

Comprehensive chromosome screening and gene expression analysis from the same biopsy in human preimplantation embryos

Diego Marin^{1,2,*†}, Yujue Wang^{3,†}, Xin Tao⁴, Richard T. Scott Jr¹, and Nathan R. Treff^{1,2}

¹Reproductive Medicine Associates of New Jersey, 140 Allen Road, Basking Ridge, NJ 07920, USA ²Thomas Jefferson College of Biomedical Sciences, Thomas Jefferson University, Philadelphia, PA 19107, USA ³Department of Genetics, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA ⁴The Foundation for Embryonic Competence, Basking Ridge, NJ 07920, USA

*Correspondence address. Reproductive Medicine Associates of New Jersey, Basking Ridge, NJ, USA. Tel: +908-350-7775; E-mail: dmarin@rmanj.com

Submitted on January 12, 2017; resubmitted on February 27, 2017; editorial decision on March 6, 2017; accepted on March 7, 2017

STUDY QUESTION: Can simultaneous comprehensive chromosome screening (CCS) and gene expression analysis be performed on the same biopsy of preimplantation human embryos?

SUMMARY ANSWER: For the first time, CCS and reliable gene expression analysis have been performed on the same human preimplantation embryo biopsy.

WHAT IS KNOWN ALREADY: A single trophectoderm (TE) biopsy is routinely used for many IVF programs offering CCS for selection of only chromosomally normal embryos for transfer. Although the gene expression profiling of human preimplantation embryos has been described, to date no protocol allows for simultaneous CCS and gene expression profiling from a single TE biopsy.

STUDY DESIGN, SIZE AND DURATION: This is a proof of concept and validation study structured in two phases. In Phase 1, cell lines were subjected to a novel protocol for combined CCS and gene expression analysis so as to validate the accuracy and reliability of the proposed protocol. In Phase 2, 20 donated human blastocysts were biopsied and processed with the proposed protocol in order to obtain an accurate CCS result and characterize their gene expression profiles using the same starting material.

PARTICIPANTS/MATERIALS, SETTING AND METHOD: A novel protocol coupling quantitative real-time PCR-based CCS and gene expression analysis using RT-PCR was designed for this study. Phase 1: six-cell aliquots of well-characterized fibroblast cell lines (GM00323, 46,XY and GM04435, 48,XY,+16,+21) were subjected to the proposed protocol. CCS results were compared with the known karyotypes for consistency, and gene expression levels were compared with levels of purified RNA from same cell lines for validation of reliable gene expression profiling. Phase 2: four biopsies were performed on 20 frozen human blastocysts previously diagnosed as trisomy 21 (10 embryos) and monosomy 21 (10 embryos) by CCS. All samples were processed with the proposed protocol and re-evaluated for concordance with the original CCS result. Their gene expression profiles were characterized and differential gene expression among embryos and early embryonic cell lineages was also evaluated.

MAIN RESULTS AND THE ROLE OF CHANCE: CCS results from cell lines showed 100% consistency with their known karyotypes. $\Delta\Delta C_t$ values of differential gene expression of four selected target genes from the cell lines GM4435 and GM0323 were comparable between six-cell aliquots and purified RNA (Collagen type I alpha-1 (*COL1A1*), $P = 0.54$; Fibroblast growth factor-5 (*FGF5*), $P = 0.11$; Laminin subunit beta-1 (*LAMB1*), $P = 1.00$ and Atlastin-1 (*ATL1*), $P = 0.23$). With respect to human blastocysts, 92% consistency was reported after comparing embryonic CCS results with previous diagnosis. A total of 30 genes from a human stem cell pluripotency panel were selected to evaluate gene expression in human embryos. Correlation coefficients of expression profiles from biopsies of the same embryo ($r = 0.96 \pm 0.03$ (standard deviation), $n = 45$) were significantly higher than when biopsies from unrelated embryos were evaluated ($r = 0.93 \pm 0.03$, $n = 945$) ($P < 0.0001$).

[†]Equal contribution.

Growth differentiation factor 3 (*GDF3*) was found to be significantly up-regulated in the inner cell mass (ICM), whereas Caudal type homeobox protein-2 (*CDX2*), Laminin subunit alpha-1 (*LAMA1*) and DNA methyltransferase 3-beta (*DNMT3B*) showed down-regulation in ICM compared with TE. Trisomy 21 embryos showed significant up-regulation of markers of cell differentiation (Cadherin-5 (*CDH5*) and Laminin subunit gamma-1 (*LAMC1*)), whereas monosomy 21 blastocysts showed higher expression of genes reported to be expressed in undifferentiated cells (Gamma-Aminobutyric Acid Type-A Receptor Beta3 Subunit (*GABRB3*) and *GDF3*).

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: Gene expression profiles of chromosomally normal embryos were not assessed due to restrictive access to euploid embryos for research. Nonetheless, the profile of blastocysts with single aneuploidies was characterized and compared. Only 30 target genes were analyzed for gene expression in this study. Increasing the number of target genes will provide a more comprehensive transcriptomic signature and reveal potential pathways paramount for embryonic competence and correct development.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first time that CCS and gene expression analysis have been performed on the same human preimplantation embryo biopsy. Further optimization of this protocol with other CCS platforms and inclusion of more target genes will provide innumerable research and clinical applications, such as discovery of biomarkers for embryonic reproductive potential and characterization of the transcriptomic signatures of embryos, potentially allowing for further embryo selection prior to embryo transfer and therefore improving outcomes.

STUDY FUNDING AND COMPETING INTERESTS: This study was funded by the Foundation for Embryonic Competence, Basking Ridge, NJ, USA. No conflicts of interests declared.

Key words: comprehensive chromosome screening / gene expression / preimplantation embryo / ART / trophectoderm biopsy

Introduction

Advancements in preimplantation genetic testing methodologies have led to significantly improved outcomes in IVF (Haapaniemi Kouru *et al.*, 2012; Tan *et al.*, 2013; Fiorentino *et al.*, 2014; Chen *et al.*, 2015; Chang *et al.*, 2016). Comprehensive chromosome screening (CCS) is now a commonly used strategy that allows physicians to select only euploid embryos for transfer (Fiorentino *et al.*, 2014; Chen *et al.*, 2015; Chang *et al.*, 2016). Although published randomized trials have demonstrated an improvement in implantation and delivery rates by selecting euploid embryos for transfer, many still fail to implant and progress to delivery (Forman *et al.*, 2012; Yang *et al.*, 2012; Scott *et al.*, 2013a).

Accumulating evidence in both human and mice has shown that the successful development and implantation of embryos may be highly dependent on the stable expression of many genes at the preimplantation stage of development (Constantinou *et al.*, 2015; Hasegawa *et al.*, 2015; Cheng *et al.*, 2016). For example, one recent study identified the expression of C-X-C Motif Chemokine Receptor 4 (*CXCR4*) in human trophectoderm (TE) cells as a potential biomarker of reproductive competence (Bao *et al.*, 2016). Since *CXCR4* is just one of the many genes involved in early embryo development, it is very likely that additional gene expression biomarkers can be identified.

Other groups have reported differences in gene expression between aneuploid and normal embryos on Day 3 of development *in vitro* (Bazrgar *et al.*, 2014; Chavez *et al.*, 2014), even suggesting a prediction model for aneuploidy based on morphokinetics and gene expression profiles (Vera-Rodriguez *et al.*, 2015). Although very promising results have been reported in these studies, the cells used for gene expression experiments were different from those used for CCS, given the absence of a methodology that could provide simultaneous analysis of gene expression and DNA constitution from the same sample. Thus, the reported observations in the aforementioned studies rely on the assumption that all cells from the same embryo have the same genetic constitution, which has been discovered not to be always the case, and

that genetic mosaicism is present among blastomeres from the same embryo (Munne *et al.*, 1994; Wells and Delhanty, 2000).

Therefore, one of the challenges associated with identification of transcriptional biomarkers of human embryonic reproductive potential is access to appropriate material. Given that the primary objective of new biomarkers is to enhance selection beyond conventional aneuploidy screening, the ideal situation would be to characterize gene expression within euploid embryos and then compare profiles from successful and unsuccessful embryos. In order to do this, the present study presents a new methodology that can simultaneously evaluate euploidy status and gene expression levels from the same TE biopsy from human blastocysts.

Several studies have focused on developing protocols that allow for simultaneous gene expression profiling and DNA sequencing or copy number variation detection (Xu *et al.*, 2008; Macaulay *et al.*, 2015; Hou *et al.*, 2016). Here, we propose a variation of a routinely used quantitative PCR (qPCR)-based CCS (Treff *et al.*, 2012) protocol to obtain a gene expression profile from the same embryo biopsy, by varying the lysis buffer and adding a reverse transcription step for cDNA syntheses of target genes. In Phase 1 of the study, the proof of principle of simultaneous CCS and gene expression analysis was validated using cell lines. In Phase 2, aneuploid human embryos were biopsied in order to further validate the technique. Differential gene expression among embryos and early embryonic cell lineages was also evaluated in this study, as well as differential expression profiles of human blastocysts diagnosed as trisomy 21 and monosomy 21 by CCS.

Materials and Methods

Design

This study was completed in two phases. In Phase 1, two well-characterized cell lines were used to model the amount of material from a TE biopsy and were subjected to the proposed protocol for combined

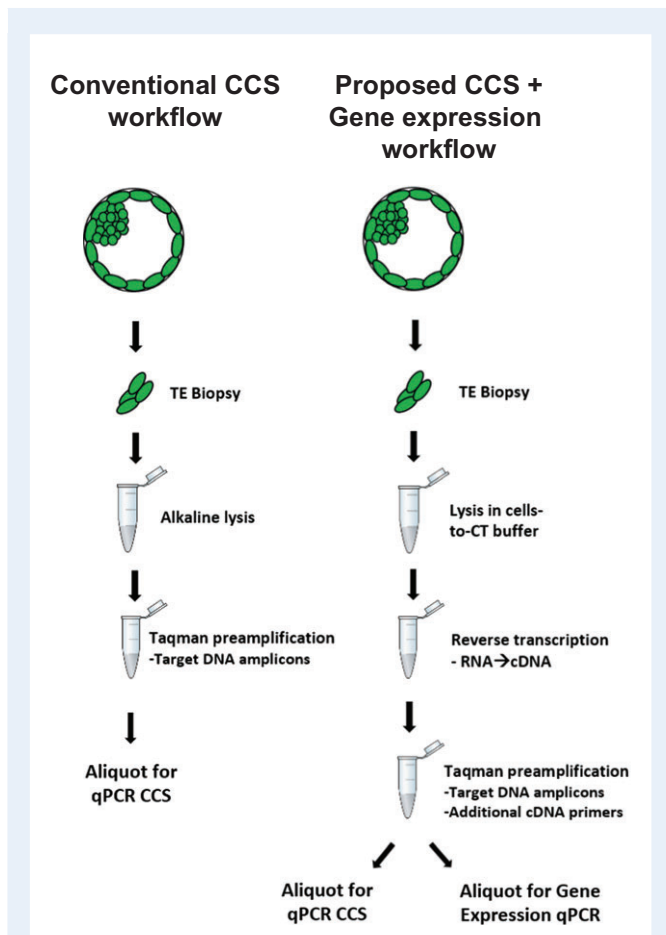


Figure 1 Comparison between the workflow of conventional comprehensive chromosome screening (CCS) on trophoctoderm (TE) biopsies and our proposed protocol with simultaneous gene expression profiling. Both protocols start with a TE biopsy of ~6–10 cells. In the CCS + gene expression protocol, lysis is performed with Single-cell-to-CT buffer (see Materials and Methods), and reverse transcription is performed immediately for cDNA syntheses of target genes. The preamplification step is present in both workflows, however, additional primers for the targeted cDNAs are added in CCS + Gene Expression. Finally, an aliquot is taken from each sample to perform CCS, and in the proposed protocol another aliquot from the same reaction tube is taken for quantitative PCR (qPCR) gene expression profiling.

CCS and gene expression analyses from the same cells (Fig. 1). Six-cell aliquot CCS results were compared with the known karyotypes, and gene expression levels were compared with levels observed in purified RNA from a large quantity of cells. In Phase 2, frozen human blastocysts previously diagnosed as aneuploid and available for research were rebiopsied, processed with the proposed protocol, re-evaluated for concordance with the original CCS result, and their gene expression profile was characterized using 30 selected genes from the Human Stem Cell Pluripotency Panel (ThermoFisher, MA, USA, Supplementary Table 1).

Ethical approval

Vitrified human blastocysts used in this study were approved for research under the institutional review board (IRB) approval # 20050731 with written informed patient consent.

Cell lines

Two established and stable fibroblast cell lines GM00323 (46,XY) and GM04435 (48,XY,+16,+21) were purchased from the Coriell Cell Repository (Camden, NJ, USA) and cultured as recommended by the supplier. Previous studies have suggested that the typical TE biopsy contains ~6 cells (Neal et al., 2016). To model this in cell lines, six cells were collected from cell culture under a dissected microscope using a 100- μ m stripper tip and pipet (Midatlantic Diagnostics, Mount Laurel, NJ, USA) in a nuclease-free 0.2 ml PCR tube (Ambion, Austin, TX, USA) with 1 μ l of cell culture media. Ten six-cell-aliquot samples from each cell line were used in subsequent qPCR analysis in order to mimic the starting material of a TE biopsy (Neal et al., 2016). In addition, purified RNA samples from each cell line were prepared by using Qiagen RNeasy Mini Kit (Qiagen, CA, USA) following the manufacturer's instructions. Concentration and purity of purified RNA was assessed with a NanoDrop 8000 8-sample spectrophotometer (ThermoFisher).

Embryos

Twenty frozen human blastocysts available for research were used in this study (IRB #20050731). All embryos were previously diagnosed by qPCR-based CCS as abnormal, 10 embryos with a single trisomy of chromosome 21 and 10 embryos with a single monosomy of chromosome 21. Embryos were thawed and incubated at 37°C with 5% oxygen tension for ~2 h before rebiopsy. Three TE and one ICM biopsy were taken per embryo as described previously (Scott et al., 2013b) and each was transferred to a 0.2-ml PCR tube.

Simultaneous CCS and gene expression profiling

Figure 1 shows the main steps of the protocol for simultaneous CCS and gene expression analysis, and how the workflow differs from conventional CCS. Cell lysis was performed with 9.7 μ l of Single-cell-to-CT buffer and 1 μ l of Superase RNase inhibitor (Single Cell Lysis Kit, ThermoFisher). Reverse transcription was performed using SuperScript[®] IV First-Strand Synthesis System (ThermoFisher). First, the primer pool containing the primers for all targeted genes was prepared and added to each sample. In Phase 1 of the study, the primer pool for reverse transcription was prepared by mixing five TaqMan gene expression assays of genes known to be expressed in human fibroblasts (ThermoFisher, Supplementary Table 2) and diluting with nuclease-free water to 0.2X. The primer pool for Phase 2 was prepared the same way, in this case mixing 30 selected TaqMan gene expression assays from the Human Stem Cell Pluripotency Panel (ThermoFisher, Supplementary Table 1). We selected these gene expression assays because their primers span more than one exon of the gene, which makes it unlikely that the assays will generate amplicons from genomic DNA and therefore will quantify exclusively mRNA expression. This was of special concern since we aim to obtain CCS results from the same sample reaction tube, and thus no DNase treatment is present in the protocol. Deoxynucleotides were added and samples were incubated at 65°C for 5 min. Next, 5X First-Strand Buffer, dithiothreitol and 10X Superscript IV Reverse Transcriptase (ThermoFisher) were added to each sample following the manufacturer's instructions. Samples were incubated at 55°C for 10 min and followed by a further incubation at 80°C for 10 min.

Preamplification

Multiplex amplification of both 96 genomic DNA loci for qPCR-based CCS (Treff et al., 2012) and 5 or 30 cDNA loci was performed using primer pools of TaqMan Copy Number and Gene Expression Assays. TaqMan Preamplification Master Mix was added to reach a final volume of 80 μ l. Samples were subjected to 18 cycles of preamplification as described

previously (Treff *et al.*, 2012) using an Applied Biosystems 2720 thermocycler. Real-time qPCR was performed in quadruplicate for each of the individual 96 loci for CCS and duplicate for the gene expression targets using TaqMan Gene Expression Master Mix (Applied Biosystems IL, USA), a 5- μ l reaction volume, 384-well plates and a 7900 HT sequence detection system, as recommended by the supplier (Applied Biosystems). A standard delta delta threshold cycle ($\Delta\Delta$ Ct) method was applied to determine the relative quantification of chromosomal copy number and gene expression level, as previously described (Treff *et al.*, 2012). All samples subjected to the proposed protocol were collected in ice and followed immediately by lysis and reverse transcription reactions in order to avoid introduction of additional variation due to RNA degradation. In addition, all experiments were performed with RNase-free equipment and work stations were treated with RNase decontamination solution.

All negative controls in all experiments consisted of equivalent aliquots of nuclease-free water. All detectors for all target genes showed Ct values above the set threshold, thus being categorized as undetected.

Data analysis

Data from gene expression experiments were analyzed using the RealTime Statminer (Integromics, Madison, WI, USA), version 20. Ct values above 36 were classified as undetected. Detectors with values below the detection limit in all samples were flagged and excluded from the analysis. Five samples presented values below the detection limit in 20% or more of the detectors and were excluded from the analyses. The reference genes used for normalization of Ct values were selected from among four reference genes (Actin beta (*ACTB*), Catenin beta-1 (*CTNNB1*), Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Raf-1 Proto-Oncogene, Serine/Threonine Kinase (*RAF1*), Supplementary Table 3) by the Statminer software based on the GeNorm score, a gene-stability measurement among all samples in each experiment.

Analyse-it for Microsoft Excel (version 2.20) software (Microsoft, CA, USA) was used for all additional statistical tests. The non-parametric Mann–Whitney *U* test was used to compare $\Delta\Delta$ Ct values between six-cell aliquots and bulk RNA samples. Pearson correlation coefficients of Δ Ct values for all target genes were calculated between samples, and a Student's *t*-test was performed to compare correlation coefficient means between samples from the same embryo or unrelated embryos. Student's *t*-test was also used to compare gene expression profiles of monosomy 21 versus trisomy 21 embryos. In this case, average Δ Ct values were calculated by subtracting experimental Ct values from an average Ct value of six endogenous controls (*ACTB*, *CTNNB1*, *GAPDH*, *RAF1*, 18S ribosomal RNA (*18S*), Eukaryotic Translation Elongation Factor one Alpha-1 (*EEF1A1*)). Since the detector for *CDH5* was not detected in any of the monosomy 21 samples, Δ Ct values for these samples were calculated by subtracting the average Δ Ct value of 6 endogenous controls from 40.

Results

Phase I—cell lines

First, we tested the combined CCS and gene expression analyses methodology in cell lines of known karyotype. We collected 10 six-cell-aliquot samples of human fibroblasts for each of the two cell lines (GM04435 and GM00323) in order to mimic the limited starting material of a TE biopsy. After performing the protocol, CCS results were all consistent with the expected karyotype of each cell line in all samples (Fig. 2, Supplementary Table 3), indicating that CCS is not compromised when coupled with a different lysis protocol and additional steps of reverse transcription for RNA expression profiling.

Next, a 20- μ l aliquot was taken from each sample containing the synthesized cDNA of five genes and was quantified using qPCR. Four

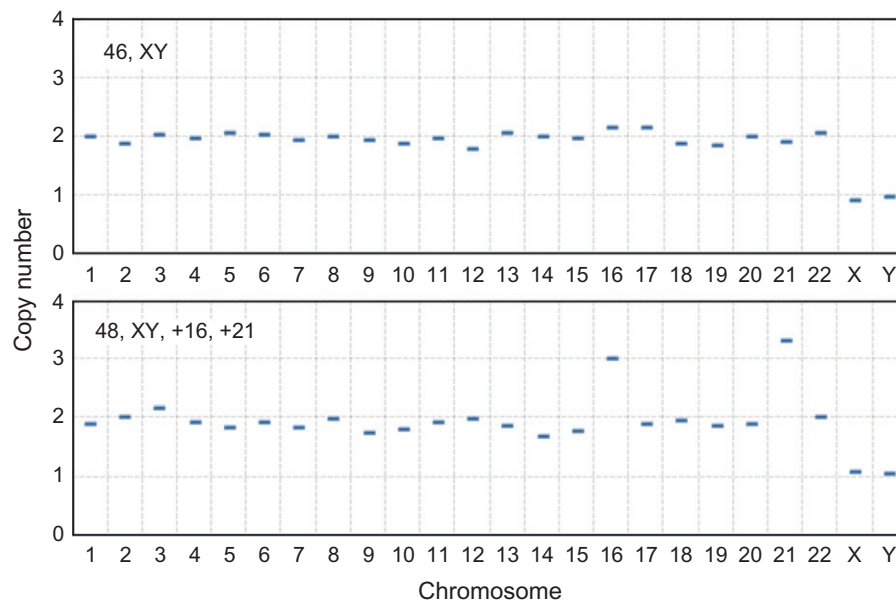


Figure 2 Representative chromosome copy number plots after CCS were performed in six-cell samples from two well-characterized fibroblast cell lines. Upper plot refers to a sample from GM0323 (46, XY, Coriell Repository) and the lower plot to a sample from GM04435 (48, XY, +16, +21, Coriell Repository).

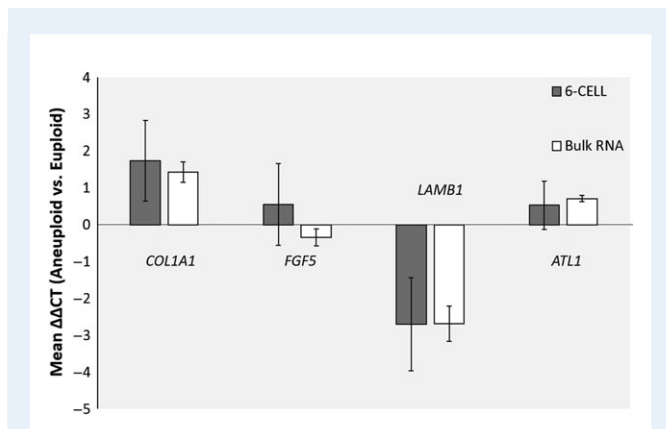


Figure 3 Differential gene expression between GM4435 (48, XY, +16, +21) and GM0323 (46, XY) fibroblast cell lines for four selected genes. Average $\Delta\Delta C_t$ values did not differ between six-cell aliquots and bulk RNA samples for any of the selected genes (collagen type I alpha-1 (*COL1A1*), $P = 0.54$; Fibroblast growth factor-5 (*FGF5*), $P = 0.11$; Laminin subunit beta-1 (*LAMB1*), $P = 1.00$ and Atlatin-1 (*ATL1*), $P = 0.23$).

of these genes were selected for this initial stage since previous experiments showed different expression levels between the two tested cell lines, therefore similar levels of differential expression were expected after gene expression profiling. The fifth gene was the housekeeping gene and endogenous control *GAPDH* commonly used for normalization. In addition, gene expression profiling was carried out in parallel on four samples of 'bulk' RNA (purified RNA from a large quantity of cells from each cell line) in order to validate that differential expression detected in six-cell aliquots was consistent with purified RNA. $\Delta\Delta C_t$ values of differential gene expression between the fibroblast lines GM4435 and GM0323 were comparable between six-cell aliquots and bulk RNA (*COL1A1*, $P = 0.54$; *FGF5*, $P = 0.11$; *LAMB1*, $P = 1.00$; *ATL1*, $P = 0.23$, Fig. 3), indicating that this protocol coupled with CCS results in reliable gene expression profiling, even with very small amounts of starting material (Fig. 3). Purified RNA from GM00323 showed a concentration of 14.68 ng/ μ l and a 260/280 nm ratio of 2.09, while for GM04435 these values were 33.87 ng/ μ l and 1.91, respectively. These were normalized to a final concentration of 1 ng/ μ l with nuclease-free water.

Phase 2—embryos

We next moved to test the protocol on human blastocyst biopsies. We obtained three TE biopsies and one ICM biopsy from 10 human embryos diagnosed with monosomy 21, and 10 with trisomy 21. After all samples were processed and a 25- μ l aliquot was taken from each sample for CCS, 71 samples generated a valid karyotype whereas 9 generated no result (unamplified or nonconcurrent). In addition, among the 71 samples with a valid karyotype, high consistency was obtained with the original diagnosis for each embryo (92%, 65/71). For those six samples found non-consistent with the previous diagnosis, three were reported as euploid (46,XY) and two were reported as having other types of aneuploidy (Supplementary Table 4) consistent with previous estimates of the frequency of mosaicism (Capalbo et al., 2016).

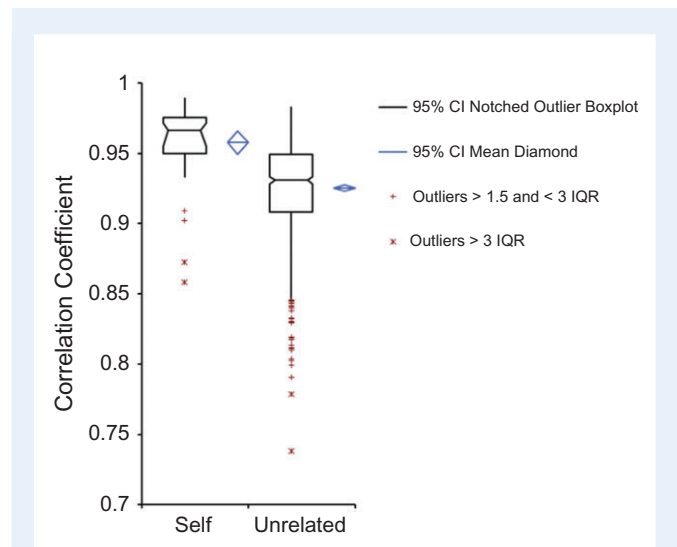


Figure 4 Gene expression profiles are more similar if embryo biopsies come from the same embryo. The Pearson's correlation coefficients of expression profiles from samples from the same human embryo ('Self', $r = 0.96 \pm 0.03$) were significantly higher than when samples from 'Unrelated' embryos were evaluated ($r = 0.93 \pm 0.03$) ($P < 0.0001$). IQR, interquartile range.

With respect to gene expression profiling, 30 genes related to pluripotency were evaluated (Adewumi et al., 2007) based on the idea that trisomy 21 embryos may have different profiles than monosomy 21 embryos, the well-established differences in their respective reproductive potential and that the ICM may have a profile unique from TE owing to their well-established differences in differentiation. After gene expression was quantified by qPCR, we compared gene expression profiles, first among samples from the same embryo and then among unrelated samples. The Pearson correlation coefficients (with standard deviation, SD) of expression profiles from samples from the same embryo ($r = 0.96 \pm 0.03$, $n = 45$) were significantly higher than when samples from unrelated embryos were evaluated ($r = 0.93 \pm 0.03$, $n = 945$) ($P < 0.0001$, Fig. 4). The fact that samples from the same embryo have more similar gene expression profiles than samples from different embryos provides further evidence that differences in gene expression obtained by this method were potentially capable of detecting biological changes in gene expression rather than random technical variation.

Furthermore, we performed a cluster analysis on all TE samples coming from 10 monosomy 21 embryos (Supplementary Fig. 1) and 10 trisomy 21 embryos (Supplementary Fig. 2). Surprisingly, the gene expression profiles of the selected target genes alone were sufficient to cluster together samples from the same embryo in seven cases of trisomy 21 embryos. Gene expression profiles of ICM and TE biopsies were evaluated in order to verify that our differential expression profiles of these two cell lineages were consistent with previous findings. Seven genes showed significant differential expression (Fig. 5, Supplementary Table 5), of which six genes showed significant down-regulation in the ICM (*CDX2*, $P = 0.00087$; *DNMT3B*, $P < 0.0001$; *GATA* binding protein 6 (*GATA6*), $P = 0.0002$; *LAMA1*, $P = 0.0002$; *LAMB1*, $P = 0.001$; transcription factor CP2-Like 1 (*TFCP2L1*),

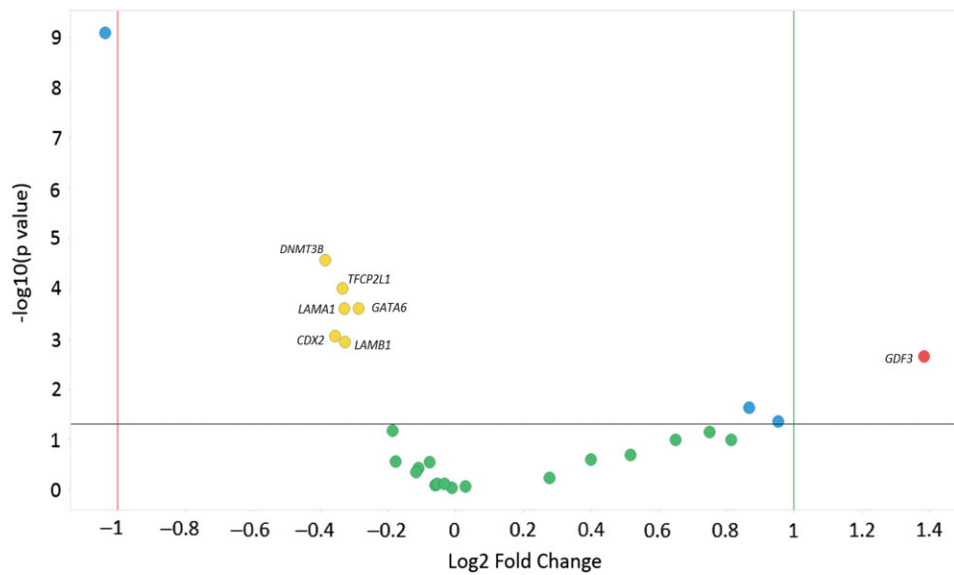


Figure 5 Volcano plot showing differentially expressed genes in inner cell mass (ICM) biopsies compared with TE. Green indicates genes with no significant differential expression. Yellow indicates genes down-regulated in the ICM compared with TE. Red indicates up-regulated genes in the ICM versus TE. Blue shows detectors that were not amplified in the majority of samples. Only the significantly up- and down-regulated genes are indicated in the plot. Caudal type homebox protein-2 (*CDX2*), DNA methyltransferase 3-beta (*DNMT3B*), GATA binding protein 6 (*GATA6*), Laminin subunit alpha-1 (*LAMA1*), Laminin subunit beta-1 (*LAMB1*), Transcription Factor CP2-Like 1 (*TFCP2L1*), Growth differentiation factor 3 (*GDF3*).

$P < 0.0001$) and only one was significantly up-regulated in the ICM (*GDF3*, $P = 0.0022$).

Finally, given that the embryos used in this study were diagnosed with either trisomy 21 or monosomy 21, and that it is well known that trisomy 21 embryos show a much higher implantation rate than those with monosomy 21 (Fragouli et al., 2013), we compared the gene expression profiles of these two groups. For this analysis, six endogenous control genes were averaged so as to calculate an average ΔCT value for each target gene for trisomy or monosomy 21 embryos. After comparing ΔCT values, four genes showed significant differential expression between monosomies and trisomies 21 (P : *GABRB3* = 0.021, *GDF3* = 0.016, *LAMC1* = 0.005, *CDH5* = 0.001, Fig. 6). Additionally, Fig. 6 depicts the calculated fold change ($2^{-\Delta\Delta\text{CT}}$) of these differentially expressed genes, where *GABRB3* and *GDF3* are significantly up-regulated in monosomy 21 blastocysts, whereas *CDH5* and *LAMC1* showed significant down-regulation (values < 1.0) in monosomy 21 compared with trisomy 21 preimplantation embryos. It is important to note that *CDH5* was not detected in any of the monosomy 21 embryos, accounting for the reported significant down-regulation.

Discussion

Here, we proposed a protocol that allows for simultaneous CCS and gene expression profiling from the same biopsy in human preimplantation embryos. In Phase 1 of the study, this new method was tested in cell lines mimicking a TE biopsy (six-cell aliquots), providing accurate karyotype calls after CCS and comparable gene expression profiles to purified RNA from the same cell cultures. In Phase 2, TE and ICM biopsies from human blastocysts were subjected to this protocol,

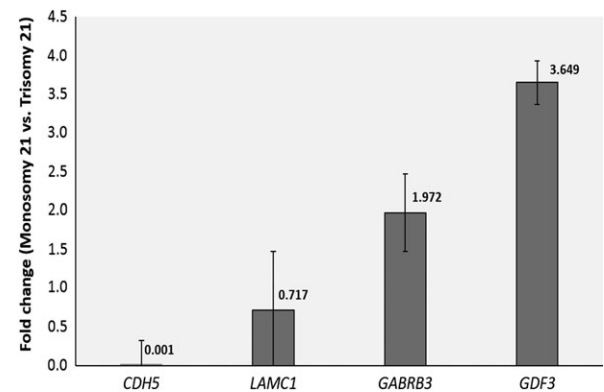


Figure 6 Significantly differentially expressed genes in human monosomy 21 embryos versus trisomy 21 embryos. Values on bars are the calculated fold changes of expression ($2^{-\Delta\Delta\text{CT}}$). All four genes showed significant differential expression after comparing average CT values (Student's t -test P : Gamma-Aminobutyric Acid Type-A Receptor Beta3 Subunit (*GABRB3*) = 0.021, *GDF3* = 0.016, Laminin subunit gamma-1 (*LAMC1*) = 0.005 and Cadherin-5 (*CDH5*) = 0.001). Error bars show SDs.

resulting in highly concordant CCS results and reliable gene expression profiles of selected target genes.

The fact that differential gene expression of two well-characterized cell lines is similar when comparing six-cell aliquots and 'bulk' purified RNA (Fig. 3) indicates that the obtained gene expression profiling

mirrors the biology of the sample and is not due to technical artifacts. More cell lines are being evaluated currently for further validation of this technology. In addition, correlation coefficients of global gene expression were higher when samples originated from the same embryo (Fig. 4), which is in agreement with the reported high gene expression heterogeneity among human preimplantation embryos (Shaw et al., 2013). These data also suggest that reliable gene expression profiles were obtained from this protocol. This was of special concern during the development of this methodology since gene expression profiling is known to vary greatly due to technical artifacts (Svec et al., 2013).

As further evidence for accurate gene expression profiling using the proposed protocol, we analyzed differences in the gene expression profiles from ICM and TE biopsies and compared our results with previous findings. Indeed, genes that presented down and up-regulation in the ICM compared with TE have been widely characterized to be expressed almost exclusively in the TE and ICM, respectively. *CDX2* for instance is a well-known marker of the TE lineage in both mouse and human embryos (Chen et al., 2009; Niakan and Eggan, 2013; Deglincerti et al., 2016). In addition, gene expression of laminins (*LAMA1*) has been characterized as enriched in TE tissue in human embryos (Bai et al., 2012), and our findings show significant down-regulation of *LAMA1* and *LAMB1* in ICM compared with TE.

Furthermore, the *de novo* DNA methyltransferase *DNMT3B* also showed down-regulation in the ICM compared with TE. Previous studies have found that expression of this gene in the mouse embryo is dynamic, but is transiently enriched in the TE lineage in the early blastocyst (Hirasawa and Sasaki, 2009). Moreover, other DNA methyltransferase genes (e.g. DNA methyltransferase 3 like) were expressed exclusively in TE of human blastocysts (Bai et al., 2012). With respect to up-regulated genes in the ICM versus TE, only *GDF3* showed significantly higher expression in the ICM, consistent with a report of *GDF3* as a marker of the epiblast in human preimplantation embryos (Yan et al., 2013).

On the other hand, although *GATA6* has been reported as a marker of the ICM, specifically of cells differentiating into the primitive endoderm (Niakan and Eggan, 2013; Deglincerti et al., 2016), our analysis showed significant down-regulation in the ICM compared with TE. Although this may bring additional concerns as to the reliability of our protocol, expression of *GATA6* has been reported to be non-specific for either ICM or TE in 6-day-old human blastocysts (Roode et al., 2012), and major protein localization in the ICM is observed later in development (Kuijk et al., 2012; Roode et al., 2012; Deglincerti et al., 2016). Also, while *TFCP2L1* has been reported to show enriched expression in the epiblast of human embryos and be responsible for maintenance of pluripotency (O'Leary et al., 2012; Blakeley et al., 2015; Qiu et al., 2015), we observed weak down-regulation in the ICM samples. A thorough and detailed characterization of *TFCP2L1* expression in the human preimplantation embryo is not yet available, as in the case of *GATA6*, and expression differences comparing embryonic cell lineages are yet to be well described for this gene.

Since embryos used for Phase 2 of this study were diagnosed to have either a single monosomy or single trisomy of chromosome 21 by CCS, we also analyzed and compared the gene expression profiles of these two different set of embryos. Since trisomies 21, in contrast to monosomies 21, are known to result in deliveries (Fragouli et al., 2013), we aimed to discover candidate genes that based on differential

expression could become biomarkers of implantation or to be very relevant in cell signaling and differentiation during early embryo development. Our analysis showed four differentially expressed genes when we compared the profiles of all biopsies from monosomy 21 embryos against trisomy 21 embryos, where *GABRB3* and *GDF3* showed significant up-regulation in monosomy 21 embryos, whereas *CDH5* and *LAMC1* were significantly down-regulated in monosomy 21 versus trisomy 21 blastocysts (Fig. 6).

As stated above, all target genes analyzed in this phase study were selected from a human stem cell pluripotency panel which characterized several human embryonic stem cells (hES) from diverse laboratories in order to determine a transcriptomic signature of undifferentiated and differentiated cells based on a comparison of hES and embryoid bodies (Adewumi et al., 2007). This panel classified *GABRB3* and *GDF3* as markers of undifferentiated cells and stemness expressed in all hES cell lines, whilst *CDH5* and *LAMC1* were categorized as markers of early differentiation. Our data indicate that monosomy 21 embryos show up-regulation of markers of undifferentiated cells when compared against trisomy 21 embryos, and that trisomy 21 blastocysts have higher expression of markers of cell differentiation. Since trisomy 21 embryos can potentially implant and result in a newborn, and monosomies 21 do not (Fragouli et al., 2013; Bianco et al., 2016), it is possible that a transcriptomic profile reflecting higher cell differentiation provides an advantage to early preimplantation development and to the implantation process itself. Furthermore, none of these four differentially expressed genes are present on chromosome 21, suggesting that single chromosome gains or losses can result in genome-wide effects (Bianco et al., 2016), and that alterations in gene expression do not follow a gene-dosage effect, as suggested by others (Mao et al., 2003; Rozovski et al., 2007).

Although high concordance was found between obtained CCS results and previous diagnoses, a CCS diagnosis was not retrieved from 9 blastocyst biopsies out of 80 processed. Given that no major changes were made to our proposed protocol in terms of number of amplification cycles or reagents used for qPCR-based CCS, it is important to note that these embryos were frozen and had to be thawed. It is possible that extra manipulation of the embryos might have caused some cells to induce apoptosis, leading to some samples not giving a CCS result. Furthermore, in order to provide a better characterization of gene expression of human embryos carrying one or more aneuploidies, gene expression profiling of chromosomally normal embryos is necessary to set them as a reference. Although we did not compare gene expression profiles with chromosomally normal blastocysts owing to restricted access to euploid embryos for research, our data show a solid differential expression of four well-characterized genes in hES. In addition, we only assessed gene expression of 30 genes (Supplementary Table 1). By increasing the number of target genes, more comprehensive transcriptomic data could be gathered providing a deeper understanding of the transcriptomic signature, allowing for pathway enrichment analyses and potentially shedding light on why some embryos possess a higher reproductive potential than others.

Finally, this is the first time that CCS and gene expression analysis have been performed on the same human embryo TE biopsy. Further optimization of this protocol and inclusion of more target genes opens a myriad of research and clinical applications for this method, such as discovery of biomarkers for embryonic reproductive potential and

characterization of the transcriptomic signatures of embryos according to their genetic constitution. Furthermore, it is important to note that this methodology could be coupled with more contemporary technologies for CCS, such as next-generation sequencing platforms, and with high-throughput gene expression techniques, such as RNA sequencing, propelling research for the discovery of new biomarkers of embryonic competence.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

D.M. wrote the manuscript and performed data analysis and interpretation. Y.W. performed experiments and preliminary analyses. X.T. performed experiments. R.T.S. contributed to study design and data interpretation. N.R.T. reviewed manuscript and lead study design and data interpretation.

Funding

Foundation for Embryonic Competence, Basking Ridge, NJ, USA.

Conflict of interest

None declared.

References

- Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 2007;**25**:803–816.
- Bai Q, Assou S, Haouzi D, Ramirez JM, Monzo C, Becker F, Gerbal-Chaloin S, Hamamah S, De Vos J. Dissecting the first transcriptional divergence during human embryonic development. *Stem Cell Rev* 2012;**8**:150–162.
- Bao S, Li T, Long X, Zhang J, Zhao H, Ren Y, Zhao Y, Li R, Tan T, Yu Y et al. Chemokine Receptor Type 4 (CXCR4) regulates migration and invasion of trophectoderm cell in the human blastocyst. *Biol Reprod* 2016;**95**:21.
- Bazrgar M, Gourabi H, Yazdi PE, Vazirinasab H, Fakhri M, Hassani F, Valojerdi MR. DNA repair signalling pathway genes are overexpressed in poor-quality pre-implantation human embryos with complex aneuploidy. *Eur J Obstet Gynecol Reprod Biol* 2014;**175**:152–156.
- Bianco K, Gormley M, Farrell J, Zhou Y, Oliverio O, Tilden H, McMaster M, Fisher SJ. Placental transcriptomes in the common aneuploidies reveal critical regions on the trisomic chromosomes and genome-wide effects. *Prenat Diagn* 2016;**36**:812–822.
- Blakeley P, Fogarty NM, del Valle I, Wamaitha SE, Hu TX, Elder K, Snell P, Christie L, Robson P, Niakan KK. Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. *Development* 2015;**142**:3151–3165.
- Capalbo A, Ubaldi FM, Rienzi L, Scott R, Treff N. Detecting mosaicism in trophectoderm biopsies: current challenges and future possibilities. *Hum Reprod* 2016;**32**:492–498.
- Chang J, Boulet SL, Jeng G, Flowers L, Kissin DM. Outcomes of in vitro fertilization with preimplantation genetic diagnosis: an analysis of the United States Assisted Reproductive Technology Surveillance Data, 2011–12. *Fertil Steril* 2016;**105**:394–400.
- Chavez SL, McElroy SL, Bossert NL, De Jonge CJ, Rodriguez MV, Leong DE, Behr B, Westphal LM, Reijo Pera RA. Comparison of epigenetic mediator expression and function in mouse and human embryonic blastomeres. *Hum Mol Genet* 2014;**23**:4970–4984.
- Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, Cowan C, Fitz-Gerald C, Zhang K, Melton DA et al. Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell* 2009;**4**:103–106.
- Chen M, Wei S, Hu J, Quan S. Can comprehensive chromosome screening technology improve IVF/ICSI Outcomes? A meta-analysis. *PLoS One* 2015;**10**:e0140779.
- Cheng EH, Liu JY, Lee TH, Huang CC, Chen CI, Huang LS, Lee MS. Requirement of leukemia inhibitory factor or epidermal growth factor for pre-implantation embryogenesis via JAK/STAT3 signaling pathways. *PLoS One* 2016;**11**:e0153086.
- Constantinou PE, Morgado M, Carson DD. Transmembrane mucin expression and function in embryo implantation and placentation. *Adv Anat Embryol Cell Biol* 2015;**216**:51–68.
- Deglinerti A, Croft GF, Pietila LN, Zernicka-Goetz M, Siggia ED, Brivanlou AH. Self-organization of the in vitro attached human embryo. *Nature* 2016;**533**:251–254.
- Florentino F, Bono S, Biricik A, Nuccitelli A, Cotroneo E, Cottone G, Kokocinski F, Michel CE, Minasi MG, Greco E. Application of next-generation sequencing technology for comprehensive aneuploidy screening of blastocysts in clinical preimplantation genetic screening cycles. *Hum Reprod* 2014;**29**:2802–2813.
- Forman EJ, Tao X, Ferry KM, Taylor D, Treff NR, Scott RT Jr. Single embryo transfer with comprehensive chromosome screening results in improved ongoing pregnancy rates and decreased miscarriage rates. *Hum Reprod* 2012;**27**:1217–1222.
- Fragouli E, Alfarawati S, Spath K, Jaroudi S, Sarasa J, Enciso M, Wells D. The origin and impact of embryonic aneuploidy. *Hum Genet* 2013;**132**:1001–1013.
- Haapaniemi Kouru K, Malmgren H, Nordenskjold M, Fridstrom M, Csemiczky G, Blennow E. One-cell biopsy significantly improves the outcome of preimplantation genetic diagnosis (PGD) treatment: retrospective analysis of 569 PGD cycles at the Stockholm PGD centre. *Hum Reprod* 2012;**27**:2843–2849.
- Hasegawa Y, Taylor D, Ovchinnikov DA, Wolvetang EJ, de Torrenté L, Mar JC. Variability of gene expression identifies transcriptional regulators of early human embryonic development. *PLoS Genet* 2015;**11**:e1005428.
- Hirasawa R, Sasaki H. Dynamic transition of Dnmt3b expression in mouse pre- and early post-implantation embryos. *Gene Expr Patterns* 2009;**9**:27–30.
- Hou Y, Guo H, Cao C, Li X, Hu B, Zhu P, Wu X, Wen L, Tang F, Huang Y et al. Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. *Cell Res* 2016;**26**:304–319.
- Kuijk EW, van Tol LT, Van de Velde H, Wubbolts R, Wellings M, Geijsen N, Roelen BA. The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. *Development* 2012;**139**:871–882.
- Macaulay IC, Haerty W, Kumar P, Li Yi, Hu TX, Teng MJ, Goolam M, Saurat N, Coupland P, Shirley LM et al. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods* 2015;**12**:519–522.
- Mao R, Zielke CL, Zielke HR, Pevsner J. Global up-regulation of chromosome 21 gene expression in the developing Down syndrome brain. *Genomics* 2003;**81**:457–467.

- Munne S, Weier HU, Grifo J, Cohen J. Chromosome mosaicism in human embryos. *Biol Reprod* 1994;**51**:373–379.
- Neal SA, Franasiak JM, Forman EJ, Werner MD, Morin SJ, Tao X, Treff NR, Scott RT Jr. High relative deoxyribonucleic acid content of trophoctoderm biopsy adversely affects pregnancy outcomes. *Fertil Steril* 2016;**107**:731–736.
- Niakan KK, Eggan K. Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. *Dev Biol* 2013;**375**:54–64.
- O'Leary T, Heindryckx B, Lierman S, van Bruggen D, Goeman JJ, Vandewoestyne M, Deforce D, de Sousa Lopes SM, De Sutter P. Tracking the progression of the human inner cell mass during embryonic stem cell derivation. *Nat Biotechnol* 2012;**30**:278–282.
- Qiu D, Ye S, Ruiz B, Zhou X, Liu D, Zhang Q, Ying QL. Klf2 and Tfcp2l1, two Wnt/beta-catenin targets, act synergistically to induce and maintain naive pluripotency. *Stem Cell Rep* 2015;**5**:314–322.
- Roode M, Blair K, Snell P, Elder K, Marchant S, Smith A, Nichols J. Human hypoblast formation is not dependent on FGF signalling. *Dev Biol* 2012;**361**:358–363.
- Rozovski U, Jonish-Grossman A, Bar-Shira A, Ochshorn Y, Goldstein M, Yaron Y. Genome-wide expression analysis of cultured trophoblast with trisomy 21 karyotype. *Hum Reprod* 2007;**22**:2538–2545.
- Scott RT Jr, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, Tao X, Treff NR. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertil Steril* 2013a;**100**:697–703.
- Scott RT Jr, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertil Steril* 2013b;**100**:624–630.
- Shaw L, Sneddon SF, Zeef L, Kimber SJ, Brison DR. Global gene expression profiling of individual human oocytes and embryos demonstrates heterogeneity in early development. *PLoS One* 2013;**8**:e64192.
- Svec D, Andersson D, Pekny M, Sjoback R, Kubista M, Stahlberg A. Direct cell lysis for single-cell gene expression profiling. *Front Oncol* 2013;**3**:274.
- Tan YQ, Tan K, Zhang SP, Gong F, Cheng DH, Xiong B, Lu CF, Tang XC, Luo KL, Lin G et al. Single-nucleotide polymorphism microarray-based preimplantation genetic diagnosis is likely to improve the clinical outcome for translocation carriers. *Hum Reprod* 2013;**28**:2581–2592.
- Treff NR, Tao X, Ferry KM, Su J, Taylor D, Scott RT Jr. Development and validation of an accurate quantitative real-time polymerase chain reaction-based assay for human blastocyst comprehensive chromosomal aneuploidy screening. *Fertil Steril* 2012;**97**:819–824.
- Vera-Rodriguez M, Chavez SL, Rubio C, Reijo Pera RA, Simon C. Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nat Commun* 2015;**6**:7601.
- Wells D, Delhanty JD. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol Hum Reprod* 2000;**6**:1055–1062.
- Xu C, Houck JR, Fan W, Wang P, Chen Y, Upton M, Futran ND, Schwartz SM, Zhao LP, Chen C et al. Simultaneous isolation of DNA and RNA from the same cell population obtained by laser capture microdissection for genome and transcriptome profiling. *J Mol Diagn* 2008;**10**:129–134.
- Yan L, Yang M, Guo H, Yang L, Wu J, Li R, Liu P, Lian Y, Zheng X, Yan J et al. Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. *Nat Struct Mol Biol* 2013;**20**:1131–1139.
- Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, Peck AC, Sills ES, Salem RD. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. *Mol Cytogenet* 2012;**5**:24.