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Isothermal whole genome amplification from single and small numbers of cells: a new era for preimplantation genetic diagnosis of inherited disease

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Preimplantation genetic diagnosis (PGD) of single gene defects following assisted conception typically involves removal of single cells from preimplantation embryos and analysis using highly sensitive PCR amplification methods taking stringent precautions to prevent contamination from foreign or previously amplified DNA. Recently, whole genome amplification has been achieved from small quantities of genomic DNA by isothermal amplification with bacteriophage ϕ29 DNA polymerase- and exonuclease-resistant random hexamer primers. Here we report that isothermal whole genome amplification from single and small numbers of lymphocytes and blastomeres isolated from cleavage stage embryos yielded microgram quantities of amplified DNA, and allowed analysis of 20 different loci, including the ΔF508 deletion causing cystic fibrosis and polymorphic repeat sequences used in DNA fingerprinting. As with analysis by PCR-based methods, some preferential amplification or allele drop-out at heterozygous loci was detected with single cells. With 2–5 cells, amplification was more consistent and with 10 or 20 cells results were indistinguishable from genomic DNA. The use of isothermal whole genome amplification as a universal first step marks a new era for PGD since, unlike previous PCR-based methods, sufficient DNA is amplified for diagnosis of any known single gene defect by standard methods and conditions.

Key words: preimplantation genetic diagnosis/single gene defects/whole genome amplification

Introduction

Preimplantation genetic diagnosis (PGD) following assisted conception is now well established clinically as an alternative to conventional prenatal diagnosis in couples at risk of having children with an inherited disease (Delhanty and Wells, 2002; Verlinsky and Kuliev, 2003). Since the first births were reported in couples at risk of X-linked conditions and cystic fibrosis (Handyside *et al.*, 1990, 1992 the range of genetic defects which can be diagnosed has expanded considerably and now includes numerical and structural chromosomal abnormalities and most of the common single gene defects (Thornhill and Snow, 2002). The scope of PGD has also been extended to screening for chromosomal aneuploidy in infertile couples (Gianaroli *et al.*, 1999; Wilton *et al.*, 2001; Wells *et al.*, 2002) and, more controversially, for HLA typing with or without single gene defect diagnosis with the aim of recovering compatible stem cells from cord blood at birth for transplantation to an existing sick child (Verlinsky *et al.*, 2001). Although precise data are not available, it is now estimated that approaching 1500 babies have been born worldwide following PGD (Verlinsky and Kuliev, 2003).

Nevertheless, PGD remains a costly, highly specialized procedure only available in a handful of centres with the necessary resources and expertise. The reason is that PGD typically relies on the genetic analysis of the first and second polar bodies and/or one or two single blastomeres biopsied from the fertilized zygote or cleavage stage embryo respectively. For the diagnosis of single gene defects, this requires the development and validation of highly sensitive amplification strategies, often using two rounds of PCR with nested primers or fluorescent PCR methods, which, because they are capable of detecting as few as one or two target double-stranded DNA molecules in a single cell, are highly susceptible to errors through contamination with foreign or previously amplified DNA. Hence isolated clean room facilities and stringent precautions are essential throughout to avoid misdiagnoses, which excludes the use of prenatal genetics laboratories where amplification of similar sequences from large numbers of samples is routine and PCR products are handled openly on the laboratory bench.

Recently whole genome amplification using the bacteriophage ϕ29 DNA polymerase for isothermal multiple displacement amplification has been reported (Dean *et al.*, 2002; Lasken and Egholm, 2003). The ϕ29 DNA polymerase has high processivity, generating amplified fragments of >10 kb by strand displacement, and has proofreading activity resulting in lower misincorporation rates compared with Taq polymerase. The random hexamer primers must be thiophosphate-modified to protect them from degradation $3'-5'$ exonuclease proofreading activity of the ϕ29 DNA polymerase (Dean *et al.*, 2001). Isothermal whole genome amplification directly from

clinical samples such as blood and buccal swabs has allowed high throughput genotyping without the need for time-consuming DNA purification steps (Hosono *et al.*, 2003). Sequence representation in the amplified DNA assessed by multiple single nucleotide polymorphism analysis is equivalent to genomic DNA when amplifying from as little as 0.3 ng target DNA, and amplification bias is superior to PCR-based methods (Lovmar *et al.*, 2003).

Here we investigate the use of isothermal whole genome amplification from single and small numbers of cells, including isolated lymphocytes and single blastomeres, as a universal first step for PGD of single gene defects. Following isothermal whole genome amplification from single lymphocytes, standard, relatively low sensitivity, PCR methods were used to analyse 20 different loci from the amplified DNA, including the ΔF508 deletion in exon 10 and two intragenic microsatellite markers in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, and, nine short tandem repeats used in DNA fingerprinting. Standard PCR amplification of the ΔF508 deletion was also compared following isothermal whole genome amplification from single and small numbers of heterozygous lymphocytes. Finally, isothermal whole genome amplification from single blastomeres and PCR analysis for ΔF508 deletion and two intragenic microsatellite markers was used to confirm the genotype of three embryos previously identified by PGD.

Materials and methods

Preparation and lysis of single cells

Lymphocytes, from an individual heterozygous for the ΔF508 deletion in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, were separated from whole blood by centrifugation over Ficoll, washed and resuspended in calcium/magnesium-free phosphate-buffered saline (PBS) with 15 mg/ml polyvinylpyrolidone (PVP), according to the manufacturer's instructions (Histopaque; Sigma–Aldrich, UK). Single lymphocytes and groups of 2, 5, 10 and 20 lymphocytes were selected by hand using a mouthpipette and placed in 3.5 μl of PBS in PCR tubes under a stereo microscope in a dedicated laboratory with positive pressure HEPA-filtered air taking precautions to avoid contamination. All sample tubes were kept in cool racks at approximately ice temperature (4–10°C) throughout. A volume of 3.5 μl of freshly prepared lysis buffer (0.4 mol/l KOH, 10 mmol/l EDTA, 100 mmol/l dithiothreiotol) was added to each sample and the tubes placed on ice for 10 min to lyse the cells. Lysis was then stopped by adding 3.5 μl of neutralizing buffer (REPLI-g 625S kit; Molecular Staging, USA).

Single blastomeres were disaggregated from three cleavage stage human embryos by gentle pipetting late on day 3 post insemination, following preincubation in calcium/magnesium-free embryo biopsy medium (EB10; Cook, Australia) for 15–30 min at 37°C and removal of the zona pellucida by incubation in acid Tyrode's solution (Medicult, UK) under a stereo microscope in a laminar flow cabinet. Each blastomere was carefully examined for interphase nuclei, washed through three drops of PBS + PVP and single blastomeres placed in 3.5 μl of PBS in PCR tubes and stored at −20°C overnight. These embryos had been biopsied earlier in the day for PGD of cystic fibrosis (ΔF508) and, following selection and transfer of two unaffected sibling embryos, were used with the patient's informed consent to confirm the diagnosis. The Assisted Conception Unit, Clarendon Wing, Leeds General Infirmary is licensed for PGD by the UK Human Fertilisation and Embryology Authority.

Isothermal whole genome amplification

Following lysis of the cells, whole genome amplification by isothermal multiple displacement amplification was achieved using bacteriophage ϕ29 DNA polymerase, exonuclease-resistant phosphorothioate-modified random hexamer oligonucleotide primers and reaction buffer according to the manufacturer's instructions (REPLI-g 625S kit; Molecular Staging, USA) in a 50 μl reaction at 30°C overnight (16 h). The reaction was terminated by incubation at 65°C for 3 min to inactivate the enzyme and the amplified DNA stored at −20°C. Genomic DNA and water blanks were included as positive and negative controls respectively in each set of samples.

PCR analysis

The yield of DNA amplified from each of the single lymphocytes following isothermal whole genome amplification was measured using a fluorochrome specific for double-stranded DNA (Picogreen; Molecular Probes, USA). In addition, real-time quantitative PCR of test loci (WIAF-1004 and WIAF-699) was run on the samples to confirm that a significant fraction of the amplified DNA was human sequence and not non-specific products amplified from the primers (data not shown).

To assess sequence representation following isothermal whole genome amplification from single lymphocytes, standard, relatively low sensitivity, PCR or fluorescent PCR (FPCR) methods were used to amplify 20 different target sequences from 1 μl aliquots: (i) multiplex fluorescent PCR (23 cycles) of exon 10 and two intragenic microsatellite repeats, IVS 17β$(CA)_n$ and IVS17β$(TA)_n$, of *CFTR*; (ii) separate PCR of variable number of tandem repeats (VNTR) in *IL1RN* (Tarlow *et al.*, 1993) (chromosome 2) and *IL4* (Mout *et al.*, 1991) (chromosome 5), and restriction fragment length polymorphisms (RFLP) in *TGFβ1* (T-I) (Syrris *et al.*, 1998) (chromosome 6) and *SCYA11* (Miyamasu *et al.*, 2001) (chromosome 17); (iii) real-time PCR analysis of three single nucleotide polymorphisms from the Whitehead Institute–Affymetrix database: WIAF-1004 (chromosome 2), WIAF-622 (chromosome 11), WIAF-525 (chromosome 18); and (iv) multiplex fluorescent PCR for nine short tandem repeat loci (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) and the amelogenin locus (AmpFLSTR Profiler Plus; Applied Biosystems, USA). Also, to compare amplification efficiency and allele drop out (ADO) following isothermal whole genome amplification from 1, 2, 5, 10 and 20 lymphocytes, a single round of PCR (30 cycles) with the inner nested primers of the single cell protocol (Handyside *et al.*, 1992) was used to amplify exon 10 of *CFTR*. Finally, following isothermal whole genome amplification from the single blastomeres, the ΔF508 deletion status of three embryos genotyped earlier by cleavage stage biopsy and single cell analysis for PGD was confirmed by PCR with the inner nested primers of the single cell protocol (Handyside *et al.*, 1992) and multiplex fluorescent PCR for exon 10 and two intragenic polymorphic repeats in *CFTR* (as above).

Results

Isothermal whole genome amplification was successful in 5/5 single lymphocytes and 10/11 single blastomeres as assessed by DNA yield and/or PCR amplification. The yield of double-stranded DNA in the single lymphocytes was 48 ± 5 μg (range 42–53 μg) in the 50 μl reaction as assessed by Picogreen labelling. However, as nonspecific products are generated even in the negative control samples, quantitative real-time PCR of two test loci was carried out, which demonstrated that a significant though variable fraction of the amplified DNA was human sequence (data not shown). For example, for lymphocyte 5, ~25% of the 53 μg of amplified DNA was estimated to be human sequence.

PCR amplification of 20 different loci from the single lymphocyte DNA was detected in 92/100 reactions (Table I; Figure 1). With polymorphic repeat sequences, allele sizes matched those amplified from genomic DNA, except in two reactions from a single lymphocyte, in which unrelated short tandem repeat alleles were amplified indicating possible contamination. However, all the other repeat sequences amplified from this cell had the appropriate allele sizes. At heterozygous loci, preferential amplification of alleles was frequent (data not shown) and ADO was detected in 22/70 (31%) reactions.

Following isothermal whole genome amplification from 1, 2, 5, 10 and 20 heterozygous lymphocytes, PCR amplification of exon 10 of *CFTR*, using only the nested inner primers of the single cell protocol and heteroduplex analysis for the ΔF508 deletion, showed a progressive reduction of preferential amplification and ADO

Table I. Amplification efficiency and allele drop-out (ADO) rates following isothermal whole genome amplification from single lymphocytes and blastomeres from cleavage stage human embryos

Single cell	*n*	No. of loci analysed	Total no. of PCR[a]	Amplification failure	No. of PCR at heterozygous loci	ADO	Unrelated allele
Lymphocytes	5	20	100	8	70	22	2
Blastomeres	10	3	40	0	25	4	0

[a]See Figures 1 and 2.

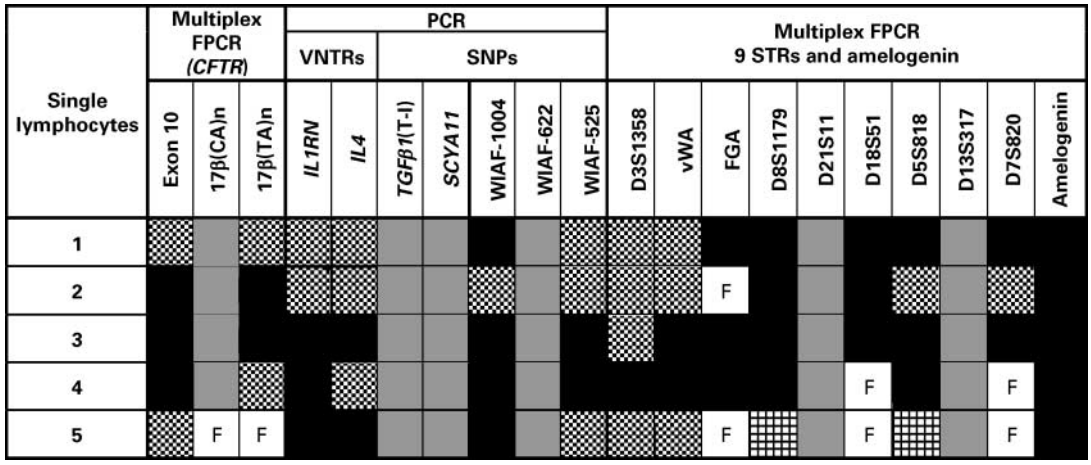

Figure 1. PCR amplification of 20 different loci following whole genome amplification from single lymphocytes: (1) multiplex fluorescent PCR of *CFTR* exon 10 and two intragenic dinucleotide repeats; (2) PCR of variable number of tandem repeats (VNTRs) in *IL1RN* (Tarlow et al., 1993) (chromosome 2), *IL4* (Mout et al., 1991) (chromosome 5) and restriction fragment length polymorphisms (RFLPs) in *TGFβ1* (T-I) (Syrris et al., 1998) (chromosome 6) and *SCYA11* [19] (chromosome 17); (3) minisequencing of single nucleotide polymorphisms (SNPs) WIAF-1004, 622, 525; (4) multiplex fluorescent PCR of 9 short tandem repeats (STRs) and the amelogenin locus: D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S81, D13S317, D7S820 and amelogenin. Heterozygous loci: both alleles amplified ■. Only one allele amplified (allele dropout) ▨. (Checkerboard) Nonheterozygous (or alleles not analysed): amplified ▩. Failed to amplify (F). Amplification of polymorphic STRs from unrelated DNA:▩ (Grid).

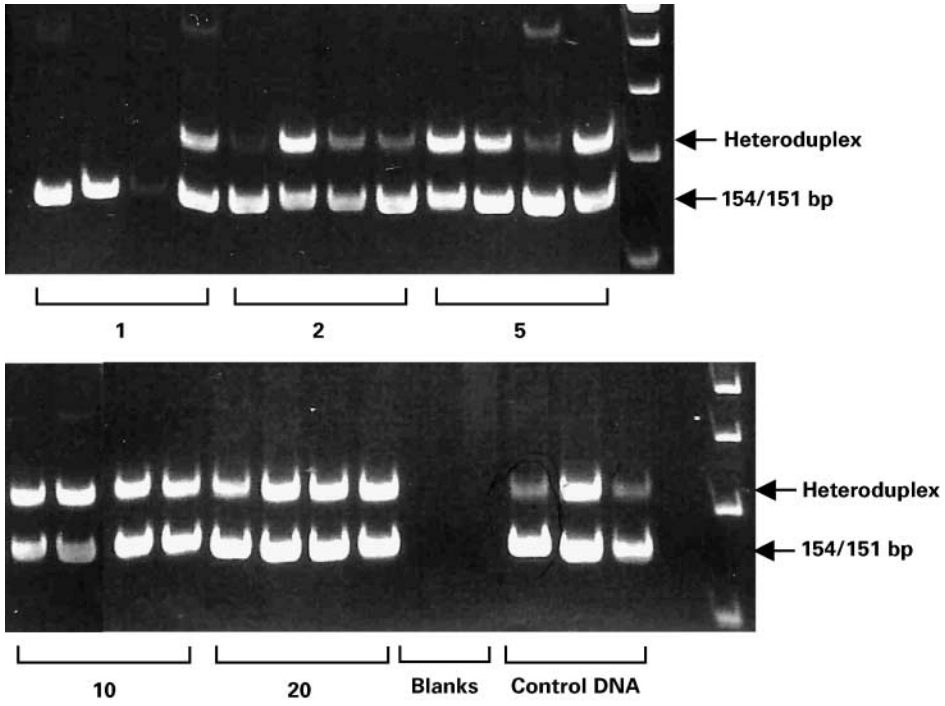

Figure 2. PCR amplification of exon 10 of *CFTR* using only the nested outer primers of the single cell protocol[4] following isothermal whole genome amplification from 1, 2, 5, 10 and 20 lymphocytes from an individual heterozygous for the ΔF508 deletion. Amplification of the normal and ΔF508 allele produces 154 and 151 base pair fragments, respectively, which run together as a broad band on the minigel. Where both alleles have amplified double stranded heteroduplex formation between the full length and deleted fragments is detected as a retarded band. Four 1, 2, 5, 10 and 20 cell samples, washdrop blank, reagent blank, control DNA, whole blood of lymphocyte donor, and control genomic DNA (unknown origin).

(Figure 2). One out of four single cell replicates only amplified weakly and the absence of a heteroduplex band indicated ADO in 3/4 reactions. With 2 cells, all four replicates amplified, but a weak heteroduplex band indicated preferential amplification of one allele in 3/4 reactions. With 5 cells, 1/4 reactions showed preferential amplification, and with 10 and 20 cells all four replicates were clearly heterozygous with strong heteroduplex bands similar to those obtained following isothermal whole genome amplification from whole blood from the heterozygous donor.

With 8/10 single blastomeres from three cleavage stage embryos, PCR amplification and heteroduplex analysis of exon 10, and multiplex fluorescent PCR amplification of exon 10 and two intragenic microsatellite repeats of *CFTR*, were successful in every case and confirmed the genotype ascertained by PGD (Table I; Figure 3). The size of the microsatellite repeat alleles was accurately determined and matched those predicted from analysis of parental and affected DNA from a previous prenatal diagnosis (data not shown), although there was frequent preferential amplification. In the other two single blastomeres from embryo 3 (Figure 3), ADO occurred either for exon 10 or the two microsatellite repeats, resulting in an overall ADO rate of 4/25 (16%) reactions. In cell 1, both PCR and multiplex FPCR failed to amplify the normal exon 10 allele, suggesting ADO during the initial isothermal whole genome amplification step. In cell 2, both IVS 17β(CA)$_n$ and IVS 17β(TA)$_n$ appeared to be homozygous for one of the parental alleles when they would have been predicted to be heterozygous. Again this was consistent with a parental IVS 17β haplotype, suggesting ADO during isothermal whole genome amplification.

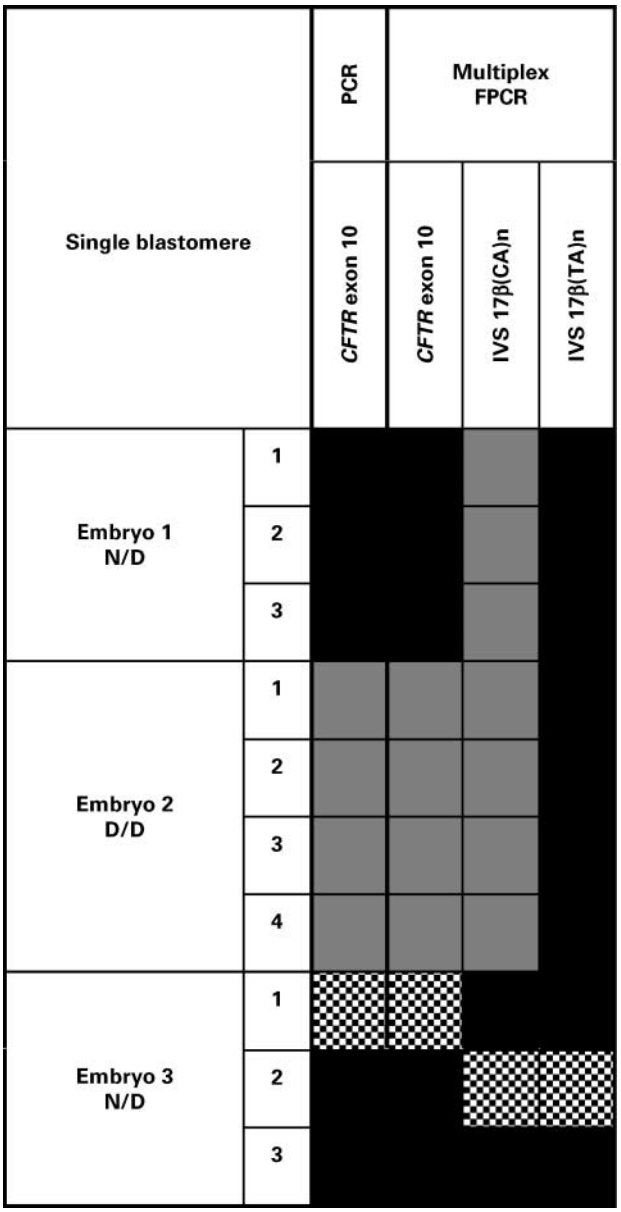

Figure 3. PCR amplification of exon 10 and two intragenic dinucleotide repeats (IVS 17β(CA)n and IVS 17ß(TA)n) of the cystic fibrosis transmembrane regulator (*CFTR*) gene following isothermal whole genome amplification from single blastomeres from heterozygous carrier (N/D) and homozygous affected cleavage stage embryos (D/D). Heterozygous loci: both alleles amplified ■. Only one allele amplified (allele dropout) ▨. Non-heterozygous (or alleles not analysed): amplified ■.

Discussion

As an initial step for PGD from single cells, whole genome amplification by primer extension preamplification (Zhang *et al.*, 1992), a PCR-based method using random primers and Taq polymerase, allows multiple PCR analysis of different gene sequences (Snabes *et al.*, 1994) and has been used, for example, to combine mutation detection with analysis of a linked marker in familial adenomatous polyposis coli, an autosomal dominant cancer-predisposing syndrome (Ao *et al.*, 1998). However, only limited amplification is achieved and sensitive methods such as nested PCR are still required to amplify specific sequences. Also, highly polymorphic microsatellite repeat sequences, particularly the common dinucleotide repeats, which are valuable as linked markers, cannot be used because of overamplification of fragments with less than the full number of repeats caused by slippage of the Taq polymerase (Wells *et al.*, 1999). Hence multiplex fluorescent PCR directly from single cells has become the method of choice for combining mutation detection and analysis of linked markers to increase the accuracy of single cell analysis (Thornhill and Snow, 2002).

Isothermal whole genome amplification from single lymphocytes with bacteriophage ϕ29 DNA polymerase and thiophosphate-modified random hexamer primers was efficient, yielded microgram quantities of amplified DNA and allowed analysis of 20 different loci using standard, relatively low sensitivity, PCR methods (Table I; Figure 1). These included multiplex fluorescent PCR of exon 10 and two intragenic repeats in the *CFTR* gene, standard PCR of several single nucleotide polymorphisms and short tandem repeats, real-time fluorescent PCR of three single nucleotide polymorphisms and a multiplex fluorescent PCR of nine short tandem repeats spread across different chromosomes and amelogenin on the X and Y chromosomes used for DNA fingerprinting. This equals or exceeds the maximum number of loci which have been amplified directly from single cells by multiplex fluorescent PCR, without any need to optimize the conditions for efficient co-amplification (Findlay *et al.*, 2001; Fiorentino *et al.*, 2004) and only using a small fraction of the amplified DNA. Furthermore, unlike PCR-based methods, the size of all the polymorphic repeat alleles, including dinucleotide and short tandem repeats, was accurately identified.

As with all PCR-based methods, some preferential amplification of alleles, ADO and, in some cases, complete amplification failure was observed (Table I, Figures 1–3). Careful optimization of primer design and PCR conditions and the use of sensitive fluorescent PCR methods have reduced but never completely eliminated these problems (Findlay *et al.*, 2001), indicating that they may be inherent to amplification from a single cell perhaps because of the rapid degradation of the target DNA (Ray and Handyside, 1996). Therefore, either two single cells are analysed independently from each embryo

or mutation detection is combined with analysis of one or more linked markers to reduce errors (Lewis *et al.*, 2001). With isothermal whole genome amplification as an initial step, not only will the analysis of multiple linked markers be completely straightforward with standard protocols, but also tests can be carried out in duplicate or repeated if necessary. Using increasing numbers of lymphocytes, PCR analysis demonstrated that, even with 2 or 5 cells, preferential amplification and ADO was markedly reduced, and with 10 and 20 cells results were indistinguishable from whole blood following isothermal whole genome amplification (Figure 2). Several groups have now established pregnancies following biopsy of up to ~20 cells excised from the outer trophectoderm layer of the human blastocyst using a non-contact infrared laser on day 5 or 6 post insemination (Handyside, 2004). If isothermal whole genome amplification and PCR analysis can be completed within 24 h, highly accurate genetic analysis should therefore be possible, although the effects of chromosomal mosaicism at cleavage and blastocyst stages will need to be assessed (Lewis *et al.*, 2001).

We believe that the use of isothermal whole genome amplification as a universal first step marks a new era for PGD, since, unlike previous PCR-based methods, sufficient DNA is amplified for diagnosis of any known single gene defect by standard methods under normal laboratory conditions. Stringent precautions to prevent contamination of cells biopsied from human embryos with foreign DNA, either from maternal cumulus cells, sperm DNA or in media and/or other reagents, will still be required in clinics prior to isothermal whole genome amplification. However, the principal advantage is that the amplified DNA can then be transferred to a normal laboratory for genetic analysis using standard, relatively insensitive, PCR methods. As these methods would not normally be sensitive enough to amplify from single cells or low levels of contamination from previously amplified products, the development of specialized single cell protocols and use of separate clean room facilities for setting up these PCR reactions should not be needed as long as good laboratory practice is followed.

Isothermal whole genome amplification will also revolutionize our ability to perform extensive genetic analysis of human preimplantation embryos (Figure 4). In addition to DNA fingerprinting, as demonstrated here, which could be useful to avoid misdiagnosis resulting from amplification of foreign DNA and to confirm the parental origin of the embryos, other possibilities include aneuploidy screening, to improve pregnancy rates in women of advanced maternal age using molecular genetic methods or comparative genomic hybridization (Wilton *et al.*, 2001; Wells *et al.*, 2002) and HLA typing for selection of embryos compatible with an existing sick child (Verlinsky *et al.*, 2001; Fiorentino *et al.*, 2004). Furthermore, with rapid advances in technology, including the use of DNA microarrays, it may be possible to generate a genome-wide 'genetic snapshot' of individual embryos. Although this raises significant social and ethical issues about the limits of genetic testing that should be allowed for embryo selection, if carefully regulated, this could bring significant benefits to couples at risk of inherited disease and those with infertility.

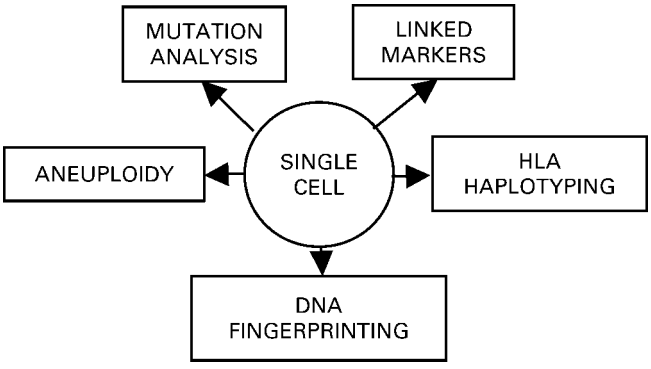

Figure 4. Examples of parallel genetic tests following isothermal whole genome amplification from single cells.

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