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Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development

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STUDY QUESTION: What is the origin and composition of cell-free DNA in human embryo spent culture media?

SUMMARY ANSWER: Cell-free DNA from human embryo spent culture media represents a mix of maternal and embryonic DNA, and the mixture can be more complex for mosaic embryos.

WHAT IS KNOWN ALREADY: In 2016, ~300 000 human embryos were chromosomally and/or genetically analyzed using preimplantation genetic testing for aneuploidies (PGT-A) or monogenic disorders (PGT-M) before transfer into the uterus. While progress in genetic techniques has enabled analysis of the full karyotype in a single cell with high sensitivity and specificity, these approaches still require an embryo biopsy. Thus, non-invasive techniques are sought as an alternative.

STUDY DESIGN, SIZE, DURATION: This study was based on a total of 113 human embryos undergoing trophectoderm biopsy as part of PGT-A analysis. For each embryo, the spent culture media used between Day 3 and Day 5 of development were collected for cell-free DNA analysis. In addition to the 113 spent culture media samples, 28 media drops without embryo contact were cultured in parallel under the same conditions to use as controls. In total, 141 media samples were collected and divided into two groups: one for direct DNA quantification (53 spent culture media and 17 controls), the other for whole-genome amplification (60 spent culture media and 11 controls) and subsequent quantification. Some samples with amplified DNA ($N = 56$) were used for aneuploidy testing by next-generation sequencing; of those, 35 samples underwent single-nucleotide polymorphism (SNP) sequencing to detect maternal contamination. Finally, from the 35 spent culture media analyzed by SNP sequencing, 12 whole blastocysts were analyzed by fluorescence *in situ* hybridization (FISH) to determine the level of mosaicism in each embryo, as a possible origin for discordance between sample types.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Trophectoderm biopsies and culture media samples (20 µl) underwent whole-genome amplification, then libraries were generated and sequenced for an aneuploidy study. For SNP sequencing, triads including trophectoderm DNA, cell-free DNA, and follicular fluid DNA were analyzed. In total, 124 SNPs were included with 90 SNPs distributed among all autosomes and 34 SNPs located on chromosome Y. Finally, 12 whole blastocysts were fixed and individual cells were analyzed by FISH using telomeric/centromeric probes for the affected chromosomes.

MAIN RESULTS AND THE ROLE OF CHANCE: We found a higher quantity of cell-free DNA in spent culture media co-cultured with embryos versus control media samples ($P \leq 0.001$). The presence of cell-free DNA in the spent culture media enabled a chromosomal diagnosis, although results differed from those of trophectoderm biopsy analysis in most cases (67%). Discordant results were mainly attributable to a high percentage of maternal DNA in the spent culture media, with a median percentage of embryonic DNA estimated at 8%. Finally,

from the discordant cases, 91.7% of whole blastocysts analyzed by FISH were mosaic and 75% of the analyzed chromosomes were concordant with the trophectoderm DNA diagnosis instead of the cell-free DNA result.

LIMITATIONS, REASONS FOR CAUTION: This study was limited by the sample size and the number of cells analyzed by FISH.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study to combine chromosomal analysis of cell-free DNA, SNP sequencing to identify maternal contamination, and whole-blastocyst analysis for detecting mosaicism. Our results provide a better understanding of the origin of cell-free DNA in spent culture media, offering an important step toward developing future non-invasive karyotyping that must rely on the specific identification of DNA released from human embryos.

STUDY FUNDING/ COMPETING INTEREST: This work was funded by Igenomix S.L. There are no competing interests.

Key words: cell-free DNA / non-invasive preimplantation genetic testing (NI-PGT) / next generation sequencing (NGS) / mosaicism / SNP sequencing

Introduction

Chromosomal aneuploidy occurs in 20–80% of human embryos (Hassold and Hunt, 2001; Vera-Rodriguez *et al.*, 2015). This phenomenon is less frequent in other species; for instance, it is found in only 1% of rodent embryos (Bond and Chandley, 1983). These aberrations can originate through both mitotic and meiotic errors. Mitotic errors lead to mosaic embryos, but most aneuploidies found at the blastocyst stage and in pregnancies are of meiotic origin. Because aneuploid embryos can reach the blastocyst stage and implant into the uterus, an affected newborn or a miscarriage can result (Rubio *et al.*, 2005; Alfarawati *et al.*, 2011; Campos-Galindo *et al.*, 2015). These potential outcomes underscore the importance of aneuploidy testing in assisted reproduction.

Current techniques used for preimplantation genetic testing of aneuploidies (PGT-A) enable the analysis of the full chromosome content of a single cell with high sensitivity and specificity (Fiorentino *et al.*, 2014). These techniques have evolved from fluorescence *in situ* hybridization (FISH) (Werlin *et al.*, 2003), which only analyzes a limited number of chromosomes, to array comparative genomic hybridization (aCGH) (Colls *et al.*, 2012; Rodrigo *et al.*, 2014), single-nucleotide polymorphism (SNP) arrays (Treff *et al.*, 2010), and, more recently, next-generation sequencing (NGS) (Zhang *et al.*, 2013). These techniques require a biopsy of either a blastomere on Day 3 or few trophectoderm cells on Day 5 of embryonic development. Furthermore, embryo biopsies require specific equipment and trained personnel that add cost and risk to the procedure. Non-invasive alternatives could improve the utility of PGT-A in assisted reproduction.

Recent studies have reported the existence of embryonic cell-free DNA, opening a new era of possibilities for non-invasive PGT. However, the percentages of informative samples vary widely, with reported values of 3.5% (Shamonki *et al.*, 2016), 27% (Feichtinger *et al.*, 2017) and 85.7% (Xu *et al.*, 2016). These discrepancies, along with the known existence of mosaicism in the human trophectoderm and the possibility of maternal DNA contamination (Hammond *et al.*, 2017), prompted us to analyze the presence and nature of cell-free DNA and to determine whether it reflects the chromosomal constitution of the human embryo. We therefore collected DNA from spent culture media and compared sequences to DNA obtained from trophectoderm biopsies. To evaluate limiting factors for non-invasive assessment, we performed (i) SNP sequencing to rule out maternal DNA contamination and (ii) whole-blastocyst FISH for evaluating chromosomal status at a single-cell level to assess mosaicism. Our results demonstrate that, using current technologies, there is poor concordance between DNA obtained from spent culture media and from trophectoderm biopsy, attributable to the high level of maternal DNA contamination in the media as well as trophectoderm mosaicism. Thus, future studies should focus on developing methods to specifically identify embryonic cell-free DNA and rule out maternal contamination to efficiently translate this non-invasive technology into clinical use.

Materials and Methods

Experimental design

One hundred thirteen human embryos from 42 couples were used for this study. Embryos were fertilized by intracytoplasmic sperm injection (ICSI) and cultured until blastocyst stage. Culture medium was changed, and assisted hatching was performed on Day 3. On Day 5, a trophectoderm biopsy was performed for PGT-A analysis as part of the clinical routine, and spent culture medium was collected from each embryo for cell-free DNA analysis. Blastocysts were vitrified until results were available. In addition to the 113 spent culture media, 28 media drops without contact with embryos were kept in parallel under the same conditions to use as controls. The 141 media samples were collected and divided into two groups: one for direct DNA quantification with 70 media samples (53 spent culture media and 17 controls) and the other for whole-genome amplification (WGA) with 71 samples (60 spent culture media and 11 controls) (Fig. 1). Subsequent analysis of the amplified samples included: (i) DNA quantification, for comparison to the non-amplified samples; (ii) aneuploidy testing by NGS sequencing, for comparison to trophectoderm biopsy results; (iii) SNP sequencing for a maternal contamination study comparing the results with the related trophectoderm biopsy and follicular fluid and (iv) FISH analysis of whole blastocysts for the analysis of mosaicism and complementary aneuploidies.

Ethical approval

The procedure and protocol for spent media collection and analysis from human embryos were approved by the local Ethical Committee at Instituto Valenciano de Infertilidad, Spain (reference 1501-IGX-003-CS).

Embryo source

All embryos included in this study were from PGT-A cycles with trophectoderm biopsy. Spent culture media from these embryos were collected with patients' written informed consent. In addition, follicular fluid samples, as well as those aneuploid blastocysts still available at the time of the study, were collected whenever possible from the patients included in the study.

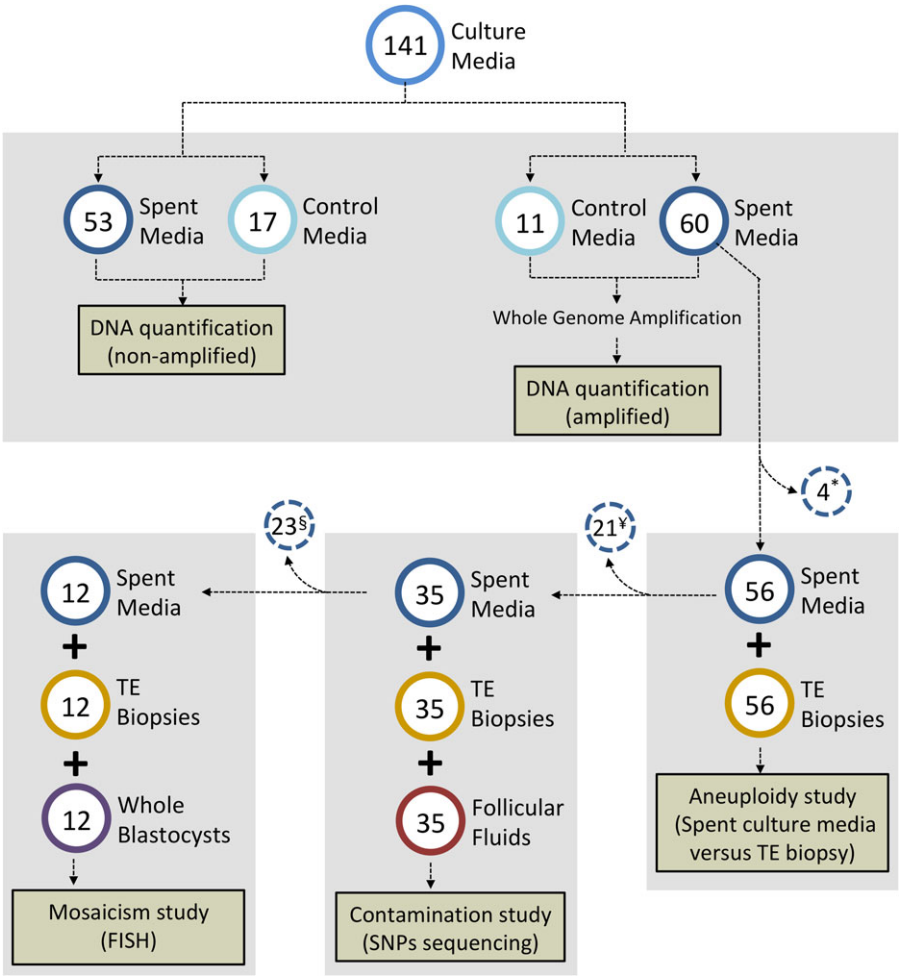

Figure 1 Flow chart including all samples in the study. Collected culture media were classified according to the type of analysis performed. Samples were differentiated between spent medium (culture medium in contact with the embryos between Day 3 and Day 5 of development) and controls (medium never in contact with embryos). Additional sample types were included for each part of the study: trophectoderm biopsies for the aneuploidy study, trophectoderm biopsies and follicular fluids for the contamination study, and trophectoderm biopsies and whole blastocysts for the mosaicism study. The same amplified DNA from spent culture media and trophectoderm biopsies were employed in multiple analyses. *Samples not included in the aneuploidy study because they derive from euploid female embryos and could not be differentiated from maternal contamination. ¥Samples not included in the contamination study because follicular fluids were not available. §Samples not included in the mosaicism study because whole blastocysts were not available.

Embryo development and culture

Immediately after ICSI, the injected oocytes were transferred to individual wells with 25 µl of pre-equilibrated Cleavage Medium (Cook Medical, USA) under mineral oil. Embryos were cultured in a time-lapse incubator (EmbryoScope®, Vitrolife AB, Sweden) with an image capture every 15 min. For each embryo and time point, stacks of five images at different focal planes were acquired to enable accurate assessment of the embryo's morphology. The images were acquired with low-intensity red light with exposure times of 15–30 ms per image. On Day 3, the medium was changed to CCM medium (Vitrolife AB, Sweden) and culture was continued at 37°C and 5.5% CO_2 in air, the standard human embryo culture conditions in our current clinical IVF practice. Also on Day 3, assisted hatching was performed as part of the clinical routine in the PGT-A program. On Day 5, embryos with good morphology underwent trophectoderm biopsy for PGT-A and were vitrified as previously described (Cobo *et al.*, 2012).

DNA quantification from spent culture media

DNA quantification was performed in non-amplified and amplified culture media samples. For the non-amplified group, we collected 20 µl of each sample—spent culture medium or control medium—and used the resDNASEQ® Human Residual DNA Quantitation Kit (ThermoFisher A26366), which is a quantitative PCR (qPCR)-based system optimized for specific detection and quantification of human DNA concentrations as low as 1.5 pg/ml in test samples. Quantification was independent of whether the samples contained high-molecular weight DNA or sheared DNA. For amplified samples, WGA DNA was quantified using the same procedure. Note that for the DNA quantification of non-amplified samples, the full volume was employed, thus amplified samples derived from different embryos.

Conventional PGT-A

Trophectoderm biopsy was performed on Day 5. The cells were washed in 5 µl of PBS 1% (v/v) PVP buffer (Cell Signaling Technology, MA, USA) and transferred to a 0.2 ml PCR tube under sterile conditions. Tubes were stored at −20°C until further analysis. DNA extraction and WGA were performed using the Ion ReproSeq PGS Kit (ThermoFisher Scientific, MA, USA), and the NGS PGT-A protocol was performed according to the manufacturer's instructions using the Ion PGM™ instrument (ThermoFisher Scientific). After sequencing, data were analyzed using Ion Reporter™ software version 5.0 (ThermoFisher Scientific) for full chromosome aneuploidies. Segmental aneuploidies >15 Mb were also reported.

Cell-free DNA chromosomal analysis

Twenty microliters of media were recovered from each Day 5 embryo culture and transferred to DNA-free/DNase-free tubes under sterile conditions. Media cultured in parallel but without contact with embryos were collected as controls. Samples and controls were stored at −80 °C. Due to the low quantity of expected DNA present in the spent culture media, the full volume was employed (20 µl) for WGA, with two consecutive amplification steps performed to increase the sensitivity of the technique. First, the Sureplex DNA amplification system (Illumina, USA) was used to amplify each sample individually. The fragment size distribution of the libraries was assessed by electrophoretic methods (2100 Bioanalyzer, Agilent Technologies, USA). Subsequently, WGA samples were diluted to 30 pg and a second round of amplification was performed using the Ion ReproSeq™ PGS Kit (ThermoFisher Scientific, USA). Ion ReproSeq™ product libraries were quantified by fluorescence-based methods (Qubit, ThermoFisher Scientific). The library fragment size distribution was assessed by electrophoretic methods (2100 Bioanalyzer, Agilent Technologies). Aneuploidies and copy number variations were analyzed using the Ion Reporter™ Software (ThermoFisher Scientific) as previously described for the trophectoderm biopsies.

SNP analysis

To determine the embryonic/maternal DNA ratio, SNP sequencing was performed using associated triads of DNA from spent culture media, trophectoderm biopsy, and follicular fluid samples. The triad groups used for SNP analysis are shown in Supplementary Table SI. The alleles identified in the trophectoderm biopsies served as references for the embryonic DNA haplotype, whereas the alleles identified in the follicular fluid were used as references for maternal contamination. In total, 124 SNPs were included in the study with 90 distributed among all autosomes (chromosomes 1 to 22) and 34 located on chromosome Y (Supplementary Table SII).

The products of WGA obtained from the trophectoderm and cell-free DNA samples were purified. For follicular fluid, DNA was directly extracted from the samples using the QIAamp DNA Blood Mini Kit (Qiagen N.V., Germany). All DNA samples were quantified and diluted to a final concentration of 0.5 ng/µl. Samples were later analyzed with the HID-Ion AmpliSeq™ Identity Panel (ThermoFisher Scientific) using the manufacturer's instructions. Briefly, targets for each SNP were amplified starting with 1 ng (2 µl) of each DNA sample. Later, adapters and individual barcodes were ligated to the amplicons and the samples were purified. Each library was quantified by qPCR to obtain an accurate measurement, and individual samples were diluted to a concentration of 20 pM (based on an average amplicon size of 218 bp). Diluted samples were pooled and diluted to a final concentration of 8 pM before template preparation using the OT2 system (ThermoFisher Scientific). The template-positive library pool was enriched using the Ion One-Touch™ ES, and samples were sequenced in the Ion S5™ instrument (ThermoFisher Scientific).

For secondary analysis, only SNPs with coverage higher than 10× in all samples from the same triad were included (Supplementary Table SIII). From the covered SNPs, we selected only informative SNPs, disregarding those for which the maternal and the embryonic genotype were the same, as this would not allow us to distinguish between the maternal or embryonic origin of the cell-free DNAs (Supplementary Table SIII). Although all SNPs showed a mix of DNAs, we classified them according to the genotype observed in 80% of the reads, calling a SNP as 'embryo', when the distribution of alleles in the media was in concordance with the embryonic reference, and 'mother', when the allele distribution was similar to the one observed in the maternal reference. Additionally, for each SNP we calculated the embryonic fraction using the number of alleles that were exclusively from the mother or the embryo. When the embryonic fraction was below zero, the SNP was considered 'non-informative' and excluded from further calculations (Supplementary Table SIII).

Fluorescence *in situ* hybridization

Chromosomally abnormal blastocysts available at the time of the study were fixed as described (Mir *et al.*, 2010). Before analysis, slides were stored at −20°C for a maximum of three days. FISH was performed using DNA probes (Abbott, USA) for the chromosomes of interest according to the manufacturer's instructions. Nuclei with unclear or tetraploid signals were excluded from the study. Cells were considered informative when they did not overlap, had a well-defined outline, and showed signals of similar size separated by a minimal distance corresponding to twice the diameter of each of the cells. In total, 12 blastocysts were assessed with a total of 24 chromosomes analyzed, of which 18 were informative.

Statistical analyses

Data analysis, including the statistical tests, was performed using IBM SPSS Statistics Version 19. The Shapiro–Wilk test was first performed to check for normal distribution of the variables. For normally distributed variables, a *t*-test (for two groups) or one-way ANOVA (for more than two groups) was performed. When not normally distributed, non-parametric tests (Mann–Whitney *U*-test and Wilcoxon) were performed to assess differences between groups.

Results

Determining and quantifying the presence of cell-free DNA in spent culture media

To confirm the presence of cell-free DNA in spent culture media, we analyzed 20 µl samples of media from 113 individual human embryos that were cultured from Day 3 to Day 5 of development, and 28 samples of culture media with no previous contact with embryos as controls (Supplementary Table SIV). The median quantity of DNA in 20 µl of non-amplified spent culture media was 6.7 pg [$N = 53$, interquartile range (IQR) 3.2–12.6 pg]. This was significantly different from the controls, which had a median quantity of 1.4 pg ($N = 17$, IQR 1.0–4.4 pg) ($P = 0.001$, Mann–Whitney test) (Fig. 2a) The median mass of DNA isolated from our samples was similar to that found in single cells, which is estimated to be around 6.5 pg (Serth *et al.*, 2000). However, approximately half of the samples analyzed were under that level. Regular protocols for single-cell analysis would not be efficient for spent culture media analyses; thus, we decided to apply a double-amplification strategy.

We performed double WGA in a separate set of spent culture and control media to see if the differences detected in the raw samples

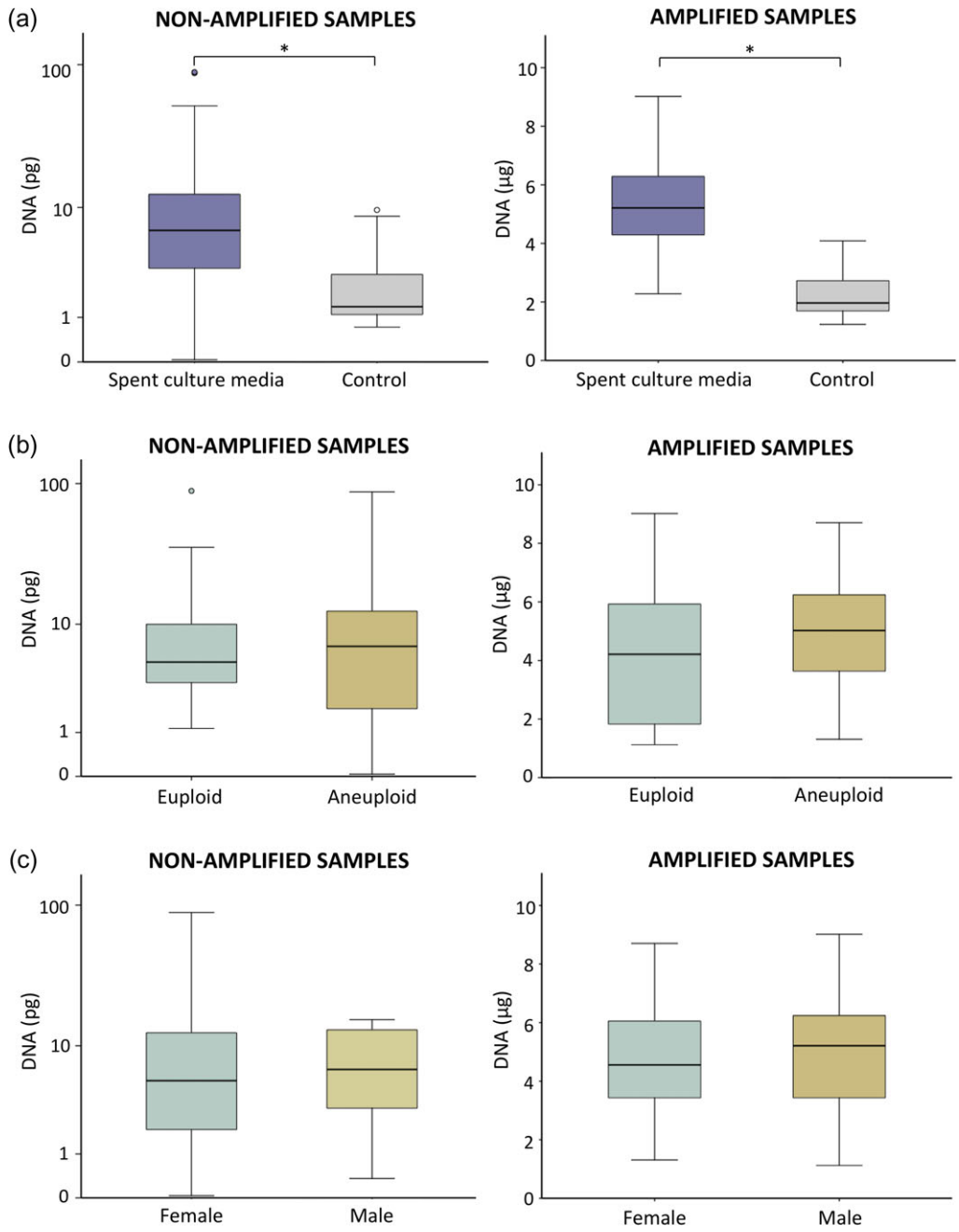

Figure 2 Cell-free DNA quantification in control and spent culture media. (**a**) Box plots for the DNA quantification performed before and after amplification in samples of culture medium with no previous contact with embryos (Control) and medium collected on Day 5 from cultures of Day 3 embryos (Spent culture media). *$P < 0.005$, Mann–Whitney test. A plot represents gene expression values between quartile 1 and 3, the black line inside the box is the median value and the circles are outliers. (**b**) Box plots for the DNA content of euploid and aneuploid Day 5 samples comparing before and after amplification, with no significant differences between groups. (**c**) Box plots for the DNA content of female and male Day 5 samples before and after amplification, with no significant differences between groups. It is important to note that different samples were used for the pre- and post- amplification analyses, as the entire volume of the sampled media was necessary for each assay.

remained after amplification. We found that statistically significant differences between the median DNA quantity of spent culture media ($N = 60$, median 4.9 µg, IQR 3.4–6.2 µg) and control samples ($N = 11$, median 1.9 µg, IQR 1.5–2.8 µg) remained after amplification ($P < 0.001$, Mann–Whitney test) (Fig. 2a).

To determine whether the quantity of cell-free DNA could be related to the ploidy of the embryo in culture, we classified the samples according to the ploidy information obtained from the trophectoderm biopsy. We found no statistically significant differences in the amount of cell-free DNA between euploid and aneuploid samples

before amplification ($N = 17$, median 5.1 pg, IQR 3.3–11.3 pg; $N = 30$, median 6.8 pg, IQR 1.9–13.0 pg, respectively), or after amplification ($N = 12$, median 4.2 µg, IQR 1.7–6.0 µg; $N = 48$, median 5.0 µg, IQR 3.6–6.2 µg, respectively) (Fig. 2b). Similarly, no differences were found when comparing the cell-free DNA amount between female and male embryos before amplification ($N = 26$, median 5.3 pg, IQR 1.9–14.2 pg; $N = 16$, median 6.6 pg, IQR 3.0–13.4 pg, respectively), or after amplification ($N = 26$, median 4.5 µg, IQR 3.3–6.1 µg; $N = 33$, median 5.2 µg, IQR 3.3–6.4 µg, respectively) (Fig. 2c).

Concordance between trophectoderm biopsy and cell-free DNA analysis

To analyze the reliability of the cell-free DNA found in the spent culture media, we sought to determine whether chromosomal abnormalities in the cell-free DNA reflected the anomalies found in the trophectoderm biopsy. For that, we collected samples from embryos undergoing PGT-A as part of the clinical routine and collected their spent culture media. This allowed us to investigate the concordance between trophectoderm biopsy DNA and cell-free spent culture media DNA from the same Day 5 human blastocysts ($N = 56$) (Fig. 3). Results were classified into four different categories:

(a) Concordant: Concordance was defined when both samples were aneuploid, with 30.4% ($N = 17$) of the samples in this category (Fig. 3, Table I). Among the concordant samples, three embryos showed aneuploid-mosaic results, meaning that they had a pure aneuploid pattern in the trophectoderm biopsy, whereas in the cell-free DNA the same aneuploidy appeared with a mosaic pattern (Table I). Six embryos were aneuploid-complementary, meaning the aneuploidies detected in the two sample types were complementary in term of loss versus gain of chromosomes (Table I). Five embryos showed aneuploid-complex results, in which at least one abnormal chromosome was diagnosed in both samples, but other unshared aneuploidies were detected (Table I). Finally, three embryos had both aneuploid trophectoderm and cell-free DNA but for different chromosomes (Table I).

(b) Partial maternal contamination: Trophectoderm biopsies diagnosed as euploid male but with 46 XXY DNA in the spent culture media or as aneuploid female but with euploid DNA in the spent culture media ($N = 17$, 30.4%; Table I) were classified as having partial maternal contamination.

(c) Full maternal contamination: All embryos diagnosed as euploid or aneuploid XY in the trophectoderm DNA but as euploid XX in the spent culture media were considered to have full maternal contamination as the Y chromosome could not be detected in the cell-free DNA ($N = 17$, 30.4%; Table I).

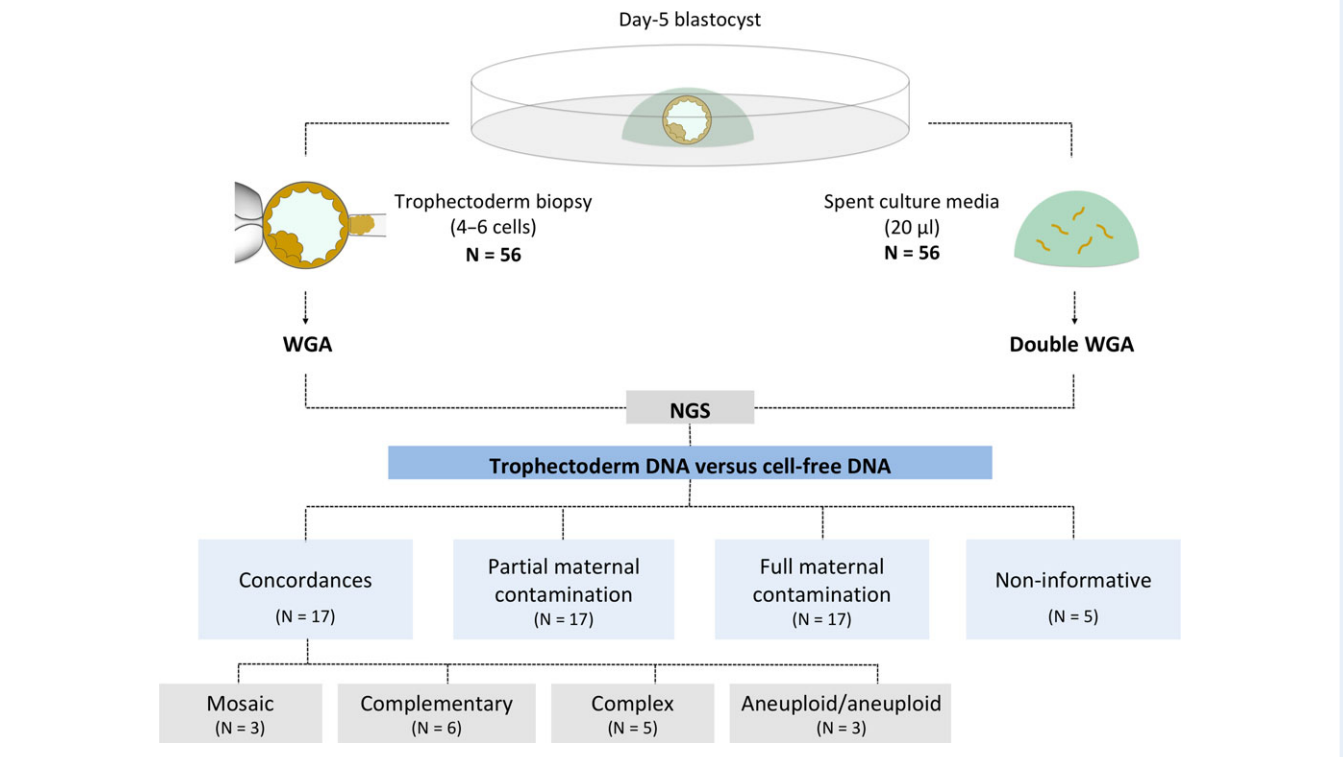

Figure 3 NGS comparing trophectoderm biopsy DNA to cell-free DNA. Fifty-six embryos were cultured until blastocyst stage when trophectoderm biopsies were performed, spent culture media were collected, and whole blastocysts vitrified. Samples were amplified (WGA), sequenced, and the results from trophectoderm DNA and cell-free DNA compared and classified according to the observed patterns. The results were classified into 'Concordances' when both sample types were aneuploid. This group was again classified into four different subgroups according to the type of aneuploidies found. When the pattern observed in spent culture media indicated a potential maternal contamination, the results were classified as 'Partial maternal contamination'. When the contamination was extensive, samples were classified as 'Full maternal contamination'. Finally, in some samples, results were not informative corresponding to a profile indicating amplification failure.

Table I Concordance of trophectoderm DNA and cell-free DNA (*N* = 56).

Sample	Trophectoderm DNA	Cell-free DNA
Concordances		
Aneuploid-mosaic		
CL640^{+}	−15 XY	−15mos XY
CL660^{+}	−21 XY	−21mos XY
CL838	−4 XY	−4mos XYY
Aneuploid-complementary		
CL668^{+*}	−15 −16 XY	+15mos +16mos XX
CL676^{+*}	+10 +15 XX	−10 −15 XX
CL678^{+*}	+19 XX	−19mos XX
CL696^{+*}	+15 XY	−15mos XX
CL842^{+*}	−11 −13 XX	+11 +13 XX
CL852^{+*}	−15 XX	+15 XX
Aneuploid-complex		
CL710^{+*}	+16 −19 XX	−1 +16 XX
CL750^{+}	+16 −22 XY	+3 +4 +5 +6 −14p −22 XX
CL776^{+*}	+14 −22 XY	−1p +20 +22 XX
CL850^{+*}	−16 XY	−16 −17 XX
CL870	+2 −21 +22 XY	−22mos XX
Aneuploid-aneuploid		
CL628^{+}	+6 −20 XX	+10 X0
CL700^{+*}	+13q XX	+19 −22 XX
CL836^{+*}	+17mos+22 XY	+16 +20q XX
Partial maternal contamination		
CL654^{+}	−12 −21 XY	46 XXY
CL674^{+}	46 XY	46 XXY
CL734^{+}	+13 +15 +22 XY	46 XXY
CL760^{+}	46 XY	46 XXY
CL770	+14 +22 XY	46 XXY
CL634	−7 XX	46 XX
CL636	+12 −15 −22 XX	46 XX
CL692^{+}	−16 −21 XX	46 XX
CL694^{+*}	+2mos XX	46 XX
CL712^{+}	−20p XX	46 XX
CL720^{+}	−11p XX	46 XX
CL736^{+}	+11 XX	46 XX
CL752	+19 XX	46 XX
CL764	+8 −16 XX	46 XX
CL782	−14 −15 +22 XX	46 XX
CL790	−16 −21 −22 XX	46 XX
CL820	−5mos −16p XX	46 XX
Full maternal contamination		
CL662^{+}	46 XY	46 XX
CL702	46 XY	46 XX
CL708^{+}	46 XY	46 XX
CL718^{+}	46 XY	46 XX
CL746^{+}	46 XY	46 XX
CL758^{+}	46 XY	46 XX
CL638	−16 XY	46 XX
CL646^{+}	+14 XY	46 XX
CL648^{+}	+16 XY	46 XX
CL698	+5p XY	46 XX
CL728^{+}	+13q +15 XY	46 XX
CL742^{+}	+20 XY	46 XX
CL748	−16 XY	46 XX
CL774^{+}	−22 XY	46 XX
CL792	−13mos XY	46 XX
CL822^{+}	+20mos XY	46 XX
CL844	−5mos XY	46 XX
Non-informative		
CL726	−9 −19 X0	Amplification failure
CL762	−10q XX	Amplification failure
CL828	+3 +5 +6 +10 XX	Amplification failure
CL830	−2 −14 XY	Amplification failure
CL840	+16 XX	Amplification failure

$^{+}$Samples included in the SNP analysis.
*Samples also with FISH analysis.

(d) Non-informative samples: Embryos with an amplification failure in the spent culture media, probably due to a low amount of DNA, were considered non-informative (*N* = 5, 8.9%; Table I).

Finally, 11 controls (culture media with no previous contact with embryos) were also sequenced and generated an amplification-failure pattern, confirming the absence of DNA in the control culture media.

Detection of maternal DNA contamination in spent culture media

The high percentage of discordant results between the DNA from trophectoderm biopsies and cell-free DNA in the spent culture media suggested that a mix of different DNA sources might be present in the media after embryo culture. This result was expected since a high concentration of female DNA originating from oocyte/embryo-associated cells and organelles, such as granulosa cells or polar bodies, was recently reported for the culture media of male embryos (Hammond *et al.*, 2017). Thus, to examine the origin of the cell-free DNA found in the spent culture media, SNP sequencing was performed on sample triads (Supplementary Table SI). In total, we analyzed 35 triads, each one including trophectoderm DNA, to obtain information about the embryonic haplotype, follicular fluid DNA to obtain information about the maternal haplotype, and embryo spent culture media DNA, in which an unknown mix of embryo and maternal haplotypes was expected (Supplementary Fig. S1). For each sample, 124 SNPs were compared, 90 for chromosomes 1–22 (autosomes), and 34 for chromosome Y. Only SNPs with coverage over 10× for the three samples of each triad were considered to avoid false results. Initially, only SNPs included on autosomes were considered (*N* = 3150 SNPs

from the 35 samples) since the Y chromosome was not present in all samples due to sex differences.

The proportion of covered SNPs per triad was 48% on average (N = 1512), with an average of 16.7% SNPs informative (N = 526) (Fig. 4a). Although we found high variability in the percentage of covered SNPs between triads, we found no statistically significant differences in covered or informative SNPs between concordant (41.4% covered SNPs, 14.5% informative SNPs) and maternal-contaminated samples (52.9% covered SNPs, 18.3% informative SNPs) (Fig. 4b). Additionally, we analyzed the coverage between sample types and found that most SNPs and chromosomes were successfully covered in trophectoderm DNA and follicular fluid samples; in contrast, for spent culture media samples, we found a higher variability (Supplementary Figs S2 and S3).

The fraction of embryonic DNA in each spent culture media sample was also calculated according to the alleles detected using the haplotypes of the maternal DNA (follicular fluid) and embryonic DNA (trophectoderm biopsy) as references. There was considerable variability in the percentage of embryonic DNA observed in the spent culture media samples (N = 35) (Fig. 4c). The median percentage of embryonic DNA estimated in the spent culture media was 8%, with the lowest percentage of embryonic DNA for CL712 (0%) and the maximum percentage of embryonic DNA for CL850 (100%) (Fig. 4c). There was a higher percentage of embryonic DNA in the concordant samples (14%, N = 170 SNPs) than in the maternally-contaminated samples (6%, N = 289 SNPs), although this difference was not statistically significant (Fig. 4d).

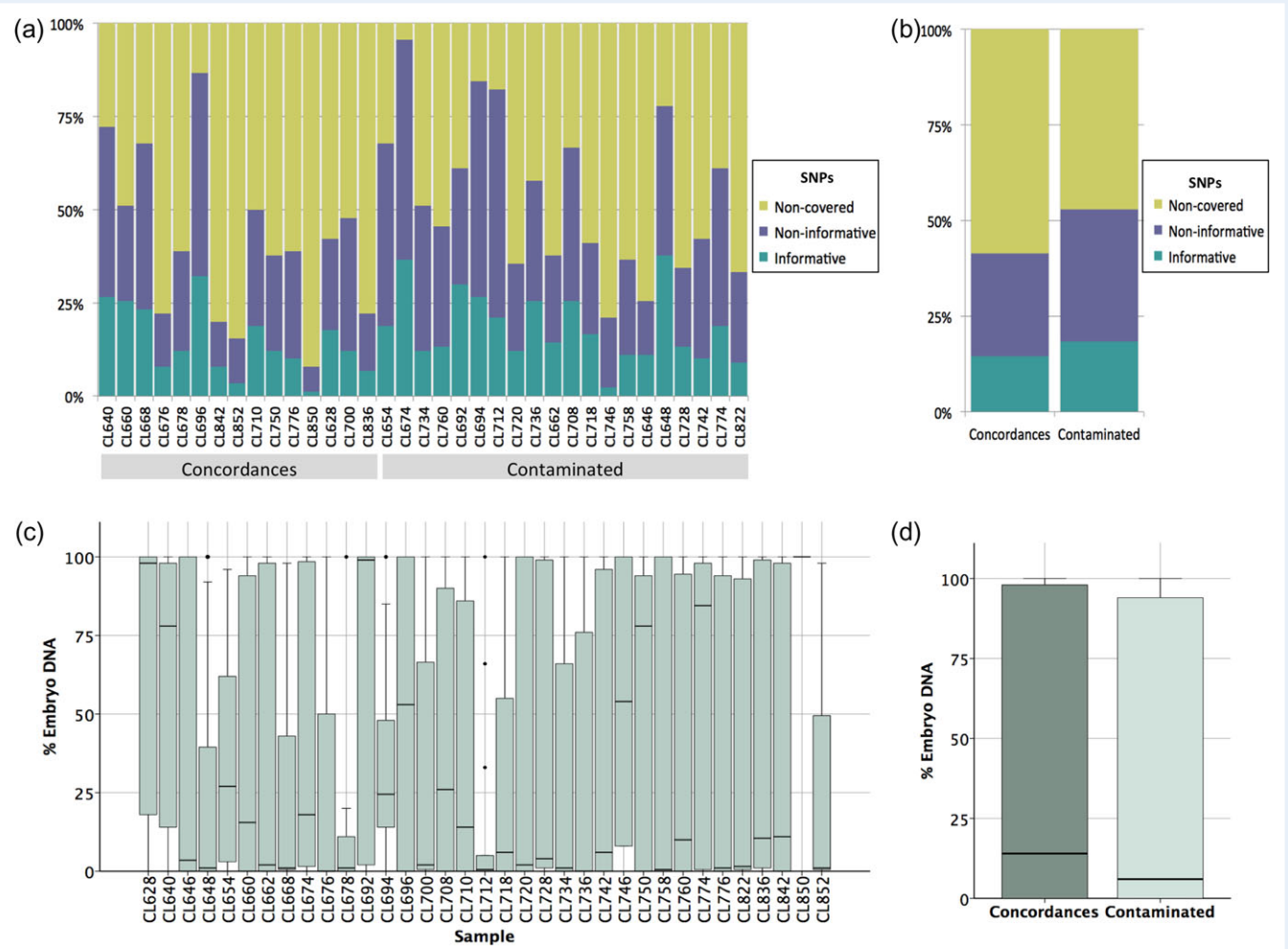

Figure 4 Classification of SNPs from spent culture media and percentage of embryonic DNA in each sample. (**a**) Distribution of the SNPs in each triad (N = 35), defined as 'non-covered' when the SNP coverage was below 10×, 'non-informative' when the haplotype of the follicular fluid and the trophectoderm DNA was equal, and 'informative' for the remaining SNPs. (**b**) SNP distribution according to sample designation: 'concordances', when the spent culture media and the trophectoderm were both aneuploid, and 'contaminated', when a chromosomal profile with partial/full maternal contamination was identified. (**c**) Box plots for the percentage of embryonic DNA per spent culture media sample (N = 35). Only informative SNPs were included. The embryonic fraction was calculated according to the division between the embryo and the maternal haplotype. A plot represents gene expression values between quartile 1 and 3, the black line inside the box is the median value and the black circles are outliers. (**d**) Box plots for the percentage of embryonic DNA according to the results of the concordance analysis.

Finally, in the analysis of SNPs on the Y chromosome (34 SNPs), 23 out of the 35 included samples were originally identified as male from the trophectoderm biopsy, and 47.8% ($N = 11$) had at least one Y-chromosome SNP detected in the spent culture media (Supplementary Fig. S4a). Four additional samples that were identified as female from the trophectoderm biopsy had Y-chromosome SNPs in DNA from the spent culture media (Supplementary Fig. S4a). While these data showed that, for Y chromosome detection in spent culture media, SNP sequencing had a higher sensitivity compared to aneuploidy testing analysis (47.8% versus 29.6%, respectively), but it also decreased the specificity for Y chromosome detection (66.7% versus 100%, respectively) (Supplementary Fig. S4).

Mosaicism as the origin for discordant results

In addition to maternal contamination, mosaicism could have a significant impact on the outcomes when comparing trophectoderm biopsy and cell-free DNA analyses. Thus, to examine mosaicism, we analyzed embryos available at the time of the study that exhibited different chromosomal constitutions when their trophectoderm and cell-free DNAs were compared. In total, 12 aneuploid human blastocysts were reanalyzed by FISH for the chromosomes of interest (Fig. 5a). Eleven exhibited aneuploid results for both trophectoderm and cell-free DNA. More specifically, six of them were classified as *complementary aneuploidies* (showing monosomy in the spent culture media and trisomy in the trophectoderm biopsy or vice-versa), three of them were classified as *aneuploid-complex* (exhibiting shared aneuploidies but also unshared aneuploidies), and two were classified as *aneuploid-aneuploid* (presenting different chromosomal defects in the trophectoderm and cell-free DNAs) (Fig. 5a). One additional sample whose spent culture medium was originally detected as *partial maternal contaminated* was also analyzed (Fig. 5a). In summary, of the 12 whole embryos analyzed by FISH, 11 of them (91.7%) showed a mosaic pattern, and of the 24 analyzed chromosomes, the majority were in concordance with the trophectoderm DNA diagnosis (75%; Table II). As these 12 embryos were also included in the SNP sequencing analysis, we analyzed whether there was any specific pattern that could correlate the proportion of embryonic DNA in the cell-free fraction with the FISH results. Thus, the percentage of cell-free embryonic DNA in the spent media was analyzed (Fig. 5b), and the samples were grouped into four categories according to the degree of FISH concordance (Fig. 5c). In total, 129 SNPs were informative in all 12 embryos, with a median percentage of embryonic DNA of 8% (IQR 0–93%). The group with the lowest proportion of embryonic DNA in the culture media (median of 2%; $N = 84$ SNPs, IQR 0–98%, Fig. 5c) was the one with the highest

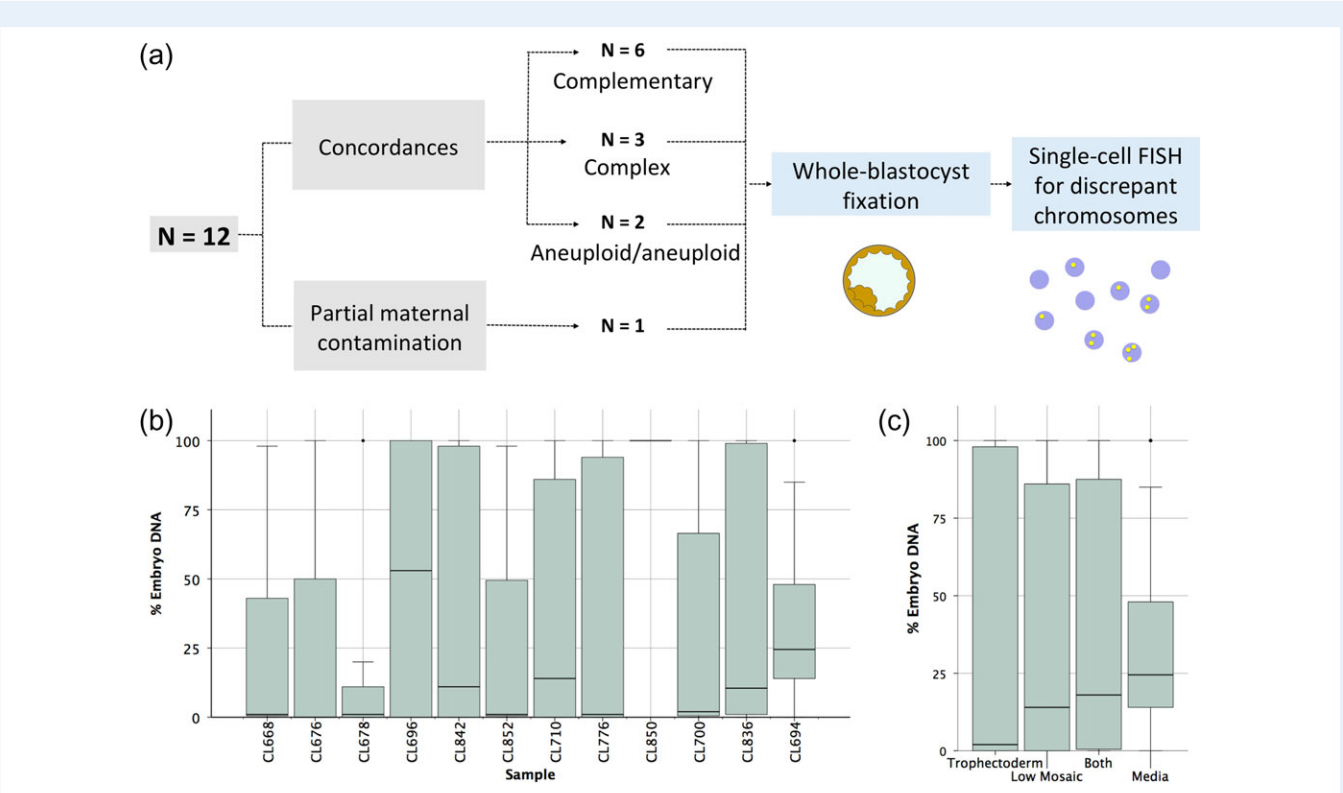

Figure 5 FISH analysis of embryos and percentage of embryonic DNA in spent media. (**a**) Twelve samples were selected for FISH analysis, 11 with concordant results between trophectoderm biopsy and spent culture media and one with partial maternal contamination. Whole blastocysts were fixed and analyzed by FISH for the discrepant chromosomes. (**b**) Box plots showing the distribution of the percentage of embryonic DNA based on informative SNPs for each sample included in the FISH study. A plot represents gene expression values between quartile 1 and 3, the black line inside the box is the median value and the circles are outliers. (**c**) Box plots representing the distribution of the percentage of embryonic DNA in the different groups according to FISH result of concordance to the trophectoderm biopsy ('Trophectoderm'), to spent culture media ('Media'), to both in similar proportions ('both') or 'low mosaic' when most of the cells' karyotypes were different from both sample types.

Table II FISH analysis on fixed whole blastocysts at a single-cell level (*N* = 12).

Sample	Troph. biopsy	Spent culture media	Whole-blastocyst FISH					
			Chrom. analyzed	Number of analyzed cells	% cells one copy	% cells two copies	% cells three copies	Concordance
Concordances								
Aneuploid-complementary								
CL668	−15 −16	+15mos +16mos	15	32	81.3	18.8	0	Trophectoderm
			16	13	53.8	46.2	0	Trophectoderm
CL676	+10 +15	−10 −15	15	4	25	0	75	Trophectoderm
			10	3	0	0	100	Trophectoderm
CL678	+19	−19mos	19	8	25	12.5	62.5	Trophectoderm
CL696	+15	−15mos	15	10	10	0	90	Trophectoderm
CL842	−11 −13	+11 +13	11	8	100	0	0	Trophectoderm
			13	8	100	0	0	Trophectoderm
CL852	−15	+15	15	10	80	10	10	Trophectoderm
Aneuploid-complex								
CL710	+16 −19	−1 +16	1	6	0	33.3	66.7	Mostly discordant
			16	7	0	71.4	28.6	Mostly discordant
CL776	+14 −22	−1p +20 +22	22	9	77.8	11.1	11.1	Trophectoderm
			20	4	50	50	0	Trophectoderm
			14	7	85.7	14.3	0	Mostly discordant
CL850	−16	−16 −17 −20mos	20	17	41.2	58.8	0	Spent media
			16	16	81.3	18.8	0	Both
			17	14	71.4	28.6	0	Spent media
Aneuploid-aneuploid								
CL700	+13q	+19 −22	13q	12	0	50	50	Both
			22	18	0	100	0	Trophectoderm
CL836	+17mos +22	+16 +20q	20	5	20	20	60	Spent media
			17	13	0	30.8	69.2	Trophectoderm
			16	16	0	87.5	12.5	Trophectoderm
			22	16	0	18.8	81.3	Trophectoderm
Partial maternal contamination								
CL694	+2mos	46 XX	2	10	0	80	20	Trophectoderm

Chromosomes detected with a mosaic pattern are indicated with the suffix 'mos'. FISH results are considered concordant according to the highest percentage of cells exhibiting the same chromosomal copy number. FISH results were considered concordant to mosaic chromosome calling when showing exactly the same mosaic pattern. Non-informative chromosomes by FISH or non-informative cells are not included. Troph, trophectoderm; Chrom, chromosome.

concordance with the trophectoderm biopsy diagnosis. In contrast, the group with the highest percentage of embryonic DNA in the culture media (median of 24.5%; *N* = 18 SNPs, IQR 10.5–53%, Fig. 5c) was the one with the highest concordance with the spent culture media DNA diagnosis.

Finally, analysis of time-lapse movies of the embryos identified some fragments/cells outside of the zona pellucida in five of them (CL676, CL710, CL836, CL850, and CL852); four embryos were partially hatched out of the zona pellucida (CL668, CL678, CL842 and CL836; Supplementary Fig. S5). However, there were no statistically significant differences in the proportions of embryonic DNA found in the culture media when comparing samples with some fragments/cells outside of the zona pellucida, hatched samples, or samples with enclosed blastocysts (data not shown).

Discussion

Recent advances have demonstrated the feasibility of chromosomal diagnosis from spent culture media, but results reported from different groups have been variable (Shamonki *et al.*, 2016; Xu *et al.*, 2016; Feichtinger *et al.*, 2017). This is the first study to combine chromosomal analysis of the DNA from spent culture media, SNP sequencing to identify maternal contamination, and whole-blastocyst analysis for mosaicism. Thus, the results shown here provide a better understanding of the origin of cell-free DNA in spent culture media, promoting a new insight toward future development of non-invasive diagnostic techniques.

In accordance with previous findings (Hammond *et al.*, 2017), we observed that the amount of cell-free DNA in spent media after

embryo culture is significantly greater than in media that have not been exposed to embryos. This information supports the hypothesis that DNA molecules are secreted into the culture media by the embryo or other associated sources, as has been suggested (Hammond *et al.*, 2017). Such DNA is proposed to derive from cells discarded by the embryos as a correction mechanism for aneuploidies (Hammond *et al.*, 2017). However, we found that the amount of cell-free DNA was not significantly greater in media from aneuploid *versus* euploid embryos, ruling out this hypothesis. The comparison between the chromosomal component of the spent culture media and the trophectoderm DNA from the same embryo revealed two main clues: (i) 61% of the paired samples analyzed showed a pattern consistent with maternal DNA contamination and (ii) in most concordant samples—aneuploid in both spent culture media and trophectoderm biopsy—the affected chromosomes differ depending on the DNA source. Thus, we performed a more detailed analysis of the samples to better understand the origin of the cell-free DNA in the spent media.

When we used SNP analysis to quantify the embryonic *versus* maternal DNA ratio found in the spent culture media, we discovered that maternal contamination was significant in all samples, including those in which a contaminated pattern was not predicted by NGS (86% maternal DNA), and for the samples predicted to be contaminated, the maternal DNA fraction was even higher (94% maternal DNA). Note that NGS can identify mosaic samples with as little as 20% mosaicism (Vera-Rodriguez and Rubio, 2017), which is crucial for making a clinical diagnosis from a trophectoderm biopsy. However, this may not be sensitive enough to analyze DNA from spent culture media where the median fraction of embryonic DNA is 8%, according to our results.

By SNP analysis, we identified individual samples of spent media with percentages of embryonic DNA ranging from 0% to 100%. This as an important finding, suggesting that the embryonic genome may not be uniformly represented in the spent culture media of all embryos. Our analysis of the SNP coverage according to the sample type supports this hypothesis. We observed that, unlike the cell-free DNA samples, most samples derived from follicular fluid or trophectoderm biopsy had uniform SNP coverage at all positions and on all chromosomes. This is not surprising since this DNA was directly extracted from the cells and represents them accordingly. This finding was not reported in the previous studies of spent culture media and might explain the false-positive results found in those studies (Feichtinger *et al.*, 2017; Xu *et al.*, 2016).

A recent study showed that the accuracy of aneuploidy testing based on cell-free DNA from spent culture media was increased when the sample had been in contact with the embryos from Day 4 to Day 5, in comparison to samples from Day 3 to Day 5 (Lane *et al.*, 2017). The authors also reported a high concordance in sex chromosomes and a lower rate of false-negative results in spent culture media from Day 4 to Day 5 versus samples from Day 3 to Day 5 (Lane *et al.*, 2017). A potential explanation for these findings could be that the ratio of embryonic DNA to maternal DNA is increased at later stages, as the number of embryonic cells increases exponentially with development. Interestingly, this study also found a high rate of complementary aneuploidies between spent culture media and trophectoderm biopsy in samples from Day 3 to Day 5 (Lane *et al.*, 2017). This is in concordance with the data presented herein, where 35% of the non-contaminated samples were identified as aneuploid-complementary. Nonetheless, the underlying biology remains to be determined.

Even when maternal contamination is prevented, mosaicism remains an important factor to consider for its potential influence on the composition of cell-free DNA. Trophectoderm mosaicism has been found in ~5% of cultured embryos (Vera-Rodriguez and Rubio, 2017). In our study, a subset of whole embryos was reanalyzed using FISH; most samples exhibited aneuploidies in the spent culture media that differed from the aneuploidies detected in the trophectoderm biopsy. The whole-blastocyst analysis revealed that most of these embryos were mosaic. This feature could explain why we observed different chromosomal alterations in the spent culture media. It is important to note that, for the majority of the embryos, most of the cells were in concordance with the trophectoderm diagnosis. This result raises a new question about the DNA present in spent culture media: Why does the cell-free DNA appear to reflect the makeup of the minority of cells from mosaic embryos? This could represent a new corrective mechanism by the embryo, but it remains unclear why embryos would release only certain types instead of all aneuploid cells into the media.

In conclusion, this study revealed that there is an increase in cell-free DNA in media following embryo culture. However, hurdles must be overcome before using cell-free DNA as a representative source for non-invasive PGT-A. First, either *in vitro* or *in silico* methods for reducing maternal contamination should be developed. Recent advances in prenatal sampling have shown that DNA fragments from the fetus differ in size and contain different preferred ends than maternal fragments (Chan *et al.*, 2016). Thus, future studies should focus on determining whether similar differences exist at the preimplantation stage. These differences could be used to discriminate between embryonic and maternal DNA in spent culture media. This must be achieved before non-invasive aneuploidy testing can be used for diagnosing human embryos because this technique requires the specific use of embryonic cell-free DNA.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Author's roles

C.S. and C.R. designed and supervised the project. A.M. and I.M. collected the samples and M.V.R., A.D.J., S.M., and V.P. performed experiments. J.J.A. and R.N. analyzed the sequencing data. D.B. and M.M. provided materials and expertise. D.V. performed the statistics. M.V.R. analyzed data and wrote the manuscript. C.S., C.R., A.D.J., J.J.A., I.M. and D.V. helped with editing the manuscript.

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Conflict of interest

None declared.

References

Alfarawati S, Fragouli E, Colls P, Stevens J, Gutierrez-Mateo C, Schoolcraft WB, Katz-Jaffe MG, Wells D. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. *Fertil Steril* 2011;**2**:520–524.

Bond DJ, Chandley AC. *Aneuploidy: The Origins and Causes of Aneuploidy in Experimental Organisms*. UK: Oxford Univ Press, 1983.

Campos-Galindo I, Garcia-Herrero S, Martinez-Conejero JA, Ferro J, Simon C, Rubio C. Molecular analysis of products of conception obtained by hysteroembryoscopy from infertile couples. *J Assist Reprod Genet* 2015;**5**:839–848.

Chan KC, Jiang P, Sun K, Cheng YK, Tong YK, Cheng SH, Wong AI, Hudecova I, Leung TY, Chiu RW *et al*. Second generation noninvasive fetal genome analysis reveals de novo mutations, single-base parental inheritance, and preferred DNA ends. *Proc Natl Acad Sci USA* 2016;**50**: E8159–E8168.

Cobo A, de los Santos MJ, Castello D, Gamiz P, Campos P, Remohi J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: evaluation of 3,150 warming cycles. *Fertil Steril* 2012;**5**:1138–46.e1.

Colls P, Escudero T, Fischer J, Cekleniak NA, Ben-Ozer S, Meyer B, Damien M, Grifo JA, Hershlag A, Munne S. Validation of array comparative genome hybridization for diagnosis of translocations in preimplantation human embryos. *Reprod Biomed Online* 2012;**6**:621–629.

Feichtinger M, Vaccari E, Carli L, Wallner E, Madel U, Figl K, Palini S, Feichtinger W. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of-concept pilot study. *Reprod Biomed Online* 2017;**34**:583–589.

Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, Kokocinski F, Michel CE. Development and validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of embryos. *Fertil Steril* 2014;**5**:1375–1382.

Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, Chamley LW, Cree LM. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. *Fertil Steril* 2017;**1**:220–228.e5.

Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* 2001;**4**:280–291.

Lane M, Zander-Fox D, Hamilton H, Jasper MJ, Hodgson BL, Fraser M, Bell F. Ability to detect aneuploidy from cell free DNA collected from media is dependent on the stage of development of the embryo. *Fertil Steril* 2017;**3**:e61.

Mir P, Rodrigo L, Mateu E, Peinado V, Milan M, Mercader A, Buendia P, Delgado A, Pellicer A, Remohi J *et al*. Improving FISH diagnosis for preimplantation genetic aneuploidy screening. *Hum Reprod* 2010;**7**:1812–1817.

Rodrigo L, Mateu E, Mercader A, Cobo AC, Peinado V, Milan M, Al-Asmar N, Campos-Galindo I, Garcia-Herrero S, Mir P *et al*. New tools for embryo selection: comprehensive chromosome screening by array comparative genomic hybridization. *Biomed Res Int* 2014;**2014**: 517125.

Rubio C, Pehlivan T, Rodrigo L, Simon C, Remohi J, Pellicer A. Embryo aneuploidy screening for unexplained recurrent miscarriage: a minireview. *Am J Reprod Immunol* 2005;**4**:159–165.

Serth J, Kuczyk MA, Paeslack U, Lichtinghagen R, Jonas U. Quantitation of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. *Am J Pathol* 2000;**4**:1189–1196.

Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril* 2016;**6**: 1312–1318.

Treff NR, Levy B, Su J, Northrop LE, Tao X, Scott RT Jr. SNP microarray-based 24 chromosome aneuploidy screening is significantly more consistent than FISH. *Mol Hum Reprod* 2010;**8**:583–589.

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.

Vera-Rodriguez M, Rubio C. Assessing the true incidence of mosaicism in preimplantation embryos. *Fertil Steril* 2017;**5**:1107–1112.

Werlin L, Rodi I, DeCherney A, Marello E, Hill D, Munne S. Preimplantation genetic diagnosis as both a therapeutic and diagnostic tool in assisted reproductive technology. *Fertil Steril* 2003;**2**: 467–468.

Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, Wang H, Song X, Ma T, Bo S *et al*. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci USA* 2016;**42**:11907–11912.

Zhang C, Zhang C, Chen S, Yin X, Pan X, Lin G, Tan Y, Tan K, Xu Z, Hu P *et al*. A single cell level based method for copy number variation analysis by low coverage massively parallel sequencing. *PLoS One* 2013; **1**:e54236.