinically[10]. In addition, *in situ* hybridization techniques, based either on autoradiography[11] or on fluorescent markers[12], facilitated PGD from single interphase nuclei.

This review describes the current status of PGD. We have included relevant technical aspects to facilitate an understanding of both the clinical and laboratory practice of PGD. We also discuss aneuploidy screening and the ethical dilemmas that might arise from the current practice of PGD, and anticipate some future developments in clinical practice and technology.

Reproductive risk and options

Couples in which both partners carry the same autosomal-recessive gene disorder, in which the female carries an X-linked disorder or in which one partner carries a balanced chromosome rearrangement have a reproductive risk (BOX 1). Embryos that are affected by a single-gene disorder are often viable, but the children might subsequently suffer from significant physical abnormalities or developmental delay. Those who inherit unbalanced chromosome rearrangements might be similarly affected or miscarried. In the absence of a strong family history, such couples might not be aware of their risk until they are investigated after obstetric problems or the birth of an affected child. Couples in which one partner carries a dominant mutation will usually be aware of their risk, and might wish to avoid transmitting the disease to their offspring.

PGD can be applied to three groups of genetic disorder. The first category encompasses single-gene disorders. These can be either autosomal dominant, autosomal recessive or X-linked recessive (BOX 1) — in which the specific mutation that is associated with the disease is known and can be amplified using PCR, or in which embryos that are likely to be unaffected can be identified using genetic linkage. The second category includes X-linked disorders in which the specific gene defect might not be known or where there is considerable genetic heterogeneity (for example, Duchenne muscular dystrophy; DMD), but the disorder can be avoided by sex selection. Chromosomal rearrangements, such as reciprocal or Robertsonian translocations fall into the third category (see below for further discussion).

Before the development of PGD, limited options were available to couples with a reproductive risk. Fertile couples, or infertile couples who are following assisted conception treatment (such as IVF), might opt for some form of prenatal diagnosis of their condition once a pregnancy is established, either by AMNIOCENTESIS or CHORIONIC VILLUS SAMPLING (CVS) with the option of terminating an affected pregnancy. This decision is not taken lightly, as termination, especially late in the second TRIMESTER, can have substantial psychological and even physical morbidity. Some couples will not contemplate termination because of religious or personal principles, whereas others, after a succession of terminations, might feel unable to accept further abnormal pregnancies.

Other couples might consider the use of gametes from a donor who is not a carrier of the disorder. In most countries, sperm donation is more easily available than egg donation owing to the difficulty in recruiting egg donors and to the rigours of ovarian stimulation and egg retrieval (see below for details). For X-linked disorders, there is the possibility of sorting spermatozoa before insemination or *in vitro* fertilization. Although several methods have been reported[13], only FLUORESCENCE ACTIVATED CELL SORTING (FACS) produces a significant enrichment of the desired type of spermatozoa[14]. If donor gametes are unavailable or unacceptable for moral or religious reasons, adoption might be an alternative. Other couples opt to remain childless rather than risk having an affected child, or passing on their genetic condition or carrier status.

Preimplantation diagnosis provides an alternative way forward, not only for couples who have such reproductive risks, but also for couples who are unable to establish a viable pregnancy because of miscarriage caused by chromosome rearrangements. It is essential that any couple contemplating PGD receives genetic counselling to ensure that they have a good understanding of the nature of the genetic disorder that could affect their child, and of the implications of its pattern of inheritance. They should also be fully

Box 1 | Genetic risk

Autosomal-dominant disorders, such as Huntington disease, Marfan syndrome and myotonic dystrophy affect anyone who inherits one copy of the mutant allele. Phenotypes of such disorders can be quite variable, and any child of an affected individual has ~50% chance of inheriting the condition. This figure is important in calculating the overall chance of success during a preimplantation genetic diagnosis (PGD) cycle, as 50% of embryos that are successfully fertilized and tested will not be suitable for implantation. So, the odds of achieving a successful pregnancy are lower than for couples who undergo PGD for an autosomal-recessive condition. Autosomal-recessive disorders, such as cystic fibrosis, spinal muscular atrophy and sickle cell disease only affect individuals who inherit two mutant alleles, one from each parent. Carriers (heterozygotes), who have just one copy of the altered gene, are usually asymptomatic, but if both parents are carriers, then each pregnancy has a 25% chance of being homozygous and is therefore affected. Three out of four tested embryos, on average, should be suitable for implantation. X-linked recessive disorders affect males who inherit a mutant allele on their single X chromosome, whereas female carriers, who have two X chromosomes, are usually phenotypically normal.

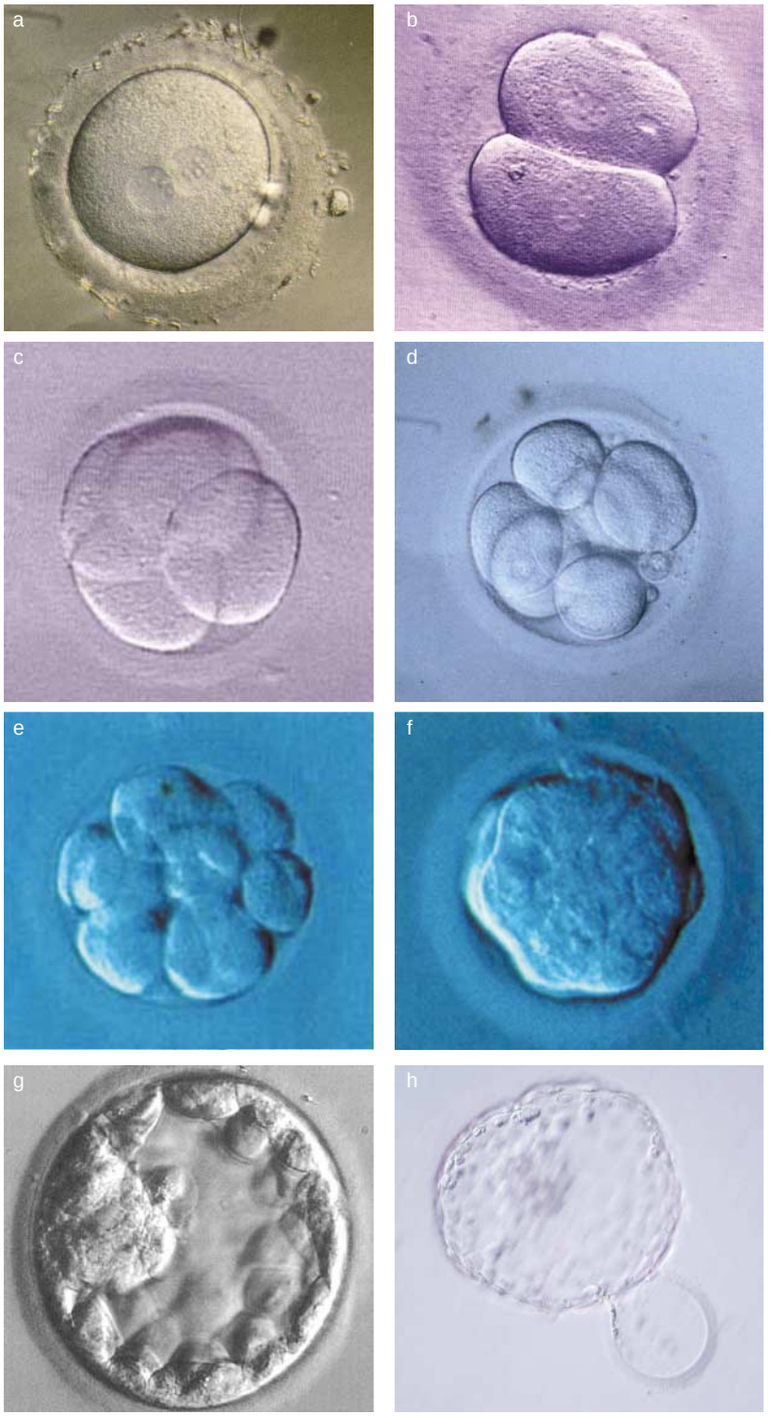

Figure 2 | **Early human preimplantation development *in vitro*. a** | The first cleavage takes place ~22–30 hours post-fertilization (a post-fertilization, pronuclear stage embryo is shown), and the embryo divides at ~18 hour intervals thereafter. **b–d** | Individual BLASTOMERES can be seen clearly until the 8-cell stage, at which point they begin to flatten on each other in a process known as 'compaction'. **e** | Tight junctions form around the 16–32-cell stage of development, and the embryo becomes a tight ball of cells known as a 'morula' (**f**). **g** | The embryo continues to divide, now more quickly, and fluid begins to accumulate inside the embryo forming the blastocoelic cavity, which subsequently expands, giving rise to an expanded blastocyst. **h** | At about six days of development, the blastocyst hatches from the zona pellucida to begin implantation. The blastocyst is composed of two different cellular types, the outer trophectoderm, which is destined to give rise to extra-embryonic tissues, and a small compact ball of cells on the inside, the inner cell mass, which protrudes into the blastocoel and will give rise to the fetus[114].

informed about all alternative reproductive options. Couples in which one partner carries a lethal or debilitating progressive genetic disorder, such as Huntington disease (HD), need to consider the welfare and arrangements for the care of any child who is born following PGD. This is a statutory requirement of the UK Human Fertilisation and Embryology Authority (HFEA), which issues licences for all new conditions that are considered for PGD[15]. However, as there is still a possibility of misdiagnosis using PGD (discussed in more detail below), couples should be encouraged to consider prenatal diagnosis to confirm preimplantation diagnosis. Couples who choose PGD need to be highly motivated, as the process is complicated, expensive and, in some cases, associated with a lower chance of having a healthy baby than conceiving conventionally (discussed in more detail below).

Clinical procedures and embryology

Stimulation and oocyte retrieval. The increasingly sophisticated technology available in the assisted reproduction clinic is harnessed to provide oocytes or embryos for genetic testing in PGD. Controlled stimulation of the ovaries with exogenous GONADOTROPHINS leads to the recruitment of many FOLLICLES, and the process can be monitored by pelvic ULTRASONOGRAPHY[16]. When the number and size of the developing follicles is deemed appropriate, oocyte maturation is hormonally triggered. Between 34 and 38 hours later, the oocytes are collected by transvaginal ultrasound-guided aspiration of the follicular fluid. The oocytes are transferred to suitable culture medium and are either inseminated and left overnight to fertilize (IVF), or fertilized by intracytoplasmic sperm injection (ICSI), whereby single spermatozoa are injected directly into mature oocytes[17] (see ICSI in Online links box). ICSI is required for patients with reduced sperm quality (low numbers, poor motility or abnormal sperm morphology) or where IVF is not likely to occur successfully, for example, because of previous poor success in fertilization. ICSI is also recommended in all cases in which PCR is required for PGD, as the presence of supernumerary sperm, buried in the ZONA PELLUCIDA after IVF, might lead to a contamination of PCR reactions with paternal DNA and, therefore, to a possible misdiagnosis. In some PGD centres, ICSI is used routinely to avoid the unexpected failure of fertilization[18,19]. The day after oocyte retrieval, embryos are examined for the presence of two PRONUCLEI that indicate normal fertilization (FIG. 2a). These embryos are separated from the failed or abnormally fertilized oocytes and are returned to culture for further development. A biopsy sample for genetic testing can then be obtained at various stages of development.

Polar body biopsy. A mature oocyte is characterized by the presence of a first POLAR BODY that contains a complement of 23 BIVALENT maternal chromosomes. This discrete structure can be removed and used for genetic testing or for aneuploidy screening of the oocyte before fertilization[20,21]. On fertilization, a second polar

GONADOTROPHINS
Hormones that are produced by the pituitary gland, which act on the gonads to control endocrine functions. Examples include follicle stimulating hormone and luteinizing hormone.

FOLLICLES
Structures in the ovary in which primary oocytes develop into mature oocytes before ovulation.

ULTRASONOGRAPHY
A technique in which sound waves are bounced off tissues and the echoes are converted into a picture (a sonogram).

ZONA PELLUCIDA
The glycoprotein coat that surrounds the oocytes and the early embryos of mammals.

PRONUCLEUS
The haploid nucleus of an egg or sperm.

BLASTOMERE
A cell that results from embryonic cleavage.

POLAR BODY
A small haploid cell that is produced during oogenesis and that does not develop into a functional ovum.

BIVALENT
A chromosome that has undergone replication. The two identical sister chromatids remain joined at the centromere.

PREDIVISION OF CHROMATIDS
The abnormal separation of chromatids during meiosis I (normally, sister chromatids separate during meiosis II) usually gives rise to gametes with a genetic imbalance.

TIGHT JUNCTION
A connection between individual cells in an epithelium that forms a diffusion barrier between the two surfaces of an epithelium.

TOTIPOTENTIALITY
The capacity of an undifferentiated cell to develop into any type of cell.

TROPHECTODERM
The outer layer of the blastocyst-stage embryo.

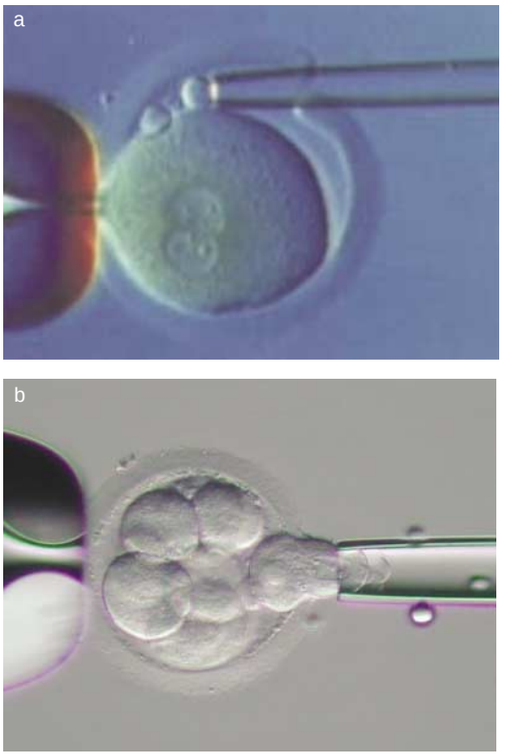

Figure 3 | **Polar body and cleavage stage biopsies.** **a** | Polar body biopsy. Around 14–20 hours after normal fertilization, the zona pellucida of the zygote is breached by partial zona dissection using a microneedle and then a small aspiration capillary is introduced under the zona and the first and second polar bodies removed by gentle suction (reproduced with permission from Reproductive Genetics Institute, Chicago). **b** | Cleavage stage biopsy. Cleavage stage embryos are taken ~72 hours post-fertilization and held stationary on a glass micropipette by gentle suction. The zona pellucida is breached either by laser beam or by a jet of acidified Tyrodes solution. A sampling pipette is introduced into the embryo and a single nucleated blastomere is removed by suction.

body, containing a complement of 23 maternal chromatids, is extruded from the oocyte and can also be tested to provide further confirmation (FIG. 3a). Polar body biopsy has the advantage that it samples extra-embryonic material and is therefore less likely to affect detrimentally subsequent embryonic development, and it might be considered ethically preferable by some. However, as it can only provide information about the maternal genotype, it cannot be used in cases of paternally derived disorders. In addition, where PREDIVISION OF CHROMATIDS or undetected recombination between markers has taken place, a reliable diagnosis might not always be possible[22,23].

Cleavage stage biopsy. Individual cells of the cleaving embryo are distinct and discernible until around the 8–16-cell stage (day 3) when the embryo begins to undergo the process of compaction (FIG. 2f). From the 16-cell stage, TIGHT JUNCTIONS begin to form[24] and cellular apposition becomes too great to separate individual cells. Biopsy at the two- or four-cell stage (FIG. 2b,c) involves removal of a large proportion of the cellular mass of the embryo, with detrimental effects on further developmental potential[25,26]. However, at the 8–12-cell stage (FIG. 2d,e), 3 days after oocyte retrieval, blastomeres retain TOTIPOTENTIALITY, and the embryo can be biopsied successfully even when compact[27]. Biopsy at this stage is, at present, the preferred option for many PGD centres. The biopsy of compact embryos is facilitated by a short pre-incubation in calcium- and magnesium- free medium, which reduces cellular apposition (FIG. 3b).

The decision as to whether one or two cells should be removed is controversial. Removing two cells reduces the cellular mass of the embryo and, therefore, might reduce its developmental capacity. The accuracy of the diagnosis, however, is likely to be enhanced if embryos are replaced only when the results from both cells are concordant[28,29]. As the likelihood of pregnancy is, in part, dependent on the quality and number of embryos replaced, discordant results between cells could also reduce the number of embryos deemed suitable for replacement[28], and might decrease pregnancy rates. After genetic diagnosis, suitable embryos are usually transferred to the uterus on day four or day five (the blastocyst stage) of development[30]. The efficacy and safety of cleavage stage biopsy were first shown in studies using mouse embryos[25], and this technique has since been used in many clinical procedures around the world[19,27].

Blastocyst biopsy. A major problem with polar body and/or cleavage stage biopsy is the paucity of material that is available, which might lead to an inaccurate and unreliable genetic diagnosis (see below for further discussion). Biopsy of the embryo at the blastocyst stage obviates many of these problems as the embryo can contain up to 300 cells — depending on the exact stage of development — so more cells can be removed without apparent detrimental effect (FIG. 2g,h). In addition, because blastocyst biopsy involves the preferential removal of the more accessible TROPHECTODERM cells, the inner cell mass that is destined to become the fetus proper is unlikely to be damaged[31], thereby reducing possible ethical concerns. Blastocyst biopsy normally takes place on day five or six after fertilization and involves making a hole in the zona pellucida before the removal of cells. The cells are biopsied either by gentle teasing using needles or by induced herniation of a trophectodermal vesicle[31], which can then be separated by physical means using needles or by a laser[32]. Blastocyst biopsy has been used successfully in mice, with a high survival rate of embryos, and live pups after re-implantation[33]. So far, it has not been extensively used in humans because of the difficulty in culturing embryos to the blastocyst stage. However, the development of sequential media that have been specifically designed for the long-term culture of embryos[34], and the recent report of a human live birth after blastocyst biopsy[35] might encourage the increased use of this promising technique.

Cryopreservation after biopsy. Cryopreservation of surplus embryos with good morphology and that have regular cleavage is now routine in IVF/ICSI procedures[36]. However, cryopreservation of surplus embryos after biopsy is more difficult as the zona pellucida has been breached. Usual protocols for cryopreservation were applied to embryos at the pronucleate, cleavage or blastocyst stages of development and depend on the slow diffusion of the cryoprotectant through an intact zona. These protocols might be suboptimal when used on biopsied embryos, and the initial attempts at cryopreservation after biopsy resulted in a reduced survival rate compared with non-biopsied embryos at the same stage of development[37]. Recently, however, pregnancies have been reported after polar body biopsy[38] and after the cryopreservation of cleavage-stage biopsied embryos, which had been frozen using a modified protocol[39]. In addition, Lalic *et al.* have reported excellent survival rates of biopsied cleavage-stage embryos that were allowed to develop to the blastocyst stage before freezing and an impressive implantation rate of 25% after embryo transfer[40]. These reports indicate that it will soon be possible to cryopreserve surplus biopsied embryos routinely after genetic diagnosis.

Applications of PGD

Single-gene disorders. Many genetic disorders can now be diagnosed using DNA from single cells[19,41,42]. However, having only one or two cells for analysis imposes several limitations on the genetic diagnosis. There are also severe time constraints, as results must be available within 12–48 hours of embryo biopsy to allow the transfer of suitable embryos at an appropriate preimplantation stage. So, there is great emphasis on the development of increasingly rapid, but robust, diagnostic assays that are effective at the level of a single cell. This situation is in complete contrast with that often experienced in prenatal diagnosis (PND), in which relatively large quantities of pure genomic DNA can be extracted from biopsied tissue samples, which are made up of many hundreds of cells — or primary cell cultures derived from them. This DNA can be used directly for a diagnosis that, if confirmation is required, can be repeated several times over a period of several days.

As it is not possible to detect directly the presence of a specific mutation in the DNA from a single cell, all single-gene PGD assays that have been developed so far rely on PCR to amplify the relevant DNA sequence from the biopsy sample[2]. The sample is lysed to release the nuclear DNA solution and the region of interest is amplified using specific primers in ~35–60 cycles of PCR. Amplified fragments can then be analysed according to the requirements of the assay. Several techniques have been used, including restriction digestion, sequencing and analysis of FRAGMENT LENGTH POLYMORPHISMS[41].

Single-cell PCR was first applied clinically in the pioneering work of Handyside and colleagues, to sex embryos that were at risk of X-linked disease[1]. The basis of the test was the successful amplification of a Y-chromosome-specific repeat sequence in blastomeres from male embryos only[43]. Despite this early clinical success, several problems with the technique were encountered subsequently. First, stringent precautions — such as using gown, mask, gloves, filtered air, separate specific laboratory and equipment — were required to avoid contamination with extraneous DNA of non-embryonic origin[44], amplification of which could lead to misdiagnosis. Second, it soon became clear that in some cases, the target sequence failed to amplify although it was shown by other methods that it was present in the sample. Indeed, failure of amplification of the Y-chromosome-specific sequence in a male embryo led to the first report of PGD misdiagnosis[45,46].

Modifications were introduced in an attempt to improve the technique, including the development of a two-step NESTED PCR procedure that considerably improved sensitivity and specificity[47]. However, results in several independent laboratories indicated that, despite rigorous optimization of the procedure, sequences still occasionally failed to amplify when a single or a few cells were used as a source of DNA. Also, often only one allele at a heterozygous locus would amplify successfully, leading to the false assumption that the sample was homozygous[44,48,49]. So, for single-gene disorders, depending on which particular allele failed to amplify, heterozygous embryos could potentially be genotyped as either homozygous affected, in which case they were lost from the cohort of available embryos, or as homozygous normal and, therefore, as suitable for replacement[48,49]. Although this approach is acceptable in autosomal-recessive disorders, in which there is no abnormal phenotype in heterozygous carriers, in autosomal-dominant disorders or in embryo sexing, the problems of undetected ALLELE DROP-OUT (ADO) or amplification failure make the misdiagnosis too likely for the single locus diagnosis to be acceptable in routine clinical use.

To overcome these problems, Findlay and colleagues applied the relatively new technique of fluorescent PCR to single-cell genetic analysis. PCR amplification with fluorescently tagged primers was shown to be highly sensitive (~1,000-fold more sensitive than in the conventional analysis systems), reliable and accurate, and fewer PCR cycles were required, thereby reducing the time taken to reach diagnosis[50,51]. In addition, if different fluorescent tags are used, or different sized amplicons are designed, several different sequences can be amplified simultaneously from an individual cell (multiplex PCR). The simultaneous amplification of two or more fragments, one containing the mutation that is associated with the disorder and one or more containing polymorphic markers that are closely linked to that mutation, identifies which parental allele the embryo has inherited and indicates cases where ADO is likely to have taken place[41,48,52]. This approach substantially decreases the possibility of misdiagnosis[28] and provides the added assurance of a partial 'fingerprint' of the embryo, confirming that the amplified fragment is of embryonic origin[44,53]; in addition, because only a single round of PCR is required, the overall time taken by the procedure is substantially reduced. Multiplex PCR also affords the opportunity to develop a generic diagnostic

FRAGMENT LENGTH POLYMORPHISMS
The individual variation in the length of a particular region of DNA (such as a dinucleotide repeat), which, if the DNA is cut with a restriction enzyme or amplified using PCR, gives rise to the generation of differently sized fragments.

NESTED PCR
A technique for improving the sensitivity and specificity of PCR by the sequential use of two sets of oligonucleotide primers in two rounds of PCR. The second pair (known as 'nested primers') are located in the segment of DNA that is amplified by the first pair.

ALLELE DROP-OUT
(ADO). The failure to detect an allele in a sample or the failure to amplify an allele during PCR.

strategy for a particular disease, which is independent of the mutation present. Linkage analysis of polymorphic markers that are closely linked to the disease locus allows the identification of embryos at high risk in a broad range of patients who might be carrying different mutations in the same gene[54].

Pregnancy rates after PGD for single-gene disorders vary with the type of disorder and its pattern of inheritance. The cumulative data from the 1,197 cycles received by the ESHRE PGD consortium during 1999–2001 data collection for all forms of embryo biopsy for genetic diagnosis (excluding screening and social sexing) showed an overall clinical pregnancy rate of 22.4% per embryo transfer (17.3% per oocyte retrieval procedure undertaken)[19]. Biopsy was successful in 97% of cases, and the diagnosis was obtained in 86% of successfully biopsied blastomeres[19]. For single-gene disorders, 575 cycles resulted in 119 pregnancies (21% per egg retrieval and 25% per embryo transfer procedure). Five misdiagnoses using PCR were reported, two of which were for embryo sexing using PCR — a method that is now considered obsolete.

Box 2 | 'Designer babies' and the ethics of PGD

The ability to select an embryo after genetic testing sometimes raises accusations of choosing a child to order, as a commodity that has been designed simply to meet the needs and desires of the parents. This view ignores the fact that most couples make the difficult choice of undergoing preimplantation genetic diagnosis (PGD) as their only hope of a viable pregnancy and of having a healthy child. Real ethical dilemmas arise in a small number of unusual cases[94,95]. For example, a couple both of whom have the dominant condition of achondroplasia might request PGD to avoid the homozygous affected embryos, which are generally lethal *in utero*, but wish to select only heterozygous embryos (which would give rise to children with achondroplasia), rather than unaffected embryos, to fit in with their lifestyle. Consideration of a case such as this must give paramount importance to the welfare of the child, but this is not always easy. Despite the medical problems that are associated with this condition, would an unaffected child in an achondroplastic family suffer more than an affected child in such an environment? A similar dilemma might occur in inherited deafness where a non-hearing child might be preferred. In a recent high-profile case, a non-hearing child was deliberately conceived using donor insemination by a male with a substantial genetic history of deafness, to be deaf like its lesbian parents (REF. 96).

In other genetic conditions, a couple might request not to replace carrier embryos to try and eliminate the disease from their family. For example, a couple in which the male partner suffers from haemophilia requested the selection and transfer of male embryos only, to avoid fathering carrier daughters[97]. In this case, there was no risk of a serious genetic disease in the following generation, only in his grandchildren. Some might view this as a departure from the purpose of PGD — to prevent the birth of an affected child — and as a move towards positive eugenics.

The sex-linked condition incontinentia pigmenti (IP) is also problematic. IP is lethal to affected male fetuses, which inevitably miscarry. The PENETRANCE in female carriers is variable, and daughters might have a far more severe phenotype than their mothers. A possible strategy for PGD in this case might therefore involve the selection and transfer of male embryos only, as those that inherit the X chromosome with the IP mutation will not survive, and all the survivors will be free of the disease[98]. However, some might feel that deliberately transferring embryos with a 50% risk of carrying the mutation is not an appropriate use of PGD.

The ability to sex embryos using preimplantation embryo biopsy and FISH is another area of PGD that fuels substantial debate and controversy[19,99–101]. When there is one child or more of one sex in a family, the wish for a child of the other sex (referred to as 'family balancing') has been viewed sympathetically by some in the United States[102,103] but remains controversial[104], and many consider that this is not a legitimate use of PGD[105]. Particular concerns arise when selection in a population is predominantly for one sex, where only one child is allowed, or where male offspring are favoured over female offspring for cultural and economic reasons. PGD for male embryo selection is practised in some Middle Eastern and Asian countries, as an alternative to prenatal diagnosis and abortion on gender grounds alone[101,106,107]. In the United Kingdom, sex selection of embryos for non-medical reasons using PGD is forbidden by the HFEA. However a new public consultation exercise is in progress, which seeks views on this issue to see if the code of practice should be changed. PGD was taken into a new dimension by the highly publicised case of Adam Nash. This boy was born, having been selected by preimplantation HLA typing, so that he could become a donor of haematopoietic stem cells for his sister who suffered from Fanconi anaemia (FA)[108]. As only 3 in 16 embryos would be both unaffected and also be a full HLA match (three out of four unaffected for the recessive disorder, and one in four a full HLA match), substantial numbers of embryos are likely to be discarded in the search for an embryo that is suitable for transfer. With only ~25% chance of pregnancy following embryo transfer, several attempts at PGD might be required to achieve a match and a pregnancy. This case has been followed by the birth of a child selected by PGD for tissue type compatibility with a sibling who suffered from leukaemia. The attempt to save the life of a sibling by having another child who might provide a suitable tissue match has been practised for years but in a rather hit and miss fashion, as never before has there been the opportunity for precise diagnosis. In the leukaemia case, there was no genetic risk to the new baby, and the PGD was carried out solely for the purpose of tissue matching[109]. Statistically, only one in four embryos is likely to be a suitable match, with an expectation that ~75% of the created embryos might be unsuitable, and possibly discarded. Although the merits of saving a sibling can be rationalized and commended, this process has met with great controversy[110]. Issues of consent and protection of children's autonomy become paramount in these cases and should form the focus for giving an approval in cases or in countries where more general methods for regulation of PGD are in force.

PENETRANCE
The proportion of affected individuals among the carriers of a particular genotype. If all individuals with a disease genotype show the disease phenotype, then the disease is said to be 'completely penetrant'.

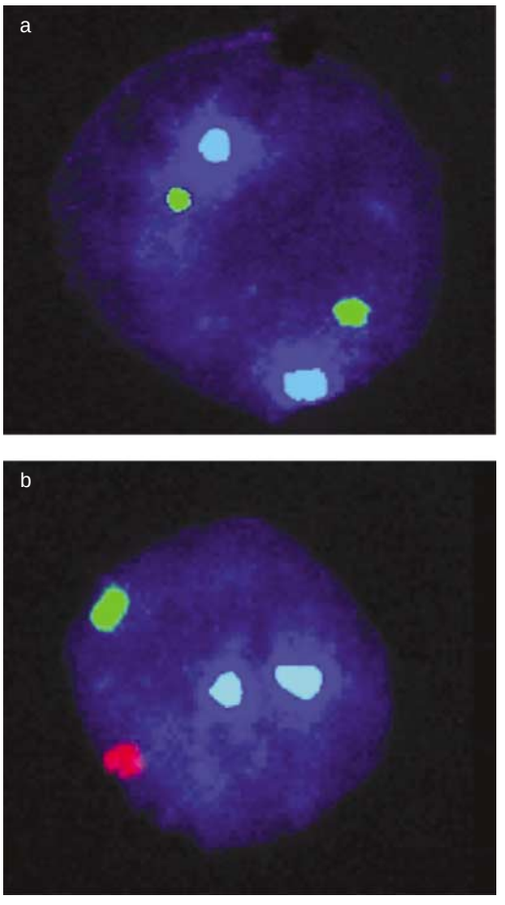

Figure 4 | **PGD of X-linked disorders using FISH.** Two nuclei that have been hybridized with probes that are complementary to sequences on chromosomes X (green), Y (red) and 18 (blue). **a** | A nucleus from the blastomere of a normal female embryo has two green and two blue signals, whereas **b** | a nucleus from a normal male has one red, one green and two blue signals.

This rate is higher than would be acceptable for molecular PND following CVS or amniocentesis, and reflects the difficulty of reaching a certain diagnosis using only one or two cells.

X-linked disorders. Any X-linked disease for which no specific single-cell PCR test is available, can be considered appropriate for PGD sex selection, although particular situations might cause ethical dilemmas (BOX 2). According to the most recent report of the ESHRE PGD Consortium, the highest number of referrals in this category was for Fragile X syndrome (despite the complication that carrier females can be clinically affected), followed by Duchenne or Becker muscular dystrophies and haemophilia. Only 4% of the referrals were turned down by the PGD centre on ethical grounds[19].

On average, half of the embryos in any sex selection cycle will be unsuitable for transfer on the grounds of sex alone. It should be appreciated that, on average, half of the discarded male embryos will be normal, a fact that gives rise to ethical criticism of this method. In addition, some embryos might be aneuploid, triploid, haploid and so on. Collecting sufficient oocytes[55], and hence generating enough embryos to give a reasonable chance of having at least two embryos for transfer, might be difficult to achieve in women who do not respond well to ovarian stimulation. Enrichment of the sperm sample for X-bearing spermatozoa by FACS (see above) would be a step towards improving PGD success rates, especially in women who produce only a small number of eggs.

Although determination of the embryo's sex by PCR was one of the earliest achievements of PGD, the possibility of misdiagnosis and the advent of *in situ* hybridization techniques have encouraged the development of more robust and reliable assays[12,56]. Since then, most centres have used fluorescence *in situ* hybridization (FISH) for sex determination[19] (FIG. 4). This assay has the added advantage of detecting abnormalities of sex chromosome copy number, so avoiding the transfer of such embryos.

Like all techniques, FISH is not without problems. Signals can go undetected arising from two signals of the same colour overlying each other (signal overlap) or the failure of hybridization, whereas extra signals can appear, arising from signal splitting or anomalous fluorescence[57,58]. However, sex selection that uses probe sets such as that shown in FIG. 4 — typically, green (G) for the X-chromosome centromere, red (R) for the Y-chromosome centromere and blue (B) for chromosome 18 centromere — is very robust. To misdiagnose a normal male embryo (RGBB) as a normal female embryo (GGBB), two errors must occur: a red signal must be lost, and an extra, anomalous green signal must be generated. This provides an effective internal check, ensuring that the chance of transferring a normal male embryo in error is very low. In some cases, embryos might be mosaic, or chaotic — in this case, cells that make up the embryo have different random chromosome constitutions[59,60], probably as a result of defective cell cycle surveillance mechanisms. The biopsied cell (or cells) might not then be representative of the whole embryo[61]. However, grossly mosaic or chaotic embryos are unlikely to be viable, making the theoretical chance of establishing a normal pregnancy with a fetus of the 'wrong' sex extremely low. Nevertheless, one FISH misdiagnosis occurred among the 78 cycles of social sexing, as reported to the ESHRE consortium[19].

Chromosome translocations. Reciprocal translocation (FIG. 5a), an exchange of two terminal segments from different chromosomes, is the commonest form of chromosome abnormality, which occurs ~1 in every 500 live births. With few exceptions, each reciprocal translocation is effectively unique to the family or individual in which it occurs. Robertsonian translocation (FIG. 5b), the centric fusion of two ACROCENTRIC CHROMOSOMES, is less common and occurs in only ~1 in 1,000 individuals. Carriers of balanced familial translocations are nearly always phenotypically normal, as there is no

ACROCENTRIC CHROMOSOME
A chromosome with the centromere located at one end.

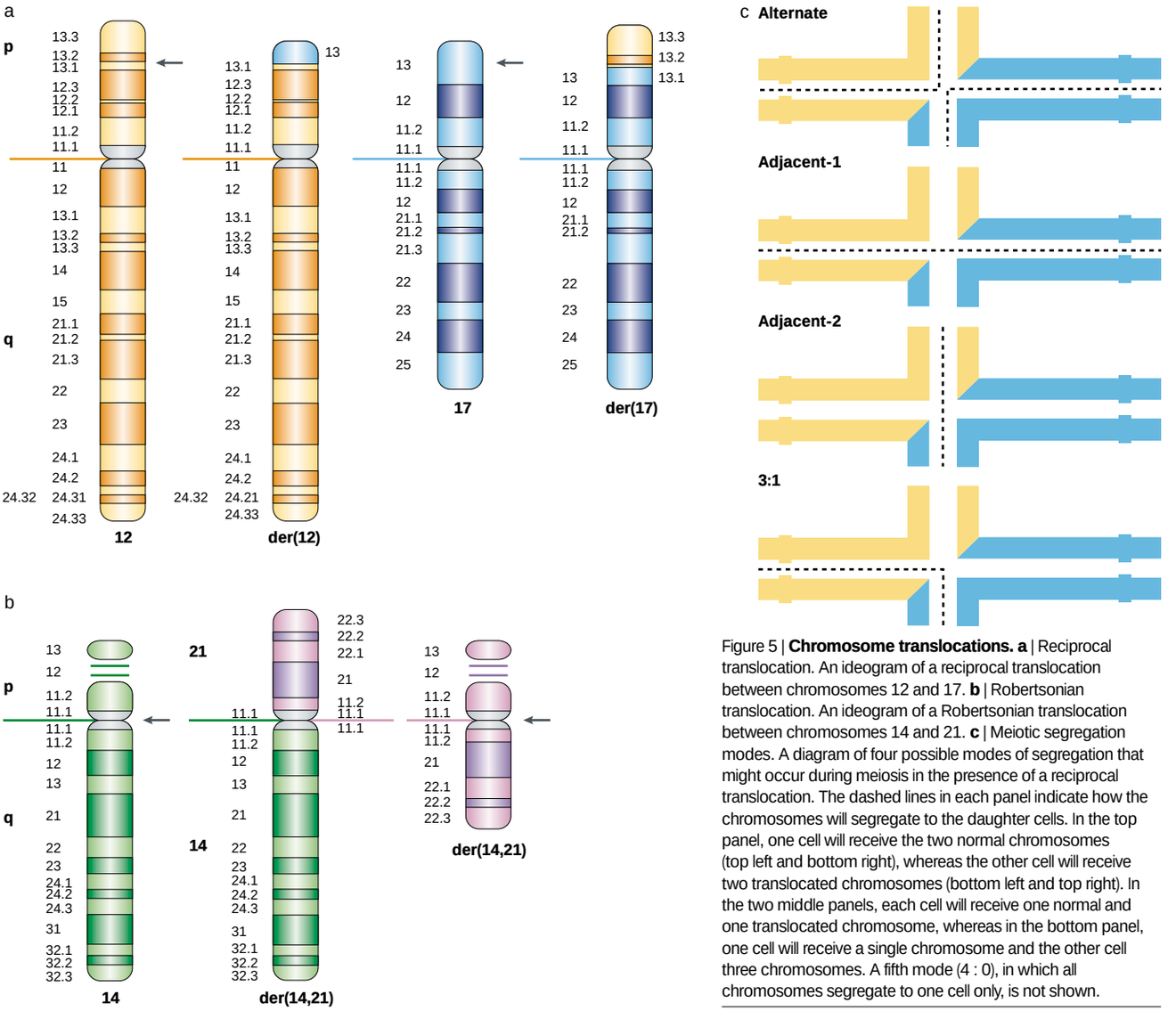

Figure 5 | **Chromosome translocations. a** | Reciprocal translocation. An ideogram of a reciprocal translocation between chromosomes 12 and 17. **b** | Robertsonian translocation. An ideogram of a Robertsonian translocation between chromosomes 14 and 21. **c** | Meiotic segregation modes. A diagram of four possible modes of segregation that might occur during meiosis in the presence of a reciprocal translocation. The dashed lines in each panel indicate how the chromosomes will segregate to the daughter cells. In the top panel, one cell will receive the two normal chromosomes (top left and bottom right), whereas the other cell will receive two translocated chromosomes (bottom left and top right). In the two middle panels, each cell will receive one normal and one translocated chromosome, whereas in the bottom panel, one cell will receive a single chromosome and the other cell three chromosomes. A fifth mode (4 : 0), in which all chromosomes segregate to one cell only, is not shown.

net loss of genetic material. Translocations are usually diagnosed when a family member is found to be infertile, suffers from recurrent pregnancy loss or has phenotypically abnormal offspring arising from the production of genetically unbalanced gametes (in which chromosomal material has been lost or gained as a result of the translocation). An ideal PGD test for patients with balanced translocations would discriminate unambiguously between different meiotic outcomes[62]. If this is not possible, then the priority must be to increase the individual's chance of a successful pregnancy and of having phenotypically normal offspring.

As for sex selection, FISH is the method of choice for diagnosing chromosome rearrangements. To detect Robertsonian translocations, chromosome enumerator probes are used to count the chromosomes in the interphase nuclei of biopsied cells — these probes can be chosen to bind at any point on the long arm of each chromosome that is involved in the translocation[63]. FISH strategies for detecting reciprocal translocations initially involved probes that spanned[64] or flanked[65,66] translocation breakpoints. These strategies had the advantage that embryos that had a normal chromosome complement could be discriminated from those that carried a balanced translocation, but were limited by the time required to develop specific probes for each translocation carrier.

An alternative approach for determining the chromosomal rearrangement status of the oocyte relies on polar body biopsy that uses whole chromosome-specific painting probes, sometimes in combination with α-SATELLITE repeat- and locus-specific probes[67]. First polar bodies that are biopsied shortly after oocyte retrieval contain highly condensed, metaphase chromosomes, and chromosome-specific paints can therefore be used to show the relative position of the regions that are

α-SATELLITE DNA
Repetitive DNA sequences arranged in tandem arrays that usually lie near the centromere.

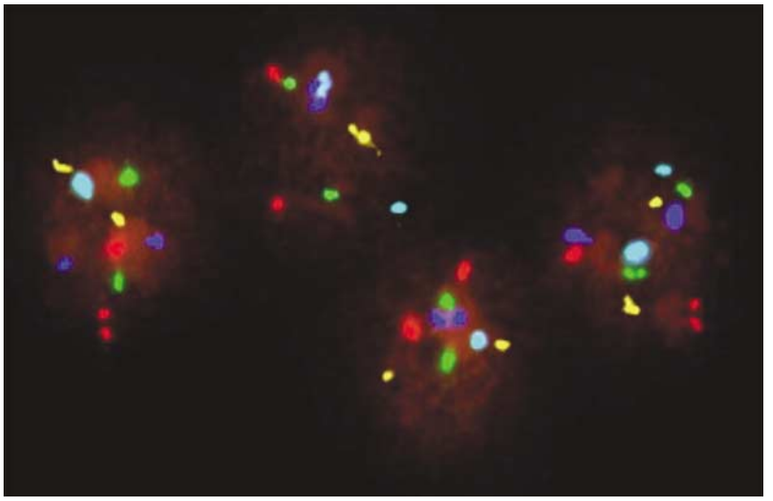

Figure 6 | **Aneuploidy screening using FISH.** Fluorescence *in situ* hybridization (FISH) of a single four-cell embryo. The embryo has been hybridized with probes to chromosomes 13, 16, 18, 21 and 22, each of which is labelled with a different fluor. Signal splitting and differences in signal size within and between blastomeres show the difficulty of interpretting FISH results.

involved in the translocation. This approach cannot be used in biopsied blastomeres because the cells might not be in metaphase and therefore the chromosomes might not be sufficiently condensed. Polar bodies with an unbalanced chromosome complement imply an unbalanced chromosome complement in the oocyte; polar bodies with a normal complement indicate an oocyte with a balanced chromosome rearrangement, which will give rise to phenotypically normal offspring. Once fertilized, normally progressing embryos are transferred to the uterus, assuming they were of sufficiently good morphology. In the absence of such embryos, those with the balanced translocation could be transferred to the patient. As this method uses polar bodies, it is of course only applicable to translocations in female carriers.

In 1998, a more general strategy for testing biopsied cells from cleavage-stage embryos was developed. It used probes that are specific for the subtelomeric regions of the translocated segments[62,68]. In this procedure, two differentially labelled probes that are specific for the chromosome arms that are involved in the translocation can be combined with a centromeric probe (or any probe that maps proximal to the breakpoint on either chromosome). The test does not discriminate between non-carrier embryos and those that carry the balanced form of the translocation, both of which should give rise to phenotypically normal offspring.

For any chromosome rearrangement, genetically unbalanced gametes are likely to be produced during meiosis. For reciprocal translocations, the prevalence of these unbalanced gametes is estimated to be between 50% and 70% (REF. 69). It is likely that each unique translocation will give rise to different proportions of possible segregation products, of which there are 32, including those that result from errors at meiosis II (REF. 62). The abnormal gametes produced by some translocations will be incompatible with a viable pregnancy, so probe combinations can be chosen to give an internal check (see above) on the abnormal products that are likely to be the most frequent. In this case, two mistakes would be needed to misdiagnose the product as normal or balanced. For those translocations that carry a significant risk of viable abnormal pregnancies, empirical data indicate that only one segregation mode (FIG. 5c) per translocation will give rise to a viable pregnancy. This is probably because only one mode will give rise to a level of genetic imbalance that can be tolerated up to such a relatively advanced stage of fetal development[69]. Algorithms for analysis of reciprocal translocations have been published[70] and can be used to establish the most likely viable mode of any such translocation. Probe combinations can then be chosen to give an internal check for products of that segregation mode.

One potential pitfall of PGD for chromosomal translocations is that some FISH probes cross-hybridize with other loci in the genome, and in some cases this cross-hybridization might be patient-specific[71]. In addition, clinically insignificant polymorphisms, in which the target sequence for one FISH probe is absent, might occur in some individuals, potentially leading to misdiagnosis. FISH probes should therefore always be tested on blood samples from both partners before being used for PGD.

As 50–70% of gametes, and hence of embryos, might carry unbalanced chromosomes arising from the translocation, embryo cohort size is even more important in this case than in sex selection PGD. For the same reasons, the embryo-transfer stage might not be reached due to the absence of normal or balanced embryos. Ideally, a clear-cut result should be obtained on each embryo to avoid excluding potentially normal embryos for technical reasons.

PGD for chromosome translocations has resulted in the birth of normal babies[19,65,72,73]. Success rates vary between centres, but a recent review of data from three large centres (two in the United States and one in Italy) reports an overall pregnancy rate of 29% per oocyte retrieval, increasing to 38% when calculated per embryo transfer[42]. The percentage discrepancy is caused by a substantial number of embryos found to be unsuitable for transfer.

Numerical chromosome abnormalities. For any one couple, the recurrence of the same autosomal trisomy (for example, trisomy 21) in pregnancy losses, terminations or live births is rare[74]. Although such recurrences might arise by chance, the possibility of mosaicism in the germ line of one partner can seldom be completely excluded. PGD has been used to test embryos for the copy number of the chromosome that was aneuploid in the previous pregnancies[75] and is usually considered appropriate — especially for couples who have religious or ethical objections to pregnancy terminations. An ideal test for chromosome copy number would include two probes, each labelled in a different colour, both for the 'at risk' chromosome, combined with a third probe for a different chromosome, to control for ploidy.

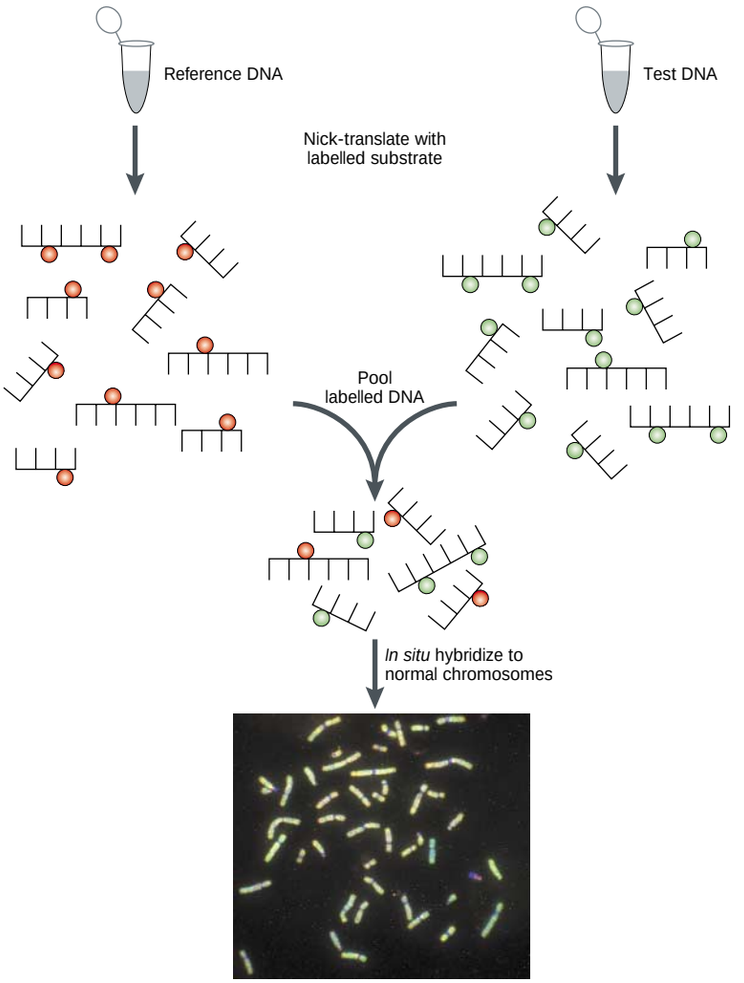

Figure 7 | **Comparative genomic hybridization**. In this technique, reference and test DNA samples are fluorescently labelled in NICK-TRANSLATION reactions. After hybridization of labelled probe mixes to normal chromosome spreads, relative fluorescent intensity is detected by capture of fluorescence using a cooled charge-coupled device camera. Dedicated software is used to compare ratios of green to red fluorescence along each chromosome and, hence, to identify genome imbalance in the test DNA. The figure is based on images supplied by Vysis, Inc. (Downers Grove, Illinois, USA).

Other chromosome abnormalities. Couples in which one partner carries a chromosome abnormality might wish to avoid transmitting this abnormality to their offspring. This is particularly true in cases such as the deletion of 22q11 that leads to Velocardiofacial syndrome (VCSF) or DiGeorge syndrome (DGS) in which the phenotype is variable and unpredictable[76]. Even monozygotic twins that carry the same deletion might have discordant phenotypes[77]. This deletion can be detected in biopsied blastomeres using FISH probes. However, 50% of embryos from such an affected individual are likely to carry a chromosome abnormality, and there is no internal check in this assay, therefore, only one mistake would be required to misdiagnose an abnormal embryo as normal. In these circumstances, most centres would consider it necessary to biopsy two cells from each embryo, and only transfer embryos with concordant results from both cells.

Enhancing in vitro *fertilization success.* Preimplantation screening for chromosome aneuploidy is carried out at many centres throughout the world. It is the most common reason for embryo diagnosis, with more than 2,000 treatment cycles reported for this purpose[42]. However, some feel that it is important to delineate between PGD for inherited genetic diseases, and PGD for the detection of sporadic chromosomal abnormality, to enhance IVF success. For this reason, the latter procedure has been designated as PGD-AS (aneuploidy screening) by the ESHRE consortium[19] or PGS (preimplantation genetic screening) by the HFEA, but it has been included in the definition of PGD in the United States[78]. The couples who opt for this test are infertile and undergoing IVF/ICSI to overcome their infertility — the success of which is heavily influenced by maternal age and the previous reproductive history of the couple[79]. Advanced maternal age or repeated IVF failure might indicate that the infertility is caused by the production of aneuploid gametes, and the test is designed to identify embryos that have a normal chromosome complement. In addition, women over 36 years of age, who are at increased risk of producing a child with Down syndrome, or with other age-related chromosomal abnormalities, and who have already opted for IVF/ICSI because of their infertility, might wish to have their embryos screened for these more common viable abnormalities rather than go through PND and possible abortion. Individual embryos are biopsied, and biopsy samples are examined for numerical chromosomal abnormality using 5–14 FISH probes (FIG. 6). One multicentre study investigating the efficacy of PGD-AS showed a decrease in miscarriage rate from 25.7% per patient in the control group to 14.3% in the group undergoing PGD-AS testing[80]. This difference was not statistically significant, although the difference was significant when the miscarriage rate was expressed as a percentage of fetal heartbeats detected[80] (24.2% miscarried in the control group and 9.6% miscarried in the tested group). There was also a significant increase (from 10.5% to 16.1%) in the ongoing pregnancy and 'delivered baby' rate in the PGD-AS group compared with the control group. Three centres (two from the United States and one from Italy) contributed to this data, and although the matching of couples for comparison was not consistent between the centres (for example, age, previous cycles and number of follicles), the trends were similar in all three centres[80].

One of the pitfalls of PGD-AS is that some normal embryos might be excluded from the cohort that is considered suitable for embryo transfer because of errors in the test, which, especially in older women who might have small embryo cohorts, could result in the failure to reach embryo transfer. The ESHRE consortium data on PGD-AS during the past 12 months in participating centres[19] showed a 28% pregnancy rate in PGD-AS for advanced maternal age (>35 years), but only a 7% pregnancy rate in women with recurrent IVF failure (excluding couples with

NICK TRANSLATION
A method for *in vitro* DNA labelling. Nicks are introduced into the DNA by an endonuclease and are subsequently repaired using labelled residues.

KARYOTYPE ANALYSIS
The ascertainment of chromosome constitution by the light microscopy analysis of stained metaphase chromosomes.

METAPHASE SPREADS
The result of a cytogenetic method in which dividing cells are artificially arrested at metaphase, when chromosomes are shortened and condensed. The fixed material from such preparations is dropped onto microscope slides, where the chromosomes from individual cells form clusters or spreads, which can be stained and analysed.

PLOIDY
The number of sets of chromosomes in a cell (*n*). Normal human somatic cells are diploid (2*n*), with 2 sets of 23 chromosomes.

translocations), indicating that, in the latter group, factors other than aneuploid gametes might be likely to be the main cause of infertility. More than 1,000 cycles of PGD-AS have been carried out in one US centre[81], but clear benefits of this technique in terms of live birth rate per initiated cycle have yet to be shown in any large-scale prospective controlled study. It is important that this technology is properly evaluated[78]. An expensive test of unproved or limited efficacy might be readily taken up by women who are desperate to establish a pregnancy (the so-called 'technological imperative'; REF. 82), when in some cases the test might reduce their chances of pregnancy by excluding normal embryos from the cohort available for transfer. Future research into the incidence of individual chromosome aneuploidies in early embryos might provide the means to design a more specific test that uses FISH probes for the most common early abnormalities. Alternatively, other technologies, such as comparative genomic hybridization (CGH) or microarrays might be used in future to allow the screening of the whole genome for genetic imbalance[68] (see below).

Future developments

Comparative genomic hybridization. Although PGD-AS has been shown to improve implantation rate in some patient groups, at present, only a limited subset of chromosomes can be screened using traditional FISH protocols[80,83]. Ideally, cytogenetic tests would involve a full KARYOTYPE ANALYSIS on metaphase chromosomes. Unfortunately, preparing METAPHASE SPREADS directly from embryonic blastomeres by traditional methods has proved difficult, with only a small proportion of the blastomeres in any one embryo giving interpretable results[84,85].

Alternative methods for karyotyping, including fusing polar bodies or blastomeres with enucleated human oocytes or with bovine oocytes to induce mitosis[86,87] have been reasonably successful, but CGH now seems to be the method of choice for enumerating the whole chromosome set in blastomeres[88,89]. Although the chromosomes are not visualized directly, copy number of every chromosomal region of 20 Mb or more can be assessed using CGH[90] (FIG. 7). Whole-chromosome aneuploidies and even small structural aberrations have been detected in blastomeres using this method, but the technique is limited to detecting relative imbalance and, therefore, changes in whole PLOIDY cannot be seen[88,89]. The technique requires two to three days for diagnosis and is therefore, at present, unsuitable for routine use on cleavage-stage embryos or blastocysts without a cryopreservation step between biopsy and transfer. Improvements in the protocols might shorten this time and allow the diagnosis and transfer of fresh material. Until now, several ongoing pregnancies and the birth of one healthy child have been reported using this technique[39,91].

DNA microarray analysis is a rapidly evolving method of molecular analysis that could find several potential uses in PGD[92,93]. Although it is primarily used for gene expression analysis, microarrays could be used in routine PGD in screens for mutations in any one gene, or screens of several genes for several mutations. Embryos could then potentially be tested for serious susceptibility traits loci, such as the breast cancer 1 (*BRCA1*) gene. Microarrays could also be useful in PGD of specific diseases that are severely genetically heterogeneous and for which there are few common mutations, such as Duchenne muscular dystrophy. Such an approach could provide a useful generic testing procedure that is applicable to all patients that carry this disease. Finally, microarrays could replace the metaphase spreads that are now used to assess chromosome imbalance during CGH. At present, technical limitations, such as the paucity of material that is available for hybridization, sensitivity and reliability of the data, and the cost of producing appropriate microarrays are likely to hinder their application in PGD for some considerable time.

Box 3 | Regulation of preimplantation genetic diagnosis

There are substantial differences in the control of preimplantation genetic diagnosis (PGD) worldwide[111]. These differences are linked to the prevailing attitudes to assisted conception, invasive procedures on human embryos and the eugenics of embryo selection. In the United Kingdom, PGD, like all reproductive technology that involves *in vitro* human embryo manipulation, is strictly regulated by the HFEA (see the section entitled 'Clinical procedures and embryology ') under the terms of the Human Fertilisation and Embryology Act (1990). According to this Act, and the consequent HFEA Code of Practice[15], embryos may only be used for PGD and for research to develop new diagnostic methods under licence from the HFEA. The HFEA provides reassurance to the public that PGD is being undertaken only for serious genetic diseases and not for social purposes (BOX 2). PGD has only recently been allowed in France[112], and then limited to three centres, whereas it is not allowed in Argentina, Austria, Switzerland and Taiwan[113]. In Germany, only those procedures that are of direct benefit to the embryo can be undertaken[111]. As PGD might result in the destruction of affected embryos, PGD is now not allowed due to deep-seated fears arising from that country's sad history of eugenics. Although, encouragingly, the future use of PGD is now being debated in the German Parliament, more restrictive legislation is being proposed in Italy. There is no federal regulation of PGD in the United States[42].

Conclusions

PGD is a sophisticated form of early prenatal diagnosis that is carried out in a few specialized centres. However, the rapid advances in molecular genetics are likely to stimulate further the use of PGD and to encourage a substantial change in the way that genetic conditions in the offspring of certain patients are prevented. It is becoming apparent that the main demand for embryo biopsy will come from infertile patients seeking to improve their chances of successful IVF treatment and to reduce the risk of conceiving a child with an age-related aneuploidy. Indeed, it is likely that a combination of approaches will be made possible by the molecular examination of the entire chromosome complement, at the same time testing for common genetic diseases, such as cystic fibrosis. The challenge will be to regulate the use of PGD technology (BOX 3) for medical purposes and to limit or prevent its use for eugenic selection.

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