Recycling the single cell to detect specific chromosomes and to investigate specific gene sequences

Alan Thornhill, Cathy Holding and Marilyn Monk

Molecular Embryology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

Introduction

Preimplantation diagnosis is a new procedure which has been developed in the past few years. As with other forms of prenatal diagnosis, its primary aim is the avoidance of severe genetic disease in the offspring of couples who have a high probability of passing on mutant genes. However, diagnosis of genetic disease in the embryo has the advantage that it avoids termination of pregnancy following preimplantation diagnosis for a specific gene defect by identifying embryos with chromosomal abnormalities and eliminating them from the transfer procedure.

Key words: fluorescent in-situ hybridization/human embryo/preimplantation diagnosis/polymerase chain reaction/single cell

Materials and methods

Mice

Mouse blastomeres isolated from 8-cell mouse embryos were used as a model system for investigation of defective preimplantation embryos in the human. Female mice (CD1, Swiss outbred albino from Charles River, UK), 8–10 weeks old, were mated to CD1 males. In the morning or early afternoon of the third day of pregnancy (day of plug is the first day), 8-cell embryos were flushed from the oviducts with phosphate-buffered saline (PBS) containing bovine serum albumin (BSA, 4 mg/ml). After removal of the zona pellucida with acid Tyrode’s solution (pH 2.4), the embryos were washed in PBS/BSA and disaggregated using a finely drawn glass pipette to separate individual blastomeres.

Blastomere fixation

Miniature glass slides (termed ‘dipsticks’) were cut from coverslips, using a diamond pen, and flame-sterilized. The blastomeres (measuring 20 mm x 3 mm) were transferred to the mother to initiate pregnancy.

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without a blastomere but treated with both washing medium (from the droplet containing the blastomeres) and fixative. After allowing the fixative to dry, the dipsticks were transferred to microcentrifuge tubes using flame-sterilized forceps.

**PCR detection of a β-globin sequence**

The conditions and primers for the amplification of the β-globin sequence were those published by Holding and Monk (1989) with some minor modifications. Sufficient amplification reaction mixture was prepared on ice for all samples and controls. Blank dipsticks and those with attached single cells were put into labelled microcentrifuge tubes. To each tube was added 50 µl of the reaction mixture [50 pmol of outer primers, 5 µl of 10× PCR buffer I (Perkin-Elmer/Cetus, Applied Biosystems Ltd, Warrington, Cheshire, UK), 0.2 mM dATP, dCTP, dGTP and dTTP (Pharmacia, Biotech, St Albans, Herts, UK), 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer/Cetus) and overlain with 100 µl mineral oil (BDH). PCR amplification was carried out using a Techne PHC II thermal cycler programmed to cycle at 93°C for 1 min, 45°C for 1 min and 72°C for 3 min. After 30 cycles, 1 µl from each of the first-round amplifications was transferred to a fresh tube containing 49 µl of freshly prepared amplification mixture. The second-round mixture was identical with 100 µl mineral oil (BDH). PCR amplification was carried out using a Techne PHC II thermal cycler programmed to cycle at 93°C for 1 min, 45°C for 1 min and 72°C for 3 min. After 30 cycles, 1 µl from each of the first-round amplifications was transferred to a fresh tube containing 49 µl of freshly prepared amplification mixture. The second-round mixture was identical to the first but contained the nested primer set. The second round of PCR amplification was carried out exactly as above. Aliquots of 10 µl of the final amplification mixtures were loaded with 2 µl of bromophenol-blue loading dye, into 2% agarose (BRL, Life Technologies Ltd, Paisley, UK) and overlain with 1 µg/ml ethidium bromide. Following electrophoresis at 80 V for 25 min, the gel was removed from the tank and photographed under short-wave ultraviolet light.

**Precautions against contamination**

In this sort of sensitive PCR analysis, the main contaminant is the amplification product, which may be carried over in the droplet containing the blastomeres and single fixed blastomeres. The RNase, protease and formaldehyde (permeabilization and refixation) steps (Griffin et al., 1992) were eliminated from this protocol.

Dipsticks with attached single cells were immersed in 100 µl of PBS for 5 min. After gently removing the PBS, the blastomeres were dehydrated using an alcohol series (100 µl each of 70, 85 and 100% ethanol for 2 min each). Hybridization mix (1–2 µl) containing X and Y chromosome-specific probes (each at a final concentration of 2 ng/µl and labelled with either biotin or digoxigenin, see below) was applied as a droplet to the marked area on the dipstick. Probe and target DNA were simultaneously denatured by placing the microcentrifuge tube (containing the dipstick) in a waterbath at 85°C for 5 min. Hybridization was carried out in the same tubes for 2.5 h in a waterbath at 37°C. Post-hybridization washes included 3 x 5 min 50% formamide/2× SSC (standard saline citrate, 50 µl) at 42°C, 2 x 5 min 2× SSC at 42°C, followed by 1 x 5 min 4× SSCT (SSC plus 0.05% Tween-20) at room temperature. Prior to detection with fluorochromes, the dipsticks were preincubated with 4× SSCT (SSC plus 5% Marvel milk powder as blocking reagent) for 15 min. The detection process consisted of three stages: avidin-FITC (fluoroscein isothiocyanate, 1:200 dilution) was followed by anti-avidin (1:100) and finally a mixture of avidin-FITC (1:200) and sheep anti-digoxigenin (1:10) conjugated to Texas red fluorochrome. Each of the detection compounds was diluted in 4× SSCT (50 µl per dipstick) and incubated in a waterbath at 37°C for 20 min. Between each detection stage, the dipsticks were washed three times with 4× SSCT (50 µl each). After the final washes, the dipsticks were removed from the tubes, mounted face up on regular glass slides in antifade solution (Vectashield, Vector Labs, Peterborough, UK) containing 2 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei. Nuclei were examined using DAPI, FITC and Texas red filters on a Zeiss Axioscope with a cooled CCD camera attachment (Photometrics) and IPLab software (Apple Computer, Inc.).

**DNA probes**

The probe pY353/B (Bishop et al., 1985) is a 1.5 kb fragment which hybridsizes specifically to a series of repetitive sequences distributed along the mouse Y chromosome. Probe DXSml5 (Nasir et al., 1990) is a 1.5 kb EcoR1 fragment from a long complex repeat unit located in multiple copies just below the centromere on the mouse X chromosome. The entire plasmids were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Lewes, E. Sussex, UK) and biotin-7-dATP (BRL) respectively using the BRL nick translation kit followed by purification through a Sephadex G50 column (Pharmacia) and ethanol precipitation. The resulting DNA pellet was resuspended, at a final concentration of 20 ng/µl, in 50 µl of Tris—EDTA solution and stored at -20°C.

**Preliminary experiments**

Experiments designed to minimize possible damage to the cell caused by PCR were conducted during the course of this study. Early attempts to perform FISH analysis on single cells prior to PCR were abandoned as they were prone to contamination (presumably due to both DNA and PCR products present in the reagents used for FISH). In order to minimize the possible disintegration of the blastomere nucleus prior to FISH, PCR procedures were performed with reduction of the number of cycles in the first-round amplification reaction (to 10, 15, 20 or 25) and lowering the denaturation temperature (to 72 or 80°C). However, these strategies did not allow efficient amplification of the β-globin sequence (data not shown).

A common problem with single-cell in-situ analysis is the loss
of, or damage to, the cell. Factors which might account for these losses include the difficulty of relocating the single cell on the dipstick after PCR, or real loss due to the method of fixation and performance of FISH analysis within a microcentrifuge tube. The problem of relocating the cell was, in part, solved by careful marking of the location of the cell on the dipstick. The problem of cell loss may be resolved by improved methods of fixation and pretreatment of the dipsticks with an adhesive, e.g. polylysine (Harper et al., 1994). Another potential problem in our experiments may be loss of DNA from the cell during PCR. This is suggested by the observation that nuclei were less intensely DAPI-stained following the PCR analysis. However, this did not affect the efficiency of the FISH analysis, which was the same for FISH following PCR as it was for FISH alone (see below).

Results

The protocol for cell recycling is illustrated in Figure 1. The results for six identical and consecutive experiments are shown in Tables I (PCR) and II (FISH). Figure 2 shows a typical example (experiment 2 in Tables I and II) of PCR amplification of the β-globin sequence in a series of 10 single blastomeres fixed to glass dipsticks followed by two examples of FISH analysis of sex chromosome constitution in the same blastomeres (a and c). In all cases, a blastomere from the same embryo (presumably having an identical genotype) was subjected to FISH analysis alone (Figure 2b and d). These controls were necessary to assess both the efficiency of FISH on single fixed blastomeres within a microcentrifuge tube and the accuracy of the FISH diagnosis obtained following PCR.

Out of 57 blastomeres fixed to dipsticks for PCR and subsequent FISH analysis, 40 (70%) gave a positive PCR amplification signal for the β-globin sequence (see Table I). Although there was some variation in the strength of the signal seen, in all experiments the amplification product obtained was the expected single band of 204 bp (Holding and Monk, 1989) and no non-specific amplification was seen. Contamination for control (cell-free) dipsticks was low (2/53).

One of the difficulties of this cell recycling procedure, and of single-cell FISH in general, is locating the cell on the dipstick following the PCR analysis. Of the 57 blastomeres analysed for the β-globin sequence, 43 (75%) were relocated for the FISH analysis. Of those relocated blastomeres, the efficiency of the FISH procedures following PCR was high (see Table II). In all, following β-globin PCR, 32 of the 43 blastomeres (74%) gave sex chromosome signals after FISH on the same single blastomere.

This efficiency compares favourably with the FISH-only control blastomeres: 29 of 39 (74%) single blastomeres, fixed individually on dipsticks, gave positive signals for the sex chromosomes and confirmed the sex diagnosed in the dual analysis. Clearly, the prior PCR analysis does not compromise the efficiency of the FISH procedures.

Discussion

One of the major disadvantages of preimplantation diagnosis, compared with prenatal diagnosis during pregnancy, is the relatively low efficiency of pregnancy associated with the in-vitro fertilization (IVF) procedure. The efficiency of pregnancy in IVF programmes for treatment of infertility is ~20–30% per transfer of 2–3 embryos (P. Serhal, personal communication). In as many

### Table I. Polymerase chain reaction amplification of the β-globin sequence

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dipstick + cell</th>
<th>Dipstick blank</th>
<th>RM control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
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<td>0</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>6</td>
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</tr>
<tr>
<td>5</td>
<td>3</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>17</td>
<td>51</td>
</tr>
</tbody>
</table>

RM = reaction mixture which contains primers, polymerase chain reaction buffer and enzyme but no DNA or dipstick.

### Table II. Fluorescent in-situ hybridization (FISH) analysis of X and Y chromosomes in single cells following the polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>FISH after PCR</th>
<th>FISH only</th>
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</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>7</td>
<td>7</td>
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<tr>
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</tr>
<tr>
<td>6</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>43</td>
</tr>
</tbody>
</table>

*One of the difficulties with the recycling procedure is location of the cell on the dipstick. In this series of experiments, 14 out of 57 (24%) of the fixed cells were not located following the PCR (see Results).
Recycling the single cell

Fig. 2. (Legend overleaf)
as 50% of cases, pregnancy failure is due to observable lethal chromosome abnormalities in the embryo (Boué et al., 1985). These abnormalities may pre-exist in the gametes or arise in the early embryo following fertilization. The frequency of chromosome abnormalities (particularly aneuploidy) is surprisingly high in oocytes: up to 30% (Plachot et al., 1988). In sperm, the frequency is lower, at -0.7% (Jacobs, 1992). It is not, therefore, surprising that human reproduction is comparatively inefficient. Many women in IVF programmes suffer considerable heartache and expense as a result of repeated pregnancy failure.

The pregnancy success rate in infertility treatment and preimplantation diagnosis programmes could be increased if embryos with chromosomal abnormalities were identified and eliminated from the transfer procedure. In the case of infertility, this could be achieved by single-cell biopsy of the embryo and chromosome analysis of that cell. However, for preimplantation diagnosis of genetic disease, diagnostic procedures are needed to give information on both chromosome constitution and the specific single-copy gene associated with the genetic disease under test.

Cell recycling, using PCR for the detection of specific gene sequences, followed by FISH to study specific chromosomes (e.g. sex chromosomes or common aneuploidies) in the single biopsied cell combines the power of these highly sensitive techniques to provide even greater potential for the efficiency and accuracy of preimplantation diagnosis. The current efficiency of cell recycling is 49% (blastomeres from which both a PCR and a FISH result have been obtained). This is the expected frequency of dual analysis when each analysis is occurring independently at ~70% efficiency (see below). However, it is reasonable to expect these efficiencies to increase with practice.

The efficiency of PCR amplification of the β-globin sequence in a fixed cell in these experiments was 70%. Previously, Holding and Monk (1989) had reported 83% efficiency. Presumably, the efficiency for single cells in suspension could be increased further to 90%, in line with other well-studied single-cell PCR systems (Liu et al., 1992; Chong et al., 1993). The nested amplification, which does not require the presence of the cell (‘solution PCR’), relies on amplified sequences diffusing away from the cell. Therefore, it may be possible to increase efficiency by proteinase K treatment to permeabilize the cell further prior to PCR. In addition, the potential of cell recycling could be extended using multiplex PCR with different sets of primers to examine between 5 and 10 specific gene sequences simultaneously.

Previously, PCR has been carried out on fixed tissue sections and cells to amplify low-copy-number sequences or viral DNA for visualization in situ (Haase et al., 1990; Nuovo et al., 1991; Weiss et al., 1991). We assume that our cell recycling procedure would also allow in-situ visualization of the attached product from the first round of PCR, using a labelled complementary probe, as well as providing the substrate for the second-round solution PCR analysis. In other words, it would provide two opportunities to look at the same single-copy sequence in the single cell. We are currently performing preliminary experiments using a digoxigenin-labelled β-haemoglobin probe to identify the first-round PCR product in situ within the single cell. Such highly sensitive in-situ procedures are becoming increasingly popular for gene mapping and may acquire a diagnostic role (particularly for single cells or mixed cell populations).

The efficiency of the FISH procedure in cell recycling was 74%. Control experiments of FISH alone on the single cell showed that prior PCR analysis did not appear to compromise the FISH efficiency. Preliminary experiments, using the same FISH analysis on blastocysts, and several modifications of the procedures, suggested that the FISH efficiency reported here may be improved up to ~95% (A. Thornhill, unpublished data). The FISH analysis can also be extended to employ up to seven specific chromosomal DNA probes (Ried et al., 1992) using combinatorial labelling.

If the efficiency of PCR performed for cell recycling from a fixed cell DNA template can be shown to be equal to that obtained using a single cell in solution, then the cell recycling PCR step has the considerable advantage that it offers the possibility of further information on the embryo under test. For example, a single blastomere biopsied from an embryo which tested normal for the gene under test can be analysed further for specific chromosomal abnormalities (e.g. the common trisomies, 13, 18, 21 etc.) using FISH. However, it must be borne in mind that the chromosome constitution of a single blastomere may not be representative of the whole embryo. Mosaicism for chromosomal abnormalities has been shown for individual blastomeres from cleavage stage human embryos (Delhanty et al., 1993; Munne et al., 1993). We are currently investigating chromosomal mosaicism in preimplantation embryos with these procedures.

The current 70% efficiency of PCR analysis and 74% efficiency of FISH analysis in the same single cell is quite remarkable and we are convinced that the efficiencies of these procedures of cell recycling will be improved still further to a level acceptable for diagnostic use.

We are currently performing the appropriate quality control experiments in human cells using large numbers of readily available buccal cells or lymphocytes for PCR detection of sickle-cell disease and Duchenne muscular dystrophy, followed by FISH detection of human chromosomes X, Y, 13, 18 and 21.
Acknowledgements

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References


