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Quality management of stem cells and establishment of standard operating protocol for cell culture

줄기세포 품질경영 및 세포배양을 위한 표준 실험지침 확립

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Abstract

Of various kinds of contamination sources of cell culture, mycoplasma infection incurs unexpected or confused results in research such as cell proliferation, metabolism and differentiation. Also, it is not easy for researchers to become aware of the existence of mycoplasma except for direct mycoplasma detection. So, it is important to manage the research environment conformed with standard operation protocols (SOPs) in the grade of good laboratory practice (GLP) to ensure the maintenance and quality control of any contamination-free cell culture. In this study, to evaluate effects on the mycoplasma contamination in cell culture, we compared some experimental parameters between a mycoplasma positive (+) group and a mycompasma negative (-) group in culture of human induced pluripotent stem cells (hiPSCs). In addition, we observed effects of antibiotics treatments against mycoplasma using Plasmocin and BM-cyclin, in culture of several cell lines. In the results, (1) mycoplasma contamination interfered with the accurate measurement of proliferation rate and differentiation potential in hiPSCs. (2)

BM-cyclin antibiotics showed substantial improvement on mycoplasma elimination compared to Plasmocin antibiotics in hiPSCs culture. (3) We also detected a recontamination case in spite of mycoplasma elimination using BM-cyclin. To sum up, we confirmed that mycoplasma contamination in cell culture have negative effects, containing both common and uncommon things, on experimental results and have the riskiness of mycoplasma recontamination as well. Finally, we propose SOPs based on information acquired from literature review and experiments.

Keywords : Mycoplasma, mycoplasma detection, mycoplasma elimination, antibiotics, BM-cyclin, Plasmocin, antibiotic resistance, recurrence, human induced pluripotent stem cell, standard operating protocol.

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LIST OF ABBREVIATIONS

551-8 : detroit 551-iPS8

AA : antibiotic-antimycotic solution

AP : alkaline phosphatase

bFGF : basic fibroblast growth factor

CCK-8 : cell counting kit-8

DAPI : 4‘,6’-diamidine-2'-phenylindole dihydrochloride

DMEM : Dulbecco’s Modified Eagle’s Medium

DMEM/F12 : Dulbecco’s Modified Eagle’s Medium/F12

DMSO : dimethyl sulfoxide

DNA : deoxyribonucleic acid

DPBS : Dulbecco's phosphate buffered saline

DW : distilled water

E13.5 : embryonic day 13.5

EBs : embryonic bodies

ELISA : enzyme linked immunoassay

F5 : fib2-iPS5

FACS : fluorescence activated cell sorter

FBS : fetal bovine serum

FS-1 : foreskin 1

hESCs : human embryonic stem cells

hiPSCs : human induced pluripotent stem cells

KSR : knockout serum replacement

LIF : leukemia inhibitory factor

MEFs : mouse embryonic fibroblasts

mESCs : mouse embryonic stem cells

MMC : mitomycin C

N2 : liquid nitrogen

NEAA : nonessential amino acid

NIH : national institutes of health

PCR : polymerase chain reaction

PS : penicillin-streptomycin

pESCs : porcine embryonic stem cells

RNA : ribonucleic acid

RT : room temperature

SOPs : standard operating protocols

srRNA : small rDNA-derived RNA

SSEA-4 : stage specific embryonic antigen -4

UV : Ultraviolet Ray

WT-iPS1 : Inducible pluripotent stem cell wild type 1

CHAPTER 1.

General introduction

1. General introduction

1) Definitaion and characteristics of mycoplasma

Mycoplasma which is the smallest and simplest prokaryotic microorganism is widespread in nature as parasite of humans, mammals, reptiles, insects and plants (Razin et al., 1998). Mycoplasma belongs to the family Mollicutes and more than 180 species have been identified (Momynaliev and Govorun, 2001). Normally they do not cause serious damage; however, many of which are considered proven pathogens.

[Higher order taxonomy] (Widipedia; Wolf et al., 2004)

Bacteria > Firmicutes > Mollicutes > Mycoplasmataceae > Mycoplasma

Mycoplasma is a very small type, 0.2um−0.8um in diameter, as about one−tenth the size of regular bacteria. Extremely, even it can be as small as 0.1um and can be seen only under the electron microscope (Stanbridge, 1981; Drexler and Uphoff, 2002; Mayer, 2011). In addition, mycoplasma is the smallest self−replicating organism with the smallest genomes (a total of

about 500 to 1000 genes); there are low in guanine and cytosine. And the mycoplasma contains the minimum set of organelles essential for growth and replication: a plasma membrane, ribosomes, and a genome consisting of a double-stranded circular DNA molecule (Razin., 1996). Unlike all other prokaryotes, mycoplasma which is surrounded by a single plasma membrane lacks a rigid cell wall and therefore has no definite shape (Razin; Drexler and Uphoff, 2002). They usually attach to the external surface of hosts and rely heavily on their hosts for many nutrients as their biosynthetic capabilities are limited. Especially, they most require sterols for the stability of their cytoplasmic membrane. The sterols need to be acquired from the environment, usually as in the form of cholesterol, meaning that mycoplasmas are always in need of hosts (Razin et al., 1982; Dahl, 1993).

2) Mycoplasma contamination

Mycoplasma is a common and serious contaminant of cell cultures, and one of the major problems in biological materials and research using cultured cells (Drexler and Uphoff, 2002). Because of their extremely small size and slow growth rate, it is never easy to discern readily the mycoplasma

presence by routine procedures in a laboratory (Rottem and Barile, 1993; Boslett and Nag, 2014). In detail, mycoplasma is not removed routinely by a filtration (bacterial retentive) as well as even at very high concentration (> 10^7 cfu/ml) of mycoplasma is not visible by microscopy (Folmsbee et al.; Folmsbee et al., 2010). Moreover, they may not cause macroscopic alterations of cells, such pH or turbidity changes of culture media by bacterial and fungal contamination (Bates and Wernerspach, 2011). And mycoplasma's lack of cell wall causes it to be resistant to certain antibiotics such as penicillin, which interfere with bacterial cell wall synthesis (Taylor-Robinson and Bebear, 1997). It has been shown that on average between 15-35% of all continuous cell cultures are contaminated with mycoplasma, some estimates are even higher (up to 80% in some countries). In addition, various studies show that the percentage of contaminated cultures in cell banks is 10-80% (McGarrity et al., 1984; Rottem and Barile, 1993). The most prevalent species of mycoplasma detected in contaminated cell cultures include *M. fermentans*, *M. hyorhinis*, *M. arginini*, *M. orale*, *M. salivarium*, *M. hominis*, *M. pulmonis* and *M. pirum* (Dvorakova et al., 2005). Mycoplasma contamination

can originate from all direction associated with cell cultures. The typical routes of infection are as listed below (Table 1). As mycoplasma competes with cells for the nutrients in culture media, one of the visible indications is a slow-down in cell proliferation. Also, other observable symptoms of mycoplasma contamination include morphological changes, suspension cells clumping together, and poor transfection efficiencies with cells that originally transfected well. Thus, their contamination can interfere with experiments in cell culture (Stanbridge, 1971; Doersen and Stanbridge, 1981; McGarrity et al., 1984; Sokolova et al., 1998; Gong et al., 1999; Ben-Menachem et al., 2001; Drexler and Uphoff, 2002; Darin et al., 2003; Miller et al., 2003; Rottem, 2003). To support this fact, a wide range of adverse effects which are cellular abnormalities by mycoplasma contamination have been consistently reported (Table 2).

Table 1. The sources of mycoplasma contamination.

Laboratory employees
−. By people handling cells −. By direct infection from the researcher
Contaminated materials
−. By culture reagents − Medium, serum, enzyme and additives −. By contaminated instrument and equipment − Pipette, pipette tips, culture vessel, hood, water bath, etc.
Cross contamination
−. By other contaminated cultures − Feeder cells used for supporting stem cell cultures − Use of same media bottles of different cell types − Aerosols generated during pipetting − Handling of more than one cell type at one time
Others
−. By cells purchased from outside − Untested infected cells −. By liquid nitrogen for storage of cells

Table 2. Adverse effects by mycoplasma contamination.

Mycoplasma effects
– Potentially harmful morphological change
– Alterations in cell growth rate
– Inhibition of cell metabolism
– Interference with protein, RNA, and DNA synthesis
– Chromosomal abnormalities (aberrations)
– Change of gene expression patterns
– DNA fragmentation due to mycoplasma nucleases
– Altered enzyme actions
– Changes in cell surface antigenic characteristics
– Inhibited or stimulated cellular transformations
– Reduction of transfection efficiencies
– Reduced or increased virus yields
– Decreased malignancy of tumor cells
– Increased sensitivity to apoptosis
– Cell death

3) Mycoplasma detection and elimination

Mycoplasma contamination can severely impact the reliability, reproducibility, and consistency of experimental results, representing a critical problem for basic research as well as for the manufacturing of bio-products (Hay et al., 1989; Nikfarjam and Farzaneh, 2012). Therefore, in cell culture there is nothing more important than both detection and treatment of infected cells by mycoplasma. Several testing methods for the detection of mycoplasma have been published as below (Studzinski et al., 1973; Chen, 1977; Bereiter et al., 1990; Jung et al., 2003; Nikfarjam and Farzaneh, 2012) (Table 3). In addition to those methods in the table below, a number of other alternative methods for mycoplasma detection have been developed : histochemical staining, electron microscopy, infrared spectroscopy, and immunological methods, e.g., immune-fluorescence and immunoblotting (Brown et al., 1974; Washburn et al., 1996; Melin et al., 2004; Dawo and Mohan, 2008). Especially, of a variety of ways for its detection, PCR testing techniques are based on amplification of a DNA fragment using primers prepared in advance, and fragment identification is usually carried out with electrophoresis.

Specific primers have been designed from DNA that is coded to the ribosomal RNA(16SrRNA). The gene sequences for RNA are considered conserved sequences and are similar in the various mycoplasma species, which have not undergone significant mutation. Consequently, primers can be designed for these areas, which are specific to the mycoplasma and will not detect bacterial or animal DNA sequences (Mattsson and Johansson, 1993; Olarerin-George and Hogenesch, 2014). So, PCR-based technique has become increasingly widespread and is suitable for routine mycoplasma detection in cell culture laboratories because of its characteristics, which is a sensitive, fast, and specific detection method (Hopert et al., 1993; Uphoff and Drexler, 1999, 2002). Besides, commercially available diagnostic kits can be used more easily for the same purpose (ATCC; InvivoGen; LifeTechnologies; Lonza; R&Dsystems). A number of experimental trials for the elimination of mycoplasma contamination in cell cultures have been reported, such as (Nikfarjam and Farzaneh, 2012) :

A. Exposure to analogs of nucleic acids that prevent reproduction of mycoplasma

B. Exposure of contaminated cells to mouse macrophages

C. Passage of contaminated cells in thymus-deficient mice

D. Treatment with antibiotics

Although the best strategy for mycoplasma elimination is prevention, once mycoplasma infection is found in cell culture it is general for contaminated cells to either be discarded or be treated with antibiotics in laboratories (Invitrogen; Zakharova et al., 2010; Uphoff et al., 2012). Since mycoplasma-infected cells cannot always be discarded, the most preferred method of many others is to treat with antibiotics, which has advantages in that its simplicity and they do not damage or alter cells (Taylor-Robinson and Bebear, 1997; Drexler and Uphoff, 2002). There are many antibiotics that commonly used in cell cultures as a below list. (Table 4) Most routine antibiotics in cell cultures are ineffective against mycoplasma because the organisms are unresponsive to antibiotics that specifically inhibit bacterial cell wall synthesis (Widipedia; Kohanski et al., 2010). However, several antibiotics show the effect on mycoplasma elimination, e.g., Ciprofloxacin, BM-Cyclin, Erythromycin, Gentamicin, and Plasmocin (Uphoff and Drexler; Nikfarjam and Farzaneh, 2012). The mycoplasmas are sensitive to tetracyclines, macrolides, and the newer quinolones. And

there are three strategies to use them (Nikfarjam and Farzaneh, 2012).

A. Using quinolones as a single antibiotic compound.

B. Application of two different antibiotics such as plasmocin.

C. Applying a combination of minocycline (in tetracycline group) and tiamulin (in macrolide group) in alternating cycles with BM-Cyclin.

To more effective treatment of contaminated cells from mycoplasma, the manners like strategy A and strategy B have accounted for 90% of the contamination situation found in cell cultures. Furthermore, various commercial products related detection, elimination and prevention of mycoplasma contamination are available in easy (Lonza, 2013).

A. Mycoplasma detection kit

B. Mycoplasma elimination reagent

C. Spray for disinfection of laboratory surfaces and apparatus

D. Combinations kit of innovative antibiotics against mycoplasma

E. Antibiotics against a broad range of common contaminants

including mycoplasma

Needless to say, prevention is better than cure. But realistically in cultures of laboratories, what great care is taken to ensure that the master seed is free of mycoplasmas by testing and by treatment with combinations of antibiotics (Macpherson, 1966).

Table 3. Overview of different assays to detect the mycoplasma contamination.

Detection method		Sensitivity Specifity	Advantages	Disadvantages
DNA stain (e.g., DAPI)	Direct DNA stain	Low +/−	Rapid, cheap	Can be difficult to interpret
	Indirect DNA stain with indicator cells	High +/−	Easy to interpret because contamination amplified	Indirect and thus more time−consuming
Microbiological culture		High +/−	Sensitive	Slow and may require expert interpretation
PCR	Direct PCR	High +	Rapid	Requires optimization
	Indirect PCR	High +	Rapid	More sensitive than direct PCR, but more likely to give false positives
ELISA		Moderate +/−	Rapid	Limited range of species detected
Autoradiography		Moderate .	Rapid	Can be difficult to interpret if contamination is at low level
Immunostaining		Moderate .	Rapid	Can be difficult to interpret if contamination is at low level

Table 4. Antibiotics that commonly used in cell cultures.

Antibiotics	Bacteria	Mycoplasma	Yeast	Fungi	Stability at 37℃
Ampicillin	+	−	−	−	3 days
Amphotericin B	−	−	+	+	3 days
Cabenicillin	+	−	−	−	3 days
Ciprofloxacin		+			5 days
BM−Cyclin	+	+	−	−	3 days
Erythromycin	+	+	−	−	3 days
Gentamicin	+	+	+	−	3 days
Kanamycin	+	+/−	−	−	5 days
Lincomycin	+/−	−	−	−	4 days
Neomycin	+	−	−	−	5 days
Nystatin	−	−	+	+	3 days
Penicillin G	+/−	−	−	−	3 days
Plasmocin	+/−	+			7 days
Polymixin B	+/−	−	−	−	5 days
Streptomycin	+	−	−	−	5 days

CHAPTER 2.

Research on mycoplasma contamination in human induced pluripotent stem cells

1. Introduction

In preceding part, it was described about overall aspects of mycoplasma in laboratories such as definition, characteristics, contamination routes, detection, and treatment. This part contains more specific details about mycoplasma in cell cultures of laboratories. Cell culture techniques are indispensable tools for biomedical research (Mnif et al., 2007). However, Mycoplasma contamination of continuous cell lines still has represented a major problem for biologists (Uphoff and Drexler, 2005). The surveys of cell culture laboratories and cell banks substantiate that on average, 15–35% of all cell cultures may be contaminated with mycoplasma (Drexler and Uphoff, 2002; Cobo et al., 2005; Uphoff and Drexler, 2005). Mycoplasma contamination of mouse embryonic stem cells (mESCs) led to reductions in the number of pups born, the percentage of chimeric mice, male progeny and the average contribution to tissues (Bradley, 1987; Markoullis et al., 2009). The report of Kyriaki et al. showed that mycoplasma contamination affects through the culture over 20 passages on various cell parameters that include not only growth rate, viability, chromosomal aberrations, and differentiation but also

the percentage of germline chimeras, litter born, pups born, and chimeras. Moreover, the mycoplasma had negative effects on postnatal development of the resulting chimeras, e.g., reduced body weight, nasal discharge, osteoarthropathia, and cachexia. Also, Flow cytometric (FACS) analysis revealed significant differences in the proportions of T-cells and increased levels of IgG1, IgG2a, and IgM, and rheumatoid factor (Markoullis et al., 2009). A research team that has uncovered biological differences among 15 NIH-approved hES cell lines acquired revealed one line harbored mycoplasma (Ware et al., 2006). For mycoplasma eradication, it has been used several antibiotics as commercial products (Ridgway et al., 1984; Nakai et al., 2000; Bronckaers et al., 2008). However, few studies have reported on the comparison among these antibiotics. The studies for efficacy comparison of Plasmocin, BM-cyclin, Ciprofloxacine, and Mycoplasma removal agent (MRA) were conducted in various mammalian cell clines (Molla Kazemiha et al., 2009; Molla Kazemiha et al., 2011). The rate of mycoplasma elimination was 65%, 66.25%, 20%, and 31.25%, respectively, of infected cell cultures (Molla Kazemiha et al., 2011). In recent, a study to evaluate the efficacy and cytotoxic effect of plasmocin and ciprofloxacin on hESCs reported that the two

antibiotics did not affect hESCs stemness and pluripotency nor cell viability (Romorini et al., 2013). Though the most reliable method in mycolasma elimination is antibiotic administration as described previously (Drexler and Uphoff, 2002; Uphoff et al., 2002), antibiotic resistance in mycoplasma has occurred (Table 5) (Nikfarjam and Farzaneh, 2012).

Nowadays, human induced pluripotent stem cell (hiPSCs) not only hold great promise as models for human development and disease studies like human embryonic stem cells (hESCs) but also can avoid ethical problems. The culture and differentiation of these cells are both complex and expensive, so it is essential to extreme aseptic conditions (Romorini et al., 2013). Nevertheless, very little is known about contamination and elimination of mycoplasma specifically in hiPSC cultures, while there have accumulated substantial data on mycoplasma in hESCs and various type of cell cultures. In this study, various surveys on mycoplasma contamination in hiPSC lines which can get from domestic research organizations of stem cells were fulfilled 1) mycoplasma detection 2) contamination effects by mycoplasma 3) antibiotics for treatment 4) antibiotic resistance and contamination recurrence.

Table 5. Antibiotics resistance of mycoplasma from infected cell cultures (Nikfarjam and Farzaneh, 2012).

Antibiotics	Resistance
Chloramphenicol	30%
Chlortetracycline	11%
Ciprofloxacin	15%
Erythromycin	98%
Gentamicin	80%
Kanamycin	73%
Lincomycin	28%
Neomycin	86%
Spectinomycin	14%
Streptomycin	88%
Tetracycline	14%
Tylosin	21%

2. Materials and methods

Cell lines

For hiPSCs experiments, three cell lines (551-8, F5, and FS-1) and one cell line (WT-iPS1) were provide respectively from domestic prominent research organization A and B that have studied stem cells. Also, data related with H9 as a human embryonic stem cell (hESC) are obtained from a laboratory of seoul national university dental hospital. Porcine embryonic stem cells (pESCs) were used to prove the antibiotic activity.

Cell cultures

The cells were maintained in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Gibco) supplemented with 20% knock-out serum replacement (KSR) (Invitrogen), 4ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), 1% Nonessential amino acid (NEAA) (Invtrogen), 0.1M 2-Mercaptoehanol (Sigma), and 1% penicillin-streptomycin (PS)

solution (Invitrogen). The medium should be filtered before the use. Debris removal of colonies and replacement of culture medium were performed every day. For subculture, 1mg/ml of warmed collagenase IV (Sigma) solution is added to hiPSC colonies and incubated at 37℃ for 10-15 min. After enzyme inactivation using culture mudium, clumps of desired size are scraped by hook-shaped glass pasteur- pipette and collected. Then, they are transferred to new MEF feeder dish which was prepared and incubated. The next day, culture medium is not replaced for the attachment stabilization of subcultured-cells.

Mycoplasma detection test

There are two kinds of manners for the mycoplasma detection, PCR-based method and luminometer assay. As a commercial test kit, *e*-Myco™ Mycoplasma PCR detection kit (iNtRON biotechnology) was used. Cells (over $5x10^4$ cells) as a sample were prepared in 1.5ml tube. After washing, cell pellets were resuspended in 100μl of DPBS. The samples were heated at 95℃ for 5min. And then, they were centrifuged for 2min at 13,000rpm. An aliquot of the heated supernatant was moved to

a fresh tube. This supernatant was used as the template in the PCR. 10μl of the template was added into *e*-Myco™ Mycoplasma PCR Detection tubes, and then resuspended after 10ul of sterile water for 20μl PCR reaction volume. Before the PCR reaction (Table 6), both a negative control (20μl of sterile water only) and a positive control (1μl of control DNA in kit) were prepared. For analysis by electrophoresis, it was used 5μl of each tube. PCR products should be discarded after UV irradiation to prevent carry-over contamination. The other way is based on a luminometer method and was performed by requesting to the Seoul National University Hospital Biomedical Research Institue where used Cambrex MycoAlert Mycoplasma Detection Assay kit (Promega).

Characterization between mycoplasma positive group and negative group in hiPSCs

Cell morphology and growth rate. After subculture, morphology of hiPSCs was observed at day 2, 4, and 6. And the appearance of cells of those days was stored as pictures. To

investigate the growth rate of hiPSCs through the change of colonies size, diameters of each colony were calculated by NIS-Elements Imaging software (Nikon).

Cell proliferation. Cell proliferation was examined by Cell Counting Kit-8 (CCK-8) (Dojindo). Cells were harvested to be assayed and subcultured in a 96 well microplate. 100ul of a cell suspension was added to each well and also, a well of only medium was made to measure the background. At day 2, 4, and 6, 10ul of Cell Counting Kit-8 solution was added to each well in a 96 well microplate. It was placed in a CO_2 incubator for 1-4 hours to react. The absorbance as the results was measured at 450mm with a microplate reader.

Karyotyping. To test the chromosomal abnormality, the karyotype of hiPSCs was analyzed in Gendix as a specialized agency.

Immunohistochemistry. To assess pluripotent abilities of hiPSCs, immunohistochemistry was performed by DAKO Cytomation / LSAB system-HRP kit (Dako). The staining kit contains all reagents except for fixation and permeabilization solution. Cells were passaged in a 4 well dish and cultured for about 4 days. After removal old culture medium and washing, cells were fixed as treatment of 4% paraformaldehyde solution for 15 min.

Optionally, 0.1% Triton X-100 solution was treated for 10 min in accordance with the target for staining. Peroxidase Block solution were added to dish and cells were incubated at RT. Each antibody, Nanog, SSEA-4, and Tra-1-81, was diluted and treated for 1.5 hours. After washing using DPBS, Biotinylated link solution was treated for 10 min and Streptavidin-HRP solution was added, sequentially. For color reaction, cells were treated by ACE chromogen solution for 15 min and staining of cells was observed under a microscope. All steps were conducted at room temperature (RT) and the cells were washed carefully when each step was completed.

Alkaline phosphatase (AP) activity. Cells were treated AP staining solution for about 15 min after the fixation as described above. Then, the cells were washed two twice and observed under a microscope. AP staining solution was composed of 2mg naphtha AS-MX phosphate, 200ul N,N-dimethylformide, 9.8ml Tris, and 10mg fast red (FR) salt.

Embryonic bodies (EBs) formation. To induce differentiation, hiPSCs colonies were transferred to Petri dish containing culture medium excluded a growth factor as differentiation medium. Then, they were incubated in suspension culture and observed at day 0, 5, 8, and 11.

Antibiotic treatments for mycoplasma elimination

Plasmocin treatment is used for 2 weeks at 25μg/ml which represents a 1:1000 dilution of the 25mg/ml stock solution (100μl plasmocin in 100ml culture medium). Old medium is removed and replaced with fresh plasmocin containing medium every 3-4 days for 2 weeks. If mycoplasma contamination is still present, 37.5 μg/ml of plasmocin is treated to cell cultures for a further week. How to treat the BM-cyclin (Roche) is as follows. Old culture medium is removed and replaced with new medium containing BM-cyclin 1 (4μl of stock solution/ml, final concentration 10μg/ml). The cells are cultured for 3 days in the usual way. Then, the culture medium is removed. New culture medium containing BM-cyclin 2 (4μl of stock solution/ml, final concentration 5μg/ml) is added and cultured for 4 days. Like this, the cycle is repeated twice.

Table 6. PCR reaction for mycoplasma detection test.

PCR condition		Temperature	Time
Initial denaturation		94℃	1min
35 cycles	Denaturation	94℃	30sec
	Annealing	60℃	20sec
	Extension	72℃	1min
Final extension		72℃	5min

3. Results

Mycoplasma detection of human induced stem cell lines and inspection for the source of mycoplasma contamination

Four kinds of human induced pluripotent stem (hiPS) cell lines were provided from outside, that is two domestic organizations (organization A and organization B) as suppliers of stem cells. The cells were transferred as few as once and as many as four times in two-type states, either frozen in cryovials or maintained on culture plates (Table 8). Originally, four cell lines were generated by varies strategies from research laboratories in internal or external universities (Table 7). In order to examine if each hiPS cell line is contaminated by mycoplasma, mycoplasma detection tests were performed using a PCR-based method. Unfortunately, all results of detection tests showed mycoplasma infection of all four hiPS cells (Table 8). Then, to identify where the route of mycoplasma contamination is, the tracing study was carried out and was contained a wide range of targets as a suspect such as not only hiPSCs but also culture media, reagents, and various

experimental instruments (Table 9). The results of screening by luminometer-based or PCR-based method revealed that the source of mycoplasma contamination is not environmental factors in our laboratory associated with cell cultures but hiPSCs, that is the cells provided from outside.

Mycoplasma effects on human induced pluripotent stem cells

To investigate the effects of mycoplasma infection in hiPSCs, above all, we secured the mycoplasm (-) group of hiPSCs through the treatments of the BM-cycline antibiotics before the comparison study between mycoplasma (+) group and (-) group in hiPSCs (Table 10). The only two cell lines, F5 and 551-8, out of four hiPSCs were used for this study because other two cell lines, FS-1 and WT-iPS1, were not appropriate to be maintained for long period, virtually which they have a strong tendency of differentiation or have an adaptation problem about different kinds of feeder cells. Many parameters for the comparison between mycoplasma (+) group and (-) group in hiPSCs contain morphology and growth rate of colonies, cell proliferation rate, karyotype, and abilities of

pluripotency and differentiation. At day 2, day 4, and day 6 of hiPSCs culture, the conspicuous difference in several aspects that are morphology and growth rate of colonies was not appeared (Fig. 1). Meanwhile, the comparison of cell proliferation using CCK-8 kit between two groups showed that mycoplasma (+) group in hiPSCs had even higher absolute absorbance values at 450mm than mycoplasma (-) group (Fig. 2A and 2B). The result of re-testing of CCK-8 assay on only culture media that were collected at culture day 0 and day 2 revealed such phenomena is because of mycoplasma beings in culture media (Fig. 2C). That is, once culture medium is used for hiPSCs infected by mycoplasma there are always mycoplasma beings in only culture medium used even if the cells are removed. Also, it means that an accurate measurement of proliferation rate of hiPSCs was interfered due to mycoplasma contamination. In karyotype analysis and pluripotentcy, evident difference between mycoplasma (+) group and (-) group in hiPSCs was not appeared (Fig. 3 and Fig.4). To confirm the pluripotent capability, both groups in hiPSCs were stained by pluripotent specific markers, Nanog, SSEA-4, and Tra-1-81, and were tested about alkaline phosphatase (AP) activity (Fig. 3). In addition, to check on the

stability of genetic level, the karyotyping was performed (Fig. 4). In spite of their contamination, consequently, definite effects of mycopalsma (+) group in hiPSCs were invisible in karyotype and pluripotency. On the other hand, remarkable effects of mycoplasma contamination in hiPSCs were exposed in abilities of embryonic body (EB) formation as a differentiation (Fig. 5). In EB suspension cultures, EBs of mycoplasma (+) groups in hiPSCs had much debris surrounded EBs obviously while those of mycoplasma (−) group in hiPSCs looked like a ball with a smooth surface (Fig. 5A). That was verified again through the quantification of debris (Fig. 5B). Moreover, to compare the number of EBs formed between two groups of hiPSCs, the suspension cultures were started at each 100 clumps equally in each experimental group. Generally, the capacity of hiPSCs as a kind of stem cells may be incapable slightly than human embryonic stem cells (hESCs) owing to unstable shut down of genes which are related with the transformation to establish hiPSCs originated from somatic cells. Even here, the number of EBs formed from hiPSCs was not, overall, as many as that from H9, hESCs. However, what is more important is that the number of EBs in mycoplasma (+) groups was fewer than in mycoplasma (−) groups in hiPSCs as

a contamination effect (Fig. 5C).

Antibiotic effects on human induced pluripotent stem cells infected myacoplasma

To evaluate antibiotics used for the mycoplasma elimination in hiPSCs, Plasmocin and BM-cyclin as two representative antibiotics were chosen and applied in contaminated hiPSCs, respectively. Unlike a BM-cyclin, It was impossible to eliminate the mycoplasma of both hiPSCs, F5 and 551-8, using plasmocin antibiotics (Table 10). Plasmocin was not able to cure the mycoplasma contamination of hiPSCs even though use of high concentration (37.5μg/ml) as well as common concentration (25μg/ml). In order to confirm if the antibiotics, itself, loose the antibiotic activity by any possibility, plasmocin was applied to stem cells originated from other species, porcine embryonic stem cells (pESCs,) contaminated by mycoplasma. But the activity of plasmocin antibiotics was a normal. As a result, hiPSCs which were brought from the domestic specialized agency where the stem cells are studied, in particular F5 and 551-8, undoubtedly had a tolerance to the plasmocin antibiotics. Besides, the recurrence of mycoplasma

was unfortunately detected in hiPSCs although the cells were completed the treatment using a BM-cyclin antibiotics and also, were verified the state of the mycoplasma elimination by PCR-based method (Fig. 6). In detail, out of two attempts of BM-cyclin treatment, one case showed the recurrence of mycoplasma contamination in just 2 days while the other case showed the mycoplasma-negative state up to 4 days (Fig. 6). Therefore, these results reported both the antibiotic-resistance and the contamination-recurrence in hiPSCs against mycoplasma.

Table 7. Basic information on human induced pluripotent stem (hiPS) cell lines which were supplied from outside.

Human iPS cell lines			Information of cell lines			Domestic organization as a supplier of cell lines
N.	Abbrevi.	Full name	Cell line −maker	Origin of cell lines	Strategies for the generation	
1	551-8	Detroit 551-iPS8	Daley (Harverd)	Human fibroblasts (fetus)	OKSM[1], Retrovirus	Organization A
2	F5	Fib2-iPS5	Daley (Harverd)	Human fibroblasts (male forearm)	OKSM, Retrovirus	
3	FS-1	Foreskin 1	Thomson (Wisconsin)	Human fibroblasts (neonatal foreskin)	OSNL[2], Lentivirus	
4	WT-iPS1	Inducible pluripotent stem cell wild type 1	Organization B	Human dermal fibroblasts (neonatal foreskin)	OKSM, Retrovirus	Organization B

[1] OKSM : 4 genes that inserted in order to generate human iPS cells (Oct4, Klf4, Sox2, and c-Myc)

[2] OSNL : 4 genes that inserted in order to generate human iPS cells (Oct4, Sox2, Nanog and Lin28)

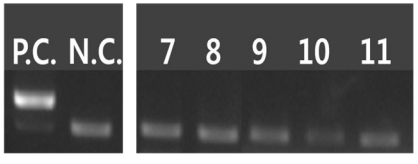
Table 8. Detection tests of mycoplasma contamination in human induced pluripotent stem cells (hiPSCs) using a PCR-based method.

	Human iPS cell line	The N. provided (Subculture N. of cells)	Supplying form	Mycoplasma test
1	551-8	1st (p.81)	Attached cell colonies (on a culture plate)	N.A.[1]
		2nd (p.85)		Contam. (+)
		3rd (p.80)		Contam. (+)
		4th (p.81)		Contam. (+)
2	F5	1st (p.54)	Attached cell colonies	Contam. (+)
		2nd (p.62)		Contam. (+)
		3rd (p.62)		Contam. (+)[2]
3	FS-1	1st (p.56)	Attached cell colonies	Contam. (+)
4	WT-iPS1	1st (p.19)	Attached cell colonies	Contam. (+)
		2nd (p.20)		Contam. (+)
		1st (p.18)	Cryopreserved	Contam. (+)

[1] N.A. (not applicable)

[2] It was observed that the visible cloudiness of cultured media by microbial contamination as well as mycoplasma infection by a PCR-based method.

Table 9. Screening tests to trace the contamination source of human induced pluripotent stem cells (hiPSCs) that infected by mycoplasma using either an luminometer method or a PCR-based method.

	Tests for mycoplasma detection		Results		The scope of the suspect as a contamination source
	Sample	Test method	result	The result levels[1]	
1	551-8 cells	Lumino	Contam. (+)	Reading A(326),B(6711)	Human iPSCs from outside
2	Feeder cells in a Forma incubator for 3days	Lumino	Contam. (-)	Reading A(126),B(62)	Each incubator, feeder, culture media for feeder cells, experimental instruments
3	Feeder cells in a Hera incubator for 3days	Lumino	Contam. (-)	Reading A(102),B(62)	
4	Feeder cells in a Sanyo incubator for 3days	Lumino	Contam. (-)	Reading A(111),B(58)	
5	Culture media for human iPSCs	Lumino	Contam. (-)	Reading A(139),B(55)	Culture media for human iPSCs, antibiotics, experimental instruments
6	Culture media supplem-ented with plasmocin	Lumino	Contam. (-)	Reading A(154),B(66)	
7	Feeder cells in a Forma incubator for 3days	PCR	Contam. (-)		Incubator, feeder, culture media for feeder or hiPSCs, antibiotics, reagent, experimental instruments
8	Culture media for feeder cells	PCR	Contam. (-)		
9	Culture media for human iPSCs	PCR	Contam. (-)		
10	Plasmocin	PCR	Contam. (-)		
11	Gelatin solution	PCR	Contam. (-)		

[1] If the level of B/A is 1 or greater in luminometer assay, the sample is contaminated by mycoplasma.

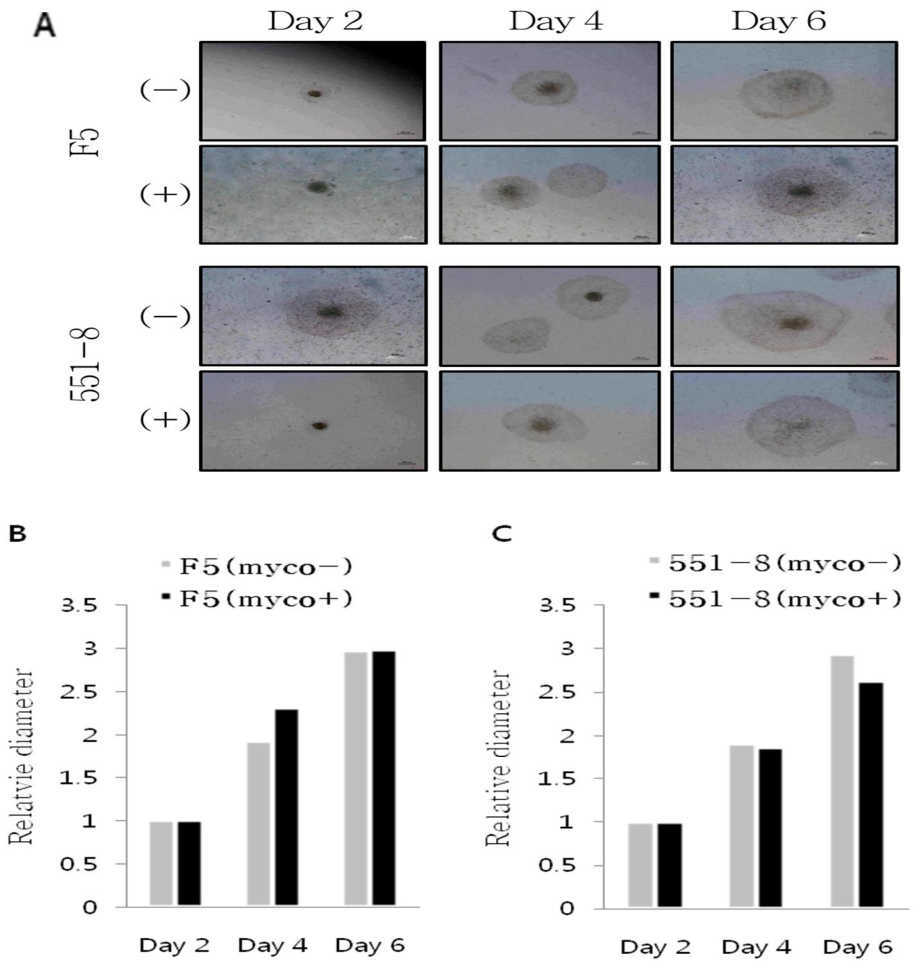
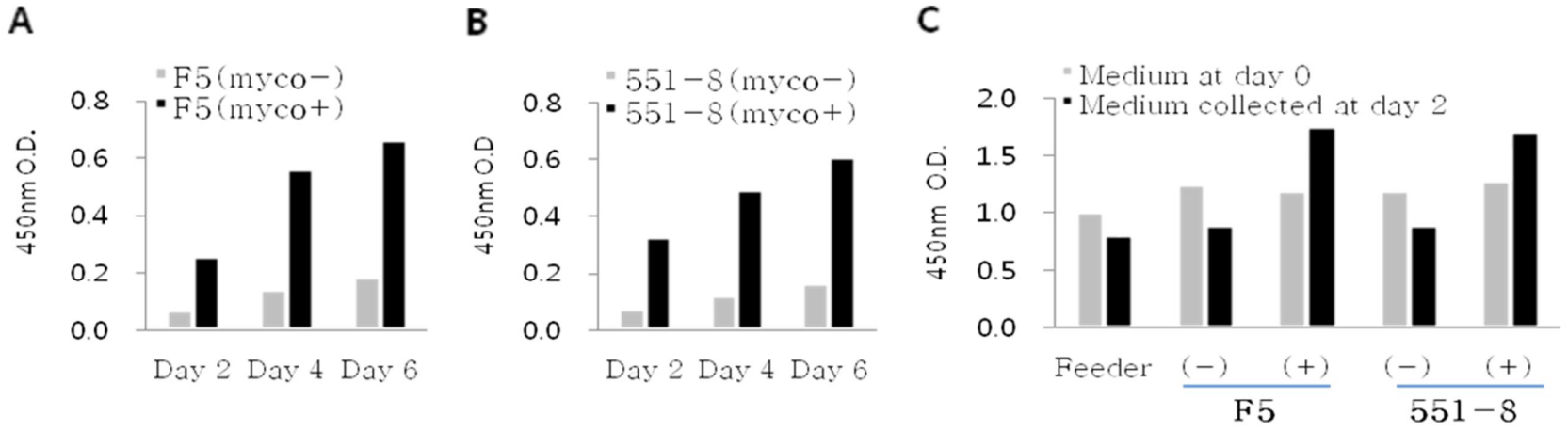


Figure 1. Comparisons of both (A) morphology and (B) growth rate of colonies between mycoplasma (+) group and (−) group in human induced pluripotent stem cells (hiPSCs). At day 2, day 4 and day 6 of hiPSCs culture, (A) difference of colony morphology of hiPSCs was not shown between mycoplasma (+) and (−) groups. Also, (B) difference of colony growth rate of hiPSCs that was relatively calculated by measuring the size of colonies at each day was not shown between mycoplasma (+) and (−) groups.



.**Figure 2.** Comparisons of proliferation rate between mycoplasma (+) group and (−) group in human induced pluripotent stem cells (hiPSCs). CCK−8 assay was performed to estimate the cell proliferation rate at day 2, day 4 or day 6 in hiPSCs culture. (A)&(B) Absolute absorbance (450nm O.D.) values of mycoplasma (+) groups are much higher than those of mycoplasma (−) groups in hiPSCs. Moreover, (C) when CCK−8 assay was performed to measure only each culture media that are collected at subculture day 0 and day 2, it was shown that the only absolute absorbance (450nm O.D.) values of (+) groups only of mycoplasma contamination are increased for 2 days because of the mycoplasm beings

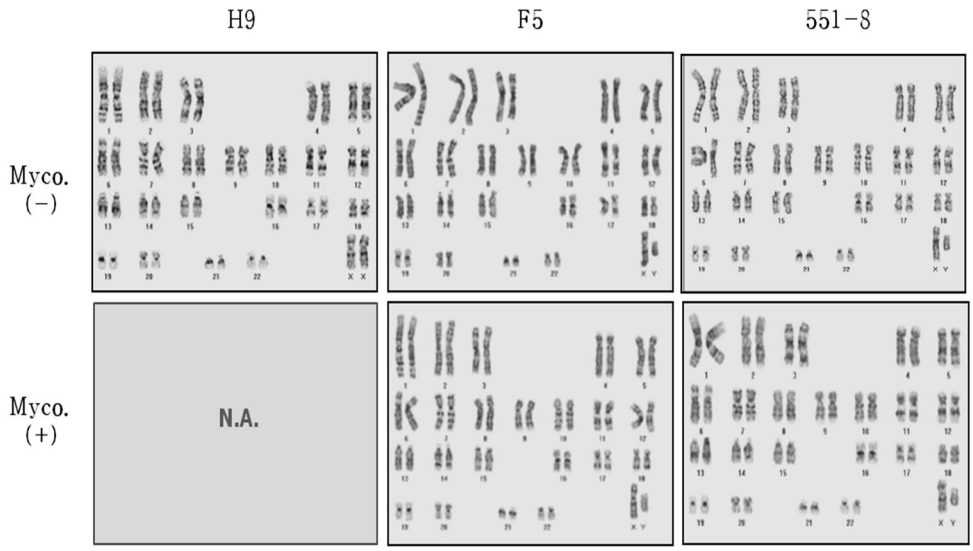


Figure 3. The karyotype analysis between mycoplasma (+) group and (−) group in human induced pluripotent stem cells (hiPSCs). Regardless of mycoplasma infection, karyotype of not only mycoplasma (−) groups but also mycoplasma (+) groups in hiPSCs is normal type, which had 23 pairs of chromosome. H9 : human embryonic stem (hES) cell line as a mycoplasma-free group (negative control).

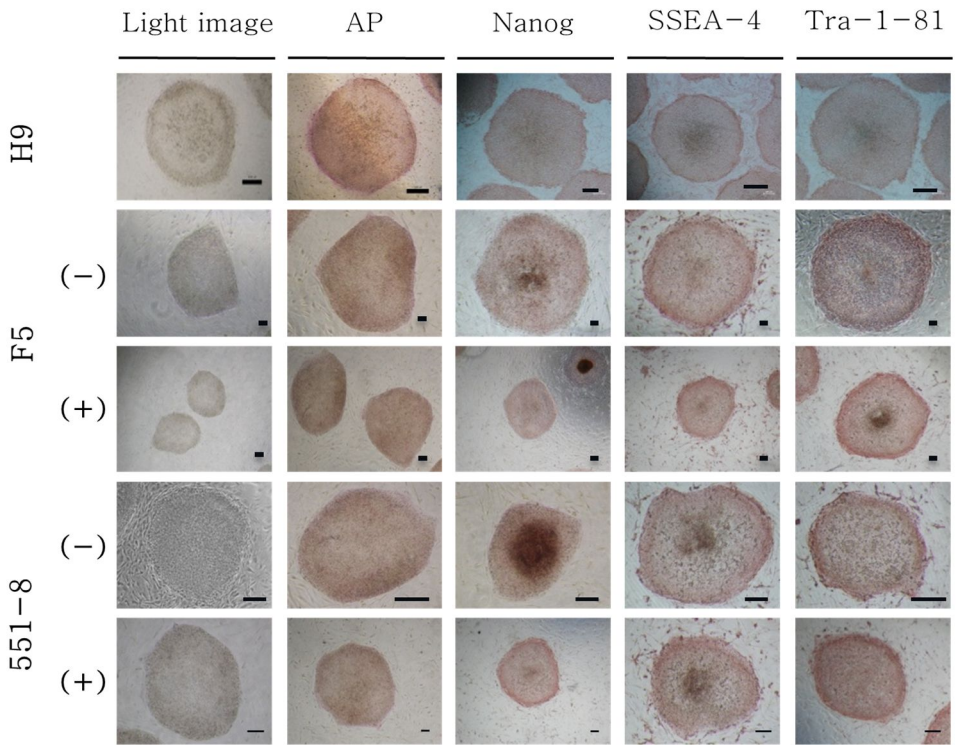


Figure 4. Comparisons of pluripotency between mycoplasma (+) groups and (−) groups in human induced pluripotent stem cells (hiPSCs). To investigate whether mycoplasma contamination effects on the pluripotent ability of hiPSCs, immunocytochemistry staining was performed by using pluripotent markers (Nanog, SSEA-4 and Tra-1-81) and alkaline phosphatase (AP) activity. Both (+) group and (−) group of mycoplasma contamination were shown the expression of poluripotent markers and AP activites of stem cells. H9 : human embryonic stem (hES) cell line as a mycoplasma-free group (negative control). Scale bar = 200㎛.

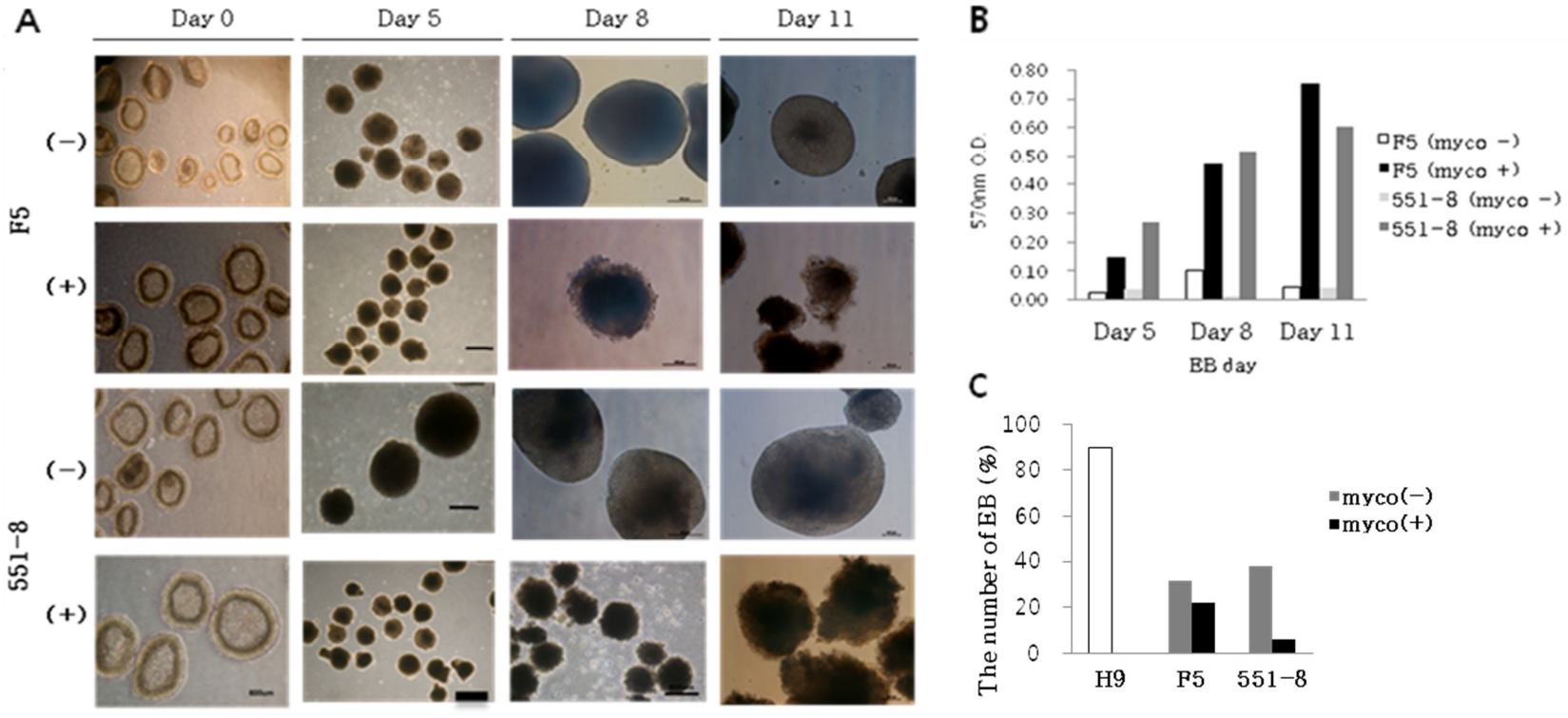


Figure 5. Comparisons of the capacity of embryonic body (EB) formation between mycoplasma (+) group and (−) group in human induced pluripotent stem cells (hiPSCs). (A) To investigate whether mycoplasma contamination

affects on the ability of EB formation of hiPSCs, EBs formed by suspension culture are observed at day 0, 5, 8 and 11. More debris, which surrounded EBs, in mycoplasma (+) groups were shown obviously than in mycoplasma (−) gourps. And (B) the quantity of debris was displayed in a graph. Also, (C) the number of EBs decreased in hiPSCs infected by mycopalsma, relatively.

Table 10. The treatment effects of two antibiotics, Plasmocin and BM-cyclin, in human induced pluripotent stem cells (hiPSCs) infected by mycoplasma.

	Before the antibiotic treatment	After the antibiotic treatment		
		Plasmocin		BM-cyclin
		Normal concentration	High concentration	
F5	Contam. (+)	Contam. (+)	Contam. (+)	Contam. (-)
551-8	Contam. (+)	Contam. (+)	Contam. (+)	Contam. (-)
pESC[1]	Contam. (+)	Contam. (-)	N.A.[2]	N.A.[2]

[1] pESC (embryonic stem cells established from porcine)

[1] N.A. (not applicable)

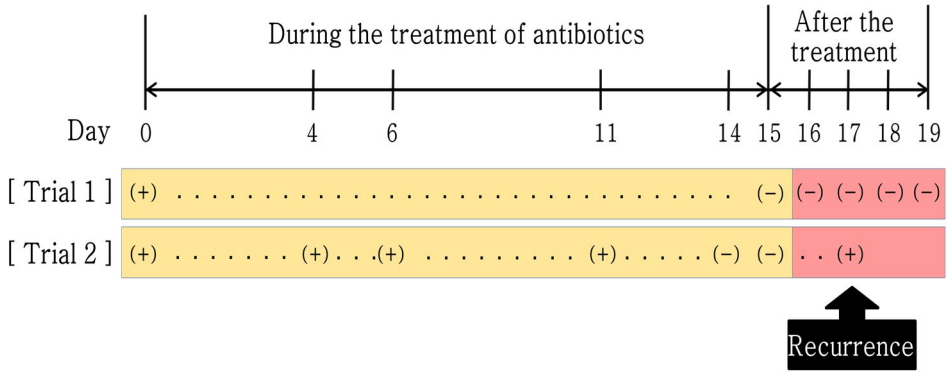


Figure 6. Recurrence of mycoplasma contamination in human induced pluripotent stem cells (hiPSCs). There are attempts twice that are antibiotic treatment using a BM−cyclin for 2 weeks in order to eliminate mycoplasma in hiPSCs (551−8). In the first case, it was confirmed that hiPSCs maintain the contamination−negative state for about 4 days even after the antibiotic treatment. In the second case, however, recurrence of mycoplasma in hiPSCs within two or three days after the antibiotic treatment occurred.

CHAPTER 3.

Guideline for cell cultures

1. Guideline for cell cultures

1) Summary

In this report, we introduce a standard protocol for stem cell self-renewal in vitro. Both fundamental and major procedures of stem cell manipulation, which are required for somatic cell coculture and self-renewal, are briefly described since they are important for stabilization and data normalization. In this chapter, information on the basic preparation of stem cell culture such as labware washing, equipment sanitization, microbe control, and mycoplasmosis prevention is provided. In addition, protocols for cell retrieval and preservation, proliferation assays, and basic manipulation techniques for the coculture of stem cells with somatic cells are described.

Keywords : Embryonic stem cell (ESC), embryonic/fetal fibroblasts, co-culture, standard operation protocol (SOP), cryopreservation, proliferation, mycoplasmosis, labware and equipment, subpassage

2) Materials

1. Preparation for aseptic cell culture

1.1. Reagent (see Note1)

A. 7X detergent (Mpbio, Cat. No. 76-670-94, Rue Geiler de Kayserberg, Illkirch, France) (see Note 2)
B. 0.25% trypsin-EDTA (Gibco, Cat. No. 25200072, Grand Island, NY) (see Note 3)
C. 70% Alcohol (see Note 4)
D. Antibiotic-antimycotic solution (AA) (Gibco, Cat. No. 15240-062, Grand Island, NY) (see Note 5)
E. Collagenase type I (Sigma-Aldrich, Cat. No. C0130, St.Louis, MO)
F. Dimethyl Sulfoxide (DMSO) (Bionichepharma, Cat. No. bip-1, Lake forest, CA)
G. Distilled water
H. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Cat. No. 10566-016, Grand Island, NY)
I. Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, Cat. No.

14190144, Grand Island, NY)

J. Fetal bovine serum (FBS) (see Note 6 and Note 3)

J-1. Non-ES-grade FBS (WelGENE, Cat. No. #S001-01, Daegu, Republic of Korea)

J-2. ES-grade FBS (Hyclon, Cat. No. SH30070.03, UT)

K. Gelatin (Sigma-Aldrich, Cat. No. G9391, St.Louis, MO)

L. Incuwater (Applichem, Cat. No. A5219, Darmstadt, Germany)

M. Leukemia inhibitory factor (LIF) (Merck millipore, Cat. No. ESG1107, Temecula, CA) (see Note 7)

N. L-Glutamine (Gibco, Cat. No. 25030081, Grand Island, NY)

O. Mitomycin C (MMC) (Sigma-Aldrich, Cat. No. m4287, St.Louis, MO)

P. Mycoplasma detection kit

Q. Nonessential amino acids (NEAA) (Gibco, Cat. No. 11140-050, Grand Island, NY)

R. Pharmacidal sprays (Biological Industries, Cat. No. IC-110110-L, Kibbutz Beit Haemek, Israel)

S. Plasmocin (Invivogen, Cat. No. ant-mpt, San Diego, CA)

T. Trypan blue solution (Sigma-Aldrich, Cat. No. T8154, St.Louis, MO)

U. β-mercaptoethanol (Gibco, Cat. No. 21985-023, Grand Island, NY)

1.2. Equipment

A. Aspiration pump (vacuum pump)

B. Autoclave

C. Biosafety cabinet class II (clean bench or laminar flow hood)

D. Centrifuge

E. ChemiDoc (UV transilluminator) (Bio-RAD)

F. Drying oven

G. Electrophoresis machine (ADVANCE, Tokyo, Japan)

H. Incubator (humid CO_2 incubator recommended, Thermo Fisher Scientific Inc., Waltham, MA)

I. Liquid Nitrogen (N_2) tank (MVE BioMedical, Ball Ground, GA)

J. Microscope

J-1. Inverted phase-contrast microscope (Olympus, Tokyo, Japan)

J-2. Stereoscopic microscope (Olympus, Tokyo, Japan)

K. Polymerase chain reaction (PCR) (Biometra, Gottingen, Germany)

L. Thermo-hygrostat

M. Ultraviolet irradiating pass box (see Note 8)

1.3. Materials

A. Alcohol lamp, aluminum foil, autoclave tape, cell culture dish (or cell culture plate), centrifuge tube, conical tube, cryo-container, cryorack, cryovial, filter system, glass bottle, latex gloves, mess cylinder, name pan, paper towel, parafilm, petri dish, pipette, pipette gun, rack, sample box, serological pipette, sterile surgical instruments, syringe and needle, timer, tip, wastebasket

2. Preparation for stem cell culture

2.1. Animals (see Note 9)

Mouse embryonic fibroblasts (MEFs) for feeder cells : 13.5-day-old fetuses retrieved from pregnant female mice (see Note 10)

2.2. Media and reagents

A. Washing solution: DPBS or DPBS containing 1% antibiotics-antimycotics (AA)

1) Composition (total 500mL) : DPBS 495mL + AA 5mL

2) Aliquot : Divide each 40~50mL into each conical tube

3) Storage : Room temperature

B. Culture medium :

a) MEFs culture medium: DMEM containing 10% FBS (non-ES-grade) and 1% antibiotic-antimycotic (AA)

1. Stock medium for MEFs culture

1) Composition (total 500mL) : DMEM 495mL + AA 5mL

2) Aliquot : Divide each 45mL into each conical tube

3) Storage : 4℃

2. Working medium for MEFs culture

1) Composition (total 50mL) : stock medium 45mL + non-ES-grade FBS 5mL

2) Storage : 4℃

b) Stem cell culture medium : DMEM supplemented with 1% nonessential amino acids (NEAA), 0.1mM β-mercaptoethanol, 1% antibiotic-antimycotic (AA), 1,000 U/mL mouse LIF, and 15% FBS (ES-grade)

1. Stock medium for stem cell culture

1) Composition (total 500mL) : DMEM 490mL + AA 5mL + NEAA 5mL + β-mercaptoethanol 910μL

2) Aliquot : Divide each 45mL into each conical tube

3) Storage : 4℃

2. Working medium for stem cell culture

1) Composition (total 50mL): stock medium 42.5mL + ES-grade FBS 7.5mL + LIF 50μL

2) Storage : 4℃

C. Dissociation solution (see Note 11):

a) Tissue dissociation for MEFs culture : 0.04% trypsin-

EDTA

b) MEFs dissociation : 0.25% trypsin-EDTA

c) Stem cell dissociation : 0.25% trypsin-EDTA

D. Enzyme inactivation solution: each culture medium

E. Freezing solution: 60% culture medium + 30% FBS + 10% DMSO (see Note 12)

F. Thawing solution: each culture medium

G. Mitotic inactivation solution: 10 μg/mL mitomycin C (MMC) in DMEM (see Note 13)

1) Composition (total 10mL) : DMEM 10mL + MMC 2g

2) Aliquot : Divide each 405μL into each centrifuge tube

3) Storage : Put all of MMC tubes into an amber-mouth bottle and store at -80℃ (see Note 14)

H. Culture plate-coating solution: 0.1% gelatin solution (see Note 15)

1) Composition (total 200mL): DW 200mL + Gelatin 0.2g

2) Dissolution: Sterilize the gelatin solution in an autoclave (121℃, 10 minutes) without a drying step (see Note 16)

3) Storage: Store at 4℃ after cooling at room temperature.

2) Methods

1. Preparation for laboratory instruments

1.1. Safety equipment

1) Depending on the experiment performed, wear proper safety equipment such as a gown, mask, goggles, and gloves for individual protection and remove them in the outer area.
2) Researchers must wear long shirts, long pants, and shoes that cover the whole foot. Sleeveless shirts, shorts, and skirts are not recommended.

1.2. Cleaning of labware

1) Wear hand protection during the washing process. Wash labware with a detergent (7X) to remove any residue and rinse them with running tap water (see Note 17).
2) Open the lid of the sonicator and wash the internal space with distilled water while opening the machine valve. Fill the sonicator with distilled water up to the operating level marked in the machine after closing the valve (see Note 18).

3) Place the washed labware into distilled water in the sonicator and ensure that it is submerged completely without any bubbles. Next, add about 10 mL of 7X detergent into the sonicator and put the lid on the machine.

4) Turn on the sonicator. Set the temperature to 60℃, degas for 5 minutes, and run the machine for 1 hour. After the run is complete, drain water from the sonicator by opening the machine valve.

5) Rinse labware with running tap water about 7 times repeatedly and then rinse again with running distilled water.

6) Wrap labware with aluminum foil and attach the autoclave tape (see Note 19). Autoclave labware at 121℃ for 20 minutes and dry for 5 minutes.

7) To completely dry labware, place the sterilized labware in a dry oven overnight and arrange the dried labware in the proper position.

1.3. Carrying labware into the cell culture room

1) Wipe the surface of experimental items with 70% alcohol when you carry them from outside the culture room. Put them into the pass box and expose the item to UV irradiation for at least 20

minutes (see Note 8).

2) Arrange the items in proper positions of the culture room well.

2. Preparing an aseptic cell culture environment

2.1. Carrying foreign cells into the cell culture room

1) Conduct a mycoplasma test prior to bringing them into the cell culture room.

2) Cells that have a negative mycoplasma detection result can be carried into the culture room. However, the cells infected from mycoplasma can be carried into the cell culture room after eradication of mycoplasma.

2.2. Clean bench

1) Before the beginning of an experiment, wipe down the work space in a clean bench with 70% alcohol and turn on the UV light and hood blower for about 20 minutes. In addition, wipe each item required for the experiment such as pipettes, reagents, and racks with 70% alcohol when you bring them to the clean bench.

2) After completing an experiment, wipe down the work space and clean the bench with 70% alcohol and turn on the UV light and hood blower for 20 minutes.

2.3. Thermo-hygrostat

1) Check the thermo-hygrostat daily by recording the state of each machine on the checklist (see Note 20).
2) Clean the thermo-hygrostat monthly. Remove dust or pollutants in the machine filter using a cleaning brush.
3) Inspect the overall condition of the machines over a half year and replace the filter annually.

2.4. Laboratory cleaning

1) Do a cleanup of the laboratory at a fixed time every week.
2) Clear the table and remove used products and organize items used for experiments. Subsequently, wipe the table with 70% alcohol.
3) After removing dust from the floor with a vacuum cleaner, wipe the floor with 70% alcohol. Also, perform cleaning using a wax treatment on the floor at a fixed time every month.

2.5. Liquid and other waste

1) To dispose of liquid waste, throw out organic and inorganic liquid waste separately. Fill in the date and main component of liquid waste on the recording tag of the waste basket (see Note 20). Be cautious of an explosion that can be caused by reactions between liquid wastes.
2) To dispose of other waste, classify and throw out each waste.
   a. Medical waste such as needles, syringes, and glassware.
   b. Infectious waste such as tips, tubes, and culture plates.
   c. Noninfectious waste such as paper, aluminum foil, and plastic.

3. Basic assays for cell culture

3.1. Cell viability and counting in a hemocytometer using the trypan blue exclusion method

1) Carefully wipe and dry both the hemocytometer and coverslip using 70% ethanol and prepare a single cell suspension in 1 mL of culture medium.
2) Prepare the sample for cell counting. Mix single cells with 0.4% Trypan Blue solution in a centrifuge tube at a 1:1 ratio. Dead

cells are then stained in blue, but live cells are not stained (see Note 21). Here, the dilution factor is "2" because of the mixed sample at a 1:1 ratio.

3) Transfer 10 μL of the mixed sample to the side of the hemocytometer and cover it with a coverslip. Count the number of viable cells and total cells within a 1−mm3 square of the hemocytometer under a microscope (see Note 22). Repeat the cell counting in two or more different squares and calculate the average.

4) Calculate the cell number (see Note 22).

a. Total number of cells (cells/mL)

= the average number of cells (unstained + stained) x dilution factor x 10^4

b. The number of viable cells (cells/mL)

= the average number of viable cells (unstained) x dilution factor x 10^4

c. Cell viability (%)

= viable cells [(unstained)/total cells (unstained + stained)] x 100

5) Wipe the hemocytometer carefully and the coverslip using 70% ethanol after cell counting.

3.2. Control of mycoplasma contamination

3.2.1. Mycoplasma detection (see Note 23)

1) Prepare at least 5 x 10^4 cells in a centrifuge tube for mycoplasma detection tests. Wash each sample using 1 mL of Dulbecco's phosphate-buffered saline (DPBS), centrifuge at 16,600 x g (typically about 13,000 rpm) for 10–15 seconds and remove supernatants.
2) Resuspend cell pellets in 100 μL of DPBS and boil them at 95℃ for 10 minutes. Transfer each supernatant to new 0.6-mL tubes for mycoplasma tests after centrifugation at 16,600 x g for about 2 minutes.
3) Prepare a positive control, a negative control, and samples to be examined in each test tube that belong to a mycoplasma kit to perform PCR.

   a. Mycoplasma positive control

      : DW (19 μL) + control DNA (1 μL)

   b. Mycoplasma negative control

: DW (20 μL)

c. Sample for test

: DW (19 μL) + supernatant of sample for checking (1 μL)

4) Perform PCR reactions as described below.

<PCR condition>		<Temperature>	<Time>
Initial denaturation		94°C	1 min
35 cycles	−Denaturation	94°C	30 s
	− Annealing	60°C	20 s
	− Extension	72°C	1 min
Final extension		72°C	5 min

5) Determine whether samples are infected through band confirmation after electrophoresis and UV irradiation.

3.2.2. Mycoplasma disinfection: "Plasmocin treatment" (see Note 24)

1) Remove old medium from cells and replace with new medium containing 25 μg/mL of plasmocin every 3–4 days for 2 weeks.

2) Culture the cells in new culture medium containing 5 μg/mL of plasmocin without changing the medium for 5 days. Next, perform the test for mycoplasma detection to confirm that mycoplasmas are eliminated.

3) If mycoplasmas are still present following treatment, treat the

cells with a high concentration (37.5 μg/mL) of plasmocin for an additional week and repeat the mycoplasma detection test. Discard the cells if no effect on mycoplasma eradication occurs despite treatment with a high concentration of plasmocin.

3.2.3. Mycoplasma recurrence and treatment

1) Perform regular monitoring on mycoplasma in cell culture even after eradication is complete. In addition, repeat the antibiotic treatment on the basis of procedure "2-B. Mycoplasma eradication" if mycoplasma contamination recurs.

2) Under conditions of recurrent mycoplasma contamination, perform evacuation of the contaminated laboratory despite the fact that researchers may expend time, cost, and labor.

   a. Discard contaminated cells and every cell line stored in liquid nitrogen.

   b. Perform disinfection and closure of the laboratory containing a recurrent mycoplasma.

      b-1. Dry fog for fumigation

      b-2. Cleaning of the wall, floor, and ceiling of the laboratory using appropriate disinfectants

c. Perform monitoring of the laboratory for a prolonged period.

4. Stem cell culture

4.1. Preparation of feeder cells

1) Prepare pregnant female mice containing E13.5-day-old fetuses (see Note 25). Euthanize the pregnant mice by cervical dislocation and harvest the uterus containing 13.5-day-old fetuses after abdominal laparotomy using sterile forceps and scissors (see Note 26 and Note 27).
2) Place the uterus into a 50-mL conical tube containing 30 mL of DPBS. Wash the uterus in a 50-mL conical tube containing 30 mL of fresh DPBS and repeat this step three times. Transfer the uterus into a 100-mm petri dish containing fresh DPBS and repeat this step twice under sterile conditions in a laminar flow hood.
3) Dissect fetuses from the uterus using two 1-mL syringes with a bent tip at an angle of about 90°. Next, place the fetuses into DPBS to remove the blood for about 5 minutes.
4) Transfer the fetuses into 60-mm petri dish containing DPBS. Remove the visceral organs, head, and extremities using sterile

forceps and 1-mL syringes with a bent tip under a stereoscopic microscope (see Note 28).

5) Chop the remaining tissues about 10 times using a 1-mL syringe with a bent tip (see Note 29) and transfer the tissues into 15-mL conical tubes containing 0.04% trypsin-EDTA (see Note 30). Invert the conical tubes about 15 times and incubate them at 37℃ with 5% CO_2 for 3 minutes. Repeat this step once (see Note 31).

6) Add 5 mL of culture medium to each conical tube containing trypsinized tissues and invert them about five times. Centrifuge at 100 x g for 1 minute and transfer the supernatants into new conical tubes (see Note 32 and Note 33).

7) Centrifuge at 320 x g for 4 minutes. Remove the supernatants and transfer the remaining pellets into new conical tubes (see Note 34).

8) Gently resuspend the pellets by adding 1-2 mL of culture medium for single cells. Seed the single cells on 100-mm culture plates containing 10 mL of culture medium (see Note 35). Next, swirl each plate to distribute the cells equally and incubate all plates at 37℃ with 5% CO_2 for 24 hours.

9) Rinse all plates using DPBS to remove the dead cells and

change the medium the next morning.

10) Cryopreserve the fibroblast cells (passage 0) when they form a 70–80% confluent monolayer (see Note 36 and Note 37).

4.2. Maintenance of MEFs

4.2.1. Cryopreservation

1) Aspirate the old medium from the fibroblast monolayer and wash twice with DPBS. Add 5 mL of 0.25% trypsin–EDTA and incubate at 37℃ with 5% CO_2 for 3 minutes.

2) Add an equal volume of culture medium for enzyme inactivation. Harvest and transfer cells from a culture plate to a 15–mL conical tube. Centrifuge at 320 x g for 4 minutes and discard the supernatant.

3) Gently resuspend the pellet by adding a freezing solution and transfer cells to cryovials (see Note 38). Put the cryovials into a cryo–container and freeze overnight at –80℃.

4) Put the cryovials into a cryorack and store in a liquid N2 tank for indefinite storage.

4.2.2. Thawing

1) Take a cryovial containing frozen cells from a liquid N_2 tank and rapidly thaw the cryovial in a 37℃ water bath (see Note 39). Transfer the contents of a cryovial to a 15-mL tube containing 5 mL of culture medium.
2) Gently resuspend cells in culture medium and centrifuge at 320 x g for 4 minutes. Remove the supernatants and resuspend gently by adding 1 mL of culture medium for single cells.
3) Seed the cells (passage 1) on 100-mm culture plates containing culture medium (see Note 37). Swirl each plate to distribute the cells equally and incubate all plates at 37℃ with 5% CO_2 in a humidified air atmosphere until use.
4) Maintain cells until they form a 70-80% confluent monolayer. Next, aspirate the old medium of cells and wash twice with DPBS. Add 3 mL of 0.25% trypsin-EDTA and incubate at 37℃ with 5% CO_2 for 3 minutes.
5) Add an equal volume of culture medium for enzyme inactivation. Harvest and transfer cells from a culture plate to a 15-mL conical tube, centrifuge at 320 x g for 4 minutes, and discard the supernatant.

6) Gently resuspend the pellet by adding 3 mL of culture medium for single cells. Seed the cells (passage 2) equally onto three 100-mm culture plates containing culture medium (see Note 37 and Note 40). Next, swirl each plate to distribute the cells equally and incubate all plates at 37℃ with 5% CO_2 for 24 hours.

7) Rinse all plates using DPBS to remove dead cells and change the medium the next morning.

8) Perform mitotic inactivation of the fibroblast cells when they form a 90% confluent monolayer.

4.3. Mitotic inactivation of MEFs

1) Aspirate the old medium from the fibroblast monolayer and rinse twice with DPBS. Add 10 μg/mL mitomycin C (MMC) solution to the cells, wrap the culture plates with aluminum foil, and allow the cells to undergo mitotic inactivation in a 37℃ incubator with 5% CO_2 for 3 hours.

2) Remove the MMC solution and rinse the cells three times with DPBS. Add the cells to 3 mL of 0.25% trypsin-EDTA and incubate at 37℃ with 5% CO_2 for 3 minutes.

3) Add an equal volume of culture medium for enzyme inactivation. Then, harvest and transfer cells from a culture plate to a 15-mL conical tube. Centrifuge at 320 x g for 4 minutes and discard the supernatant.

4) Gently resuspend the pellet by adding culture medium for single cells. Seed the cells onto 0.1% gelatin-coated 60-mm culture plates (see NOTE 41) and incubate mitotically inactivated cells at 37℃ with 5% CO_2 in a humidified air atmosphere for the next use.

4.4. Maintenance of stem cells

4.4.1. Subculture of Stem Cells

1) Change the old medium in the culture plate of feeder cells with new culture medium used for stem cells 1 hour before subculturing stem cells.

2) Remove old medium of stem cells and wash twice with DPBS when stem cell colonies reach about 80% confluency in the culture plate. Trypsinize the stem cells by adding 0.25% trypsin-EDTA and incubate them at 37℃ with 5% CO_2 for 3

minutes.

3) Add an equal volume of culture medium for enzyme inactivation. Harvest and transfer the stem cells from a culture plate to a 15-mL conical tube. Then, centrifuge at 320 x g for 4 minutes and discard the supernatant.

4) Gently resuspend the pellet by adding 1 mL of culture medium for single cells. Count the number of stem cells using a hemocytometer.

5) Seed the appropriate number of stem cells onto feeder cells prepared in advance. Change the culture medium daily and subculture repeatedly when stem cells reach approximately 80% confluency.

4.4.2. Cryopreservation

Same as Set. 4.2.1

4.4.3. Thawing

1) Prepare feeder cells a day prior to thawing stem cells and change old medium as a feeder to new culture medium for stem cells an hour before thawing the cells.

2) Remove a cryovial containing frozen stem cells from a liquid N_2

tank and rapidly thaw the cryovial in a 37oC water bath within 2 minutes (see Note 39). Transfer the contents of the cryovial to a 15-mL tube containing 4 mL of prewarmed culture medium and gently resuspend them to decrease dimethyl sulfoxide (DMSO) cytotoxicity.

3) Centrifuge at 320 x g for 4 minutes and remove supernatants. Next, resuspend the pellet by adding culture medium for single cells and seed them on feeder-cultured plates prepared in advance. Swirl each plate to distribute the cells equally and incubate at 37℃ with 5% CO_2 in a humidified air atmosphere.

4) Change the stem cell medium in a culture plate every day.

3) Notes

1. Aliquot each reagent as required. However, the amount of time required, frequency of experiments, and reagent loss can affect the aliquot volume of reagents.
2. Any detergent can be used to wash labware instead of the 7X detergent.
3. It is possible to melt fetal bovine serum (FBS; or trypsin–EDTA) at 4℃ or by incubation in a water bath with shaking.
4. To prepare 70% alcohol, mix well 99% ethanol and distilled water as a 7:3 ratio.
5. Antibiotic–antimycotic solution (AA) is a combination of 10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL Fungizone® (amphotericin B). This is effective against Gram–negative bacteria, Gram–positive bacteria, and fungi–containing yeast.
6. Non–ES–grade FBS is generally used to culture feeder cells while ES–grade FBS is used to culture stem cells. Both FBS solutions should be prepared after inactivation (56℃, 30 minutes with shaking) and filtration prior to use for cell culture.

Aliquot and store the prepared FBS at –20℃ until the next use.

7. The 10^7 units of leukemia inhibitory factor (LIF) can be diluted with 10 mL of Dulbecco's modified Eagle's medium (DMEM) to make 10^6 units of LIF solution. Diluted LIF solution is divided into each centrifuge tube covered with aluminum foil and stored at 4℃.
8. The pass box is one laboratory facility intended for material transfer with minimum cross-contamination between two areas without actual personnel movement.
9. Choose the optimal mouse strain depending on the experiment. However, the developmental rate or growth rate can differ according to the mouse strain.
10. Consider that the average litter size of outbred mice such as ICR is larger than that of inbred mice.
11. The 0.04% trypsin–EDTA is the enzyme used for primary culture of MEFs, while 0.25% trypsin–EDTA is the commonly used enzyme for subculture.
12. Add DMSO to the freezing solution prior to use.
13. MMC powder may not dissolve as well as expected and it is important to ensure sufficient mixing.

14. MMC is stored in an amber-mouth bottle because it is sensitive to prolonged exposure to light.
15. The concentration of gelatin solution (0.1%) is not absolute and it is possible to modify the concentration as required.
16. Place gelatin solution into a bigger glass bottle than the preparation volume of solution. Otherwise, the solution will boil over in the operating autoclave.
17. Pay attention to hand protection because 7X detergent is a very toxic detergent.
18. Follow the operating level in a sonicator for the proper amount of water because it is likely to damage the sonicator if the machine is not filled with water until the operating level is operated repeatedly.
19. Autoclave tape is used as a process indicator in steam sterilizers by showing the color change from beige to black.
20. Contents of recording tags.

    a. Recording tag for liquid waste includes the following contents: the date, the amount of waste and main chemical component of waste.

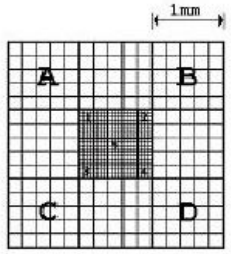
    b. Recording tag for the thermo-hygrostat includes the following contents: the date, temperature displayed on the equipment, actual temperature measured by a thermometer, and

real humidity measured using a hygrometer.

21. Prepare the mix sample (cells + Trypan Blue solution) to at least 10 μL in a centrifuge tube because the loading volume on a hemocytometer is 10 μL for one cell counting.

    a. Cells (10 μL) + Trypan Blue solution (10 μL)

    = 20 μL (Dilution factor = 2)



22. The volume of 1 mm^3 of hemocytometer is 10^{-4} mL.

    a. Length x width x height

    = 1 mm x 1 mm x 0.1 mm = 0.0001 mL = 10^{-4} mL

23. The commercial mycoplasma kits are classified into two types based on the detection principle. The first type is a PCR detection kit that has a set of primers designed to detect mycoplasma. The second type is a selective biochemical test kit that exploits the activity of mycoplasmal enzymes.

24. There are various types of antimycoplasma agents including mycoplasma removal agent (MRA), ciprobay, and BM-cyclin, as well as plasmocin. Therefore, choose the optimal antimycoplasma agent for experiments in consideration of the treatment period, ease of use, efficacy, cytotoxicity, and resistance.

25. To prepare pregnant mice, perform the plug check of mature female mice the morning after interbreeding with mature male mice. The plug check is E0.5−day. Alternatively, pregnant mice can be purchased commercially.
26. Pregnant female mice generally contain 13–14 fetuses.
27. Work should be prepared near the flame of an alcohol lamp for this step under sterile conditions.
28. Ensure removal of the placenta.
29. It is possible to use a sterile surgical blade or a wide point of a blue tip instead of a 1−mL syringe with a bent tip.
30. Use 2–4 mL of the enzyme depending on amount of tissues.
31. Keep the cap of a conical tube open slightly during the incubation.
32. Exclude the sunken mass for the following steps.
33. Resuspend the pellets in culture medium and invert the conical tube about 15 times if more cells are required. Subsequently, centrifuge at 100 x g for 1 minute.
34. Be careful not to remove the blood layer when you transfer the cell pellets.
35. Cells of one fetus should be seeded on a 100−mm culture plate.
36. It commonly takes 3–4 days for the cells to form a confluent

monolayer.

37. The passage number is the number of subcultures performed.
38. It is important that the amount of cells on a 100-mm culture plate is divided into 3-4 cryovials.
39. Thaw frozen cells of a cryovial rapidly in a water bath until only a small ice pellet remains.
40. Not only the fibroblasts of "passage 2" but also of "passage 3" can be used to prepare mitotically inactivated MEFs. If cells of "passage 3" are used for the next step, repeat the process of trypsinization and culture.
41. It is appropriate to seed about 1 x 106 cells onto a 60-mm culture plate.
42. Table 1 provides a comparison among various protocols that have minor differences.

CHAPTER 4.

General Discussion and Conclusion

1. General discussion and conclusion

One of the most common problems present in bio-laboratory work is mycoplasma infection. Despite the importance of bacterial and fungal contaminations in cell culture, they are not such a serious problem because they are usually obvious and easily detected (Hay et al., 1989; Harlin and Gajewski, 2008; Nikfarjam and Farzaneh, 2012). The most serious problem is mycoplasma contamination since there is a wide range of activity, from no detectable changes, through mild cytopathic effects, to destruction of the cells (Macpherson, 1966). Slow-growing, they can remain in the system for years and even decades, until chemicals, sizable emotional stress, injury, vaccination, or other trauma sets them off (Sylver, 2011). Consequently, Mycoplasmas can be difficult to detect in routine cell lines culture work (Hay et al., 1989; Young et al., 2010). Antibiotic therapy provides the best chance of successful decontamination. Several antibiotics, like a Plasmocin or BM-cyclin, are known to be effective against mycoplasmas in cell culture at levels which are relatively non-toxic to the cells harbouring them (Macpherson, 1966). Nonetheless, mycoplasma strains may become resistant to

certain antibiotics and such constant reports have been found in various antibiotics (Balduzzi and Charbonneau, 1964; Macpherson, 1966; Sogen et al., 2008; Uphoff et al., 2012). Mycoplasma infection in cell lines used for research poses a big problem. This insidious effect makes it sure that any data derived from mycoplasma infected cell cultures represent unreliable accuracy containing reliability, reproducibility, and consistency of experimental data (Doersen and Stanbridge, 1981). Since continuous or established cell lines are very useful tools, a number of mycoplasma data, especilly in stem cell lines, have piled up to understand and prevent them in laboratories (Markoullis et al., 2009; Romorini et al., 2013; Lee et al., 2014). Few data of mycoplasma in hiPSCs have reported relatively despite its usefulness.

In chapter 2, analysis on mycoplasma effects in hiPSCs were conducted through wide range of parameters above all. The contamination cause was the hiPSCs itself provided from other laboratories, which was confirmed by PCR-based method or luminometer method. There was no difference in appearance and growth rate of colonies in hiPSCs infected by mycoplasma as well as in pluripotency using immunostaining. Also,

karyotype analysis showed the normal chromosomes but this does not mean that genetic status of mycoplasma-infected hiPSCs is completely safe. In other parameters, mycoplasma being definitely had negative effects on hiPSCs. The accuracy measurements of a proliferation rate were interrupted with contamination. More importantly, decreased differentiation capacity was revealed by the number of EBs formed and the much debris through EB suspension cultures. To eliminate the contamination of mycoplasma, we used two types of antibiotics, Plasmocin and BM-cyclin. Plasmocin strategy is simultaneous application of two different antibiotics that are Tetracycline and Quinolone. BM-cycline principle is sequential treatment of two different antibiotics that are Macrolide and Tetracycline. BM-cyclin could cure mycoplasma contamination in hiPSCs. However, Plasmocin could not. That is, this is additional reports of the resistance of plasmocin antibiotic. In addition, we proved mycoplasma recurrence of hiPSCs is able to arise at any time in several days in spite of mycoplasma decontamination by BM-cyclin antiotics. Therefore, follow-up management is so crucial after antibiotic treatment.

In chapter 3, we suggested SOP for cell culture. SOP is a set of

written instructions that document a routine or repetitive activity followed by an organization. The development and use of SOPs minimizes variation and promotes quality through consistent implementation of a process or procedure within the organization, even if there are temporary or permanent personnel changes (EPA, 2007). Of course, our SOP is not absolute that should follow in every laboratory but can be sufficiently referred for each stem cell laboratory. Given the importance of mycoplasma contamination in the routine cell culture laboratory, and the considerable experience needed in optimization and interpretation of tests, a laboratory will wish to provide thorough training to its inexperienced personnel and confirm any positive or negative results obtained by them (Young et al., 2010). Virtually, our laboratory experienced that cell culture work had been closed down and every hiPSCs were discarded. Moreover, all surfaces were cleaned and disinfected and all old medium and reagents were discarded. The fulfillment of standard testing for mycoplasma infection is an crucial quality control that should be contained in SOPs. Unless researchers are able to discard the contaminated cell line, they must select and treat the most suitable antibiotics. However, cytotoxic effects and resistance likelihood should be taken into

consideration. Heightened awareness about mycoplasma contamination, and increased use of sensitive and effective methods for the detection and treatment of mycoplasma contaminations, will shepherd to a lower incidence of contamination (Fiorentini, 2014). Additionally, it is clear that a general improvement of aseptic techniques for cell-culturing will lead to a decline in the percentage of contaminated cell cultures (Macpherson, 1966).

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다양한 종류의 세포배양 오염원 중, 마이코플라즈마 감염은 예상치 못한 연구결과를 초래하거나 또는 혼선을 초래한다. 또한, 직접적인 테스트를 제외하고는 연구자가 그들의 존재를 쉽게 인식하기 힘들다. 그러므로, GLP 수준에서 SOPs 가 잘 지켜지고 있는 실험실 환경을 관리하는 것이 위생적인 세포 배양을 유지하고 관리하는데 매우 중요하다. 이 스터디에서는, 세포 배양에서 마이코플라즈마 오염으로 인한 영향을 평가하기 위해, 인간 유도 전능 줄기세포를 대상으로 마이코플라즈마 감염(+) 그룹과 그렇지 않은(−) 그룹을 다양한 세포학적 파라미터를 사용하여 비교해 보았다. 또한, 마이코플라즈마 오염을 치료하기 위해 항생제 (Plasmocin 과 BM−cyclin) 를 사용하였고, 그 효과를 관찰하였다. 결과는 (1) 마이코플라즈마가 감염된 상태에서는 인간 유도 전능 줄기세포의 증식률 및 분화능력의 정확한 측정이 방해되었다. (2) 치료를 위해 사용된 항생제 중, BM−cyclin 은 감염된 인간 유도 전능 줄기세포의 마이코플라즈마를 제거하는 능력을 보여 주었고, Plasmocin 항생제는 그러하지 못했다. (3) 게다가, BM−cyclin 을 이용해 마이코플라즈마 오염을 치료한 경우라 하더라도 오염 재발이 탐지될 수 있음을 확인하였다. 다시 말해, 우리는 마이코플라즈마 오염이 세포 배양 실험의 결과에 끼치는

부정적인 영향들을 관찰하였고, 그를 치료하는데 사용하는 일반 항생제에서의 내성을 발견 및 치료 후에도 나타날 수 있는 마이코플라즈마 재발 위험성을 직접적으로 확인하였다. 게다가, 추가 결과물로써 실험실에서 바로 이용 가능한 표준작업지침을 제시하여 실험실 환경의 효과적 관리를 꾀한다.