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Karyotyping of human oocytes by chromosomal analysis of the second polar bodies

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This paper describes a method for obtaining metaphase chromosomes from human second polar bodies. The second polar body nucleus was injected into the cytoplasm of an enucleated oocyte, which is activated shortly after injection. When the polar body nucleus is transformed into a haploid pronucleus, treatment with okadaic acid was used to induce premature chromosome condensation. A total of 25 analysable chromosome plates were obtained from 38 polar bodies karyotyped using this technique. Whole chromosome painting was used to detect second polar bodies (and respectively, oocytes) with unbalanced translocations. In combination with the first polar body analysis, this technique may be useful in preimplantation genetic diagnosis for patients carrying maternal translocations.

Key words: metaphase chromosomes/micromanipulations/oocyte activation/polar body/whole chromosome painting

Introduction

Currently, preimplantation genetic diagnosis (PGD) depends primarily on the reliability of the polymerase chain reaction (PCR) and fluorescent in-situ hybridization (FISH) techniques in single cell genetic analysis. However, neither of these methods can reliably detect cells with chromosomal rearrangements. In cases where detection of preimplantation embryos with unbalanced translocations is needed, at least one analysable metaphase should be obtained from each embryo. The recent development of the whole chromosome painting (WCP) has softened the criteria for analysable chromosome plates, thus making the idea of detecting chromosomal translocations using a single metaphase plausible.

In cases when the mother is a carrier of translocation, analysis of the first or second polar body could reveal the chromosomal constitution of the female pronucleus. If the first polar body is removed and fixed shortly after extrusion, before its chromosomes start to disintegrate or clump together, analysable metaphases can be obtained (Durban *et al.*, 1998; Y.Verlinsky, J.Cieslak, V.Ivakhnenko, S.Evsikov, A.Kuliev, unpublished). Unlike the first polar body, at the moment of extrusion the second polar body inherits the cytoplasm possessing chromosome-decondensing activity. As a result, its chromosomes enter interphase, form a nucleus and may even start DNA replication (Howlett and Bolton, 1985). In this article we describe a method of obtaining metaphase chromosomes from the nucleus of human second polar body. Since any one of the polar bodies gives a definite answer as to the chromosomal constitution of the female pronucleus, this method is used as complementary to the analysis of the first polar body.

The importance of karyotyping second polar bodies was first realized by Modlinski and McLaren (1980). Two different techniques of karyotyping mouse second polar bodies have been developed in our laboratory: electrofusion of the polar bodies with enucleated zygotes and polar bodies treatment by okadaic acid (OA) (Dyban *et al.*, 1992; Verlinsky *et al.*, 1994). We showed that as many as 80% of mouse second polar body nuclei can be transformed into analysable chromosomes by these methods. In this paper we propose a technique for obtaining metaphase chromosomes from the nucleus of the human second polar body, which may be used for PGD of chromosomal disorders.

Materials and methods

Micromanipulations

Second polar bodies were removed from normally fertilized zygotes of the patients, undergoing in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The use of human oocytes and preimplantation embryos for this project was approved by Internal Review Board of Illinois Masonic Medical Center, Chicago, USA.

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Chemical Co (St Louis, MO, USA). Embryo culture and micromanipulations were performed in human tubal fluid (HTF) medium with 10% plasmanate (Bayer Biological, New Haven, CT, USA). All micromanipulations were performed under Nikon Diaphot microscope equipped with a warming stage, Hoffman modulation contrast optics and epifluorescence for Hoechst stain. The double-instrument micromanipulation technique, initially proposed by Tsunoda *et al.* (1986), was used for polar body removal (PBR) and for oocyte/zygote enucleation. Both the biopsy pipette and the needle for partial zona dissection (PZD) were set in a Leica two-instrument holder, attached to Narishige MO202 micromanipulator. In order to achieve finer control over micromanipulation procedures, the syringe in the commercially available Narishige IM-16 microinjector system was exchanged for a 100 µl Hamilton syringe, the entire hydraulic system was filled with light paraffin oil (Squibb Pharmaceuticals,

Table I. Different methods of transforming human second polar body (2PB) nucleus into metaphase chromosomes

Technique	Cytoplasm recipients	No. of 2PB	Success of the procedure	Pronucleus formation	Metaphase or PCC
Okadaic acid treatment	NA	18	18	NA	0
Electrofusion + cytoplast	human and mouse	70	1	0	0
Microinjection + electrofusion	human ooplasts	7	5	0	0
Intracytoplasmic injection	human zygote cytoplasts	4	2	0	0
Intracytoplasmic injection	human ooplasts	38	34	18	25
Intracytoplasmic injection	hamster oocytes	2	2	0	0

NA = not applicable; PCC = premature chromatin condensation.

Table II. Activation of human oocytes

Activating agent	No. of oocytes	Degenerated	Activated	Entered mitosis
None (control)	135	0	19	15
Ethanol	15	0	0	0
Ionophore A23187	85	37	39	39
Electrofusion medium	9	0	0	0
Electrostimulation	23	1	8	8
Puromycin	45	4	30	12
Cycloheximide	16	1	5	5
Cycloheximide + electrostimulation	30	2	22	22

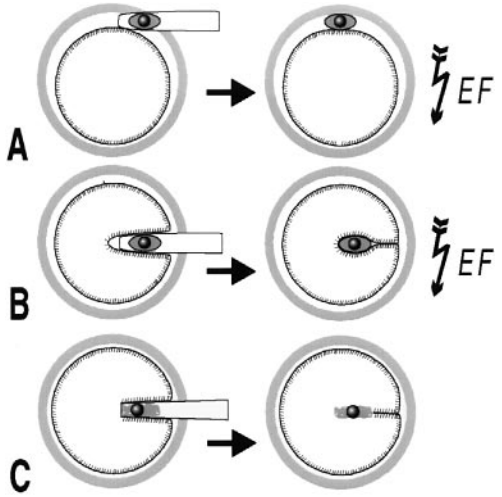

Figure 1. Different techniques for polar body nucleus transplantation into cytoplast. (**A**) Polar body is placed into the perivitelline space of the cytoplast. Electric pulse is used to induce cell fusion. (**B**) In order to achieve more tight contact between plasma membranes and to facilitate cell fusion, the polar body is injected inside the cytoplast. None of the plasma membranes should be broken. Electrofusion is performed immediately after injection, before the polar body is expelled into the perivitelline space. (**C**) Intracytoplasmic polar body injection. The polar body plasma membrane is broken during its aspiration into the pipette; pipette is moved into the perivitelline space through the previously made slit in zona pellucida. Injection procedure mimics intracytoplasmic spem injection (ICSI).

Princeton, NJ, USA), and the microtools themselves were filled with highly viscous silicone oil (125×10^{-4} m^2/s dimethylpolysiloxane). The microscope with attached micromanipulators was standing on a custom-made antivibration plate in a laminar flow hood. The technique for polar body removal was the same as described previously (Verlinsky *et al.*, 1996) with the only modification that the biopsy was performed in a medium without sucrose.

The same setup (and usually the same set of instruments) was used to produce oocyte cytoplasts as follows: metaphase II (MII) oocytes not fertilized after IVF or ICSI, or MII oocytes matured for 24–48 h *in vitro* from immature oocytes [germinal vesicle (GV) or metaphase I (MI)] were enucleated and served as ooplast recipients for the second polar body nuclei. Prior to enucleation, oocytes were incubated for 10–15 min at 37°C in medium with 1 μg/ml cytochalasin D, 0.3 μg/ml nocodazole and 0.5 μg/ml Hoechst 33342. Stock solutions of cytochalasin D and nocodazole were prepared at concentrations 2mg/ml in dimethylsulphoxide (DMSO) and stored at –20°C. 1 mg/ml stock solution of Hoechst 33342, dissolved in high purification liquid chromatography (HPLC)-grade water was stored at +4°C. First polar bodies are always removed from perivitelline space; the enucleation procedure itself was the same as described by Tsunoda *et al*. (1988) for mouse oocyte enucleation. During enucleation of the oocytes, which had failed fertilization, special attention was paid to verify that not only the meiotic MII spindle, but also sperm chromosomes had been completely removed. After enucleation, ooplasts should be washed and transferred into culture medium for at least 1 h to recover before further procedures.

Enucleation of abnormally fertilized zygotes was achieved by first exposing 1- or 3-pronuclear zygotes for 15 min to a medium containing 0.05 M sucrose, 1 μg/ml cytochalasin D and 0.3 μg/ml nocodazole. Polar bodies (if present) and pronuclei were removed in the same medium.

Intracytoplasmic polar body injection

Pipettes for intracytoplasmic polar body injection were prepared in three steps. The first step is identical to biopsy tool construction: a needle with a long attenuated tip is pulled on the pipette puller. Then a microforge is used to break the very tip of the needle, so that a resulting pipette has a tip broken perpendicularly, without any irregularities and with an inner diameter of 7–10 μm. Unlike the biopsy tools, these pipettes are not flame-polished. The same microforge is used to bend the tool at the desired angle. A few hours before micromanipulation these micropipettes are treated with non-ionic detergent (NP10) as described by Hogan *et al*. (1986).

The procedure of polar body injection is essentially the same as ICSI: the second polar body is aspirated into an injection pipette, making sure that the polar body plasma membrane is broken. The injection pipette is brought into perivitelline space of the cytoplast through the PZD slit made during oocyte enucleation, then moved into the centre of the cytoplast. Cytoplasm is aspirated into the pipette until the plasma membrane is broken, then the polar body nucleus is expelled into the cytoplast. In order to increase cytoplasm flexibility during polar body injection into zygote cytoplasts, the procedure was performed in a medium containing 0.5 μg/ml cytochalasin D.

Reconstructed haploid embryos are cultured in regular medium for at least 1 h before further processing.

Oocyte activation and electrofusion

Absolute ethanol was dissolved in plain HTF medium immediately before use; oocytes were exposed to 7% ethanol in plain HTF for 10 min at 25°C (Abramczuk and Lopata, 1990; Winston *et al.*, 1991). Stock solutions of calcium ionophore A23187, puromycin and cycloheximide were dissolved in DMSO at concentrations 10 mg/ml and stored at –20°C. Oocytes were incubated for 12–14 h in HTF medium with 20 μg/ml puromycin according to De Sutter *et al*. (1992). Different methods of calcium ionophore-induced oocyte activation were tried varying the duration of oocyte exposure to ionophore (5–15 min), varying the choice of medium [phospate-buffered saline (PBS); plain HTF; HTF + 3 mg/ml bovine serum albumin (BSA); HTF + 10% plasmanate], preincubation in medium either with or without plasmanate, and varying the temperature of activation medium (22 or 37°C), according to suggestions from different sources (Winston *et al.*, 1991; Balakier and Casper, 1993; Taylor and Braude, 1994; Tesarik and Sousa, 1995).

Human oocyte activation by exposure to electrofusion medium was performed according to Levron *et al*. (1995). Electrostimulation and electrofusion were induced with the aid of the electrofusion apparatus (Bams Manufacturers Inc, Chicago, IL, USA) in a fusion chamber consisting of two platinum wire electrodes glued to the bottom of a glass dish with a gap of 0.33 mm. Electrofusion medium consisted of 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.5% polyvinylpirrolidone, dissolved in HPLC-grade water. The pH of the medium was adjusted to 7.4 by titration with 0.1 N NaOH.

A combination of protein synthesis inhibition with electroactivation, used by Meng *et al*. (1997) for Rhesus monkeys ooplasts activation, was the last technique we assessed. Prior to electrostimulation oocytes were incubated for 1 h in medium containing 7.5 μg/ml cycloheximide. Oocyte activation was induced with three electric pulses, 1 h apart (each consisting of 2 DC pulses, 0.5 s apart, 1.5 kV/cm, 50 μs each); between pulses embryos were kept in a medium with cycloheximide.

Premature chromosome condensation

Premature chromosome condensation (PCC) was induced by exposing polar bodies or one- or three-pronuclear zygotes to OA. OA (5 or 10 μg) was first dissolved in 5 μl of DMSO, diluted to 1 mM with PBS and kept at –20°C in 5 μl aliquots. A side-effect of embryo incubation in the presence of OA was embryo cytoplasm fragmentation. To prevent this, OA was diluted to the working concentration with PBS containing 3 mg/ml BSA and 0.5 μg/ml cytochalasin D. 5 μM of OA was the most effective concentration for PCC induction in pronuclear-stage zygotes. The optimal time for inducing chromosome condensation of one-pronuclear zygotes is 1 h in OA.

Fixation and whole chromosome painting

Immediately before fixation, zonae pellucidae were removed by acid Tyrode's solution; Dyban's technique of oocyte fixation was used (Dyban, 1983). The use of ice-cold fixative was crucial during fixation of OA-treated embryos. Chromosome plates were first photographed under phase contrast and then processed for the whole chromosome painting (WCP). Vysis probes (Vysis Inc, Downers Grove, IL, USA) for the WCP and standard protocol suggested for hybridization and slide washing were used. Slides were imaged with a Nikon Microphot–

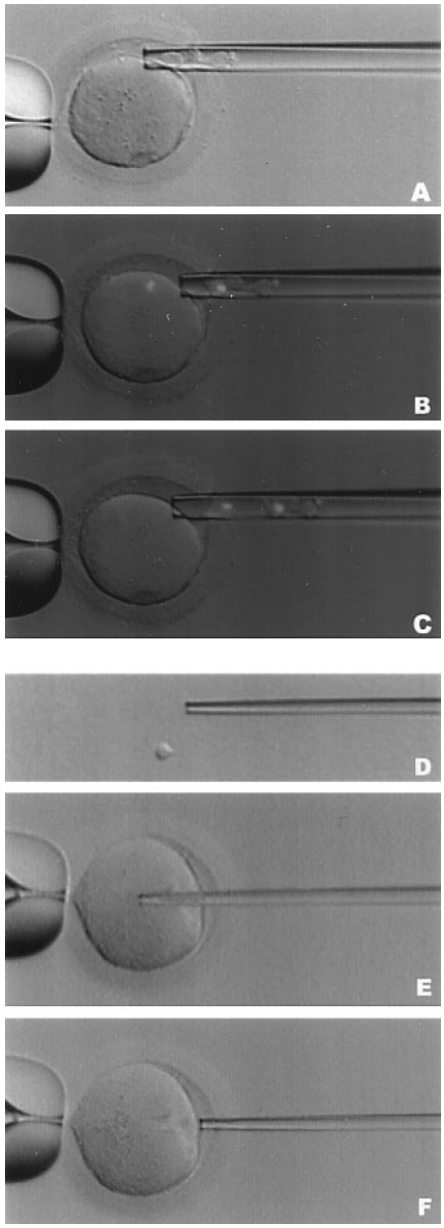

Figure 2. Stages of intracytoplasmic polar body injection. (**A**) Following partial zona dissection (not shown) a flame-polished biopsy tool is used to remove the first polar body from the perivitelline space. (**B**) To reduce cytoplasm irradiation, the Hoechst-stained metaphase is visualized by a very brief exposure to UV light, enucleation itself is performed under bright-field illumination. (**C**) Success of enucleation is verified by a second brief exposure to epifluorescence. Both the first polar body and metaphase II (MII) are inside the pipette. (**D**) Previously removed second polar body (procedure not shown) is aspirated into injection pipette. Because of the small inner diameter of the tool, the polar body plasma membrane is broken during the procedure. (**E**) Intracytoplasmic polar body injection. In order to break the plasma membrane of the ooplast, cytoplasm is first aspirated inside the tool, until the membrane is broken. (**F**) After injection the embryo should be left to recover for at least 1 h before activation.

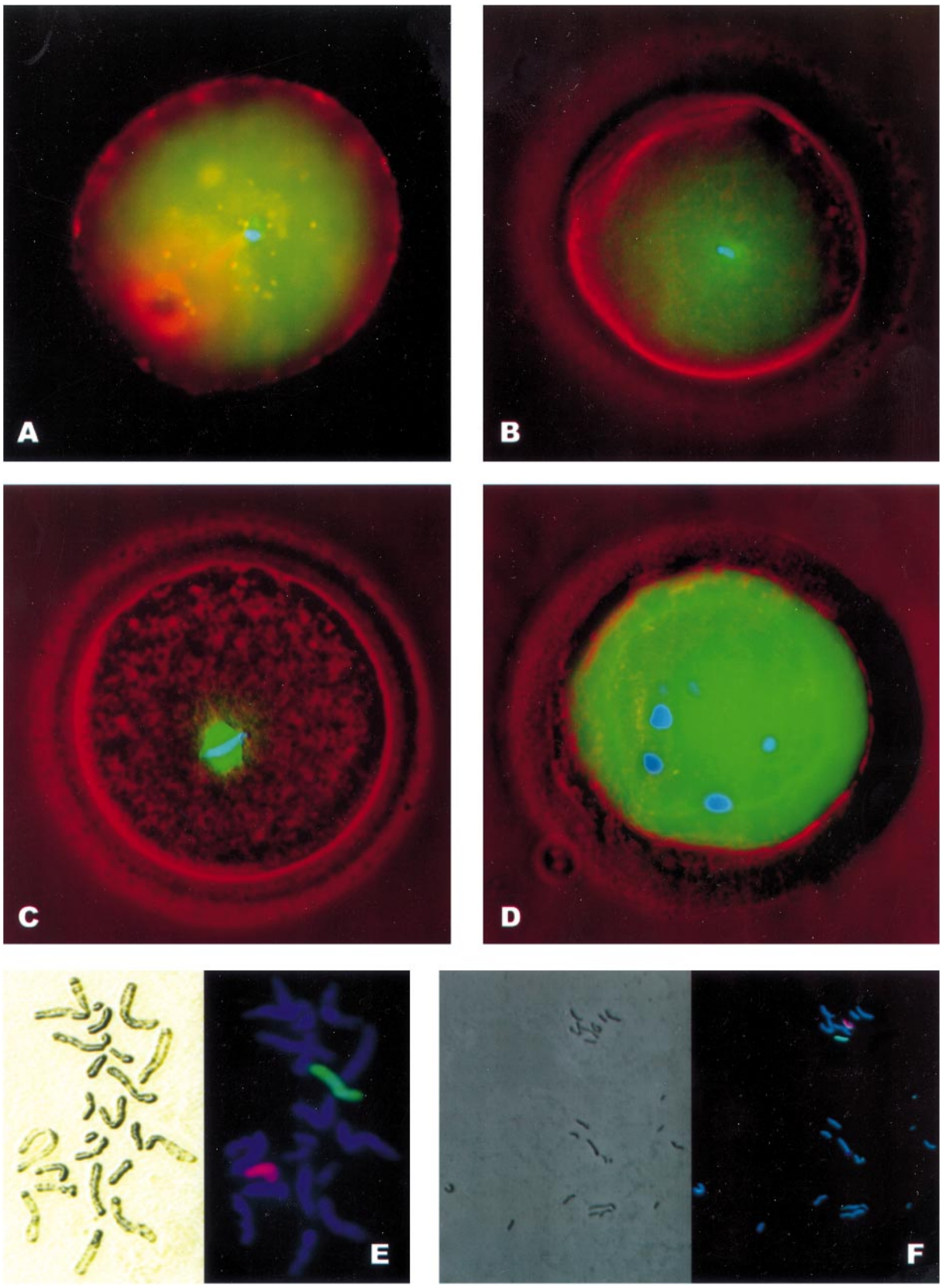

Figure 3.

FXA microscope equipped with CE 200 A CCD camera (Photometrics Ltd). Quips' Genetics Imaging Software (Vysis Inc) was used for the chromosome and embryo imaging.

For G-banding, slides were treated for 25 s with a pancreatine/Hank's balanced salt solution (HBSS; ×1) solution and stained for 1 min 15 s in a Wright and Giemsa stain.

Immunocytochemical analysis

The fixation and immunostaining method described by Emerson (1988) and Pickering *et al*. (1988) was used for immunostaining. Reconstructed haploid embryos were fixed/permeabilized with 2% formaldehyde + 0.02% Triton X-100 in PBS for 30 min at room temperature. After a short rinse in PBS the embryos were neutralized for 10 min using 0.26% ammonium chloride in PBS, then rinsed in PBS. Following a 30 min preincubation at 37°C in PBS with 1 mg/ml albumin (BSA, fraction V) embryos were immunolabelled for tubulin and/or actin, then washed at 37°C. We used monoclonal anti-α-tubulin fluorescein isothiocyanate (FITC)-conjugated antibodies (1:50) alone or in mixture with phalloidin (2 μg/ml) in PBS + 3% BSA + 0.1% Tween 20. Washing steps were for a total of 1 h in two changes of PBS + 0.1% Tween 20 and two changes of PBS. Embryos were mounted in Vectashield antifade (Vector Labortories, Burlingame, CA, USA) with 0.5 μg/ml 4′,6-diamidino-2-phelylindole (DAPI) counterstain, on glass slides with teflon-bordered wells (12 wells, from Erie Scientific, Portsmouth, NH).

Results and discussion

In contrast to the effect of OA we have observed on mouse second polar bodies, treatment of human second polar bodies with OA had led to further condensation of already picnotic nuclei (Table I). It became clear that, similar to the technique of sperm karyotyping, the nucleus of the polar body must be decondensed first.

Mouse second polar bodies can be fused with a zygote (Figure 1A). It can participate in development or, when fused with enucleated zygote, can be karyotyped after being arrested and fixed at the first mitotic division (Verlinsky *et al.*, 1994).

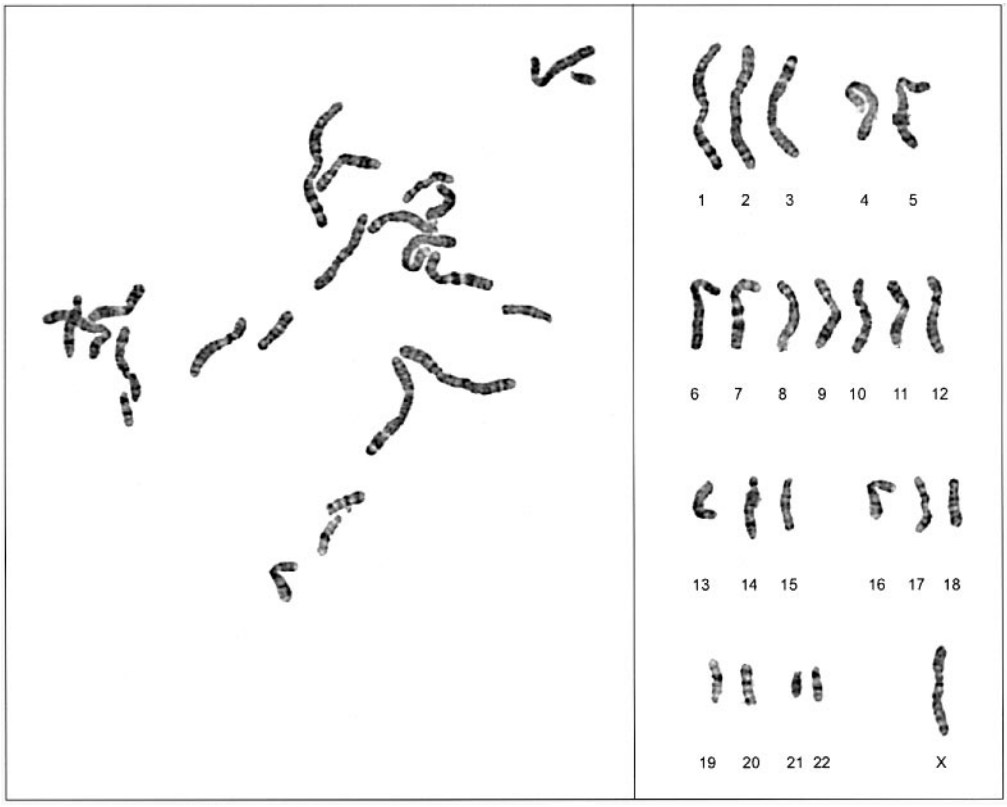

Figure 4. Karyotyping of the second polar body haploid metaphase by G-banding.

Figure 3. Immunocytochemical and cytogenetic investigation of the second polar body nucleus transformations in foreign cytoplasm. (**A**) Immediately after injection (actin is red, tubulin green, chromatin blue). Polar body nucleus is inside the cytoplast. Anti-actin antibodies (labelled red) stain subcortical layer of actin and help to visualize membranes. The cavity is still not relaxed after injection. Polar body nucleus has no plasma membrane around. However, it is surrounded by tubulin microfilaments, abundant in the polar bodies (unpublished observations). (**B**) 2 h after polar body injection (bright-field view of an embryo is shown in red, tubulin green, chromatin blue). Tubulin microfilaments, initially present only around the nucleus, started to elongate into the cytoplasm. (**C**) First mitotic division of the reconstructed embryo. Metaphase spindle of the second polar body. (**D**) When premature chromosome condensation is induced by okadaic acid, polar body chromosomes (blue) are scattered all over the cytoplasm. No spindle is formed; staining for tubulin (green) also reveals cytoplasm stratification, caused by cytochalasin D. (**E**) Bright-field (left panel) view of the haploid metaphase plate obtained from human second polar body. The same metaphase (Right panel) after whole chromosome painting (WCP). Chromosome 5 – pink, chromosome 10 – green. (**F**) Metaphase from the second polar body. WCP: chromosome 3 – orange, chromosome 13 – green. Original magnification ×400.

We have not succeeded in the fusion of human first or second polar bodies with human or mouse cytoplasts (Table I).

We have been able to introduce second polar bodies inside the cytoplasts using a technique we initially proposed for sperm injection (Evsikov *et al.*, 1990). Figure 1B shows the first two steps of this method. Although the second polar body nucleus was introduced into ooplast, pronucleus formation was never observed (Table I). Most probably, instead of fusing membranes of the polar body and cytoplast, electrofusion induced a fusion of the walls of the cavity. This led to 'pinching off' of the plasma membrane bubble with the polar body inside, simulating the process of endocytosis.

The most efficient way of introducing the second polar body nucleus into cytoplasts is the use of the procedure similar to ICSI, with the only difference being the shape of the injection pipette (Figure 1C; Figure 2D–F). After transfer into enucleated zygotes, second polar body nuclei were never transformed into pronuclei (Table I). Similar to the mouse second polar bodies fused with intact zygotes (Evsikov and Evsikov, 1994) this might have been caused by the asynchrony between cell cycle stages of a cytoplast and introduced nucleus. Consequently, in order to transform second polar body nuclei into pronuclei, it must be introduced into an enucleated oocyte. Since the cytoplasm of the second polar body does not possess oocyte-activating factors, the reconstructed embryos must be activated after polar body injection.

Table II presents our results on human oocyte activation using different techniques described in the literature (see Materials and methods). The oocytes were of highly diverse origin: MII oocytes matured *in vitro* for 24–48 h from the GV or MI stages, embryos with zero pronuclei after IVF or ICSI, and oocytes after freezing–thawing. There was no difference in activation rate among these groups of oocytes. Different approaches to oocyte activation by calcium ionophore A23187 (see Materials and methods) gave discouraging results: either low activation rates and low mortality rates, or high activation rates and very high mortality rates. We have not succeeded in determining optimal conditions for this type of activation. Pooled results from different experiments with A23187 are presented in the Table II.

Inhibition of protein synthesis in conjunction with electrostimulation, has turned out to be the most reliable technique for human oocyte activation. We are using this technique to activate ooplasts after second polar body nucleus intracytoplasmic injection. Successful second polar body injection followed by ooplast activation was initially as low as 10%, primarily because the nuclei, when stripped of surrounding cytoplasm and plasma membrane, tend to stick to the micropipette. The technique was improved to a level comparable with the efficiency of ICSI with the introduction of the blunt-end, NP10-treated injection pipette (34 out of 38 successful nuclear transfers; Table I). The micromanipulation steps of this method are shown in Figure 2.

Of 34 reconstructed embryos, 18 activated and had one pronucleus 14 h later. However, the analysis of 16 'not activated' (zero pronuclei) embryos showed that six of them contained a haploid metaphase instead of the expected picnotic nucleus. Apparently, after a brief period of activation (accompanied by pronucleus formation) these reconstructed haploid embryos returned to the initial, metaphase III-like state (Kubiak, 1989). The low activation observed in our study may be explained by the use cytoplasts from the oocytes, which matured *in vitro* or failed fertilization/activation during regular IVF/ICSI cases.

There are two ways to obtain metaphase chromosomes from the second polar body pronucleus. Starting at 24 h post-activation, embryos are checked every 30 min. By 45 min after the disappearance of pronuclei has been observed, such embryos are fixed. We did not use tubulin inhibitors, since they significantly delayed the time of embryo entry into mitosis. Alternatively, pronucleus-stage reconstructed haploid embryo may be cultured for 1 h in the presence of OA (see Materials and methods), then fixed. This method is less time-consuming and has been 100% effective in producing metaphase plates from one-pronuclear-stage haploid embryos. It should be noted that, unlike the mitotic spindle of the first cleavage division (Figure 3C), OA-treated embryos have prematurely condensed chromosomes scattered all over the cytoplasm (Figure 3D). Consequently, hypotonic treatment of such embryos can be omitted. When an embryo is transferred from the fixative to the slide the embryo should be left to dry out without any attempts to improve chromosome spreading by blowing or flooding with fixative or softening solutions. The end result should resemble the one described in the technique of gradual fixation (Kamiguchi and Mikamo, 1986).

Figures 3E and 3F show metaphase plates, obtained from human second polar bodies before and after WCP for chromosomes 5 versus 10 and 3 versus 13. As seen from Figure 4, haploid metaphases, obtained from the second polar bodies, can be karyotyped by G-banding. The effectiveness of the proposed method is limited primarily by the ineffectiveness of human ooplast activation. Unlike ICSI, the cytoplasts used for polar body injection are dispensable. Accordingly, the loss associated with ooplasts lysis during injection can be reduced to zero. Since the availability of human unfertilized oocytes is limited, presently we are investigating the possibility of using rodent oocytes as recipients for the second polar bodies.

As demonstrated below, the results of the second polar body karyotyping are obtained by the third day of embryo development, which is acceptable for the purpose of PGD:

Day 1 (zygote stage)

Biopsy of the second polar bodies
Enucleation of oocytes
Intracytoplasmic polar body injection
Activation of nuclei-transplanted haploid embryos.

Day 2, noon

Fixation of embryos without pronuclei
Incubation of one-pronuclear embryos with OA
Embryo fixation
Probe application for WCP, hybridization.

8–16 h later:

Slide washing
Slide imaging.

The timetable is perfectly acceptable for application of the method for the selection of embryos resulting from chromosomally normal oocytes. Because the second polar body has no known biological significance for pre- and post-implantation development, the method is clearly non-destructive to the oocyte. With further development and simplification the method will be of great value for PGD of maternal translocations, making it possible to detect and avoid the transfer of embryos resulting from oocytes with unbalanced chromosomal translocations.

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