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EMBRYO BIOLOGY

Re-analysis of aneuploidy blastocysts with an inner cell mass and different regional trophectoderm cells

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Abstract
Purpose The purpose of this study is to explore which part of the trophectoderm best represents the inner cell mass after aCGH analysis.
Methods Fifty-one preimplantation genetic diagnosis/preimplantation genetic screening of abnormal blastocysts diagnosed by array comparative genomic hybridization were included in this study. Blastocysts were thawed, incubated for 3 to 4 h, and then biopsied. Four regions were biopsied per blastocyst, including the inner cell mass (ICM), trophectoderm (TE) cells opposite the ICM, TE cells at the upper right of the ICM, and TE cells at the lower right of the ICM. The biopsied pieces were processed through multiple annealing and looping-based amplification cycle sequenced for 24-chromosome aneuploidy screening. The aneuploidy results were compared among the ICM and the different regional trophectoderm cells from the same blastocyst.
Results Fifty of 51 (98.04%) ICM samples were concordant with at least one of the TE biopsies derived from the same embryos. There were 43 blastocysts in which ICM and the other three TE pieces were consistent. Discordance among the four pieces occurred in eight blastocysts. Only one blastocyst was discordant between the ICM and the other three TE pieces, while seven blastocysts were discordant between one of TE and the other three biopsied pieces. There was no special region that the mosaic TE was located.
Conclusions Our findings indicate that TE aneuploidy is an excellent predictor of ICM aneuploidy. The blastocyst mosaic cells are inclined to be located in TE. Moreover, the mosaic TE was not limited to the special region.

Keywords Blastocyst · 24-chromosome aneuploidy screening · Multiple annealing and looping-based amplification cycle sequencing · Mosaic · Preimplantation genetic screening

Capsule Re-analysis of the aneuploidy blastocysts reveals that TE is an excellent predictor of ICM. And the mosaic TE was not limited to the special region.

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With the robust growth of genetic test platforms, preimplantation genetic screening (PGS) has developed rapidly [1–3]. About 10 years ago, many in vitro fertilization (IVF) laboratories performed PGS by fluorescent in situ hybridization (FISH) to select euploid embryos [4]. However, FISH is limited because only a few chromosomes can be detected simultaneously in a single biopsied cell. FISH is also less reliable, and some studies show PGS via FISH analysis failed to increase pregnancy or live birth rates [5]. Currently, PGS by the FISH technique is not recommended at cleavage stages. Wilton reported the first successful clinical application of comparative genomic hybridization (CGH)-PGS in 2001 [6], which detects all chromosomes in one biopsied blastomere. Subsequently, 24-chromosome aneuploidy screening has almost replaced FISH. Several years later, the extended technologies, namely array CGH [7, 8] and single nucleotide polymorphism array [9, 10], are widely applied to PGS cycles. Next-generation sequencing (NGS) is another new technique that has been introduced into PGS cycles. Treff and his

colleagues [3] evaluated semiconductor-based NGS for genetic analysis of human embryos. Huang [11, 12] validated multiple annealing and looping-based amplification cycle (MALBAC) sequencing for 24-chromosome aneuploidy screening of cleavage-stage embryos and blastocysts.

In addition to the genetic test platforms, there is another key step in PGS: the biopsy. The biopsy procedure should acquire enough cells to gain accurate genetic information, while resulting in the least harm to embryos (oocytes). At present, there are three stages of the biopsy procedure: polar body biopsy from the oocyte, blastomere biopsy of cleavage-stage embryos, and trophectoderm (TE) cell biopsy of blastocysts. The polar body can only predict the genetic information of maternal genome. The aneuploidy rate of oocytes is very high, ranging from 22 to 72% [13]. The blastomere can provide the genetic information for both maternal and paternal genomes, but the mosaic rate of cleavage-stage embryos is between 30 and 85% [11, 14–16]. With the development of embryos into blastocysts, the mosaic rate decreases. Currently, an increasing number of researchers prefer the blastocyst stage as the optimal time to perform biopsies for PGS [17].

It is very difficult to decide if a mosaic aneuploid embryo should be transferred or not. Some scholars reported healthy live births after the transfer of mosaic aneuploid blastocysts [18]. It is unclear whether the genetic information of TE cells reflect that of the inner cell mass (ICM). Furthermore, it is unclear which part of the TE best reflects the ICM. To clarify these aspects, we investigated blastocyst mosaics with an ICM and different regional TE cells.

Materials and methods

This study was approved by the Institutional Review Board of Peking University Third Hospital, China. Written informed consent was obtained from each couple.

A total of 51 abnormal preimplantation genetic diagnosis (PGD)/PGS blastocysts were donated by 23 couples. The ages of the female patients ranged from 24 to 44. The indications of PGD/PGS were carriers of balanced translocations and recurrent miscarriage. The genetic test technique of these PGD/PGS cycles was array CGH.

In all of these PGD/PGS cycles, fertilization was performed by intracytoplasmic sperm injection (ICSI) on the day of oocyte retrieval. Zona pellucida drilling was conducted on day 3 of ICSI, followed by blastocyst culture. All of the cycles were subjected to trophectoderm-cell-biopsy by laser. The biopsied blastocysts were vitrificated individually. The morphological criterion for a blastocyst biopsy was a score above 5 BC according to Gardner's criterion [19]. Array CGH was performed on 24 sure-plus chips (Illumina). The Sure Plex DNA amplification system was used for whole genome amplification (WGA). Samples and control DNA were labeled with Cy3 and Cy5 fluorophores and then hybridized overnight. After laser scanning, Blue Fuse software was used to analyze microarray data concerning chromatin loss/gain across all 24 chromosomes.

The 51 frozen blastocysts were thawed and incubated for 3 to 4 h. When the thawed blastocysts had expanded, the biopsy was performed. First, the ICM was held at the 9 o'clock position. Then, three regions of TE cells were biopsied, including the TE cells opposite the ICM (at 3 o'clock, region "a"), TE cells at the upper right area of the ICM (at 12 o'clock, region "b"), and TE cells at the lower right area of the ICM (at 6 o'clock, region "c"). Last, ICM cells were biopsied (region "d") (Fig. 1). TE cells from region "b" or "c" are closer to the ICM, while TE cells from region "d" are farther to the ICM.

The biopsied cells of all four regions were whole genomic amplified by MALBAC. The amplification was initiated by a pool of random primers, each of which had a common 27 nucleotide sequence and eight variable nucleotides. MALBAC can generate the micrograms of DNA required for NGS. Using an Illumina HiSeq 2500 platform, the amplified genome of each biopsied cell was sequenced at an approximate 0.04× genome depth. Therefore, we sequenced a total of approximately 40 million bases, obtaining an average genome coverage of 3% for each single cell [11, 12].

We compared the MALBAC sequencing results of the biopsied cells with the array CGH results of the same blastocyst. Furthermore, we compared the MALBAC sequencing results of the ICM cells and the three regions of TE cells from the same blastocyst.

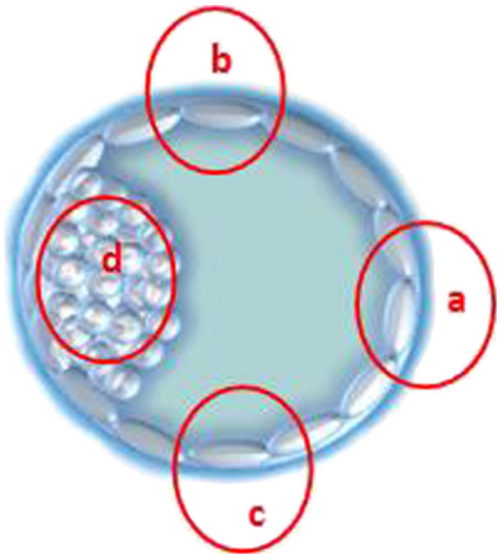


Fig. 1 Different biopsied regions of the blastocyst. *a* TE cells opposite to the ICM; *b* TE cells right upper of the ICM; *c* right down of the ICM; *d* ICM

Results

A total of 204 MALBAC sequencing results were obtained from 51 blastocysts. There were 50 blastocysts whose MALBAC sequencing results were concordant with those of the CGH diagnosis.

Fifty of 51 (98.04%) ICM samples were concordant with at least one of the TE biopsies derived from the

Table 1 The blastocysts which ICM and the other three TE pieces were consistent

Embryo no.	PGD/PGS indication	Biopsy region	MALBAC sequencing results (research results)	aCGH results (PGD/PGS results)
1	46,XY,t(10,17)	a, b, c, d	+15,XY	+15,XY
2	46,XY,t(3,12)	a, b, c, d	−22,XY	−22,XY
3	45,XY,rob(15,21)	a, b, c, d	+8q,+17,XX	+8q,+17,XX
5	46,XY,t(7,22)	a, b, c, d	−7p,XY	−7p,XY
7	45,XY,rob(13,14)	a, b, c, d	−13XX	−13XX
8	45,XY,rob(13,14)	a, b, c, d	+13,+14,XX	+13,+14,XX
9	46,XX,t(1,20)	a, b, c, d	−1p, XX	−1p,XX
12	PGS recurrent miscarriage	a, b, c, d	−19,−21,−22,XY	−19,−21,−22,XY
15	46,XY,t(11,13)	a, b, c, d	−11q,+13q,XY	−11q,+13q,XY
16	46,XY,t(11,13)	a, b, c, d	+13,XY	+13,XY
17	45,XY,rob(13,22)	a, b, c, d	−22,XY	−22,XY
18	46,XX,t(2,10)	a, b, c, d	−22,XX	−22,XX
19	46,XX,t(2,10)	a, b, c, d	−10q,+15,+16,+21,XX	−10q,+15,+16,+21,XX
20	46,XX,t(2,10)	a, b, c, d	+2q,−10q,XY	+2q,−10q,XY
21	46,XX,t(3,16)	a, b, c, d	+3q,−16,XX	+3q,−16,XX
22	46,XX,t(3,16)	a, b, c, d	+1,XX	+1,XX
24	46,XY,t(3,5)	a, b, c, d	−3q,+5q,XY	−3q,+5q,XY
25	46,XY,t(3,5)	a, b, c, d	+5,XY	+5,XY
26	46,XY,t(3,5)	a, b, c, d	−3q,+5q,XY	−3q,+5q,XY
27	46,XX,t(5,21)	a, b, c, d	XO	XO
29	46,XX,t(6,12)	a, b, c, d	−16,−22,XY	−16,−22,XY
30	46,XX,t(6,12)	a, b, c, d	−6p,+12p,XY	−6p,+12p,XY
31	47,XYY	a, b, c, d	−22,XY	−22,XY
32	47,XYY	a, b, c, d	−16,XY	−16,XY
33	47,XYY	a, b, c, d	+5,XY	+5,XY
34	46,XY,t(4,16)	a, b, c, d	+4q,−16q,XX	+4q,−16q,XX
35	46,XY,t(4,16)	a, b, c, d	+4q,−16q,XY	+4q,−16q,XY
36	46,XY,t(4,16)	a, b, c, d	−4q,+16q,XX	−4q,+16q,XX
37	46,XY,t(4,16)	a, b, c, d	+4q,−16q,XX	+4q,−16q,XX
38	46,XY,t(4,16)	a, b, c, d	−4q,+16q,XY	−4q,+16q,XY
39	PGS recurrent miscarriage	a, b, c, d	+17,XY	+17,XY
40	PGS recurrent miscarriage	a, b, c, d	−18,XX	−18,XX
41	46,XY,t(4,16)	a, b, c, d	−6q,+9q,XY	−6q,+9q,XY
42	46,XY,t(5,15)	a, b, c, d	+5q,−15q,XY	+5q,−15q,XY
43	46,XY,t(5,15)	a, b, c, d	+5q,XX	+5q,XX
44	45,XY,rob(13,14)	a, b, c, d	−14,XY	−14,XY
45	45,XY,rob(13,14)	a, b, c, d	−19,XY	−19,XY
46	45,XY,rob(13,14)	a, b, c, d	+13,+14,XX	+13,+14,XX
47	45,XY,rob(13,14)	a, b, c, d	+13,XY	+13,XY
48	46,XY,t(8,19)	a, b, c, d	−8p,XX	−8p,XX
49	46,XY,t(5,15)	a, b, c, d	−5q,XY	−5q,XY
50	46,XY,t(5,15)	a, b, c, d	+5q,XX	+5q,XX
51	46,XY,t(5,15)	a, b, c, d	+5q,XX	+5q,XX

same embryos. There were 43 blastocysts (84.31%, 43/51) in which the ICM and the other three TE pieces were consistent. That is, the MALBAC sequencing and array CGH (aCGH) results were consistent among these 43 blastocysts. Table 1 shows the details of these 43 blastocysts.

There were eight blastocysts with discordance among the four pieces (15.69%, 8/51). Only one blastocyst was discordant between the ICM and the other three TE pieces, while seven blastocysts were discordant between one of the TE regions and the other three biopsied pieces. Table 2 shows the details of these eight blastocysts. Blastocyst No. 28 was a typical mosaic embryo in which aCGH result indicated "−8p,−21q,XY," while the MALBAC sequencing results showed that TE regions "a," "b," and "c" were "XY," but the ICM was "−5q,+21q,XY" (Fig. 2). Blastocyst No. 6, 11, and 23 had multiple abnormalities in one of TE biopsy by MALBAC sequencing, which means we detected not only the same aberrations among the other TE cells and ICM but also some other aberrations.

Further analysis of the other seven mosaic blastocysts showed no special region of mosaic cells. The mosaic cells were located among the TE cells in these seven blastocysts, including mosaic cells in region "a" of two blastocysts, region "b" of two blastocysts, and region "c" of three blastocysts. The mosaic cells were multi-chromosome abnormal in three blastocysts and had either duplication or deletion of some segments in some chromosomes of the other four blastocysts.

Discussion

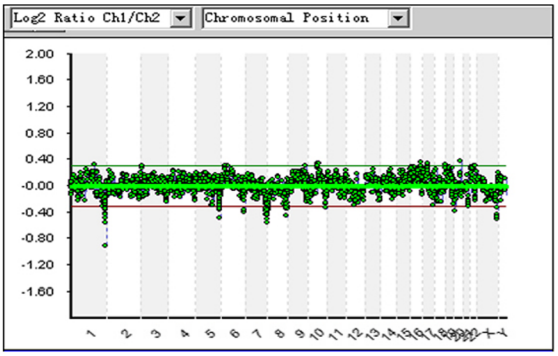
Chromosome imbalance is one of the major factors affecting the success of human IVF. Thus, PGS has become an embryo selection method in clinics. The blastocyst stage may be the optimal time to perform biopsies for PGS. Several TE cells can be obtained from a blastocyst biopsy, which improves the stability of WGA and generates more reliable genetic results [2, 7]. Another important advantage of blastocyst biopsy is that the mosaic rate of blastocysts is lower than that of cleavage-stage embryos, which objectively reflects the genetic information of the embryo.

The mosaic rate of cleavage-stage embryos is relatively high. Our previous study showed that the mosaic rate of D3 embryos is more than 50% [11], which is similar to the results in other studies [20, 21]. Some studies have focused on blastocyst mosaicism. Bradley examined discarded embryos with poor morphology. Thirteen karyotypically abnormal blastocysts were uniform (nonmosaic) [22]. Another study revealed that high proportions of aneuploid blastocysts (69.2%) were mosaic, including an aneuploid TE and euploid ICM, inconsistent anomalies between the ICM and TE or euploid TE cells, and an aneuploid ICM in the same blastocyst [23]. A study by Johnson indicated that approximately 80% of blastocysts are euploid [24].

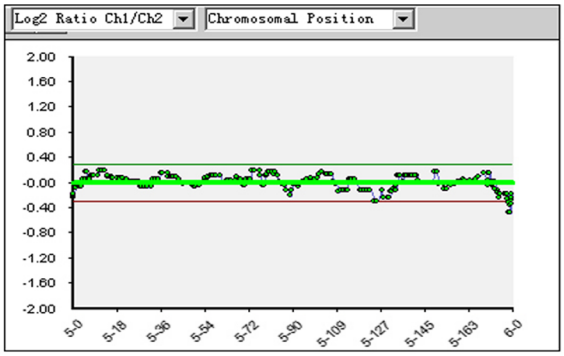
In our study, we analyzed donated PGD/PGS abnormal blastocysts. ICM cells and three selected regions of TE cells were biopsied per blastocyst. To eliminate the effects of different technologies on the results, only MALBAC sequencing

Table 2 The blastocysts which discordance among the four pieces

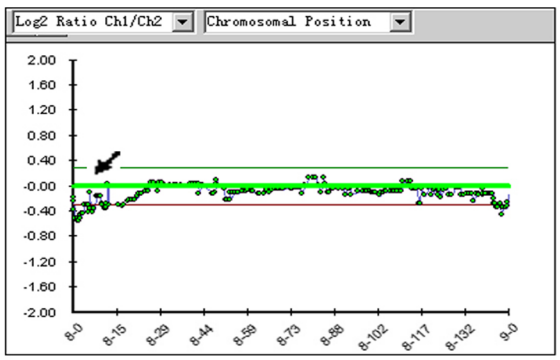
Embryo no.	PGD/PGS indication	Biopsy region	MALBAC sequencing results	aCGH results
4	46,XY,t(7,22)	a	45,XO,+7q	XO
		b, c, d	45,XO	
6	46,XY,t(15,19)	a, c, d	46,XY,+19q	+19q,XY
		b	Multi-chromosomes abnormal	
10	PGS recurrent miscarriage	a, b, d	47,XX,+22	+22,XX
		c	47,XX,+9q,+22	
11	PGS recurrent miscarriage	a	Multi-chromosomes abnormal	−21,XY
		b, c, d	45,XY,−21	
13	46,XX,t(7,13)	a, c, d	46,XX,+1,−22	+1,−22,XX
		b	45,XX,−22	
14	46,XY,t(11,13)	a, b, d	−11q,+13q,XY	−11q,+13q,XY
		c	−11q,+13q,+16p,XY	
23	45,XX,rob(14,21)	a, b, d	+21,XY	+21,XY
		c	Multi-chromosomes abnormal	
28	46,XX,t(5,21)	a, b, c	XY	−8p,−21q,XY
		d	−5q,+21q,XY	



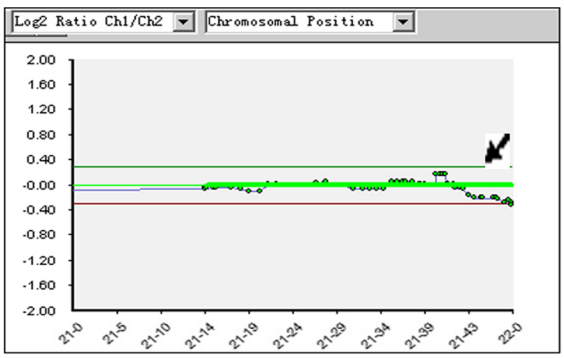
CGH result: -8p, -21q, XY



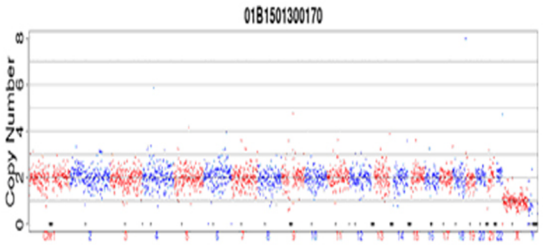
CGH result of chromosome 5:euploidy



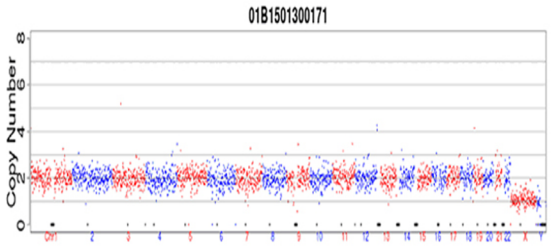
CGH result of chromosome 8: -8p



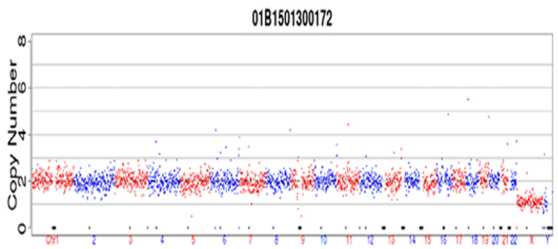
CGH result of chromosome 21:-21q



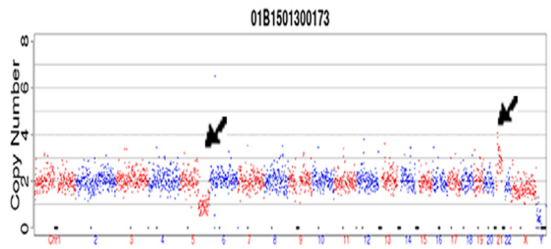
MALBAC sequencing result of TE "a" : XY



MALBAC sequencing result of TE b: XY



MALBAC sequencing result of TE "c" : XY



MALBAC sequencing result of ICM: -5q, +21q, XY

Fig. 2 The aCGH result and the four biopsied cells' MALBAC sequencing results of No. 28 blastocyst (the aCGH results show that this blastocyst is "−8p,−21q,XY"; the MALBAC sequencing results show that TE regions a, b , and c are "XY," but ICM is "−5q,+21q,XY")

was performed in this study. Our results showed that 50 blastocysts had MALBAC sequencing results that were concordant with the CGH diagnosis, and eight (15.69%) blastocysts were mosaic.

Mosaicism is a major limitation for PGS. There are several reasons that the mosaic proportion of blastocysts is much lower than that of cleavage-stage embryos. First, not all cleavage-stage embryos can develop to the blastocyst stage, especially

chromosomally abnormal embryos [25]. Second, mosaic cells in cleavage-stage embryos may develop into TE cells and not the ICM. In prenatal diagnosis, it has been found that some fetuses have normal chromosomes, while the placenta has both normal and abnormal chromosomes [26–28]. There may be a self-correction mechanism during embryonic development. Some abnormal mosaic blastomeres fail to incorporate into the blastocyst. We have previously reported an interesting translocation-PGD case [29]. An embryo was diagnosed as abnormal at the cleavage stage but was normal at the blastocyst stage according to TE cell diagnosis. This embryo was transferred with the couple's consent, and the patient delivered a healthy baby carrying a chromosomally balanced translocation (tested by amniocentesis).

Studies on mosaics between the TE and ICM are very limited. In 1983, Kalousek and Dill showed chromosomal mosaicism in natural conceptions, and the existence of chromosomal mosaics was strictly confined to tissues of extraembryonic origin [26]. It has been estimated that approximately 2% of viable pregnancies have this type of mosaicism [30]. In a blastocyst biopsy, the TE cells that will develop into the placental tissue are biopsied. Whether the genetic information of the TE can reflect that of the ICM is unknown. A study by Fragouli indicated that all TE and ICM cells were consistent in ten blastocysts according to CGH aneuploidy screening [25]. Liu obtained different results [23]. In 13 blastocysts, four blastocysts had an abnormal TE but a normal ICM. Johnson showed that 96.1% of ICM samples were concordant with TE biopsies derived from the same embryos [24]. Our data showed that 50 of 51 (98.04%) ICM samples were concordant with the other TE biopsies. Therefore, it can be concluded that the TE karyotype is an excellent predictor of the ICM karyotype.

This is a study to investigate the relationship between mosaic cells and the regions of a blastocyst. In our study, seven blastocysts were discordant between one of the TE cell regions and the other three biopsied pieces. There was no special region with mosaic cells. Mosaic cells were randomly located among the TE cells. This study may serve as a guide for blastocyst biopsy. Any region of TE cells can be biopsied, as long as damage to the ICM is avoided.

A rapidly developing technique, NGS, has been applied to PGD/PGS in recent years. Treff and his colleagues [3] investigated the applicability of NGS for PGD and PGS. Their study evaluated semiconductor-based NGS for genetic analysis of human embryos. In our study, we used MALBAC sequencing, which is a combination of WGA and NGS. MALBAC is a newly developed amplification method. Hou [31], Lu [32], and Zong [33] introduced MALBAC for genomic analysis of single human oocytes and single human sperm cells. We used MALBAC to amplify the DNA of the biopsied cells. Then, the amplified genome of each sample was sequenced at an approximate 0.04× genome depth using the Illumina HiSeq 2500 platform. Our previous study validated MALBAC sequencing as a satisfactory method for 24-chromosome aneuploidy screening of cleavage-stage embryos and blastocysts [11, 12]. In this study, MALBAC sequencing showed that 98.04% of blastocysts (50 of 51) had MALBAC sequencing results that were concordant with the aCGH diagnosis. The only discordant blastocyst was a mosaic embryo (no. 28 blastocyst).

Of course, the present study had limitations. The embryos in this study were aCGH diagnosed as imbalanced blastocysts that were donated by clinical PGD/PGS couples. The mosaic proportion of these abnormal blastocysts cannot completely represent that of all blastocysts. However, euploidy blastocysts are the available embryos in clinical IVF, and it is very difficult to use these embryos for research. Second, the blastocysts in this study were chromosomally abnormal embryos, but they had high-quality morphology (the morphological criterion for a blastocyst biopsy was a score above 5 BC according to Gardner's criterion in this study, as mentioned before). Therefore, the results of this study cannot represent all blastocysts. Moreover, when an expanding blastocyst is biopsied, the blastocyst will immediately collapse, which causes TE cells to cluster tightly. We only biopsied the ICM and three selected TE regions in each blastocyst.

In conclusion, we performed 24-chromosome screening of the ICM and various regions of TE cells in discarded imbalanced blastocysts by MALBAC sequencing. Our findings indicate that the TE karyotype is an excellent predictor of the ICM karyotype. Mosaic cells in blastocysts are inclined to be located in TE, and the mosaic cells were not limited to a specific region. These results not only reveal the genetic information of blastocysts but also provide beneficial information for PGD/PGS genetic counseling and embryo biopsy.

Compliance with ethical standards This study was approved by the Institutional Review Board of Peking University Third Hospital, China. Written informed consent was obtained from each couple.

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