

Articles

Nuclear transfer for full karyotyping and preimplantation diagnosis for translocations



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Abstract

Preimplantation genetic diagnosis (PGD) for chromosomal disorders is currently performed by interphase fluorescence in-situ hybridization (FISH) analysis. This technique is known to have limitations for detecting some translocations and complete karyotyping, so visualization of chromosomes in single cells is required to improve the PGD accuracy for chromosomal disorders. To achieve this, single blastomeres were fused with enucleated or intact mouse zygotes, followed by fixing the resulting heterokaryons at the metaphase of the first cleavage division, or treating them with okadaic acid to induce premature chromosome condensation. This method allowed a significant improvement in the accuracy of testing both maternally- and paternally-derived translocations. In all, 437 blastomeres were tested, achieving full karyotyping in as many as 383 (88%), making it possible to pre-select only normal embryos or those with balanced chromosomal complements for transfer. Overall, PGD for translocations was applied in 94 clinical cycles, resulting in 66 transfers and 20 (30.3%) clinical pregnancies, with healthy deliveries of 15 children. Fifty-two of these cycles were performed using a nuclear transfer (conversion) technique, which resulted in 38 transfers of balanced or normal embryos, demonstrating that the technique is accurate and reliable for karyotyping single blastomeres for PGD of translocations.

Keywords: blastomere nuclear conversion, chromosomal translocations, FISH, mouse oocytes, nuclear transfer, PGD

Introduction

Cytogenetic testing of single cells obtained from preimplantation embryos is currently performed by interphase fluorescence in-situ hybridization (FISH) analysis, which allows chromosome enumeration on interphase cell nuclei. However, the number of chromosomes studied by FISH is limited to the number of chromosome-specific probes available. Even with the presently available methods for re-hybridization of interphase nuclei for the second and the third time, complete karyotyping will not be realistic in the near future. The currently available technique has also clear limitations for the detection of some translocations, making it highly important to develop methods for visualization of chromosomes in single cells, including polar bodies and individual blastomeres. This would clearly improve the accuracy of preimplantation genetic diagnosis (PGD) for chromosomal disorders (International Working Group on Preimplantation Genetics, 2001).

One of the approaches to PGD for translocations is first polar body (PB1) testing, based on the fact that PB1 never forms an interphase nucleus and consists of metaphase chromosomes. It has been shown that PB1 chromosomes are recognizable when isolated 2–3 h after in-vitro culture, with degeneration beginning 6–7 h after extrusion (Verlinsky and Kuliev, 1993). Therefore, whole chromosome painting or chromosome-segment-specific probes can be applied for testing of maternally-derived chromosomal translocations in PB1 (Munné *et al.*, 1998). Although the method resulted in a significant reduction of spontaneous abortions in the patients carrying translocations, yielding unaffected pregnancies and births of healthy children, it has been shown to be sensitive to malsegregation and/or recombination between chromatids, requiring a further follow up analysis of the second PB (PB2) in order to accurately predict the meiotic outcome following the second meiotic division (Munné *et al.*, 2000; Verlinsky and Kuliev, 2000). However, despite the progress in transforming PB2 into metaphase chromosomes via electrofusion of the PB2 nucleus with a foreign one-cell human embryo, the proportion

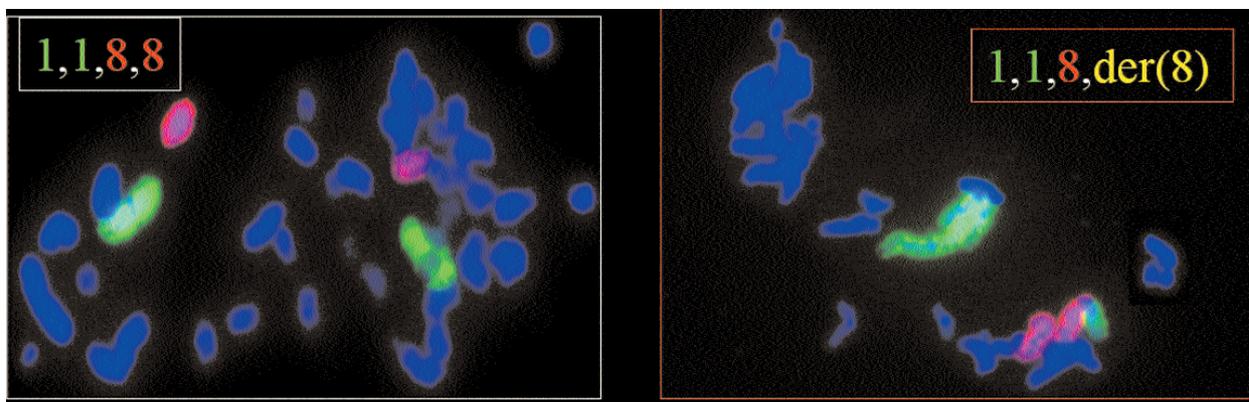


Figure 1. Preimplantation diagnosis (PGD) for paternally-derived translocation $t(1;8)(p13;q23)$ by blastomere nucleus conversion technique, distinguishing normal from balanced and unbalanced embryos (for probes see **Table 1**). Left panel: blastomere metaphase from normal 8-cell embryo, which shows two normal chromosomes 1 and 8. In interphase fluorescence in-situ hybridization (FISH) analysis, this will be indistinguishable from a balanced karyotype, which will show the same number of signals for chromosomes 1 and 8. Right panel: blastomere metaphase from the unbalanced 8-cell embryo, which shows two normal chromosomes 1 (middle), one normal chromosome 8 (lower left), and a derivative chromosome 8 (lower right).

of metaphase plates did not exceed 64%, even after enucleation of the recipient one-cell stage mouse embryo, reducing its usefulness in clinical practice (Verlinsky and Evsikov, 1999a). Therefore, alternative methods were developed to convert single blastomere nuclei into metaphase chromosomes, following the fusion of single blastomeres with murine or bovine zygotes (Verlinsky and Evsikov, 1999b; Willadsen *et al.*, 1999). These appeared to be useful for PGD for both maternally- and paternally-derived translocations.

The present paper describes the application of these methods for PGD of translocations.

Materials and methods

In total, 94 clinical cycles of PGD for translocations were performed, using a standard IVF protocol combined with PB1 and PB2 removal and/or embryo biopsy and FISH analysis (Verlinsky and Kuliev, 2000). Eighteen PGD cycles were performed for Robertsonian translocations, maternally-derived in 12 cycles and paternally-derived in six cases, and 76 for reciprocal translocations, derived maternally in 44 cases and paternally in 32 cases. Of the 94 PGD cycles, 38 were performed using PB1 and PB2 testing, four by interphase blastomere analysis, and the remaining 52 cycles using a blastomere nucleus conversion technique to metaphase chromosomes (**Figure 1**).

The visualization of single blastomere chromosomes was performed as previously reported, involving the fusion of the human single blastomeres with enucleated or intact mouse zygotes at the pronuclear stage, known to be at the S-phase of the cell cycle, and fixing the resulting heterokaryons at the metaphase of the first cleavage division (Verlinsky and Evsikov, 1999b; Verlinsky and Kuliev, 2000). The commercially available frozen mouse zygotes (Charles River Laboratories, Wilmington, MA, USA) were used as recipient cytoplasts to induce the conversion of blastomere nuclei to metaphase. One to two hours before electrofusion with the human blastomeres, mouse zygotes were thawed, freed of zona pellucida using acid Tyrode's solution, and pipetted to

separate PB2. An intact single blastomere was brought into contact with the mouse zygote by agglutination with phytohaemagglutinin (Irvine Scientific, Santa Ana, CA, USA), followed by induction of cell fusion using a single direct current pulse. Four hours after fusion, heterokaryons were monitored for signs of the disappearance of pronuclei, and fixed at mitosis following hypotonic treatment. The heterokaryons entering mitosis, as a result of the fusion of human blastomeres with the intact mouse zygotes, were identified under a dissecting microscope. To avoid monitoring and to maintain the heterokaryons in mitosis, they were cultured in the presence of microtubuli inhibitors. Heterokaryons with persisting pronuclei left in culture by the ninth hour after fusion were fixed following a 1-h treatment with ocaidaic acid. This was followed by 10–15 min incubation in a hypotonic solution (0.1% sodium citrate and 0.6% bovine serum albumin (BSA)), and then the resulting mitotic heterokaryons were fixed in a cold 3:1 solution of methanol and acetic acid. Air-dried chromosome plates were assessed by phase-contrast microscopy.

Prior to FISH, the slides were pretreated with a 1% formaldehyde solution (Vysis, Downers Grove, IL, USA), followed by digestion of residual cytoplasmic proteins with 0.5 mg/ml pepsin. The strategy of FISH analysis depended on chromosomes involved in the structural rearrangement and the size of the translocated segment, and usually involved a combination of the locus specific, centromeric and/or subtelomeric probes with the whole chromosome painting, as well as required re-hybridization. The list of translocations and the probes applied is presented in **Table 1**.

Results

Fifty-two PGD cycles for translocations were performed utilizing the nuclear conversion method. Overall, the technique was applied to 437 blastomeres, which included 333 for reciprocal and 104 for Robertsonian translocations, resulting in successful nuclear metaphase conversion in as many as 383 (88%). This made possible the pre-selection of normal embryos or those with a balanced chromosomal

Table 1. List of translocations and fluorescence in-situ hybridization (FISH) probes used in preimplantation genetic diagnosis (PGD).

<i>Patients</i>	<i>Cycles</i>	<i>Karyotype</i>	<i>FISH probes</i>
7	9	45,XX,rob(13;14)(q10;q10)	WCP 13, WCP 14, LSI 13, Tel 14 q
1	1	45,XX,rob(13;22)(q10;q10)	WCP 13, WCP 22, PB panel (chromosomes 13, 16, 18, 21, 22)
1	1	45,XX,rob(14;21)(q10;q10)	WCP 14, WCP 21, LSI 21
1	1	45,XX,rob(14;22)(q10;q10)	WCP 14, WCP 22, Tel 14 q, LSI 22
5	5	45,XY,rob(13;14)(q10;q10)	WCP 13, WCP 14, LSI 13, Tel 14 q
2	2	45,XY,rob(14;21)(q10;q10)	WCP 14, WCP 21, LSI 21
1	2	46,XX,(9;13)(q22;q14)	WCP 9, WCP 13, CEP 9, Tel 9 q, LSI 13
1	1	46,XX,t(1;15)(q32;q26)	WCP 1, WCP 15, Tel 15 q, Tel 1 q, CEP 15, CEP 1
1	1	46,XX,t(1;4)(q23;q31.1)	WCP 1, WCP 4, CEP 18
1	1	46,XX,t(1;7)(q23;p11)	WCP 1, WCP 7, CEP 1
1	3	46,XX,t(1;8)(q42;p11.2)	WCP 1, WCP 8, CEP 1, CEP 8, Tel 1 q, Tel 8 p
1	1	46,XX,t(10;15)(q23;q21)	WCP 10, WCP 15, CEP 15
1	1	46,XX,t(10;11)(p15;q23)	WCP 10, WCP 11, CEP 10, Tel 10 p, Tel 11 q
1	1	46,XX,t(11;14)(p15;q24)	WCP 11, WCP 14, CEP 11, Tel 11 p
1	3	46,XX,t(11;22)(q23;q11.2)	WCP 11, WCP 22, CEP 11, Tel 11 q, LSI 22
1	4	46,XX,t(12;18)(p13.31;q21.32)	WCP 12, WCP 18, CEP 18, Tel 12 p, Tel 18 q
1	2	46,XX,t(2;10)(q31;q11.2)	WCP 2, WCP 10, CEP 2, Tel 2 q
1	1	46,XX,t(2;13)(q22;q33)	WCP 2, WCP 13, CEP 2, Tel 13 q, LSI 13
1	1	46,XX,t(2;20)(q37;q11.2)	WCP 2, WCP 20, CEP 2, Tel 20 q
1	1	46,XX,t(3;12)(q21;q24.33)	WCP 3, WCP 12, CEP 3, CEP 12, Tel 3 q
1	1	46,XX,t(3;19)(p25;p13.3)	WCP 3, WCP 19
1	1	46,XX,t(3;7)(p21;q22)	WCP 3, WCP 7, Tel 7 q, Tel 3 p
1	1	46,XX,t(3;8)(q27;q22)	WCP 3, WCP 8, Tel 8 q, CEP 8, CEP 3
1	1	46,XX,t(4;14)(q21.3;q24.3)	WCP 4, WCP 14, Tel 14 q, CEP 4
2	3	46,XX,t(5;21)(q31;q22.1)	WCP 5, WCP 21, LSI 5, LSI 21
1	2	46,XX,t(5;10)(q11;q22.1)	WCP 5, WCP 10 CEP 10
1	1	46,XX,t(5;14)(q35;q32.2)	WCP 5, WCP 14, Tel 5 q
1	1	46,XX,t(5;7)(p14;p21)	WCP 5, WCP 7, Tel 7p
1	1	46,XX,t(7;11)(p11.2;q24.2)	WCP 7, WCP 11, CEP 11, Tel 11 q
1	1	46,XX,t(7;18)(q32;q23)	WCP 7, WCP 18 CEP 18
1	3	46,XX,t(8;10)(q24.3;q24.1)	WCP 8, WCP 10, CEP 10, Tel 10 q, Tel 8 p, Tel 8 q
1	1	46,XX,t(8;12)(q21.2;p13.3)	WCP 8, WCP 12, Tel 12 p CEP 12
1	2	46,XX,t(8;22)(q24.1;q11.2)	WCP 8, WCP 22, CEP 8
1	1	46,XX,t(9;11)(q13;q13.5)	WCP 9, WCP 11, CEP 11, Tel 11 q
1	2	46,XY,t(1;21)(p13;q11.2)	WCP 1, WCP 21, CEP 1
1	1	46,XY,t(1;3)(p36.1;q27)	WCP 1, WCP 3, CEP 1, Tel 1 p, Tel 3 q
1	7	46,XY,t(1;8)(p13;q23)	WCP 1, WCP 8, CEP 8, CEP 1, Tel 1 q
1	1	46,XY,t(10;13)(p12;p11.2)	WCP 10, WCP 13, CEP 10
1	1	46,XY,t(11;20)(p15;q13.12)	WCP 11, WCP 20, Tel 11 p
1	1	46,XY,t(11;22)(q23.3;q11.2)	CEP 11, Tel 11 q, LSI 22
1	2	46,XY,t(11;22)(q25;q12)	WCP 11, WCP 22, CEP 11, Tel 11 q
2	3	46,XY,t(13;20)(q22;q11.2)	WCP 13, WCP 20
1	1	46,XY,t(15;16)(q13;q13)	WCP 15, WCP 16, CEP 15
1	2	46,XY,t(2;18)(p13;q11)	WCP 2, WCP 18, CEP 18
1	1	46,XY,t(2;3)(q21;p13)	WCP 2, WCP 3, CEP 2, Tel 2 q, Tel 3 p
1	2	46,XY,t(3;10)(q21;p14)	WCP 3, WCP 10, CEP 10
1	1	46,XY,t(3;7)(p24;p21)	WCP 3, WCP 7, Tel 3 p
1	1	46,XY,t(6;7)(q23;q36)	WCP 6, WCP 7, CEP 7
1	2	46,XY,t(6;8)(q21;q32.1)	WCP 6, WCP 8
1	1	46,XY,t(7;10)(q36;p11.2)	WCP 7, WCP 10, CEP 10, Tel 7 q, Tel 10 p
1	2	46,XY,t(8;19)(p23.1;q13.3)	WCP 8, WCP 19, CEP 8, Tel 8 p, Tel 19 q
1	1	47,XX,+fis(19)(p10),+fis(19)(q10)	Tel 19 p, Tel 19 q

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WCP = whole chromosome paint; LSI = locus specific identifier; Tel = subtelomeric; CEP = centromeric enumeration probe.

^aTotal.

complement for transfer in 38 (73%) cycles. Of those, 54 (12%) blastomeres had no results; these included 48 (14.4%) involving reciprocal and 12 (11.5%) involving Robertsonian translocations. The conversion procedure had failed in 41 (9%), with the remaining 13 (3%) containing no nucleus in the biopsied blastomeres. Eleven (29%) pregnancies were obtained from 38 transfers, resulting in seven healthy deliveries, two spontaneous abortions and two pregnancies ongoing at the time of submission of this paper (Table 2). No pregnancy was obtained in three transfers resulting from four PGD cycles performed by interphase FISH analysis of blastomeres.

In 38 PGD cycles from couples with maternally-derived translocations, testing was performed using PB1 and PB2 FISH analysis. Of 446 oocytes from these cycles tested, FISH results were available in 351 (79%), allowing pre-selection of normal or balanced embryos for transfer in 25 (71.4%) cycles, resulting in nine clinical pregnancies, six of which resulted in the delivery of eight unaffected children and three of which were spontaneously aborted (Table 2). The confirmatory testing was possible in two of three spontaneously aborted embryos, showing the presence of de-novo translocations different from the expected meiotic outcomes (Scriven *et al.*, 1998).

The proportion of abnormal oocytes and embryos detected varied, depending on the type of translocations and their origin. The results of testing for maternally- or paternally-derived translocations in reciprocal or Robertsonian translocations are summarized in Tables 3 and 4. As seen in Table 3, testing of 382 embryos from 44 PGD cycles for maternally-derived reciprocal translocations resulted in the prediction of 292 (76.4%) unbalanced embryos, leaving only 90 (23.6%) suitable for transfer, of which 43 (11.3%) were determined to be balanced and 47 (12.3%) normal. By

Table 2. Preimplantation genetic diagnosis (PGD) for translocations by conversion of blastomere nuclei into metaphase or polar body analysis.

Method of analysis	Blastomere conversion	PB1 & PB2	Total
Cycles	52	38	90 ^a
Blastomeres/oocytes studied with results	437/383 (88)	446/351 (79)	883/734 (83)
Embryos transferred (mean)	68 (1.8)	42 (1.7)	110 (1.8)
Pregnancies/transfers	11/38 (29)	9/25 (36)	20/63 (32)
Deliveries	7 ^b	6	13 (15 ^c)
Spontaneous abortions	2	3	5

Values in parentheses are percentages unless stated otherwise.

^aIn addition, 4 PGD cycles were performed, using interphase FISH analysis of blastomeres, resulting in the transfer of eight embryos in three cycles but yielding no clinical pregnancy.

^bTwo pregnancies ongoing.

^c15 children altogether,

contrast, the testing of 214 embryos from 32 PGD cycles for paternally-derived reciprocal translocations resulted in the prediction of 146 (68.2%) unbalanced embryos, leaving 68 (31.8%) embryos suitable for transfer, of which 28 (13.1%) were determined to be balanced and 40 (18.7%) normal.

As seen in Table 4, testing of 101 embryos obtained from 12 PGD cycles for maternally-derived Robertsonian translocations resulted in the prediction of 72 (71.3%) unbalanced embryos, with the remaining 29 (28.7%) embryos suitable for transfer, which included 16 (15.8%) balanced and 13 (12.9%) normal. Similarly, the testing of 50 embryos obtained from six PGD cycles for paternally-derived Robertsonian translocations allowed identification of 28 (56%) unbalanced embryos, with the remaining 22 (44%) suitable for transfer, including 11 (22%) balanced and 11 (22%) normal ones.

The overall results for the total of 94 PGD cycles for translocation show that normal/balanced embryos were available for transfer in 66 (70%) of the clinical cycles. Twenty (30.3%) clinical pregnancies resulted from 66 transfer cycles, in which the mean number transferred was 1.8 embryos. Thirteen of these pregnancies resulted in the delivery of 15 healthy children, while two pregnancies are still ongoing and five (25%) resulted in spontaneous abortions. The data on the pregnancy outcomes prior to undertaking PGD were available in 64 couples with translocations, showing that 72 (81%) of 82 pregnancies in these patients (prior to undertaking PGD procedure) resulted in spontaneous abortions. This demonstrates the tremendous positive impact PGD has on the

Table 3. Results of preimplantation genetic diagnosis (PGD) for reciprocal translocations.

	Maternally-derived (44 cycles)	Paternally-derived (32 cycles)
Total ^a	382	214
Unbalanced	292 (76.4)	146 (68.2)
Balanced	43 (11.3)	28 (13.1)
Normal	47 (12.3)	40 (18.7)
Suitable for transfer	90 (23.6)	68 (31.8)

Values in parentheses are percentages.

^aBlastomeres/oocytes.

Table 4. Results of preimplantation genetic diagnosis (PGD) for Robertsonian translocations.

	Maternally-derived (12 cycles)	Paternally-derived (6 cycles)
Total ^a	101	50
Unbalanced	72 (71.3)	28 (56)
Balanced	16 (15.8)	11 (22)
Normal	13 (12.9)	11 (22)
Suitable for transfer	29 (28.7)	22 (44)

Values in parentheses are percentages.

^aBlastomeres/oocytes.

clinical outcome of pregnancies in couples carrying both reciprocal and Robertsonian translocations.

As already mentioned, the detection rates of embryos suitable for transfer depended on the type of the translocations tested. For example, the clinical outcome was poorer in reciprocal than in Robertsonian translocations, with the pregnancy rates of 23% and 33%, respectively, based on the analysis of data from 55 PGD cycles for maternally-derived translocations. As may be predicted, there was also a correspondence between the poorer clinical outcome and the proportion of unbalanced embryos in these PGD cycles. In 53 PGD cycles with the proportion of unbalanced complements under 75%, the pregnancy rate was 28%, compared with 12% in 41 PGD cycles with an unbalanced rate of over 75%.

Discussion

Although the application of the conversion technique to visualize chromosomes in single blastomeres improves the accuracy of the diagnosis by analysis of metaphase chromosomes using a combination of commercially available probes, a high frequency of mosaicism in the cleavage stage embryos, arising from anaphase lag or nuclear fragmentation, still presents problems for diagnosis. Follow-up analysis of 78 unbalanced embryos, including those in which chromatid mal-segregation or recombination was identified by the analysis of PB1, and subsequent testing of PB2 inferred a balanced or normal embryo, revealed a mosaicism rate of 41%. Different cell lines were present, including normal or balanced, which if investigated only by embryo biopsy, may have led to misdiagnosis. Therefore, the PGD strategy for maternally-derived translocations is still based on PB1 and PB2 testing, applying the blastomere nucleus conversion technique only if further testing is required.

It has also been observed that the embryos with unbalanced chromosome complements have the potential to reach the blastocyst stage of embryo development in extended culture. Of 538 (76%) unbalanced embryos identified from 707 embryos with FISH results, 250 were cultured for a further period, of which many (78, 31%) reached the blastocyst stage. This confirms the present authors' previous results, that some of the detected chromosomal rearrangement may not be lethal in preimplantation development, being eliminated either during implantation or post-implantation development (Evsikov *et al.*, 2000), explaining an extremely high spontaneous abortion rate in couples carrying translocations. The present data are also in agreement with previous reports suggesting as much as a six-fold reduction of spontaneous abortions in PGD cycles for translocations (Munné *et al.* 2000; Munné, 2002).

In addition to avoiding the use of the expensive and time-consuming customized breakpoint-spanning probes (Cassel *et al.*, 1997), the conversion technique used in the present study was highly accurate, based on the follow-up testing of embryos predicted to be abnormal and confirmatory prenatal diagnosis of the ongoing pregnancies or testing of the newborn babies. The method also allows balanced and normal embryos to be distinguished, something that cannot be achieved by currently available interphase FISH analysis.

Because PGD is practically the only hope for couples with translocations to have an unaffected child without fear of repeated spontaneous abortions, increasing numbers of PGD cycles for this indication have been performed. Around 400 clinical cycles has been performed at the present time, resulting in approximately more than 100 clinical pregnancies and births of unaffected children (International Working Group on Preimplantation Genetics, 2001; Proceedings of the Fourth International Symposium on Preimplantation Genetics, 2002).

Considerable progress is reported in the development of the single-cell comparative genome hybridization (CGH) technique, to enable testing for translocations based on the polymerase chain reaction (PCR) (Proceedings of the Fourth International Symposium on Preimplantation Genetics, 2002). However, the most important limitation of the present CGH protocol is still the 3-day duration of the procedure, which is incompatible with the current laboratory framework for PGD. The other possible approach may be a multiplex fluorescence PCR using polymorphic microsatellite markers, for the detection of numerical changes in chromosomes using their allelic fingerprints. DNA fingerprinting systems for Robertsonian translocations 13q14q and 14q21q were developed and applied in a PGD case, making it possible to distinguish embryos with balanced and unbalanced chromosome constitutions (International Working Group on Preimplantation Genetics, 2001).

The presented data suggest that patients with translocations who are at an extremely high risk for spontaneous abortions should be informed about the availability of PGD for this indication. Awareness of the availability of PGD will permit such couples to establish pregnancies unaffected from the onset, and to have the opportunity to have children of their own, instead of multiple unsuccessful attempts at prenatal diagnosis and subsequent termination of pregnancies. The application of the method for conversion of interphase nuclei of PB2 and blastomeres will make it possible to perform PGD for a wide variety of structural rearrangements, independent of the availability of specific fluorescent probes, which amplify the limitations of interphase FISH analysis.

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