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ORIGINAL ARTICLE

Preclinical validation of a targeted next generation sequencing-based comprehensive chromosome screening methodology in human blastocysts

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STUDY QUESTION: Can a novel targeted next generation sequencing (tNGS) platform accurately detect whole chromosome aneuploidy in a trophectoderm biopsy and provide additional information to improve testing?

SUMMARY ANSWER: Karyotypes obtained by tNGS were concordant with other validated platforms and single nucleotide polymorphism genotyping information obtained can be used for improved detection and quality control.

WHAT IS KNOWN ALREADY: qPCR-based whole chromosome aneuploidy screening is highly accurate in comparison to other common methods and has been shown to improve IVF success in two randomized clinical trials. With aneuploidy screening becoming standard of care in many IVF centres, there is a need to develop platforms with high throughput, low cost capabilities.

STUDY DESIGN SIZE, DURATION: Twelve well-characterized cell lines were obtained from a commercial cell line repository and 31 discarded human blastocysts were obtained from 17 IVF patients who underwent comprehensive chromosome screening (CCS).

PARTICIPANTS/MATERIAL, SETTING, METHODS: All samples were processed using a unique amplification strategy which directly incorporated sequencing library adapters and barcodes. Sequencing was performed on an Ion Torrent Proton. A custom bioinformatics pipeline was used to determine the karyotype for each sample. The consistency of tNGS diagnoses with either conventional karyotyping of cell lines or quantitative real-time PCR based CCS of blastocyst biopsies was evaluated.

MAIN RESULTS AND THE ROLE OF CHANCE: Overall consistency per sample of tNGS based CCS in 5-cell samples from a variety of cell lines was 99.2%. In the blinded analysis of rebiopsies of aneuploid blastocysts, an overall targeted tNGS CCS consistency of 98.7% was observed per sample. These data demonstrate the ability of tNGS based CCS to provide an accurate and high throughput evaluation of aneuploidy in the human blastocyst.

LARGE SCALE DATA: Not applicable.

LIMITATIONS REASONS FOR CAUTION: This study is limited to whole chromosome aneuploidy, as mosaicism and segmental aneuploidy have not been investigated.

WIDER IMPLICATIONS OF THE FINDINGS: These data show an accurate, high throughput method, and with the greater depth of each amplicon sequenced in comparison to commercial kits, there is greater application available for single nucleotide polymorphism based analysis for quality control.

STUDY FUNDING/COMPETING INTERESTS: This study was funded through intramural research funds provided by the Foundation for Embryonic Competence. There are no competing interests.

Key words: comprehensive chromosome screening / next generation sequencing / preimplantation genetic screening / blastocysts / aneuploidy

Introduction

Aneuploidy in the trophectoderm of human preimplantation embryos now represents the most well established molecular biomarker of reproductive potential (Gardner et al., 2015). Multiple randomized controlled trials have demonstrated improved outcomes when incorporating comprehensive chromosome screening (CCS) based preimplantation testing of aneuploidy in IVF-derived embryos. A wide variety of methods have now been developed to allow for comprehensive detection of aneuploidy (Treff et al., 2015). Methods currently in clinical use include single nucleotide polymorphism (SNP) arrays (Handyside et al., 2010; Johnson et al., 2010; Treff et al., 2010a), array-based comparative genomic hybridization (aCGH) (Fishel et al., 2010; Fragouli et al., 2011; Munne et al., 2010; Gutierrez-Mateo et al., 2011), multiplex quantitative real-time PCR (qPCR) (Treff et al., 2012b; Capalbo et al., 2014) and next generation sequencing (Treff et al., 2013b; Wells et al., 2014; Fiorentino et al., 2014a, 2014b; Huang et al., 2016).

As above, description of these methods of CCS is often limited to the downstream analysis platforms. However, one of the most critical components of CCS is the upstream amplification strategy. Indeed, there are a wide variety of amplification methods, including isothermal multiple displacement amplification (MDA), PCR based whole genome amplification (WGA) and multiplex targeted PCR (Treff et al., 2011). Each amplification method provides a unique set of capabilities and limitations. For example, MDA has been shown to be more useful for genotyping applications, such as parental support or karyomapping, while PCR-based WGA methods have improved copy number analysis capability. In contrast, targeted amplification provides the ability to simultaneously obtain accurate copy number and genotype data in a single biopsy (Zimmerman et al., 2016).

For example, targeted amplification allows for simultaneous single gene disorder screening, DNA fingerprinting, detection of triploidy and mitochondrial DNA mutation testing by simple incorporation of additional genotyping assay primers into the preamplification reaction (Treff et al., 2012a; Scott et al., 2014; Marin et al., 2017). Thus far, reports of NGS-based CCS have been limited to the use of WGA and have not involved targeted amplification strategies. One potential advantage of using next generation sequencing over qPCR is that the same amplicons used for copy number analysis may provide the opportunity for genotyping. The study presented here will investigate the use of a targeted PCR amplification (instead of WGA) followed by semiconductor based sequencing with the Ion Proton and evaluate the performance against previously validated methods of testing.

Materials and Methods

Experimental design

This study was conducted in three phases with an emphasis on avoiding the analysis of samples with the potential for biological variation (mosaicism) to fully understand the amount of technical variation the platform may have. Phase 1 was designed to define a method of amplification and analysis that could accurately determine the copy number state of whole chromosomes and would allow for the greatest capacity through barcoding multiple samples on one chip. In Phase 2, a set of known abnormal cell lines were tested and assessed for accuracy. In Phase 3, a blinded analysis was performed on whole chromosome aneuploid embryos to evaluate consistency between NGS and qPCR-based CCS. To complete this portion of the study, additional biopsies were taken from each embryo; this is in contrast to previously published reports which simply re-aliquoted DNA from the original sample (Fiorentino et al., 2014a).

Cell lines

A total of 12 well-characterized cell lines were obtained from the Coriell Cell Repository (CCR, Camden, NJ) and cultured as recommended by the provider. Cell lines included: 47,XY,+22 (GM07106), 47,XY,+18 (GM01359), 47,XY,+13 (GM02948), 46,XX (GM13119, GM00321), 46, XY,+16,+21 (GM04435), 47,XYY (GM09326), 49,XXXXY (GM00326), 45,XX,−21 (GM01201), 47,XY,+21 (GM02067), 47,XXX (GM04626), and 46,XY (GM13120). To model the sample obtained with a typical trophectoderm biopsy, 5-cell aliquots were prepared using a 100um stripper tip and pipette to place individual cells into a PCR tube under a dissecting microscope. The 5-cell aliquots and embryo biopsies were processed by alkaline lysis as previously described (Cui et al., 1989; Treff et al., 2010b). Prior to using 5-cell aliquots for these experiments, we also examined the accuracy, assay failure rate and quality of data obtained on 1, 2, 3, 4, 5, 10, 15 and 20 cells (Supplementary Fig. 2).

Blastocyst biopsies

Phase 3 included 31 embryos that had two consistent qPCR-based aneuploidy screening results from two different trophectoderm biopsies, as previously described (Scott et al., 2013). Additional biopsies, for a total of 77 samples, were taken and the samples were randomized and blinded to investigators to validate consistency between NGS and qPCR-based CCS. Use of this discarded material was approved by the Western IRB review board, protocol # RMA-00-02, which included informed consent for donation of discarded embryos to research.

Targeted NGS

Amplification for targeted NGS was performed with the use of proprietary multiplexed primers that included adapter A (5′-CCATCTCATCCCTG CGTGTCTCCGACTCAG-3′) in the forward primer, and adapter trP1 (5′-CCTCTCTATGGGCAGTCGGTGAT-3′) in the reverse primer. For access to primer sequences, please contact the authors. Barcode sequences 1–96 were added to the forward primer on the 3′ end of adapter A to make 96 unique primers in order to multiplex embryo or cell line samples as recommended by the supplier (ThermoFisher Scientific Inc., Waltham, MA). TaqMan Preamplification Master Mix was used as recommended by the supplier (ThermoFisher Scientific Inc.) in a 50-uL reaction volume with 25 cycles (95°C for 10 min, 24 cycles of 95°C for 15 s and 60°C for 4 min, then 4°C hold) using an Applied Biosystems 2720 thermocycler, generating 2679 amplicons across the genome. In the next step, DNA was quantified using the Agilent high sensitivity D1000 ScreenTape system (Agilent Technologies, Waldbronn, Germany), followed by purification utilizing the Agencourt Ampure XP Systems (Beckman Coulter, Beverly, MA), both as per manufacturer recommendations. The samples were then prepared and run on the Ion OneTouch 2 System and, upon completion, loaded onto the Ion PI Chip v2 for next generation sequencing using the Ion Proton System as per manufacturer specifications (ThermoFisher Scientific Inc.). A maximum of 96 samples per chip were run. Supplementary Fig. 1 shows the number of amplicons per chromosome used for analysis.

NGS data analysis

A custom sequencing analysis plugin was developed in-house for the Torrent Server to estimate the copy number for each chromosome. In summary, barcode-specific reads were aligned to the human reference genome (hg19) within Torrent Suite™ using tmap. Amplicon read counts

were calculated with an in-house programme that assigns each read in an aligned bam file to an amplicon specified in a bed file and determines how many reads align to each amplicon. The read counts then went through additional data processing (including within-sample read count normalization and principal-component-based read count corrections to reduce the impacts of non-biological factors on amplicon read counts) and were compared against reference amplicon read counts obtained from samples with normal karyotypes to yield a log 2 ratio for each amplicon. Whole chromosome copy number estimates were obtained based on summarizing log 2 ratio values for all amplicons. Quality metrics that include the average raw autosomal amplicon read count (or depth, DP), MAPD (the Median of the Absolute values of all Pairwise Differences between log 2 ratios of adjacent amplicons) and jscore (chromosome jumpiness score; a metric for evaluating the level of fluctuation of per chromosome copy number estimates, presumably due to reasons other than real chromosome copy number differences) can be calculated and used when assessing sample results. Higher MAPD and jscore values correspond to less consistency between amplicon results and more noise in the cross-genome chromosome copy number baseline, respectively. Typically a MAPD of over 0.5 is flagged as non-concurrent or requiring further interpretation. The final output of the custom plugin includes a scatter plot that shows the amplicon log 2 ratios across the genome and the confidence intervals for the copy number estimates of each chromosome.

Limit of detection

A subsampling study was performed to determine the minimum depth required to achieve the highest accuracy. Samples that were previously run

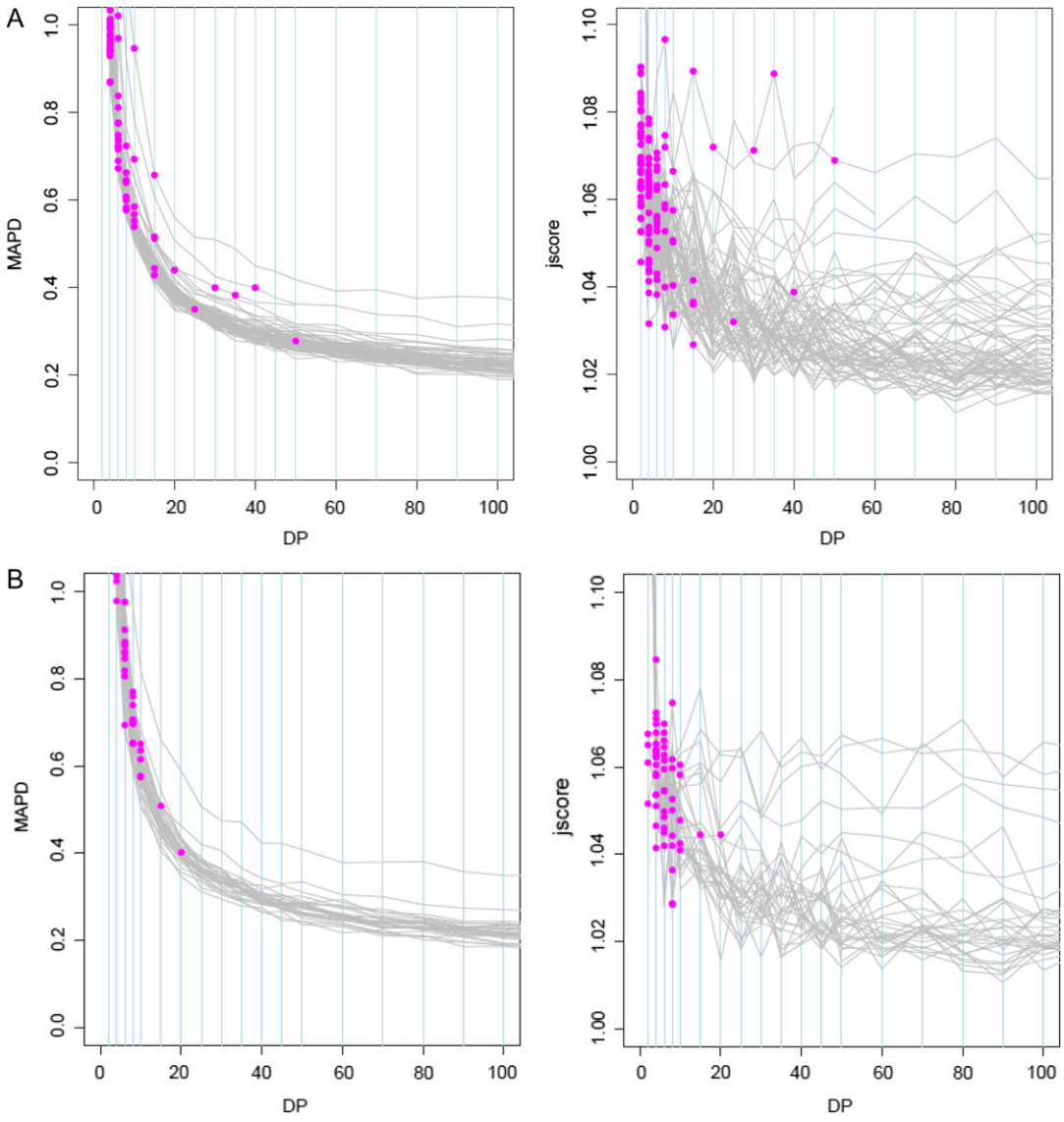

Figure 1 (**A**) Abnormal samples: 66 abnormal samples were downsampled and the depth (DP) at which the karyotype changed from what was expected is noted with a pink dot. Two quality metrics, MAPD and jscore, are plotted against DP, separately. For all 66 samples, a DP higher than 60 would yield an accurate result. (**B**) Normal samples: 20 normal samples were downsampled and the depth at which the karyotype changed from what was expected (46,XX or 46,XY) is noted with a pink dot. For these samples, a DP of over 20 yielded an accurate result.

on the NGS assay (66 abnormal and 30 normal) were bioinformatically downsampled and run through the analysis pipeline. Samples were chosen for this experiment with the following criteria: (i) a known karyotype was available from prior methods; (ii) for normal samples the embryo biopsy had a euploid result, the embryo was transferred and a baby was delivered; (iii) there were no segmental or mosaic calls in analysis results at the maximum depth; and (iv) the call at the maximum depth is the same as the known karyotype. For each of the 96 samples, the reads were randomly sampled to the following target DP values whenever possible and the analysis was performed at default settings: 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000.

Genotyping assessment

Genome regions that correspond to the set of amplicons selected for our targeted amplification were investigated for SNPs reported in the 1000 Genomes Project phase 3 variant set (20 130 502 version) in region set A. For autosomal chromosomes, allele frequencies of all SNPs were retrieved from 1367 samples with average depth higher than 200. The number of samples with alternative allele frequencies between 0.4 and 0.6 was calculated for each SNP and defined as heterozygosity rate. Only SNPs with a heterozygosity rate different from zero were kept for variant calling in our customized plugin. With respect to sex chromosomes, 639 samples with female karyotype were used to select SNPs for variant calling in chromosome X as described above. No SNPs were selected for variant calling in the Y chromosome. A customized variant calling plugin was set to retrieve discrete genotype calls from each SNP. SNPs with alternative allele frequencies higher 0.9 are considered homozygote for the alternative allele, those lower than 0.1 are the homozygote reference and any other frequency is considered heterozygote.

In order to asses reproducibility of SNP variant calling, 12 5-cell aliquots from the cell line GM0323 (46,XY, Coriell Repository, Camden, NJ) were processed with our targeted next generation sequencing platform and discrete genotype calls of all 2690 preselected SNPs form each sample were compared to genotyping data from 10 ng of isolated gDNA (Qiagen DNeasy kit, Hilden Germany) from a cell culture of the same cell line. Mismatches were divided by all genotype comparisons and an error rate per sample and an average error rate was calculated. Furthermore, genotyping data was retrieved from 40 samples derived from 10 human embryos (four biopsies per embryo) and discrete genotype calls from all four biopsies from the same embryo were compared. In this case, a mismatch was defined as any SNP where at least one biopsy differed in the discrete genotype call. An error rate per embryo was calculated as well as an average error rate.

Results

Phase 1: Targeted NGS strategy

Commercially available kits for NGS-based aneuploidy screening in blastocysts rely on a WGA approach, followed by the binning of amplicons to allow for copy number generation. Those approaches are reported to allow for multiplexing of samples in a single run; however even running 24 samples on a single run has led to a depth that is too shallow to allow for accurate detection of genotypes (Wells *et al.*, 2014; Fiorentino *et al.*, 2014b). As such, one of our primary goals was to establish a novel method that would allow for multiplexing of over 24 samples per run while also increasing the depth to be able to investigate the utilization of allele calls from the sequencing data for quality control applications and improved detection of polyploidies.

In any clinical test validation, it is important to determine quality control thresholds that can be set when reviewing clinical examples. By examining the limit of detection of next generation sequencing data, a laboratory can determine the depth at which a sample should be failed due to a risk of an inaccurate result. Simple bioinformatics tools can allow for random subsampling of sequencing reads to mimic different depths, and then those subsamples can be processed through analysis pipelines to determine the proposed karyotype. Figure 1 displays the results of the downsampling with the depth (DP) plotted against two QC measurements, MAPD and jscore, which are representative metrics of how noisy the data is. Each line represents a sample and the pink dots represent the depth at which a result for a sample was different than the known result. For samples that started with an abnormal karyotype at the maximum depth, a false negative result was observed at a depth of 50 and lower. For samples that started with a normal

Table I Cell line concordance.

Cell line ID	Expected karyotype	# Blinded samples	% Concordant
GM07106	47,XY,+22	6	100%
GM01359	47,XY,+18	5	100%
GM02948	47,XY,+13	6	100%
GM13119	46,XX	4	100%
GM04435	48,XY,+16,+21	18	100%
GM00321	46,XX	4	75% (3/4)
GM09326	47,XYY	7	100%
GM00326	49,XXXXY	6	100%
GM01201	45,XX,−21	6	100%
GM02067	47,XY,+21	6	100%
GM04626	47,XXX	5	100%
GM13120	46,XY	51	100%
		Total concordance	99.2% (123/124)

karyotype at the maximum depth, a false positive result was observed at a depth of 20 and lower. These findings may allow for quality control cut offs to be established in a clinical setting.

Given the higher sequencing depth obtained after targeted amplification, we investigated whether reliable genotyping data could be retrieved from polymorphisms present in the amplified genome regions. A total of 8584 SNPs were found in autosomal chromosomes, 471 were in chromosome X and 7 were in chromosome Y. After selecting for SNPs with heterozygosity rates higher than 0 from a preselected set of samples, our platform was set to retrieve, for every single sequenced sample, allele frequencies and discrete genotype calls from a total of 2690 SNPs, of which 2571 are autosomal and 119 are in chromosome X.

We also assessed reproducibility of genotyping data by comparing discrete genotype calls from 5-cell aliquots samples and gDNA isolated from a cell culture from the same cell line, as well as discrete calls from four biopsies from the same embryo (10 embryos in total). The average genotyping error rate found after sequencing the cell line samples was 0.1612% (0.0932–0.2292, 95% CI), with a maximum value of 0.4608% and a minimum of 0.0400%. With respect to the embryos, the average error rate was 0.0788% (0.0207–0.2655) with a maximum value of 0.1721% and a minimum of 0%. Therefore, after sequencing a trophectoderm biopsy, an average of 2, out of 2690, SNPs are incorrectly genotyped.

Phase 2: Whole chromosome aneuploidy in cell lines

A total of 128 randomized blinded samples of the 12 cell lines were evaluated for 24-chromosome copy number and compared for consistency with the cell lines' karyotype previously determined by conventional G-banding. The percent concordance for each cell line is listed in Table I. Four samples were eliminated from analysis due to non-concurrent (low quality) results. (Of note, more recent testing of

Table II Rebiopsy results by embryo.

Embryo #	qPCR Karyotype A	qPCR Karyotype B	NGS C	NGS D	NGS E
1	47,XY,+16	47,XY,+16	47,XY,+16	47,XY,+16	47,XY,+16
2	45,X	45,X	45,X	45,X	**46,XX**
3	47,XY,+16	47,XY,+16	47,XY,+16	47,XY,+16	47,XY,+16
4	45,XY,−21	45,XY,−21	45,XY,−21	45,XY,−21	45,XY,−21
5	45,XY,−22	45,XY,−22	45,XY,−22	**44,XY,−4,−22**	45,XY,−22
6	45,XX,−7	45,XX,−7	NA	45,XX,−7	45,XX,−7
7	45,XX,−18	45,XX,−18	45,XX,−18	45,XX,−18	45,XX,−18
8	45,XY,−16	45,XY,−16	45,XY,−16	45,XY,−16	45,XY,−16
9	45,XY,−22	45,XY,−22	45,XY,−22	45,XY,−22	45,XY,−22
10	45,XY,−21	45,XY,−21	45,XY,−21	45,XY,−21	45,XY,−21
11	45,XX,−21	45,XX,−21	45,XX,−21	**46,XX,+18,−21**	45,XX,−21
12	47,XY,+8	47,XY,+8	47,XY,+8	47,XY,+8	NA
13	47,XX,+20	47,XX,+20	**48,XX,+1,+20**	NA	NA
14	45,XX,−20	45,XX,−20	45,XX,−20	45,XX,−20	45,XX,−20
15	45,XX,−19	45,XX,−19	45,XX,−19	NA	NA
16	45,XX,−18	45,XX,−18	45,XX,−18	45,XX,−18	NA
17	47,XY,+19	47,XY,+19	**48,XY,+3,+19**	47,XY,+19	NA
18	47,XY,+9	47,XY,+9	47,XY,+9	47,XY,+9	47,XY,+9
19	45,XY,−20	45,XY,−20	45,XY,−20	**46,XY,+9,−20**	45,XY,−20
20	45,X	45,X	45,X	45,X	NA
21	47,XXY	47,XXY	47,XXY	47,XXY	47,XXY
22	44,X,−15	44,X,−15	NA	44,X,−15	NA
23	47,XX,+21	47,XX,+21	47,XX,+21	NA	NA
24	45,XY,−21	45,XY,−21	45,XY,−21	NA	NA
25	45,XY,−21	45,XY,−21	45,XY,−21	NA	45,XY,−21
26	47,XX,+16	47,XX,+16	47,XX,+16	47,XX,+16	47,XX,+16
27	47,XY,+19	47,XY,+19	47,XY,+19	47,XY,+19	47,XY,+19
28	47,XY,+18	47,XY,+18	47,XY,+18	47,XY,+18	47,XY,+18
29	47,XY,+15	47,XY,+15	47,XY,+15	47,XY,+15	47,XY,+15
30	45,XY,−21	45,XY,−21	45,XY,−21	45,XY,−21	45,XY,-21
31	46,XY,+20,−22	46,XY,+20,−22	46,XY,+20,−22	46,XY,+20,−22	46,XY,+20,−22

over 20 000 embryo biopsies submitted from multiple centres shows an average unamplified/non-concurrent rate of 1–2%.) Of the remaining 124 samples, 99.2% (123/124) had completely concordant results across all chromosomes. There were no false negative diagnoses or inaccurate predictions of gender. The discordant sample was a normal cell line that was called abnormal (47,XX,+2) by the algorithm suggesting a false positive rate of 0.8%. The overall consistency of chromosome copy number assignment was 99.97% (2975/2976) for all 2976 chromosomes analysed (124 samples × 24 chromosomes) and the overall diagnostic accuracy was 99.2%. Since the cell lines exhibit uniform cellularity, they were used to calculate the technical sensitivity and specificity. These calculations were done both per chromosome and per overall diagnosis. The sensitivity per chromosome and for overall sample diagnosis was calculated using all of the aneuploid cell line samples and was determined to be 100% ($n = 74$ and 65, respectively). The specificity was calculated using all of the euploid cell line samples and was 99.97% (2901/2902) per chromosome and 98.3% for overall diagnosis (58/59).

Phase 3: Blinded embryo biopsies

In Phase 3, a set of 31 whole chromosome aneuploid blastocysts (Table II), which were given consistent diagnoses from qPCR-based 24 chromosome aneuploidy screening results on two separate biopsies (to reduce the risk for mosaicism), were biopsied again (total of 77 samples) to establish consistency of diagnosis in the embryo. A consistent diagnosis of normal versus abnormal for whole chromosome aneuploidy was made in 98.7% (76/77) of samples (an example shown in Fig. 2), where 92.2% (71/77) were completely concordant, 6.5% (5/77) were concordant for the chromosome called abnormal by qPCR, but discordant for an additional chromosome and 1.3% (1/77) was completely discordant. The overall chromosome concordance was 99.83% (3536/3542). The partially concordant samples confirmed the same whole chromosome abnormality seen by qPCR; however, the NGS results detected additional abnormalities (Table II, bold results). The one completely discordant sample was called 46,XX. The qPCR results for this embryo were a clear 45,X, as well as two

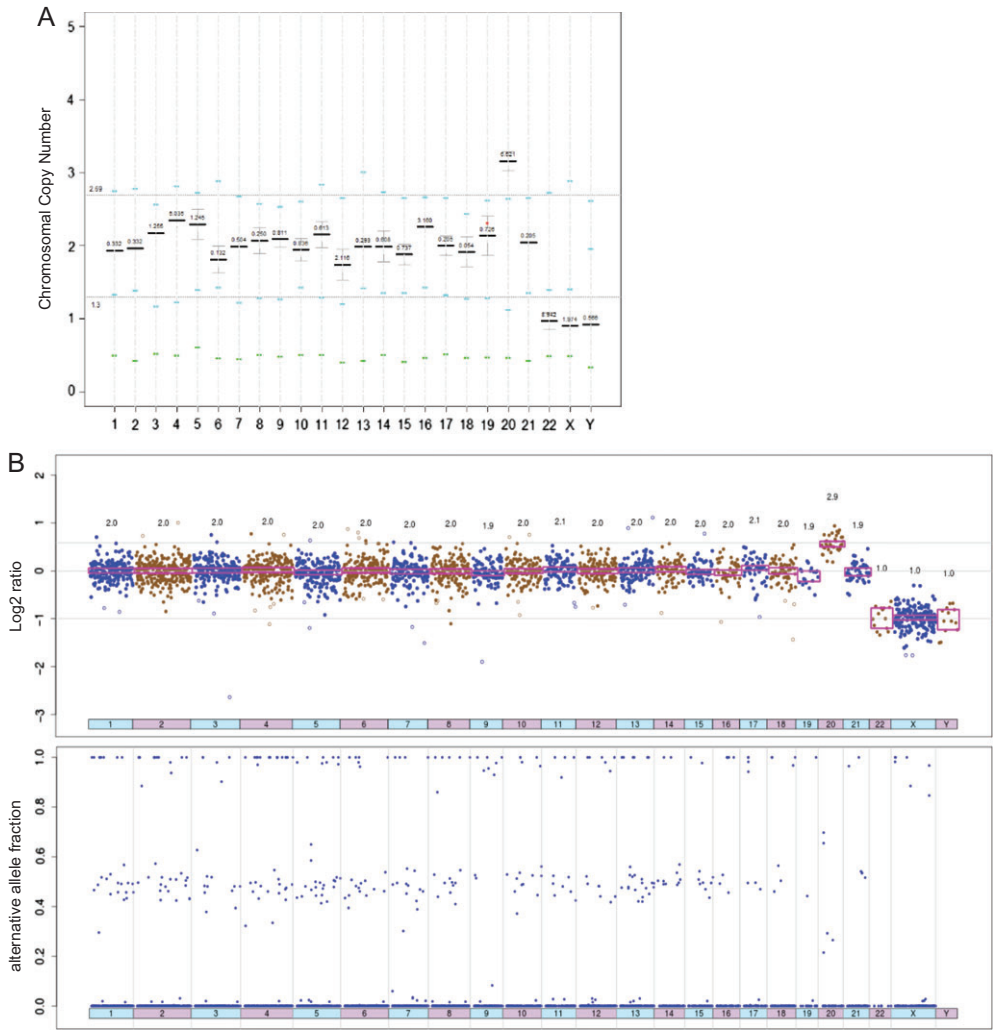

Figure 2 Results for embryo 31. (**A**) qPCR results (46,XY,+20,−22). (**B**) NGS results (46,XY,+20,−22) with copy number plots (top) and SNP allele ratios (bottom).

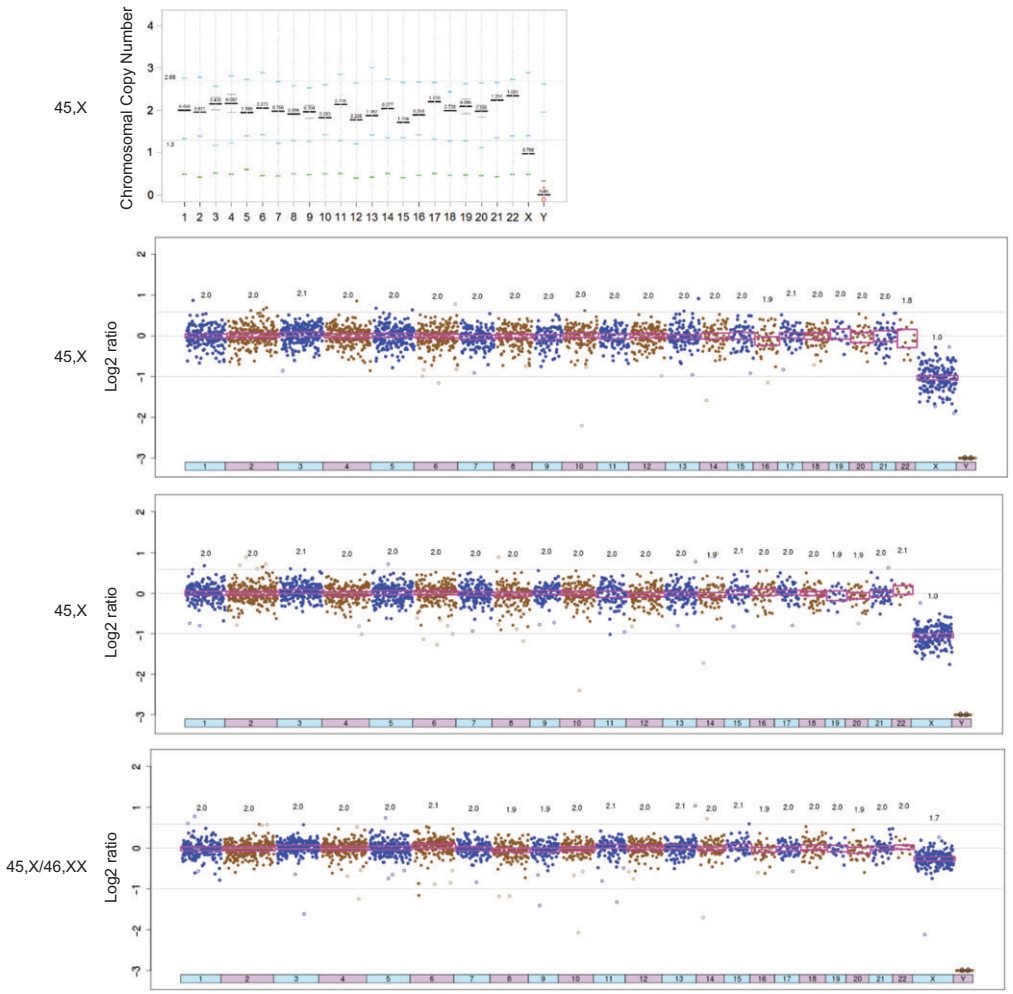

Figure 3 qPCR (top) and NGS (bottom three) results for embryo 2 showing a mosaic result for the final biopsy.

other biopsies (Fig. 3). One embryo (#5) (Fig. 4), showed two NGS biopsies with segmental aneuploidy for chromosome 4 and a third NGS biopsy with complete monosomy 4, which is consistent with recent studies showing mitotic errors detected in embryos at a significant frequency (Juneau *et al.*, 2016). The remaining biopsies with partial concordance have mosaic range or segmental aneuploidy results that were seen only in a single biopsy, which can represent low level mosaicism or technical variation. Fingerprinting analysis using SNPs that are present in the NGS amplicons showed that the rebiopsies for each embryo matched the original embryo biopsy, ruling out the possibility that a sample switch lead to the discordant results.

Discussion

Results of the present study demonstrate the preclinical validity of targeted NGS-based CCS screening in human blastocysts. Emphasis has been placed on evaluating the technique's ability to determine the correct chromosomal status by reducing the impact of mosaicism on variation in results. This was achieved in three phases by utilizing cells derived from well-characterized cell lines and blastocysts with consistent qPCR-based CCS diagnoses from two independent biopsies. With this strategy, we demonstrated an overall consistency of NGS-based CCS in cell lines with 24-chromosome diagnosis of 99.2% (123/124). In the blinded analysis of aneuploid embryos, an overall 24 chromosome diagnostic consistency of 99.83% was identified. These phases of preclinical validation have not been performed by other methods of NGS-based CCS without the use of leftover WGA products (Wells *et al.*, 2014; Fiorentino *et al.*, 2014a, b). Additionally, the accuracy reported here for tNGS is comparable to, if not better than, other platforms for which accuracy studies have been performed and published (Treff and Scott, 2012; Capalbo *et al.*, 2014)

One of the limitations of the present study is the inability to evaluate performance on euploid embryos. Embryos originally diagnosed as euploid by qPCR are not available to rebiopsy since they are instead made available for transfer. Unlike Fiorentino *et al.*, it is also not possible to split amplified DNA from the original biopsy for analysis by targeted NGS CCS since the amplification strategy for qPCR CCS is completely different. However, the main outcome of evaluating performance on euploid embryos would be to determine the false positive rate of the present method. As an alternative, one can evaluate performance on

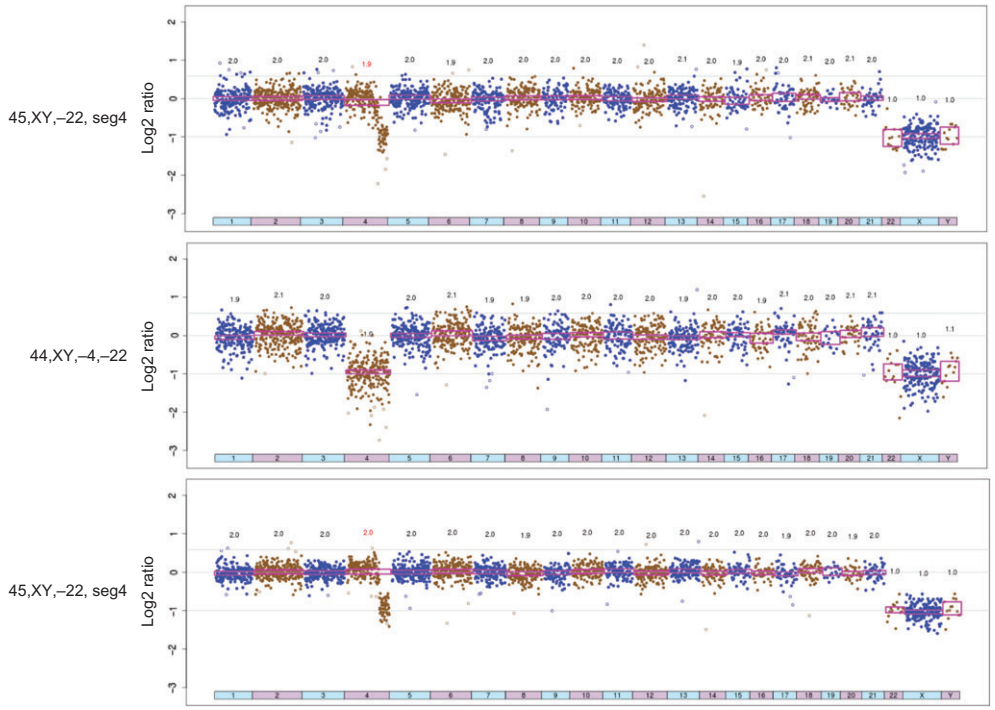

Figure 4 NGS results of three different biopsies from embryo 5 showing additional abnormalities involving chromosome 4.

euploid chromosomes within aneuploid embryos. In doing so, the present study indicates a false positive rate of 0.03% in cell lines, and 0.23% in embryos at the chromosome level. Furthermore, evaluating concordance within embryos previously diagnosed as euploid is far less powerful than performing a 'non-selection' clinical trial. In such a study, embryos are biopsied without using CCS to select embryos for transfer. Once sustained implantation is confirmed for specific embryos using DNA fingerprinting, the negative and positive predictive values of the CCS method can then be evaluated. This study design represents an additional phase of preclinical validation currently ongoing for the targeted NGS method described in this study.

A number of features of the present study make its application more advantageous than existing alternatives. First, commercially available methods of NGS-based CCS which utilized WGA involve running 24 samples in parallel (VeriSeq), while the present study was able to accurately evaluate 48–96 samples simultaneously, thus increasing throughput and reducing laboratory reagent costs. Second, since a universal PCR based master mix and cycling conditions were used here, the ability to multiplex additional primers of interest makes simultaneous analysis of single gene disorders or clinically relevant microdeletions and duplications more feasible than ever before (Treff *et al.*, 2013a).

Recent experience with CCS has demonstrated dramatic improvement in implantation and delivery outcomes (Schoolcraft *et al.*, 2011; Forman *et al.*, 2013; Scott *et al.*, 2013; Rubio *et al.*, 2017; Yang *et al.*, 2012). An important prerequisite is demonstration of preclinical consistency in cell lines and embryos as reported here, and the accuracy is equivalent to our previous qPCR-based method that has shown significant improvement in pregnancy outcomes in two randomized controlled trials.

Supplementary data

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

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Authors' roles

X.T., A.L. and D.M. performed experiments and analysed results; M. D.W. and K.H.H. were involved in data collection; J.L., D.T. and Y.Z. performed bioinformatic data analyses (Y.Z. designed the final algorithm used); R.T.S., N.R.T. and R.S.Z. were involved in the overall study design, data interpretation and article writing/review.

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Conflicts of interest

There are no conflicts of interest.

Capsule

The present study demonstrates the validation of a novel method for CCS screening in blastocysts using targeted NGS.

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