The Establishment of Mouse Embryonic Stem Cell Cultures on 96-Well Plates for High-Throughput Screening

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Embryonic stem (ES) cells can be valuable for monitoring differentiation processes and for improving applications in basic developmental biology. The application of ES cells can be a useful tool for drug discovery and toxicology. Therefore, we suggest the high-throughput screening (HTS) system based on ES cells in this study. Firstly, we optimized the feeder-free condition and seeding cell number which can be maintained for at least 7 days without over-confluency. We analyzed the system by cell viability, proliferation activity, RT-PCR and morphologic/immunohistochemical evaluations. The optimal cell seeding number was 30/well that was maintained the typical colonial morphology over 9 d with 1,000 U/ml LIF in the limited space. The cell in optimized condition expressed ALP, SSEA-1, Oct 4 and Nanog and the genetic expressions showed similar to protein expressions. The cell lineage marker expressions showed faint or none. The cell viability and proliferation activity were increased in time-dependent manner in our optimized HTS system. In conclusion, the novel HTS system using ES cells can be useful for developing models for drug discovery as well as toxicological screening in the near future.

INTRODUCTION

ES cells are derived from the inner cell mass of the mammalian blastocyst, and were initially harvested from a mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). The cells are pluripotent, immortal, and can be cultured in undifferentiated conditions. First isolated in 1998, human ES (hES) cells were shown to retain the same plasticity as mouse ES (mES) cells (Thomson and Marshall, 1998; Thomson et al., 1998).

ES cells provide a dynamic system suitable for the identification of new molecular targets and development of novel drugs, which can be tested in *in vitro* culture models. In general, cells used in culture models for drug discovery and development are derived from primary tissues such as tumors or immortalized cell lines. These cell lines are poor functional models, however,

because the cells are genetically abnormal, often having an aneuploid karyotype. Additionally, these cells typically have a phenotype unrelated to the tissue of interest, even before immortalization. Primary tissues are genetically diploid, but their survival time is limited *in vitro* (Davila et al., 2004; Pouton and Haynes, 2005). Stem cells, on the other hand, offer considerable advantages compared with tumor-derived immortalized cells or cells derived from primary tissues. Stem cells are genetically normal, demonstrate uniform physiological responses, can be maintained for long periods of time, and are harvested in large numbers. These unique strengths of ES cells afford the ability to monitor the cell differentiation processes and improve applications in basic developmental biology (Anderson et al., 2004; Bremer et al., 1999).

Biomedical research on the efficiency and toxicity of new drug candidates requires reproducible *in vitro* assay systems. The field of stem cell research is also in need of an effective molecule or material that can control self-renewal and the pluripotent capacity of stem cells. High-throughput screening (HTS) has been a successful technique in that it enables rapid and quantitative study of the interactions between cells and their microenvironments as a preliminary screening. In general, this technique requires three integrated high-throughput processes: library synthesis, data acquisition, and data processing and modeling (Jost et al., 2005; Pepperkok and Ellenberg, 2006). The use of 96-well-microtiter plates allows the performance of HTS experiments, though these plates do not provide the optimal conditions necessary for mES cell culture. Determining optimal conditions, including the ideal cell number per well on 96-well plates, is a prerequisite for the application of HTS in mES cells.

Typically, mES cells are sub-cultured every 3–4 days. However, the cells may be maintained in confined spaces, such as in 96-well plates, for longer periods (at least 7 days) without losing genetic or physiologic characteristics.

In this study, we developed optimal culture conditions and a system for analyzing mES cells on 96-well microtiter plates that maintain cellular pluripotency for HTS. This was made possible by the previously described experiment which supported moni-

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toring of the differentiation pathways of mES cells using specific growth factors, cytokines, and transcription factors. We found that the number of ES cells per well plays an important role in the cultivation of undifferentiated cells and for analyzing the physiology and characteristics of cellular reproduction.

MATERIALS AND METHODS

Mouse ES cell culture without a feeder layer

The E14TG2a (ATCC, USA) mES cell line was cultured on a feeder layer of primary mouse embryonic fibroblasts (MEF), which were pretreated with mitomycin-C for 1.5 h and then transferred to 35 mm 0.1% gelatin covered plates without feeder cells. Feeder-free cultured mES cells were plated onto 96-well 0.1% gelatin covered plates, each containing one of four different cellular concentrations (1,000, 100, 50, and 30 cells/well). The feeder-free mES cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 15% fetal bovine serum (FBS; Hyclone, USA), 1% non-essential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma-Aldrich, USA), 1% (v/v) penicillin-streptomycin (Invitrogen), and 1,000 U/ml recombinant mouse leukemia inhibitory factor (LIF; Chemicon, USA) in a 5% CO₂ incubator at 37°C. The ES cell culture medium was changed daily. mES cells were subcultured every 4 days using 0.05% trypsin-EDTA (Invitrogen).

Experimental design

In the first set of experiments, we compared the maintenance efficiencies of four different cellular concentrations (1,000, 100, 50, and 30 cells/well). Cultured E14 mES cells were analyzed for expression of markers of differentiation, ALP activity, and RT-PCR. In a second set of experiments, we examined the viabilities of different batches of mES cells which were cultured on 0.1% gelatin covered 96-well plates. Cultured E14 mES cells were examined for cell viability, cell proliferation (BrdU), and growth rate.

Immunofluorescence staining for Oct-4, Nanog, and stage-specific embryonic antigen-1 (SSEA-1)

E14 mES cell colonies were fixed in 96-well culture dishes using 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) for 10 min at room temperature, followed by 2 PBS washes (5 min each). Cells were permeabilized with 0.1% Triton X-100 for 10 min, washed 3 times with PBS, blocked with normal goat serum (Jackson Immunoresearch Laboratory, USA) for 60 min, and incubated with primary antibodies overnight at 4°C. The primary antibodies used included Oct-4 at a dilution of 1:100 (polyclonal rabbit IgG; USA), Nanog at a dilution of 1:500 (polyclonal rabbit IgG; Abcam, UK), and stage-specific embryonic antigen-1 (SSEA-1) at a dilution of 1:60 (monoclonal mouse IgM; Chemicon, USA). The cells were washed 3 times with PBS (5 min each) and probed with secondary antibodies including Alexa Fluor 488 goat anti-rabbit IgG at a dilution of 1:500 (Molecular Probes, USA) or goat anti-mouse IgM for 1 h at room temperature. After incubation with the secondary antibody, cells were washed 3 times with PBS (5 min each). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The labeled cells were viewed and photographed using a Nikon Eclipse TE2000-U fluorescence microscope and a JENA ProgRes camera.

RNA preparation and gene expression analysis

The pluripotency of mES cells cultured on 96-well plates was determined by RT-PCR analysis. Total RNA was isolated from

cells (1,000, 100, 50, and 30 cells/well) using TRIzol reagent (Invitrogen). The concentration and quality of isolated RNA were determined using an ND-1000 Spectrophotometer (Nano Drop Technologies, USA). Complementary DNA synthesis was performed using an M-MLV Reverse Transcriptase Kit (Bioneer, Korea) with oligo-dT primers. One microliter of cDNA was used as a template in each PCR reaction. The negative control contained sterilized water instead of the RNA template. PCR reactions were carried out in an Eppendorf Mastercycler (Eppendorf AG, Germany) as follows: denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s followed by final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing 0.4 μ g/ml ethidium bromide (Sigma-Aldrich) and a DNA standard.

Alkaline phosphatase activity

ALP activity was detected using an ES Cell Characterization Kit (Chemicon). The E14 mES cell colonies were fixed in 96-well culture dishes with 4% paraformaldehyde in PBS (0.01 M, pH 7.4) for 10 min at room temperature, followed by 2 PBS washes (5 min each). Cells were stained with naphthol/Fast Red Violet solution [Fast Red Violet (FRV) with naphthol AS-BI phosphate solution and water at a 2:1:1 ratio] for 5-10 min and then washed 3 times with PBS. The cells were viewed and photographed with a Nikon Eclipse TE2000-U microscope and a JENA ProgRes camera.

MTT assay

Cell proliferation was quantified by colorimetric analysis based on the metabolic cleavage of the tetrazolium salt MTT (WST-1) in viable cells. A commercial MTT (WST-1) kit (EZ CyToX; Daeil, Korea) was used. ES cell viability was examined 3, 5, 7, and 9 days after culture onto 96-well plates. A 10 μ l aliquot of WST-1 solution was added to each well. Absorbance was measured at 450 nm after a 2 h incubation using a Bio-Rad microplate reader Model-550 (Bio-Rad, USA). Error bars represent the SD from 3 independent measurements. The result was analyzed statistically by ANOVA and *t*-test.

BrdU assay

We performed a colorimetric immunoassay of cell proliferation using Cell Proliferation ELISA and BrdU (colorimetric) Kits (Chemicon) according to the manufacturer's instructions. This test measures BrdU incorporation during DNA synthesis. Samples of E14 ES cells were cultured on 96-well plates. After 3, 5, 7, and 9 days, wells were incubated in a medium containing BrdU for 20 h. The absorbance was measured at 450 nm using a Bio-Rad microplate reader Model-550 (Bio-Rad). Error bars represent the SD from 3 independent measurements. The result was analyzed statistically by ANOVA and *t*-test.

RESULTS

Morphological changes of mES cells cultured on 96-well plates

The mES cells maintained under feeder-free conditions exhibited normal cell proliferation. After being transferred to 0.1% gelatin-coated 96-well plates, the four different cellular concentrations (1,000, 100, 50, and 30 cells/well) attached to the wells and formed mES colonies. The wells with 1,000 mES cells/well did not form colonies, but did show overgrowth 3 days after seeding (Table 1). Wells with 50 and 30 cells/well contained tightly packed colonies that maintained an undifferentiated

Table 1. Mouse ES cell attachment and colony formation after seeded on 0.1% gelatin coated 96 well plate

No. of mES cell seeded	Mean $\pm$ SD numbers of	
	mES cell attached	mES cell colony formed ^a
1,000/well	447.9 $\pm$ 46.1	-
100/well	27.1 $\pm$ 3.2	18.8 $\pm$ 3.0
50/well	14.4 $\pm$ 1.9	9.6 $\pm$ 1.7
30/well	7.8 $\pm$ 1.8	5.1 $\pm$ 1.1

^amES cell colonies were counted 3 d after seeded onto 96 well plate. Data are expressed as mean $\pm$ SD (n = 8).

morphology, even after 8 days. mES cells at 1,000 and 100 cells/well differentiated into a variety of cell types. These groups appeared as merged colonies with loss of their colonial shapes.

Expression of pluripotent cell-specific markers

The undifferentiated markers, ALP and SSEA-1, were expressed in the intact normal colonies of mES cells in the 30 cells/well group at 7 days (Figs. 1D and 1P). The other cellular concentrations showed weak expression with loss of colonies (Fig. 1). These characteristics were similar for Oct 4 and Nanog expression at 7 days in mES cells grown on 0.1% gelatin-coated 96-well plates for all four concentrations in a cell-number dependent manner (Figs. 2D and 2L).

Fig. 1. ALP and SSEA-1 expression in mES cells cultured on 96-well plates. Immunohistochemical staining for ALP and SSEA-1 expression in mES cells cultured in a concentration-dependent manner on 0.1% gelatin-coated 96-well plates. The expression of AP is shown at 7 days (A-D) and 8 days (E-H). The expression of SSEA-1 is shown at 7 days (I-L) and 8 days (M-P). Images are magnified at 100 $\times$.

Fig. 2. Oct 4 and Nanog expression in mES cells cultured on 96-well plates. Immunofluorescence staining for Oct-4 and Nanog expression in mES cells cultured in a concentration-dependent manner on 0.1% gelatin-coated 96-well plates. The expression of Oct-4 is shown at 7 days (A-D) and 8 days (E-H). The expression of Nanog is shown at 7 days (I-L) and the expression of SSEA-1 is shown at 8 days (M-P). Images are magnified at 100 $\times$.

Fig. 3. Genetic expression of mES cells cultured on 96-well plates. mRNA expression of undifferentiated stem cell markers and lineage specific markers was analyzed. (A) mES cells cultured for 7 days. (B) mES cells cultured for 8 days. α -SMA = α -smooth muscle actin; α -FP = α -fetoprotein.

Fig. 4. Growth rate of mES cells cultured on 96-well plates. Growth rates of mES cells cultured in a concentration-dependent manner on 96-well plates. Cells were counted every 2 days until day 9. (A) 1,000 cells/well, (B) 100 cells/well, (C) 50 cells/well, (D) 30 cells/well. Error bars represent the SD from three independent measurements.

Genetic expression

We confirmed the expression of transcripts for markers of undifferentiated and differentiated stem cells using RT-PCR (Fig. 3). The expression patterns of stem cell markers in the four groups were slightly different, though all markers were present in each group. Tissue specific analysis of marker expression revealed differentiated marker genes from all three embryonic germ layers including the mesoderm (desmin and α -smooth muscle actin), endoderm (albumin, α -fetoprotein), and ectoderm (Ncam and nestin). RT-PCR showed that all groups expressed nestin except the 30 cells/well group (cultured for 7 days). Ncam was not expressed in either the 30 or 50 cells/well groups cultured for 7 days. Desmin, albumin, and α -fetoprotein were not detected in any group. All groups expressed α -smooth muscle actin.

Growth rate, cell viability, and proliferation activity of mES cells cultured on 96-well plates

Growth rate, cell viability, and proliferation activity of mES cells cultured in a cell-number dependent manner were measured 1, 3, 5, 7, and 9 days after plating. The growth rates of the 1,000 and 100 cells/well groups peaked at 5 days, but the 30 and 50

cells/well groups increased their total cell numbers in a time-dependent manner with a peak at 7 days (Fig. 4). Cell viability of the 1,000 and 100 cells/well groups, as measured by MTT, showed confluence at 5 days and 7 days, respectively (Figs. 5A and 5B). The viability of the 50 cells/well group was similar to that of the 100 cells/well group (Fig. 5C). Only the 30 cells/well group showed an increase in cell viability in a time-dependent manner when continuously cultured for up to 9 days (Fig. 5D).

Cell proliferation activity peaked on day 3 in the 1,000 cells/well group and on day 5 in the 100 cells/well group (Figs. 6A and 6B). The 30 cells/well group showed an increase in cell proliferation activity until day 7 (Fig. 6D).

Cell growth, cell viability, and cell proliferation activity assays indicated that mES cells in the 30 cells/well group showed optimal conditions for HTS for up to 7 days under feeder-free conditions.

DISCUSSION

In this study, we determined the optimal culture conditions for undifferentiated mES cells cultured in limited space under

Fig. 5. Cell viability assay. Cells were plated at 1,000, 100, 50, or 30 cells/well on 96-well plates and were cultured for 9 days. MTT assays were measured every 2 days until day 9. The relative absorbance measured at 450 nm is shown for each group: (A) 1,000 cells/well, (B) 100 cells/well, (C) 50 cells/well, (D) 30 cells/well. Error bars represent the SD from three independent measurements.

Fig. 6. Cell proliferation assay. Cells were plated at 1,000, 100, 50, or 30 cells/well on 96-well plates and were cultured for 9 days. BrdU assays were measured every 2 days until day 9. The relative absorbance measured at 450 nm is shown for each group: (A) 1,000 cells/well, (B) 100 cells/well, (C) 50 cells/well, (D) 30 cells/well. Error bars represent the SD from three independent measurements.

feeder-free conditions for facilitating HTS. It is important that similar conditions be reproduced in order to maintain the characteristics of mES cells in limited spaces, and to compare both expression characteristics and cellular morphology. We started with a standard number of 1,000 cells/well on a 96-well micro-titer plate (0.32 cm²), which is equivalent to the culture conditions when using a typical 35 mm (9.5 cm²) plate (5,000 cells in 1 cm²) under feeder-free conditions. The seeded cells were then reduced in a cell number-dependent manner to 100, 50, and 30 cells/well to determine the optimal cellular concentration

for maintaining the pluripotent characteristics of ES cells for at least 7 days. In screening pharmaceutical or toxicological effects of newly developed drugs on mES cells, the length of the testing period is critical. For this reason, we developed optimal HTS conditions for mES cells for a duration of 7 days.

Our results for the 1,000 cells/well group showed that these cells were unable to maintain their typical morphology for 7 or more days. Immunohistochemistry and RT-PCR analysis showed that mES cell pluripotency markers disappeared at these later time points. Furthermore, cell numbers and viability plateaued

earlier in the high-density cell cultures, with proliferation peaking at 3 days and decreasing rapidly thereafter. These data indicate that conditions similar to normal mES cell culture conditions are not suitable for the confined spaces of the 96-well plates needed for HTS. Therefore, a decreased number of cells per well was necessary. When the cell seeding number was decreased ten-fold, the results were similar to those seen under the standard culture conditions. Cells seeded in even smaller numbers such as 50 or 30 cells/well attached successfully and were maintained as undifferentiated mES cells for 7 days. Generally, mES cells are subcultured every 3-4 days, but this culture period is typically not long enough for the monitoring of a testing reagent or toxicant. We found that 30 mES cells per well is optimal for HTS for 7 days.

Cell viability and proliferation assays were successfully used to monitor HTS with mES cells in our study. The 30 mES cells/well group showed a continuous spontaneous increase in cell numbers and proliferation during the 7 days experimental period. The mES cell colony formation rate in the 30 cells/well group was 17% (5.1/30), and each well successfully formed colonies. An mES cell density that is too low may result in insufficient levels of ES cell colony formation, maintenance of the mES cells in the undifferentiated condition on a feeder-free culture system, and empty wells that cannot produce colonies. Conversely, an mES cell density that is too high (100 cells/well) may result in the physical hindrance of mES cell proliferation and increases in ES colony differentiation rates. Our results clearly showed that both the 1,000, and 100 cells/well groups exhibited overgrowth that could not sustain stem cell pluripotency (Figs. 4-6).

Generally, cell lines such as HeLa or CHO are used for HTS (Kunapuli et al., 2005; Metzger et al., 2001; Tang et al., 2004). Because these cells are genetically and morphologically (Ding and Schultz, 2004; Rohwedel et al., 2001) altered and do not represent viable and primary mammalian tissues, studies performed using these cells are not as accurate as those done with primary tissues or stem cells. ES cells are a powerful tool for the development of novel therapies for regenerative medicine and animal biotechnology using transgenic technology. ES cells have revolutionized laboratory cell biology and are expected to provide much improved cell culture models for discovery and development of drugs, fundamental studies of the genetic basis of disease, and toxicity testing (Vogel, 1993). In the future, ES cells may be used to monitor cell differentiation processes and improve applications in basic developmental biology (Anderson et al., 2004; Bremer et al., 1999). While the use of hES cells may be safer and more effective for understanding human diseases, basic human developmental biology, and drug discovery, most ES cell studies to date have been done using mouse ES cells.

In this study, an optimal culture system for undifferentiated mES cells cultured on 96-well plates was developed as a tool for monitoring the interactions between cells and their microenvironments using a cell-based HTS assay. These HTS assays may serve as models for drug discovery and development, studies in cell biology, and toxicity testing. In particular, this culture condition is expected to improve monitoring systems for mES differentiation pathways using specific growth factors, cytokines, and transcription factors.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

- Anderson, D.G., Levenberg, S., and Langer, R. (2004). Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. *Nat. Biotechnol.* 22, 863-866.
- Bremer, S., van Dooren, M., Paparella, M., Kossolov, E., Fleischmann, B.K., and Hescheler, J. (1999). Effects of Embryotoxic Chemicals on the *In vitro* differentiation of genetically engineered embryonic stem cells into Cardiac cells. *Toxicol In Vitro* 13, 645-650.
- Davila, J.C., Cezar, G.G., Thiede, M., Strom, S., Miki, T., and Trosko, J. (2004). Use and application of stem cells in toxicology. *Toxicol. Sci.* 79, 214-223.
- Ding, S., and Schultz, P.G. (2005). Small molecules and future regenerative medicine. *Curr. Top Med. Chem.* 5, 383-395.
- Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-156.
- Jost, M.M., Budde, P., Tammen, H., Hess, R., Kellmann, M., Schulte, I., and Rose, H. (2005). The concept of functional peptidomics for the discovery of bioactive peptides in cell culture models. *Comb. Chem. High Throughput Screen.* 8, 767-773.
- Kunapuli, P., Zheng, W., Weber, M., Solly, K., Mull, R., Platchek, M., Cong, M., Zhong, Z., and Strulovici, B. (2005). Application of division arrest technology to cell-based HTS: comparison with frozen and fresh cells. *Assay Drug Dev. Technol.* 3, 17-26.
- Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* 78, 7634-7638.
- Metzger, R., Deglmann, C.J., Hoerlein, S., Zapf, S., and Hilfrich, J. (2001). Towards *in-vitro* prediction of an *in-vivo* cytostatic response of human tumor cells with a fast chemosensitivity assay. *Toxicology* 166, 97-108.
- Pepperkok, R., and Ellenberg, J. (2006). High-throughput fluorescence microscopy for systems biology. *Nat. Rev. Mol. Cell Biol.* 7, 690-696.
- Pouton, C.W., and Haynes, J.M. (2005). Pharmaceutical applications of embryonic stem cells. *Adv. Drug Deliv. Rev.* 57, 1918-1934.
- Rohwedel, J., Guan, K., Hegert, C., and Wobus, A.M. (2001). Embryonic stem cells as an *in vitro* model for mutagenicity, cytotoxicity and embryotoxicity studies: present state and future prospects. *Toxicol In Vitro* 15, 741-753.
- Tang, Y., Luo, J., Fleming, C.R., Kong, Y., Olini, G.C. Jr., Wildey, M.J., Cavender, D.E., and Demarest, K.T. (2004). Development of a sensitive and HTS-compatible reporter gene assay for functional analysis of human adenosine A2a receptors in CHO-K1 cells. *Assay Drug Dev. Technol.* 2, 281-289.
- Thomson, J.A., and Marshall, V.S. (1998). Primate embryonic stem cells. *Curr. Top Dev. Biol.* 38, 133-165.
- Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147.
- Vogel, R. (1993). *In vitro* approach to fertility research: genotoxicity tests on primordial germ cells and embryonic stem cells. *Reprod. Toxicol.* 7, 69-73.