A novel single-cell DNA fingerprinting method successfully distinguishes sibling human embryos

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Objective: To validate a novel system for embryonic DNA fingerprinting which can reliably distinguish sibling embryos from each other.
Design: Prospective, randomized, and blinded study.
Setting: Academic center for reproductive medicine.
Patient(s): Blastomeres were obtained from discarded and transferred embryos from six patients undergoing IVF treatment.
Intervention(s): None.
Main Outcome Measure(s): Single lymphocytes from sibling cell lines and blastomeres from sibling day 3 human embryos were evaluated for accurate assignment of relationship using whole genome amplification and single-nucleotide polymorphism microarrays.
Result(s): Assignment of single-cell relationships was accomplished with 100% accuracy. We also observed complete agreement between the molecular karyotype and DNA fingerprint–based identification of embryos implanted in three clinical IVF cases after multiple embryo transfer.
Conclusion(s): These data demonstrate the first single-blastomere DNA fingerprinting technology capable of unequivocal discrimination of sibling human embryos. (Fertil Steril® 2010;94:477–84. ©2010 by American Society for Reproductive Medicine.)

Key Words: Blastomere, DNA fingerprinting, DNA microarray, single nucleotide polymorphism, whole genome amplification

Advances in assisted reproduction technologies (ART) have produced significant increases in delivery rates over the last 20 years. However, more than one-half of all babies born in the USA after in vitro fertilization (IVF) are from multiple gestations (1). Development of laboratory techniques which would allow precise assessment of the reproductive potential of individual embryos has therefore become the most important challenge of contemporary human embryology. One of the major methodologic limits to development of diagnostics of reproductive potential has been a need to know with absolute certainty if an embryo with a particular developmental, genomic, proteomic, or metabolomic marker actually implants and develops into a healthy infant. In the case of a multiple embryo transfer followed by the implantation of a single embryo (the most common scenario), it is not possible to know with certainty which embryo implanted. Single embryo transfer may overestimate the predictive value of a marker of interest as a result of the inability to control for numerous and significant variables, such as follicular stimulation, sperm quality, culture media, laboratory conditions, endometrial receptivity, and embryo transfer technique.

The ability to simultaneously transfer multiple embryos in a single transfer and then know with a high degree of certainty which embryos did and did not implant would be an enormously valuable investigative tool. When multiple embryos are transferred together, they are typically subjected to the same environmental conditions. That is, they come from a single cohort of developing follicles following a single gonadotropin stimulation, have been inseminated from a single prepared semen sample, develop in identical laboratory conditions, and undergo the exact same transfer into the same endometrium. This eliminates many of the nonembryonic variables which might affect outcome and provides the most precise opportunity for evaluating markers of, or interventions to improve, embryonic reproductive competence.

We have previously applied DNA fingerprinting techniques to discriminate unrelated individuals from a single somatic cell nuclear transfer–derived blastomere (2), but application of DNA fingerprinting in the IVF setting will require the ability to discriminate siblings from a single cell. The purpose of the present study was to develop and validate a highly reliable methodology for sibling embryo DNA fingerprinting which investigators could use in future studies evaluating markers or treatments which may affect reproductive competence.

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MATERIALS AND METHODS

Cell Lines

Human B lymphocytes were obtained from 18 individuals (parents and four offspring from each of three families) through the Coriell Cell Repository (CCR, Camden, NJ) (repository nos. GM13113, GM13114, GM13118, GM13119, GM13120, GM10858, GM10859, GM11870, GM11871, GM11872, GM11875, GM07554, GM07554, GM07440, GM07441, GM07555, and GM07556). Cell lines were cultured as recommended by the supplier (CCR). Single cells were removed using a 100 μm stripper tip and pipette (Midatlantic Diagnostics, Mount Laurel, NJ) under a dissecting microscope and placed in a nuclease-free 0.2 mL polymerase chain reaction (PCR) tube (Ambion, Austin, TX) in a volume of 1 μL medium for subsequent whole genome amplification (WGA). Genomic DNA (gDNA) was also obtained from each cell line as described below.

Embryos

Two sets of embryonic cells were obtained for this study. The first set comprised 72 blastomeres which were derived from 12 abnormally developing day 3 embryos (not suitable for transfer) donated by three IVF patients. Forty-seven of these blastomeres were evaluated by microarray analysis. The second set of cells was obtained from 13 embryos from three IVF patients as part of an ongoing clinical trial to determine the predictive value of single-nucleotide polymorphism (SNP) microarray–based 24-chromosome aneuploidy screening. Individual embryos were placed into calcium/magnesium–free human tubal fluid–Hepes media (Sage In Vitro Fertilization, Trumbull, CT) for embryo biopsy. Embryos were positioned so that a nucleated cell was adjacent to the anticipated biopsy site. A 25–30 μm hole was opened in the zona pellucida with a series of 3–5 single pulses from an infrared 1.48 μm diode laser using a 1 ms pulse duration at 100 % power (Hamilton-Thorne Research, Beverly, MA). Nucleated blastomeres were removed through the opening by applying gentle pressure with a blastomere biopsy pipette (Humagen, Charlottesville, VA) to the adjacent intact zona pellucida to eject individual blastomeres. Before processing, the nuclear status of isolated blastomeres from each embryo was verified by light microscopy using Hoffman optics (Nikon). Blastomeres were placed into PCR tubes as described above for lymphocytes. Discarded follicular fluid and sperm or peripheral whole blood were also obtained to develop parental genotypes after isolation of gDNA as described below. All material was collected with patient consent and under Institutional Review Board (IRB) approval.

Chorionic Villi

Dilatation and curettage procedures were performed as a result of clinical pregnancy loss. Chorionic villi were dissected from products of conception (POC) and used to isolated gDNA as described below. A conventional G-banding karyotype report from a commercial laboratory or a comparative genomic hybridization (CGH) report (see method below) was also obtained. All material was collected with patient consent and under IRB approval.

Genomic and WGA DNA

Five to ten milliliters of follicular fluid with resuspended cellular debris, between 3 and 5 million excess sperm, 10 mL peripheral whole blood, approximately 5×10^6 cells from cell lines, or dissected villi from POC were used to isolate gDNA using the QIAamp DNeasy Tissue kit as recommended for cell cultures (Qiagen Inc., Valencia, CA). Lysis was conducted with the addition of 2-mercaptoethanol (Fisher Scientific, Pittsburgh, PA) to the lysis buffer for sperm. Single-cell WGA was performed using a Sigma WGA4 GenomePlex Whole Genome Amplification Kit (Sigma Aldrich, St. Louis, MO). The WGA DNA was purified using GenElute PCR purification columns as recommended by the supplier (Sigma Aldrich). Genomic DNA isolation and WGA yields were calculated from the concentration as determined using a Nanodrop spectrophotometer (Nanodrop Inc., Wilmington, DE).

Microarrays

NspI or StyI GeneChip Mapping 500K microarrays were used as recommended by the supplier (Affymetrix Inc., Santa Clara, CA). The dynamic model mapping algorithm (3) was used to assign a confidence score measuring the reliability of each genotype call made (Gtype version 4.1; Affymetrix). The SNP calls were exported as a text file and imported into Excel (Microsoft, Redmond, WA) to determine the overall concordance between samples with and without the use of informative SNPs (potential for sibling discrimination) based on parental gDNA genotypes and mendelian inheritance rules. Molecular karyotyping was performed by copy number analysis of the microarray data using the Copy Number Analysis Tool (CNAT) version 4.0.1 (Affymetrix).

Comparative Genomic Hybridization

Genomic DNA was extracted from chorionic villi and fluorescently labeled using nick-translation. The CGH probes were prepared and washed as previously described (4). The fluorescence ratios (green/red) for at least ten of each autosome and seven of each sex chromosome were obtained per slide. The CGH profiles were compared with a dynamic standard reference interval based on an average of normal cases (5). The standard reference interval was scaled automatically to fit the individual test case. The mean ratio profile of each case with 99.9% confidence was compared with the average ratio profile of the normal cases with similar confidence intervals. The genetic diagnosis was assigned using results obtained at the 99.9% confidence interval. A positive finding was considered to be when the confidence intervals of the patient profile and normal averaged profile did not overlap. Digital image analysis was performed using a Cytovision Probe system and high-resolution CGH software (Applied Imaging Corp., Santa Clara, CA).

Statistical Analyses

Receiver operating characteristic (ROC) curves, sensitivity and specificity, box-and-whisker plots, and standard deviations were calculated using Analyse-It (Analyse-It, Leeds, UK) for Microsoft Excel. Student *t* test was also used for