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Novel strategy with potential to identify developmentally competent IVF blastocysts

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BACKGROUND: Currently there are no markers fully predictive of developmental competence of human IVF embryos. The present study investigated a novel strategy involving blastocyst biopsy and DNA fingerprinting to link developmental competence with gene expression patterns. METHODS: Patient's blastocysts were biopsied to remove 8–20 trophectoderm (TE) cells for molecular analysis prior to transfer. Biopsy samples were amplified and gene expression was evaluated using microarrays. Sibling TE biopsies and cells from resulting offspring were subjected to DNA fingerprinting to identify which blastocyst(s) in the transfer cohort developed to term. RESULTS: Blastocyst biopsy did not appear to impair developmental competence. Comparative microarray analysis of cDNA from pooled 'viable' and 'non-viable' TE samples identified over 7000 transcripts expressed exclusively in 'viable' blastocysts. The most significant of these included transcripts involved in cell adhesion and cell communication, key processes that have been associated with mammalian implantation. DNA fingerprinting of three cohorts of sibling blastocysts identified those blastocyst(s) that produced term pregnancies. CONCLUSIONS: The combination of blastocyst biopsy, microarray gene expression profiling and DNA fingerprinting is a powerful tool to identify diagnostic markers of competence to develop to term. This strategy may be used to develop a rapid diagnostic assay or for refining existing criteria for the selection of the single most viable blastocyst among a cohort developing *in vitro*.

Keywords: blastocyst biopsy; DNA fingerprinting; microarrays; developmental competence; implantation

Introduction

Assisted reproductive technology (ART) developed over 30 years ago (Steptoe and Edwards, 1978) is now a well-established treatment for human infertility. Despite numerous advances in the practice of ART in the past three decades, implantation rates following the transfer of *in vitro* produced embryos have remained <30% (Edwards and Beard, 1999). Elective single-embryo transfer has been employed in several IVF clinics worldwide, with implantation rates approximating 50%. However comparative trials between elective single- and elective double-embryo transfers have been conducted in selected patient groups, and the success of live-birth is only similar when transfer of frozen embryos in subsequent frozen–thaw cycles is considered (Bergh, 2005; Pandian *et al*., 2005). Therefore the identification of the single most viable embryo among a cohort available for transfer is the ultimate aim to achieve a live-birth within the fresh IVF cycle.

Implantation failure is believed to be due to a range of factors including chromosomal abnormalities which are common in the female (15–61% of oocytes and embryos generated for ART) (Munne *et al*., 1998; Clouston *et al*., 2002; Plachot, 2003) and typically further increased in women of advanced reproductive age (Munne *et al*., 1995; Kuliev *et al*., 2003; Pellestor *et al*., 2003; Rosenbusch, 2004), asynchrony between embryo development and uterine receptivity (Norwitz *et al*., 2001; Margalioth *et al*., 2006) and factors associated with treatment interventions including the potential adverse effects of *in vitro* culture conditions (Jones, 2000) and the technique of embryo transfer (Levi Setti *et al*., 2003; Margalioth *et al*., 2006). However, a large proportion of the failed implantations must be ascribed to the embryo, as it is not uncommon for a single implantation to result from the transfer of two or more embryos of good morphological quality.

Despite numerous informative correlations for morphological, metabolic or biochemical criteria, no accurate predictive markers of the embryo's developmental capacity to term have been revealed. Morphological criteria are routinely used

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to select the 'best' embryos for transfer and these criteria have taken on greater importance, as the IVF community is moving towards a single embryo transfer policy (Bergh, 2005; Pandian *et al*., 2005). These criteria include pronuclear morphology and orientation at 17–19 h post-insemination (Scott and Smith, 1998; Tesarik and Greco, 1999), early cleavage at 25–27 h post-insemination (Shoukir *et al*., 1997; Sakkas *et al*., 2001), scoring systems for Days 2–3 embryos that incorporate embryo cleavage rates and synchrony of cleavage as well as the degree and pattern of fragmentation (Cummins *et al*., 1986; Puissant *et al*., 1987; Steer *et al*., 1992; Giorgetti *et al*., 1995; Alikani *et al*., 1999) and grading systems for blastocysts that incorporate the timing of blastocyst formation, the degree of expansion of the blastocyst and the appearance of the inner cell mass and trophectoderm (TE) cell lineages (Dokras *et al*., 1993; Schoolcraft *et al*., 1999). These criteria are currently the most predictive markers of embryo developmental competence and are widely used in many IVF laboratories. However these criteria are not fully predictive of an embryo's ability to develop to term in a receptive uterus, and combinations of the various scoring systems do not significantly improve the predictive value. Metabolic studies have found that both glucose uptake and glycolytic activity are unsuitable markers of blastocyst viability because the specific blastocysts that implant show a wide variance of metabolic activities (Jones *et al*., 2001). Biochemical markers of blastocyst viability such as CD30 ligand (Pera *et al*., 1997), LIF (Cullinan *et al*., 1996) and matrix metalloproteinases MMP-2 and MMP-9 (Turpeenniemi-Hujanen *et al*., 1995) have also been examined but these systems have lacked the required sensitivity to detect small quantities of these molecules in the spent embryo culture media. More recently, chromatographic assays of amino acid consumption (Brison *et al*., 2004), secretion of soluble human leukocyte antigen (HLA)-G molecules (Sher *et al*., 2004) and spectroscopic analysis of spent culture media (Katz-Jaffe *et al*., 2006; Brison *et al*., 2007) show some promise as a means of assessing embryo viability non-invasively but these methods are technically difficult and not sufficiently developed for routine application.

The power of all these observations has been frequently confounded by the practice of transferring multiple embryos which does not enable identification of the actual embryo responsible for the establishment of pregnancy. DNA fingerprinting is the ultimate form of biological identification but has not been used for the purposes of identifying the embryo(s) among a cohort transferred that is responsible for the pregnancy. Informative DNA fingerprints from low-template DNA samples can now be derived either by the analysis of highly polymorphic short tandem repeats (STRs) with high heterozygosity indices or using a large panel of single-nucleotide polymorphisms (SNPs). To aid pre-implantation genetic diagnosis (PGD) of single-gene disorders, linked STR markers have been used extensively to identify allele dropout (ADO), monitor external DNA contamination and indirectly track the inherited affected and non-affected parental alleles in biopsy samples from both cleavage and blastocyst stage embryos (Sermon *et al*., 2004; Kokkali *et al*., 2005). Furthermore, DNA fingerprinting of single blastomeres isolated from aneuploid cleavage-stage IVF embryos using a minimum of four STR markers has been shown to discriminate between sibling embryos (Katz *et al*., 2002). Hence, the application of DNA fingerprinting at the pre-implantation embryo stage and at birth could potentially identify the specific embryo(s), in a multiple transfer cohort, that is responsible for the establishment of pregnancy.

Pre-implantation development of mammalian embryos is governed by maternal transcripts until the time of zygotic genome activation. Recently, it has been demonstrated in the mouse that there are two independent and distinct waves of transcription. One which occurs at zygotic genome activation (one- to two-cell stage) and one which occurs at the morula-blastocyst transition (Hamatani *et al*., 2004; Wang *et al*., 2004). Therefore molecules important for implantation events may only be detectable at the blastocyst stage of development, precluding relevance of data from early cleavage stage embryos or blastomeres biopsied from these embryos. Molecular and immunohistochemical studies in humans, primates and laboratory animal species, together with data from experimental animal models such as gene knockout mice, have identified candidate growth factor ligands/receptors and adhesion molecules that are likely to play a key role in implantation and early placentation (Aplin, 1997). However, much of the data have been accumulated from studies of the endometrium, not the blastocyst, and the information regarding molecules involved in the early stages of blastocyst apposition and attachment for implantation is virtually non-existent (Simon *et al*., 2000). Even less information is available on the expression of molecules by human peri-implantation embryos. Further, although there are some published data on early gene expression through the molecular analysis of whole-human pre-implantation embryos (Adjaye *et al*., 1999, 2005; Holding *et al*., 2000; Dobson *et al*., 2004; Wells *et al*., 2005; Li *et al*., 2006), the data are difficult to relate to viability and implantation competence because the embryos were never transferred for confirmation.

The observed variance in viability among a cohort of a patient's embryos suggests that some *in vitro* cultured embryos are not receiving adequate autocrine and paracrine signalling to achieve full implantation and developmental competence. We hypothesize that the reduced viability of otherwise morphologically good quality ART embryos is a result of a transcriptome that is not compatible with implantation and/or growth to term. The present study describes a novel strategy that utilizes a combination of blastocyst biopsy (Kokkali *et al*., 2005, 2007), DNA fingerprinting (Katz *et al*., 2002) and microarray mRNA analysis (Jones *et al*., 2007) to identify viable blastocysts among the cohorts transferred to patients, with the ultimate aim of deriving a gene expression profile associated with blastocyst developmental competence to a healthy baby at term. This is the ultimate objective of ART and the infertile couples seeking treatment for infertility.

Materials and Methods

ART treatment

Patients undergoing infertility treatment at the Centre for Human Reproduction, Athens, Greece in 2003 whose ovarian ultrasound scan indicated the development of a large number of follicles in response to controlled ovarian hyperstimulation (Pantos *et al.*, 1994) were approached to provide informed consent to participate in this study. Patients consented to transfer at the blastocyst stage of development with TE biopsy prior to transfer. The study was approved by the First Regional Health and Welfare System of Attica (PESYAT), Department of Research and Development Ethics Committee. Molecular analysis of the RNA generated from biopsied material was approved by the Monash University Standing Committee on Ethics in Research Involving Humans, Clayton, Australia (SCERH 2005/272ED).

Following their retrieval, oocytes were cultured and fertilized as described previously (Jones *et al.*, 1998). According to the policy of the Centre for Human Reproduction, if a patient has more than 15 zygotes, a proportion are frozen at the pronucleate stage using a slow cooling protocol (Testart *et al.*, 1986) for use in a subsequent cycle if required. Zygotes were cultured to Day 3 in 10 μl microdrops of G1.3 culture medium (Vitrolife, Goteborg, Sweden) and then transferred to 20 μl microdrops of G2.3 (Vitrolife) for culture to the blastocyst stage (Jones *et al.*, 1998) under standard incubation conditions.

TE biopsy

Early on Day 5 after oocyte retrieval, embryos were assessed for blastocyst development. TE biopsy of approximately 8–20 cells was performed on all blastocysts regardless of the degree of expansion as described previously (Kokkali *et al.*, 2005). In brief, this involved making a small hole in the zona pellucida opposite the inner cell mass using a non-contact laser (Zilos-tk, Hamilton Thorne Biosciences, Beverley, MA, USA) and allowing the TE to herniate through the hole during a period of 1–4 h pre-incubation. Herniating TE was aspirated into an embryo biopsy pipette and TE was teased out of the zona opening using a combination of suction and gentle mechanical pulling. Once 8–20 TE cells had been aspirated into the biopsy pipette, the TE was dissected free of the blastocyst mass by laser ablation (ZILOS-tk). All TE biopsy samples were immediately transferred to PCR tubes containing lysis buffer (0.8% Igepal, MP Biomedicals, Co., Irvine, CA, USA; 5 mM DTT, Gibco BRL, Invitrogen Corporation; 1 U/μl rRNAsin, Promega Corporation, Madison, WI, USA) and frozen at −80°C to preserve the DNA/RNA fraction. Biopsied blastocysts were transferred to fresh microdrops of G2.3 and cultured singly overnight prior to transfer on Day 6 after oocyte retrieval. All biopsied blastocysts were re-assessed on Day 6 prior to transfer and 1 to 4 blastocysts were selected for transfer on the basis of the number of blastocysts available, the medical history and the patient's age and wishes and in accordance with the assisted reproduction regulations in Greece in 2003. It should be noted that the number of blastocysts transferred was determined by consultation between doctor and patient and was not dictated by participation in the research study. Biopsied blastocysts were selected for transfer in preference to non-biopsied blastocysts. Blastocysts were transferred to the patient using a Wallace catheter and remaining blastocysts were frozen (Jones *et al.*, 1998).

Clinical outcomes

Pregnancy was determined by serum βhCG levels ≥50 IU/l on Day 16 after oocyte retrieval and confirmed by demonstration of a gestational sac by ultrasound scan performed 4 weeks after embryo transfer. Pregnancy was defined as the presence of a fetal heart beat beyond 7 weeks gestation. Pregnancies resulting from biopsied blastocysts and the normality of fetal development were confirmed in follow-up ultrasounds during the first and second trimesters. Where possible, cord blood or a cheek buccal cell sample was kept from all resultant offspring for the purposes of identifying which of the transferred sibling blastocysts developed to term.

Isolation of genomic DNA

Genomic DNA was purified from offspring cell samples using a Qiagen DNA Extraction Kit (Qiagen). Genomic DNA samples were sent to Melbourne on dry ice and stored at −80°C prior to molecular analysis.

Generation of TE cDNA libraries

TE biopsy lysates were shipped to Melbourne on dry ice and stored at −80°C prior to molecular processing. Exponential or linear amplification of the poly A^+ fraction to generate T7-tagged cDNA was performed as described previously (Jones *et al.*, 2007). For exponential amplification, single biopsy samples, or samples pooled to represent eight biopsied blastocysts, were subjected to first- and second-strand cDNA synthesis using the SMART III cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. For linear amplification, an aliquot of 200 μl of Picopure extraction buffer (Arcturus Bioscience Inc., Mountain View, CA, USA) was added to a 40 μl pooled sample representing eight biopsied blastocysts and cDNA generated following two rounds of synthesis using the RiboAmp HS RNA Amplification Kit (Arcturus Bioscience Inc.).

Microarray analysis

Biotinylated cRNA for microarray analysis was generated from T7-tagged cDNA by *in vitro* transcription using the Codelink Expression Assay Reagent Kit (GE Healthcare Biosciences, Piscataway, NJ, USA) and purified using a QIAGEN RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The quantity and purity of the cRNA were determined by UV spectrophotometry at 260 and 280 nm. A total of 10 μg of labelled cRNA was hybridized to CodeLink^{TM} Human Whole Genome Bioarrays printed with discovery probes for 54 840 transcripts (GE Healthcare Biosciences) (Jones *et al.*, 2007). Following hybridization and washing, arrays were scanned on a GenePix Array Scanner (Molecular Devices Co., Sunnyvale, CA, USA) and images were captured as TIF files. Codelink Expression Analysis version 4.2 software (GE Healthcare Biosciences) was used to analyse images for each slide. Spots with intensities below that of the negative control (absence of an oligonucleotide probe) were excluded, as were those with irregular shapes or near-background intensity or oligonucleotides masked as part of the quality control process during manufacture. Spot quality and signal intensities were exported to Genespring compatible report format. The Codelink Expression Analysis output was loaded into Genespring GX 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) and values below 0.01 were set to 0.01 with per chip normalization to the 50th percentile and per gene normalization to the median and these data were used for all comparisons and analyses. A principal components analysis using Genespring software based on all genes was applied to all microarrays generated from individual TE biopsies to identify any samples that showed significant variability and to determine discrimination between groups of blastocysts. Genespring GX 7.3.1 Bioscript Library 2.2 Biological Pathways analysis was used to identify the probes associated with particular GO biological processes represented on the microarrays that were significantly over-represented at the 5% level within the list of probes identified as being expressed uniquely by viable blastocysts.

Table I. Primers used for DNA fingerprinting

Gene/STR	Primer sequence (5′–3′)	Size range (bp)	Repeat type
LAMP2	6-fam-TGTTCTACCGTGGATTTGTACTT AGGTCAAATATGCAGCAATGCCA	∼140	$(TG)_n$
SREB3	6-fam-CCTGTAATCTAGGCACCTTT TTGAATTGTCACTTCTGAGAGA	∼200	$(TC)_n$
BASP1	6-fam-CTAATTCCGACCAAACCGTAA TGATGGGACAACATCCTGTAT	∼250	$(TC)_n$
HNRPC	6-fam-ACGATCTGCAGCGGAGATGT CCTGATACACGCTGACATTT	∼190	$(CCT)_n$
AMELX/Y	6-fam-CCCTCCGCTCTGTAAAGAATAGTG ATCAGAGCTTAAACTGGGAAGCTG	105, 110	N/A
HPRT	hex-ATGCCACAGATAATACACATCCCC CTCTCCAGAATAGTTAGATGTAGGTAT	268–296	$(TCTA)_n$
D13S258	hex-ACCTGCCAAATTTTACCAGG GACAGAGAGAGGGAATAAACC	231–267	$(AGAT)_n$
D18S535	ned-CAGCAAACTTCATGTGACAAAAGC CAATGGTAACCTACTATTTACGTC	455–500	$(GAAA)_n$
D18S386	hex-TGAGTCAGGAGAATCACTTGGAAC CTCTTCCATGAAGTAGCTAAGCAG	330–400	$(AGAT)_n$
D21S11	6-fam-TATGTGAGTCAATTCCCCAAGTGA GTTGTATTAGTCAATGTTCTCCAG	214–242	$(TCTA)_n$
D21S1413	hex-TTGCAGGGAAACCACAGTT TCCTTGGAATAAATTCCCGG	152–184	$(ATAG)_n$
D21S1442	hex-CACTGCAGACGGCATGAACTTCC CCAGAATCACATGAGCCAATTCC	225–261	$(ATAG)_n$

DNA fingerprinting

Fluorescent PCR (F-PCR) based DNA fingerprinting was performed using either the TE cDNA library or a fraction of the TE cell biopsy lysate as a starting template. The DNA fingerprint from each sibling blastocyst was matched to the DNA fingerprint(s) generated from the cell sample of any resulting offspring to identify the specific blastocyst(s) that implanted and developed to term.

For cDNA fingerprinting, genes that were expressed by the TE of a single pool of eight blastocysts known to have implanted (classified as viable) and a single pool of eight blastocysts from young patients who had undergone few or no previous assisted reproduction cycles with tubal disease or male factor as the aetiology of infertility that failed to implant (classified as non-viable) were analysed to identify genes expressed in common. These genes were screened against the H-Invitational Database (www.h-invitational.jp) to identify cDNAs containing STR sequences suitable for analysis. A panel of 24 cDNAs that were expressed at high levels in both microarrays were identified as potential candidates for cDNA fingerprinting. Oligonucleotide primers spanning the polymorphic repeat regions of each gene (Table I) were designed specifically to exonic sequences so that they could amplify the equivalent region in genomic DNA from any resulting babies.

For genomic DNA fingerprinting, half the volume of the TE cell lysate was subjected to whole-genome amplification (WGA) using Genomiphi V2 DNA amplification kit (GE Healthcare). The volume of the TE cell lysate was reduced to 1 μl using a vacuum concentrator. The resulting lysate was mixed with 9 μl of Genomiphi sample buffer, denatured at 95°C for 3 min and then cooled on ice. Following the addition of 9 μl of reaction buffer and 1 μl of enzyme mix, the WGA reaction (20 μl) was subsequently incubated at 30°C for 2 h and then inactivated at 65°C for 10 min and stored at −20°C prior to F-PCR analysis. DNA fingerprinting of the WGA product was based on the analysis of a panel of chromosomal STR markers (Table I).

F-PCR and genescan analysis

F-PCR amplification was performed in 25 μl of PCR reaction mix containing 2.5 μl of 10× *Taq* PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl and 25 mM $MgCl_2$), 0.5 μl of 10 mM dNTP, 0.5 μl *Taq* polymerase (5 U/μl) (Roche Applied Science, Indianapolis, IN, USA), 18.5 μl molecular biology grade water (Scientifix P/L, Australia), 1 μl of each of 10 μM 6-FAM labelled forward primer and 10 μM unlabelled reverse primer (Sigma-Proligo, Australia) and either 2 μl of the TE cDNA library or 2 μl of the WGA product as the DNA template. A total of 35 thermal cycles were performed consisting of denaturation for 45 s at 94°C, annealing for 45 s at 50–60°C and extension for 45 s at 72°C. A 1 μl aliquot of the F-PCR reaction was mixed with 1 μl of formamide containing Genescan 500 ROX size standards (Applied Biosystems, Foster City, CA, USA). Samples were denatured at 95°C for 3 min, placed on ice and 1 μl subjected to capillary electrophoresis in POP4 resin on the ABI Prism 3100 DNA Sequencer. Fluorescent bands were analysed and sized by Genescan software (Applied Biosystems) and the number of allelic repeats calculated for each STR sequence.

Results

Strategy to identify viable blastocysts

The strategy devised to identify viable human blastocysts and potentially reveal genes associated with viability is schematically represented in Fig. 1. Embryos produced for an ART cycle were grown to the blastocyst stage and a TE biopsy was performed to remove a small number of cells. The poly A^+ mRNA fraction from the biopsied material was subsequently used to generate cDNA expression libraries for downstream molecular analyses. Biopsied blastocysts were then transferred to establish a pregnancy and once the baby was born, cord blood or a buccal cell sample was collected. Comparison of DNA fingerprints derived from the blastocyst biopsy samples and the baby's cells therefore identifies which of the transferred blastocysts produced the pregnancy. This approach minimized maternal effects because sibling blastocysts transferred to the same uterine environment

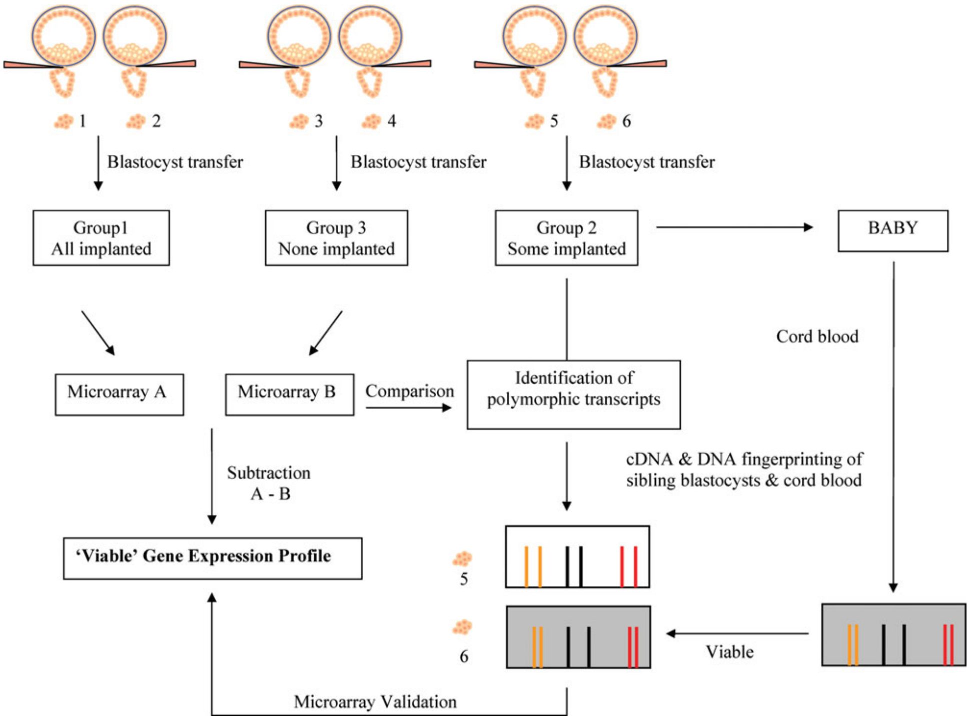

Figure 1: Strategy for identifying developmentally competent IVF embryos using a combination of blastocyst biopsy, microarray analysis and DNA fingerprinting.

should have the same opportunity to implant. When all of the blastocyst(s) transferred implanted and produced term pregnancies, the blastocysts were classified as viable (all implanted, Group 1). When some of the blastocyst(s) transferred implanted and produced a term pregnancy, the origin of which could clearly be identified by DNA fingerprinting, these blastocysts were classified as viable and the ones that did not match by DNA fingerprinting were classified definitively as non-viable (some implanted, Group 2). When none of the blastocyst(s) transferred implanted, the blastocysts were classified as non-viable or the uterus was deemed to be non-receptive (none implanted, Group 3). The availability of cDNA libraries from the viable and definitively non-viable blastocysts provides a means to identify genes associated with implantation and developmental competence using microarray expression profiling.

Blastocyst biopsy and clinical outcomes

There were 48 patients recruited for blastocyst biopsy and all patients produced at least one blastocyst by Day 5. Approximately, 8–20 TE cells were biopsied from 153 blastocysts, some of which were cryopreserved for a later treatment cycle.

The biopsy technique did not appear to impact on the blastocyst's ability to re-form a blastocoele cavity and continue to grow and hatch from the zona pellucida, as all blastocysts had a clearly visible blastocoele cavity within 1–2 h of biopsy and all blastocysts had a larger diameter on Day 6 compared with Day 5 (Fig. 2). In a small number of blastocysts, usually early blastocysts, few TE cells herniated from the hole in the zona pellucida following several hours incubation after laser-assisted hatching (Fig. 2B and C). At all instances, it was possible to apply suction around the opening and encourage a sufficient number of cells into the biopsy pipette using a combination of suction and gentle mechanical pulling. The ease of biopsy, however, was far greater for those blastocysts that had herniated a significant number of cells during the pre-incubation period (Fig. 2A). There were no blastocysts early on Day 5 that could not be biopsied later in the day and no blastocysts failed to re-form a blastocoele cavity following biopsy. No blastocyst showed major signs of morphological damage at the light microscopic level although occasional debris and individual TE cells could be identified in the perivitelline space and this material was always excluded from the re-sealed blastocyst and often remained within the empty zona pellucida when the blastocyst had completely hatched (data not shown). Transfer of biopsied blastocysts resulted in a similar pregnancy rate (52%) to that following transfer of non-biopsied blastocysts to patients not participating in the trial during the same time period ($n = 50$ patients; pregnancy rate 46%). Implantation rates were significantly higher following transfer of biopsied blastocysts (35 versus 19%; $P < 0.004$). However the validity of this comparison is questionable because patients were not randomly allocated to the biopsy or non-biopsy groups.

All 48 patients proceeded to blastocyst transfer of one or more blastocysts. Twenty-five patients became pregnant, with evidence of at least one fetal heart on ultrasound scan. The implantation outcome for all 48 patients is summarized in Table II. In seven patients (Group 1), all transferred blastocysts implanted indicating the blastocysts were all viable. In 18 patients (Group 2), some blastocysts implanted, whereas others did not, giving a powerful internal control for uterine

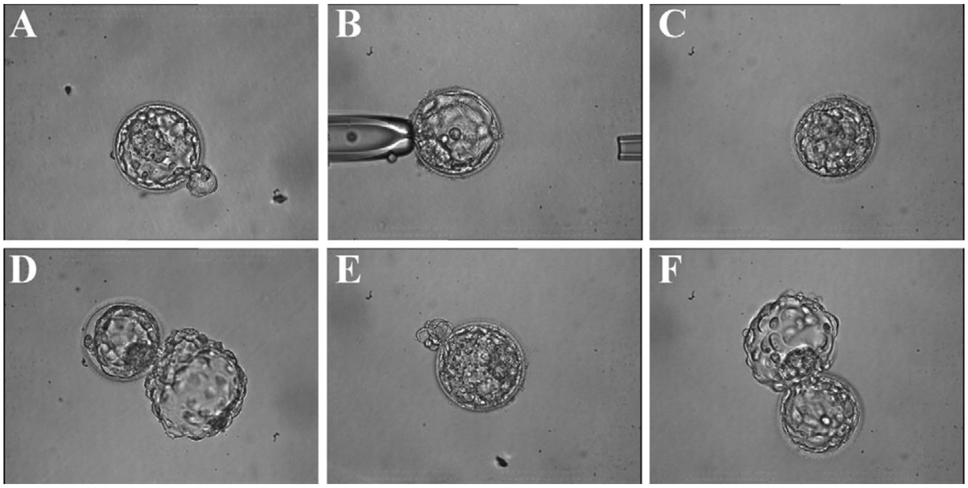

Figure 2: Micrographs of a sibling cohort of blastocysts pre- and post-TE biopsy that resulted in two live-births following blastocyst transfer. (**A–C**) Sibling blastocysts on Day 5 post-zona opening and immediately prior to TE biopsy. Note that no significant herniation of TE cells from the opened zonae in blastocysts (B and C). (**D–F**) Sibling blastocysts on Day 6 post-biopsy and culture overnight and immediately prior to transfer. Note that blastocysts (B and C) required more invasive suction to enable biopsy of sufficient cells and did not show any morphological evidence of damage or growth inhibition.

Table II. Summary of clinical outcome following transfer of biopsied blastocysts to 48 patients

Type of implantation	Number of patients	Number of biopsied blastocysts implanted	Number of biopsied blastocysts that failed to implant	Number of babies born
Group 1—all implanted	7	18	0	11
Group 2—some implanted	18	34	27	26
Group 3—none implanted	23	0	70	NA

receptivity. In the remaining 23 patients (Group 3), none of the blastocysts implanted, indicating that the blastocysts were either all non-viable or that the uterus was not receptive. A small number of patients from Group 2 undertook fetal reduction to reduce a multiple pregnancy, which meant that the blastocyst(s) resulting in the offspring could be identified by DNA fingerprinting but not all the blastocysts transferred could be definitively diagnosed as viable or non-viable. In total, 37 healthy babies were delivered.

Microarray analysis of cDNA libraries from biopsied TE

In order to identify candidate genes associated with viability, we have performed comparative microarray expression analysis using separate pools of TE biopsies from Group 1 and Group 3. Pools of eight TE biopsies were chosen to provide a representative cRNA template for microarray analysis and to reduce the effects of any heterogeneity between blastocysts. Group 1 blastocysts were those in which all blastocysts transferred implanted in the uterus and established ongoing pregnancies. Group 3 blastocysts were chosen from patients in which all blastocysts transferred failed to implant and also in whom implantation failure rather than endometrial deficiency was the more likely cause of infertility. Accordingly, blastocysts from young patients who had undergone few or no previous assisted reproduction cycles and had tubal disease or male factor as the aetiology of infertility were selected to generate pools of Group 3 blastocysts. Two pools of Group 1 blastocysts and two pools of Group 3 blastocysts were subsequently generated. One pool from each group was subjected to linear amplification and the other to exponential amplification and cRNA products were analysed on whole-genome arrays. Both types of amplification procedures were used to maximize the identification of genes expressed by such a small number of TE cells in the biopsy sample in the event that one of the procedures was less efficient or showed some degree of bias in the amplified cDNA. Comparison of Group 1 and Group 3 microarray profiles identified similarities and differences in gene expression profiles. A total of 44 947 gene transcripts were commonly expressed in TE. Comparison of Group 1 and Group 3 results identified 7317 and 1896 transcripts, respectively, which were unique. Of the 7317 transcripts identified to be expressed uniquely by viable embryos, 4208 (58%) were detected in the microarrays generated by both linear and exponential amplification techniques. Genespring analysis of the GO biological processes overrepresented in these 7317 transcripts compared with the total number of probes on the array identified cell adhesion, cell communication, cellular metabolic processes and response to stimuli (Table III) as the most significant ($P < 0.05$).

In an attempt to refine the 7317 transcripts associated with viability, cDNA expression libraries were generated by exponential amplification of the TE biopsy RNA from eight

Table III. Gene ontology biological pathways over-represented in the gene list expressed uniquely by viable blastocysts

	Subcategory	*P*-value
Cell adhesion	Homophilic cell adhesion	1.2×10^{-4}
	Calcium-independent cell adhesion	1.3×10^{-2}
	Neuron adhesion	3.5×10^{-2}
	Calcium-dependent cell adhesion	4.1×10^{-2}
Cell communication	Cell–cell signalling	
	Synaptic transmission	1.7×10^{-4}
	Nerve ensheathment	1.4×10^{-2}
	Signal transduction	
	Adenylate cyclase activation	1.0×10^{-2}
	G-protein signalling	1.4×10^{-2}
	Transmembrane receptor protein tyrosine kinase activation	1.9×10^{-2}
	Acetylcholine receptor signalling	1.9×10^{-2}
	Glutamate signalling pathway	3.0×10^{-2}
	Cell surface receptor-linked signal transduction	3.3×10^{-2}
	Activation of MAPK activity	4.1×10^{-2}
Cellular metabolic process	Positive regulation of interleukin-13 biosynthesis	2.4×10^{-3}
	Positive regulation of interleukin-6 biosynthesis	8.5×10^{-3}
	Alanyl-tRNA aminoacylation	1.9×10^{-2}
	Cyclic nucleotide metabolism	3.5×10^{-2}
Response to stimuli	Defence response to bacteria	5.6×10^{-3}

individual blastocysts that implanted (Group 1) and 19 individual blastocysts that failed to implant (Group 3). Microarray analysis of these 27 TE cDNA libraries identified a significantly lower number of transcripts (10 222 ± 6614) compared with the four pooled samples (43 255 ± 10 009), indicating that the individual cDNA libraries derived from 8–20 TE cells were not completely representative of global gene expression. Nevertheless, the individual TE biopsy data were subjected to principle components analysis to identify any trends in the gene expression patterns that could discriminate between Group 1 and Group 3 blastocysts. Clustering of Group 1 and of Group 3 blastocysts on the basis of trends defined by principal components X (9.3% variance), Y (7.1% variance) and Z (5.5% variance) was indeed found, although there was a small degree of overlap between the two groups (Fig. 3).

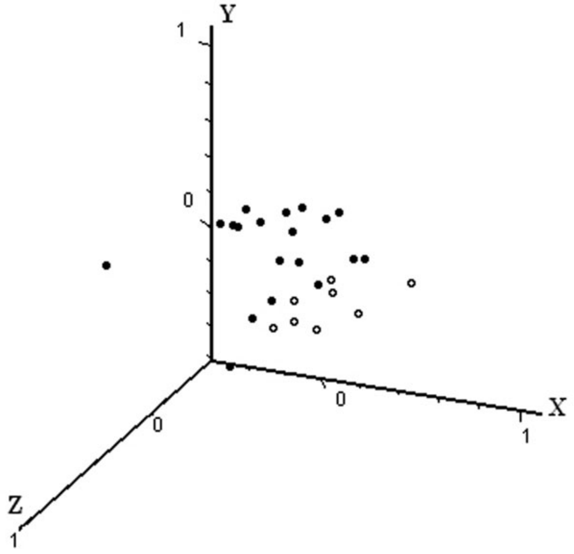

Figure 3: Principal components analysis of gene expression in Group 1 and Group 3 blastocysts.
Individual Group 1 ($n = 8$) and Group 3 blastocysts ($n = 19$) are represented by open and closed circles, respectively. Differences in gene expression trends between the two groups are defined by principal components X (9.3% variance), Y (7.1% variance) and Z (5.5% variance).

DNA fingerprinting to define viable and non-viable blastocysts transferred to the same uterus (Group 2)

To unequivocally discriminate between viable and non-viable blastocysts, we first investigated the possibility of using the information provided by the cDNA libraries generated from the pooled Group 1 and Group 3 TE biopsy microarrays to define polymorphic cDNAs for DNA fingerprinting. The 44 947 transcripts expressed in common from Group 1 and Group 3 blastocysts were compared with the H-invitational database (http://www.jbirc.aist.go.jp/hinv/index.jsp) to identify transcripts with STR sequences in their exonic regions. A list of 24 polymorphic genes expressed at relatively high levels in both Group 1 and Group 3 TE was identified as initial candidate transcripts for fingerprint analysis. Analysis of the polymorphic nature of the STR region of these 24 transcripts across sibling blastocysts identified only four genes (*LAMP2, SCEB3, BASP1* and *HNRPC*) with allelic heterozygosity indices of >0.5 that were suitable for unique discrimination. Eight cohorts of sibling blastocysts from Group 2 that had previously been fully processed to cDNA libraries together with the corresponding offspring cell sample were analysed. The resulting DNA fingerprints derived from the four genes listed earlier (data not shown) were not sufficiently informative in all cases to uniquely identify the embryonic origin of the baby.

As an alternative strategy, DNA fingerprinting of the genomic DNA fraction of the TE biopsy samples combined with WGA was investigated. For this purpose, we used TE biopsy lysates from an additional three sibling blastocyst cohorts from Group 2 that had not been previously processed to cDNA libraries. Half of the sibling blastocyst TE biopsy

Table IV. DNA fingerprinting of TE samples from sibling-transferred blastocysts and cells from resulting offspring

Sample	Amelogenin	HPRT	D13S258	D18S386	D18S535	D21S11	D21S1442	D21S1413	Viable embryo
Patient 33									
TE 1	105/111	288/?	244/?	368/?	–	227/235	256/?	154/?	
TE 2	105	276/288	236/276	352/?	482/?	227/235	228/256	154/?	TE 2
TE 3	105/111	–	236/?	–	–	235/?	256/?	–	
Baby	105	276/288	236/276	352/368	478/482	227/235	228/256	154/154	
Patient 44									
TE 1	–	280/288	–	360/?	478/482	235/?	–	154/174	
TE 2	105	280/?	–	364/?	482/?	227/235	232/235	174/?	TE 2
TE 3	105	288/?	–	–	–	231/?	228/?	168/174	
TE 4	105	–	260/?	–	–	235/?	–	–	
Baby	105	280/280	264/268	364/372	478/482	227/235	232/236	174/174	
Patient 46									
TE 1	105/111	288/?	236/?	352/368	466/?	223/227	236/248	174/?	TE 1 (Baby B)
TE 2	–	–	–	–	–	–	248/?	–	TE 2 (Baby A)
TE 3	105	280/288	236/?	360/?	486/?	223/227	236/248	154/174	
TE 4	–	288/?	240/?	–	–	227/?	236/248	154/178	
Baby A	105	276/280	236/236	360/368	482/486	223/227	236/248	154/174	
Baby B	105/111	288/288	236/236	352/368	466/474	223/227	236/248	154/174	

The numbers represent allelic size calculated from Genescan analysis; '–', total amplification failure; '?', homozygous or allele drop out.

lysate was subjected to WGA, and amplified products together with the matching offspring cell sample(s) were subjected to DNA fingerprinting using the sexing amelogenin marker and seven STR markers which have heterozygosity indices of >0.8. The DNA fingerprinting outcomes for the three sibling blastocyst cohorts are summarized in Table IV, and the DNA fingerprint profiles of one of these sibling blastocyst families and the resultant offspring are depicted in Fig. 4.

Discussion

The hallmark of a successful ART program is a consistent and sustainable high pregnancy rate balanced by a low incidence of multiple gestations. Although several clinics have moved towards single-embryo transfers, a paradigm shift from multiple embryo to single-embryo transfers worldwide may be contingent on a reliable method to select the most viable embryo in the cohort with the ability to implant in a receptive uterus and develop to term. In the present study, we have developed and validated a new strategy on the basis of blastocyst biopsy and DNA fingerprinting that links blastocyst viability with gene expression profiles. The strategy was premised on previous observations that removal of 2–4 TE cells from early blastocysts for the purpose of PGD resulted in healthy babies unaffected with genetic disease (Kokkali *et al*., 2005; McArthur *et al*., 2005), indicating that TE biopsy was safe. The present study demonstrates that removal of approximately 8–20 TE cells from Day 5 human blastocysts, representing ~10–20% of the blastocyst mass (Dokras *et al*., 1993; Fong and Bongso, 1999), using a non-contact mode laser is compatible with implantation and normal development to term following blastocyst transfer on Day 6. Further, it is possible

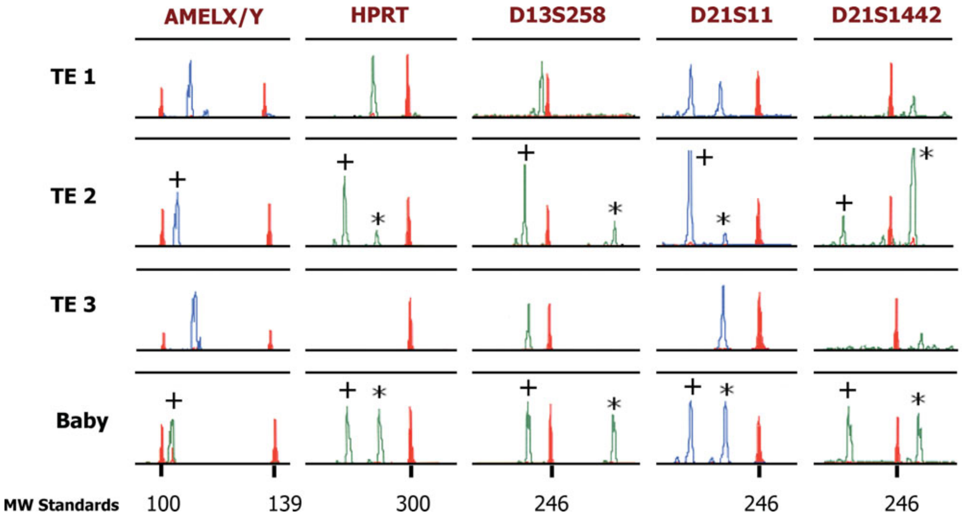

Figure 4: DNA fingerprint profiles from three sibling biopsied and transferred blastocysts (TE 1, 2 and 3) and the resulting baby (Patient 33). Results for markers *AMELX/AMELY*, *HPRT*, D13S258, D21S11 and D21S1442 are shown. Marker alleles are represented by open peaks, and molecular weight (MW) standards by closed peaks (sizes in nucleotides indicated below). Symbols '+' and '*' indicate matching alleles between sibling blastocyst TE 2 and the baby.

using the methodology described to biopsy all blastocysts on Day 5 regardless of developmental stage.

Pre-implantation development is governed by two independent and distinct waves of transcription in mammals, the second of which occurs at the morula–blastocyst transition (Hamatani *et al*., 2004; Wang *et al*., 2004). Therefore molecules important to implantation events may be expressed only at the blastocyst stage of development, emphasizing the relevance of gene expression data derived from TE biopsies. Prior to implantation, adhesion of the blastocyst is mediated by cell–cell interactions involving cell surface molecules expressed by TE cells and luminal uterine epithelial cells (Giudice, 1999). Molecular pathology and immunohistochemical studies in humans, primates and rodent species, together with data from experimental animals models such as gene knockout mice, have identified candidate growth factor ligands/receptors and homo- and heterophilic adhesion molecules expressed by the endometrium that are likely to play a key role in implantation and placentation (Aplin, 1997). However, from the perspective of the pre-implantation primate blastocyst, our understanding of the molecules involved in apposition and attachment is limited (Simon *et al*., 2000). Bioinformatics analysis of the gene families over-represented in the 7317 transcripts uniquely expressed in blastocysts that implanted identified cell adhesion as the most significant, in particular homophilic cell adhesion and calcium dependent and independent adhesion interactions. Thus, cell adhesion mediated by a combination of these molecules may be a critical event for attachment of the human blastocyst to the endometrium. The incorporation of additional gene expression profiles from pooled TE biopsy samples from blastocysts identified to be viable by DNA fingerprinting with the data generated in the present study followed by bioinformatics analyses will assist in refining genes and cellular pathways that are important in the implantation process in the human. Experiments to generate additional known viable TE biopsy samples are underway, as insufficient samples were available to both develop the described technology and perform sufficient replicate microarrays to identify genes that might be differentially expressed between viable and non-viable blastocysts. Genes differentially expressed may be equally or more important than those genes identified to be uniquely expressed. Furthermore the genes identified to be uniquely expressed by viable blastocysts are not provided, as it is likely that this list will be refined to a smaller list once more replicate microarrays have been completed. When the gene data set is refined, it will be possible to develop real-time PCR assays for specific genes expressed in single TE biopsy samples to select the most viable blastocyst in the cohort prior to transfer. Further, the identification of genes critical for implantation and developmental competence should expand the potential targets for contraception and expand our understanding of the basis of some aetiologies of female infertility.

The identification of a large set of genes associated with viability from pooled TE biopsy samples and separation of individual blastocysts that implanted from those that did not implant by principle components analysis demonstrate for the first time that viable blastocysts do express a different transcriptome that is compatible with implantation competence. Microarray analysis of individual TE biopsy samples however revealed far fewer genes expressed than when TE biopsy samples were pooled and arrayed, which is typical of this methodology when the starting template of mRNA is low (Jones *et al*., 2007). Nevertheless principle components analysis separated viable from non-viable blastocysts even though the combined principle components explained only 22% of the variance. This highlights the need to accumulate and analyse additional TE biopsy samples where the implantation outcome is known and this is currently being undertaken. Although a large number of transcripts were identified in pooled samples to be expressed only by viable embryos, these data rely on single replicates amplified by two different amplification procedures, so statistical analysis of genes expressed in common between viable and non-viable blastocysts could not be undertaken. Nevertheless, the majority of genes found to be uniquely expressed by viable blastocysts were detected in microarrays generated using both linear and exponential amplification providing a further level of confidence in the viability gene list.

The reason for the difference in gene expression between viable and non-viable blastocysts is not clear given that the cohort from a patient's ART cycle is grown in the same culture media under the same conditions *in vitro*. It has been proposed that chromosomal aneuploidy and/or the failure to properly activate the embryonic genome are two key inherent abnormalities that reduce the implantation potential of good quality IVF embryos (Braude *et al*., 1988; Margalioth *et al*. 2006). Meiosis I and II errors in the oocyte and mitotic errors in the first, second and third cleavage divisions of early embryos are common (Katz-Jaffe *et al*., 2004; Kuliev *et al*., 2003) and lead to the development of aneuploid and mosaic embryos, respectively. Depending on the chromosome(s) involved, these embryos either undergo early arrest or grow normally to the blastocyst stage (Magli *et al*., 2000). Therefore, altered gene dosage caused by either a monosomy or trisomy could potentially lead to an abnormal transcriptome culminating in implantation failure. However, the fingerprinting analysis for the three sibling cohorts of embryos using gene markers on chromosomes X, Y, 13, 18 and 21 did not reveal any evidence of aneuploidy even in blastocysts that failed to implant. On the other hand, the failure of euploid embryos to implant in a receptive uterus could be related to abnormalities in the oocytes from which they were derived. It is well known that within a cohort of mature oocytes retrieved from a standard stimulated cycle, only a proportion are developmentally competent. It has been proposed that during oocyte maturation, de-adenylation of specific transcripts required for early pre-implantation development in a time dependent manner is critical to maintain developmental competence (Brevini-Gandolfi *et al*., 1999; Zheng *et al*., 2005; Su *et al*., 2007). Thus, premature degradation of these transcripts could lead to a deficiency of developmental proteins that are required for proper activation of the embryonic genome and an altered transcriptome that is incompatible with implantation.

DNA fingerprinting has been a useful ART technique to assist in PGD of single-gene disorders (Sermon *et al*., 2004).

Incorporation of polymorphic STR markers linked to specific mutations into the multiplex PCR reaction enables the identification of DNA contamination and ADO and thus assists with making a more accurate diagnosis. More recently, PGD cases have also been performed where disease-free embryos are also selected for an HLA match using STR markers across the HLA region on chromosome 6 (Fiorentino *et al*., 2004; Verlinsky *et al*., 2004), so that if the pregnancy reaches term, the cord blood from the donor child can be used for transplantation in an attempt to save the life of a sick sibling. Apart from these applications, DNA fingerprinting has not been used to track transferred embryos to term for the purpose of identifying developmentally competent embryos. In the present study, a novel strategy is described whereby a portion of the blastocyst TE biopsy sample was used to obtain unique sibling embryo DNA fingerprints to identify the specific blastocyst(s) in the transfer cohort that implanted and developed to term. Some of the embryonic DNA fingerprints derived from the TE lysate were incomplete due to sporadic instances of total amplification failure and ADO for one or more STR markers, indicting that the DNA template in half the TE biopsy samples (4–10 cells) was limiting. Nevertheless, the combined STR profiles established from all markers were sufficiently informative to confidently identify the sibling blastocyst(s) that produced the offspring in all three families examined.

Tracking of transferred blastocysts to term to reveal the viable sibling blastocyst among a cohort transferred represents a new approach for refining existing criteria for selecting viable blastocysts for transfer. This approach is particularly powerful when multiple blastocysts are transferred and a pregnancy results because the uterus must have been receptive. In contrast, single-blastocyst transfers where DNA fingerprinting is not required to identify the origins of the pregnancy remain confounding in cases where the blastocyst does not implant, as it is not known whether this is due to the transfer of a non-viable blastocyst or a non-receptive uterus. Using the method of DNA fingerprinting TE biopsy samples developed in the present study, it will now be possible to conduct controlled experiments whereby the test and control can be conducted within one patient's cohort of embryos, i.e. the performance of two different culture media or two different culture conditions on pregnancy outcome can be analysed by transferring a biopsied blastocyst from each arm of the experiment, and the blastocyst(s) responsible for any resulting offspring can be identified by DNA fingerprinting. DNA fingerprinting will also assist in the re-assessment of classical morphological grading as a non-invasive tool for selecting the most viable blastocyst for transfer. For example, embryologists could record morphological observations of oocytes and resulting embryos up to the blastocyst stage and correlate the findings with those blastocysts that implanted or failed to implant in a receptive uterus. This approach may identify a set of morphological characteristics that is more highly predictive of blastocyst viability. DNA fingerprinting could also be used to improve the predictive value of other metabolic and biochemical non-invasive markers of viability. Furthermore, once additional TE biopsy samples have been accumulated and analysed, it will be possible to refine the set of viability genes to produce a smaller subset of genes more predictive of competence to develop to term. These molecular markers could then be measured using rapid PCR-based assays on TE biopsy samples to prospectively select the single most viable blastocyst among the cohort developing *in vitro* for transfer back to the patient.

If a simple non-invasive assay of blastocyst viability can be developed, it will permit the identification of the single most viable blastocyst among the cohort developing *in vitro* and enable single blastocyst transfers without a concomitant reduction in pregnancy rates. This would effectively reduce the incidence of multiple gestations, which is currently a priority in the field of ART medicine.

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