2	111	1	1	irrad-HPF	WB	20% SR+bFGF	Genbacev *et al.* (2005)
3	7	3	0	mitoC-HUE	IS	20% SR+bFGF	Lee *et al.* (2005)
3	10	1	2	mitoC-STO	IS/WB	20% SR+bFGF	Oh *et al.* (2005)
1	5	1	0	free[e]	IS	8% SR+8% plasmanate+ LIF+bFGF	Klimanskaya *et al.* (2005)
2	19	1	1	mitoC-MEF	IS	20% FBS+bFGF	Chen *et al.* (2005)
2	55	0	2	irrad-MEF	IS	20% FBS+LIF+bFGF	Mateizel *et al.* (2005)
3	14	2	1	irrad-MEF	IS	20% SR+bFGF	Mateizel *et al.* (2005)
4	14	2	2	irrad-MEF	IS	20% SR+bFGF	Hong-mei and Gui-an (2006)
1	33	1	0	inact-MEF	WB	20% FBS+bFGF	Sun *et al.* (2006)
2	5	0	2[f]	free[g]	IS	TeSR1[h]	Ludwig *et al.* (2006)

[a] irrad, irradiated; mitoC, mitomycin C; MEF, mouse embryonic feeders; HFM, human fetal muscle; HFF, human foreskin fibroblasts; STO, STO cells; HPF, human placental fibroblasts; HUE, human uterine endometrial cells; IS, immunosurgery; WB, whole blastocyst; FBS, fetal bovine serum, HS, human serum; SR, Serum Replacement; ITS, insulin transferring selenium; HA, hyaluronic acid.

[b] Information not available from published sources.

[c] XX karyotype with a trisomy 13.

[d] One line was a triploid, 69,XXY.

[e] Mouse extracellular matrix coated.

[f] One line was a 47,XXY.

[g] Human extracellular matrix coated.

[h] TeSR1was highly defined medium, which was composed of a DMEM/F12 base supplemented with human serum albumin, vitamins, antioxidants, trace minerals, specific lipids, and growth factors.

Preparation of Mouse Embryo Fibroblasts (MEFs)

We use primary MEF cells, which have been mitotically inactivated by γ-irradiation, for derivation and propagation of hES cells. MEFs are harvested from 12.5-day postcoitum (dpc) fetuses of ICR mice (Cowan *et al.*, 2004). The following reagents are required to follow our protocol for preparing MEFs.

Sterile phosphate-buffered saline (PBS), pH 7.2
MEF medium (90% Dulbecco's modified Eagle's medium [DMEM], 10% fetal bovine serum [FBS], 50 units/ml penicillin, and 50 μg/ml streptomycin)
0.25% trypsin
0.1% gelatin (made by dissolving 1 g of gelatin in 1000 ml of Milli-Q quality water, followed by sterile filtering)
Freezing medium (90% FBS, 10% dimethyl sulfoxide [DMSO])
10- and 15-cm tissue culture dishes
Sterile single-edged razor blade

Dissection and Primary Culture of MEFs

Prior to dissecting the mouse embryos, several 15-cm tissue culture plates (seven to eight plates per pregnant ICR female) should be coated with 0.1% gelatin. We typically cover the plates with a minimal amount of the gelatin solution (5 to 7 ml) and incubate them for 20 min at 37° with 5% CO_2. Using a microscope placed in a laminar flow hood, 12.5-dpc embryos are dissected into a 10-cm Petri dish containing sterile PBS solution. The embryos are then stripped of any maternal or extraembryonic tissues and eviscerated. Eviscerated embryos are transferred to a 15-cm dish and, using a sterile blade, minced. Ten milliliters of warm 0.25% trypsin is added per 10 to 14 minced embryos and collected in a 50-ml conical tube. The embryos are homogenized further by trituration (pipetting up and down) until no large pieces remain. This partially dissociated mixture is then incubated at 37° for 1 min followed by further trituration (pipetting 5 to 10 more times). Forty milliliters of prewarmed MEF medium is added to the dissociated embryos and the mixture is centrifuged for 10 min at 500 to 600*g* at room temperature. Aspirate media and then resuspend the pelleted cells with 30 ml prewarmed MEF medium. Plating density is 1.5 to 2 embryos per 15-cm gelatin-coated plate. The final volume of medium on each plate should be 20 ml. The primary MEFs are incubated at 37° with 5% CO_2 until confluent (typically 5 to 6 days). MEFs are expanded once after the initial plating (1:3 to 1:5 split) and then frozen (passage 1). Freeze MEFs in freezing medium (90% FBS and 10% DMSO) at a rate of $-1°$/min and store at $-80°$ or in liquid nitrogen.

γ-Irradiation and Plating

Thawed MEFs are only passaged once (passage 2) for expansion purposes prior to γ-irradiation. MEFs are trypsinized and resuspended in a volume of MEF medium that will be accommodated by the γ-irradiator. Irradiate the MEFs for 25 min at 247.3 rad/min for a total exposure of 6182.5 rad. After irradiation, spin cells in MEF medium for 5 min at 500 to 600*g*. To ensure a confluent monolayer, plate MEFs at a concentration of approximately 50,000 cells/cm^2. If there is no immediate need for mitotically inactivated MEFs, they can be frozen at a concentration of 4×10^6 to 1.2×10^7 cells/vial. MEFs feeder layers should be prepared and used within 3 days.

Preparing hES Derivation Medium

During the isolation and early stages of ES cell cultivation, hES derivation medium is used, which consists of 75% knockout DMEM (Invitrogen GIBCO), 10% KO-Serum Replacement (Invitrogen GIBCO), 10% plasmanate (Bayer), 5% fetal bovine serum (Hyclone), 2 m*M* Glutamax-I (Invitrogen GIBCO), 1% nonessential amino acids (Invitrogen GIBCO), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen GIBCO), 0.055 m*M* β-mercaptoethanol (Invitrogen GIBCO), 12 ng/ml recombinant hLIF (Chemicon International), and 5 ng/ml bFGF (Invitrogen GIBCO). The medium is sterilized by 0.22-μm filtration. Screening of FBS, plasmanate, and Serum Replacement should be done and is described elsewhere (Klimanskaya and McMahon, 2004).

Isolation of Inner Cell Mass

Fresh or frozen-thawed human embryos are cultured to the blastocyst stage in sequential media, G1.2 and G2.2 (Gardner *et al.*, 1998). We have derived several new human ES cell lines at relatively higher efficiency from blastocysts cultured in modified KSOM media. Blastocysts are treated with acid tyrodes (Specialty Media) for 30 to 90 s to dissolve the zona pellucida. When the zona pellucida starts to dissolve, remove the embryo and wash it three times in fresh hES derivation medium. The zona-stripped embryos are then cultured in hES derivation medium at 37° with 5% CO_2 until immunosurgical isolation of the ICM. The process of immunosurgery includes several stages and is performed essentially as described by Solter and Knowles (1975). Initially, the embryo is incubated for approximately 30 min in rabbit antihuman RBC antibodies (resuspended as per manufacturer's instructions, aliquoted, and stored at −80°, freshly diluted 1:10 in derivation medium, Inter Cell Technologies). Penetration of the antibodies into the blastocyst is prevented because of cell–cell connections within the outer layer of the

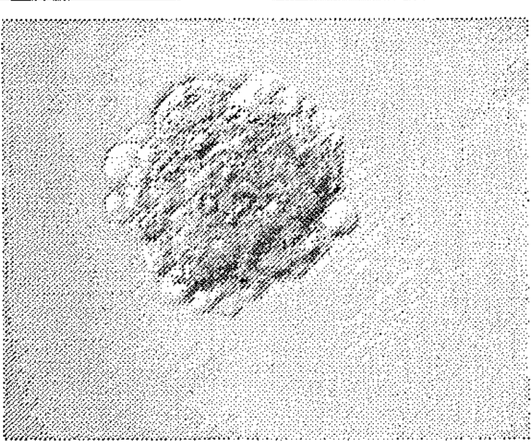

FIG. 1. "Bubbling" of trophoblast cells. Blastocyst after exposure to guinea pig sera complement is lysed and stop the incubation followed by removing the lyses trophoblast cells.

trophoblasts, leaving the ICM intact. After rinsing off any antibody residue (at least three washes with hES derivation media), the blastocyst is transferred into a guinea pig sera complement (resuspended as per manufacturer's instructions, aliquoted, and stored at −80°, freshly diluted 1:10 in derivation medium, Sigma), diluted in hES derivation medium, and incubated until cell lysis is notable, indicated by an apparent "bubbling" of the trophoblast cells (Fig. 1). Following selective removal of the trophectoderm cells by gentle mouth pipetting of the embryo in and out of a glass capillary, the intact ICM is cultured on MEF feeders plated on gelatin (Sigma)-coated tissue culture plates at a density of approximately 50,000 cells/cm^2. After 2 days add a few fresh drops of hES derivation medium and then every other day change one-half the total medium (e.g., for 500 μl total medium and then remove 250 μl of medium and add 250 μl of fresh medium to a final volume of 500 μl).

Dispersion of Inner Cell Mass

Six to 10 days after the initial plating, ICM outgrowths require mechanical dissociation. Two to three pieces are cut from the initial outgrowth using a narrow glass capillary and are left in the same well or moved to a new well (Fig. 2). When doing the initial dispersion, a part of the original colony should be left untouched as a backup, especially if the picked pieces are transferred into a new well. At this stage, it is better to concentrate on expanding the number of hES cell colonies versus freezing or proceeding to any downstream experiments. When the colonies are growing steadily, FBS is omitted from the culture media. Usually, mechanical passaging needs to be done every 5 to 6 days, but several larger colonies may need to be dispersed daily.

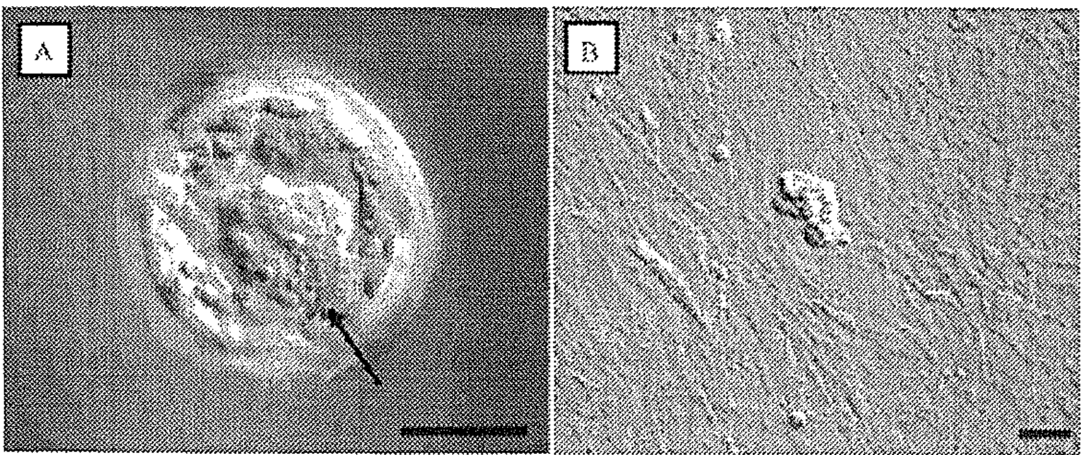

FIG. 2. Blastocyst and ICM outgrowth. (A) Cultured blastocyst is grade 4AA. Arrow indicates ICM. (B) Isolated ICM from the blastocyst (A) is just grown ICM at day 4 after plating on mitotically inactivated MEFs. Black bar: 50 μm.

Maintenance of hES Cells

Variability among human ES cell lines has been reported by several groups, including differences in growth characteristics, differentiation potential, karyotype, and gene expression pattern. In part, these differences might reflect the genetic heterogeneity of hES cell lines derived, as they are from a genetically diverse, outbred population (Abeyta *et al.*, 2004; Bhattacharya *et al.*, 2004). Further confounding researchers is the fact that human ES cell cultures are often heterogeneous because they contain both undifferentiated stem cells and spontaneously arising differentiated derivatives. While no single uniform protocol exists for the maintenance of hES cells in culture that adequately addresses all researchers' concerns, we will attempt to present an overview of the techniques currently used by a number of laboratories around the world. Again, we describe in detail our method for maintaining undifferentiated hES cell growth in culture and briefly review several alternative protocols.

Enzymatic Dissociation with Trypsin

Human ES cell colonies are passaged by mechanical dissociation until there are sufficient colonies (50 to 100 average-sized colonies) or cells (usually 1×10^5 cells) to passage enzymatically. Thereafter, hES cells are propagated by enzymatic dissociation with 0.05% trypsin/EDTA (Invitrogen GIBCO). During the first three passages with trypsin, it is a good idea to keep a backup well of mechanically passaged cells. A mechanical backup should always be maintained until the cells are frozen. Subconfluent cultures are generally split at a 1:3 ratio (i.e., one culture well is split into three new culture wells). It is important to split colonies prior to excessive differentiation.

Materials

For the routine culture of hES cell by enzymatic dissociation with trypsin we recommend the following media and reagents.

hES medium (80% knockout DMEM, 10% KO-Serum Replacement, 10% plasmanate, 2 m*M* Glutamax-I, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.055 m*M* β-mercaptoethanol, and 5 ng/ml bFGF
Trypsin 0.05%
Sterile PBS, pH 7.2.

Trypsinization

1. Warm hES medium and trypsin in a 37° water bath and keep them warm until ready for use.
2. Place MEF plate from incubator in the hood and aspirate off the medium from the well followed by 1 ml prewarmed hES medium. Set the plate aside in the hood.
3. Carefully aspirate the hES medium from the culture to be split. Gently rinse the cells with a sufficient volume of PBS to completely cover the bottom of the culture dish (e.g., 5 ml for a 10-cm dish).
4. Aspirate the PBS and add a small volume of trypsin (usually 0.3 ml for a 35-mm well or 2 ml for a 10-cm dish) to the cells. Incubate in the hood at room temperature, frequently checking the cells under the microscope. MEFs surrounding the colonies should begin to retract (Fig. 3). When the MEFs are sufficiently shrunk and the borders of the colonies are roughly rounded up, add 10 volumes of prewarmed hES medium to the trypsinized colonies. Gently pipette up and down five to seven times until the MEF monolayer has completely detached. Extensive pipetting should be avoided.
5. Aliquot the hES cell solution dropwise, making sure to distribute the drops evenly about the well. Without shaking the plate, carefully return to the cells to a 37° incubator overnight to let the colonies seed.

The time in trypsin required for the cells to detach varies depending on the hES cell density, age of MEF monolayer, etc. We recommend checking the appearance of the hES culture under a stereomicroscope and determining the best incubation time for each well empirically.

Freezing hES Cells

1. Trypsinize the cells; see trypsinization section. Centrifuge the cells at 600*g* in 10 volumes of hES culture media.
2. Resuspend the pellet in cold freezing medium, which consists of 90% FBS and 10% DMSO.

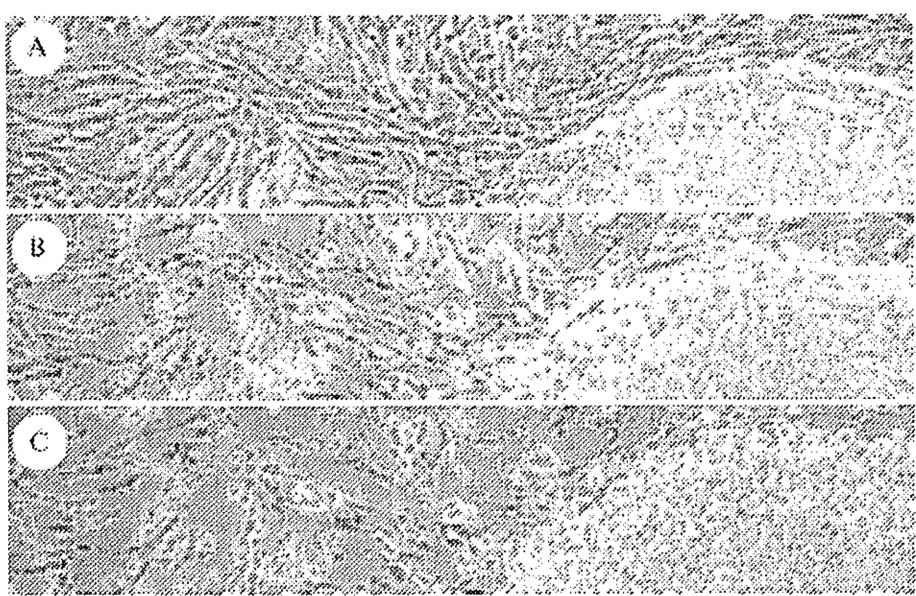

FIG. 3. Time-lapse series of photographs showing dissociation of hES cells and MEF feeder layer with trypsin. (A) Prior to addition of trypsin. (B) Approximately 30 s after addition of trypsin. (C) Approximately 60 s after addition of trypsin. Trypsinization should be stopped when cells appear as in C.

3. Aliquot the cell suspension into prechilled freezing vials and sandwich the vials between two Styrofoam racks, taping to prevent them from separating. Transfer to a −80° freezer overnight. Cryovials should be placed in liquid nitrogen for long-term storage.

Thawing hES Cells

Ensure that the MEF plate prepared is confluent and in good condition before thawing hES cells. Prewarm hES medium to 37°. Aliquot 10 ml hES medium into a sterile and labeled 15-ml conical tube for each cell line.

All procedures should be done quickly.

1. Thaw the vial in a 37° water bath. (Do not overthaw; the vial should be removed from the water bath with a small ice crystal still remaining.) It should take about 45 to 60 s before the cells are 80% thawed.
2. Bring the tube to a laminar flow hood; spray down with 70% isopropanol. Gently transfer cells to the 10 ml of prewarmed medium.
3. Centrifuge the 15-ml conical tube at 500 to 600*g* for 5 min.
4. Remove preplated MEFs from incubator to the hood. Aspirate off the MEF medium and aliquot prewarmed hES medium into each well of the plate, being careful not to disturb the attached MEFs.
5. After the spin is complete, carefully remove the medium without disturbing the pellet.

6. Gently resuspend the pellet in a small volume of prewarmed hES medium.

7. Transfer the hES cell solution, in a dropwise manner, to a prepared MEF plate well that already contains hES medium. Carefully return the plate to avoid swirling to a 37° incubator overnight to allow the hES cells to seed the MEFs.

8. The colonies usually begin to appear in 3 to 4 days and can be ready for splitting in 5 to 10 days.

Alternative Methods

Several alternative methods exist for the culture of hES cells, but few have been examined rigorously over a long period of time. We will attempt to summarize some of the more common alternative methods for maintaining hES cells in culture. For detailed protocols, we advise referring to the primary literature. In addition, several alternatives, such as feeder and serum-free culturing of hES cells, are described elsewhere in this volume.

Dissociation with Collagenase or Dispase

Quite possibly the most widespread method for maintaining hES cells in culture depends on their dissociation with either collagenase or dispase. For a detailed protocol, please see http://www.geron.com/PDF/scprotocols.pdf. The reported advantages of culture with these enzymes are reduced cell death and perhaps greater karyotypic stability. The disadvantages of enzymatic dissociation with collagenase or dispase include the inability to accurately assess cell number and the failure to generate definitive single cell clones.

Culture with Human Feeder Cells

Mouse embryonic fibroblast cells have generally been used as feeder layers to support the unlimited growth of hES cells, but the use of animal feeder cells is associated with risks such as pathogen transmission and viral infection (Amit *et al.*, 2003, 2004; Richards *et al.*, 2002; Rosler *et al.*, 2004). Martin *et al* (2005) reported that hES cells could incorporate foreign sugars into the glycoproteins on the cell surface. They also showed that an immune reaction could occur following exposure of the cells to serum from adults with high level of the antibody. These reports and other concerns have prompted many researchers to seek alternatives to mouse feeder layers.

Several groups have reported that feeder layers composed of cells originating from human fetal and adult tissues support unlimited proliferation of hES cells without differentiation. The cell types used include human

fetal skin fibroblasts, human muscle cells, adult fallopian tubal epithelial cells (Richards *et al.*, 2002), adult marrow cells (Cheng *et al.*, 2003), foreskin fibroblasts (Amit *et al.*, 2003; Hovatta *et al.*, 2003), human uterine endometrium cells, and breast parenchyma cell abortus fetus fibroblasts (Lee *et al.*, 2004). In perhaps the most comprehensive study, Richards *et al.* (2003) reported on the evaluation of various human adult, fetal, and neonatal tissues as feeder cells for supporting the growth of hES cells. In addition, feeder cells derived from hES cells can be used as an autogenic feeder system that efficiently supports the growth and maintenance of pluripotency of hES cells (Stojkovic *et al.*, 2005; Yoo *et al.*, 2005).

Conclusion

Human ES cells are viewed by many as a novel and unlimited source of cells and tissues for transplantation for the treatment of a broad spectrum of diseases (reviewed by Keller, 2005). Moreover, human ES cells represent an unprecedented system suitable for the identification of new molecular targets and the development of novel drugs, which can be tested *in vitro* or used to predict or anticipate potential toxicity in humans. Finally, human ES cells can yield insight into the developmental events that occur during human embryogenesis, which are, for ethical reasons, nearly impossible to study in the intact embryo (reviewed by Dvash and Benvenisty, 2004).

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Possible Involvement of CD81 in Acrosome Reaction of Sperm in Mice

MAYA TANIGAWA,[1,2] KIYOKO MIYAMOTO,[1] SATOSHI KOBAYASHI,[3] MASAHIRO SATO,[4] HIDENORI AKUTSU,[1] MASARU OKABE,[2,5] EISUKE MEKADA,[3] KEIICHI SAKAKIBARA,[1] MAMI MIYADO,[1] AKIHIRO UMEZAWA,[1] AND KENJI MIYADO[1,6,7]*

[1]*Department of Reproductive Biology and Pathology, National Center for Child Health and Development, Tokyo, Japan*
[2]*Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan*
[3]*Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
[4]*Frontier Science Research Center, Kagoshima University, Kagoshima, Japan*
[5]*Genome Information Research Center, Osaka University, Osaka, Japan*
[6]*PREST, Japan Science and Technology Agency, Saitama, Japan*
[7]*School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan*

ABSTRACT Tetraspanin CD81 is closely homologous in amino acid sequence with CD9. CD9 is well known to be involved in sperm–egg fusion, and CD81 has also been reported to be involved in membrane fusion events. However, the function of CD81 as well as that of CD9 in membrane fusion remains unclear. Here, we report that disruption of the mouse CD81 gene led to a reduction in the fecundity of female mice, and CD81–/– eggs had impaired ability to fuse with sperm. Furthermore, we demonstrated that when CD81–/– eggs were incubated with sperm, some of the sperm that penetrated into the perivitelline space of CD81–/– eggs had not yet undergone the acrosome reaction, indicating that the impaired fusibility of CD81–/– eggs may be in part caused by failure of the acrosome reaction of sperm. In addition, we showed that CD81 was highly expressed in granulosa cells, somatic cells that surround oocytes. Our observations suggest that there is an interaction between sperm and CD81 on somatic cells surrounding eggs before the direct interaction of sperm and eggs. Our results may provide new clues for clarifying the cellular mechanism of the acrosome reaction, which is required for sperm–egg fusion. *Mol. Reprod. Dev.* © 2007 Wiley-Liss, Inc.

Key Words: CD9; acrosome reaction; fertilization; mice; zona pellucida

INTRODUCTION

Fertilization is accomplished by the direct interaction of sperm and eggs, a process mediated primarily by predicted, but yet unidentified gamete membrane proteins. In fertilization, the acrosome reaction is a change in sperm that is required for penetration into the zona pellucida, the egg coat, and facilitates the subsequent fusion with the egg plasma membrane (Moreno and Alvarado, 2006). Zona pellucida protein 3 (ZP3), one of the components forming the meshwork of the zona pellucida, has been considered to be the prime physiological inducer of the acrosome reaction in sperm, although the frequency of acrosome reaction is low after incubation with recombinant ZP3 (Beebe et al., 1992). This discrepancy suggests that, besides ZP3, unknown major factor(s) might be responsible for the acrosome reaction. To date, despite the importance of the acrosome reaction in fertilization, the underlying cellular mechanisms that regulate the acrosome reaction remain unclear.

Two tetraspanins, CD9 and CD81, are known to be important in the membrane fusion events in various biological systems. In virus-host cell fusion, human CD81 has been identified as a co-receptor for hepatitis C virus (Higginbottom et al., 2000; Cormier et al., 2004). Both CD9 and CD81 have been implicated in myoblast fusion (Tachibana and Hemler, 1999; Schwander et al., 2003) and monocyte/macrophage fusion in mice (Takeda et al., 2003). Recent studies using gene-targeting techniques demonstrated that female mice carrying a deletion of the CD9 gene produce eggs that mature normally but are defective in sperm–egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Takahashi et al., 2001). CD81 has also been reported to be expressed on the plasma membrane of unfertilized mouse eggs (Takahashi et al., 2001). Furthermore, CD81–/– mice have been reported to have defects in reproduction after several generations of backcrossing (Deng et al., 2000).

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*Correspondence to: Kenji Miyado, National Center for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan. E-mail: kmiyado@nch.go.jp

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Recently, Rubinstein et al. (2006) provided more detailed data showing that eggs of CD81−/− mice are unable to be fertilized with sperm, although the degree of the defect appeared not to be severe compared with that of CD9−/− eggs. Moreover, injection of CD9−/− eggs with mouse CD81 mRNA revealed that mouse CD81 was only moderately effective at reversing the infertility of CD9−/− eggs (Kaji et al., 2002). These findings taken together indicate that CD81 and CD9 each have different roles in fertilization.

Here we studied the role of CD81 in fertilization by in vitro fertilization (IVF) and immunohistochemical analysis, and propose a possible role of CD81 in the acrosome reaction in sperm.

MATERIALS AND METHODS

Animals

CD81−/− mice (Miyazaki et al., 1997) were kindly provided by Dr. Miyazaki and were backcrossed to C57BL/6 mice. Genotyping was carried out using polymerase chain reaction as previously described (Miyazaki et al., 1997). To visualize acrosome-intact sperm, EGFP-transgenic mice expressing EGFP in the acrosomes were generated by pronuclear injection of constructs carrying the EGFP gene driven by the mouse acrosin promoter (Nakanishi et al., 1999) and the DsRed2 gene tagged with a mitochondrial transport signal and driven by the CAG promoter into fertilized eggs of BDF1 mice (unpublished information). After the sperm were acrosome-reacted, EGFP was lost from the sperm heads and DsRed remained in the mitochondria of the mid-piece region. All animal procedures were performed according to protocols approved by the National Center for Child Health and Development and use committees.

Egg Collection

Female mice (aged 8–15 weeks) were injected with 5 U of hCG (Gonatropin; Aska Pharmaceutical Co., Ltd, Tokyo, Japan) 48 hr after administration of 5 U of PMSG (Serotropin; Aska Pharmaceutical Co., Ltd). Ovulated eggs were collected from the oviductal ampulla 13.5–15 hr after hCG injection, and placed in 100-µl drops of TYH medium equilibrated with 5% CO_2 in air at 37°C. Cumulus cells were removed with 300 IU/ml of hyaluronidase (H-3506, Sigma-Aldrich, Missouri, MO), and eggs were incubated with a defined number of sperm.

Sperm Preparation and In Vitro Fertilization

Sperm were collected by squeezing two cauda epididymides of 8- to 10-week-old B6C3F1 or transgenic male mice in a well containing 100-µl of TYH medium. Sperm were incubated at 37°C in 5% CO_2 for 90 min before being mixed with eggs derived from wild-type or CD81−/− female mice. The final concentration of sperm added to an egg-containing drop was 1.5×10^5 sperm/ml. To examine the rate of fertilization, we counted the number of eggs at the two-cell stage 24 hr after incubation with the sperm. For counting the number of fused sperm, the zona pellucida was removed from the eggs by a brief incubation in acid Tyrode solution, and sperm were incubated with eggs preloaded with 4′,6-diamidino-2-phenylindole (DAPI) for counting the number of sperm fused with eggs (Yamagata et al., 2002). For counting the number of acrosome-intact sperm, EGFP-expressing sperm were incubated with zona-intact CD81+/+ or CD81−/− eggs. The eggs were all subjected to confocal microscopic analysis for the presence of sperm exhibiting red and green fluorescence or red fluorescence alone within the perivitelline space 4 hr after incubation.

Immunostaining

For immunostaining of cryostat sections, ovaries from 8- to 10-week-old wild-type C57BL/6 females were fixed in 2% paraformaldehyde in PBS (−) for 2 days at 4°C, and then immersed in 30% sucrose in PBS (−) for more than 2 days at 4°C, embedded in Tissue-Tek OCT compound (Sakura Finetek Co., Tokyo, Japan), and finally frozen before serial cryostat sectioning (8 µm in thickness). Slides were fixed in an acetone and incubated with anti-CD81 antibody (Eat-1) diluted 1:300 in PBS (−) containing 0.1% bovine serum albumin (BSA), BSA/PBS (−), or anti-ZP3 antibody diluted 1:300 in BSA/PBS (−), overnight at 4°C. After washing three times with BSA/PBS (−), the samples were incubated with Alexa 546-conjugated goat anti-hamster IgG (A-21111, Invitrogen, California, CA) or Alexa 488-conjugated goat anti-rat IgG (A-11006, Invitrogen) for 2 hr at room temperature. After extensive washing, the slides were inspected for fluorescence using LSM 510 META confocal microscope.

Immunoblotting

Samples containing equal amounts of eggs were dissolved in nonreducing sample buffer and subjected to 12% SDS–PAGE according to procedures described previously (Miyado et al., 2000). After electrophoresis, the gels were transferred to PVDF membranes for immunoblot analysis. The blots were blocked in 1% nonfat dry milk, and were probed with the primary antibodies, anti-mouse CD81 antibody (Eat-1, BD Biosciences, California, CA) or anti-mouse CD9 antibody (KMC8, BD Biosciences). After washing in TBS-Tween buffer, the membranes were incubated with HRP-labeled secondary antibodies; goat anti-rat antibody or goat anti-hamster antibody. The expression level of immunoreacted products was determined by treatment of the blots with an ECL or ECL Plus Detection Kit (GE Healthcare Bio-Science Co., New Jersey, UK) and exposure to X-ray film at room temperature.

Statistical Analysis

Data from different groups were tested by the t-test for the significance of the difference between the means of two independent samples using the computer software KaleidaGraph (version 3.6, Synergy Software, Inc., Pennsylvania, PA).

RESULTS

Female Fertility Impaired by CD81 Deficiency

Figure 1A depicts the average litter size in matings of three genotypes of females, wild-type, CD81+/− and CD81−/− mice, with CD81+/− males over a period of 6 months. Although these females displayed normal mating behavior with the males (data not shown), the average litter size of CD81−/− females was markedly reduced relative to those of CD81+/− and wild-type mice (on average, 1.3 ± 2.5 vs. 11.3 ± 1.3 and 11.0 ± 0.8) (Fig. 1A). To examine the oocyte maturation and ovulation, we also collected the eggs from mice superovulated by stimulation with exogenous gonadotropin. The eggs collected from CD81−/− mice were indistinguishable with regard to morphology and number (on average, 18.0 ± 2.8) from those wild-type and CD81+/− mice (on average, 19.9 ± 1.7 and 21.7 ± 2.8) (Fig. 1B). Therefore, the reduction in fertility of CD81−/− females did not seem to be due to defects of ovulation or oocyte maturation.

Involvement of CD81 in Fertilization

To clarify the cause of the reduced fertility of CD81−/− females, the function of CD81−/− eggs was further examined by IVF. When cumulus oocyte complexes (COCs) collected from CD81−/− or wild-type control mice were incubated with the wild-type sperm, the sperm could disperse cumulus cells, somatic cells surrounding eggs, and reach and apparently penetrate the zona pellucida of CD81−/− and wild-type eggs. However, the average rate of eggs developing to the two-cell stage was substantially decreased for CD81−/− eggs (on average, 15.0 ± 2.5%) compared with that for wild-type eggs (on average, 65.0 ± 10.8%) 24 hr after incubation with the sperm (Fig. 1D). Furthermore, in CD81−/− eggs, several sperm were observed in the perivitelline space (Fig. 1C). The delayed formation of two-cell embryos and the accumulation of more than one sperm within the perivitelline space in CD81−/− eggs demonstrate that CD81−/− eggs have impaired ability of fertilization. Subsequently, to examine the cause of the impaired fertilization, we performed IVF for CD81−/− eggs and wild-type eggs after the zona pellucida was removed using acid Tyrode solution (Fig. 2A,B). To measure the number of sperm fused with eggs, both types of eggs were preloaded with DAPI before incubation with wild-type sperm (Yamagata et al., 2002). One hour after insemination, estimation of the average number of sperm fused with one egg by measurement of DAPI fluorescence revealed that CD81−/− eggs showed a decreased number of fused sperm (on average, 1.21 ± 0.23) in comparison with the wild-type eggs (on average, 1.95 ± 0.27). Those results suggest that CD81 is involved in sperm–egg fusion, either directly or indirectly.

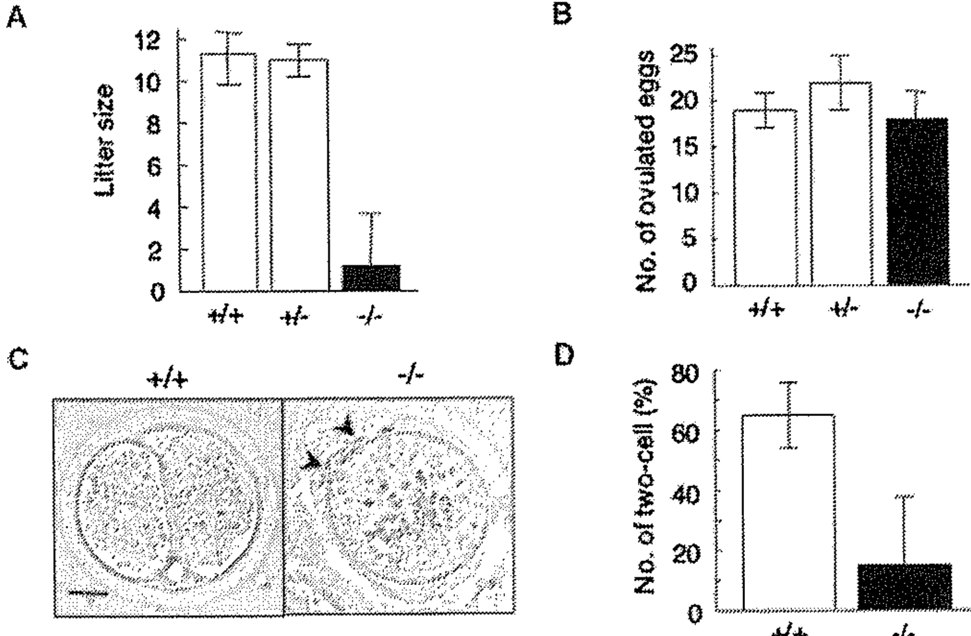

Fig. 1. Female infertility caused by CD81 deficiency. A: Average litter sizes of crosses between CD81+/− males and three types of females, wild-type, CD81+/− and CD81−/− mice. Each of the mating pairs was kept in a separate cage, and births over a 6-month period were monitored. Data of births during successive 2-month periods were grouped together, and the average litter size of wild-type, CD81+/− and CD81−/− females was calculated from data recorded for five mating pairs 8–15 weeks of age at the start of the experiment. B: Average number of ovulated eggs from wild-type, CD81+/− and CD81−/− female mice. The eggs were collected 13.5–16 hr after hCG treatment, and counted. C: Representative micrographs of CD81+/+ and CD81−/− eggs. The eggs were obtained 24 hr after incubation with the wild-type sperm. D: Average number of eggs that developed to the two-cell stage 24 hr after incubation with the wild-type sperm. The black bars show the results for CD81−/− eggs (A,B,D). Error bars represent SEM (A,B,D). Scale bar, 20 μm (C).

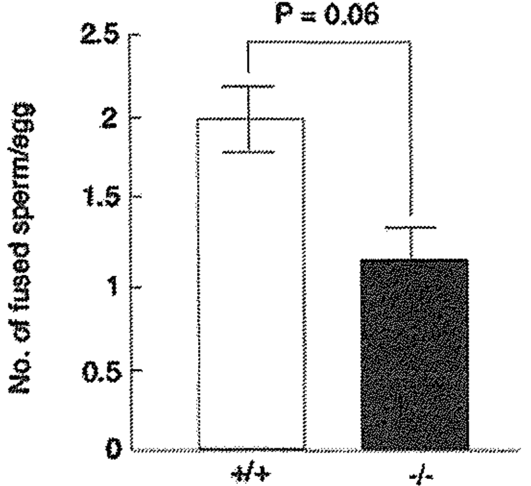

Fig. 2. In vitro sperm–egg fusion. Average number of sperm fused with wild-type or CD81−/− eggs after 3 hr of incubation. Error bars represent SEM. Data from different groups were tested by the *t*-test for the significance of the difference between the means of two independent samples ($P = 0.06$).

Expression of CD9 in CD81−/− Eggs

The mechanisms by which CD81 acts are still unclear. However, CD81 tends to form multimolecular complexes in which tetraspanins associate with specific proteins depending on the type of cell. In B cells, CD81 directly associates with CD19, taking part in the CD19-CD21-CD81 signaling complex (Pileri et al., 1998), which accords with the evidence that the expression of CD19 in bone marrow, spleen, and peripheral B cells is reduced in CD81−/− mice (Miyazaki et al., 1997). As previously mentioned, CD9 on the egg plasma membrane is required for fusion with sperm, and the impaired fusibility of CD81−/− eggs with sperm may likely be dependent on the expression of CD9. To investigate whether CD81 deficiency may cause downregulation of CD9 expression, the expression level of CD9 was examined (Fig. 3). We collected three types of eggs,

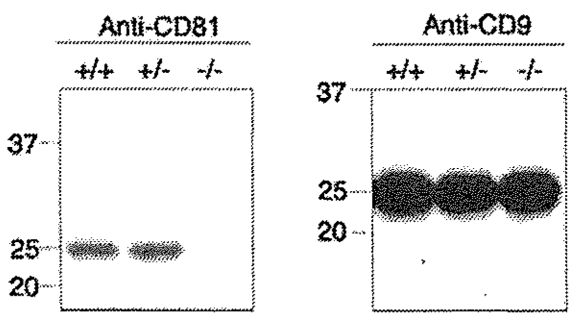

Fig. 3. The expression of CD9 in wild-type, CD81+/− and CD81−/− eggs. Proteins were isolated from the types of eggs indicated and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions. The proteins were electrophoretically transferred to a membrane, where they were probed with antibodies to CD81 (panel A) and CD9 (panel B). The proteins corresponding to each 110 egg (panel A) and 10 eggs (panel B) were analyzed.

wild-type, CD81+/− and CD81−/− eggs, 13.5–15 hr after hCG injection into mice, and examined the expression level of CD9 in comparison with that of CD81 by SDS–PAGE. The amounts of CD81 were invariable in wild-type and CD81+/− eggs, but CD81 was lost in CD81−/− eggs. By contrast, there were no significant differences in the expression of CD9 among these eggs. Therefore, the impairment of fertilization caused by CD81 deficiency cannot be attributed to decreased expression of CD9 in eggs.

Expression of CD81 During Folliculogenesis

The expression and localization of CD81 in ovarian tissues were immunohistochemically assessed using cryostat sections of adult wild-type ovaries. The follicles consist of immature eggs and granulosa cells that surround the egg; a single follicle usually grows to the preovulatory stage and releases the egg for potential fertilization (Buccione et al., 1990). Immunohistochemical staining with anti-CD81 mAb demonstrated that CD81 was continuously expressed in the egg and surrounding follicles (Fig. 4), and in cumulus cells surrounding ovulated eggs (data not shown). These data indicate that the sperm may encounter CD81 on the somatic cells surrounding eggs before direct interaction of sperm and eggs.

Possible Involvement of CD81 in Acrosome Reaction

Based on the localization of CD81 and the impaired fertilization of CD81−/− eggs, we speculated that the inability of wild-type sperm to fuse CD81−/− eggs might be due to impairment of prefusional stages, including the acrosome reaction. To examine the involvement of CD81 in the acrosome reaction of the sperm, CD81−/− eggs or wild-type eggs were incubated with the sperm collected from transgenic mice specifically expressing enhanced green fluorescent protein (EGFP) in the acrosomes (Fig. 5). The acrosome corresponds functionally to a lysosome and thus contains lysosomal enzymes (Moreno and Alvarado, 2006), and acrosin is a sperm acrosomal serine proteinase that is lost from the sperm head after the acrosome reaction is completed (Baba et al., 1994). Therefore, sperm expressing EGFP at the acrosomes in the heads are useful for the detection of acrosome-intact sperm. After 3 hr of incubation, we counted the number of acrosome-intact sperm within the perivitelline spaces. To count the sperm that had penetrated into the zona pellucida, the eggs were incubated with 3.0×10^5 sperm/ml. When the number of sperm within the perivitelline space were counted 3 hr after incubation with the eggs, we observed that an increased percentage ($8.5 \pm 2.3\%$) of the sperm that had penetrated into the perivitelline space of CD81−/− eggs exhibited EGFP fluorescence in their head portion. In contrast, very few sperm that had penetrated into the perivitelline space of wild-type eggs exhibited green fluorescence ($1.4 \pm 1.0\%$). These results suggest that the sperm that penetrated into the zona pellucida of the CD81−/− eggs were impaired in the acrosome reaction.

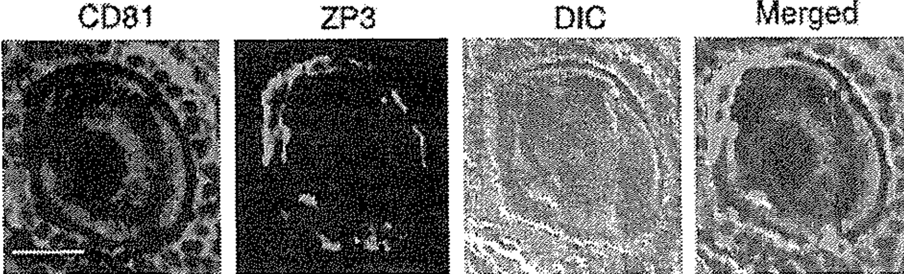

Fig. 4. CD81 is expressed at high levels in granulosa cells during oogenesis. Frozen sections of ovaries from wild-type mice were stained with anti-mouse CD81 mAb and with anti-ZP3 mAb. DIC represents a photograph taken by differential interference contrast. Scale bar, 20 μm. [See color version online at www.interscience.wiley.com.]

DISCUSSION

CD81 has been suggested to be a protein playing a role in membrane fusion events, but the function of CD81 in sperm–egg fusion remains unknown. As suggested by Rubinstein et al. (2006), CD9 and CD81 may have different roles in sperm–egg fusion. This notion is supported by the following facts: (1) deletion of a single gene, CD9 or CD81, causes impaired fertilization, and the expression of CD9 on eggs is not perturbed by CD81 deficiency, and (2) CD9−/− eggs injected with mRNA encoding CD81 cannot be fully rescued to the same degree as those injected with CD9 mRNA (Kaji et al., 2002).

Generally, the acrosome reaction is a change in the membrane of sperm that are activated for penetration into zona pellucida and facilitates the subsequent fusion with the egg membrane (Baba et al., 1994). During the acrosome reaction, the disruption of the acrosome covering the sperm head causes the release of acrosin and other proteolytic substances. As previously reported (Moreno and Alvarado, 2006), these materials included in the acrosome are important for the penetration of sperm into the zona pellucida and for sperm–egg fusion, but the molecular mechanism underlying the acrosome reaction is largely unknown. When wild-type eggs were incubated with sperm expressing EGFP in the acrosomes, we found the presence of acrosome-intact

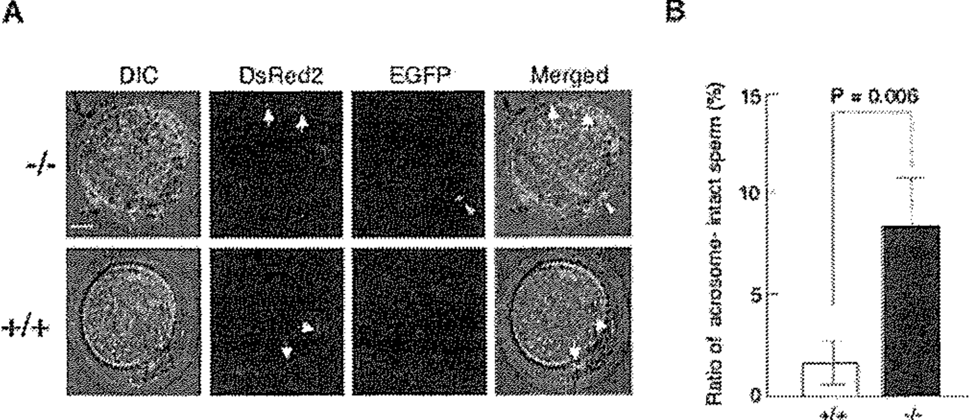

Fig. 5. In vitro fertilization assay for acrosome reaction. A: Representative photographs. CD81−/− eggs were incubated with transgenic sperm expressing EGFP at acrosomes in the sperm heads. Eggs from wild-type females were also subjected to fertilization using the AR-GFP transgenic sperm as controls. Four hours after insemination, the eggs were inspected for fluorescence using a confocal microscope. As shown in the upper panel, some CD81−/− eggs had sperm with green fluorescence (indicated by arrowheads) in their head region in the perivitelline space, while almost no wild-type eggs had such types of sperm (lower panel). Photomicrographs taken under light (DIC); photomicrographs taken for detecting DsRed2 translocated to mitochondria by the retention signal (Mt-DsRed2) and specifically expressed in the mid-piece of sperm (indicated by arrows); photomicrographs taken for detecting EGFP-derived green fluorescence specifically expressed in the head region of sperm (indicated by arrowheads); merged images. Scale bar, 20 μm. B: Examination of acrosome reaction using EGFP-expressing sperm. CD81−/− or wild-type eggs were fertilized in vitro with epididymal sperm expressing EGFP in the acrosomes. Four hours after insemination, the sperm entering into the perivitelline space were inspected for fluorescence using a confocal microscope. Note that the number of sperm carrying intact acrosomes (exhibiting green fluorescence in the sperm head region, as shown in A) and entering into the perivitelline space of CD81−/− eggs was significantly higher than that of acrosome-intact sperm entering into the perivitelline space of wild-type eggs. Acrosome-intact sperm can easily be detected since they exhibit bright green fluorescence in their head region. The total number of sperm entered into perivitelline space can be counted by inspection for red fluorescence in the mid-piece of the sperm. [See color version online at www.interscience.wiley.com.]

sperm in the outer layer of the zona pellucida (data not shown), but almost all sperm that penetrated into the perivitelline space had lost the acrosome caps (Fig. 5). These findings suggest that the acrosome reaction may occur in the perivitelline space and/or inner layer of the zona pellucida.

Another possible reason for the failure of the acrosome reaction of EGFP-expressing sperm in CD81−/− eggs is that "zona hardening" in CD81−/− eggs may not be sufficient compared to that in wild-type eggs. The weakened zona hardening might permit the penetration of some acrosome-intact sperm into CD81−/− eggs. However, since proteins other than components forming the zona pellucida may be triggers for preventing polyspermy and zona hardening (Sun, 2003), it would be of interest to test whether CD81 and ZP3 interact with each other.

In conclusion, the results of our IVF experiments suggest the possible involvement of CD81 in the acrosome reaction of zona pellucida-penetrated sperm prior to the fusion of sperm with eggs. Extensive attempts to elucidate the role of CD81 in the acrosome reaction are now underway.

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Human ES cells research progress in USA

阿久津英憲(写真)　梅澤明弘
Hidenori AKUTSU and Akihiro UMEZAWA
国立成育医療センター研究所生殖医療研究部

◎ヒト胚性幹細胞は無限増殖能と分化多能性というユニークな性質をもち，基礎医学研究から再生医療応用へと大きな可能性を社会へ提示している．しかし，この細胞は胚を破壊して作成するために，社会性の強い研究分野である．アメリカにおけるヒト胚性幹細胞研究の法的および倫理的枠組みとその現状について述べてみたい．

Key word：ヒトES細胞，アメリカ，再生医療，生命倫理

胚性幹細胞(embryonic stem cells：ES 細胞)はある特定の培養環境下で2つのユニークな性質を同時に保持している細胞である．ひとつは際限なく増殖するということ(自己増殖能)であり，もうひとつはすべての成体細胞に分化する能力(分化多能性)があるということである[1]．これらの特性により，ES 細胞は Parkinson 病や糖尿病などの変性疾患に対する細胞移植，または脊髄損傷，心筋梗塞などの組織損傷に対する組織再生に必要な細胞の無尽蔵な供給源となりうる．

アメリカでは慢性変性疾患に罹患している患者が約1億2,500万人にのぼり，その経済的支出は全医療費の80%近くを占めている．多くの人びとが有効な治療法を待ち望んでいるなか，ヒト ES 細胞の再生医療応用へ大きな期待が寄せられている．ある調査結果ではアメリカ国民の約60%は ES 細胞を用いた再生医学研究を支持している[2]．1990年代後半からの5年間に，体細胞クローン動物作製[3]，ヒト ES 細胞樹立の成功[4]，そしてヒトゲノム解読(概要版)[5,6]など，生命化学に関する大きな研究成果が立て続けに報告され，幹細胞研究も難治疾患治療に対する医療的関心のみならず，経済，法律，生命倫理，政治，宗教と連関して社会全体での関心が非常に高まっていった．そのなかでアメリカでのヒト ES 細胞研究はどのような状況にあり，どこへ向かおうとしているのか．著者は2004～2005年にハーバード大学にてヒト ES 細胞研究に携わってきたので，そのときの経験も交えアメリカでのヒト胚性幹細胞研究事情を述べていきたい．

ヒト ES 細胞の樹立には必然的にヒト胚の破壊を伴うため，その是非には個人的・宗教的，そして文化的な信条によってさまざまな見解がある．アメリカではヒト ES 細胞研究が政治的駆け引きの場に持ち込まれ，実際に ES 細胞研究のおかれている状況や方向性は新聞の見出しからだけでは非常にわかりにくい．ヒト ES 細胞が樹立されたのは1998年で，まだ10年もたっていないが，その研究支援政策はその間の2回の大統領選挙を経て大きく変動している．そこで本稿では，ジョージ・W・ブッシュ大統領が就任した2001年1月を起点に述べていきたい．

ヒトES細胞をめぐる状況——1998～2001年

それまでマーモセット，サルで ES 細胞樹立に成功していたアメリカ・ウィスコンシン大学のトムソン博士らが1998年にヒト ES 細胞の作成を報告した[4]．当時ヒト胚を用いた研究には政府資金

援助が認められていなかったので(The Dickey Amendment；研究目的のためにヒト胚を作出することやヒト胚を滅失して行う研究にはNIH研究資金が認められない．1997〜2005年有効)，トムソン博士らは，バイオベンチャー企業のGeron Corp.とthe Wisconsin Alumni Resarch Foundationの援助のもと，ヒトES細胞研究を行う研究室を別個に立ち上げ，NIH研究費フリーの施設でヒトES細胞樹立を成功させたのである．すぐさまクリントン大統領は国家生命倫理諮問委員会に対し，ヒトES細胞研究に関連する課題について倫理的・医学的観点をバランスさせた綿密なレビューを下命した．国家生命倫理諮問委員会のヒトES細胞研究に関する倫理的側面の考察結果の答申を受けて，2000年8月に国立衛生研究所(National Institutes of Health：NIH)が行政指針“National Institute of Health Guidelines for Research Using Human Pluripotent Stem Cells”によりヒト幹細胞研究に対する資金提供の容認を発表した．これは胚を滅失させるヒトES細胞樹立にはNIH研究資金は許可しないが，私的資金を用いて樹立されたヒトES細胞を使用する研究にはNIH研究資金を提供できるというものであった．つまり，研究者はヒトES細胞使用研究に対してNIH研究費を獲得し，研究を進めることが可能になったわけである．ちなみに，一般的にアメリカでの研究者の研究費・人件費は約50%がNIHの研究費より捻出されている．NIH研究費が申請できない分野では当然，研究の進歩や後進の育成などが滞る可能性が出てくる．

クリントン大統領はヒトES細胞の件より以前の1997年に，国家生命倫理諮問委員会に対しクローニング技術の利用に関する法的・倫理的課題のレビューを諮問しており，ヒトクローニングの生殖応用を禁止する大統領令を発している．

“National Institute of Health Guidelines for Research Using Human Pluripotent Stem Cells”を受けて，NIHにはヒトES細胞研究の研究費申請が集まってきたが，その年の大統領選挙のキャンペーン期間中，ブッシュ大統領候補はヒト胚の破壊のうえに成り立つES細胞研究に対して政府資金援助を与えない方針だと声高に訴えていた．2000年の大統領選挙は近年まれにみる激戦の末，共和党のブッシュ大統領が第43代大統領に就いたのである．

この時期，実際の研究領域では，最初のヒトES細胞樹立の報告以後ようやく2番目のヒトES細胞樹立が2000年にシンガポールとオーストラリアの共同研究グループから報告された．2001年ごろまではヒトES細胞に関しては樹立・培養に関する報告が大半を占めていたが，それ以降，徐々にヒトES細胞に関する研究報告が活発になってきた．

ヒトES細胞をめぐる状況——2001〜2005年

2001年1月，ブッシュ大統領が就任すると，政権の宗教的理念を背景にした生命倫理観がヒトES細胞研究政策に投影されてきた．4月，NIHは予定していた第1回のヒトES細胞研究の研究費申請レビューを中止した．メディアでもヒトES細胞研究に関する注目が集まってきたが，科学的な関心に加え，ヒトES細胞を樹立する際に利用する初期胚に関して宗教観の相違からくる“ヒト生命のはじまり”論争が再燃してきた．そして僅差勝負だった大統領選挙を引きずった民主党と共和党との対立にも利用された感が出てきた．難治疾患の患者グループ，科学者，そしてバイオテクノロジー産業界はヒトES細胞研究を認めるように，反対に中絶反対グループやカトリック教会はヒトES細胞研究を認めないようにと，激しいロビー活動が起こった．その間，ヒトES細胞研究自体は推進すべきであるとして，ブッシュ大統領の共和党内部からもヒトES細胞研究を支持する大物議員も出てきた．

8月，最終的にブッシュ大統領は全国民に向かってテレビ演説を行い(http://www.whitehouse.gov/news/releases/2001/08/20010809-2.html)，その時点で世界中よりNIHに登録された64ヒトES細胞株を用いた研究にのみ政府資金を援助するという決定を下した．しかし，研究者にとっては不満の残る決定であった．なぜなら，実際に研究に利用可能な質をもったNIH登録ES細胞は約20%の細胞株数で，多くの研究者が利用できる数ではなかった．さらに，アメリカ国内で樹立し