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Validation of a targeted next generation sequencing-based comprehensive chromosome screening platform for detection of triploidy in human blastocysts

Diego Marin [a,b,*], *Rebekah Zimmerman* [c], *Xin Tao* [c], *Yiping Zhan* [c], *Richard T Scott Jr* [a,b], *Nathan R Treff* [d]

[a] *IVI-RMA New Jersey, Basking Ridge, NJ, USA*
[b] *Thomas Jefferson College of Biomedical Sciences, Thomas Jefferson University, Philadelphia, PA, USA*
[c] *The Foundation for Embryonic Competence, Basking Ridge, NJ, USA*
[d] *Rutgers, The State University of New Jersey, Department of Genetics, Piscataway, NJ, USA*

Diego Marin is a PhD candidate at IVI-RMA Global Basking Ridge, New Jersey and Thomas Jefferson University in Philadelphia. He completed his Bachelor's Degree in Biology at the University of Costa Rica. He obtained his Master's Degree in Clinical Embryology at the Valencian Infertility Institute (IVI) in Valencia, Spain.

KEY MESSAGE

In this study a tNGS-based CCS platform for detection of triploidy in human blastocysts from a TE biopsy was validated. It was established that ICSI-derived triploid blastocysts are rare, with a frequency of approximately 0.5%, and that, as expected due to virtual exclusion of polyspermy, are mostly of maternal origin.

A B S T R A C T

Triploidy accounts for ~2% of natural pregnancies and 15% of cytogenetically abnormal miscarriages. This study aimed to validate triploidy detection in human blastocysts, its frequency and parental origin using genotyping data generated in parallel with chromosome copy number analysis by a targeted next generation sequencing (tNGS)-based comprehensive chromosome screening platform. Phase 1: diploid and triploid control samples were blinded, sequenced by tNGS and karyotype predictions compared for accuracy. Phase 2: tNGS was used to calculate the frequency of triploidy in 18,791 human blastocysts from trophectoderm (TE) biopsies. Phase 3: parental origin of the inherited extra alleles was evaluated by sequencing parental gDNA to validate triploidy predictions from Phase 2. All karyotypes and ploidy in controls from Phase 1 were correctly predicted by two independent methods. A blastocyst triploidy frequency of 0.474% (89/18,791) was observed in Phase 2 of the study. Finally, five suspected triploid blastocysts with parental DNA available were confirmed to be triploid and of maternal origin. tNGS provides higher sequencing depth in contrast to other contemporary NGS platforms, allowing for accurate single nucleotide polymorphism calling and accurate detection of triploidy in TE biopsies. Triploidy in intracytoplasmic sperm injection-derived blastocysts is rare and mostly of maternal origin.

* Corresponding author.
E-mail address: dmarin@rmanj.com (D Marin).

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Introduction

Chromosomal aneuploidy is the most common genetic abnormality in humans and the main genetic cause of infertility (Treff and Zimmerman, 2017). The prevalence of aneuploidy in pre-implantation embryos increases dramatically with maternal age (Franasiak et al., 2014; Hassold et al., 2007), which in turn explains why infertility rapidly declines after 35 years of age in female patients. Therefore, several technologies have been developed in order to perform comprehensive chromosome screening (CCS) from a single biopsy of the pre-implantation embryo allowing for selection of euploid embryos for transfer. All randomized controlled trials published to date highlight the benefit of performing this intervention to increase implantation rates and reduce miscarriage rates (Dahdouh et al., 2015; Forman et al., 2013; Rubio et al., 2017; Scott et al., 2013; Yang et al., 2012). In addition, single embryo transfers coupled with CCS have been reported to be more cost-effective than transfers of unscreened embryos (Neal et al., 2016a). Nevertheless, although improvements in clinical outcomes seem unquestionable, current CCS platforms still face the challenge of detecting polyploidy from a single trophectoderm (TE) biopsy (Marin et al., 2017), a phenomenon known to result in negative clinical outcomes (Hassold et al., 1980; Levy et al., 2014).

Triploidy refers to the presence of an extra copy of every chromosome, resulting in 69 instead of the normal 46 chromosomes in humans. The prevalence of triploidy in natural pregnancies ranges from 1 to 3% (McFadden and Robinson, 2006; Rosenbusch, 2008). Its frequency in spontaneous abortions rises to around 8% (Hassold et al., 1980; Jacobs et al., 1978; Wang et al., 2014) and accounts for approximately 15% of all cytogenetically abnormal miscarriages (Hassold et al., 1980; Levy et al., 2014; Wang et al., 2014). Furthermore, triploid pregnancies can present aberrant phenotypes depending on the origin of the extra set of chromosomes. If the origin happens to be paternal (diandric), pregnancies may develop into partial hydatidiform moles, whereas if maternal in origin (diginic), they most commonly result in asymmetric intrauterine growth restriction, marked adrenal hypoplasias, or non-molar placentas (McFadden and Robinson, 2006). While triploid gestations culminating in live births are extremely rare, they present numerous congenital abnormalities and do not survive after the neonatal period (Takabachi et al., 2008).

In addition, the prevalence of zygotes with three pronuclei after IVF is not negligible and may result in a triploid pregnancy or failure to implant if transferred. The prevalence of three-pronucleated (TPN) zygotes ranges from 6.6 to 8.1% in conventional IVF cycles (Balakier, 1993; Porter et al., 2003) and from 2.5 to 6% in intracytoplasmic sperm injection (ICSI) cycles (Porter et al., 2003; Sachs et al., 2000). As routine practice in IVF laboratories, TPN zygotes are discarded. Nevertheless, there is strong evidence that around half of the TPN zygotes fertilized by ICSI can result in true diploids (Grau et al., 2011, 2015). Consequently, evaluating ploidy status in embryos developed from TPN zygotes might result in increasing the number of transferable embryos per patient (Capalbo et al., 2017). Therefore, a technology capable of discriminating between polyploid and normal embryos may be important to improve clinical outcomes in patients with infertility.

Given the considerable prevalence of this phenomenon in pre-implantation embryos, pregnancies and miscarriages, and its detriment to obstetric outcomes, it is clinically relevant to develop technologies that can detect this abnormality and potentially avoid pregnancies with a triploid karyotype. Although CCS platforms based on single nucleotide polymorphism (SNP) microarrays may be capable of identifying triploidy based on their genotyping data (Doody et al., 2016), most contemporary CCS platforms rely purely on copy number to evaluate chromosomal content. Furthermore, commercially available next generation sequencing (NGS) methods that involve whole genome amplification (WGA) only provide shallow sequencing in the standard protocols, preventing accurate genotyping and genotyping-based polyploidy assessment (Treff and Zimmerman, 2017), and only allow for the diagnosis of triploidy if the X and Y copy numbers fall into a step-wise pattern, which could be interpreted as two copies of the X chromosome, one of the Y and three for the rest of the autosomes.

Here we present a validation study of an NGS platform for CCS that instead of WGA follows an initial targeted amplification approach (targeted next generation sequencing, or tNGS), allowing for enough sequencing depth per locus to generate accurate genotyping data (Zimmerman et al., 2017). We demonstrate that SNP genotyping data generated in parallel to chromosome copy number analysis by tNGS accurately detects triploidy in human blastocysts from a single TE biopsy. In addition, we calculate the frequency of triploid embryos derived by ICSI and develop an independent test to confirm the presence and origin of triploidy using sequencing data from parental DNA.

Materials and methods

Study design

Phase 1 – validation of detection of triploidy by tNGS

In Phase 1, a validation step was performed in order to evaluate whether our proprietary tNGS platform could discriminate between diploid and triploid samples based on genotyping data obtained in parallel with chromosome copy number analysis. In this phase, genomic DNA isolated from triploid products of conception (POC) and isolated genomic DNA from normal individuals were processed with tNGS. In addition, six-cell aliquots of well-characterized diploid and triploid cell lines were also processed with the same platform, in order to model the approximate number of cells in a TE biopsy (Neal et al., 2016b). All samples were processed blindly. Genotyping data from 2690 SNP, from which 2571 are in autosomes and 119 in sex chromosomes, were retrieved in variant call format files (.vcf) in order to analyse the alternative allele frequencies provided for each SNP and to predict whether the sample was diploid or triploid.

Predictions of polyploidy were performed following two independent methods. *Method 1-allele ratio (AR)*: First, from all 2571 autosomal SNP genotyped by tNGS, only those with a heterozygous call were selected for each sample. For each heterozygote SNP and for each sample, the allele with the highest read count was considered the major allele, and the one with the lowest the minor allele. A value referred to as the 'average allele ratio' was calculated for each sample. Because diploid specimens have two copies of each autosome, a 1:1 ratio is expected between the major and minor allele genotypes for a given heterozygous SNP. In contrast, if three copies of all chromosomes are present, then a 2:1 ratio is expected. Therefore, the average AR may be sufficient to discriminate between a diploid and a triploid sample irrespective of any $\log_2$ ratio values obtained after copy number analysis for aneuploidy. *Method 2-high throughput*: A second high-throughput set of criteria was also used whereby alternative allele frequencies in the range from 0.30 and 0.36 were predicted as triploid. All blinded samples were analysed using these

criteria and ploidy predictions were subsequently compared with the known karyotypes after unblinding.

Phase 2 – frequency of triploidy in human blastocysts

Once our tNGS platform was properly validated for discrimination between diploid and triploid specimens, we proceeded to retrospectively estimate the frequency of triploid blastocysts among all trophectoderm biopsies processed at our centre between July 2016 and May 2017, accounting for 18,791 blastocysts. All trophectoderm biopsies were from embryos generated by ICSI.

Phase 3 – parental DNA triploidy confirmation and determination of origin

In Phase 3, a third method was developed to further confirm triploidy predictions. Blood samples from both parents of alleged triploid embryos were requested and obtained under Institutional Review Board (IRB) approval (13 March 2017, reference #RMA1-09-165) and DNA was isolated as described below. Parental DNA samples were sequenced by our tNGS-based CCS platform and genotyping data were retrieved in .vcf files. In addition, tNGS sequencing data from TE biopsies of diploid sibling embryos of the alleged triploid embryos were also analysed as diploid controls.

In a true triploid karyotype where three copies of each chromosome are present, it is expected that two copies of each chromosome share the same parental origin, and that this will also be the same across all chromosomes. To evaluate this hypothesis a % similarity between the embryo's more common allele (2:1 ratio) and the genotypes of each parent was calculated for all SNP categorized as informative. This was calculated using only a subset of SNP that were categorized as informative by presenting homozygosity on each parent and having an opposite genotype (e.g. mother AA and father BB, or mother BB and father AA). Each major allele for each embryonic SNP was identified and compared with both the maternal and paternal genotypes. If for a given SNP the embryo had an AAB genotype, then its major allele would have been A for that locus. By the principles of chromosome inheritance, a true triploid embryo would have the same parental origin of the extra allele in all evaluated SNPs. Therefore, by comparing all major alleles for all informative loci, triploid embryos may have a 100% similarity to the parent of origin. By doing this, not only is the triploid condition confirmed, but the parental origin of triploidy could also be elucidated. In the case of diploid embryos, the embryonic major alleles would be randomly distributed because for heterozygous SNP the allele frequencies will be close to 0.5, but because of sequencing artefacts will rarely present exact frequencies of 0.5 for each allele. Thus, a random distribution of percentage embryo major allele similarity is expected between the maternal and the paternal genotypes (around 50% for each) in non-triploid embryos.

Finally, we obtained data from a blastocyst that was re-biopsied four times from an unpublished study executed at our centre and that, based on the described algorithm, presented an alleged triploid pattern. Three biopsies from the TE and one from the inner cell mass (ICM) were processed by tNGS and both CCS results and ploidy calls were compared among samples.

Cell lines, products of conception, and DNA isolation

In order to mimic the amount of DNA in a TE biopsy, six-cell aliquots of the following cell lines were collected in 0.2 ml PCR tubes along with 1 µl of Gibco minimal essential media (MEM) alpha (Thermofisher, Waltham, MA, USA): GM00323 (46,XY), GM04435 (48,XY,+16,+21), GM04939 (69,XXX) and AG05025 (69,XXY) (Coriell Repository, Camden, NJ, USA). Twelve genomic DNA (gDNA) samples of POC previously diagnosed as triploid by a whole genome SNP microarray platform were donated by the CLIA-certified laboratory GeneDx (Gaithersburg, MD, USA) for the validation phase of the study.

For Phase 3 of the study, parental gDNA was isolated from blood using the QIAmp DNA Blood Maxi Kit (Qiagen, Germany) following the manufacturer's instructions. DNA samples were stored at –80°C.

Targeted next generation sequencing

A proprietary tNGS-based CCS platform was used for sample sequencing as described elsewhere (Zimmerman et al., 2017). Briefly, six-cell aliquots were subjected to lysis as previously described (Cui et al., 1989), whereas gDNA samples were normalized to a DNA concentration of 1 ng/µl with nuclease-free water. DNA amplification was performed in 50 µl reaction volume with the use of proprietary multiplexed primers and TaqMan Preamplification Master Mix following the manufacturer's instructions (ThermoFisher Scientific Inc.) for 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. Next, preamplified DNA from each sample was quantified with D1000 ScreenTape system with the Agilent 4200 Tapestation (Agilent Technologies, Waldbronn, Germany) and purified using Agencourt AMPure XP Reagent beads (Beckman Coulter, Beverly, MA). Pooled libraries with up to 48 samples were sequenced on one Ion PI Chip V3 (Thermofisher). Ion Sphere particles containing clonally amplified DNA were prepared, enriched and loaded to each PI chip using the Ion Chef instrument (Thermofisher). Loaded chips were briefly centrifuged before being sequenced using the Ion PI Hi-Q Sequencing Kit on the Ion Proton instrument (Thermofisher) following the manufacturer's instructions.

A total of 2679 amplicons were generated across the genome. Chromosome copy number estimations were performed by a proprietary custom sequencing analysis plug-in as described elsewhere (Zimmerman et al., 2017). Briefly, sequencing reads were aligned to the human reference genome (hg19) and amplicon read counts were compared against reference counts obtained from samples with normal karyotypes.

SNP genotyping

Allele frequencies were determined based on the proportion of read counts mapped to each specific locus. For example, if 100 read counts were obtained for a specific SNP and 70 presented allele A and 30 allele B, then the allele frequencies were 0.7 and 0.3, respectively. Sequencing reads were aligned to the human reference genome (hg19), thus sequencing reads that did not match the hg19 for a specific locus were considered alternative allele reads (non-reference). Customized thresholds for alternative allele frequencies were set to generate discrete genotype calls. Loci with alternative allele frequencies lower than 0.10 were called as homozygous reference, higher than 0.9 as homozygous non-reference, and the remaining range as heterozygous. A minimum coverage of five reads was required to generate a variant call; otherwise genotyping information was considered unreliable and therefore excluded from analyses.

Data analysis

Student's t-test was performed to compare average AR between known diploid and triploid samples for the validation phase and to compare % embryo major allele similarities to both parents. Analyse-it software version 2.20 (Microsoft, CA, USA) was used for statistical tests and figures.

Results

For Phase 1 of the study, a total of 23 six-cell aliquots of diploid (GM0323 = 12, GM04435 = 11) and 11 triploid cell lines (GM04939 = 6, AG05025 = 5) were sequenced, in addition to gDNA from 12 triploid POC and 12 healthy individuals. All CCS results predicted the correct karyotype for all samples. Average AR was significantly different between diploid and triploid samples, and proved sufficient to correctly identify ploidy for both cell lines (diploid 1.47 ± 0.10, triploid 2.12 ± 0.10, $P < 0.0001$) and isolated gDNA (diploid 1.30 ± 0.03, triploid 2.04 ± 0.07, $P < 0.0001$) (**Figure 1**). Moreover, when our second method was applied to each sample's .vcf file, all samples were correctly categorized as either diploid or triploid, rendering 100% specificity and sensitivity when applying the algorithm for the second method in sequencing data from control samples. **Figure 2** shows an example of how copy number and genotyping data are visualized after sequencing data are analysed for a diploid and triploid sample.

After our platform was validated for accurate detection of triploidy in Phase 1 of this study, we applied the second high-throughput method on 18,791 tNGS experiments of TE biopsies to calculate the frequency of triploidy at the blastocyst stage in in-vitro derived human embryos. Eighty-nine blastocysts were categorized as triploid, resulting in a triploidy frequency of 0.474% in human blastocysts. From these 89, 26 represented a 69,XXY karyotype, 16 a 69,XXX karyotype and the remaining 47 had additional chromosomal abnormalities (Supplementary Table S1). Interestingly, from the remaining 18,702 samples, our algorithm was able to identify 10 samples (0.053% frequency) that were called neither diploid nor triploid due to complete loss of heterozygosity in all chromosomes, which could be explained by either being haploid blastocysts or cases of complete uniparental disomy (UPD) or uniparental diploidy (**Figure 3**).

Table 1 – Parental DNA method. Percentage similarities between embryonic major alleles and parental genotypes are shown for each embryo. Bold represents suspected triploids; *italic* represents respective siblings.[a]

Sample	Predicted karyotype	Embryo major allele similarity (%)	
		Mother	Father
Embryo #1	68,XXX,–16	**100**	**0**
Sibling of embryo #1	46,XX	61.8	38.2
Embryo #2	68,XY	**93.3**	**6.7**
Sibling of embryo #2	46,XY	43.3	56.7
[a]**Embryo #3**	–	**–**	**–**
Sibling of embryo #3	46,XX	57.5	42.5
Embryo # 4	67,XXX,–2,–16	**94.9**	**5.2**
Sibling of embryo #4	46,XX	48.7	51.3
Embryo # 5	69,XXY	**100**	**0**
Sibling of embryo #5	46,XX	59.4	40.6
Embryo # 6	69,XXX	**95.3**	**4.7**
Sibling of embryo #6	46,XY	44.2	55.8

[a] Sequencing data from embryo #3 was uninterpretable and was excluded from analysis.

Furthermore, for the third phase of the study, gDNA from both parents was available from six suspected triploid unrelated embryos, thus a total of 12 parental DNA samples. After these samples were sequenced and analysed, five embryos presented an average percentage major allele similarity to maternal genotype of 96.7 ± 3.1%, whereas it was 3.3 ± 3.1% to the respective paternal genotypes ($P < 0.0001$), confirming not only that previous methods correctly identify triploid samples, but also that these were all maternal in origin (**Table 1**). One diploid sibling embryo of each alleged triploid was similarly analysed and the % major allele similarities averaged 47.5 ± 8.1% to paternal genotypes and to 52.5 ± 3.5% maternal, as expected by

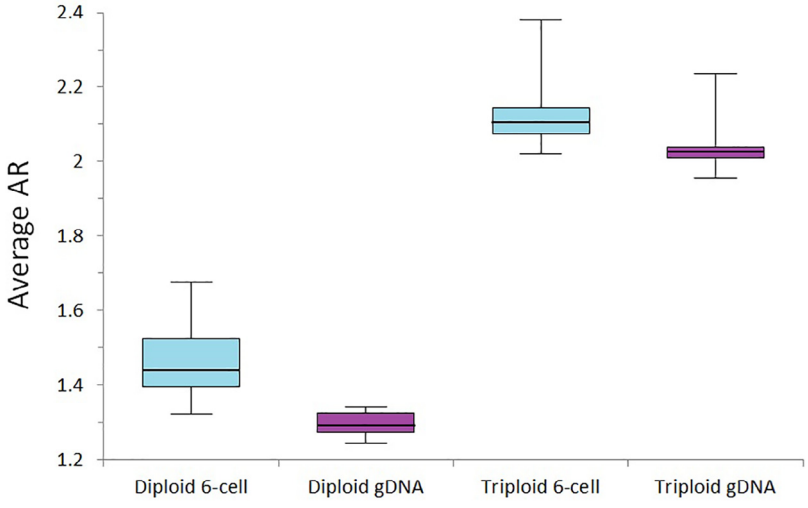

Figure 1 – Calculated average allele ratios (AR) between known diploid and triploid samples. Box plots in light blue refer to data from six-cell aliquots of diploid ($n = 23$) and triploid cell lines ($n = 11$). Those in purple show data from isolated gDNA from products of conception (POC) ($n = 12$) or blood from healthy individuals ($n = 12$). In both cases $P < 0.0001$ for diploid versus triploid. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

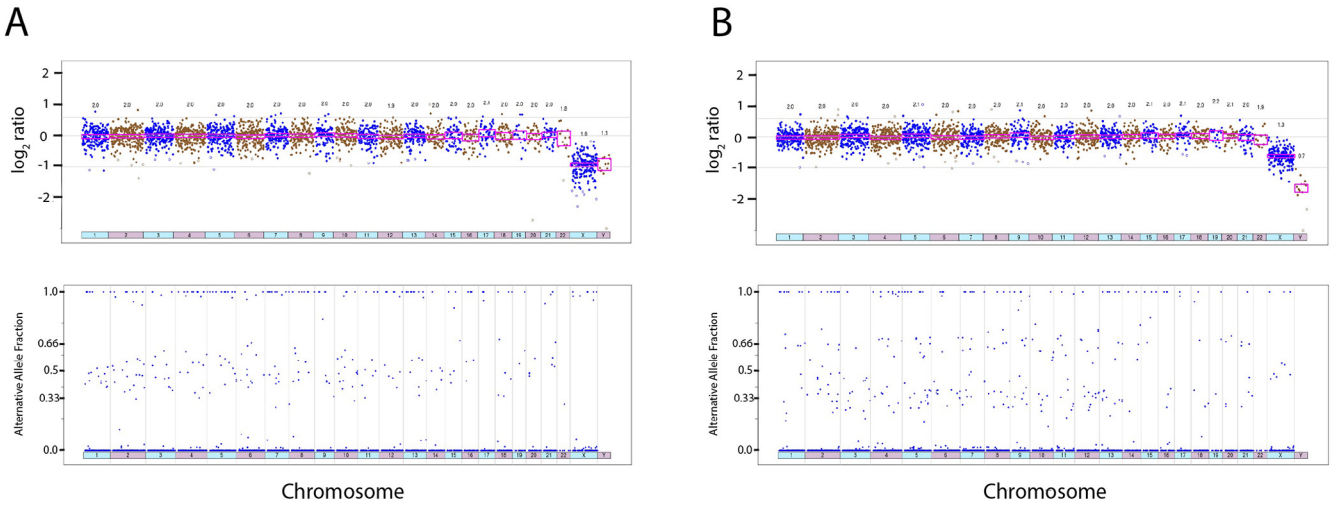

Figure 2 – Chromosome copy number and alternative allele frequency plots for samples processed by a proprietary targeted next generation sequencing-based comprehensive chromosome screening platform. Each filled dot in the upper plots (copy number) represents an amplicon. The *y*-axis shows log_2 ratio scores for each amplicon used to estimate chromosome copy numbers (small numbers above amplicons). All amplicons from the same chromosome share the same colour. The lower plots show the alternative allele frequencies of 2690 single nucleotide polymorphisms (SNP) genotyped (each represented by a dot). (A) Six-cell aliquot from diploid cell line (GM00323, 46,XY). (B) Six-cell aliquot from triploid cell line (AG05025, 69,XXY). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

random distribution for a diploid sample (**Table 1**). One embryo with available parental DNA sequenced resulted in uninterpretable results after sequencing.

Finally, one blastocyst with an alleged triploid call was re-biopsied four times, from which three were performed on the TE and one on the ICM (Supplementary Figure S1). All four biopsies showed a consistent pattern with triploidy and was called as such by the algorithm mentioned above, providing an additional alternative method to confirm triploid status and showing evidence that triploidy can be evenly distributed in the human blastocysts.

Discussion

To date, several CCS platforms have been validated for chromosome copy number analysis from a TE biopsy. However, polyploidy has remained a challenge for the majority of platforms because chromosome copy number estimations are derived from the relative amount of DNA present in the biopsy and not by individually quantifying the number of chromosomes present. These methods are useful when detecting gains or losses of individual chromosomes, but fail to identify the overall ploidy of the cell. In this study we validated a tNGS-based CCS platform for detection of triploidy in human blastocysts from a TE biopsy. In addition, we established that ICSI-derived triploid blastocysts occur at a frequency of approximately 0.5%, and that, as expected due to virtual exclusion of polyspermy, are mostly of maternal origin.

Although other CCS platforms have reported polyploidy detection along with aneuploidy testing using SNP arrays (Doody et al., 2016), these platforms are rapidly being replaced by novel technologies with markedly decreased costs per sample and with higher data resolution, extending their applications beyond aneuploidy screening. The common denominator of these novel platforms is the use of NGS technologies. In addition, the majority of these platforms involve a WGA step in their protocols in order to amplify the miniscule amounts of DNA in a TE biopsy and to proceed to library preparation and sequencing. Although cheaper, these platforms still fail to detect polyploidy. For instance, attempts have been made to diagnose a sample as triploid based on sex to autosomal chromosome ratios (Maxwell et al., 2016), which not only fail to account for cases with three X chromosomes (**Figure 3**), but could also be a product of technical error such as sample contamination. The tNGS protocol presented here, on the other hand, uses an initial step of targeted genome amplification that allows for deep sequencing of several reads spanning targeted loci in the genome. As a result, the sequencing depth obtained at specific loci is deep enough to retrieve genotyping data across all 24 chromosomes, and as shown in the Results section, the data generated in parallel to copy number estimations are pivotal for determining the actual number of chromosomes present per cell and therefore indicate cases of triploidy. In addition, cases of sample contamination that could potentially lead to a false positive call for triploidy can be readily identified by our high-throughput algorithm because it is not expected that SNPs in a contaminated sample will follow an allele frequency distribution of a true triploid karyotype (**Figure 2**). Nevertheless, here we proposed two alternative methods that can help in confirming a polyploidy diagnosis and rule out a contamination issue.

Besides a platform validation with known triploid samples, here we report two alternative methods to confirm the triploid status of a TE biopsy. One is based on patterns of inheritance deduced after sequencing parental DNA and comparing SNP allele frequencies between embryonic and parental DNA. This method is particularly useful because our analysis did not account for the specificity (false positives) of the proposed algorithm given that due to the nature of our samples it was technically impossible to know the true ploidy status, because embryos were either transferred, cryopreserved for further cycles or discarded if abnormal. Therefore, if applied clinically, it is recommended to request sequencing of parental DNA so as to provide further evidence to report an embryo as triploid and thus unsuitable

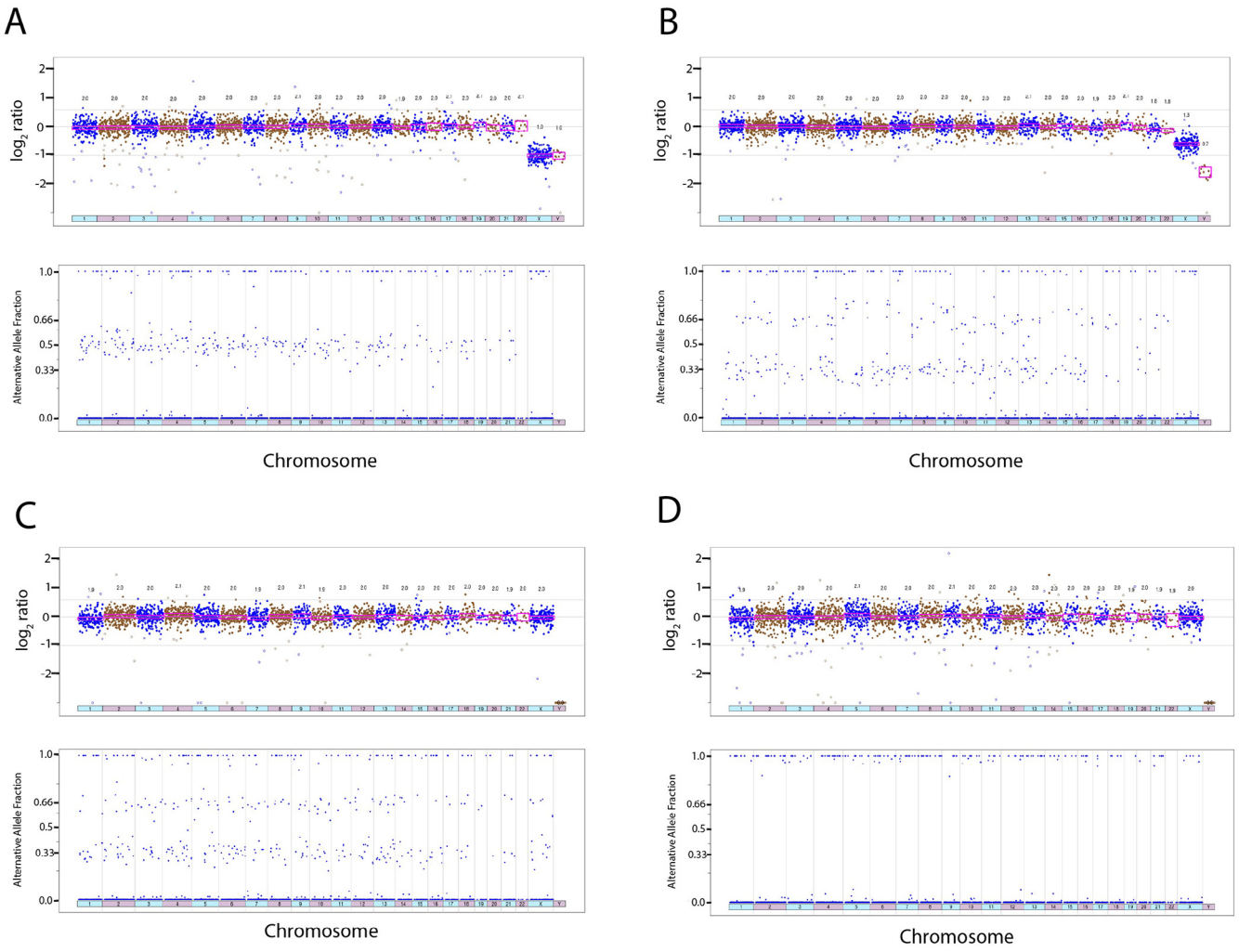

Figure 3 – SNP genotyping patterns generated by targeted next generation sequencing reveal cases of triploidy and putative haploidy or total uniparental disomy (UPD) (uniparental diploidy) in human blastocysts from a trophectoderm biopsy. (A) Results from a diploid embryo with 46,XY karyotype. (B) Results from an alleged triploid embryo (69,XXY). (C) Alleged triploid embryo with 69,XXX karyotype. (D) Case with complete loss of heterozygosity, indicating either a haploid embryo or total UPD.

for transfer. In addition, one abnormal embryo that was selected for a parallel study at our centre underwent multiple biopsies. Repeating biopsies can be considered as an alternative approach because of other issues that can potentially cause distortion from a diploid SNP allele frequencies pattern, such as sample contamination or poor sequencing depth. A second diagnosis from a second biopsy and independent sample processing may prove useful in reaching a diagnosis.

An immediate adjunct benefit of detecting triploidy in parallel with CCS is the ability to avoid transfer of triploid embryos that would have otherwise been considered normal. As we report in this study, the phenomenon of triploidy appears to be rare, at least in ICSI-derived embryos, which may raise concerns regarding the diagnosis of triploidy in the clinical setting. The predictive value of every diagnostic test is dependent on the prevalence of the condition in question. Therefore, screening for phenomena with low prevalence leads to poor clinical predictive values, which translates to an unacceptably high rate of false positive diagnoses (Grimes and Schulz, 2002). Nonetheless, it is also important to stress that the applications of this technology go beyond deselecting blastocysts for transfer. For instance, several groups have suggested that abnormally fertilized zygotes with one or three pronuclei can result in a normal diploid blastocyst (Capalbo et al., 2017; Grau et al., 2011, 2015). A platform capable of discriminating between diploid and triploid samples is of immense value because in many IVF centres zygotes with an abnormal number of pronuclei are immediately discarded, reducing the number of potentially viable embryos per patient. Moreover, by obtaining genotyping data from parents by our proposed platform the issue of heteroparentality can be further studied and confirmed, because it is also an important concern raised after putative self-correction of mono- and tri-pronucleated zygotes.

To our knowledge, this is the first time that a NGS-based CCS platform has been validated for detection of triploidy. Although we have validated this diagnosis for a limited amount of DNA, as is the case in TE biopsies, this platform can be applied for confirmation of polyploidy in other types of tissues provided that isolation of gDNA is plausible. More studies assessing the various additional applications for this platform mentioned above are encouraged in order to further understand embryonic pre-implantation development and improve outcomes in a clinical setting.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2017.12.015.

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