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—Review—

Serious Limitation of Preimplantation Genetic Diagnosis

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Abstract: *Preimplantation genetic diagnosis (PGD) is the technology used to avoid inheritance of genetic disease by selecting unaffected embryos. Furthermore PGD has made genetic screening of embryos possible. The limited number of cells available for genetic tests has been a serious problem with PGD. The first solution was the application of molecular technologies such as PCR, FISH and cell recycling. The next solution was the visualization of metaphase plates and application of advanced FISH technologies. The ideal solution was in vitro culture of blastomeres to increase the number of cells because it would be easy to reexamine them to assure the accuracy of genetic test results and obtain additional genetic information. The culture system for ES cells was applied to culturing mouse isolated blastomeres because it met two requirements: rapid proliferation and maintenance of the normal karyotype during culture. Our trial with mouse embryos successfully demonstrated these two requirements.*
Key words: *Preimplantation genetic diagnosis, Blastomere, Embryonic stem cell, Mouse, Co-culture*

Preimplantation genetic diagnosis (PGD) is the technology used to avoid inheritance of genetic disease by transferring unaffected embryos [1]. It is the advantage of PGD over classical prenatal diagnosis (PND) by means of such methods as chorionic villi sampling (CVS) and amniocentesis that provide genetic information before conception. If classical PND reveals that genetic disease has affected a fetus, artificial abortion could be one option for the patient. Some carrier patients have been choosing artificial abortion

repeatedly and this might be the cause of mental and physical damage to them. Unaffected conception and avoidance of artificial abortion can be expected by means of PGD.

The avoidance of genetic disease has become the classical purpose of PGD, and it has made genetic screening of embryos possible. The chromosomal abnormalities have been the cause of implantation failure and abortion. Embryo loss could be reduced by aneuploid screening, which is one form of genetic screening [2]. In future, gene screening of embryos could become a reality.

Typically only one or two blastomeres are biopsied from cleavage stage embryos without spoiling their viability [3] and analyzed by fluorescence in situ hybridization (FISH) and/or polymerase chain reaction (PCR). The limitation of the number of cells available for genetic tests has been a serious problem with PGD. In classical PND cases, a lot of cells are available for genetic tests and it is easy to reexamine them to assure the accuracy of results and obtain the additional genetic information. Many researchers who face this limitation problem have been developing some diagnostic strategies. In this mini-review, we introduce some of these strategies including our attempt to solve this problem.

PCR and FISH

The primary solution of the cell number limitation was the application of PCR, the technology of molecular biology. The sensitivity of PCR is extremely high and even a set of genome DNA can be analyzed. At the beginning of PGD, the gender is detected to avoid inheritance of X-linked genetic disease by amplification of the region specific to chromosome Y [1]. Although

PCR is able to detect the existence of the target sequence, it is impossible to specify the number of target chromosomes. Therefore it has been recommended to use FISH for gender determination [4]. Recently PCR has been mainly used to diagnose single gene defect disorder. Furthermore multiplex nested PCR, which was able to analyze a number of regions simultaneously, was applied to increase the amount of genetic information obtained from single blastomeres.

The second solution of the limitation was the application of FISH. The first application of FISH was gender determination to substitute for PCR. Then FISH was used to diagnose numeric chromosomal abnormalities such as aneuploidy. Recently FISH has been applied to structural chromosomal abnormalities such as reciprocal translocation and Robertsonian translocation. Furthermore multiple-probe FISH made it possible to analyze 2 or more chromosomes simultaneously, even in an interphase nucleus.

PCR is required for the preparation of primer sets to amplify target sequences and FISH is required for the preparation of probes to hybridize target regions. In other words, both PCR and FISH are able to detect only target genes and chromosomes. Therefore they do not meet the requirements for screening purposes.

Cell recycling of single blastomeres

Thornhill *et al.* described a powerful technique called 'cell recycling' which combined PCR and FISH in the same blastomeres for PGD [5]. This technique made it possible to obtain information on both genes and chromosomes from the same cells. Biopsied blastomeres were fixed to the tips of miniature slides designed for insertion into PCR tubes. The first step in cell recycling was PCR. Then the miniature slides used for FISH and PCR products were used for genetic diagnosis. If the amount of PCR products is not enough for analysis, nested PCR can be applied. The genomic DNA fixed on the miniature slides can be a template for amplification and then the same genomic DNA can be a target for hybridization. We developed this technique for PGD of X-linked diseases, such as Duchenne muscular dystrophy (DMD). Mutation of the dystrophine gene could be detected by PCR and gender and/or numeric chromosomal abnormality by FISH.

Benadiva *et al.* described another kind of 'cell recycling', which performs sequential FISH on the same blastomeres [6]. The biopsied blastomeres were fixed on regular slides. Then 2 step FISH with two different sets of probes was applied to the same blastomeres.

This technique makes it possible to increase the amount of information on chromosomes. We developed this technique for numerical abnormality of 7 chromosomes. The FISH probes for 7 chromosomes were divided into 2 groups. The first step was performed for chromosomes 13, 18, 21, X and Y and the second step for chromosomes 16 and 22 [7].

These two kinds of cell recycling are increasing the amount of genetic information obtained from same single cells. The limitations of cell recycling are the same as those of PCR and FISH. In other words, because cell recycling is able to detect only target genes and chromosomes, it is not suitable for screening purposes.

Visualization of Metaphase Plates

The chromosome analysis on good-quality metaphase plates makes it possible to diagnose both inherited and *de novo* chromosomal abnormalities, but a large proportion of blastomeres isolated from cleavage stage embryos would be in the interphase and rare blastomeres in the metaphase. Even after overnight culture of isolated blastomeres in the presence of colcemid, metaphase plates suitable for karyotyping could be obtained from only one-third of the cells.

Willadsen *et al.* reported a procedure for visualization of metaphase chromosomes in single blastomeres by fusion with bovine oocytes [8]. Similar research on human, hamster and mouse zygotes and oocytes was reported by Verlinsky *et al.* [9, 10]. The oocytes or zygotes were used as recipient cytoplasm and the blastomeres were electrically fused with them to transform into the metaphase [11]. Although this technique was a novel idea for obtaining the metaphase, skillful techniques for nuclear transplantation were required. Improvements, for example simplification of the procedures, success rates and preparation of recipient cells, would be expected. Furthermore it was not easy to obtain good-quality metaphase plates suitable for G-banding from single metaphase cells. Instead of G-banding, advanced FISH techniques, such as whole chromosome painting or spectral karyotyping (SKY) [12], could be applied. Those techniques on metaphase plates made possible the screening of numerical chromosomal abnormalities and several kinds of structural chromosomal abnormalities. Screening of 'limited' *de novo* chromosomal abnormalities was realized.

In Vitro Culture of Single Blastomeres

The methods described above increase the sensitivity of diagnostic tools or the amount of genetic information but are not a fundamental solution of the cell number limitation. The ideal solution for the limitation will be *in vitro* culture of single isolated blastomeres. This solution makes it possible to reexamine to assure the accuracy of genetic test results and obtain the additional genetic information. Wilton *et al.* reported that isolated blastomeres from mouse 4 cell embryos were cultured in wells the bottoms of which were coated with 4 kinds of extracellular matrix for 6 days [13]. Plaque consisting of approximately twenty cells was obtained in all fibronectin wells.

Meanwhile embryonic stem (ES) cell lines were established from inner cell masses of blastocyst embryos [14]. They are able to subculture continuously without differentiation and transmit genetic characteristics accurately. It is also presumed that the culture medium for ES cells includes many factors to proliferate cells efficiently. We utilized the culture system for ES cell lines because its characteristics are suitable for our purpose.

Delhaise *et al.* described the establishment of ES cell lines from mouse single blastomeres at the 8-cell stage [15]. This culture system was applied to the proliferation of mouse isolated blastomeres in our trial. In brief, single isolated blastomeres were obtained from BDF1 mouse 4 cell embryos fertilized *in vitro*. Each blastomere was placed in Dulbecco's MEM/Ham's F-12 (50/50, v/v) to which were added 10% fetal bovine serum, 10% newborn calf serum, 0.1mM beta-mercaptoethanol and 5,000 unit/ml leukemia inhibitory factor (LIF) with STO cells as feeder cells for 4 days (co-culture). Control blastomeres were cultured in G1.2 medium alone (control). On day 4 of the culture, colonies of proliferated blastomeres were observed under the microscope and then fixed on slides to count the numbers of nuclei. On day 4 of culture, 69.8% of 'co-culture' blastomeres had formed colonies and the mean number of cells in them was approximately 460 (Fig. 1, A). While 28.6% of 'control' blastomeres formed a cyst similar to a typical blastocyst (Fig. 1, B-a), multiple cysts similar to atypical blastocysts (Fig. 1, B-b) and cell masses (Fig. 1, B-c) were composed of 10–15 cells. Explosive proliferation of isolated blastomeres was successfully demonstrated. It was one of the notable advantages of this culture system.

Another important aspect was the genetic normality of proliferated cells. The culture system was required not to accumulate genetic abnormalities. At the middle of our trial, we realized that the colonies were classified into two different shapes. Then the colonies were classified into 2 types (Type A and B, Fig. 2). Type A were colonies composed of relatively small cells at high density with a smooth surface and clear borderline similar to the ES cell line. Type B were the colonies composed of relatively large cells at low density with a rough surface and unclear borderline similar to epithelial or trophectodermal cells. The frequencies of type A and type B colonies were irregular depending on the experiment series. FISH analysis with probes specific for mouse chromosome X and Y revealed that type A colonies were mainly composed of cells presumed to be diploid and type B colonies be often composed of abnormal karyotype cells. It was an important characteristic of ES cells to maintain normal and stable karyotypes during continuous passages. The culture system for ES cells was supposed to inhibit differentiation and accumulation of genetic abnormalities. It was presumed that type A colonies successfully inhibited differentiation and maintained genetic normality and type B colonies failed to do so. Therefore when type A colonies were obtained from biopsied blastomeres, the cells composing them could be suitable for genetic analysis. Although the proliferated cells in our trial were not confirmed to be ES cells, we expected that they had the potential to be established as ES cell lines.

Discussion

The limited number of cells available for genetic diagnosis promoted us to develop various ways to overcome this disadvantage. The technologies described in this review such as cell recycling and visualization of metaphase plates were developed to increase the amount of genetic information from biopsied blastomeres. Cell recycling with PCR and FISH was able to investigate only target genes or chromosomes and did not meet screening purposes for *de novo* chromosomal abnormalities. Meanwhile the visualization of metaphase plates made it possible to investigate numerical and several kinds of structural chromosomal abnormalities and met the screening purpose of 'limited' *de novo* chromosomal abnormalities. The fundamental solution of the limitation could be *in vitro* culture of single isolated blastomeres. This method made it possible to obtain metaphase plates suitable for G-banding without cell fusion and completely met our needs in screening *de*
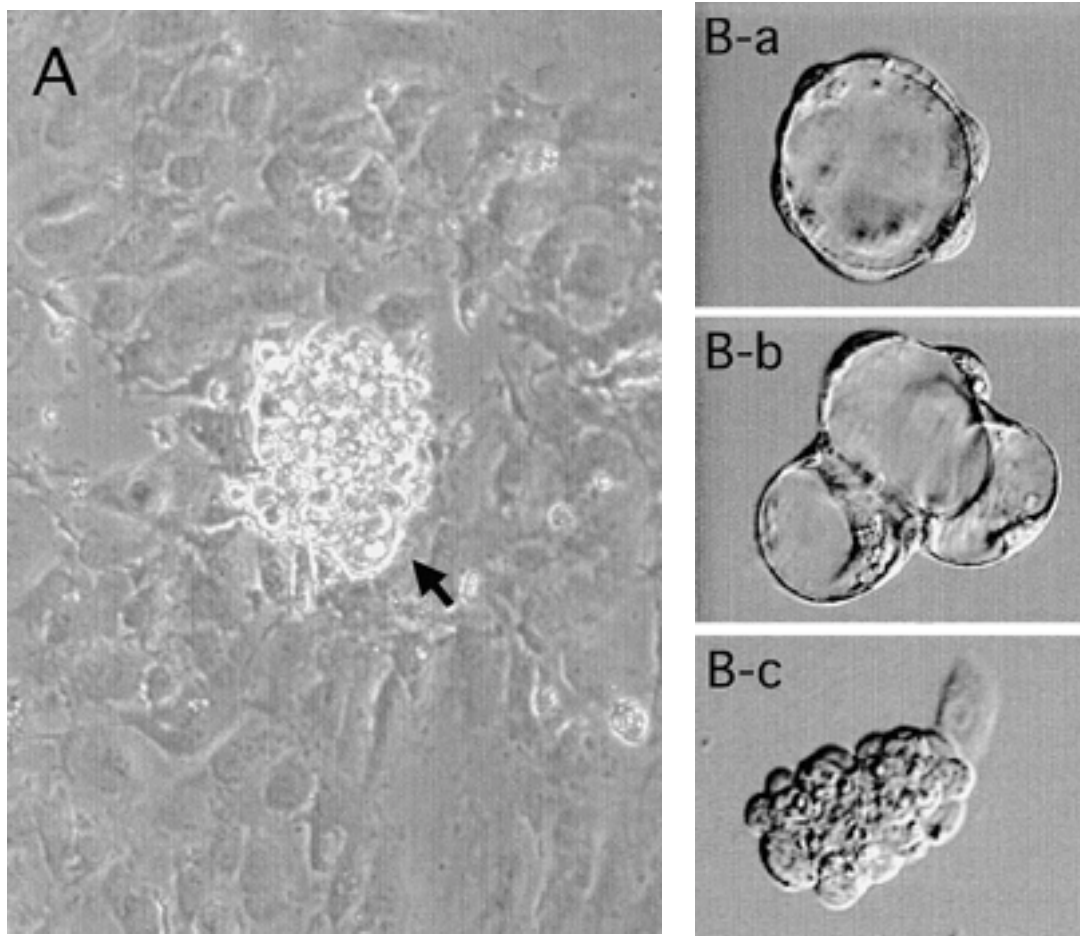
Fig. 1. Cell mass and cyst formation derived from isolated blastomere on day 4 of culture. A: a colony derived from a 'co-culture' blastomere (black arrow indicating type A colony). B: a cyst formation (a), multiple cyst formation (b) and a cell mass (c) derived from a 'control' blastomere.
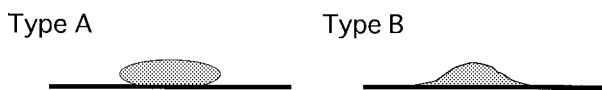
Type A   Type B

Fig. 2. Classification of colonies derived from 'co-culture' blastomeres. Type A: Colony composed of relatively small cells at high density with a smooth surface and clear borderline resembling ES cell lines. Type B: Colony composed of relatively large cells at low density with rough surface and unclear borderline resembling epithelial or trophectodermal cells.

novo chromosomal abnormalities. In the future, a certain amount of DNA extracted from proliferated blastomeres would be applied to DNA chips for genetic screening.

The culture system for the establishment of ES cell lines was applied to proliferate isolated blastomeres in our trial. It indicated that ES cell lines might be established from isolated blastomeres without donor enucleated oocytes. In future babies conceived after PGD could have their own ES cell lines at birth.

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