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Isolation and Culture of Human Embryonic Stem-like Cells from Abnormal Blastocysts

Chun Kyu Lim¹, Ji Hye Sung¹, Jong Hyuk Park², Sun Jong Kim²,
Hyun Soo Yoon², Mi Kyoung Koong¹, Jin Hyun Jun¹

¹Laboratory of Reproductive Biology & Infertility, Samsung Cheil Hospital & Women's Healthcare Center,
Sungkyunkwan University School of Medicine, Seoul, Korea

²Division of Stem Cell Biology, Medical Research Center, MizMedi Hospital, Seoul, Korea

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(n=20)
immunosurgery
PMEF STO feeder
Miz-hES1 cell line
colony
colony가
Miz-hES1 cell line
karyotyping (46, XY)
immunophenotyping (positive in SSEA-3 and -4)
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Key Words: Human ES cells, Abnormal blastocyst, Undifferentiated hES cell, Normalcy of blastocyst

Thomson et al (1998) firstly established a human embryonic stem (hES) cell line, which have the potential to form virtually any cell type in the body

and can be propagated *in vitro* indefinitely in an undifferentiated state.¹ Several studies were reported that establishment of hES cell lines and it could be

100-380 1-19,
Tel: (02) 2000-7590, Fax: (02) 2265-5621, e-mail: junjh55@hanmail.net
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differentiated into various cell types, such as pancreatic cells, neural cells, endothelial cells and cardiomyocytes by various conditions of *in vitro* culture.²⁻⁷ These characteristics of hES cells indicate that they are valuable and useful for regenerative medicine, pharmacokinetic screening, functional genomics and also researches on the development and differentiation mechanisms of specific organs and tissues.

For the functional analysis of specific genes *in vivo*, knock-out mouse models have been produced by screening and cloning of knock-out ES cells.^{8,9} However, the functional analyses of human genes are limited by the species difference between mouse and human. We speculate that *in vitro* differentiation of knock-out human ES cells may provide the advanced knowledge of functional genomics in human system.

In human IVF-ET program, normal zygotes present two pronuclei (2-PN) after *in vitro* fertilization, however, a few zygotes show one (1-PN) or more than three pronuclei (3-PN) as abnormal fertilization. Pre-implantation genetic diagnosis (PGD) has been widely applied for couples with high risk of offsprings with chromosomal aberrations or genetic defects.^{10,11} After PGD, normal embryos are transferred to the mother's uterus, and abnormal embryos are further cultured or analyzed. These abnormally fertilized and aneuploid embryos may develop to the blastocyst stage and the abnormal conceptus.

This study was performed to evaluate the effect of chromosomal abnormality on the establishing efficacy of hES cell line from abnormal blastocysts, and to derive the defective hES cell line as a pilot model of knock-out human ES cell line.

MATERIALS AND METHODS

1. Collection of human blastocysts

Human zygotes and pre-implantation embryos were donated for use in this study following informed consent by couples undergoing IVF-ET program. After *in vitro* fertilization, the PN status of zygote

observed with an inverted microscope and classified 2-PN as normal and 1-PN or 3-PN as abnormal fertilization. The abnormally fertilized zygotes were cultured to blastocysts with the sequential media, G1.2 and G2.2 medium (Vitrolife Inc, Sweden). In the PGD using fluorescent *in situ* hybridization (FISH), the blastomere biopsy and diagnosis of biopsied nucleus were undergone by previously described methods.^{10,11} The embryos with normal or balanced FISH signals were transferred to the mother's uterus and abnormal embryos were cultured to the blastocyst with the sequential media.

2. Isolation and culture of inner cell mass

The inner cell mass (ICM) of blastocyst was isolated by immunosurgery as previously described.^{12,13} Briefly, blastocysts were treated with anti-human whole serum (Sigma, USA) for 10–15 minutes, followed by 5–10 minutes exposure to guinea pig complement (Life technologies, Germany) in 20 μ l droplets at 37 °C in 5% CO₂. Isolated ICMs were cultured with Dulbecco's modified Eagle medium (DMEM) high glucose containing 20% fetal bovine serum or serum replacement, 1 mM glutamine, 0.1 mM β -mercaptoethanol, 1% non-essential amino acids, 4 ng/ml human basic fibroblast growth factor, 100 U/ml penicillin G and 100 μ g/ml streptomycin on the feeder cells. Primary mouse embryonic fibroblast (PMEF) or STO feeder layer which was treated 10 μ g/ml mitomycin C for mitotic inactivation, were used as feeder cells. The mitomycin C- treated PMEF or STO cells were extensively washed with PBS and replated at 75,000 cells/cm² on gelatin- coated tissue culture dishes. To prove our facility of hES cell culture system, already established Miz-hES1 cell line was cultured on the same condition for fresh isolated ICMs, simultaneously. We subcultured the hES cell colonies by mechanical dissociation using a micropipette every 7 days.

3. Characterization of undifferentiated hES cells

Alkaline phosphatase (APase) activity is one of the

features of undifferentiated cells. For APase localization and immunocytochemistry, cultured hES cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -PBS and then fixed with 4% paraformaldehyde at 4 °C for 30 minutes. APase staining was performed using a kit containing NBT/BCIP as the substrate (Roche, Germany). The dark blue staining was visualized by light microscopy and it was considered positive. Cell surface monoclonal antibodies, SSEA-1, SSEA-3 and SSEA-4, were purchased from the Developmental Studies Hybri-

doma Bank (University of Iowa, USA). The antibodies were diluted with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -PBS containing 1% BSA to block non-specific binding. Primary antibodies were localized using biotinylated secondary antibody, followed by a complex of avidin and horseradish peroxidase and a Vector NovaRED or DAB substrate kit (Vector laboratory, USA). The red staining was considered positive under a light microscope (Nikon, Japan).

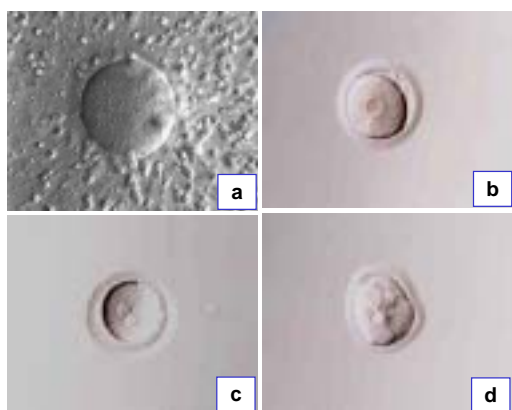


Figure 1. The oocyte-cumulus complex of a mature oocyte (metaphase-II), 1-pronucleus (1-PN), 2-pronucleus (2-PN) and 3-pronucleus (3-PN) zygote in human IVF-ET program.

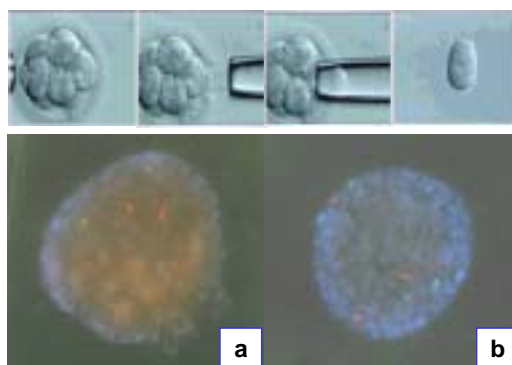


Figure 2. Blastomere biopsy procedure of PGD and FISH signals. (a) A normal nucleus with two green (TelVision 5p) and two orange (TelVision 5q) signals, and (b) an abnormal nucleus with three green and three orange signals in a chromosomal translocation case.

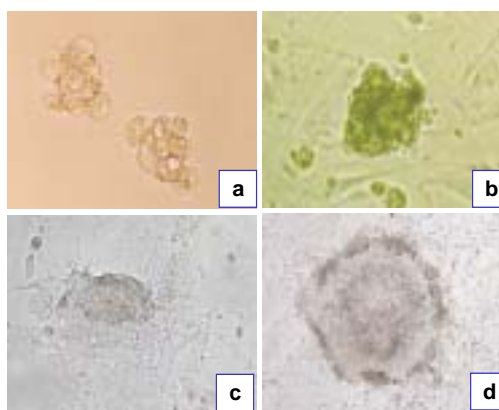


Figure 3. Isolation and culture of ICMs and the Miz-hES1 cell line. (a) Isolated ICMs by immunosurgery, (b) cultured an ICM after 2 days, (c) a hES cell-like colony from a blastocyst of abnormal zygote after 6 days of culture, (d) morphology of undifferentiated Miz-hES1 cell line during subculture.

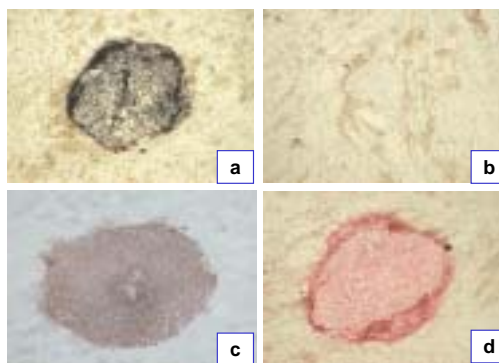


Figure 4. Characteristics of undifferentiated Miz-hES1 cell line after subculture. (a) Localization of alkaline phosphatase and immunohistochemistry of (b) SSEA-1, (c) SSEA-3 and (d) SSEA-4.

RESULTS

In human IVF-ET program, oocyte-cumulus complexes (OCCs) are aspirated from ovarian follicles, and then they are fertilized by conventional insemination or intracytoplasmic sperm injection. Observation of pronucleus status is essential step for selection of normal zygotes with two pronuclei (2-PN) from both parents. We selected 1-PN and 3-PN zygotes, and they were cultured blastocysts. The OCC, 2-PN, 1-PN and 3-PN zygote are illustrated in Figure 1. A total of 20 blastocysts from abnormal zygotes were obtained and used for this study. After immunosurgery, we could not find any ICMs in some blastocysts (n=7) from abnormal zygotes, although the morphology of blastocysts was fair. Isolated ICMs were cultured on the mitotically inactivated PMEF or STO cells. Only one hES cell-like colony was formed from an abnormal zygote, and the colony was dissociated and mechanically disaggregated using a micropipette after 6 days of culture. The colony was not maintained further culture, and we failed to establish the hES cell line from abnormally fertilized blastocysts.

Blastomere biopsy procedure of PGD and FISH signals of blastomeres' nuclei are shown in Figure 2. After FISH, the aneuploid embryos were cultured to blastocysts. A total of 27 blastocysts from aneuploidy embryos were prepared for isolation of ICMs. The isolated ICMs from aneuploid blastocysts were quietly small and poor. Two cell colonies of isolated ICMs were subcultured three times, respectively, but they did not show any morphological characteristics undifferentiated hES cells, such as Miz-hES1 cell line. The colony was transformed trophoblast-like cells after passage number three, and we could not establish the hES cell line from aneuploid blastocysts.

Established Miz-hES1 cell line (passage number 45) was provided from MizMedi Hospital, Seoul. Miz-hES1 cell line was maintained its characteristics of undifferentiated hES cells after more than 30 passages of subculture in our facility. They showed

normal karyotypes (46, XY) and positive APase activity. In the immunophenotyping of the subcultured hES cells, SSEA-3 and SSEA-4 were stained red, while SSEA-1 was not detected (Figure 4).

DISCUSSION

The knock-out or aneuploid hES cell line may provide the advanced knowledge of development and differentiation of specific tissues and organs in human system. Blastocysts from abnormal zygotes and aneuploid embryos are occasionally observed in human IVF-ET program.^{14,15} In this study we want to establish the hES cell line from abnormally fertilized and aneuploid blastocysts as a pilot trial for the establishment of knock-out human ES cell line. However, the ICMs from abnormal blastocysts showed very low potency of establishing hES cell line.

Blastocysts from abnormal fertilized zygotes had well differentiated trophoblasts and expanded blastocoels, but its ICMs were poor. After immunosurgery, we could not find any specific cell clusters as ICMs, it might be related to the normal trophoblast development and differentiation of tetraploidy embryos in mouse and rat.^{16,17} In human, anembryonic pregnancy, only trophoblasts, was clinically occurred and reported.^{18,19} One ES cell-like colony was formed from isolated ICM but it was not maintained undifferentiation status after subculture. Generally, haploid or polyploid embryos cannot survive to offsprings in human because they have many defects in tissues and organs. Thus hES cell lines with haploidy or polyploidy might be useful for the human developmental biology.

The detection of chromosomal abnormalities in human preimplantation embryos and PGD using FISH has been successfully applied for couples who are at high risk of transmitting chromosomal abnormalities to their offsprings.^{8,9} We have reported the successful outcomes and more than 90% reliability of diagnosis by PGD-FISH.^{20,21} Two ICMs among 27 aneuploid

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