Blastocoel fluid from differentiated blastocysts harbors embryonic genomic material capable of a whole-genome deoxyribonucleic acid amplification and comprehensive chromosome microarray analysis

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Objective: To obtain embryonic molecular karyotypes from genomic DNA (deoxyribonucleic acid) isolated from blastocoel fluid (BF) and to compare these karyotypes with the karyotypes from the remaining inner cell mass (ICM) and trophectoderm (TE) of the blastocyst.
Design: Prospective cohort study.
Setting: Academic center and preimplantation genetics laboratory.
Patient(s): Ninety-six donated cryopreserved embryos.
Intervention(s): Embryo biopsy, BF aspiration, DNA analysis using a comparative genomic hybridization microarray (aCGH).
Main Outcome Measure(s): The aCGH of a single blastomere, BF-DNA, and ICM-TE.
Result(s): The BF-DNA samples resulted in a successful aCGH in 63% of cases. Discordance in karyotypes was found between the BF-DNA and the ICM-TE in 52% of cases. A total of 70% of aneusomic (mosaicism), cleavage-stage embryos differentiated into euploid blastocysts. Probabilities for diagnostic accuracy were calculated and demonstrated the following: sensitivity of 0.88 (95% confidence interval [CI]: 0.62–0.98); specificity of 0.55 (95% CI: 0.39–0.70); positive predictive value of 0.41 (95% CI: 0.25–0.60); negative predictive value of 0.92 (95% CI: 0.75–0.99).
Conclusion(s): Genomic DNA from the BF can be amplified and characterized by comprehensive chromosome microarrays. The results demonstrated that aneusomic cleavage-stage embryos differentiated into euploid blastocysts, possibly using a mechanism that marginalizes aneuploid nuclei into the blastocoel cavity. In addition, owing to the high discordance between the karyotypes obtained from the BF-DNA and the ICM-TE, using BF-DNA for preimplantation genetic testing is not yet advised. (Fertil Steril® 2015;104:418–25. ©2015 by American Society for Reproductive Medicine.)
Key Words: Blastocoel fluid, preimplantation genetic testing, microarray, comparative genomic hybridization, preimplantation genetic screening

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Human embryogenesis is a complex process that is not fully understood. Since the advent of in vitro fertilization (IVF), the fundamentals of embryo development continue to expand. After activation of the embryonic genome, the embryo

can undergo compaction and differentiation into a blastocyst (1). After differentiation, the blastocyst consists of 3 principal morphological components: the inner cell mass (ICM) or future fetus: the trophectoderm (TE); and the blastocoel fluid (BF). Although investigations of the ICM and TE have contributed to the understanding of embryogenesis and affected clinical IVF outcomes (2–6), the potential use of the BF-DNA (deoxyribonucleic acid) has been largely neglected, with relatively few studies completed.

Preliminary investigations of human BF have been limited in scope and number. In 1 BF study, mass spectrometry was performed on BF-metabolites that were directly aspirated from the blastocoel cavity, using techniques similar to those previously completed on residual embryo culture media. They identified multiple metabolic proteins that have been suggested to correlate with embryo metabolism (7). However, apart from identifying the metabolic products, no direct clinical or embryologic quality outcomes were assessed. An additional BF study used proteomic analysis to identify numerous proteins within the BF (8); however, these were not found to be correlated with embryologic quality or clinical outcomes. Although these studies demonstrated that BF can be analyzed in a manner similar to that for embryo culture media, using metabolomics or proteomics, current findings have not yielded any correlation with clinical utility.

In an attempt to use BF for direct clinical applications, 4 prior studies (9–12) obtained embryonic BF-DNA and analyzed it for single genes and/or molecular karyotypes. Palini et al. (9) used a method described by D'Alessandro et al. (7) to obtain BF by creating a micropuncture through the TE, using an intracytoplasmic sperm injection (ICSI) pipette, followed by aspiration of the fluid until the blastocoel cavity fully collapsed around the pipette. The study targeted the glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) gene as well as *TSPY1* and *TBC1D3*, using real-time polymerase chain reaction analysis. They next attempted a whole-genome microarray on 5 amplified DNA products obtained from the BF, and found a karyotype in only 2 samples.

Perloe et al. (10) reported preliminary data, at the American Society of Reproductive Medicine (ASRM) annual meeting in 2013, from an attempt to validate the use of BF-DNA for preimplantation genetic testing (PGT) using comparative genomic hybridization microarrays (aCGH). They analyzed DNA from the BF of 32 embryos; however, only 9 (28%) produced an interpretable molecular karyotype, and of those 9, only 3 karyotypes were concordant with the corresponding embryo's TE microarray analysis. In an additional study presented at the 2013 ASRM annual meeting, Poli et al. (11) reported results on the use of DNA fingerprinting for 8 chromosomes from 11 paired samples (BF-DNA separated from the whole-embryo DNA) to determine the concordance of polymorphic loci between paired samples. These results showed that 100% of the loci from the whole embryo successfully amplified, but that the DNA from the BF was of inferior quality and insufficient to complete DNA fingerprinting analysis.

Additionally, they performed aCGH on 4 paired samples, and obtained a karyotype on each sample analyzed. Their results showed discordance between the DNA from the BF and the whole embryo in 3 of 4 samples analyzed. They concluded that BF contains a variable amount of DNA and that the results were insufficient for reliable single-gene preimplantation genetic diagnosis.

In contrast to the studies just described, which were mostly unsuccessful attempts at BF-DNA analysis, Gianaroli et al. (12) recently published their successful attempt at investigating the potential of BF and PGT. This group analyzed BF that had been removed from differentiated blastocysts, and compared the ploidy status of the BF-DNA with either polar bodies or a single blastomere and TE cells. They used 51 blastocysts from 17 couples, which resulted in DNA detection from the BF in 76.5% of the samples. They identified a total ploidy concordance of 94.9% between the BF-DNA and either polar body or blastomere cells. Additionally, they identified a 97.4% ploidy concordance between the BF-DNA and the TE cells. They concluded that BF represents an accurate alternative source of embryonic DNA that could be used for chromosome testing.

As these preliminary studies demonstrated inconsistent and conflicting results, additional studies are required to determine the potential role of BF-DNA in PGT.

The aim of this study is to determine if embryonic DNA can be isolated from cells within the BF, and thereby a whole-genome microarray obtained. The results of this study provide insight into embryogenesis during differentiation and data on the feasibility of using BF-DNA for PGT.

MATERIALS AND METHODS

This study used 96 cryopreserved embryos that were donated for research, primarily owing to their unsuitability for clinical use, by patients who had undergone previous IVF cycles. Institutional review board (IRB) approval from Johns Hopkins Medical Institutions was obtained. As required by the IRB, all samples were deidentified at the time of informed consent and donation, and no patient demographics were available.

All embryos used in this study had been previously cryopreserved using vitrification at either the cleavage stage or the blastocyst stage of embryo development. Cryopreserved embryos first underwent a slow-thaw laboratory protocol. After thawing, those embryos that were initially cryopreserved at the cleavage stage were biopsied, using a laser, to remove a single blastomere. The embryos were continued in culture (continuous single culture, with Gentamicin, Irvine Scientific) until they differentiated to the blastocyst stage of embryo development. After differentiation to the expanded blastocyst stage, approximately 1 μL of BF was removed from each embryo, using an ICSI micropipette (5.5 μm, 30 degrees, Wallace, Smith Medical), via an established technique; the BF was placed in 2.5 μL of 1x phosphate buffered saline (7, 9).

Figure 1A–1C demonstrates this technique in 3 separate stages. Only those embryos that had expanded with an identifiable blastocoel cavity were aspirated. The remaining ICM-TE were removed from the media droplet and placed in a separate tube. All embryos that failed to differentiate to the blastocyst stage after thaw, or did not have a discernable blastocoel cavity, were excluded from the study.

FIGURE 1

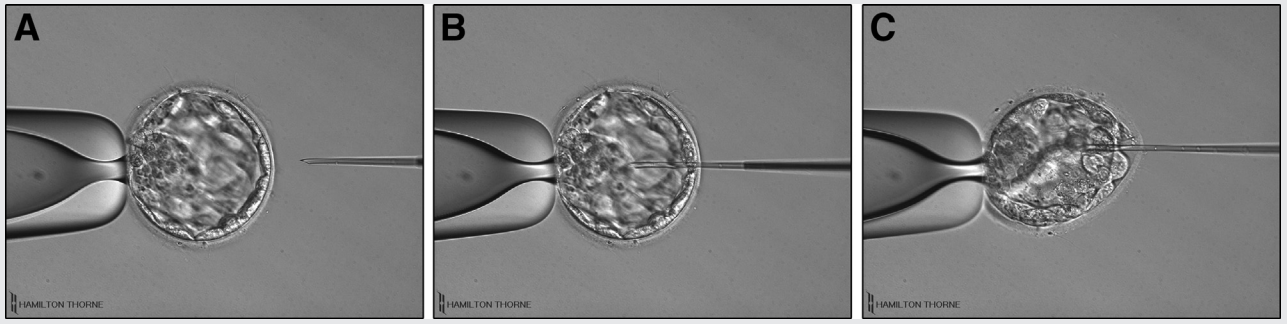

Image of ICSI micropipette removing BF from a differentiated blastocyst: (**A**) The ICSI needle is (**B**) inserted through the mural wall opposite the side of the ICM, followed by (**C**) gentle aspiration to allow the blastocoel cavity to collapse around the needle while simultaneously withdrawing the needle.

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The cells within the BF were isolated from the fluid by high-speed centrifugation for 3 minutes. The remaining blastocyst was placed in a separate tube with 2.5 μL of 1x phosphate buffered saline. To reduce contamination risk, all sampling aspiration was performed under a hood, within a temperature- and air quality–controlled IVF laboratory at a large academic institution with high quality-assurance standards.

The presence of embryonic DNA within the BF was confirmed by 4′, 6-diamidino-2-phenylindole (DAPI) staining. In brief, 1 μL of the BF samples was placed on glass slides, and permitted to air dry; 4 uL of DAPI was added, and the sample was reviewed using an Olympus BX-61 epi-fluorescence microscope.

All 3 specimens, the single blastomere, the BF-DNA, and the remaining ICM-TE were analyzed separately, using aCGH. First, all cells were lysed, and a modified whole-genome amplification protocol was accomplished using a Klenow fragment and a random priming protocol (SurePlex DNA Amplification System, Illumina). Next, the amplified DNA was labeled using Cy3 and Cy5 fluorophores, condensed, and hybridized to 24-Sure array chips (Illumina) at 47°C for at least 3 hours. Each 24-Sure array contained approximately 4,000 bacterial artificial chromosome clones run in duplicate. Next, we performed stringency washes that removed unhybridized and non–specifically bound DNA fragments. The microarray chips were scanned using a Tecan Array Reader (Tecan US, Inc), and raw data analysis was accomplished using BlueGnome BlueFuse software (BlueGnome, Ltd). Clinical data were compared to male and female reference DNA for copy number gains or losses.

RESULTS

A total of 96 embryos were analyzed. Of those, 63% (60 of 96) of the BF samples had embryonic DNA, identified with DAPI, and resulted in successful amplification and an aCGH molecular karyotype. Twenty-nine of 60 embryos included separate analysis of a blastomere, BF-DNA, and the ICM-TE of each embryo. Thirty-one of the 60 embryos included an analysis of only the BF-DNA and the ICM-TE, as they were cryopreserved at the blastocyst stage before being donated for research. Table 1 lists all 60 embryos, and the aCGH karyotype obtained from the various cell types: a single blastomere, BF-DNA, and the ICM-TE. Thirty-six of the 96 (37%) blastocoel fluid samples failed to show any adequate DNA amplification, and no molecular karyotype was obtained.

Next, we compared the aCGH karyotype results obtained from the various cell types taken from the same embryo, to observe how the BF-DNA compared to the precursor blastomere and the concurrent ICM-TE. The BF-DNA karyotype was discordant from the ICM-TE karyotypes in 52% (31 of 60) of embryos analyzed. Only 2 embryos had a euploid karyotype obtained from the BF sample but an aneuploid ICM-TE. In comparing the karyotypes of the blastomere, the BF-DNA, and the ICM-TE, we noted that all 3 DNA sources were euploid and concordant in only 20% (6 of 29) of embryos analyzed. Most remarkable was that 70% (14 of 20) of aneusomic cleavage-stage embryos (diagnosed from a single blastomere) normalized by resolving their aneusomy during blastulation. Of these 14 normalized blastocysts, 86% (12 of 14) had aneuploid nuclei within the BF, whereas 2 were euploid. All 9 cleavage-stage embryos diagnosed as euploid remained euploid at the blastocyst stage, as shown by analysis of the ICM-TE cells; this represents 100% concordance between the cleavage stage and the blastocyst stage. Of the euploid cleavage-stage embryos that differentiated into euploid blastocysts, remarkably, 3 of the BF samples harbored aneuploid DNA. Additionally, all but 2 of the BF samples would have successfully predicted the gender of the embryos.

To determine if BF-DNA could be used as a less-invasive method for PGT, we calculated the diagnostic accuracy of the BF-DNA karyotype to represent the karyotype of the whole embryo (ICM-TE). Diagnostic accuracy was quantified using the ploidy status (euploid or aneuploid) of the ICM-TE as the standard, to determine whether it was true positive, true negative, false positive, and false negative. We considered a "positive" test to be aneuploidy, and a "negative" test to be euploid. Probabilities for diagnostic accuracy were calculated, and demonstrated the following: sensitivity of

TABLE 1

Comparative genomic hybridization microarray results from 3 different cells from the same embryo.

Embryo #	Single blastomere	Blastocoel fluid DNA	Inner cell mass and trophectoderm
Euploid embryos (n = 23)			
1	46, XX	46, XX	46, XX
2	46, XX	46, XX	46, XX
3	46, XX	46, XX	46, XX
4	46, XX	46, XX	46, XX
5	46, XY	46, XY	46, XY
6	46, XX	46, XX	46, XX
7	No blastomere cell	46, XX	46, XX
8		46, XX	46, XX
9		46, XY	46, XY
10		46, XY	46, XY
11		46, XY	46, XY
12		46, XY	46, XY
13		46, XY	46, XY
14		46, XY	46, XY
15		46, XY	46, XY
16		46, XY	46, XY
17		46, XY	46, XY
18		46, XX	46, XX
19		46, XX	46, XX
20		46, XY	46, XY
21		46, XY	46, XY
22		46, XY	46, XY
23		46, XX	46, XX
Aneuploid embryos (n = 4)			
1	No blastomere cell	45, XY, −21	45, XY, −21
2		45, XX, −13	45, XX, −13
3		46, XY, −11, +20	46, XY, −11, +20
4		47, XX, +19	47, XX, +19
Aneusomy (n = 21)			
1	46, XY	45, XY, −6	46, XY
2	46, XY	45, X	46, XY
3	46, XY	45, X	46,XY
4	45, XX, −15	46, XX	46, XX
5	45, XY, +18	46, XY	46, XY
6	45, XY, −12	47, XY, +12	45, XY, −12
7	46, XX, +16, −20	43, XX, −3, −7, −16, +20, −22	46, XX, +16, −20
8	47, XY, +13	46, XY	47, XY, +13
9	45, XY, −11, +20, −21	45, XY, −11, +20	46, XY, −11, +20, −21
10	48, XY, +6, +15	48, XY, +15, +18	48, XY, +6, +15
11	49, XX, +1, +5, +15	48, XX, +5, +15	47, XX, +22
12	No blastomere cell	45, XX, −16	46, XX
13		53, XYY, +2, +4, +6, +7, +9, +20	46, XY
14		45, XY, −20	46, XY
15		49, XX, +13, +15, +19	44, XX, −16, −22
16		47, XX, +15	45, XX, −21
17		46, XX, −13, +16	46, XX, +5, −16
18		46, XX	46, XX, −13, +17
19		53, XYY	46, XY
20		48, XY, −1, +10, +12, +22	47, XY, +17
21		48, XY, +15, +18	48, XY, +6, +15
Resolution of aneusomy during differentiation from an aneuploid cleavage-stage embryo to a euploid blastocyst with aneuploid DNA within the blastocoel fluid (n = 12)			
1	45, X	47, XXX	46, XX
2	47, XX, +1	47, XX, +1	46, XX
3	47, XX, +13	45, XX, −13	46, XX
4	47, XX, +16	47, XX, +16	46, XX
5	47, XX, +17	45, X	46, XX
6	47, XX, +7	48, XX, +7, +17	46, XX
7	46, XY, +1, −10	48, XY, −1, +10, +12, +22	46, XY
8	47, XY, +5	48, XY, +5, +21	46, XY
9	48, XY, +17, +18	47, XY, +17	46, XY
10	48, XY, +8, +9	47, XY, +9	46, XY
11	46, XX, +19, −21	46, XX, +19, −21	46, XX
12	50, XX, +1, +2, +5, +22	49, XX, +2, +5, +19	46, XX

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0.88 (95% confidence interval [CI]: 0.62–0.98); specificity of 0.55 (95% CI: 0.39–0.70); positive predictive value of 0.41 (95% CI: 0.25–0.60); and negative predictive value of 0.92 (95% CI: 0.75–0.99) (see Table 2).

DISCUSSION

This study demonstrates that genomic DNA from the BF can be successfully isolated, amplified, and assessed. The molecular karyotypes obtained from a single blastomere at the cleavage stage, and from the BF and ICM-TE of blastocysts, provide insight into the ploidy status of various cell types during embryo differentiation. These data show that approximately 70% (14 of 20) of cleavage-stage embryos with an aneuploid blastomere differentiated into euploid blastocysts, which included analysis of both the TE and ICM. Additionally, the aneuploid BF karyotype from the majority of these embryos suggests that the aneuploid cells may have been marginalized during blastulation. Furthermore, we demonstrated that, in this cohort of embryos, the karyotypes obtained from the DNA isolated from the BF were discordant to the ICM-TE in 52% of the embryos analyzed; thus, based on this study's finding, the use of BF-DNA does not adequately represent the remaining embryo (ICM-TE) and should not be used as an alternative biopsy modality.

In this study, we identified embryonic DNA, within the BF, that resulted in complete genomic molecular karyotypes, using aCGH analysis. Given that the karyotypes were complete, we suspected that the embryonic DNA resulted from intact nuclei, rather than from fragmented DNA or cell-free DNA. Additionally, whole cells were most likely not the source of embryonic DNA, because the size of intact cells would preclude their capture by the ICSI needle used in obtaining the BF.

The presence of embryonic DNA within the BF, whether in the form of intact nuclei, fragmented DNA, or cell-free DNA, suggests that some mechanism allows lysed or partially lysed cells to extrude their nuclei into the BF during differentiation. The presence of the aneuploid nuclei within the BF from euploid blastocysts that harbored aneuploid blastomeres at the cleavage stage (aneusomy) provides some evidence of a possible mechanism that marginalizes aneuploid cells into the blastocoel cavity; such a mechanism could explain the previously described observation that aneusomic, cleavage-stage embryos can differentiate into euploid blastocysts and fetuses.

TABLE 2

Both the molecular karyotype and ploidy (aneuploid vs. euploid) status of BF-DNA, compared with the ICM-TE of all embryos and the quantitative parameters for the diagnostic accuracy of BF-DNA to represent the ICM-TE (whole embryo).

Variable	Data
Concordant karyotypes	48% (29/60)
Discordant karyotypes	52% (31/60)
Sensitivity	0.88 (95% CI: 0.62–0.98)
Specificity	0.55 (95% CI: 0.39–0.70)
Positive predictive value	0.41 (95% CI: 0.25–0.60)
Negative predictive value	0.92 (95% CI: 0.75–0.99)

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In possible further support of our data that suggest marginalization of aneuploid cells away from the ICM during blastulation, time-lapse cinematography studies have shown a dynamic process of cellular movement during differentiation. This movement could include the marginalization of aneuploid cells away from the ICM during blastulation. In alignment with our data, 1 such study suggested the movement of aneuploid fragments that were marginalized to the periphery of the differentiating embryo (13). As time-lapse cinematography is still at the initial stages of investigation, monitoring of cellular movement toward the blastocoel cavity during blastulation has yet to be reported.

Our study suggests that aneuploid cells marginalize into the BF during blastulation, leaving a euploid ICM from a previously identified aneuploid, cleavage-stage embryo. The concept of embryo normalization has been postulated previously (14, 15). The results of these previous studies have suggested that embryo normalization may exist; however, the evidence has been indirect or incomplete, and therefore inconclusive. Previous PGT studies, with fluorescence in situ hybridization (FISH) or aCGH on cells obtained from embryo biopsy comparing single cells from cleavage-stage embryos, with a few, to several, TE cells from blastocyst-stage embryos failed to show normalization using standard IVF culture conditions (5, 16, 17). Other studies had significant experimental limitations, including, but not limited to: use of FISH technology that evaluated only 9–14 chromosomes; evaluating only a small (<10 cells) sample size from developing embryos; failing to differentiate the ICM from the TE in evaluating blastocyst cells; failing to discriminate between different stages of embryologic development; and using laboratory conditions that were not representative of standard IVF culture conditions, including the use of growth factors and the culture of embryos beyond 6 days of development (14, 15).

The high prevalence of aneusomy observed in this cohort of embryos, evidenced by the high rate of aneuploid cleavage-stage embryos that resulted in euploid blastocysts, provides an additional argument that a cleavage-stage biopsy is suboptimal for conducting PGT. This finding is not isolated to our study, and blastocyst-stage vs. cleavage-stage biopsy used for PGT has been correlated to multiple clinical outcomes that demonstrate significant improvement with the blastocyst biopsy and PGT (18–20).

In addition to furthering our understanding of the ploidy status during embryogenesis, our study reports on the potential use of BF-DNA as a diagnostic measure of the karyotype of the entire embryo. Our study observed that the karyotype identified from DNA within the BF was discordant to the karyotype of the ICM-TE in 52% of cases. In addition, the measures of diagnostic accuracy (sensitivity, specificity, positive predictive value, negative predictive value) were all too low to be considered acceptable for use as a diagnostic test.

The negative predictive value of 0.92, or the predictive probability of euploid BF-DNA to identify a euploid embryo, was considerably higher than all other parameters calculated, but it is still unacceptable for demonstrating a clinical diagnostic utility. In contrast, as many as 41% (18 of 44) of euploid blastocysts would have been incorrectly diagnosed

as aneuploid, owing to the high frequency of false-positive results. At this time, BF-DNA, as studied through this cohort of embryos, does not seem to be an adequate replacement for the TE biopsy for embryonic genetic analysis.

Of note, the only other previously published report on successfully identifying BF-DNA, and obtaining karyotypes at various stages of embryonic development, is by Gianaroli et al. (12), and their results contrast significantly with our findings. Most notably, they reported that the ploidy condition had a concordance of 97.4% (38 of 39) between the BF-DNA and TE samples, and it was 98.1% concordant for the specific karyotype. The findings from our study, by contrast, showed a 62% concordance between ploidy status, and a 48% concordance between the specific karyotypes. A similar finding in the 2 studies was the low efficiency of obtaining a karyotype from the BF-DNA: 76.5% vs. 63%; these results may have come from use of: a similar technique to obtain the BF (an ICSI needle, puncturing away from the ICM between cells of the TE); and the SurePlex WGA amplification kit (Illumina); and the same aCGH microarray platform (24-Sure; Illumina).

However, the studies were completed in different laboratories, so despite the use of similar techniques and materials, differing results are possible. However, a potential explanation for the differences in aneuploidy, and the high level of discordance in our embryos from the various stages of embryonic development, which contrasts with the work of Gianaroli et al. (12), is the difference in the population of embryos. Gianaroli et al. (12) used embryos from a relatively small pool of patients. Unfortunately, our study was blinded to the number of patient couples who generated the 96 embryos analyzed. However, our embryos could plausibly have come from a group of patients that differed in their demographics from the cohort used by Gianaroli et al. (12).

As with any diagnostic or screening test that uses measures of sensitivity, specificity, positive predictive value, and negative predictive value to compare the "test" value with the "true" value, the prevalence of a condition within a population will change that predictive value. The prevalence of embryonic aneuploidy is well known to increase with age, and other variables are suggestive of increasing embryonic aneuploidy; thus, embryos resulting from different populations can result in different calculated test measures. If the test being studied errs on the side of euploidy, and the prevalence of aneuploidy is very low, then despite the proportion of errors the test results in, it will still align itself with the "true" test value, owing to the population's prevalence of the tested condition.

Additionally, embryos in this study were supernumerary, and were donated for research because of their unsuitability for clinical use, which may have skewed the population to one of lower-quality embryos. At the time of the study, morphological grading was not performed to determine quality level. A follow-up study of BF-DNA using high-quality embryos would potentially settle this question.

As an increasing number of IVF centers continue embryo culture to the blastocyst stage for both transfer and cryopreservation, an abundance of BF is available for analysis with minimal invasion to the embryo. Currently, the most effective form of vitrification requires the blastocoel cavity to be drained and compressed (21, 22). This technique of draining the cavity was developed originally for blastocyst cryopreservation and has been demonstrated to be very effective, with excellent clinical outcomes. As blastocyst vitrification becomes ever more prevalent, with many centers that cryopreserve at only the blastocyst stage and even advocate freeze-all cycles (23–25), the availability of BF will be abundant and potentially useful if harnessed by the correct analytic tool. Although the goal of obtaining BF-DNA is to create a less-invasive form of PGT analysis, we recognize that this procedure involves aspiration of BF from the blastocoel cavity, which potentially could alter embryo dynamics. However, the impact seemed to be less invasive, compared with the currently used blastocyst TE biopsy procedure, which imposes more-intense stress to the embryo by removal of multiple cells from the embryo. Nonetheless, the potential of the procedure to impair embryo viability and implantation potential requires further investigation.

Additionally, this study demonstrated that the amplification rates of embryonic DNA from the BF were significantly lower than the generally accepted 2% failed amplification rate that results from TE or blastomere cells. Our study demonstrated an amplification rate of 63%, which is comparable to, although slightly lower than, the failed amplification rate of 79.5% demonstrated by Gianaroli et al. (12). Our results, as well as those from the Gianaroli et al. (12) study, are a significant improvement compared with the initial attempts, discussed in the first section of this paper. However, the rate is much lower than amplification rates when an intact cell(s) is used.

Ultimately, we do not know why such a high proportion of BF samples did not result in DNA amplification. We do state, in the Materials and Methods section, that the embryos used in this study were unsuitable for clinical use; however, all were expanded blastocysts and potentially viable embryos, and none of the embryos were arrested or embryonic fragments. Errors in amplification of embryonic DNA occur at a low rate when the DNA is extracted from whole cells, as the complete complement of DNA is potentially available for amplification. Within the fluid aspirated from the blastocoel cavity are no intact cells. Given the size of the ICSI needle, an intact cell would not be aspirated out. All DNA analyzed is cell-free DNA harbored within the BF. We did identify nuclei with the BF with DAPI staining from which did correlate with the BF-DNA amplification rates; however, positive staining for DAPI, does not necessarily correlate with intact nuclei.

Given that the DNA is cell free, in a very small compartment, with limited exposure to cells, the quantity of DNA likely varies. If the quantity of DNA is too low, amplification is likely to be unsuccessful, and we hypothesize that the low quantity of BF-DNA is most likely a result of: (1) nucleus degradation as it is released into the BF, with subsequent nuclear DNA fragmentation; or (2) lack of any movement of nuclei into the BF during blastocyst differentiation. Additionally, the failure could have resulted from laboratory technique, although this is unlikely given that we used the same laboratory personal, equipment, and techniques as we did for our clinical preimplantation genetic testing, which has

excellent amplification rates (failed amplification well below 2%). Although 1 objective for this study was met by successful obtainment of aCGH molecular karyotypes from BF-DNA, the diagnostic accuracy from this cohort of embryos is insufficient for the application of BF-DNA as a diagnostic source for PGT.

Limitations that may have contributed to our findings include the following factors. First, this cohort of embryos was donated to research because they were unsuitable for clinical use. Second, the karyotypes obtained from the BF-DNA may represent contamination that results from embryo culture media or other human sources; however, this possibility is unlikely because precautions were taken to avoid contamination. In addition, not all the cryopreserved blastocysts were inseminated by ICSI; thus, sperm contamination is possible, although the number of cells from a full differentiated blastocyst (approximately 100 cells [26]) would presumably dominate the influence that several sperm cells would have on the overall karyotype.

Finally, the karyotypic makeup of the blastocysts may include mosaicism that is not detected by aCGH, given that the cells are analyzed as a pool in the microarray. This problem is intrinsic to use of PGT to "deduce" the karyotype of an embryo from a set (4–8 cells) of cells biopsied from the TE of the embryo. Additionally, the remaining embryo is untested, because it would destroy the embryo to obtain DNA from it for analysis. The fact that each cell is not individually tested creates potential for error, because the tested cell(s) may not accurately reflect the untested portion of the remaining embryo.

In conclusion, genomic DNA within the BF can be amplified, and aCGH results can be obtained. Our results demonstrate that marginalization of aneuploid cells can occur as part of a process of resolving aneusomy during differentiation. Our study additionally showed that the level of karyotypic discordance between the BF and the ICM-TE is high, making its diagnostic accuracy unacceptable at this time. Additional studies of the potential clinical uses of BF are required, using best-quality embryos for clinical outcomes.

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