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A simplified and efficient method for obtaining metaphase chromosomes from individual human blastomeres

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Objective: To develop a reliable and cost-effective technique for karyotyping single human blastomeres for preimplantation diagnosis of chromosomal translocations.

Design: Controlled laboratory study.

Setting: Preimplantation genetic diagnosis and IVF program, Reproductive Genetics Institute/IVF Illinois, Chicago, Illinois.

Patient(s): Patients undergoing IVF and preimplantation genetic diagnosis.

Intervention(s): Individual human blastomeres were fused with enucleated or intact mouse zygotes. After blastomere–cytoplast fusion, heterokaryons were fixed at metaphase of the first cleavage division or treated with okadaic acid to induce premature chromosome condensation.

Main Outcome Measure(s): Percentage of analyzable metaphase plates and ease and reliability of the procedure.

Result(s): The effectiveness of the proposed technique with blastomeres from day 3 diploid embryos was 91%. Sixty-three metaphases were obtained from 69 blastomeres; 3 blastomeres had not fused, 1 heterokaryon had no chromatin (an anucleated cytoplasmic bleb was biopsied and fused), and 2 heterokaryons cleaved before they were fixed.

Conclusion(s): Human blastomere fusion with an intact mouse zygote is an efficient and technically undemanding method for obtaining metaphase chromosome plates from individual human blastomeres for preimplantation testing for chromosomal translocations and aneuploidy. (Fertil Steril® 1999;72:1127–33. ©1999 by American Society for Reproductive Medicine.)

Key Words: Chromosomal translocations, preimplantation genetic diagnosis, karyotyping, visualization of chromosomes, whole chromosome painting

Carriers of reciprocal or robertsonian translocations have a high risk of conceiving an infant with an unbalanced chromosomal complement. Unbalanced translocations may have a deleterious effect even on an embryo's ability to reach the blastocyst stage (1). The use of fluorescence in situ hybridization on interphase nuclei of individual blastomeres already has been shown to be useful for preimplantation genetic diagnosis (PGD) of unbalanced translocations (2–4). However, the major limitation of the technique is the limited availability of locus-specific DNA probes, which are needed for each particular case of translocation.

The development of a reliable method for obtaining a metaphase chromosome plate from a biopsied blastomere would allow the use of standard probes for whole chromosome painting. Such a technique also would allow differentiation between embryos with balanced and normal chromosomal complements. Although analysis of the first and second polar bodies can reveal chromosomally unbalanced oocytes, this is only applicable to maternally derived translocations (5–7). Blastomere analysis would allow the detection of paternally derived translocations and could be used in conjunction with analysis of the polar bodies.

In our previous report (8), we assessed different techniques for obtaining metaphase chromosome plates from individual human blastomeres. We showed that the most reliable way of transforming blastomere nuclei into metaphase chromosomes is through blastomere fusion with an enucleated human zygote. This technique relies on the ability of zygote cyto-

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plasm to reprogram the introduced nucleus according to the zygote's cell cycle, making it possible to predict the timing of mitosis of such a nuclear-cytoplasmic hybrid. Although it recently has proved effective in clinical cases (unpublished data), the availability of fresh or frozen-thawed, abnormally fertilized or donated human zygotes limits the use of this technique. In this study, we showed that commercially available frozen mouse zygotes can be used instead as a readily available source of cytoplasts.

MATERIALS AND METHODS

Human embryos at the 4-cell to 16-cell stage were used as blastomere donors. These embryos were 3–5 days old and were derived from abnormally fertilized (zero, one, or three pronuclei) oocytes that had been donated or found to be abnormal by polar body analysis of diploid zygotes. The use of human preimplantation embryos for this project was approved by the institutional review board of the Illinois Masonic Medical Center in Chicago, IL. Frozen mouse zygotes were purchased from Charles River Laboratories (Wilmington, MA).

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Embryo culture and micromanipulations were performed in human tubal fluid medium with 10% Protenate (Baxter Healthcare Corp., Glendale, CA).

A double-instrument micromanipulation technique initially proposed by Tsunoda et al. (9) was used for mouse zygote enucleation and human blastomere biopsy. Before enucleation, the zygotes were incubated for 10–15 minutes at 37°C in medium with 1 μg/mL of cytochalasin D and 0.1 μg/mL of Demecolcine; enucleation was performed in the same medium. Unlike blastomere biopsy for fluorescence in situ hybridization, only intact blastomeres can be used for cell fusion. Several precautions were taken to ensure the integrity of the blastomere plasma membrane during the biopsy procedure. Before blastomere biopsy, some embryos were preincubated for 10–20 minutes in Ca^{2+}, Mg^{2+}-free medium (e.g., Hank's balanced salt solution with 10% Protenate). Biopsy was performed in medium containing 0.05 M of sucrose. A "t"-shaped partial zona dissection slit (10) made it possible to use a biopsy tool with an internal diameter of approximately 40 μm. To obtain individual blastomeres, some embryos were dissociated in Ca^{2+}, Mg^{2+}-free medium after removal of the zona pellucida with acidic Tyrode's solution.

Initially, blastomeres were inserted microsurgically into the perivitelline space of enucleated mouse zygotes. This step of the procedure proved difficult and, in some cases, even traumatic for human blastomeres because of the small size of mouse embryos compared with human blastomeres and blastomere biopsy pipettes. Subsequently, this step was replaced with blastomere–zygote agglutination by phytohemagglutinin (Irvine Scientific, Santa Ana, CA) (11).

Mouse zygotes or cytoplasts were freed of zonae pellucidae with acidic Tyrode's solution. To separate the second polar bodies, zona-free zygotes were pipetted through flame-polished Pasteur pipettes with an internal diameter of 80 μm. Using flame-polished Pasteur pipettes with an internal diameter of 100 μm, blastomere–zygote pairs were brought together and agglutinated in 300 μg/mL of phytohemagglutinin in protein-free human tubal fluid buffered with 20 mM of HEPES in a four-well plastic dish (#176740, Nunc, Naperville, IL).

Electrofusion was induced with the aid of the electrofusion apparatus (Bams Manufacturers Inc., Chicago, IL) in a custom-made fusion chamber. Electrofusion medium consisted of 0.3 M of mannitol, 0.1 mM of $MgSO_4$, 0.05 mM of $CaCl_2$, and 0.1% polyvinylpyrrolidone molecular weight 360,000 (kd). Blastomere–zygote pairs were oriented between electrodes by hand; the final orientation was achieved with alternating current (500 kHz; 0.2 kV/cm for 2 seconds). Cell fusion was induced with a single direct current pulse (1 kV/cm for 500 μs). The success of the procedure was assessed 20 minutes later.

When human blastomeres were fused with intact mouse zygotes (Fig. 1A), the heterokaryons entering mitosis were identified and separated under a dissecting microscope. Because of the transparency of mouse cytoplasm, the disappearance of pronuclei and the formation of the joint metaphase plate was clearly visible (Fig. 1B).

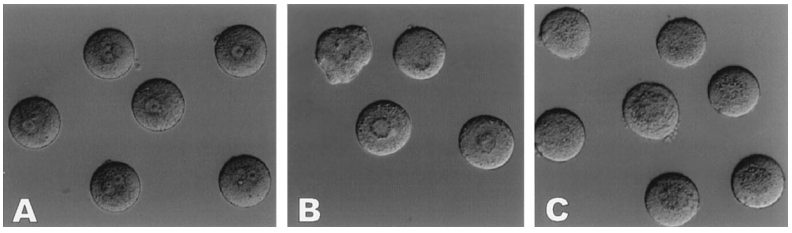
Premature chromosome condensation (PCC) was induced by exposure of the cells for 1 hour to 5 μM of okadaic acid (OA) in phosphate-buffered saline containing 3 mg/mL of bovine serum albumin and 0.5 μg/mL of cytochalasin D (7) (Fig. 1C).

A modification of Dyban's technique of oocyte fixation was used (12). Heterokaryons were incubated for 10–15 minutes in a hypotonic solution (0.1% sodium-citrate and 0.6% bovine serum albumin), then fixed by placing them in a cold 3:1 solution of methanol and acetic acid in a four-well plastic dish. When the cytoplasm cleared, heterokaryons were transferred onto slides and air-dried.

Chromosome plates were assessed first using phase contrast followed by pretreatment with formaldehyde and pepsin, as suggested by Vysis, Inc. (Downers Grove, IL). Vysis probes and standard protocols for hybridization and slide-washing were used for the whole chromosome painting. Slides were imaged with a Nikon Microphot-FXA microscope equipped with a SenSys CCD camera (Photometrics, Tucson, AZ). Quips Smart Capture Software (Vysis, Inc.) was used for chromosome imaging. Five-color fluorescence in situ hybridization is used routinely at our institution to detect maternally derived aneuploidy for chromosomes 13, 16, 18, 21, and 22. When embryos found to be abnormal by

FIGURE 1

Heterokaryons between mouse zygotes (not enucleated) and 1/8 human blastomeres a few hours after fusion (**A**) and in mitosis of the first cleavage division (**B**). The transparency of the mouse cytoplasm and the number of chromosomes make the chromosomes visible even under the dissecting microscope. Telophase is seen in the irregularly shaped cleaving embryo. (**C**). Embryos treated with okadaic acid do not form metaphase spindle, the chromosomes are visible only after fixation.



Verlinsky. Metaphase chromosomes from blastomeres. Fertil Steril 1999.

polar body analysis were used for blastomere biopsy or blastomere segregation, whole chromosome painting probes for the same five chromosomes were used after conversion of the blastomere nuclei into the metaphase chromosomes.

For G-banding, slides were treated for 25 seconds with a solution of pancreatine plus Hank's balanced salt solution (×1) and stained for 1 minute and 15 seconds in a Wright and Giemsa stain.

RESULTS

The overall success rate of the electrofusion technique was 94%; of 191 individual human blastomeres, 180 were fused successfully with intact or enucleated mouse zygotes (Table 1). The triploid and (presumably) haploid embryos (Table 1) that were used in the first stages of this project provided two important insights.

First, the success of the procedure did not depend on whether mouse zygotes were enucleated before fusion with human blastomeres. From 191 blastomeres (Table 1), only 71 were fused with enucleated zygotes, resulting in 62 metaphases. Because the metaphases obtained by this method were used exclusively for human chromosome painting, the presence of mouse chromosomes did not interfere with the final analysis.

Second, triploidy of the human blastomere shifts the nuclear-cytoplasmic ratio of the resulting heterokaryon beyond the point at which mouse cytoplasm can lead it reliably into the first mitosis, even with the help of OA treatment (Table 1). Consequently, diploid human embryos found to be genetically abnormal by polar body analysis were used for further improvement of the technique.

We expected that, similar to blastomere fusion with enucleated human zygotes (8), human–mouse heterokaryons would follow the zygote's time schedule. Of 70 control mouse zygotes that were treated and cultured under the same conditions as heterokaryons, only 1 cleaved by the seventh hour after thawing; by the ninth hour after thawing, 10% of the zygotes had cleaved. Because of the contribution of the

TABLE 1

Number of metaphases obtained after human blastomere fusion with intact or enucleated mouse zygotes.

Number of PN	Blastomere age (d)	Blastomere stage	No. of blastomeres biopsied	No. of blastomeres fused	No. of heterokaryons cleaved	Mitoses before OA treatment	Total no. of metaphases (%)
3	4	1/4–1/16	22 (−4 blebs*)	20 (−4 blebs)	1	0	5 (28)
0 or 1	3 or 4	1/4–1/16	30	29	5	4	21 (70)
2	3	1/8–1/16	69 (−1 bleb)	66 (−1 bleb)	2	18	63 (93)
2	3	1/4	7	7	3	1	3 (43)
2	4	1/4–1/16	44	41	1	19	40 (91)
2	5	1/4–1/16	19	17	1	3	6 (32)
0–3	3–5	1/4–1/16	191 (−5 blebs)	180 (−5 blebs)	16	42	138 (74)

Note: Heterokaryons were treated with OA 6–11 hours after fusion. PN = pronuclei.

* No human chromatin was found after heterokaryon fixation; an anucleated cytoplasmic bleb was fused.

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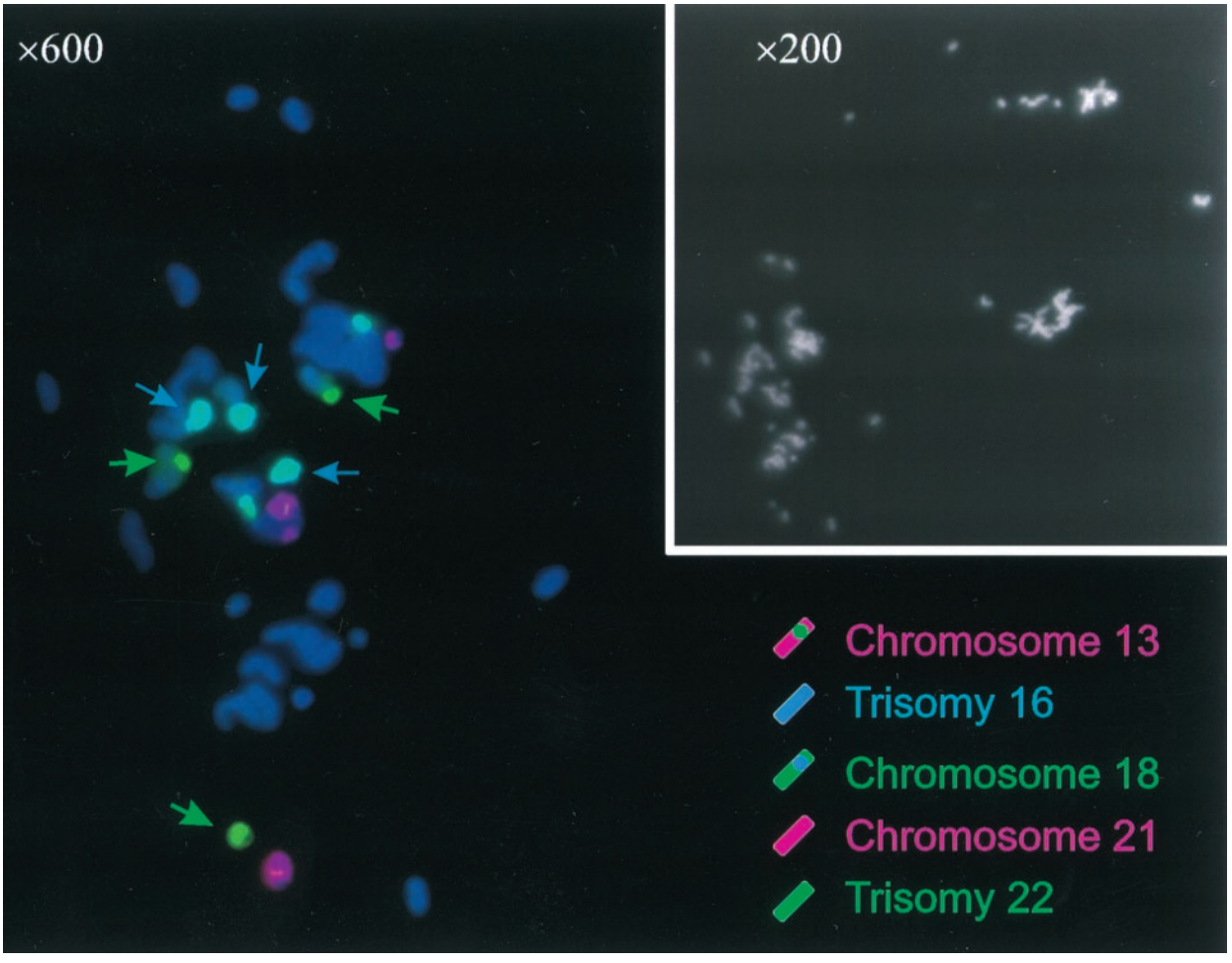
FIGURE 2

Whole chromosome painting of the human metaphase, obtained after blastomere fusion with a mouse zygote and induction of PCC with OA. A schematic of the color probes used for this case (orange, aqua, and green) is provided on the right. A locus-specific probe for chromosome 13 and a centromeric probe for chromosome 18 were added to the whole chromosome painting probes. The *inset* shows the same metaphase under low magnification with 6-diamino-2-phenylindole fluorescence; two metaphases from the mouse pronuclei are shown on the right.



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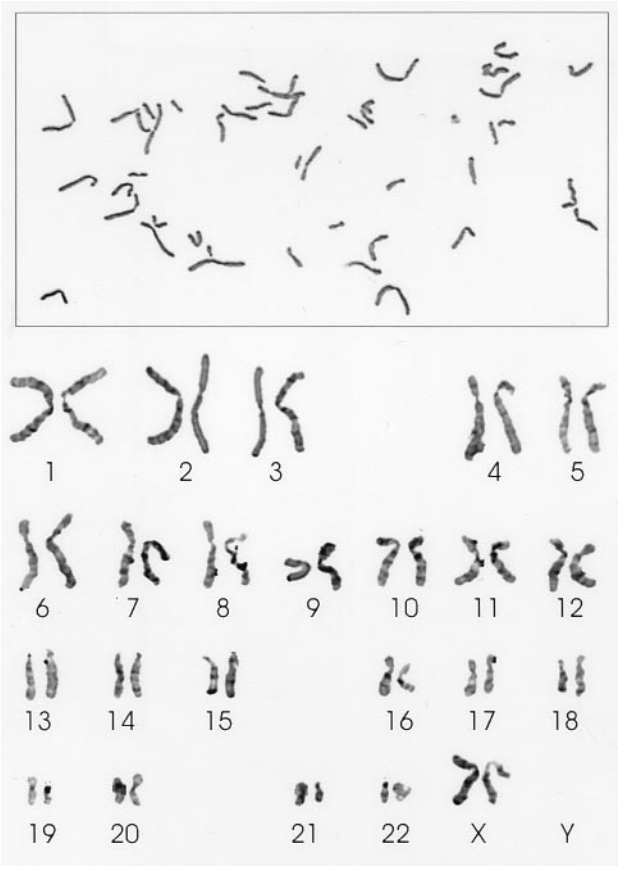
blastomere cytoplasm, a small percentage of human–mouse heterokaryons started to cleave as early as 5–6 hours after the mouse zygotes thawed. A few embryos, fixed during telophase of the first mitosis, showed that human and mouse chromosomes form a joint metaphase. After cleavage, however, besides the two hybrid nuclei, a substantial number of micronuclei were found in the cytoplasm. It should be noted that none of the 24 heterokaryons that were left in culture cleaved beyond the two-cell stage.

Treatment of the two-cell embryos with OA does not lead to PCC and the formation of analyzable metaphase plates. Therefore, for the purpose of PGD, heterokaryons entering mitosis must be fixed immediately. On the other hand, if all heterokaryons are treated with OA as early as 6 hours after the mouse zygotes thaw (and approximately 3–4 hours after fusion), a significant percentage of human blastomeres form S-phase PCC nuclei.

The technique, developed in the process of this study, is as follows. Mouse zygotes should be thawed, enucleated if necessary, and freed of zonae pellucidae and second polar bodies no more than 1–2 hours before electrofusion with human blastomeres. Starting at 4 hours after fusion (or 6 hours after zygote thawing, whichever comes first), heterokaryons should be checked at hourly intervals for signs of the disappearance of pronuclei. Embryos in mitosis (Fig. 1B) should be selected and fixed immediately. By the ninth hour after fusion, all the embryos still left in culture should be treated for 1 hour with OA (Fig. 1C) and fixed immediately

FIGURE 3

G-banding of the human metaphase obtained after blastomere fusion with an enucleated mouse zygote.



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afterward. Depending on the quality of the metaphase plate, the slides can be processed for either G-banding or fluorescence in situ hybridization using whole chromosome painting.

Figure 2 shows a metaphase plate from an embryo that was predicted to be abnormal by polar body analysis. Double trisomy for chromosomes 16 and 22 was confirmed in all six blastomeres. In this particular blastomere, PCC was induced with OA. Treatment with OA induces chromosome condensation in mouse pronuclei and human nuclei independently and no joint spindle is formed (7). Unlike the heterokaryons, which enter mitosis spontaneously, fixation of OA-treated embryos leads, in most cases, to three separate chromosome plates (Fig. 2, *inset*).

For chromosome counting or chromosome banding, the human blastomere should be fused with the enucleated mouse zygote. G-banding of human chromosomes obtained from such heterokaryons after the induction of PCC with OA is shown in Figure 3.

As seen in Table 1, metaphase plates can even be obtained from blastomeres of four-cell embryos. It should be noted, however, that such blastomeres tend to accelerate the process of heterokaryon cleavage (in this group, 3 of 7 cleaved 3 hours after fusion). Alternatively, if it is decided to biopsy only 1/8-blastomeres, the delayed embryos may be left in culture and biopsied on day 4. The success rate of the procedure was the same as with day 3 embryos: 40 metaphases from 44 individual blastomeres were obtained (Table 1).

This technique does not work efficiently on day 5 arrested embryos: of 17 blastomere nuclei, only 7 (6 metaphases plus 1 cleaved) (Table 1) converted to metaphase chromosomes.

DISCUSSION

Our previous results (8) showed that the techniques of PCC are not efficient enough for use in cases of PGD. To date, we have obtained 23 metaphases from 38 human blastomeres fused with enucleated human oocytes. The major limitation of such an approach lies in the inability of a replicating nucleus to form metaphase chromosomes after the induction of PCC. The transfer of such a nucleus into a metaphase II oocyte leads to chromatin pulverization (8, 13, 14), which is of no value for PGD. By introducing a human blastomere nucleus into a bovine oocyte, Willadsen et al. (15) were able to obtain 64 analyzable metaphases from 87 blastomeres analyzed. Our experience suggests that the high efficiency of the PCC technique reported in that study reflects the low percentage of blastomeres that were at the S-phase of the cell cycle.

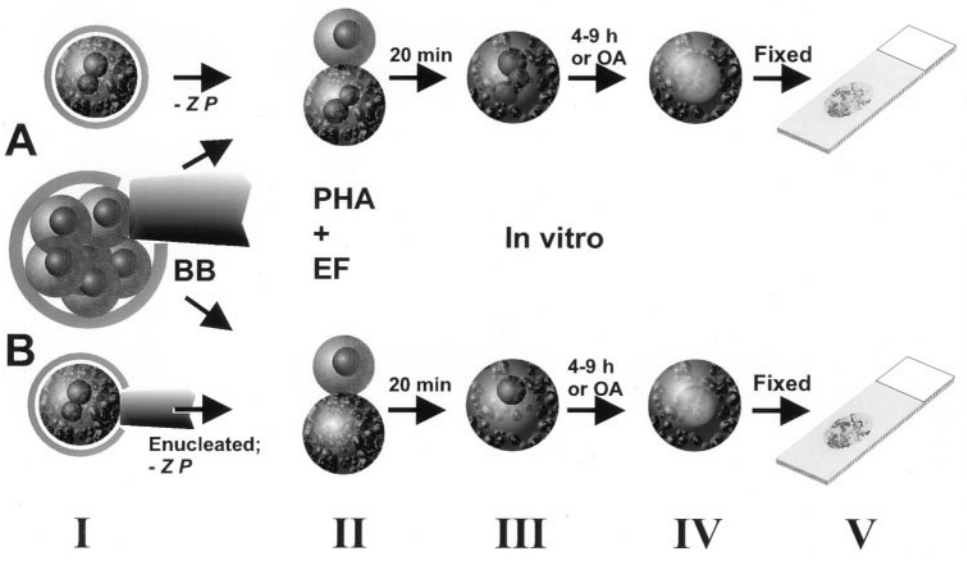
This study addressed the possibility of obtaining metaphase chromosomes from an individual human blastomere nucleus after its fusion with an intact or enucleated mouse zygote. The need for such an approach is based on the fact that the biopsied blastomere can be at any stage of the cell cycle: ready to cleave (G2), just cleaved (G1), or at the stage of DNA replication (S). As seen in Table 1, there were a few cases in which cytoplasmic, anucleated blebs had been fused, indicating that at the moment of biopsy and fusion, it sometimes was difficult to ascertain even the presence of a blastomere nucleus.

Determining the cell-cycle stage of a biopsied blastomere and thus the chance of obtaining a metaphase by fusion of a blastomere with an oocyte or by treatment of a blastomere with OA or demecoleine is practically impossible. On the other hand, animal cloning experiments have shown that it is possible to control, to some extent, the timing of mitosis of the nucleus by artificially introducing it into the cytoplasm of a cell at a known cycle stage. When fused with a pronuclear-stage zygote, which is in the S-phase of its cell cycle, the blastomere nucleus, irrespective of its own cell-cycle stage, proceeds to mitosis according to the zygote's time schedule.

Because of the necessity for pretreatment with cytoskeletal inhibitors, removal of the blastomere karyoplast may be traumatic for the analyzed embryo. Moreover, even high

FIGURE 4

The major steps of the proposed technique. See text for explanations. BB = blastomere biopsy; ZP = zona pellucida removal; PHA = phytohemagglutinin-assisted cell pairing; EF = electrofusion; OA = okadaic acid treatment.



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concentrations of actin and tubulin depolymerizing agents do not always have the necessary "softening" effect on some human blastomeres (unpublished observations). The only option is to fuse the whole blastomere with the zygote. Unlike the "cloning" procedure, the amount of cytoplasm in a human blastomere (e.g., 1/4-blastomere) sometimes equals the volume of the mouse zygote; thus, it more closely resembles somatic cell hybrids than classic "cloning" procedures.

Therefore, it was surprising that all heterokaryons proceeded into mitosis and that most of them followed the zygote's time schedule. Cell fusion was accomplished 5–8 hours before the zygote's cleavage, at the S-phase of the first cell cycle. Apparently, irrespective of its own cell-cycle stage, the blastomere nucleus had enough time to complete DNA replication and enter mitosis along with the mouse pronuclei.

The schematic of the proposed technique is presented in Figure 4. To count chromosomes or attempt chromosome banding, the human blastomere may be fused with an enucleated mouse zygote (Fig. 4). Otherwise, blastomere fusion with an intact zygote is recommended (Fig. 4). This facilitates the detection of heterokaryons spontaneously entering mitosis (by the disappearance of mouse pronuclei) and also simplifies the whole procedure.

From a methodologic viewpoint, the most demanding steps of the procedure are blastomere biopsy (Fig. 4, step I) and embryo fixation (Fig. 4, step IV). On the other hand, the major loss in the course of this study occurred during steps II and III. Although electrofusion is an easy and very efficient cell fusion technique (compared with Sendai virus– or polyethylene glycol–induced fusion), even repeated fusion attempts do not always provide 100% fusion efficiency. The "overlooked" heterokaryons, which cleaved ahead of the (expected) zygote's time schedule, derived from the delayed human blastomeres in the G2 phase of their cell cycle. During clinical cases, however, heterokaryons are monitored starting 2 hours after fusion and the rate of loss has been reduced to zero.

The technique recently has been simplified by culturing the heterokaryons in the presence of the microtubuli inhibitors vinblastine and podophyllotoxin (16). This eliminates the necessity for constant embryo monitoring. After 9 hours of culture, or until at least 50% of the control mouse zygotes cleave, heterokaryons without pronuclei are fixed. The rest are treated with OA before fixation, as described previously.

The use of the proposed technique on 120 blastomeres of day 3 and 4 diploid embryos resulted in 106 metaphases (Table 1). Six blastomeres did not fuse with mouse zygotes. There were no losses during embryo fixation. Eight heterokaryons did not produce human metaphase chromosomes: one had no human chromatin, indicating that an anucleated cytoplasmic bleb was biopsied and fused; one had human chromatin resembling prometaphase or prematurely condensed nuclei in the S-phase of the cell cycle; and six cleaved before they were fixed. Given that the biopsy of anucleated blebs and cleaved heterokaryons represents an

omission on the part of the operator and not a failure of the procedure itself, the success of this method, based on our preliminary results, is nearly 100% (94%) and is limited primarily by the effectiveness of blastomere–zygote fusion.

We conclude that the efficiency and technical ease of the proposed technique (the most demanding step, in our opinion, is the blastomere biopsy) makes it suitable for the detection of chromosomal translocations in cases of PGD.

References

1. Ménézo YJR, Bellec V, Zaroukian A, Benkhalifa M. Embryo selection by IVF, co-culture and transfer at the blastocyst stage in case of translocation. Hum Reprod 1997;12:2802–3.
2. Pierce KE, Fitzgerald LM, Seibel MM, Zilberstein M. Preimplantation genetic diagnosis of chromosomal balance in embryos from a patient with a balanced reciprocal translocation. Mol Hum Reprod 1998;4:167–72.
3. Iwarsson E, Ährlund-Richter L, Inzunza J, Rosenlund B, Fridström M, Hillensjö T, et al. Preimplantation genetic diagnosis of a large pericentric inversion of chromosome 5. Mol Hum Reprod 1998;4:719–23.
4. Munné S, Scott R, Sable D, Cohen J. First pregnancies after preconception diagnosis of translocations of maternal origin. Fertil Steril 1998;69:675–81.
5. Conn CM, Harper JC, Winston RML, Delhanty JDA. Infertile couples with robertsonian translocations: preimplantation genetic analysis of embryos reveals chaotic cleavage divisions. Hum Genet 1998;102:117–23.
6. Munné S, Morrison L, Fung J, Márquez C, Weier U, Bahçe M, et al. Spontaneous abortions are reduced after preconception diagnosis of translocations. J Assist Reprod Genet 1998;15:290–6.
7. Verlinsky Y, Evsikov S. Karyotyping of human oocytes by chromosomal analysis of the second polar bodies. Mol Hum Reprod 1999;5:89–95.
8. Evsikov S, Verlinsky Y. Visualization of chromosomes in single human blastomeres. J Assist Reprod Genet 1999;16:133–7.
9. Tsunoda Y, Yasui T, Nakamura K, Uchida T, Sugie T. Effect of cutting the zona pellucida on the pronuclear transplantation in the mouse. J Exp Zool 1986;240:119–25.
10. Cieslak J, Ivakhnenko V, Wolf G, Sheleg S, Verlinsky Y. Three-dimensional partial zona dissection for preimplantation genetic diagnosis and assisted hatching. Fertil Steril 1999;71:308–13.
11. Czolwska R, Tarkowski A. First meiosis of early dictyate nuclei from primordial oocytes in mature and activated mouse oocytes. Zygote 1996;4:73–80.
12. Dyban AP. An improved method for chromosome preparation from preimplantation mammalian embryos, oocytes or isolated blastomeres. Stain Technol 1983;58:69–72.
13. Campbell KHS, Loi P, Otaegui PJ, Wilmut I. Cell cycle co-ordination in embryo cloning by nuclear transfer. Rev Reprod 1996;1:40–6.
14. Campbell KHS. Maternal effects upon the development of embryos created by nuclear transfer. In: Lauria A, Gandolfi F, Enne G, Gianaroli L, eds. Gametes: development and function. Rome: Serono Symposia, 1998:439–56.
15. Willadsen S, Levron J, Munné S, Schimmel T, Márquez C, Scott R, et al. Rapid visualization of metaphase chromosomes in single human blastomeres after fusion with in-vitro matured bovine eggs. Hum Reprod 1999;14:470–5.
16. Kamiguchi Y, Mikamo K. An improved, efficient method for analyzing human sperm chromosomes using zona-free hamster ova. Am J Hum Genet 1986;38:724–40.