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ARTICLE

Karyomapping identifies second polar body DNA persisting to the blastocyst stage: implications for embryo biopsy

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Abstract Blastocyst biopsy is now widely used for both preimplantation genetic screening (PGS) and preimplantation genetic diagnosis (PGD). Although this approach yields good results, variable embryo quality and rates of development remain a challenge. Here, a case is reported in which a blastocyst was biopsied for PGS by array comparative genomic hybridization on day 6 after insemination, having hatched completely. In addition to a small trophectoderm sample, excluded cell fragments from the subzonal space from this embryo were also sampled. Unexpectedly, the array comparative genomic hybridization results from the fragments and trophectoderm sample were non-concordant: 47,XX,+19 and 46,XY, respectively. DNA fingerprinting by short tandem repeat and amelogenin analysis confirmed the sex chromosome difference but seemed to show that the two samples were related but non-identical. Genome-wide single nucleotide polymorphism genotyping and karyomapping identified that the origin of the DNA amplified from the fragments was that of the second polar body corresponding to the oocyte from which the biopsied embryo developed. The fact that polar body DNA can persist to the blastocyst stage provides evidence that excluded cell fragments should not be used for diagnostic purposes and should be avoided when performing embryo biopsies as there is a risk of diagnostic errors.

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Introduction

Blastocyst biopsy, by excision of small numbers of herniating trophectoderm cells, is now widely used, and is increasingly replacing cleavage stage biopsy, for both preimplantation genetic screening (PGS) for aneuploidy and preimplantation genetic diagnosis (PGD) of single gene defects and other abnormalities (Thornhill et al., 2012). The main reasons for this are the availability of improved culture media and the widespread use of blastocyst culture to select normally developing embryos for transfer with improved implantation and live birth rates. Also, the original protocol, which used microneedles for partial zona dissection to promote herniation as the blastocyst expands, and mechanical excision of trophectoderm cells (Dokras et al., 1990, 1991), has now been superseded by the use of non-contact infrared lasers (Boada et al., 1998; Kokkali et al., 2005; Veiga et al., 1997). Zona drilling by laser allows precise control of the position of the herniating trophectoderm cells, away from the inner cell mass, and laser assisted excision causes minimal damage to the biopsied cells and the embryo (Scott et al., 2013).

Nevertheless, blastocyst biopsy remains challenging because of variability in embryo quality, particularly in the number of cells in the trophectoderm layer and the rate of development to the expanded blastocyst stage. Although most normally developing embryos reach the six- to 10-cell stage on the morning of day 3 after insemination, allowing cleavage-stage biopsy, the timing of blastocyst expansion can vary by over 24 h and occur on days 5, 6 or even day 7. Increasingly, therefore, vitrification is being used to cryopreserve biopsied blastocysts allowing more flexibility in the timing of blastocyst biopsy (Liebermann, 2015). One strategy is to biopsy any embryos reaching the expanded blastocyst stage on day 5, carry out the genetic analysis within 24 h and transfer unaffected fresh blastocysts on day 6. Embryos reaching the expanded blastocyst stage on day 6 or day 7 are biopsied later, vitrified, tested and unaffected embryos are transferred in a subsequent cycle. Alternatively, all biopsied blastocysts can be vitrified and replaced in subsequent cycles. Indeed, recent evidence indicates that a strategy involving trophectoderm biopsy, vitrification and PGS is highly effective clinically, as euploid blastocysts all have similarly high implantation and clinical pregnancy rates despite differences in morphology and developmental rate (Capalbo et al., 2014).

Here, we report a case in which a slow developing embryo only reached the blastocyst stage on day 6 after insemination, and had hatched completely before being biopsied for PGS by array comparative genomic hybridisation (CGH). Biopsy of hatched blastocysts is more difficult and, because the trophectoderm sample obtained was relatively small, excluded cell fragments present in the subzonal space were also sampled. Excluded fragments and cells, some of which are nucleated, are commonly observed at the morula stage onwards, and are a potential source of DNA for genetic analysis. Unexpectedly, however, the array CGH results from the fragments and trophectoderm samples were non-concordant in this case. To investigate the cause of this non-concordance, DNA fingerprinting using a panel of informative short tandem repeat (STR) markers, including amelogenin to determine the sex and single nucleotide polymorphism (SNP) genotyping and karyomapping analysis, was carried out on all samples. The results of both tests were completely concordant, and karyomap analysis identified beyond doubt that the origin of the DNA amplified from the fragments was exclusively that of the second polar body corresponding to the fertilized oocyte that gave rise to the embryo from which the trophectoderm had been biopsied. The implications for blastocyst biopsy and the risk of diagnostic errors are discussed.

Materials and methods

Patient history and IVF treatment cycle

Following genetic counselling, a couple (maternal age 40 years; paternal age 34 years) requested IVF with PGS by array CGH for advanced maternal age after failing to conceive naturally for more than a year. The woman had never previously been pregnant but otherwise had no known cause for infertility. The man had never fathered a child and had a history of surgery to correct undescended testicles. Semen analysis, however, showed that he was normozoospermic.

Ovarian stimulation was achieved by a standard antagonist regimen with HCG trigger after 12 days of stimulation. Oocyte collection followed 35 h afer HCG, and six mature metaphase II oocytes were retrieved. All oocytes underwent IVF with the male partner's sperm and all six fertilized normally, with two pronuclei visible the next day (day 1 after insemination). The embryos were cultured in a time-lapse incubator (Embryoscope; Fertilitech, Denmark) to enable continuous observation. On day 6, three embryos had reached the expanded blastocyst stage, with one of the three embryos having hatched completely from the zona pellucida. The remaining three embryos arrested at the cleavage stage and were discarded.

Blastocyst biopsy and vitrification

The two expanded, non-hatched blastocysts were biopsied by first making a small hole in the zona pellucida using a laser (Saturn; Research Instruments, Penryn, UK) opposite to the position of the inner cell mass. Three to 10 cells were then drawn through the breach in the zona into a sampling pipette and excised using a series of laser pulses across the join between adjacent trophectoderm cells. With the hatched blastocyst, the embryo was immobilized directly by suction onto the holding pipette and biopsied by drawing a small number of trophectoderm cells into a sampling pipette as above. In addition, excluded fragments left behind in the subzonal space (**Figure 1**) were also sampled separately. Finally, all three biopsied blastocysts were vitrified using a commercial kit (Kitazato, Japan) following a previously published protocol (Kuwayama, 2007).

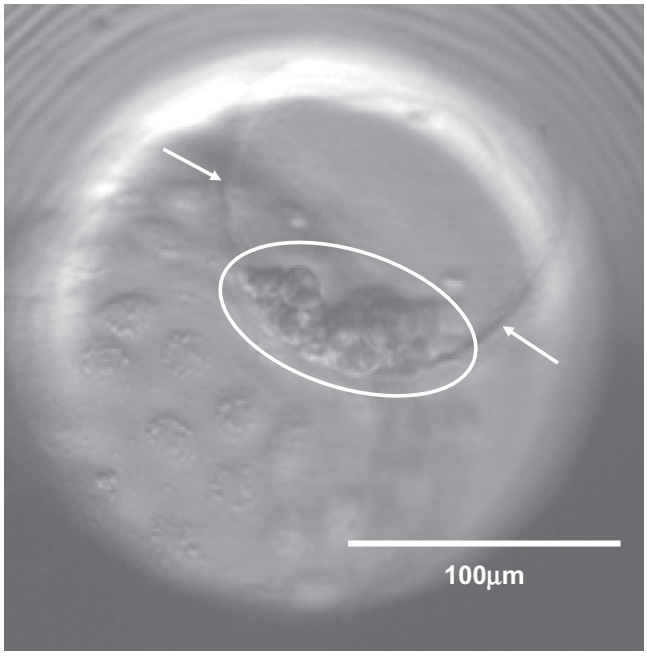

Figure 1 Hatched blastocyst (embryo 5) on day 6 after insemination before biopsy. Note the large group of excluded cell fragments (circled) remaining within the zona pellucida (arrows).

Array comparative genomic analysis

The biopsied cells and fragments were each placed in 2 μl of phosphate buffered saline in 0.2 ml polymerase chain reaction (PCR) tubes and whole genome amplification (WGA) carried out using a PCR-library based method (Sureplex, Illumina, Cambridge). The WGA products were then used for 24 chromosome copy number analysis by array CGH (24Sure; Illumina, Cambridge) with dedicated software (Bluefuse Multi v3; Illumina, Cambridge) according to the manufacturer's instructions. For detailed methodology see Fragouli et al. (2011).

DNA fingerprinting

All of the WGA products and parental genomic DNAs were DNA fingerprinted by analysis of five informative STR markers on different autosomes (D2S389, D3S1581, D4S2964, D7S2847, D15S659) and on the sex chromosomes (AMELX/Y). The STR markers were amplified in separate single-plex fluorescent PCRs (Hot Master Taq DNA polymerase; 5PRIME, Hilden, Germany), and the amplified fragments sized by capillary electrophoresis (3130 Genetic Analyzer (Applied Biosystems, USA) with dedicated software (GeneMapper v4.0; Applied Biosystems, USA).

Single nucleotide polymorphism genotyping and karyomapping

All of the WGA products and parental genomic DNAs were also genotyped at about 300K SNP loci genome wide for karyomap analysis using a dedicated beadarray (Human Karyomap; Illumina, USA) as described previously (Natesan et al., 2014) using a 24 h protocol (Konstantinidis et al., 2015). Genotype data were then exported into Microsoft Excel and karyomap analysis carried out using a dedicated Visual Basic for Applications (VBA) macro. To phase heterozygous SNPs, one of the embryo samples was used as a reference, and this was then repeated using another embryo to check the analysis. Finally, the positions of meiotic crossovers between parental chromosomes for each chromosome were located and marked (excluding reference crossovers) and other custom VBA macros used for processing.

Ethical approval

No ethical approval was required for this study. All work for the study was performed within the remit of the Bridge Center's HFEA licence.

Results

Array comparative genomic analysis

Whole genome amplification and array CGH was successful with all four samples biopsied from the three biopsied blastocysts (**Table 1**). One embryo (embryo 3) was missing a copy of chromosome 22 (monosomy 22), one embryo was euploid and the two samples from the third embryo were non-concordant. The trophectoderm biopsy (sample 5a) was euploid and male (46,XY), whereas the excluded fragment sample (sample 5b) had an extra chromosome 19 (trisomy 19) and was female (47,XX,+19) (**Figure 2**).

DNA fingerprinting

Short tandem repeat (STR) analysis at five loci on different chromosomes demonstrated that all four samples had unique combinations of parental alleles indicating they were related but distinct individuals (**Table 2**). Furthermore, amelogenin analysis confirmed the sex of each sample as originally ascertained by array CGH. There was a high allele dropout (ADO) rate across the samples, as typically observed for STR analysis applied to WGA products. In addition, however, the excluded fragment sample was anomalous because only a single maternal allele was detected at each of the three STRs which amplified and no paternal alleles were observed for any of the STRs. Furthermore, the three maternal alleles were the opposite of those seen in the corresponding trophectoderm sample.

Single nucleotide polymorphism genotyping and karyomapping

All four WGA products underwent successful SNP genotyping and karyomapping. Conventional analysis of the SNPs on the X and Y chromosomes confirmed the sex indicated by array CGH in all cases. In addition, karyomapping confirmed the absence of the maternal copy of chromosome 22 in embryo 3

Table 1 24 chromosome copy number analysis of biopsied blastocysts by array comparative genomic hybridization and single nucleotide polymorphism genotyping and karyomapping.

Sample ID	*Sample type*	*Array CGH*	*Karyomapping*
3	Trophectoderm	45 XX, −22	45 XX, −22 (maternal)
5a	Trophectoderm	46 XY	46 XY
5b	Excluded cell fragments	47 XX, +19	23 X haploid (maternal)
6	Trophectoderm	46 XY	46 XY

CGH, comparative genomic hybridization.

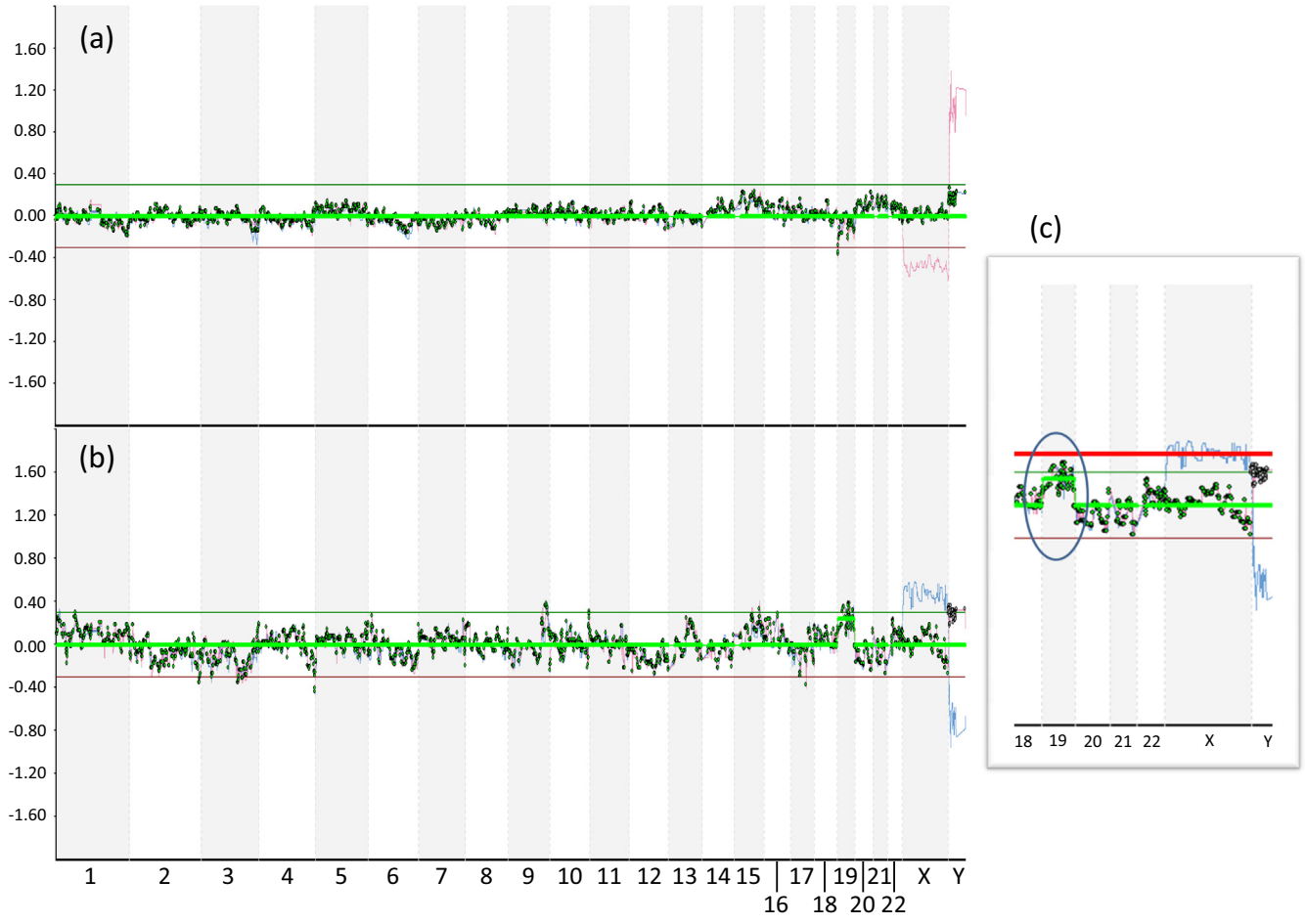

Figure 2 Array comparative genomic hybridization ratio plots for the trophectoderm biopsy sample (a), and the excluded cell fragments (b) from embryo 5. Note that both samples have normal copy number for all chromosomes except that the ratio of probes on chromosome 19 are consistently raised in (b). Also the gender of the trophectoderm sample (a) is identified as male, as there is only a single copy of the X chromosome compared with the sex-mismatched female DNA and a single Y chromosome (pink line). The fragment sample (b) is identified as female with the same copy number for autosomes and the X chromosome and missing the Y chromosome compared with sex-mismatched male DNA (blue line). (c) Magnified portion of (b) showing the elevated ratio for chromosome 19 (circled) not reaching the level of the internal X chromosome control (bold red line) on the array.

(Table 1) and, in agreement with the euploid array CGH result, failed to detect any meiotic trisomies or missing chromosomes in the trophectoderm sample from embryo 5 (sample 5a) and embryo 6. For the excluded fragment sample from embryo 5 (sample 5b), however, only maternal SNP markers for a single maternal chromosome were detected across all chromosomes (including chromosome 19) indicating that it was haploid.

Comparing the karyomaps and the positions of the crossovers in the excluded fragment sample (sample 5b) and the results for maternal chromosomes in the trophectoderm sample (sample 5a), it was clear that all 22 autosomes and

Table 2 DNA fingerprinting at five short tandem repeat markers on different chromosomes and amelogenin.

Marker	Samples											
	Paternal gDNA		Maternal gDNA		3		5a		5b		6	
D2S389	197	209	199	216	209	216	197	**216**		199	209	216
D3S1581	113	142	142		142		142			ADO	113	142
D4S2964	179	182	187	191	ADO	187	ADO	187		ADO	182	191
D7S2847	178	184	184	192	184		178	**184**		**192**	184	
D15S659	176	200	180	192	176	192	176	**192**		**180**	ADO	192
AMELX/Y	105	110	105		105		110	105		105	105[a]	

[a]Presumed allele dropout of AMELY (110).
Paternal alleles, blue and red; maternal alleles, green and yellow; semi- informative alleles, orange; uninformative alleles, no shading.
Samples 3, 5a and 6 are trophectoderm samples. Sample 5b is the excluded cell fragment sample related to sample 5a.
Bold type highlights the opposite maternal alleles present in samples 5a and 5b.

the X chromosome were derived from the same maternal homologue in both samples, i.e. the maternal haplotype (yellow or green) detected around the centromere was identical (**Figure 3**). This proves that the maternal chromosome sets from the two samples are derived from the same oocyte. Moreover, the presence of only a maternal set of chromosomes and the pattern of crossovers identifies the origin of the DNA in the excluded fragment sample as being derived exclusively from the second polar body i.e. most of the crossovers were in different positions, except for 12 distal crossovers in closely similar positions, as would be expected for reciprocal crossovers between sister chromatids (Ottolini et al., 2015). These genetic data rule out the possibility of the two samples originating from sibling embryos. Similar comparisons with the maternal chromosomes in the other two embryos demonstrated that they had distinct maternal chromosome sets and crossover patterns (data not shown). Finally, the maternal haplotypes identified with karyomapping in the two samples from embryo 5 on the relevant chromosomes were concordant with the results of the STR analysis, which showed opposite alleles at each locus.

Discussion

A major advantage of blastocyst biopsy is that multiple trophectoderm cells, in the range of 3 to 10 cells, can be biopsied from each embryo. Compared with genetic analysis of single cells, whole genome amplification bias and chromosome copy number artefacts are reduced in multiple cell samples and also ADO at, for example, mutation sites decreases dramatically (Handyside et al., 2004; Piyamongkol et al., 2003). On the other hand, a disadvantage is that chromosomal mosaicism arising through abnormal processes of nuclear and cell division, mainly during cleavage, can persist at the blastocyst stage. In the context of PGS of aneuploidy by any quantitative method, this can result in intermediate chromosome copy number changes, which may be difficult to interpret. Although most multiple trophectoderm biopsies are concordant, a few give results consistent with mosaicism (Capalbo et al., 2013b).

In the case reported here, one of the embryos only reached the blastocyst stage on day 6 but had hatched completely from the zona before biopsy. Biopsy of fully hatched blastocysts is technically challenging and, in this situation, the blastocyst has to be held directly by gentle suction to the holding pipette while the trophectoderm cells to be biopsied are drawn up into a sampling pipette and a laser used to excise them. The blastocyst then collapses initially preventing any second attempt to obtain more cells if deemed necessary. As only a small number of cells were biopsied from this particular embryo, a cluster of excluded cell fragments that had remained within the zona pellucida were also biopsied separately for analysis. Although no evidence has been published, these cells were sampled on the assumption that they were the remnants of arrested cells not incorporated into the developing blastocyst. The array CGH results for the fragments (47,XX,+19) and the trophectoderm sample (46,XY), however, were non-concordant. Although it was assumed that the more reliable result for this embryo was the latter, despite rigorous witnessing protocols, we could not rule out that the samples had been mixed up as the embryo biopsied following this one was also a euploid male (46,XY). To exclude this possibility, therefore, all samples were DNA fingerprinted using a panel of informative STR markers and amelogenin to determine the sex and subsequently SNP genotyped for karyomapping.

The results of the DNA fingerprinting and amelogenin analysis confirmed the sex of three of the samples determined by array CGH and ruled out any sample mix up as the sampled fragments had a distinct set of STR alleles. In the fourth sample, only AMELX amplified from embryo 6, presumably as a result of ADO (**Table 2**). Furthermore, the absence of any paternal alleles and the presence of only a single maternal allele at each locus suggested that the DNA may have originated in a polar body with a haploid set of maternal chromosomes. Finally, genome-wide SNP genotyping and karyomapping identified beyond doubt that the DNA from the fragments was exclusively that of the second polar body corresponding to the embryo from which the trophectoderm cells had been sampled. The evidence for this is threefold: no paternal SNP markers were detected for any of the chromosomes; the grandparental origin of each of the 23 maternal chromosomes in both samples was identical (theoretically the chance of an identical set is 2^{23}:1); and, although most crossovers were in different positions, there were 12 crossovers in identical positions in both sets of maternal chromosomes consistent with distal crossovers between sister chromatids (Ottolini et al., 2015)

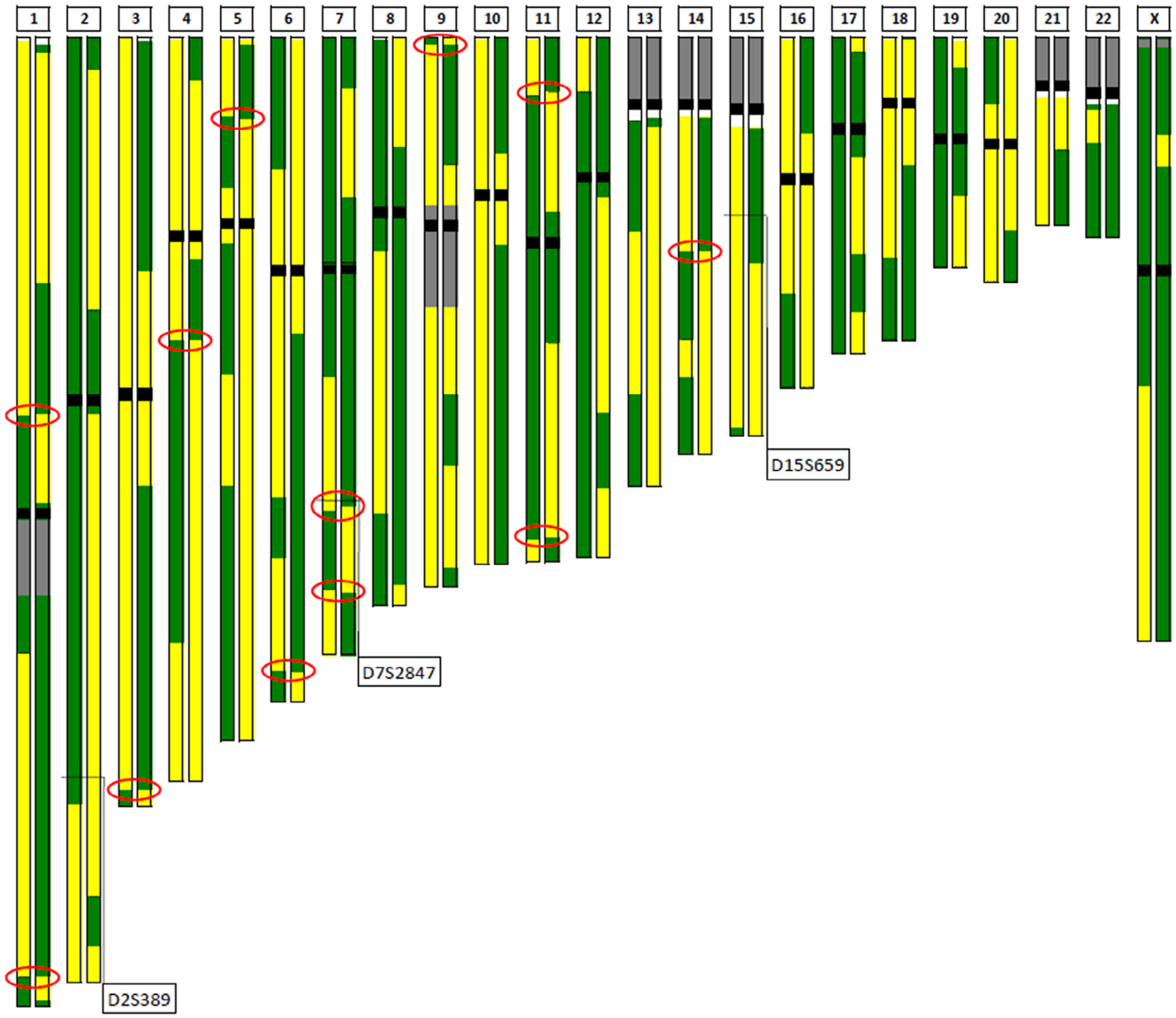

Figure 3 Comparison of the karyomaps for the maternal chromosome sets for the excluded cell fragments (on the left in each case) and the trophectoderm biopsy (right). Maternal haplotypes, yellow and green; centromeres, black; satellite regions, grey. Note the crossovers from one maternal haplotype to the other are mostly different except for 12 common crossovers between sister chromatids (red ellipses). The positions of the three STR markers which amplified in both samples are indicated on the relevant chromosomes. Note that the maternal haptotypes are different at these positions.

(**Figure 3**). Furthermore, the maternal haplotypes identified by karyomapping at the STR loci are all concordant with the alleles detected by direct analysis (**Table 2**).

Interestingly, contrary to the array CGH result, only a single maternal chromosome 19 was detected by karyomap analysis in both samples from embryo 5. The trisomy 19 in the fragment sample, however, was reported on the assumption that it was a multiple cell sample, which could include mosaic copy number abnormalities. Close inspection of the array CGH plot for sample 5b (**Figure 2**) reveals that this is most likely the explanation for this discrepancy. The ratio of all of the probes on chromosome 19 is raised consistently as indicated by the software (green line). The ratio shift, however, is much less than the X chromosome internal control compared with the male sex mismatched control DNA that would qualify as a trisomy in a single cell sample. In this case, therefore, this may be an example of whole genome amplification bias, which is known to occur with polar body samples (Capalbo et al., 2013a; Christopikou et al., 2013). Knowing that this can occur, experienced array CGH laboratories typically use more stringent criteria for calling aneuploidies in polar bodies, especially those affecting chromosome 19.

The persistence of polar body DNA for almost a week after extrusion following fertilization is unexpected as both polar bodies seem to fragment and are generally not visible at the blastocyst stage. In this case, a relatively large group of excluded fragments has been shown to have DNA originating exclusively from the second polar body. The origin of most of the fragments, therefore, was presumably anucleate fragments, which commonly appear during cleavage divisions.

Larger studies of such fragments will be needed to assess how frequently this occurs and whether there is evidence of the persistence of DNA from the first polar body also or excluded nucleated cells, which have subsequently fragmented.

In conclusion, the implications of the persistence of polar body DNA in excluded fragments in the sub-zonal space up to the blastocyst stage are important for both cleavage and blastocyst stage embryo biopsy. Clearly, the assumption that these fragments are representative of the embryo is not always the case. Therefore, there is a risk of misdiagnosis for several reasons. As demonstrated here for the three STR loci, PGD for single gene defects by conventional targeted haplotype and mutation analysis, for example, may give the opposite result for maternal loci in distal regions of the affected chromosome. Polar body DNA may be more prone to WGA bias artefacts for chromosome copy number analysis (Capalbo et al., 2013a; Christopikou et al., 2013). Biopsy samples that inadvertently include these fragments could be contaminated with DNA, which is not representative of the embryo, potentially giving false results or appearing to be mosaic. Until further studies have been conducted, harvesting samples of excluded fragments for diagnostic purposes should be avoided and efforts made to prevent them contaminating any embryo biopsy samples.

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