Preembryo biopsy and analysis of blastomeres by in situ hybridization

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We developed a method for the biopsy of preimplantation mouse embryos (preembryos) at the four- to eight-cell stage, which uses partial zona pellucida dissection. The preembryos were collected in calcium- and magnesium-free phosphate-buffered saline solution with 0.01% ethylenediaminetetraacetic acid, 0.1 mol/L sucrose, and 4 mg/ml of bovine serum albumin to facilitate removal of blastomeres. This allows entry of a fine micropipette into the perivitelline cavity with subsequent removal of a single blastomere by gentle suction. The majority of embryos (75%) from which biopsy specimens were obtained in this fashion developed to the blastocyst stage. The blastomeres obtained were mainly intact and they were fixed to glass slides. After permeabilization, in situ hybridization was performed with chromosome X– and chromosome 3–specific probes. Human unfertilized eggs and blastomeres from human polyspermic embryos also have been analyzed by in situ hybridization with chromosome specific probes. The combination of nondestructive embryo biopsy and in situ hybridization is a possible approach for preimplantation genetic diagnosis. (AM J OBSTET GYNECOL 1990;163:2013-9.)

Key words: Preembryo biopsy, in situ hybridization, blastomere

Recent advances in molecular genetics have improved the sensitivity of methods used for genetic diagnosis. Two techniques in particular, the polymerase chain reaction and in situ hybridization permit the analysis of the genetic material of a single cell. The sensitivity of polymerase chain reaction is achieved by enzymatic amplification of target deoxyribonucleic acid (DNA) sequences by a factor of 10^5-fold or more.[1] In contrast, in situ hybridization allows the direct visualization of single genes in metaphase chromosomes or interphase nuclei.[2,3] The micromanipulation of preembryos could allow specific preimplantation diagnoses to be made. Currently, genetic diagnoses are limited to the postimplantation period and are performed by chorionic villus sampling or amniocentesis. If genetic diagnoses could be achieved in preimplantation embryos with no untoward effects then in vitro fertilization technology could be used for fertile couples at genetic risk. Because a broad range of diseases can be diagnosed by polymerase chain reaction or in situ hybridization, the technique of in vitro fertilization coupled with preembryo biopsy could provide the conduit from which these technologies can be applied to the human species.

Edwards[4] first suggested that sex-linked recessive disorders could be diagnosed by sampling trophoblast cells removed from blastocysts. Sex of bovine preembryos has been determined by chromosome analysis[5,6] or Y chromosome–specific DNA probes.[7] Monk et al.[8] have diagnosed a deficiency of the X-linked enzyme hypoxanthine phosphoribosyl transferase in mouse preembryos before transfer. However, analyses of this nature are limited to enzyme deficiency diseases that can be detected by microenzyme assay. Trophectoderm biopsy has been successfully carried out in marmoset monkey. These cells were cultured for analysis and the embryos were transferred with resultant live-born offspring.[9] Sex of the human preembryo has been successfully determined with chromosome-specific probes and in situ hybridization. However, this used a tritiated Y chromosome–specific probe that required a long time for diagnosis and the analysis used the whole embryo in a destructive fashion.[10] Whereas this method is successful, it would provide no clinical application. Recently, Handyside et al.[11] determined the sex of the human preembryo in a nondestructive fashion with embryo biopsy in conjunction with polymerase chain reaction. They were able to remove a single blastomere, amplify Y-specific DNA repeat sequences, and culture embryos from which biopsy specimens were obtained to blastocysts at the same rate as that of embryos from

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which biopsy specimens were not obtained. Holding and Monk[12] diagnosed β-thalassaemia by polymerase chain reaction in single blastomeres from mouse preimplantation embryos. The unique feature of this study is that they were able to amplify a region of a gene in a single diploid blastomere by a method that is theoretically applicable to any single copy genetic disorder in which a mutation or deletion is the cause. Wilton and Trounsen[13] recently described a method of mouse preembryo biopsy with successful cryopreservation. In addition, they developed a method for culture of blastomeres that after 6 days resulted in 20-cell nuclei per cultured blastomere.[14] Whereas in situ hybridization can be applied to a single cell, the ability to culture blastomeres would facilitate a more reliable diagnosis. In situ hybridization allows numeric chromosome analysis and is not limited by contamination. These are distinct advantages over polymerase chain reaction for analysis of single cells.

In this article we describe a method of embryo biopsy that uses partial zona dissection and we describe the first successful in situ hybridization with blastomeres obtained by embryo micromanipulation. In addition, we performed in situ hybridization with both unfertilized human eggs and with polyspermic human embryos.

Material and methods

Embryo collection. Female mice B6xC3/F1 were superovulated by intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (Gestyl, Diosynth) followed 48 hours later by 5 IU of human chorionic gonadotropin (Pregnyl, Organon) and mated with male B6xC3/F1 mice. Mated females were killed approximately 48 hours later and embryos were flushed from the oviducts with either Hoppe-Pitts media or phosphate-buffered saline solution with 4 mg/ml bovine serum albumin. Embryos were obtained at the two-cell to four-cell stage and 80% to 90% achieved the blastocyst stage when cultured in our system. Four- to eight-cell embryos were incubated for 30 minutes in calcium- and magnesium-free phosphate-buffered saline solution with 0.01% sodium ethylenediaminetetraacetic acid (EDTA) to reduce intercellular contacts. They were transferred to a small drop on a Lab-Tek microculture slide with the plastic chamber removed and covered with paraffin oil equilibrated with phosphate-buffered saline solution. Human polyploid embryos and oocytes that failed to fertilize were obtained from the in vitro fertilization program (Human Investigation Protocol 5443).

Micromanipulation. A single blastomere was removed from four- to eight-cell embryos with an inverted microscope and Narishige micromanipulators (Fig. 1). A holding pipette of approximately 80 μmol/L outside diameter with a 30 μmol/L inside diameter was fire polished with a Nikon microforge. A sharpened dissection pipette was used for zona dissection and a microbiopsy pipette was pulled to a 10 to 20 μmol/L outside diameter with a 5 to 15 μmol/L inside diameter. The preembryo was stabilized with gentle suction through the holding pipette and a slit was made through the zona pellucida with a sharpened dissection pipette similar to the method of Malter and Cohen.[15] The dissection pipette was removed and replaced by a biopsy pipette that was placed into the zona slit, and a blastomere was aspirated partially into the pipette under controlled suction. The blastomere was removed from the zona and the preembryo was placed into the Hoppe-Pitts media for continued culture after three passes into fresh media droplets. Embryo culture was done in microdrops under paraffin oil equilibrated with media.

Analysis of blastomeres. Blastomeres obtained in media droplets under oil were fixed directly onto the tissue culture slides under a Zeiss dissecting microscope in the following fashion. Two fixation protocols were used, one with 4% paraformaldehyde in 1/2X phosphate-buffered saline solution the other with 0.075 mol/L potassium chloride solution to swell the cell. Media was replaced with 4% paraformaldehyde in 1/2X phosphate-buffered saline solution or 0.075 mol/L potassium chloride and blastomeres incubated for 15 to 30 minutes. The oil was removed by suction and the solution surrounding the blastomere was removed without dislodging it. As the blastomeres started to air dry methanol/acetic acid (3:1) was dropped on to further fix them to the slide. This last step was repeated after air drying and finally 100% methanol was applied to remove excess oil and debris. Blastomeres were stored fixed at 4° C. Later experiments showed no need for paraformaldehyde fixation and this step was omitted. When this was done no permeabilization was required either. Human oocytes and polyploid embryos were processed in a manner similar to mouse preembryos; however, polyploid preembryos were not cultured after biopsy because all blastomeres were fixed to glass slides. The polyploid embryo shown in Fig. 2 had five blastomeres in it and only three were retained on the glass slide after the in situ hybridization procedure.

Permeabilization. Blastomeres were equilibrated in phosphate-buffered saline solution with 20% glycerol for 30 minutes and then 0.5% triton (vol/vol), 0.5% saponin (wt/vol) in phosphate-buffered saline solution for 10 minutes. The slides were immersed in liquid nitrogen for 5 seconds and allowed to thaw. Two additional freeze-thaw steps were performed. This permeabilized the cell to allow biotinylated DNA probes access to the nucleus during in situ hybridization. When it was omitted variable results were obtained with in situ hybridization in paraformaldehyde-fixed blas-