Trophectoderm DNA fingerprinting by quantitative real-time PCR successfully distinguishes sibling human embryos

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Abstract

Purpose To validate a novel and more practical system for trophectoderm DNA fingerprinting which reliably distinguishes sibling embryos from each other.

Methods In this prospective and blinded study two-cell and 5-cell samples from commercially available sibling cell lines and excess DNA from trophectoderm biopsies of sibling human blastocysts were evaluated for accurate assignment of relationship using qPCR-based allelic discrimination from 40 single nucleotide polymorphisms (SNPs) with low allele frequency variation and high heterozygosity.

Results Cell samples with self relationships averaged 95.1 ± 5.9 % similarity. Sibling relationships averaged 57.2 ± 5.9 % similarity for all 40 SNPs, and 40.8 ± 8.2 % similarity for the 25 informative SNPs. Assignment of relationships was accomplished with 100 % accuracy for cell lines and embryos.

Conclusions These data demonstrate the first trophectoderm qPCR-based DNA fingerprinting technology capable of

unequivocal discrimination of sibling human embryos. This methodology will empower research and development of new markers of, and interventions that influence embryonic reproductive potential.

Keywords Trophectoderm · DNA fingerprinting · DNA microarray · Single nucleotide polymorphism

Introduction

The ability to identify the oocyte or embryo with neonatal potential remains one of the greatest challenges in reproductive medicine [1]. One hurdle to identifying new markers of reproductive competence has been the difficulty with obtaining an appropriate control group since implantation depends upon a significant number of embryo independent known and unknown variables. A potential solution has been proposed which involves the ability to track which embryo implanted after a multiple embryo transfer by using DNA fingerprinting [2]. More specifically, tracking which embryo implanted would allow paired analysis of putative markers within sibling oocytes and embryos. Those oocytes/embryos with proven reproductive potential (case) could be compared to sibling, co-transferred oocytes/embryos with proven lack of reproductive potential (control), and eliminate all patient and laboratory specific variables from the comparison. This may represent an extremely powerful approach to finding and validating new markers of reproductive competence. In fact, there are now a number of preliminary studies involving the application of DNA fingerprinting to help determine the impact of embryo biopsy [3] or oocyte vitrification [4] on reproductive competence, and to characterize the predictive value of cumulus cell gene expression profiles [5], telomere DNA content [6], or mitochondrial DNA copy number [7] for reproductive potential.

Capsule A method of quantitative real-time PCR that distinguishes sibling cell lines and embryos was developed. This inexpensive and powerful technology represents a new more practical solution to support the validation of markers and the evaluation of interventions that may influence embryonic reproductive competence.

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Accurate DNA fingerprinting and embryo tracking methodologies have been developed and include using either the 1st polar body [8], or a single blastomere or trophectoderm biopsy [9], and either newborn DNA from buccal cells or cell-free fetal DNA from maternal circulation [10]. Each method demonstrated 100 % accuracy in discrimination between sibling and self relationships in order to successfully determine which embryo(s) implanted. However, the high costs and specialized capital equipment associated with the necessary components of these methods, namely whole genome amplification (WGA) and single nucleotide polymorphism (SNP) microarrays [2], could prevent more widespread application. An alternative to WGA and microarray analysis, such as quantitative real-time (q) PCR, may represent a less expensive and therefore more practical methodology for general use in IVF research. Indeed, DNA fingerprinting of large quantities of cells using qPCR of 40 SNPs has already been reported [11]. However, this methodology has not been applied to limited starting material which is necessary when considering its application to DNA fingerprinting of preimplantation stage embryos. Therefore, we set out to develop and validate the application of qPCR-based DNA fingerprinting to limited starting material as an important new and more practical tool for distinguishing sibling human embryos in the IVF research setting.

Materials and methods

Experimental design

A set of 40 previously published SNPs with high heterozygosity and low allele frequency variation were utilized throughout this study [11]. These 40 SNPs were originally characterized against ~2,070 individuals from 40 populations. The estimated probability that siblings will have identical genotypes for these 40 SNPs is 0.5 raised to the power of the number of SNPs used (40) since all of these markers have alleles close to 50:50 in the population. Therefore, the likelihood of observing identity of two full siblings is approximately 1 in 1×10^{12} . An experimental estimate of likelihood of siblings with full identity was obtained using publically available and newly obtained data from 405 samples. A leave-one-out analysis was performed to determine the minimum number of informative SNPs needed to successfully distinguish siblings.

The evaluation of sibling discrimination precision of these 40 SNPs on limiting starting material was conducted in 2 phases. In phase 1, cell lines were used to model the clinical test situation where a trophectoderm biopsy (2 to 5 cells) from an embryo would be compared to a genomic DNA sample obtained from a buccal swab from a newborn. Phase 2 involved evaluating the accuracy of performing DNA

fingerprinting on embryos from 3 cases to demonstrate utility in a relevant tissue type of the intended application.

Sibling identity likelihood

Genotype data for the 40 SNPs were compiled from 405 samples as follows. A total of 90 DNA samples were obtained upon request from Life Technologies Inc. (Foster City, CA), including 45 Caucasian and 45 African American individuals (Coriell Cell Repository, Camden, NJ; human variation panel—Caucasian panel and African American panel). Since data for only 38 of the 40 SNPs were available for these samples, a “no result” genotype was assigned to the remaining 2 (rs447818 and rs7205345). Genotypes from 270 HapMap samples (Caucasian, Han Chinese, Yoruban, and Japanese) were downloaded from www.hapmap.org. Missing or inconsistent data was similarly replaced with a “no result” genotype in all cases. Genotypes from the 5 siblings described in the following cell line DNA section were included to model the situation where siblings mate. DNA samples from a random selection of 40 individuals (20 couples) undergoing IVF treatment at the Reproductive Medicine Associates of New Jersey (Morristown, NJ) were also included, and isolated from peripheral blood as described in the following cell line DNA section, and genotyped as described in the following qPCR section.

Cell line DNA

Human B-lymphocytes from CEPH/Utah pedigree #1347 including maternal, paternal, and 5 sibling offspring cell lines were obtained from the Coriell Cell Repository (CCR, Camden, NJ) (Repository numbers GM10858, GM10859, GM11870, GM11871, GM11872, GM11873, and GM11875). Cell lines were cultured as recommended by the supplier (CCR). Approximately 5×10^6 cells from each cell line were used to isolate genomic (g) DNA using the QIAamp DNeasy Tissue kit as recommended for cell cultures (QIAGEN Inc., Valencia, CA). Each of the 40 SNPs was evaluated in each of the DNA samples by qPCR as described below. Those SNPs in which one parent was heterozygous and one parent was homozygous or both were heterozygous were considered informative since Mendelian rules of biallelic inheritance allow for unique genotypes in the offspring. The same process was used for determining informative SNPs from the parental DNA associated with the embryo samples described below. Two-cell ($n=12$) or 5-cell ($n=12$) samples to model the number of cells obtained from trophectoderm biopsy were removed from 3 of the sibling cell lines (GM11870, GM11872, GM11873) using a 100 μm stripper tip and pipet (MidAtlantic Diagnostics), under a dissecting microscope, and placed into a nuclease-free 0.2 ml PCR tube (Ambion Inc., Austin, TX) in a volume of 1 μl media for

subsequent qPCR. Cell line samples were processed by alkaline lysis as previously described [12]. All cell line DNA samples were blinded before qPCR analysis as described below.

Embryonic DNA

Excess embryonic trophectoderm DNA PCR product from 3 cases of qPCR based 24 chromosome aneuploidy screening [13] where DNA from products of conception was also available, were used as template for qPCR based DNA fingerprinting. The first case (maternal age 41) involved the transfer of one blastocyst. Excess DNA from an additional 5 cryopreserved embryos was also evaluated by qPCR. The second case (maternal age 36.5) involved the transfer of one blastocyst. Excess DNA from an additional 4 cryopreserved embryos was also evaluated by qPCR. The 3rd case (maternal age 32.2) involved transfer of 2 blastocysts where only one implanted. Products of conception were obtained for qPCR based analysis of the 40 SNPs for comparison with embryonic DNA in all cases. All embryonic DNA samples were blinded before qPCR analysis as described below.

qPCR

For the 2-cell and 5-cell cell line and the embryonic DNA, multiplex amplification of the 40 previously identified SNP loci [11] was performed using the corresponding TaqMan SNP Genotyping Assays and TaqMan PreAmplification Master Mix as recommended by the supplier (Applied Biosystems Inc., Foster City, CA), in a 50 μ l reaction volume for 18 cycles using a 2720 thermocycler (Applied Biosystems Inc.). TaqMan assay IDs can be found in supplementary Table 1. For either the multiplex amplification PCR product or the purified genomic DNA, real-time PCR for allelic discrimination was then performed in duplicate reactions for each of the individual 40 loci. Each reaction used the individual TaqMan SNP Genotyping Assays, TaqMan Genotyping Master Mix (Applied Biosystems Inc.), a 5 μ l reaction volume, a 384-well plate, and a 7900 HT sequence detection system, as recommended by the supplier (Applied Biosystems Inc.). Informative SNP genotypes were compared between samples to determine the levels of similarity and standard deviations from the mean were calculated using Microsoft Excel (Microsoft Inc., Redmond, WA). Distributions were plotted using Analyse-It software for Excel (Analyse-It Inc., Leeds, UK).

Ethics

All research material was obtained with patient consent and under Institutional Review Board approval.

Results

Discrimination of self and sibling cell lines

Allelic discrimination analysis of 40 SNPs in the parental DNA from CEPH/Utah pedigree #1347 identified 25 SNPs which were informative. Blinded similarities of isolated total genomic DNA genotypes of the 5 sibling offspring were clearly distinguishable as either self (replicates) or sibling relationships. All samples with self relationships (replicates) were 100 % similar ($n=5$), while sibling relationships averaged 61.5 ± 8.1 % similarity when evaluating all 40 SNPs, and 41.9 ± 8.6 % similarity when evaluating only the 25 informative SNPs ($n=10$).

To demonstrate applicability to limited numbers of cells from the embryo, 2- ($n=12$) or 5-cell samples ($n=12$) from each of 3 of the sibling cell lines were blinded and evaluated. The 2-cell and 5-cell sample genotypes were compared to the genotypes observed in purified genomic DNA from large quantities of the same or sibling cells in order to model the situation where blastocyst biopsy DNA (2 to 5 cells) is compared to purified genomic DNA from the conceptus. Two-cell samples with self relationships averaged 95.1 ± 5.9 % similarity ($n=12$ comparisons) while sibling relationships averaged either 57.2 ± 5.9 % similarity for all 40 SNPs, or 40.8 ± 8.2 % similarity for the 25 informative SNPs ($n=24$ comparisons). Importantly, no overlap between the self and sibling distributions were observed with or without the use of parental information (Fig. 1). Five-cell samples with self relationships averaged 99.8 ± 0.7 % similarity ($n=12$ comparisons), while sibling relationships averaged either 60.6 ± 6.1 % similarity for all 40 SNPs, or 42.7 ± 8.4 % similarity for the 25 informative SNPs ($n=24$ comparisons). Again, no overlap between the self and sibling distributions were observed with or without the use of parental information (Fig. 1). Locus dropout (no amplification of either allele) was observed in 2.9 % (14/480) of the 2-cell sample assays and 0.2 % (1/480) of the 5-cell sample assays. Allele dropout (no amplification of one allele) was observed in 4.7 % (22/466) of the 2-cell sample assays, and 0.2 % (1/479) of the 5-cell sample assays. To define an applicable threshold for distinguishing genotype similarities predictive of a self or sibling relationship, the minimum similarity of a self relationship (83 %) was added to the maximum similarity of a sibling relationship (65 %) and divided by 2 to give the midpoint of 74 %.

Among the samples, the lowest heterozygosity rate was 0.2, which correlates to 8 total SNPs among the 40. As such, the rate at which two siblings will receive the same alleles from a given parent is 0.39. If both parents are heterozygous at 8 SNPs the rate at which two siblings will receive the same alleles from a given parent is 1.5×10^{-5} .

Fig. 1 DNA fingerprinting performance on limited starting amounts (2 or 5 cells) of sibling cell lines using a panel of 40 SNPs. Using only the 25 informative SNPs based upon parental genotypes improves separation but is not necessary for discrimination between self and sibling relationships

Discrimination of self, sibling, and unrelated human embryos

To demonstrate applicability to the relevant tissue type, excess trophectoderm DNA from 13 blastocysts (6 from one case, 5 from another, and 2 from the 3rd) were blinded, evaluated, and compared to DNA from products of conception from the 3 cases. Since cell lines were discriminated successfully without the use of parental information, embryos were evaluated using all 40 SNPs and the previously defined threshold of 74 %. Genotypes of the embryos predicted to be self relationships ($n=3$) were 100 % similar to the genotypes observed in the conceptuses (Fig. 2). Nine of the 10 remaining embryos were not transferred and were therefore known siblings, while 1 of the 10 was transferred together with an embryo with 100 % identity to the products of conception. These predicted and known sibling embryos averaged 59 ± 6.4 % similarity and unrelated embryos averaged 40.3 ± 7 % similarity when compared to the products of conception. No overlap between the self and sibling distributions was observed (Fig. 2). After decoding the blinded identities, the embryos with 100 %

identity were confirmed as transferred embryos. Locus dropout was observed in 0.4 % (2/520) of the embryo DNA assays.

Discussion

These results demonstrate the first robust qPCR based DNA fingerprinting method for distinguishing sibling human embryos. Locus and allele dropout were minimal in both the cell line samples and the excess embryonic DNA and did not impact the performance of DNA fingerprinting. For example, the lowest similarity of a self relationship occurred in a 2-cell sample with 17 % allele dropout, giving 83 % similarity. However, this was still sufficient given that the highest percent similarity of a sibling relationship was 65 %. The ability to discriminate sibling cells from either 2 cells, 5 cells, or excess DNA from trophectoderm biopsies was similar, such that a threshold of 74 % similarity was able to provide 100 % accuracy in assignments of self and sibling relationships. Furthermore, the use of parental information was not necessary since 100 % discrimination was achieved without incorporating selection of informative SNPs. Therefore, the additional time and expense of evaluating parental DNA can be avoided with this methodology in contrast to previously developed methods [8, 9].

In addition, the ability to selectively evaluate a specific subset of cases in an ongoing clinical trial will significantly reduce the costs associated with applying DNA fingerprinting to research in this setting. More specifically, many cases may not require the incorporation of DNA fingerprinting to determine embryo specific reproductive potential. For example, when no pregnancy occurs or when dizygotic twins result from a 2 embryo transfer, the reproductive potential of the embryos can be confirmed without DNA fingerprinting. Instead, DNA fingerprinting would only be necessary when a fewer number of those embryos transferred to one patient actually implanted. By demonstrating that DNA fingerprinting can be performed on normally discarded material (i.e. the

Fig. 2 Results of 40 SNP fingerprinting of excess DNA from 13 blastocyst trophectoderm biopsies from 3 cases compared to 3 corresponding products of conception samples

excess DNA available after completing aneuploidy screening), this method provides an opportunity to retrospectively select these specific cases where DNA fingerprinting is necessary and thus avoid the additional costs associated with prospectively performing DNA fingerprinting on all cases in a clinical trial.

In the clinical setting, the ability to perform genetic fingerprinting of the excess embryo DNA from aneuploidy screening cases also represents an important tool in the event of a suspected laboratory error. For example, if it is suspected that the incorrect embryo was selected for transfer, the genotypes of the fetus could be compared to genotypes of the cohort of embryos originally screened prior to the transfer.

The fact that excess DNA from clinical cases of aneuploidy screening could be used also provides a unique and powerful opportunity to control for this significant variable in a research setting. That is, the well documented and significant impact that aneuploidy has on reproductive competence could be controlled for in order to better identify aneuploidy independent markers of embryonic reproductive potential. For example, in the event that 2 euploid embryos were transferred and one implanted, the excess DNA from the transferred embryos could be evaluated and compared to newborn DNA to determine which embryo implanted. Subsequent comparison of the cumulus cell transcriptome or the spent media metabolome (normally discarded material) could then be evaluated for differences which may be predictive of reproductive potential. The differences identified between the two embryos could be considered as markers of reproductive potential that are independent of the contribution of aneuploidy since they were both originally identified as euploid.

In conclusion, this study has resulted in the validation of a new more cost effective method for tracking which embryo implanted after multiple embryo transfer using qPCR-based allelic discrimination of 40 SNPs. This method will be instrumental in the development and validation of new markers of reproductive potential by allowing for paired analysis of sibling oocytes and embryos within one patient. Future work incorporating this powerful and inexpensive qPCR-based DNA fingerprinting technology may provide a critical stimulus towards enhancing euploid embryo selection and improving the success of IVF.

References

1. Jones Jr HW. Seven roads traveled well and seven to be traveled more. *Fertil Steril*. 2011;95:853–6.
2. Scott Jr RT. Using the “omics” to assess the reproductive competence of individual embryos. Chicago: Midwest Reproductive Symposium; 2011.
3. Treff NR, Ferry KM, Zhao T, Su J, Forman EJ, Scott RT. Cleavage stage embryo biopsy significantly impairs embryonic reproductive potential while blastocyst biopsy does not: a novel paired analysis of cotransferred biopsied and non-biopsied sibling embryos. *Fertil Steril*. 2011;96:S2.
4. Forman EJ, Ferry KM, Benson MR, Campos J, Treff NR, Scott RT. Embryos derived from vitrified oocytes are not at an increased risk of aneuploidy and demonstrate excellent implantation rates: a paired, randomized controlled trial (RCT) in an infertile population. *Fertil Steril*. 2011;96:S44.
5. Treff NR, Su J, Lonczak A, Taylor D, Scott Jr RT. A subset of the cumulus cell transcriptome is predictive of euploid human oocyte reproductive potential. *Fertil Steril*. 2010;94:S34–S5.
6. Treff NR, Su J, Taylor D, Scott Jr RT. Telomere DNA deficiency is associated with development of human embryonic aneuploidy. *PLoS Genet*. 2011;7:e1002161.
7. Su J, Tao X, Baglione G, Treff NR, Scott RT. Mitochondrial DNA is significantly increased in aneuploid human embryos. *Fertil Steril*. 2010;94:S88–S9.
8. Treff NR, Su J, Kasabwala N, Tao X, Miller KA, Scott Jr RT. Robust embryo identification using first polar body single nucleotide polymorphism microarray-based DNA fingerprinting. *Fertil Steril*. 2010;93:2453–5.
9. Treff NR, Su J, Tao X, Miller KA, Levy B, Scott Jr RT. A novel single-cell DNA fingerprinting method successfully distinguishes sibling human embryos. *Fertil Steril*. 2009;94:477–84.
10. Treff NR, Tao X, Su J, Lonczak A, Northrop LE, Ruiz A, et al. Tracking embryo implantation using cell-free fetal DNA enriched from maternal circulation at 9 weeks gestation. *Mol Hum Reprod*. 2011;17:434–8.
11. Pakstis AJ, Speed WC, Kidd JR, Kidd KK. Candidate SNPs for a universal individual identification panel. *Hum Genet*. 2007;121:305–17.
12. Cui XF, Li HH, Goradia TM, Lange K, Kasasian Jr HH, Galas D, et al. Single-sperm typing: determination of genetic distance between the G gamma-globin and parathyroid hormone loci by using the polymerase chain reaction and allele-specific oligomers. *Proc Natl Acad Sci U S A*. 1989;86:9389–93.
13. Treff NR, Tao X, Ferry KM, Su J, Taylor D, Scott RT, Jr. Development and validation of an accurate quantitative real-time PCR based assay for human blastocyst comprehensive chromosomal aneuploidy screening fertility and sterility 2012;In Press.