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SHORT COMMUNICATION

CHICAGO, ILLINOIS

Visualization of Chromosomes in Single Human Blastomeres

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INTRODUCTION

Preimplantation genetic diagnosis (PGD) makes it possible to select genetically normal embryos for patients at risk of having children with genetic and chromosomal disorders. The major limitation of this technique is the amount of material available for genetic analysis. It usually involves either the first and second polar bodies or only one or two blastomeres biopsied at the cleavage stage (1,2). FISH analysis of the first polar bodies has been used to detect chromosomal translocations in human oocytes (3,4). However, this technique is limited for maternally derived translocations and since, at present, there is no reliable method for visualizing blastomere chromosomes, paternally derived translocations cannot be detected at the preimplantation stage.

Nuclear transplantations have been extensively used for the study of nucleocytoplasmic interactions, cell differentiation, and animal and embryo cloning (reviewed in Ref. 5). It is usually expected that heterologous nuclei, if transferred into enucleated eggs and zygotes, would undergo remodeling in accordance with the developmental program of the ooplasm. In this study, we investigated the possibility of applying nuclear transplantation techniques for karyotyping a single blastomere of preimplantation human embryo. Our expectations were based on the fact that individual blastomeres, when fused with ooplasts, will be involved in the cell cycle, characteristic for recipient ooplasm and thus, the timing of mitosis could be manipulated and predicted.

The present work describes our results from employing these techniques for visualization of the chromosomes from single human blastomeres and application of this method for the detection of translocations.

MATERIALS AND METHODS

The use of human oocytes and preimplantation embryos for this project was approved by IRB of Illinois Masonic Medical Center, Chicago, IL. The use of hamsters was approved by ACC of the University of Illinois at Chicago. Frozen mouse zygotes were purchased from Charles River Laboratories (Wilmington, MA).

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Embryo culture and micromanipulations were performed in HTF medium with 10% Plasmanate (Bayer Biological, New Haven, CT). Micromanipulations were performed under a 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.* (6), was used for oocyte-zygote enucleation and for blastomere biopsy. MII oocytes not fertilized after IVF or ICSI, or MII oocytes matured for 24–48 hrs in vitro from immature oocytes (Germinal Vesicle or MI) were enucleated and served as ooplast recipients. Prior to enucleation, oocytes were incubated for 10–15 min at 37°C in medium with 1 μg/ml Cytochalasin D, 0.2 μg/ml Demecolcine, and 0.5 μg/ml Hoechst 33342. Enucleation procedure was basically the same as described by Tsunoda *et al.* (7) for mouse oocyte enucleation.

Enucleation of abnormally fertilized zygotes was achieved by first exposing 1- or 3-pronuclear zygotes for 10 min to a medium containing 1 μg/ml Cytochalasin D and 0.2 μg/ml Demecolcine. Then zygotes were transferred for 3–5 min into the medium con-

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taining 0.05 M sucrose, 1 μg/ml Cytochalasin D, and 0.2 μg/ml Demecolcine. Enucleation was performed in the same medium (Fig. 1A and B).

Prior to blastomere biopsy cleavage-stage embryos were incubated in the medium containing 0.05 M sucrose. Biopsy was performed in the same medium. To reduce the chances of blastomere damage, blastomere biopsy was performed using a pipette with a internal diameter of approximately 40 μm.

Electrofusion was induced with the aid of the electrofusion apparatus (Bams Manufacturers Inc, Chicago, IL) in a fusion chamber consisting of two platinum wire electrodes glued to the bottom of a glass dish with a gap of 0.4 mm (Fig. 1C). Electrofusion medium consisted of 0.3 M Mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.5% polyvinylpyrrolidone. Cell fusion was induced with a single DC pulse at 40 V for a duration of 500 μs. Cell fusion was assessed 30 min after electrofusion (Fig. 2D).

Premature chromosome condensation (PCC) was induced by cell exposure to 5 μM of okadaic acid (OA) diluted to the final concentration with PBS containing 3 mg/ml BSA and 0.5 μg/ml Cytochalasin D (8). The optimal time for inducing PCC in one-pronuclear zygotes was 1 hr.

Blastomere arrest at the metaphase of cleavage division was achieved by 5–15 hr blastomere or whole embryo incubation in the culture medium containing 0.1 μg/ml Demecolcine, or 3 μg/ml Nocodazole (9), or 0.04 μg/ml Podophyllotoxin and 0.04 μg/ml Vinblastine (10).

Immediately before fixation, zonae pellucidae were removed by Acid Tyrodes solution; Dyban's technique of oocyte fixation was used (11). Chromosome plates were first assessed using phase contrast and then processed for the whole chromosome painting (WCP). Vysis probes (Vysis, Inc., Downers Grove, IL) and standard protocol suggested for hybridization and slide washing were used for the WCP. Slides were imaged with a Nikon Microphot-FXA microscope equipped with CE 200A CCD camera (Photometrics Ltd.). Quips™ Genetics Imaging Software (Vysis, Inc.) was used for the chromosome imaging.

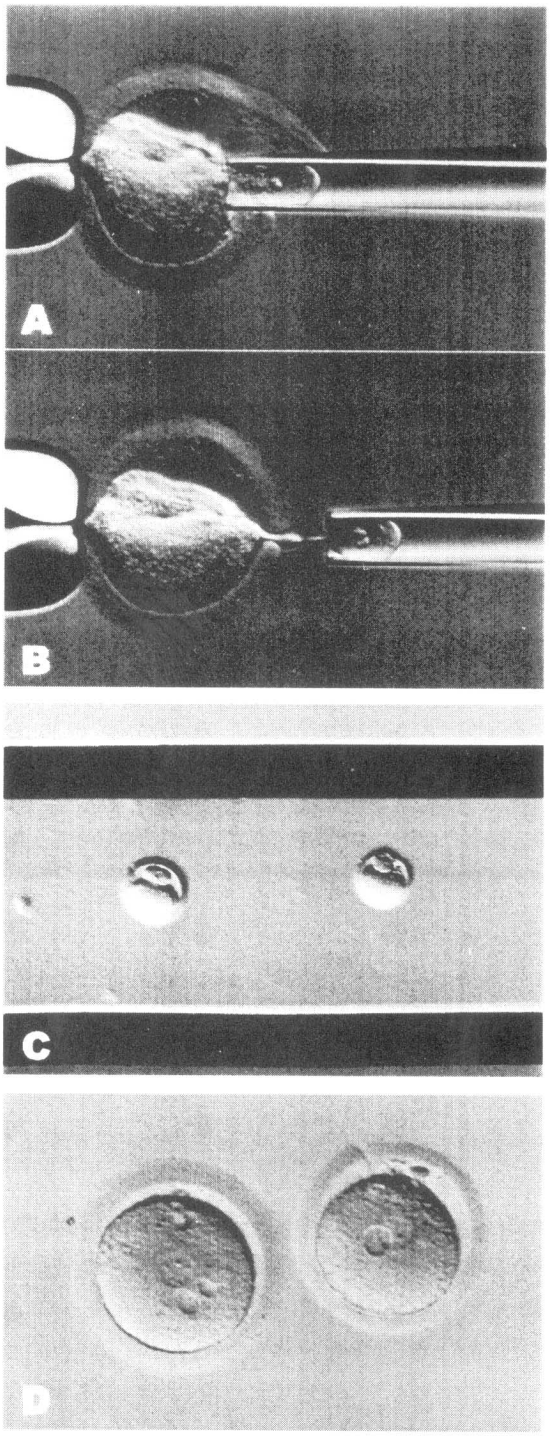

Fig. 1. Nucleocytoplasmic hybrids between enucleated haploid zygote and 1/8-blastomere. (A, B) Zygote enucleation (×200; reduced at 90% for reproduction), (C) cytoplast and blastomere electrofusion (×50; reduced at 90% for reproduction), (D) embryos 1 hr after fusion (×200; reduced at 90% for reproduction).

RESULTS AND DISCUSSION

Our attempts to obtain metaphases by incubating blastomeres in the presence of the inhibitors of microtubuli (Nocodazole, Demecolcine, Vinblastine, and Podophyllotoxin) supported the previous observation

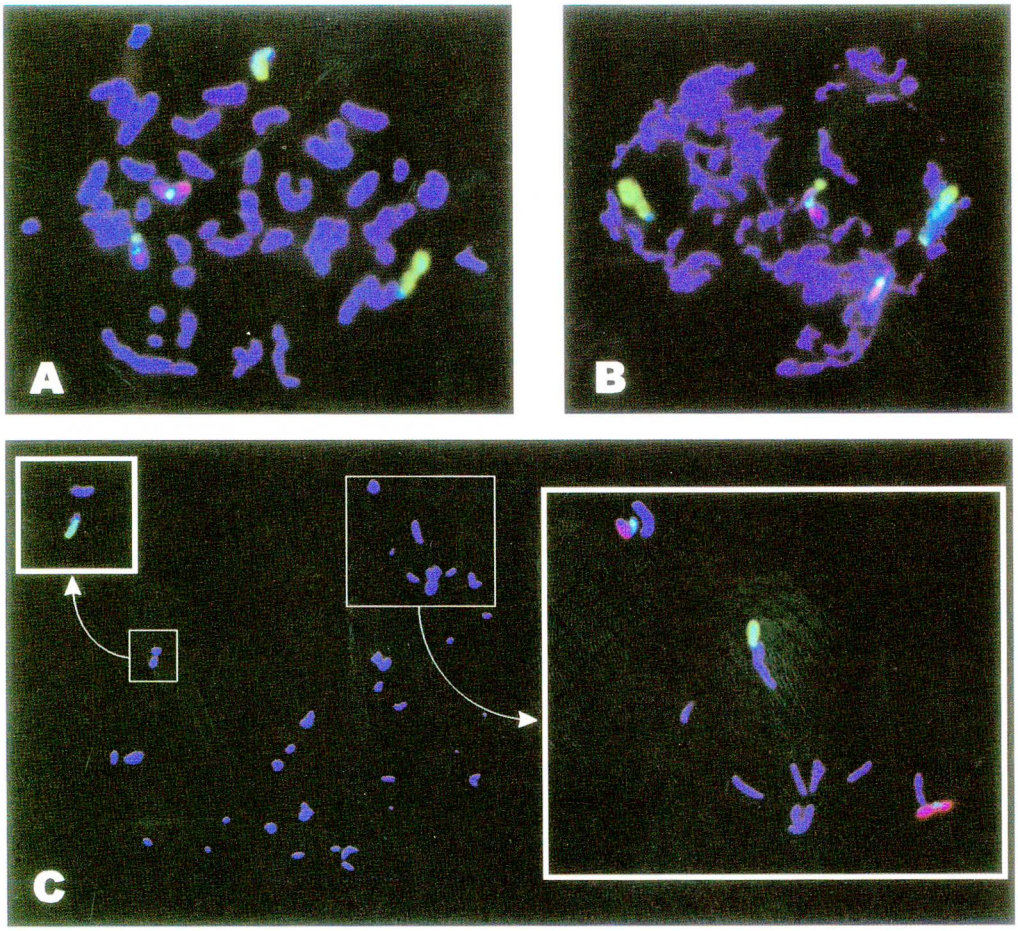

Fig. 2. Three metaphases obtained from a single eight-cell embryo, carrier of unbalanced, maternally derived chromosomal translocation. Whole chromosome painting (WCP). Chromosome 11 painted orange, chromosome 14 painted green, centromeric region of chromosome 11 stained aqua. Note the tiny telomeric region of chromosome 14 (green) translocated onto chromosome 11. (A) Metaphase (original magnification ×600), (B) prometaphase (original magnification ×600), (C) a metaphase (magnifications: metaphase plate at ×200, WCP inserts at ×600).

(12) that the standard technique of cell karyotyping is not applicable for PGD.

Okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, has been widely used to induce PCC in both somatic and embryonic cells (13–15). Blastomere incubation in the presence of OA resulted in 20 metaphases from 89 individual blastomeres (Table I). The success rate of this technique was too low for use in PGD cases. In most cases, OA treatments had either no noticeable effect on the interphase nucleus, or induced nucleus picnotization, or have led to chromatin pulverization (characteristic for PCC of the nucleus in the S-phase of the cell cycle; see Ref. 16).

Our results on blastomere fusion with enucleated human and intact hamster oocytes are presented in Table I. Out of 29 nuclear transplants, ooplasm (with or without additional treatment by OA) induced PCC in 23 nuclei, 16 metaphases with discernable chromosomes were obtained. In order to avoid the (rather time consuming) necessity for oocyte enucleation, we have attempted to use hamster oocytes as recipients for human blastomere nuclei. The result was clearly negative: hamster ooplasm has never induced PCC in the nucleus of the fused human blastomere. Further experiments show that, unlike human oocytes, hamster oocytes are activated by the electric impulse used for oocyte–blastomere fusion. Instead of inducing PCC in the introduced blastomere, hamster ooplasm led its nucleus into the beginning of a cell cycle. As a result, when such embryos have been treated with OA 2–3 hr after fusion, morphology of the introduced nucleus was typical for PCC of a nucleus in the S-phase of

Table I. Analyzable Metaphase Plates Obtained After Blastomere Fusion with Heterologous Cytoplasm, as Compared to Blastomere Treatment by Okadaic Acid (OA)

Number of blastomeres	Cytoplast donors	OA treatment (hrs)	Number of metaphases
89	none	1–3	20
29	enucleated human oocytes	0 or 1	16
23	intact hamster oocytes	0 or 1	0
16	enucleated human zygotes	1	14

the cell cycle. From the practical point of chromosome painting, such nuclei are of little value.

The results of human blastomere fusion with enucleated human oocyte show that about 45% of the nuclei cannot be transformed into analyzable chromosomes by the techniques of PCC induction. Since the embryo nuclei have been randomly picked from their fourth cell cycle, this figure reflects the percentage of nuclei in the S-phase, when PCC results in pulverized, not analyzable chromosomes. This leads to the conclusion that before attempting to induce PCC in any given nucleus, it should be led into G2-phase. Blastomere fusion with an oocyte, followed by ooplasm activation and in vitro culture, is a well-proven method to guide the blastomere nucleus into G2 and subsequently into metaphase of the cleavage division. However, unless blastocyst transfer is considered, this technique is incompatible with embryo transfer in the same cycle.

The use of zygote cytoplasm seems to be more appropriate for the IVF time frame. As seen from Table I, out of 16 blastomeres analyzed by this technique, 14 metaphases, one prometaphase (analyzable by WCP, see Fig. 2B), and one nonanalyzable pulverized chromosome plate, characteristic for S-phase PCC were obtained. The technique seems to be compatible with the routine IVF time scale:

Day 3, noon: enucleation of 1PN or 3PN human zygotes (fresh or, if unavailable, frozen-thawed);
14:00: biopsy of single 1/8 blastomeres, which are immediately inserted into perivitelline space of enucleated cytoplasts;
16:00: blastomere-cytoplast fusion;
23:00: embryo incubation in OA;
24:00: embryo fixation, probes application for WCP;
Day 4, 08:00: slides washing, slides imaging;
11:00: embryo transfer.

This technique was applied for confirmation of a PGD case of maternally derived translocation *t*(11,14), determined by the first polar body analysis (unpublished data). Three blastomeres of the embryo predicted to have unbalanced translocation, which was not transferred, were fused with zygote cytoplasts. Eight hours later, hybrids were transferred into OA and fixed 1 hr later. Two metaphases and one prometaphase were obtained, all showing the presence of unbalanced translocation, thus confirming the first polar body diagnosis (Fig. 2).

Our preliminary attempts to use frozen-thawed mouse zygotes as cytoplast donors for human blastomeres gave promising results. The next day, after fusion, three hybrids were at the pronuclear stage and eight hybrids cleaved and had two nuclei, indicating that the metaphase of the first cleavage of such hybrids can be obtained.

The proposed technique provides the possibility for obtaining metaphase chromosomes from human blastomeres. However, its application for human PGD will depend on the availability of human abnormally fertilized zygotes, fresh or frozen-thawed, which might be replaced by commercially available frozen mouse zygotes as a source for zygote-stage cytoplasts.

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