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Authors: Liu, Jianqiao, Wang, Weihua, Sun, Xiaofang, Liu, Lian, Jin, Hua, et al.

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DNA Microarray Reveals That High Proportions of Human Blastocysts from Women of Advanced Maternal Age Are Aneuploid and Mosaic[1]

Jianqiao Liu,[3] Weihua Wang,[2,4] Xiaofang Sun,[3] Lian Liu,[5] Hua Jin,[5] Man Li,[5] Craig Witz,[4] Dan Williams,[4] Jason Griffith,[4] Josh Skorupski,[4] Gus Haddad,[4] and Jimmy Gill[4]

[3]*Key Laboratory of Major Obstetrics Diseases of Guangdong Province, Guangzhou Medical College, Guangdong, China*
[4]*Houston Fertility Institute/New Houston Health, Houston, Texas*
[5]*Pacgenomics, Inc., Village Medical Center, Thousand Oaks, California*

ABSTRACT

Trophectoderm (TE) biopsy and DNA microarray have become the new technologies for preimplantation genetic diagnosis in humans. In this study, we comprehensively examined aneuploid formation in human blastocysts produced in vitro with microarray and investigated the clinical outcome after transfer of euploid embryos. Biopsied cells from either TE or inner cell mass (ICM) were processed for microarray to examine the errors in 23 pairs of chromosomes and the consistency between TE and ICM. It was found that 56.6% of blastocysts were aneuploid. Further analysis indicated that 62.3% of aneuploid blastocysts had single and 37.7% had multiple chromosomal abnormalities. Chromosome errors could occur in any chromosome, but errors in chromosome 21 accounted for the most (11.3%) among the 23 pairs of chromosomes. Transfer of array-screened blastocysts produced high pregnancy (70.2%) and implantation (63.5%) rates. Microarray of TE and ICM cells in the same blastocysts revealed that high proportions of aneuploid blastocysts (69.2%) were mosaic, including aneuploid TE and euploid ICM, inconsistent anomalies between ICM and TE, or euploid TE cells and aneuploid ICM in the same blastocyst. These results indicate that high proportions of human blastocysts produced in vitro from women of advanced maternal age are aneuploid and mosaic. Errors can occur in any of the 23 pairs of chromosomes in human blastocysts. Biopsy from TE in blastocysts does not exactly predict the chromosomal information in ICM if the embryos are aneuploid. Some mosaic blastocysts have euploid ICM, which may indicate important differentiate mechanism(s) of human preimplantation embryos.

aneuploidy, embryo, human, microarray, mosaicism

INTRODUCTION

Preimplantation genetic diagnosis (PGD) is a technology to screen abnormal chromosomes in oocytes, early-stage embryos, and blastocysts, and is widely applied in human in vitro fertilization (IVF) laboratories. Chromosomal anomaly (the aneuploidy) in embryos is one of the major factors affecting the success of human IVF; thus, embryo screening by PGD has become one of the embryo selection methods in clinics. Previous studies by fluorescence in situ hybridization (FISH) of 5–12 chromosomes revealed that about half of human embryos produced by IVF were aneuploid [1–4]. However, the implantation rates were not significantly increased after transfer of embryos screened by FISH-based PGD [5–8]. The reason is that limited numbers of chromosomes were examined in the FISH-based PGD procedures [7]. It has been found that anomalies can occur in any chromosome [3, 8], but it is difficult to use FISH technology to examine all chromosomes, especially for clinical purposes. Such a technical limitation or shortage can be overcome with DNA microarray.

DNA microarray technology has revolutionized life science research and medical diagnosis. Researchers can use DNA microarray to examine the full sequence of a genome in a single cell, allowing them to study the details of its genetic map. DNA microarray has been widely applied to basic research, such as gene expression, genome mapping, single-nucleotide polymorphism (SNP) discrimination, and transcription factor activity [9]. It has also been used in clinical diagnoses, such as toxic detection, pathogen detection, and genetic disease detection [9]. Comparative genomic hybridization (CGH) and whole-genome arrays can accurately measure DNA copy number in a sample [10]. Because CGH and array-based CGH can be used to detect DNA copies in a few cells or a single cell, such technologies have been recently applied to PGD in human embryos produced by IVF [11–17], and there is an increased tendency for them to be used more commonly in infertility clinics. It has been found that transfer of array-screened normal blastocysts could significantly increase implantation of embryos [16].

Recently, more clinicians and laboratory scientists have tended to perform microarray by using trophectoderm (TE) cells biopsied from blastocysts instead of blastomere biopsy from Day 3 cleavage embryos [16]. Because more than one cell can be biopsied from a blastocyst, it is expected that more accurate information can be obtained with blastocyst biopsy compared with one-cell biopsy from Day 3 embryos [16, 18, 19]. Due to its accuracy, the array-based PGD has been used not only in patients with advanced ages, but also in young patients [16, 19], and a higher pregnancy rate was obtained in the patients who underwent transfer of array-screened embryos than in those without screened embryos [19].

Currently, use of a TE cell biopsy from a blastocyst is encouraged in IVF laboratories [16, 19]. However, due to mosaicism of human embryos, it is possible that chromosomes are different among TE cells or between TE cells and inner cell mass (ICM) cells. Because the mosaic rate in human embryos at cleavage stage (Day 3) is extremely high [3, 8, 15], it is possible

[1]Supported in part by a grant from the Third Hospital of Guangzhou Medical College.
[2]Correspondence: Weihua Wang, Houston Fertility Institute, 2500 Fondren Rd., Suite 350, Houston, TX 77063.
E-mail: wangweihua11@yahoo.com

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that such mosaicism is still present in blastocysts, and microarray of biopsied TE cells may not exactly predict the ICM cells. Therefore, it is necessary to examine not only aneuploid formation, but also the mosaicism in the human blastocysts. In the present study, we initially examined the detailed chromosome abnormalities in human blastocysts from the patients undergoing IVF, and then we examined whether chromosomes were consistent between TE and ICM cells in the same embryos. In order to assure the accuracy in this study, we used two different array platforms: one is a bacterial artificial chromosomal (BAC)-based microarray CGH that has already been applied to human PGD services [11–17, 19, 20], and another is an oligonucleotide (oligo) NimbleGen microarray provided by Roche [21], which is a more sensitive and higher-resolution platform that has been used in some research fields [22, 23].

MATERIALS AND METHODS

Patient Preparation for Egg Retrieval and PGD

All patients undergoing IVF and PGD signed written consent forms for all kinds of laboratory manipulations and tests of the resulting embryos. When the patients signed the consent forms, they were aware that biopsy and PGD were investigational procedures requiring removal of one or more cells from embryos, and the genetic analysis of their samples would be used for investigational purposes only. As promulgated by the U.S. Department of Health and Human Services, this study was exempted from Institutional Review Board approval because it involved the review of existing data, documents, records, and diagnostic specimens in such a manner that participants could not be identified, either directly or through identifiers linked to the participants.

Patients received PGD service because they either had previous infertility experience with recurrent miscarriage and/or advanced maternal age. For oocyte collection, patients were treated with long protocol or antagonist protocol depending on the infertility diagnosis. Follistim (Organo USA), Gonal-F (EMD Serono), and/or Menopure (Ferring Pharmaceuticals) were usually started within the first 2–3 days after the period began, with a starting dose between 150 and 375 IU per day. The dose may have been adjusted during the stimulation process based on the follicle growth and hormone levels. Human chorionic gonadotropin (hCG) at a dose of 5000–10 000 units was injected to cause final maturation of the eggs when at least two dominant follicles reached a diameter of >18 mm. Eggs were retrieved via transvaginal ultrasound between 35 and 37 h after hCG administration.

Fertilization, Embryo Culture, and Blastocyst Biopsy

Oocytes were cultured for 4–5 h before the denuding of the surrounding cumulus cells in a Hepes-buffered medium containing 80 IU hyaluronidase (IVFonline), and the mature (metaphase II) oocytes were inseminated by intracytoplasmic sperm injection. Fertilization was examined 16–18 h after intracytoplasmic sperm injection, and zygotes were cultured in a Global medium (IVFonline) supplemented with 10% serum protein substitute (SPS; IVFonline) at 37°C in a humidified atmosphere of 5.5% CO_2, 5% O_2, and balanced nitrogen until Day 6 after insemination. At Day 3, a hole of about 20 μm was opened on the zona pellucida with a laser generated by a ZILOS-tk laser system (Hamilton Thorne Bioscience Inc.). On Day 5, embryos were examined to check whether a blastocyst was formed and whether some cells started to hatch from the opening in the zona pellucida. If some cells started to hatch, approximately three to five TE cells were biopsied using a 20-μm polished biopsy pipette with assisted cutting by laser. Blastocyst biopsy was done on TE cells at Days 5 and 6 depending on blastocyst development. After biopsy, the embryo proper was cultured in the Global medium supplemented with 10% SPS for 1–2 h before vitrification. The biopsied cells were washed with a washing buffer, placed in tubes with cell lysis buffer, and then frozen before being processed for microarray.

Blastocyst Vitrification

Blastocysts were vitrified after the blastocoele completely collapsed according to the previous method [24], with with Cryotop (Kitazato Biopharma) and Irvine vitrification kit (Irvine Scientific). Briefly, blastocysts were equilibrated in the equilibration solution containing 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethyl sulfoxide for 2 min at warm stage. The blastocysts were then transferred into the vitrification solution that was composed of 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulfoxide, and 0.5 M sucrose, and then loaded onto the Cryotop within 30–45 sec. The Cryotops were immediately plunged into the protective straw inside liquid nitrogen for cryopreservation. All embryos were vitrified individually and then stored in the liquid nitrogen until warming for frozen embryo transfer (FET).

Rebiopsy of Frozen-Thawed Blastocysts and Separation of ICM and TE

Thirteen abnormal blastocysts after the initial biopsy and microarray were warmed and rebiopsied. For warming, blastocysts were exposed to 1 M sucrose that had been warmed at 37°C. After 1 min in the sucrose solution, blastocysts were transferred to 0.5 M sucrose for 3 min and then to a basic solution (Hepes-buffered tissue culture medium 199 supplemented with 20% SPS) for 10 min with a solution change after 5 min at room temperature. After warming, blastocysts were washed with the Global medium supplemented with 10% SPS and then cultured in the same medium overnight before biopsy. For the biopsy, cells in ICM and TE were separated with aspiration by biopsy pipette and assisted laser cutting, and both samples were placed separately in the sample tubes. Samples in each tube were simultaneously processed for the microarray with two microarray platforms. After biopsy, the remaining cells were cultured for 2–4 h and were refrozen if they survived or discarded if they degenerated.

Microarray of ICM and TE Cells with BAC BlueGnome Platform

Biopsied ICM and TE cells were lysed and the cell's genomic DNA was amplified using a Rubicon whole-genome amplification kit (Rubicon). Amplified samples were labeled with Cy3 or Cy5 using a fluorescent labeling system (BlueGnome). Labeled samples were mixed with COT human DNA, dried, dissolved, and loaded onto BlueGnome 24Sure V3 arrays following the BlueGnome 24Sure V3 protocol. After overnight hybridization, arrays were washed following the BlueGnome 24Sure V3 protocol. Arrays were dried and scanned with a NimbleGen MS200 scanner (Roche Nimblegen) at 10 μM. Scanned images were analyzed by ImageViewer and Bluefuse software (BlueGnome) following BlueGnome 24Sure V3 data analysis protocol.

Microarray of ICM and TE Cells with Oligo NimbleGen Platform

Biopsied ICM and TE cells were lysed and the cell's genomic DNA was amplified using a Rubicon whole-genome amplification kit (Rubicon). Amplified samples were purified with a GenElute PCR Clean-Up Kit (Sigma). The DNA concentration of purified samples was measured using NanoDrop 2000 (Thermo Scientific). Purified samples were then labeled with Cy3 using an NG dual-color labeling kit (Roche Nimblegen). Labeled samples were mixed with Cy5 control labeled samples, dried, dissolved, and loaded onto Nimblegen 6X630K CGH tiling array following Nimblegen hybridization protocol. After overnight hybridization, arrays were washed following Nimblegen washing protocol. Arrays were dried and scanned with a NimbleGen MS200 scanner (Roche NimbleGen) at 2 μM. Scanned images were analyzed by Deva 1.1 software (Roche NimbleGen), and the normalized ratio of each sample versus the control was retrieved following NimbleGen CGH data analysis protocol. Finally, the normalized ratio of each sample was input into Nexus 6.1 software (Biodiscovery), and the result of each sample's whole genome view is presented.

Blastocyst Warming and FET

Euploid blastocysts after microarray were warmed for FET based on the same methods mentioned above. After warming, blastocysts were washed with the Global medium supplemented with 10% SPS and then cultured in the same medium for 2–3 h before transfer.

All patients for FET received gonadotropin-releasing hormone agonist and supplementary estrogen pills (Warner Chilcott Laboratories) for preparation of the endometrium. Intramuscular administration of progesterone oil was initiated 6–7 days before embryo transfer and was continued to be applied until the first serum β-hCG test 2 wk after transfer.

Pregnancy Diagnosis

After 14 days of embryo transfer, the serum β-hCG was checked. When the β-hCG showed a positive pregnancy, the patients were regarded as having a biochemical pregnancy. Four weeks after the successful embryo transfer, when

TABLE 1. Microarray analysis of human blastocysts.

Observation	Age (yr)				Total
	<35	35–37	38–40	≥41	
No. of cases	9	10	20	12	51
Total number of blastocysts biopsied and examined	47	59	100	52	258
No. of blastocysts with DNA signals	43	57	94	50	244
Percentage of blastocysts with DNA signals	91.5	96.6	94.0	92.2	94.6
No. of blastocysts with normal chromosomes	24	32	41	9	106
Percentage of blastocysts with normal chromosomes	55.8[a]	56.1[a]	43.6[b]	18.0[c]	43.4
No. of blastocysts with abnormal chromosomes	19	25	53	41	138
Percentage of blastocysts with abnormal chromosomes	44.2[a]	43.9[a]	56.4[b]	82.0[c]	56.6
No. of cases with transferable embryos	8	8	15	6	37
Percentage of cases with normal embryos for transfer	88.9	80.0	75.0	50.0	72.5
No. of cycles that had FET	8	8	15	6	37
No. of clinical/ongoing pregnant	6	5	10	5	26
Clinical pregnancy rate (%)	75.0	62.5	66.7	83.3	70.2
No. of embryos transferred	11	14	20	7	52
No. of embryos implanted	7	7	13	6	33
Implantation rate (%)	63.6	50.0	65.0	85.7	63.5

[ab] $P < 0.05$.
[ac, bc] $P < 0.01$ in the same row.

a gestational sac and the primitive heart tube appeared under ultrasonography, the patients were diagnosed as having a clinical pregnancy.

Statistical Analysis

The data were analyzed by ANOVA and differences between groups were analyzed by chi-square test. If the *P* value was less 0.05, it was considered to be statistically different between groups.

RESULTS

High Proportions of Human Blastocysts Are Aneuploid

A total of 258 blastocysts from 51 cycles were analyzed after biopsy and microarray. As shown in Table 1, 244 blastocysts (94.6%) had intact DNA signals after microarray. Of these samples with DNA signals, 43.4% were euploid and 56.6% were aneuploid that had single or multiple (complex) chromosomal abnormalities. The aneuploid rates (56.4%–82.0%) were significantly higher in patients ages 38 yr or older compared with patients ages 37 yr or younger (43.9%–44.2%). It was found that 82.0% of blastocysts were aneuploid in patients ages ≥41 yr, and only 18.0% of the embryos were euploid. Of the cases performed, 72.5% had at least one normal embryo for FET, whereas other patients did not have an embryo for transfer because all embryos were aneuploid. The proportion of patients with transferable embryos decreased as maternal age increased, from 88.9% for patients ages <35 yr to 50.0% for patients ages ≥41 yr (Table 1).

High Pregnancy and Implantation Rates after Transfer of Microarray-Screened Blastocysts

All embryos survived after vitrification/warming, showing re-expansion after 2–3 h of culture. High pregnancy rates (average, 70.2%) were observed in the present study, and there were no age differences. A high implantation rate (63.5%) was observed in the patients who had FET.

Chromosomal Errors Occur in Any Chromosome

When we further analyzed 138 aneuploid blastocysts, we found that 62.3% of aneuploid blastocysts had a single chromosome error and 37.7% had multiple or complex chromosome errors. As shown in Figure 1A, single chromo-

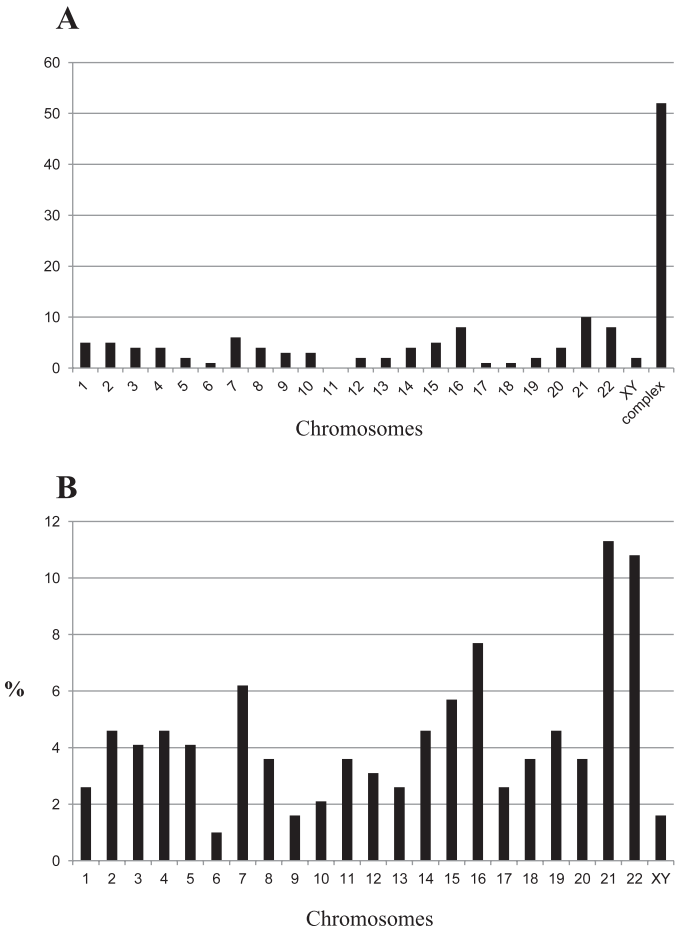

FIG. 1. Presence and distribution of abnormal chromosomes in the aneuploid blastocysts detected by TE biopsy and DNA microarray. **A**) Data represent the numbers of aneuploid blastocysts with a single abnormal chromosome and multiple (complex) abnormal chromosomes. **B**) Data show the proportions of aneuploid embryos with combined single and multiple chromosome abnormalities. Data were based on 138 aneuploid blastocysts.

TABLE 2. Consistency examination of TE and ICM in human blastocysts by DNA microarray analysis.

Sample no.	Embryo type*	BlueGnome[§] TE	BlueGnome[¶] TE	NimbelGen[¶] TE	BlueGnome[¶] ICM	NimbelGen[¶] ICM	Mosaic
1	II	45, XX; −22	45, XX; −22	45, XX; −22	45, XX;-22	45, XX; −22	No
2		46, XY; +18	47, XY; +18	47, XY; +18	47, XY; +18	47, XY; +18	No
3		45, XX; −2	45, XX; −2	45, XX; −2	45, XX; −2	45, XX; −2	No
4		45, XO	45, XO	45, XO	45, XO	45, XO	No
5	III	47, XXY	46, XX	46, XX	46, XX	46, XX	Yes
6		45, XX; −3	45, XX; −3 Ch-6 partial del	46, XX; −3 Ch-6 partial del	46, XX	46, XX	Yes
7		46, XX del4	46, XX	46, XX	46, XX	46, XX	Yes
8		47, XX; +16	47, XX; +16	47, XX; +16	46, XX	46, XX	Yes
9	IV	48, XXY; +22	48, XXY; +22 Ch-19 del	48, XXY; +22 Ch-19 del	48, XXY; +22	48, XXY; +22	Yes
10		47, XY; +21	47, XY; +21 Dup 9p	47, XY; +21 Dup 9p	47, XY; +21	47, XY; +21	Yes
11		45, XX; −8	45, XX; −8 Ch-18 del	45, XX; −8 Ch-18 del	45, XX; −8	45, XX; −8	Yes
12		47, XX; +8	46, XX; +8,-6	46, XX; +8,-6	46, XX; +8,-6	46, XX; +8,-6	Yes
13		45, XX; −7	45, XX	45, XX	45, XX; −7	45, XX, −7	Yes

* Type I: Normal chromosomes in both TE cells and ICM cells, and data were not available in the table. Type II: Abnormal but consistent chromosomes were detected in both TE and ICM cells. Type III: Abnormal or mosaic cells (normal and abnormal) were detected in TE cells, but ICM cells had normal chromosomes. Type IV: Normal or abnormal chromosomes were detected in TE cells, but abnormal chromosomes were detected in the ICM cells.
[§] The first biopsy and microarray. Only TE cells were biopsied and BlueGnome platform was used.
[¶] The second biopsy and microarray. Both TE and ICM cells were biopsied and BlueGnome and NimbleGen platforms were used.

some error occurred in most chromosomes except chromosome 11. However, when single and multiple chromosome errors were analyzed together, as shown in Figure 1B, we found that chromosome errors could occur in any of the 23 pairs of chromosomes, but errors in chromosome 21 (11.3%) were the most frequent chromosome anomaly, followed by chromosomes 22 (10.8%), 16 (7.7%), 7 (6.2%), and 15 (5.7%).

When we analyzed chromosomes 13, 18, 21, and XY, which are the most common chromosomes examined by FISH, we found that only 12.7% of the blastocysts had these chromosome errors, and the rate increased to 29.5% if 12 chromosomes were analyzed (8, 9, 13, 14, 15, 16, 17, 18, 21, 22, and XY). However, if all chromosomes were examined with microarray, 56.6% of the blastocysts had chromosome errors (Table 1). These results indicate that chromosome errors in many blastocysts cannot be detected if a 5- or 12-probe FISH is used.

Mosaicism in Human Blastocysts

When cells from the TE and ICM of the same blastocysts were analyzed in 13 samples from four patients (ages 35–39 yr), as shown in Table 2 and Figures 2–5, the following four types of chromosome distributions were observed:

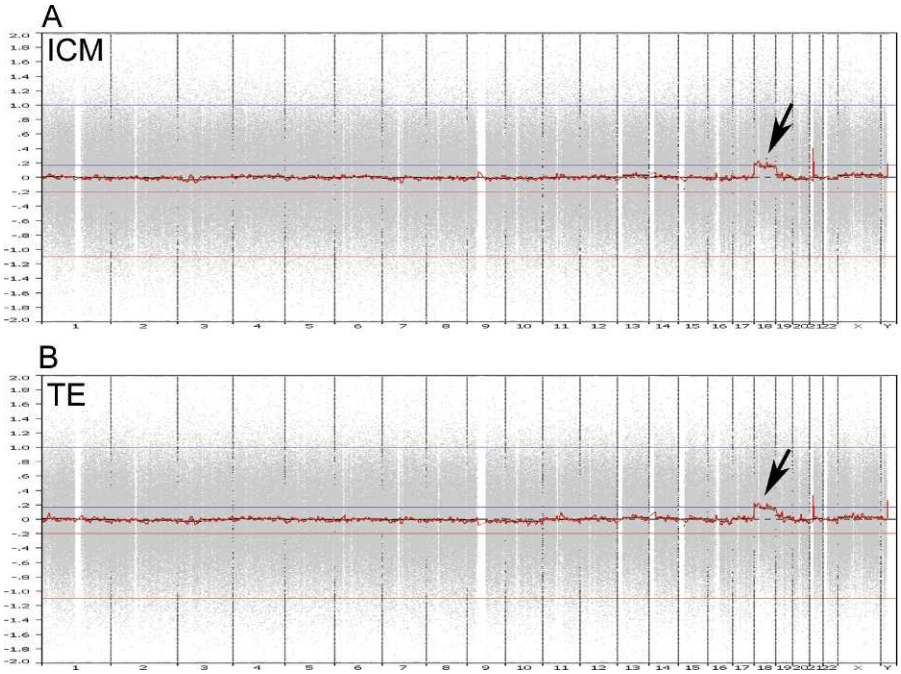

FIG. 2. NimbleGen preimplantation genetic screening (PGS) charts of an embryo with consistent chromosomal data in both TE and ICM. Both ICM (**A**) and TE (**B**) cells had 47, XY; +18.

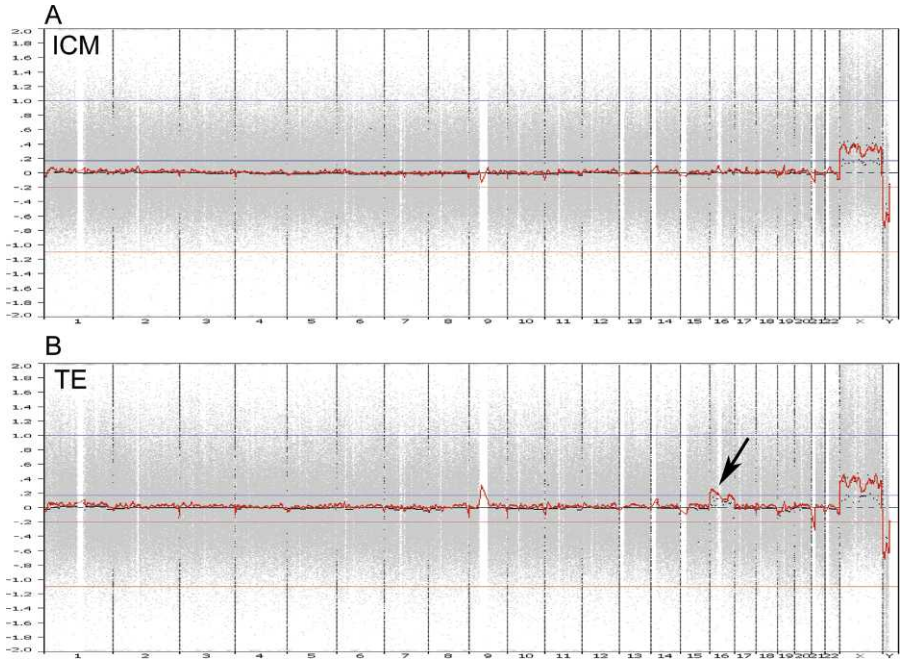

FIG. 3. NimbleGen PGS charts of an embryo with inconsistent chromosomal data in both TE and ICM. ICM (**A**) had 46, XX, but TE (**B**) had 47, XX; +16 (arrow).

Type I: Normal chromosomes in both TE cells and ICM cells. Most transferred embryos should be type I blastocysts in the present study. Due to the clinical use of euploid blastocysts, rebiopsy of euploid blastocysts and microarray were not done in the present study.

Type II: Abnormal but consistent chromosomes were detected in both TE and ICM cells, which was observed in 4 of 13 embryos (Fig. 2).

Type III: Abnormal or mosaic cells (normal and abnormal) were detected in TE cells, but ICM cells had normal chromosomes (Fig. 3). This was observed in 4 of 13 blastocysts.

Type IV: Normal or abnormal chromosomes were detected in TE cells, but abnormal chromosomes were detected in ICM cells (Fig. 4), which were observed in 5 of 13 embryos. The chromosomes in ICM cells were the same or not the same as those in TE cells.

Type III and IV embryos were mosaic embryos, which accounted for 69.23% (9 of 13) of abnormal blastocysts. Four of nine mosaic blastocysts had normal ICM cells. Diagrams of

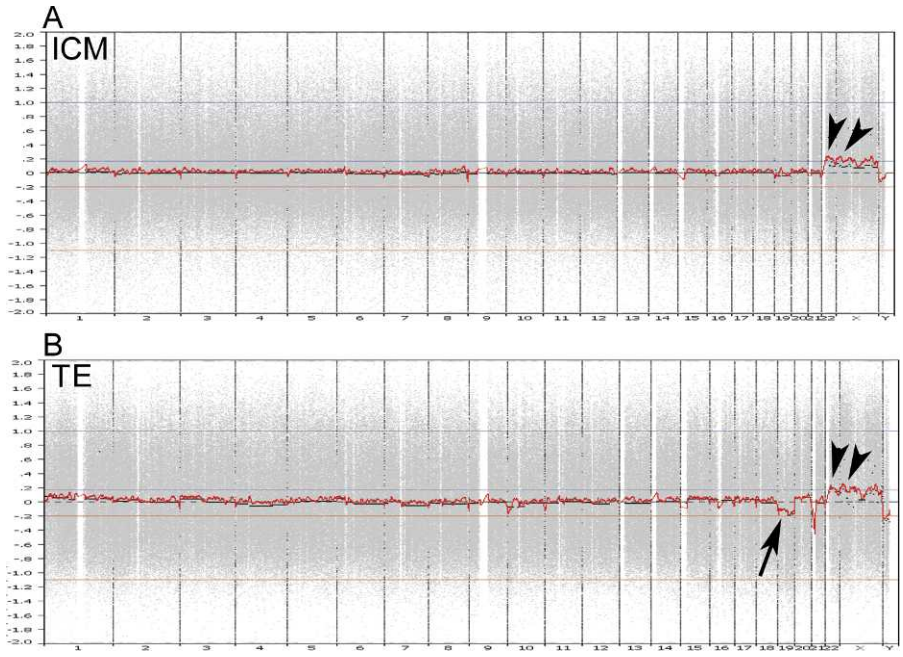

FIG. 4. NimbleGen PGS charts of an embryo with inconsistent chromosomal data in both TE and ICM. ICM (**A**) had 48, XXY; +22 chromosomes (arrowheads), but TE (**B**) had 48, XXY; +22 and chromosome 19 deletion (arrow).

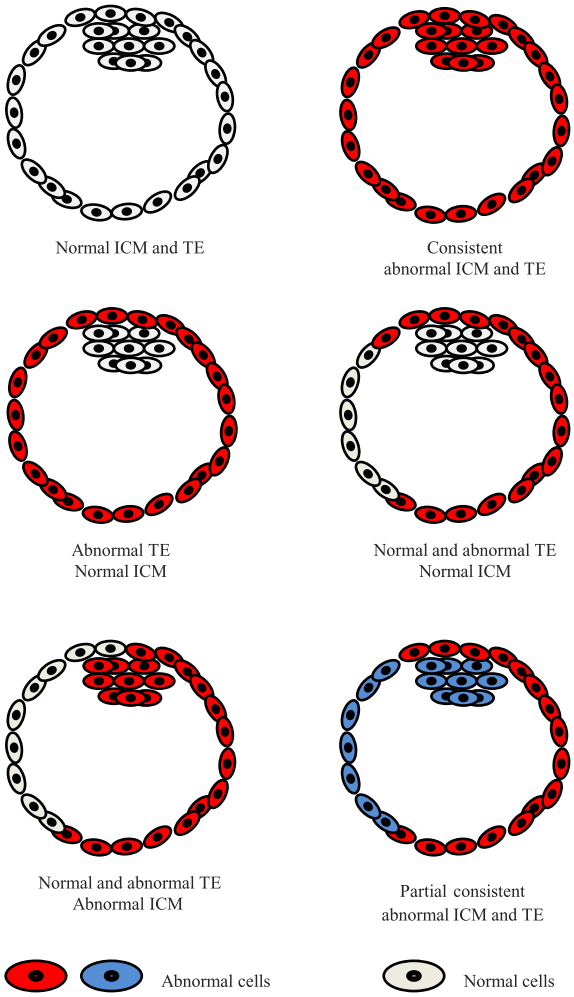

FIG. 5. Diagrams of types of mosaicism in human blastocysts. Type I (top left): Euploid ICM and TE, a normal blastocyst. Type II (top right): Consistent aneuploid TE and ICM, an abnormal blastocyst. Type III: Euploid ICM cells and aneuploid TE (middle left) or inconsistent (euploid and aneuploid) TE (middle right). Both are mosaic embryos with euploid ICM. Type IV: Inconsistent (euploid and aneuploid) TE (bottom left) or mosaic TE (bottom right) with aneuploid ICM. Both are mosaic embryos.

these types of mosaicism in human blastocysts are shown in Figure 5.

NimbleGen Platform Is Another Microarray Platform for Human PGD

In the present study, we used 13 embryos and 26 samples (TE and ICM cells), and we performed 52 microarray tests using BlueGnome and NimbleGen platforms. As shown in Table 2 and Figure 6, the results between BlueGnome and NimbleGen platforms matched exactly in the same samples, indicating that the NimbleGen microarray platform can provide the correct chromosome information for PGD in human embryos.

DISCUSSION

Since microarray technology has been used for human embryo diagnosis, numerous studies have been performed to examine the accuracy of this technology. For example, it has been reported that polar body array CGH can predict the status of the corresponding eggs [25–27]. Johnson et al. [28] also reported a high rate of concordance between TE cells and ICM cells in selected blastocysts. In the present study, we found that chromosomes in 9 of 13 aneuploid embryos were not consistent between TE and ICM, and the mosaic rate was as high as 69.23%, which was higher than the rate (20%) observed by Johnson et al. [28]. The higher mosaic rate observed in the present study may be due to the fact that all embryos were already known to be aneuploid.

Mosaicism was mainly noticed in cleavage embryos [3, 8, 15], and it was found that about half of cleavage embryos were mosaic by DNA microarray [15]. According to a previous study using FISH [2], 20% of aneuploid (possibly mosaic) Day 3 embryos could develop to blastocysts, and of these blastocysts, 40% were euploid. It is still unknown how these aneuploid embryos became euploid blastocysts. It would appear that mosaicism must be present in these blastocysts, but due to the limited FISH technology, detailed mosaicism in those blastocysts was not detected. It is impossible for these aneuploid embryos to do a self-correction within this short developmental period from Day 3 to Day 5 [2]. Our results may suggest that mosaic embryos observed at Day 3 would still be mosaic embryos if they were able to develop to blastocysts at Day 5 or Day 6. However, most mosaic embryos cannot be detected by single microarray with a sample containing one or a few cells.

In the present study, we used DNA microarray to examine all chromosomes from biopsied cells in both TE and ICM and found some interesting mosaic blastocysts. First, one embryo had euploid TE in one sample and aneuploidy TE in another sample, but it had aneuploid ICM cells, which indicates that there were mosaic cells in the TE. Second, two blastocysts had both euploid and aneuploid TE cells but had euploid ICM cells, and another two blastocysts had completely aneuploid TE cells but had euploid ICM cells. Although these embryos were aneuploid/mosaic, transfer of these embryos might produce a normal pregnancy and healthy birth because they had normal/euploid ICM cells. These kinds of mosaicism were first observed in the human blastocysts in the present study, but similar mosaicisms were observed in prenatal diagnosis [29–33]. For example, it has been found that some fetuses had normal chromosomes while the placenta had both normal and abnormal chromosomes [29–31]. It was estimated that there were approximately 2% of viable pregnancies with this kind of mosaicism [32]. Furthermore, it was reported that there was about a 10% of risk of fetal mosaicism when placental mosaicism was diagnosed in natural conception [33]. In the present study, we found that aneuploid blastocysts had either aneuploid ICM or euploid ICM, but it is unknown whether implantation of those embryos would cause fetal mosaicism. From the present results, it suggests that mosaic embryos at early stages (before differentiation) may have the potential to differentiate euploid blastomeres into ICMs and leave aneuploid blastomeres in TE.

In the present study, we did embryo biopsy twice, and the second biopsy was performed in the abnormal blastocysts in which the first biopsy and microarray had been performed. Although the data were obtained from a limited number of embryos ($n = 13$) and patients ($n = 4$), the present study was the first to use DNA microarray to report these phenomena. Further studies remain necessary to use more embryos in different patient populations and/or embryos from donor eggs. In order to avoid diagnostic errors by microarray, for the test of the samples from the second embryo biopsy, we used two different microarray platforms to examine the same samples. We found that the same results were obtained by two separate microarray platforms.

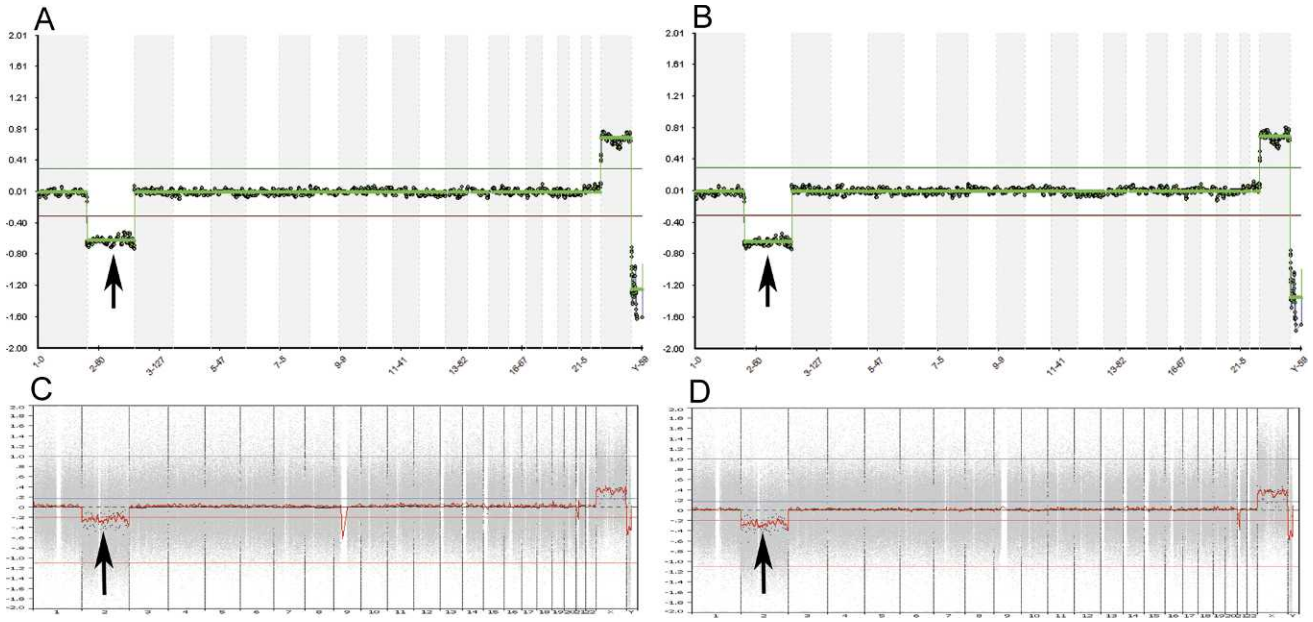

FIG. 6. The PGS charts of TE and ICM cells from a blastocyst. Charts show microarray information of ICM (**A** and **C**) and TE (**B** and **D**) cells from a blastocyst detected by BlueGnome (**A** and **B**) and NimbleGen (**C** and **D**) microarray platforms. The PGS charts show the same chromosomal information in all samples, which are 45, XX; −2.

Microarray of TE cells from blastocyst can predict ICM information if the embryos are either euploid or aneuploid, but they should not be mosaic. Trophectoderm biopsy and array cannot detect the following two types of mosaic embryos: 1) embryos with euploid TE cells and aneuploid ICM cells—transfer of this kind of embryo would result in abnormal pregnancy and/or birth defect; and 2) embryos with aneuploid TE cells and euploid ICM cells—this kind of diagnosis would result in disposal of a possibly normal embryo. Both phenomena are present in microarray of TE cells, and the rates may be different among patient ages; thus, further studies with more samples are necessary. A second embryo biopsy and microarray may be necessary if the initial microarray shows abnormal chromosomes in the embryos and the patients do not have a transferable embryo in the cycle. However, if the patients have normal embryos for transfer, these aneuploid embryos may be kept in storage, and the final disposition can be made by the patients.

It has been expected that PGD by FISH will vanish from human IVF services because only partial chromosomes were examined by FISH in most cases [2–4]. As found in the present study, chromosomal anomalies can occur in any chromosome, and abnormalities in the most frequently examined five chromosomes accounted for only 12.7% of blastocysts tested. This indicates that high proportions of chromosome abnormalities cannot be detected by a five-probe FISH due to technical limitations. This may explain why transfer of embryos screened by FISH does not increase embryo implantation [5–7].

Although there are many advantages of blastocyst microarray compared with Day 3 embryo microarrays, patients who have blastocyst microarray usually do not have fresh embryo transfer due to the time-consuming nature of array technology. It is estimated that 20–24 h are necessary to complete microarray procedures [34]. Blastocysts usually are formed on Days 5 and 6 after fertilization; therefore, there is a 24-h period for blastocyst formation in a single complete IVF cycle. It is difficult to manage fresh blastocyst transfer if all blastocysts need to be biopsied and analyzed. This problem can be solved by current blastocyst cryopreservation technology. It has been found that blastocyst cryopreservation by vitrification can result in 95%–100% survival rates, and the transfer of vitrified/warmed human blastocysts can produce clinical pregnancy and implantation rates equal to those for fresh embryos [24, 35]. Thus, the combination of blastocyst microarray, blastocyst vitrification, and FET would not cause additional difficulty for establishing a clinical pregnancy. Schoolcraft et al. [16] reported a high pregnancy rate with this kind of combination. In the present study, we also vitrified all blastocysts after biopsy and FET were scheduled later for all patients. We obtained a 100% survival rate and a 70.2% clinical pregnancy rate in the present study. Implantation rate of the transferred blastocysts was as high as 63.5%, which was similar to that reported previously [16]. Our results indicate that PGD by microarray is especially beneficial to patients with advanced maternal ages because aneuploidy is the major cause of unsuccessful embryo implantation in this group of patients.

Aneuploid rate is extremely high in patients undergoing IVF, especially in cases of patients with previous aneuploid conceptions [36], recurrent miscarriage couples [37], repeated implantation failure [38], and advanced maternal age [3, 4, 6, 39, 40]. Recently it has also been noticed that high rates of maternal chromosomal abnormalities were also repeatedly found in young patients undergoing IVF [8]. Although the exact reasons for this are unknown, it is suggested that chromosome instability [41, 42] and spindle checkpoint defects [43, 44] in human oocytes and embryos are the main reasons [40, 41], which may be related to oxidative stress caused by environmental pollution [45], mental stress [46], obesity [47], and other factors [42–44]. Although current assisted human reproduction cannot prevent the occurrence of aneuploidy, screening of the embryos and transfer of the euploid blastocysts are the practical approaches to increase embryo implantation, as reported in the previous studies [16, 19] and observed in the present study.

The patient population reported in the present study was mainly the women with advanced maternal ages. Recently, a study with younger IVF patients (mean age of 31 yr) also showed the benefits when the embryo biopsy and DNA

microarray were performed, but the authors also found a 44.9% aneuploid rate in their study [19]. It would appear that this rate was much lower than those from women with advanced maternal age (Schoolcraft et al. [16] and the present study). However, because the data were still obtained from infertile couples, it would be very informative if the microarray could be performed in the blastocysts from donated oocytes. In this case, we may get to know whether chromosome instability, as suggested by Vanneste et al. [41], of embryos under in vitro conditions is also a main cause of aneuploidy in humans.

It is expected that more IVF clinics will use blastocyst microarray for PGD in humans; thus, more technical information about this technology will be obtained in the future. Currently, two microarray platforms are mainly used for PGD in human IVF. One is oligo SNP array, and another is BAC array technologies [9, 16, 19]. They differ in the number of the probes and SNP amplification, the number of color channels, labeling procedures, and the time of the procedure [9]. In the present study, we used 630 000 probes in the NimbleGen microarray, and all samples had DNA signals and the data coordinated between NimbleGen and BlueGnome platforms. These results indicate for the first time that the oligo NimbleGen platform can provide accurate chromosome information in samples and that this platform can be reliably used to perform PGD in human IVF.

The key features of oligo NimbleGen microarray are ultrahigh density and long oligo probes that enable the highest resolution and most comprehensive array CGH platform for whole-genome analysis [48]. In a previous study comparing performance of different platforms for DNA array, it was found that NimbleGen had the best performance after consideration of the dosage sensitivity, precision, specificity, sensitivity, and copy number variation border definition [48]. The NimbleGen new platform with 4.2 million probes per array is able to detect copy number variations down to ~5 kb. Thus, it may be better to identify small errors in the chromosomes.

In conclusion, our results indicate that high proportions of human blastocysts from women of advanced maternal age are not only aneuploid but also mosaic. Transfer of microarray-screened human blastocysts can significantly increase clinical pregnancy and embryo implantation, indicating that DNA microarray from the biopsy of TE cells in human blastocysts can predict the chromosome information in ICM in most embryos. Some aneuploid/mosaic blastocysts have euploid ICM cells, which suggest that early human embryos may have the potential to differentiate euploid blastomeres into ICM cells. We also suggest that a second embryo biopsy and microarray may be necessary if patients do not have transferable embryos in the initial test.

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