A Thesis for the Degree of Master of Engineering

Reprogramming of Mouse Embryonic Fibroblast by External Stimuli and Vitamin C

외부자극들과 비타민 C에 의해 리프로그램된 쥐의 배아 섬유아세포

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이 논문을 공학석사 학위논문으로 제출함

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Abstract

After estamblish reprogramming concept, many methods were developed such as using transgenes, synthesized small molecule, and recombinant protein. Induced pluripotent stem cells (iPSCs) can proliferate without limitation and differentiate into three germ lines. Reprogramming cells such as iPSC are good medicine resource because of differentiated to various tissues or cells. However, iPSCs which are generated by traditional methods that transgenes or virus vectors are utilized during generating iPSCs are not appropriate for clinic because of safety and risk of tumor.

This research attempted to develop a new method for reprogramming using small molecule and external stimuli without transgene. First, 4 groups were selected; condition with both acid and shear stress, with acid, with shear stress , without acid and shear stress. In acid experiment, cells were soaked at

pH 5.4 solution for 25min. In shear stress experiment, pipetting was carried out cells twice per day for 2 minutes under suspension culture and it continued for 7 days. 500ul of embryonic stem (ES) medium was added in 60mm the petri dish every day for 7 days. After 7 days, spheres were picked up and they were utilized in alkaline phosphate (ALP) staining immunocytochemistry to confirm pluripotency. Spheres of all group showed positive- ALP and pluripotent proteins but aspects of staining were not perfect. This experiment show that acid or shear stress assisted to generate spheres compared with group without any stimulation but acid induced cell death.

For improving efficiency, stimulated period was modified much longer and vitamin C was selected instead of acid. Vitamin C had been reported that it promoted to accelerate reprograming and increased efficiency of generating iPSCs. 2 groups were established; shear stress under suspension culture and combination of shear stress and vitamin C under suspension culture. ES medium or ES medium with 10uM vitamin C was added into experimental group every day for 14 days according to experimental design. For 14 days, pipetting was carried out cells twice per day for 2 minutes. After 14 days, all spheres were picked up and were performed experiments for confirming pluripotency and capacity of differentiation such as ALP staining, immunocytochemistry with pluripotent markers and 3 germ layers' marker, western blot, fluorescence activated cell sorter (FACS) and teratoma formation in vivo.

Both groups were observed positive results in ALP staining, immunocytochemistry with pluripotent markers, western blot, FACS,

differentiation in vitro although difference of degree existed. However, any group did not form teratoma as in vivo experiment.

Although shear stress group and group of combination of shear stress and vitamin C were not significantly different in FACS data, most of data demonstrated the spheres under shear stress and 10uM vitamin C had better. In synthesizing all data, combination of 10uM vitamin C and shear stress under suspension culture was appropriate for generating spheres which had capacity of differentiation.

This research showed possibility that methods using external stimuli and small molecule without transgenes can reprogram from somatic cells to differentiated cells. This method would be utilized in direct cell reprogramming field.

주요어 : mouse embryonic fibroblast, reprogramming, sphere formation, shear stress, vitamin C, nitric oxide

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List of Terms and Abbreviations

ALP	Alkaline phosphatase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
BAEC	Bovine aortic endothelial cell
BSA	Bovine serum albumin
CE	Ciliary body epithelial
CHIR	CHIR99021
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's Medium
Dnmt3b	DNA methyltransferase 3b
DPBS	Dulbecco's phosphate-buffered saline
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial- to-mesenchymal transition
eNOS	Endothelial nitric-oxide synthase
ESC	Embryonic stem cell
Esrrb	Estrogen related receptor, beta
FACS	Fluorescence activated cell sorter
FSK	Forskolin
HBSS	Hanks' balanced salt solution
HCAEC	Human coronary artery endothelial cell
HCCEC	Human corpus cavernosal endothelial cell
hESC	Human Embryonic stem cell
HUVEC	Human umbilical vein endothelial cell
ICC	Immunocytochemistry
Id	Inhibitor of differentiation
IP	Intraperitoneal
iPSC	induced pluripotent stem cell
IRS	Insulin receptor substrate

Klf4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
MAF	Mouse adult fibroblast
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cell
MET	Mesenchymal to epithelial transition
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
NEAA	Non-essential amino acids solution
NO	Nitric oxide
NOS	Nitric oxide synthase
Oct4	Octamer-binding transcription factor 4
SC	Subcutaneous injection
Sox2	Sex determining region Y-box 2
STAP	Stimulus-triggered acquisition of pluripotency
STAT3	Signal transducer and activator of transcription 3
Tet1	Ten-eleven translocation 1
Tet2	Ten-eleven translocation 2
Tuj1	Neuronal class III b-tubulin
Zeb1	Zinc finger E-box binding homeobox 1
2-Me-5HT	2-methyl-5-hydroxytryptamine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine

1. Introduction

In 1957, Waddington suggested the concept of an “epigenetic landscape” which is involved in reprograming today [1]. Gurdon replaced UV-treated nucleus of a frog egg by differentiated nucleus in 1962 [2]. This experiment proved cells can be reprogrammed. Yamanaka first generated induced pluripotent stem cells (iPSCs) from somatic cells derived from mouse in 2006 and human in 2007 [3, 4]. Developing iPSCs innovative event in cancer therapy and regenerative field since pluripotent stem cell can differentiate most of cell or tissue type [5, 6]. However, it has limitation for clinic right now because of low efficiency of generating iPSCs and safety issues involved in the generation of iPSCs including methods using virus or transgenes [6]. For improving the efficiency or developing risk-free methods, iPSCs can be generated by genetic methods or chemical methods such as various combinations of DNA, virus-free methods, external proteins, and small molecule [7-14].

In 2014, Obokata introduced stimulus triggered acquisition of pluripotency (STAP) stem cells which CD+45 lymphocyte were obtained pluripotency by treating mild acid but it was proved that treating transient acid was no effect [15, 16]. This paper suggested external stimulus could assist reprogramming of cells even though the paper was fabricated and retracted.

Low-frequency electromagnetic fields could support to generation of iPSC from somatic cells [17]. Graphene based substrate result in increasing efficiency of iPSC through promoting mesenchymal to epithelial transition (MET) process [18].

This study select three factors which are sphere formation, nitric oxide (NO) and vitamin C to help reprogramming from somatic cells. Sphere formation, low concentration of nitric oxide (NO) and vitamin C which were reported to increase maintain pluripotency and iPSC's efficiency [19-26]. NO can be induced by shear stress [27-32].

This thesis was attempt to confirm effect of acid and shear stress on MEF in advance. Then, there was verified that shear stress or combination of shear stress and vitamin C under suspension culture could help reprogram from MEF to cell types of three germ layers.

2. Objectives

The objective of this thesis was a new method to acquire differentiation capacity of MEF. For this, MEFs were cultured under acid, shear stress and vitamin C on suspension culture. Then, MEF spheres under each condition were compared for confirming optimized reprogramming environment.

Detailed objectives were as follows:

1) To optimize the environment for generating MEF spheres.

2) To investigate quality of spheres whether they are capable to differentiate into three germ layers under shear stress and combination of shear stress and vitamin C

3. Literature Review

3.1. Cellular reprogramming from somatic cell to pluripotent stem cell (PSC)

Yamanaka group first created artificial pluripotent stem cell called iPSC from MEF with Oct4, Sox2, Klf4, c-Myc which are called Yamanaka factors [3]. Adult human fibroblasts were reprogrammed to iPSC by Yamanaka factors [4]. New combinations of gene factors were developed. Thomson's group succeeded to produce iPSC from human somatic cells by another gene combination which consists of Oct4, Sox2, Nanog, Lin28 [33]. Jaenisch and his colleagues found a new combination of Sall4, Nanog, Esrrb and Lin28 was better iPSC's efficiency than Yamanaka factors in MEF [34]. Another method to generate iPSC is to decrease the number of transgenes. Only Oct4 and Sox2 induced iPSC from human fibroblast [7]. In addition to transgenes, small molecule, for example RSC133, raise efficiency of iPSC [14]. To avoid risk of virus, various methods were utilized. There were reported minicircle vector, plasmid with calcium phosphate or gene carrier such as poly-beta-amino ester, magnetic nanoparticle, Lipofectamine2000, nucleofector kit [8-10, 12]. Except for genetic methods, proteins and small molecule cocktail can reprogram from somatic cells to pluripotent stem cells. Recombinant proteins of Oct4, Sox2, Klf4 and c-Myc produced iPSC although time of generating iPSC was longer than virus including transgenes methods [11]. Seven small molecules which are Forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me-

5HT), and D4476, VPA, CHIR99021 (CHIR), 616452, Tranylcypromine generate iPSC without any transgene and efficiency was up to 0.2% [13]. This paper proposes only chemical treatment is possible to generate iPSC.

3.2. Influences on sphere formation through suspension culture

Sphere formation of embryonic stem cell (ESC) and iPSC have an effect on yield. Suspension culture not only increased yield of pluripotent cells but also kept undifferentiated state. These result pluripotent cells. These results indicate possibility of scale-up processes [19].

Sphere formation of mouse adult fibroblast (MAF) and MEF led to stable reprogramming and immortalization. Cell signaling by forming sphere induced to decrease Zeb1 which was involved in Epithelial- to-mesenchymal transition (EMT). There were also reported expressions of Oct4 and Nanog were weakened compared with sphere formation when MAF and MEF were culture in monolayer [20].

Sphere formation drove iPSC generation from ciliary body epithelial (CE) with only Oct4. Compared with sphere and monolayer of CE, Sall4, c-Myc, Lin28, Glis1, Klf4 and Nanog which were related to reprogramming were significantly upregulated in sphere. Also, the levels of Dnmt3a, Dnmt3b, Tet1, and Tet2 which are necessary genes in EMT process were higher in sphere. These results proposed forming sphere partially caused reprogramming [21].

3.3. Effects of shear stress in cells

Shear stress induces to synthesize nitric oxide (NO) from endothelial NOS (eNOS) in certain cell types ; human corpus cavernosal endothelial cell (HCCEC), human coronary artery endothelial cell (HCAEC), human umbilical vein endothelial cell (HUVEC), bovine aortic endothelial cell (BAEC), MEF [27]. Fluid shear stress-induced [29, 31-32].

Shear stress not only encouraged the intensity and phosphorylation of eNOS but also activated eNOS in endothelial cell [30]. Detailed mechanism was below. Shear stress lead to AMP-activated protein kinase (AMPK) activation which mediated phosphorylation of eNOS Ser633/635. Phosphorylated Ser633/635 resulted in NO production [29]. Shear stress also induced AMPK and SIRT1 but these were stimulated independently. Both protein activated eNOS and increased NO bioavailability [28].

When a range of shear stress was from 0 dynes/cm^2 to 25 dynes/cm^2, strong shear stress induced more expression of eNOS mRNA in BAEC [32]. In addition, No which was produced by eNOS maintained at low level [27].

3.4. Character and biological effects of Nitric oxide (NO)

NO is an ephemeral free radical molecule, which is synthesized by nitric oxide synthase (NOS) to produce L-citrulline and NO from L-arginine in mammalian cells [23]. NOS has three isoform; neuronal NOS (nNOS), inducible NOS (iNOS) and eNOS [23]. NO is involved in survival,

proliferation and angiogenesis [24, 26, 30].

Also, NO plays important roles but the roles are different depending on concentration in stem cells [22]. When concentration of NO was high, it caused differentiation process and apoptosis in embryonic stem cells (ESC) [24, 26]. During differentiation, the expression of eNOS decreased and the expression of iNOS increased [26]. On the other hand, low concentration whose range is from 2uM to 20uM of NO prohibited to disappear expression of self-renewal genes which were Oct4, Sox2, Nanog and SSEA-1 in mouse embryonic stem cell (mESC) and SSEA-4 in human embryonic stem cell (hESC) [26]. In addition, 5uM of NO is maximum Nanog expression [25]. However, Oct4 and Nanog decreased with 50uM of NO [26].

The revealed mechanism was vitamin C activated Tet enzyme which triggered to demethylate many gene promoters and upregulate demethylated germline genes through converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [35, 36].

3.5. Properties and biological function of Vitamin C

Vitamin C is essential element in cells but it is sensitive about light [37, 38]. Vitamin C also facilitated to improve efficiency of pluripotency. Vitamin C improved iPSC generation from mouse and human somatic cells. Vitamin C alleviated cell senescence during reprogramming but it is another property of vitamin C not as antioxidant. Over 10ug/ml of vitamin c increased expression of pluripotent genes. Vitamin C also accelerated partial reprogramming state to fully reprogramming state [39].

Treating Vitamin C downregulated genes involved in differentiation genes and upregulated genes related to pluripotent genes. It indicated that vitamin C inhibited mESC differentiation and support to maintain pluripotency [40].

4. Materials and Methods

4.1. MEF isolation and cell culture

Pregnant mice of strain ICR (E13.5) were dissected for isolating MEF. Embryos were separated by uterine horns. They were washed with 70% ethanol (Duksan, Korean) and Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (Welgene, Korea). Then, they were removed head and red organs. After the remnants of the embryos were minced by a sterile razor blade, they were treated by 0.05% trypsin-EDTA(Welgene, Korea) including 100units of DNase (Promega, USA) and incubated for 15 minutes at 37℃. MEF medium which consisted of Dulbecco's Modified Eagle's Medium (DMEM) (Welgene, Korea), 10% Fetal bovine serum (FBS) (Gibco, USA), 10uM L-glutamine (Sigma-aldrich, USA), 1% antibiotic antimycotic solution and 1% non-essential amino acids solution (NEAA) (Gibco, USA) was added for inactivating trypsin-EDTA. After centrifuging them, supernatant was removed and they were transferred to a T75 flask coated 0.2% gelatin (Sigma-aldrich, USA) with fresh MEF medium at 37℃ and in 5% CO_2. Passage 0 of the fibroblasts were subcultured until passage 2.

4.2. Cytotoxicity assay

Cytotoxicity of Vitamin C was evaluated by MTT assay in MEFs for 24 hours and 72 hours. MEFs were seeded on $3X10^4$ per well of 24-well plate in 500ul MEF medium. After 48 hours, cells were treated at diverse concentration of vitamin C (10, 50, 100, 250, 500uM) for 24 hours or 72 hours. 24 hours or 72 hours later, 500 ㎕ MTT solution which was mixed thiazolyl blue tetrazolium bromide (Sigma-aldrich, USA) and DMEM medium was added per each well and the final concentration was 0.5mg/ml. They were incubated for 4 hours at 37℃ and in 5% CO_2 then the medium was aspirated. The remainders of purple formazan crystals on well were dissolved in 500 ㎕. 100 ㎕ of 500 ㎕ dissolved formazan from each well were transferred in 96-well plate and absorbance was measured at 540nm using a Sunrise absorbance reader (Tecan, Austria).

4.3. Acid stimulation

1 X 10^6 MEFs of passage 2 were seeded in in 60mm non-coated petri dish (SPL, Korea) containing pH 5.4 of Hanks' Balanced Salt Solution (HBSS) (Welgene, Korea) and were incubated at 37℃ for 25 minutes. They were harvested in 15ml conical tube and centrifuged. Supernatant was aspirated in suspension. ES medium which constitutes DMEM, 15% Knockout serum

replacement (Gibco), 10uM L-glutamine (Sigma-aldrich, USA), 2-mercaptoethanol(Sigma-aldrich, USA) 1% antibiotic antimycotic solution and 1% non-essential amino acids solution (NEAA) (Gibco, USA) and 5X 10^5 unit leukemia inhibitory factor (LIF) (Millipore, USA) was added in a pellet. After the pellet was resuspended in ES media and transferred in 60mm the petri dish. The petri dish plated in an incubator at 37℃ and 5% CO_2 for one week. 500ul of ES medium was added in 60mm the petri dish per every day during incubation.

4.4. Shear stress

MEF cells of passage 2 were seeding 1 X 10^6 in 60mm non-coated petri dish (SPL, Korea) or 4 X 10^6 in 150mm the petri dish (SPL, Korea) at D0. The cells were cultured in ES medium. After that, the cells were stimulated with 1ml pipet (Eppendorf, Germany) and 1ml pipet tips (Axygen, USA) for 2min twice a day. Gap time between first stimulation and second stimulation is over 5 hours. Experiment time was one week according to Vacanti and Kojima's first revised protocol using acidic condition and shear stress, and two weeks compared to combination of shear stress and vitamin C. After incubation, spheres were picked up for next experiments.

4.5. Shear stess and vitamin C treatment

This process was same in shear stress part. Medium was ES medium including 10uM L-ascorbic acid (Sigma-aldrich, USA). After adding ES meida containing vitamin C, cells were applied by shear stress in dark room and it continued for 14days. After 14 days, spheres were picked up for next experiments.

4.6. Coomassie staining

For isolating protein, cells were incubated in 1X RIPA lysis buffer (Millipore, USA) with phosphatase inhibitor cocktail solution (GenDEPOT, USA) and protease inhibitor cocktail solution (GenDEPOT, USA) for 20 min on ice. After centrifuging for 20min at 4℃, proteins in supernatant was measured by Pierce™ BCA protein assay kit (Thermo scientific, USA). Total proteins and Xpert 2 prestained protein marker (GenDEPOT, USA) were load on 4-12% Bis-Tris gel (Invitrogen, USA) for 35min in XCell *SureLock*® Mini-Cell (Invtrogen, USA). The Bis-Tris gel was soaked under coomassie staining solution comprised of 0.66g coomassie Brilliant blue R-250 (Acros organics, USA) and 150ml Methanol (Carlo Erba reagents, France), 30ml acetic acid glacial (Duksan, Korea) and 150ml distilled water. 4-12% Bis-Tris gels (Invitrogen) for 2 hours. The gel was

placed under destaining solution which were consisted of 100 ml methanol, 50 ml acetic acid glacial, and 350 ml distilled water overnight.

4.7. Alkaline phosphatase (ALP) staining

ALP staining was performed using Alkaline Phosphatase Blue Membrane Substrate Solution (Sigma-aldrich, USA) according to the manufacturer's instructions. The MEF spheres were observed by Nikon ECLIPSE Ti with DS- U3 (Nikon, Japan) and NIS-Elements AR. 4.20 program.

4.8. Immunocytochemistry (ICC)

The MEF spheres were picked up at D15. They were transferred on 4 well plate (Nunc, USA) coated hESC-qualified matrigel (BD Biosciences, USA) at 37℃ for 2hour. When spheres were attached on 4-well plate, the spheres were fixed by 4% paraformaldehyde (Sigma-aldrich, USA) for 15minutes then washed by DPBS twice. They were soaked in DPBS including 0.2% Tween 20 (Amresco, USA) for 10min at room temperature. After washing them with DPBS twice, they were placed in 10% bovine serum albumin (BSA) (Bio basic canana Inc., Canada) for blocking nonspecific binding for 1 hour at room temperature then BSA was removed. They were incubated in each 10% BSA solution including ratio 1 : 20 of anti-Oct4

(Stanta Cruz biotechnology, USA), anti-Nanog (Abcam, USA), anti-SSEA-1 (Abcam, USA) and ratio 1 : 200 of anti-Neuronal Class III b-Tubulin (TUJ1) (Covance, USA), anti-Sarcomeric Alpha Actinin (Abcam, USA) and anti-alpha 1 fetoprotein (NOVUS Biologicals, USA) overnight at 4℃. They were washed twice with DPBS then incubated in each 10% BSA solution of ratio 1 : 50 for pluripotent markers or 1 : 400 for differentiation markers of Alexa Fluor 488 goat anti-mouse IgG (H+L) (Life Technologies, USA) or Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Life Technologies, USA) at room temperature for 2 hours. They were washed with DPBS then 2mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-aldrich, USA) was added. After 10minutes incubation at room temperature, they were washed with DPBS once. They were placed on slide glass and mounted using aqua poly/mount (Polysciencs Inc., USA). Images of Oct4, Nanog, SSEA-1 in results of shear stress and vitamin C were acquired by LSM710 (Carl Zeiss, Germany) with Zen 2009 LE and images of results of acid and shear stress and three germ layers in vitro were obtained by ECLIPSE Ti (Nikon, Japan) with Zyla sCMOS (Andor technology, UK) camera and NIS-Elements AR. 4.20 program.

4.9 Fluorescence activated cell sorter (FACS)

The spheres were harvested in 15ml tube. They were washed with DPBS and treated 0.05% trypsin-EDTA (trypsin-ethylenediaminetetraacetic acid) for 5 minutes at 37℃. After adding medium including FBS, rinse them with cold

DPBS once. They were incubated with 10% BSA for 1hour on ice to block Fc receptor. After 1 hour, each 10ug/ml of primary antibodies of anti-Oct4 (Santa Cruz, USA) and anti-SSEA-1 (Abcam, USA) in 3% BSA solution was added and incubated for 1 hour on ice in dark. The spheres were fixed with 1% paraformaldehyde for 10 minutes on ice. After fixation, added secondary antibody in 3% BSA solution of ratio 1 : 100 of Alexa Fluor 488 goat anti-mouse IgG (H+L) (Life Technologies, USA) for 1 hour on ice under dark. After rinse them with DPBS, they were passed through a cell strainer of 35μm nylon mesh (BD, USA) to make single cells. They were analyzed by FACS Aria II (BD, USA) with FACSDiva software.

4.10 Western blotting

It was followed from isolating protein to loading protein in 4-12% Bis-Tris gel in coomassie staining method. The proteins were transferred to nitrocellulose (Invitrogen, USA) to use iBlot Dry Blotting System (Invitrogen, USA) for 7min. The membrane were blocked in DPBS (Welgene, Korea) containing 5% of blotting grade blocker (Bio-Rad, USA) and 0.1% Tween 20 (Amresco, USA) for one hour. Each primary antibodies of Oct4 (Santa Cruz, USA), SSEA-1 (Abcam, USA) in skim milk solution were incubated on membrane at 4℃ overnight. The membrane was washed and incubated with secondary antibody of anti-mouse horseradish peroxidase (Life technologies) for two hours at room temperature. Enhanced chemiluminescence (ECL)

chemiluminescent Substrate Reagent (Life technologies) was spread on the membrane and observed by chemidoc (Bio-rad, USA)

4.11. Self-renewal test and spontaneous hetero-differentiation in vitro

At 2 days before spheres were transferred on matrigel-coated 35mm dishes, MEF feeder cells by mitomycin C (Sigma, USA) had been seeded on T-75 (Thermo scientific, USA). Next day, Medium was exchanged to ES medium or ES including 10uM of vitamin C which would change into conditioned medium. After picking spheres up and place them on the 35mm dishes, they were cultured under the conditioned media which were exchanged daily.

4.12. In vitro differentiation

For neuron differentiation, cell spheres transferred on matrigel-coated plate. The spheres were culture in neuron differentiation medium which included DMEM (Welgene, Korea), L-glutamine. Medium was changed every day.

Cell spheres placed on 0.2% gelatin coated plate. For cardiomyocyte differentiation, the spheres were cultured in ES medium which was supplemented 100 uM L-ascorbic acid removed LIF. Medium was changed

every 2 days. For endoderm differentiation, the spheres were cultured under ES medium without LIF. Medium was changed every 2 days.

4.13. Teratoma formation

After collecting cells of 1 X 10^5/100ul, the cell solution in DPBS mixed matrigel in the same amount on ice. The Bal/bc nude mice were anaesthetized by Intraperitoneal (IP) injection with anaesthetic which Zoletil (Virbac, France) and Rompun (Virbac, France) mixed of 4:1 ratio then were diluted 8 times in DPBS. Cell and matrigel solution was injected by subcutaneous injection (SC). After injection the solution, mice laid on 37℃ plate for 10 min.

8 weeks later, mice were sacrificed and teratomas were confirmed whether they existed or not.

4.14. Nitric oxide (NO) assay

For manufacturing standard solution, different concentration of sodium nitrite (Sigma-aldrich, USA) added in each ES medium and ES medium with vitamin C.

Different concentration of standard solution (0 uM, 2 uM, 4 uM, 6 uM, 8 uM, 10uM, 12uM, 14uM, 16uM, 18uM and 20uM) and supernatant medium of control and experiment groups mixed with equal volumes of 40 mg/ml Griess reagent (modified) (sigma-aldrich, USA). After incubation at 37℃ for 15min,

absorbance was measure at 540nm using a Sunrise-basic microplate reader (Tecan, Austria). All experiments were conducted in triplicate.

4.15. Statistical data analysis

All data were triplicated and analyzed using one-way analysis of variance (ANOVA) test using Originlab version 9. P values less than 0.05 were considered significant.

5. Results and Discussion

5.1. Effects of acid or shear stress

5.1.1. Morphologhy

First, Vacanti and Kojima's first revised protocol demonstrated acid is one of important factor. To confirm it, 4 group were established; condition with acid and shear stress, with acid without shear stress, without acid and with shear stress and with acid and shear stress.

Figure 1 were under each condition with or without acid and shear stress. Very few spheres were formed under condition without both acid and shear stress, they were no big different under the other condition. Also, few spheres were generated under only acid condition. This results indicated acid had trend to interrupt to form spheres.

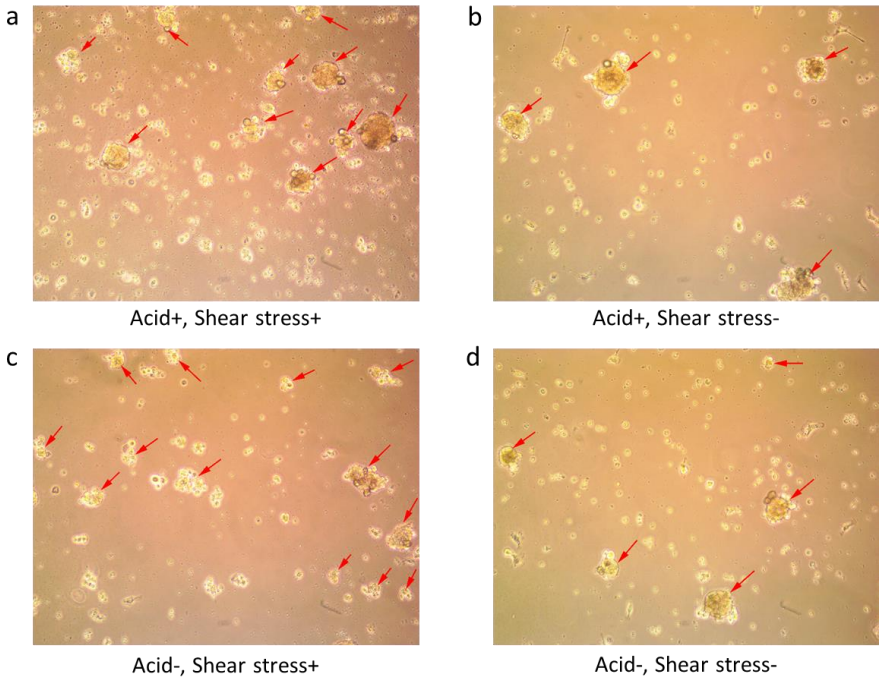


Figure 1. Morphology under acid or shear stress. To generating shear stress, there was pipetting for 2min and twice per day and it was continued for 7 days. a: Condition of acid and shear stress. b: Condition of acid. c: Condition of shear stress. d: Condition without both acid and shear stress. (Magnification: 100X, Arrow: sphere)

5.1.2. Alkaline phosphatase (ALP) staining

Figure 2 showed ALP staining. Some spheres were stained in all of conditions (figure 2. a-d). However, small amount of spheres in condition under without acid and shear stress were stained.

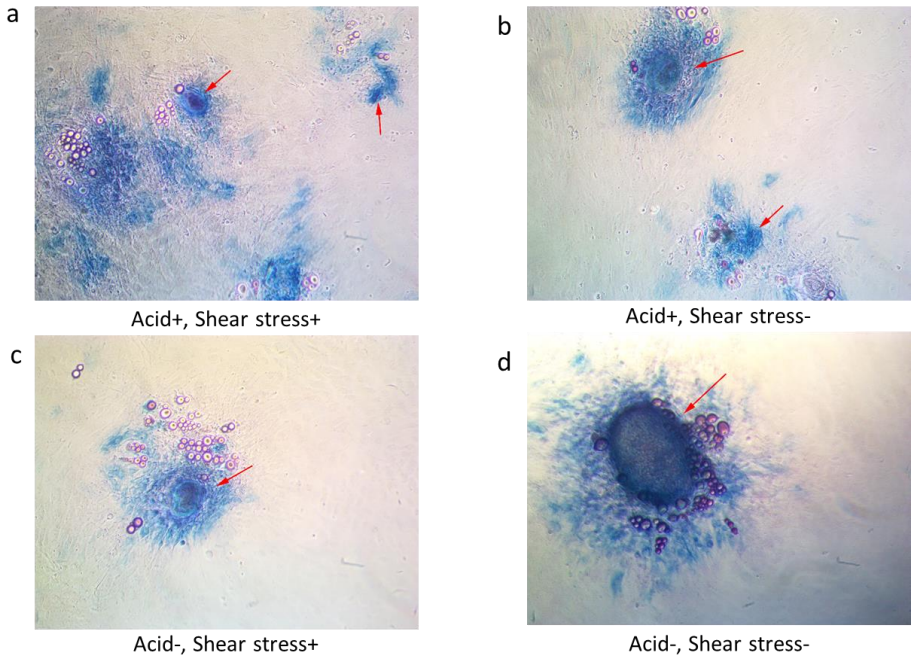


Figure 2. ALP staining under acid or shear stress. a: Condition of acid and shear stress. b: Condition of acid. c: Condition of shear stress. d: Condition without both acid and shear stress. (Magnification: 100X, Arrow: sphere)

5.1.3. Immunocytochemistry (ICC)

To confirm pluripotency, ICC was a method with antibodies of Oct4, Nanog and SSEA-1 which are pluripotent markers. Four groups showed expressions of pluripotent genes were positive.

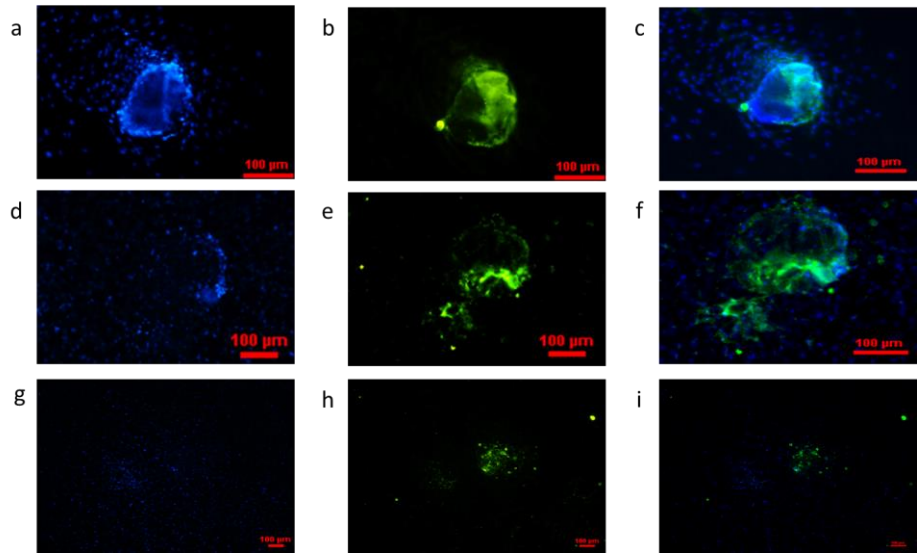


Figure 3. Immunocytochemistry under condition of both acid and shear stress. a, d, g : DAPI image for nucleus staining, b : Image of Oct4 staining, c : Merge image of DAPI and Oct4, e : Image of Nanog staining, f. Merge image of DAPI and Nanog, h : Image of SSEA-1 staining, i : Merge image of DAPI and SSEA-1. (Magnification: 100X)

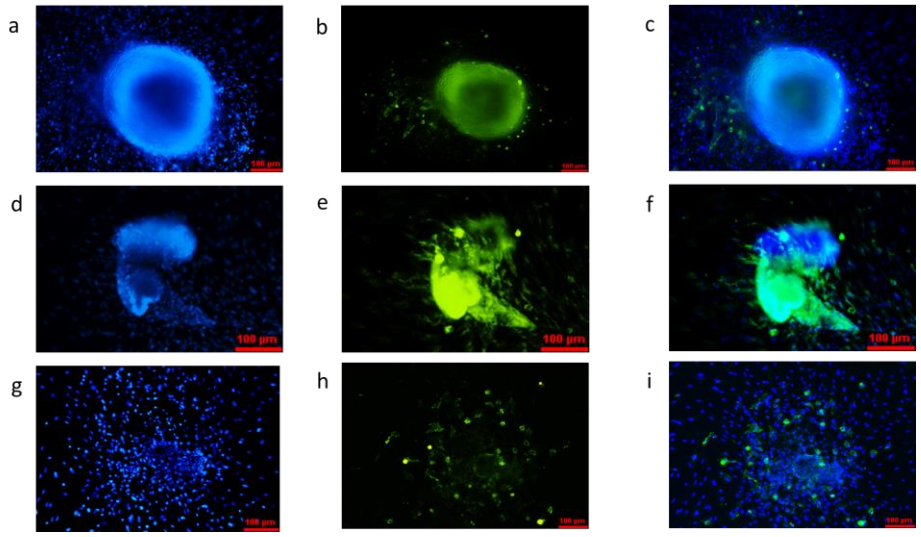


Figure 4. Immunocytochemistry under condition of acid. a, d, g : DAPI image for nucleus staining, b : Image of Oct4 staining, c : Merge image of DAPI and Oct4, e : Image of Nanog staining, f. Merge image of DAPI and Nanog, h : Image of SSEA-1 staining, i : Merge image of DAPI and SSEA-1. (Magnification: 100X)

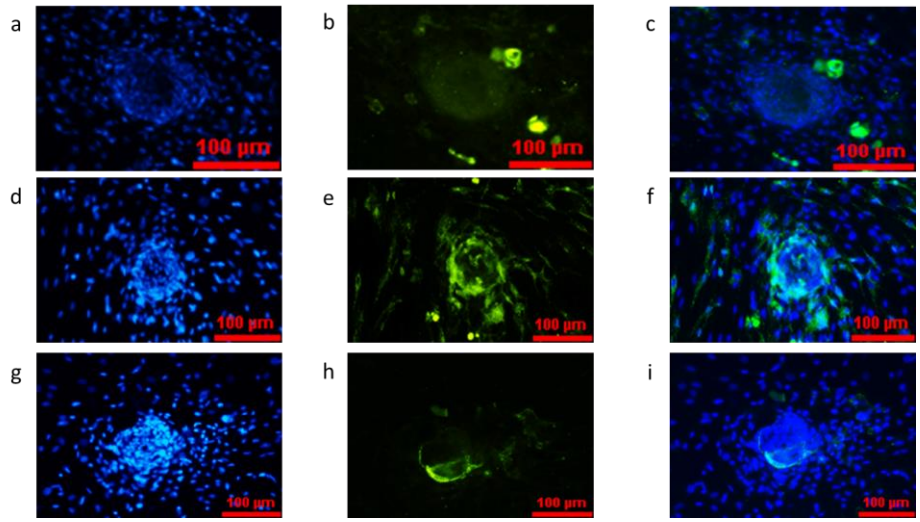


Figure 5. Immunocytochemistry under condition of shear stress. a, d, g : DAPI image for nucleus staining, b : Image of Oct4 staining, c : Merge image of DAPI and Oct4, e : Image of Nanog staining, f. Merge image of DAPI and Nanog, h : Image of SSEA-1 staining, i : Merge image of DAPI and SSEA-1. (Magnification: 100X)

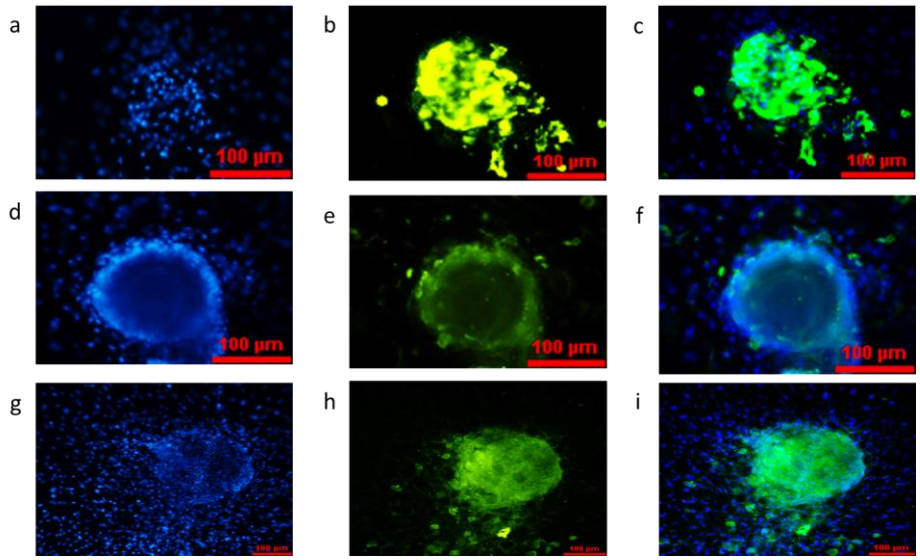


Figure 6. Immunocytochemistry under condition of neither shear stress nor, acid. a, d, g : DAPI image for nucleus staining, b : Image of Oct4 staining, c : Merge image of DAPI and Oct4, e : Image of Nanog staining, f. Merge image of DAPI and Nanog, h : Image of SSEA-1 staining, i : Merge image of DAPI and SSEA-1. (Magnification: 100X)

5.2. Effects of shear stress and vitamin C

5.2.1. Cytotoxicity of vitamin C

Vitamin C was selected because it reported vitamin C enhanced stemness. Before reprogramming from MEF, cytotoxicity of vitamin C was measured through MEF viability.

Figure 7 showed MEF viability about vitamin C. After vitamin C was treat at 24 hours, MEFs treated 10uM of vitamin C decreased proliferation rate. But, after 72 hours when vitamin C was treat in MEF, it was no significant different of all concentration of vitamin C.

10uM vitamin C was chosen because concentration from 10uM vitamin C improved efficiency of iPSC generation and reprogramming process.

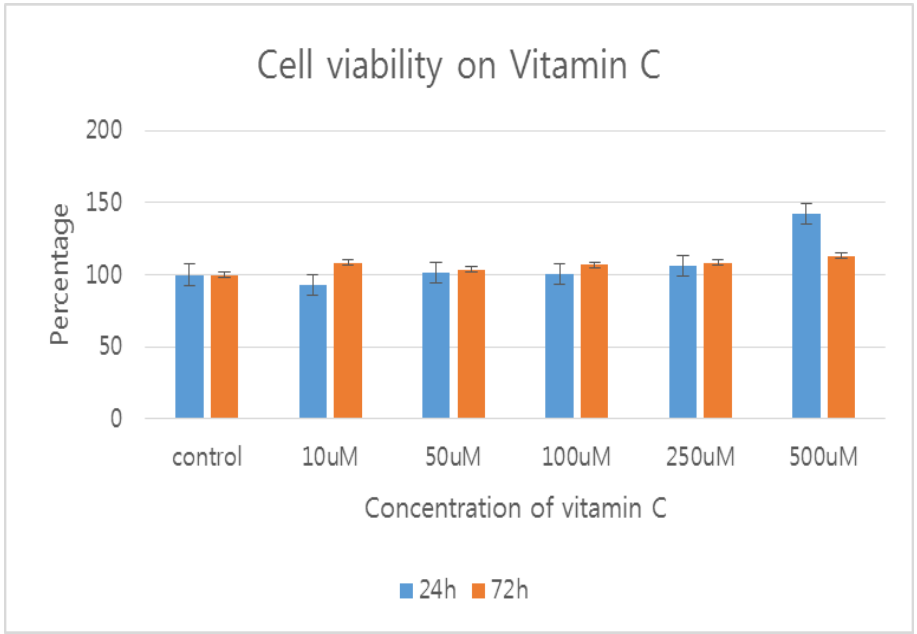


Figure 7. Cell viability on vitamin C. Error bars represent standard deviation, n=3.

5.2.2. Process to generate spheres

MEFs were culture in gelatin-coated plate had spreading shape. However, MEFs were culture in low attached plate formed spheres. Spheres under shear stress or combination of shear stress and vitamin C increased the area of spheres after passing time.

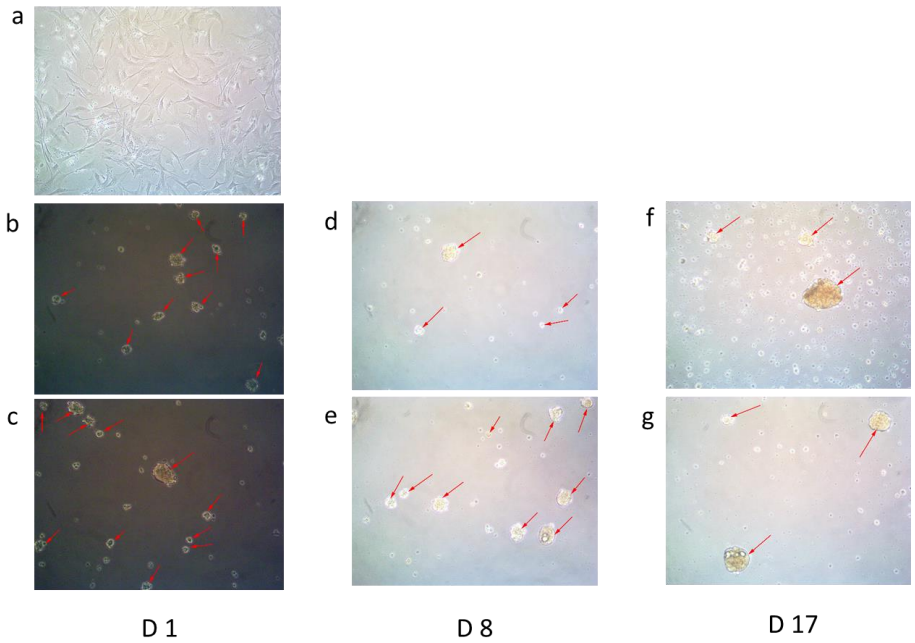


Figure 8. Process of generate spheres. a. MEF culture in gelatin-coated plate. b. MEFs were seeded on low attached plate under shear stress at D1. c. MEFs were seeded on low attached plate under shear stress and vitamin C at D1. d. MEFs were under shear stress at D8. e. MEFs were under shear stress and vitamin C at D8. f. MEFs were under shear stress at D17. g. Under shear stress and vitamin C at D17. (Magnification: 100X)

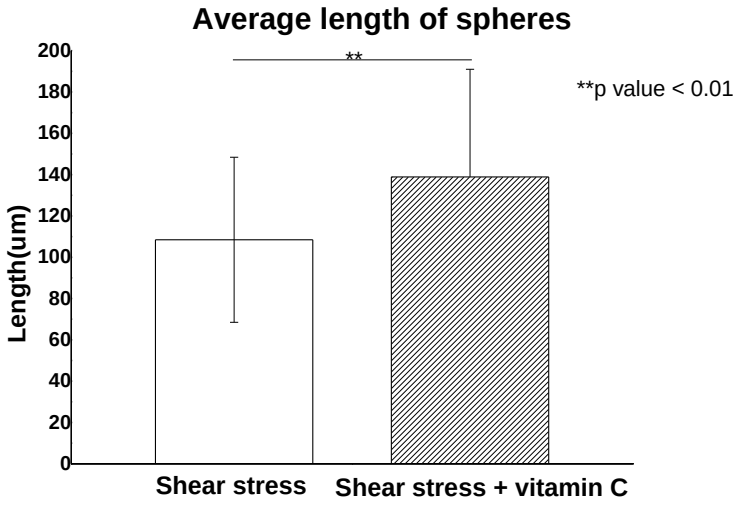


Figure 9. Average length of spheres. Error bars represent standard deviation, n=3, **P values < 0.01.

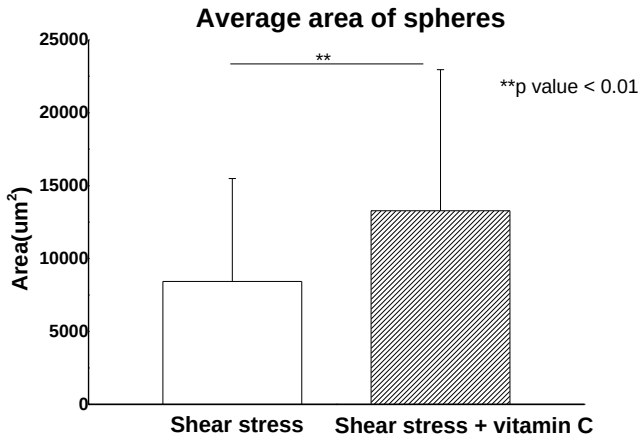


Figure 10. Average area of spheres. Error bars represent standard deviation, n=3, **P values < 0.01.

After picking each spheres, they were measured of length and area. Group of combination shear stress and vitamin c was longer and larger than one of shear stress. This may suggest combination shear stress and vitamin c had high efficiency of forming cell per sphere.

5.2.3. Coomassie staining

Figure 11 presented protein patterns of shear stress, shear stress + vitamin C and MEF. Protein patterns in shear stress group and shear stress + vitamin c group were different from MEF. This data suggested expression pattern of protein by shear stress and vitamin C.

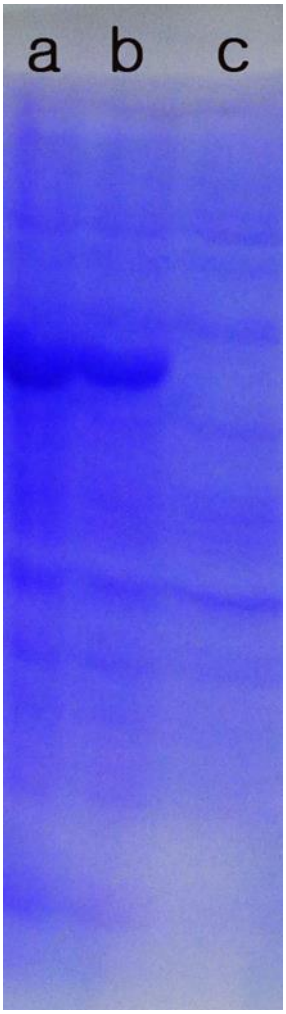


Figure 11. Coomassie staining of shear stress, shear stress with vitamin C and MEF as negative control. a. Shear stress. b. group of combination of shear stress and vitamin C. c. MEF as negative control.

5.2.4. Alkaline phosphatase (ALP) staining

Figure 12 showed ALP staining each condition under shear stress and combination of shear stress and vitamin C. MEF spheres under shear stress responded partially with ALP solution. On the other hand, MEF spheres which cultured under combination of shear stress and vitamin C responded strongly

with ALP solution. These data demonstrated only shear stress is not enough to reprogramming fully.

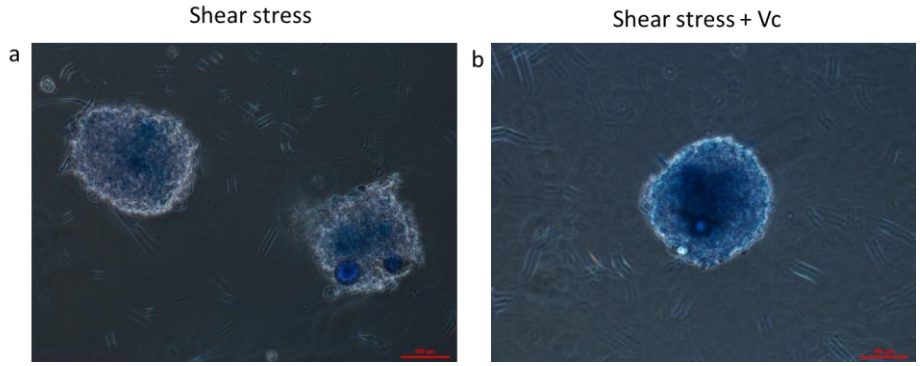


Figure 12. ALP staining of group of shear stress and group of combination of shear stress and vitamin C. a. Spheres were cultured under shear stress. b. Spheres were cultured under shear stress and vitamin C. (Magnification: 100X)

5.2.5. Immunocytochemistry (ICC)

To identify MEF spheres were pluripotent, we observed expression of Oct4, Nanog, SSEA-1 through ICC. Both groups were observed pluripotent markers although MEF did not show any pluripotent expression. This indicated shear stress, vitamin C and sphere formation assisted reprogramming to cells owning capacity of differentiation.

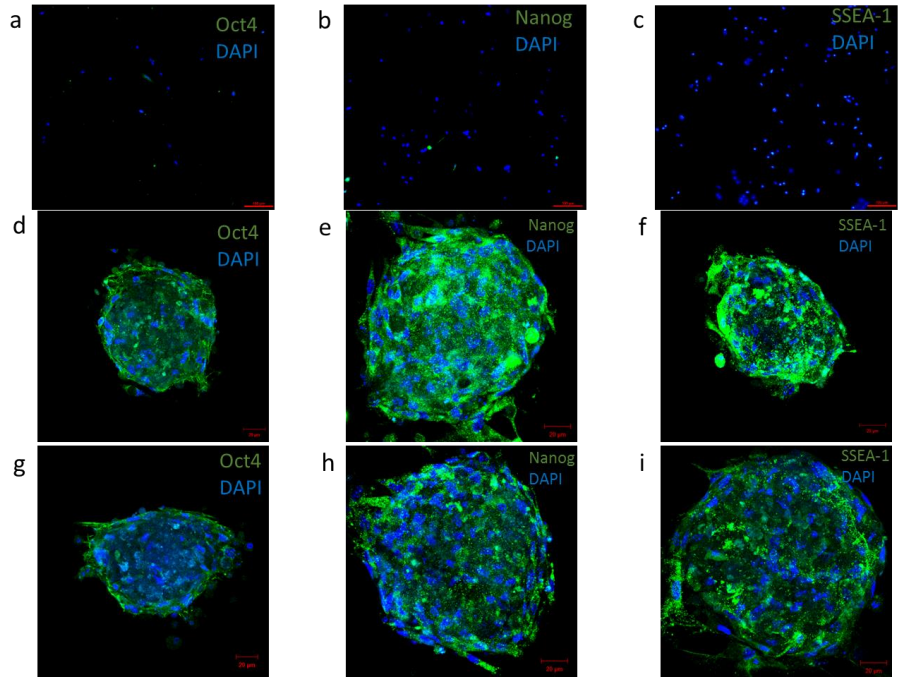


Figure 13. Immunocytochemistry of MEF, experimental groups on 2D images. a. Oct4 of MEF. b. Nanog of MEF. c. SSEA-1 of MEF. d. Oct4 of shear stress group. e. Nanog of shear stress group. f. SSEA-1 of shear stress group. g. Oct4 of group of combination shear stress and vitamin C. h. Nanog of group of combination shear stress and vitamin C. i. SSEA-1 of group of combination shear stress and vitamin C. Scale bar: 100um in MEF (a-c) and 20um in experimental group (d-i). Magnification: 200X in MEF, 400X in Oct4 of experimental group, 480X in Nanog and SSEA-1 of experimental group.

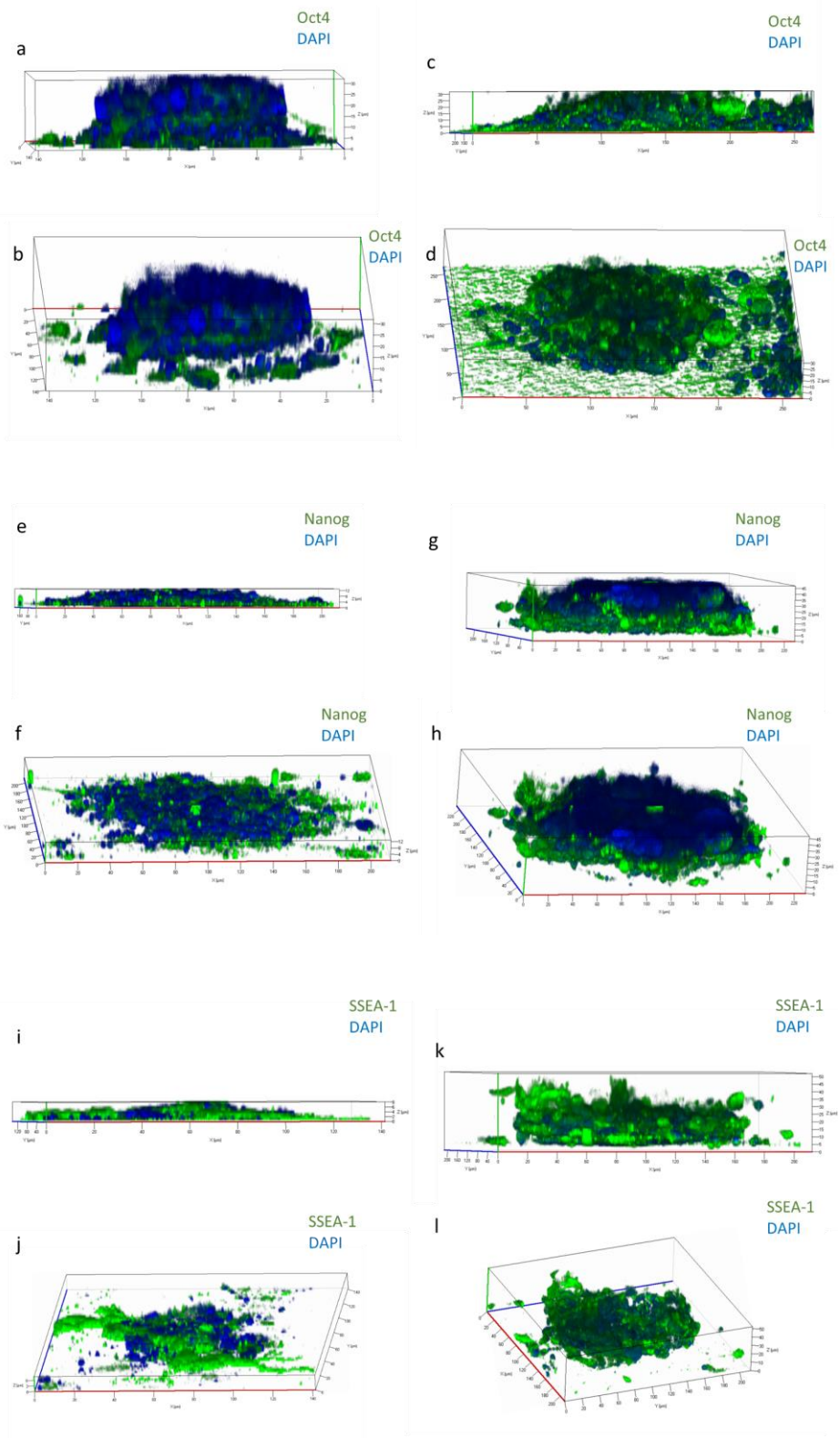


Figure 14. Immunocytochemistry of MEF, experimental groups on 3D

images. a. Shear stress group in Oct4 with side view. b. Shear stress group in Oct4 with up view. c. Group of combination of shear stress and vitamin C in Oct4 with side view. d. Group of combination of shear stress and vitamin C in Oct4 with up view. e. Shear stress group in Nanog with side view. f. Shear stress group in Nanog with up view. g. Group of combination of shear stress and vitamin C in Nanog with side view. h. Group of combination of shear stress and vitamin C in Nanog with up view. i. Shear stress group in SSEA-1 with side view. j. Shear stress group in SSEA-1 with up view. k. Group of combination of shear stress and vitamin C in SSEA-1 with side view. l. Group of combination of shear stress and vitamin C in SSEA-1 with up view.

5.2.6. Western blotting

Western blot data confirmed Oct4 and SSEA-1 were detected. Shear stress and vitamin C group showed deeper band than stimulation group. This data supported shear stress and vitamin C affected expression of Oct4 and SSEA-1 in sphere.

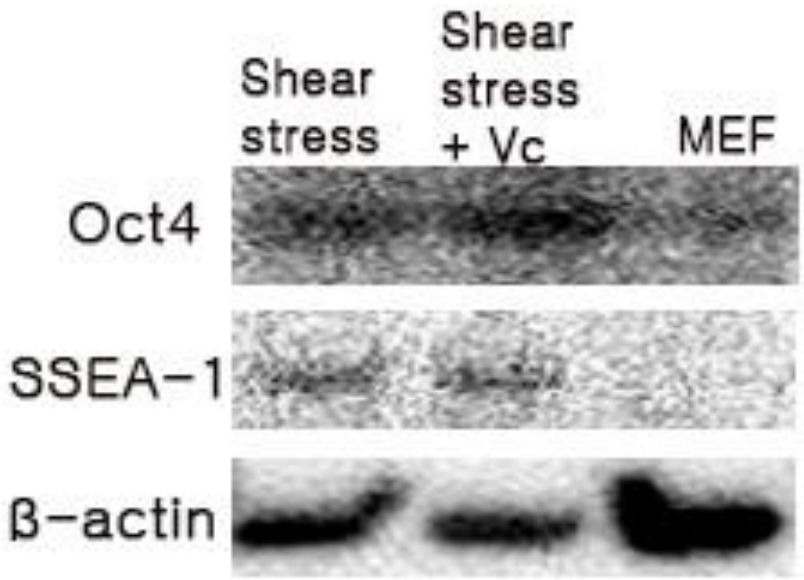


Figure 15. Western blot of shear stress, shear stress + vitamin C and MEF as negative control.

5.2.7. Fluorescence activated cell sorting (FACS)

Figure 16 and 17 demonstrated expression of SSEA-1 and Oct4 were shown in group of shear stress and combination of shear stress and vitamin C compared with MEF. Most of data showed expressions of SSEA-1 and Oct4 were over 50%. Figure 18 indicated experimental groups had significant different from MEF but difference of each experimental groups did not exist. Although it was impossible to analyze Oct 4 whose experiments were duplicated, it also showed Oct4 expression of experimental group was higher than the expression of control group. This results supported ICC data but effect of vitamin C remained a question.

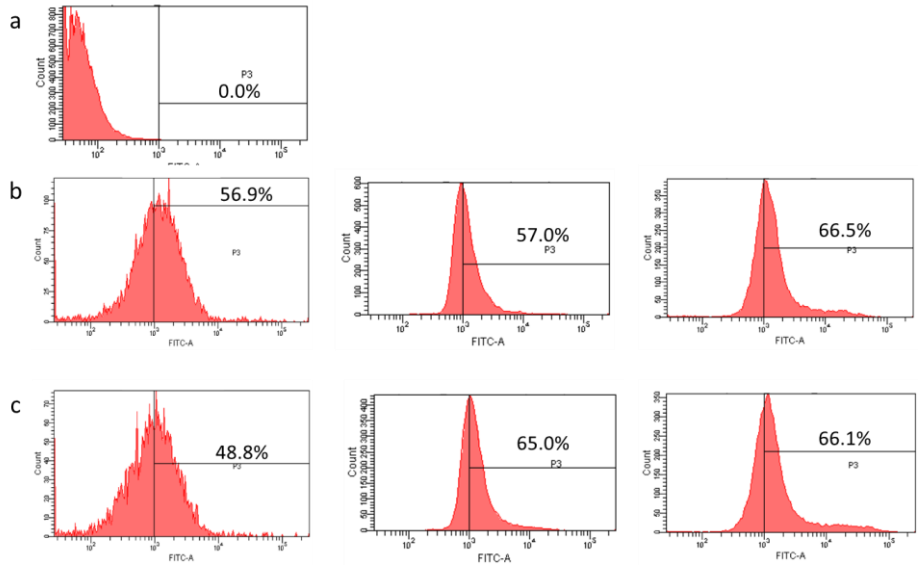


Figure 16. FACS data of MEF and experimental groups. Marker was SSEA-1. a. MEF as negative control. b. Group of shear stress. C. Group of combination of shear stress and vitamin C.

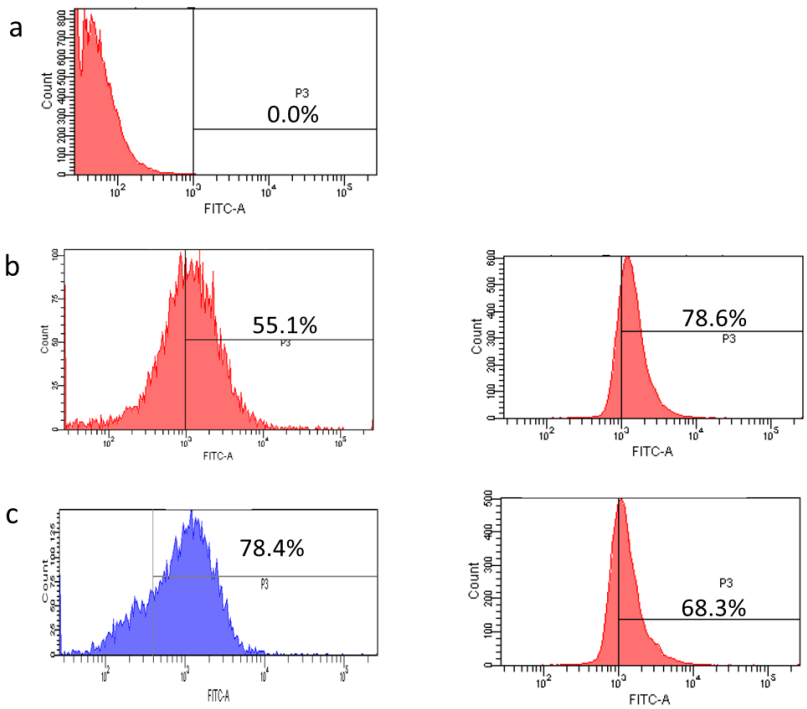


Figure 17. FACS data of MEF and experimental groups. Marker was Oct4. a. MEF as negative control. b. Group of shear stress. C. Group of combination of shear stress and vitamin C.

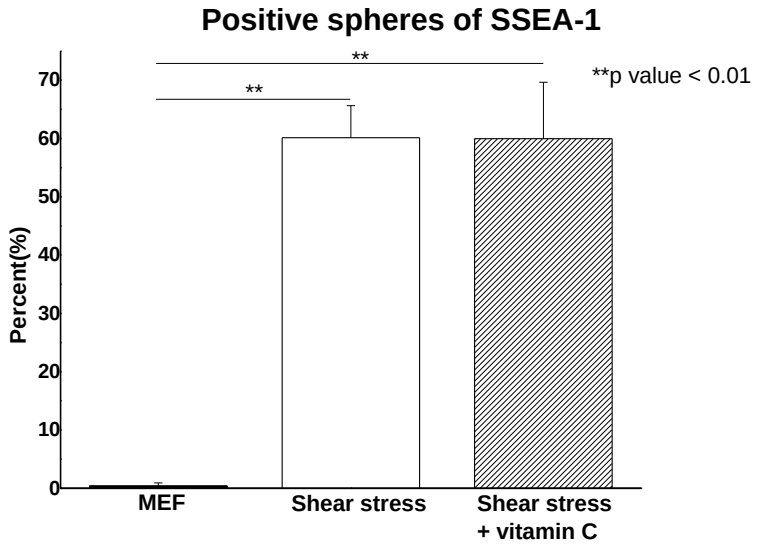


Figure 18. Statistical analysis on FACS data of SSEA-1. Error bars represent standard deviation, n=3, **P values < 0.01.

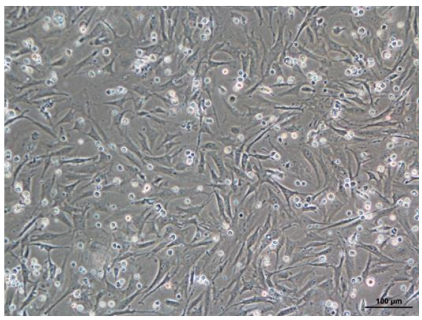
5.2.8. Self-renewal test and spontaneous hetero-differentiation in vitro

Spheres were cultured on matrigel-coated plate to investigate capacity of self-renewal. The spheres immediately showed several types of morphology neuron-like cells or muscle-like cells as soon as they were placed on the plate. It indicated that the sphere were differentiated and capability of self-renewal did not be verified under these conditions.

It has reported that LIF signal is important but not sufficient to maintain self-renewal. Under presence of LIF, expression of inhibitor of differentiation (Id) gene which was induced by Bone morphogenetic proteins (BMPs) via Smad pathway resulted in sustaining self-renewal of mES cells [41]. Also, function of retinol was revealed that it overexpressed Nanog, which induced to maintain self-renewal independently on LIF/STAT3, BMPs, Oct3/4-Sox2

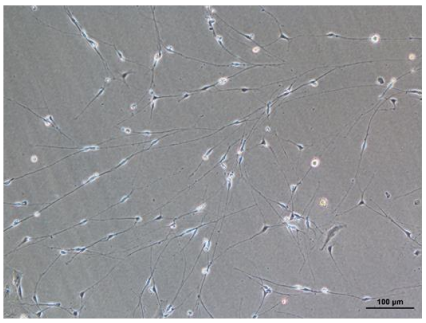
pathways [42]. In addition, insulin receptor substrate (IRS)-1 showed synergy effect with LIF to sustain self-renewal through suppression of Id-1 and Id-2 in mES [43]. It requires further study whether the spheres have self-renewal.

a

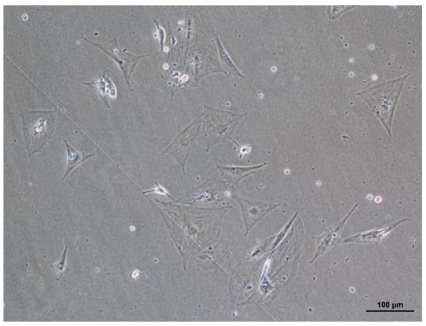


MEF

b



Type 1



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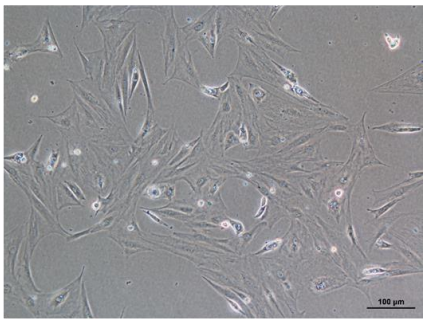

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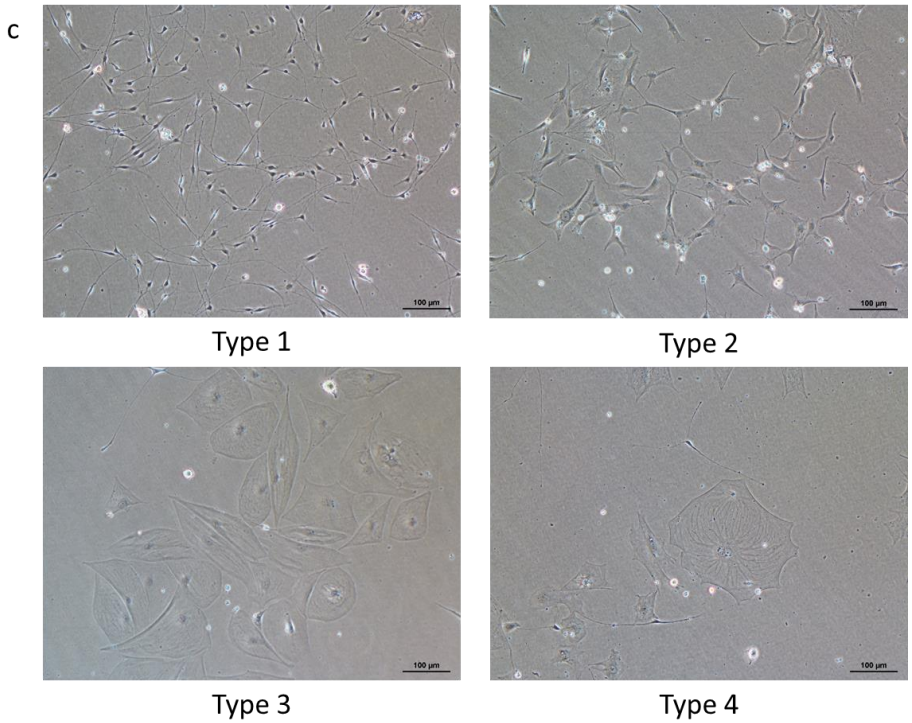

Figure 19. Spontaneous differentiation of spheres. a. MEF as negative control. b. Several morphologies in group of shear stress. c. Diverse morphologies in group of combination of shear stress and vitamin C.

5.2.9. Differentiation in vitro

To confirm capacity of differentiation, Neuronal Class III b-Tubulin (Tuj1) which is neuron marker in ectoderm, sarcomeric alpha actinin which is cardiomyocyte marker in mesoderm and alpha 1 fetoprotein which is one of endoderm markers were used. The spheres of shear stress group and group of shear stress with vitamin C differentiated to neuron, mesoderm and endoderm partially. This results suggested sphere formation, shear stress and vitamin C without transgenes induced partial reprogramming.

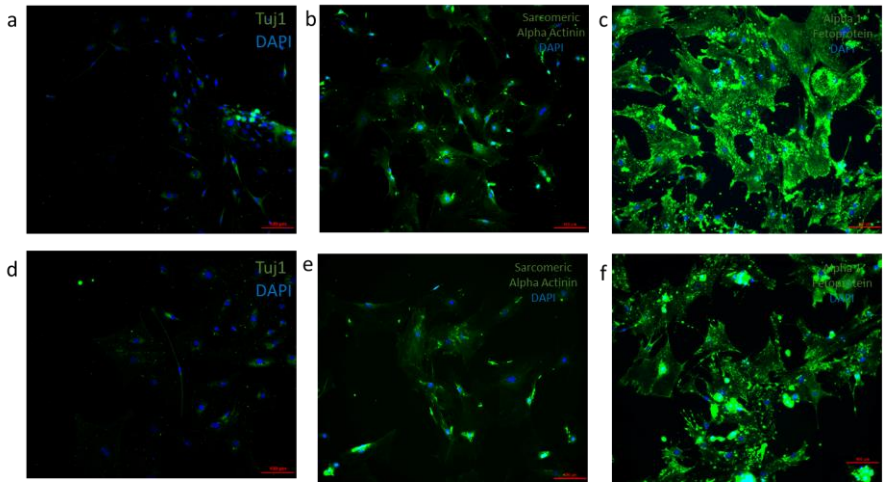

Figure 20. Immunocytochemistry on differentiation in vitro with experimental groups. a. Tuj1 in ectoderm marker in shear stress group. b. sarcomeric alpha actinin in mesoderm marker in shear stress group. c. alpha 1 fetoprotein in endoderm marker in shear stress group. d. Tuj1 in ectoderm marker in group of shear stress and vitamin C. e. sarcomeric alpha actinin in mesoderm marker in shear stress and vitamin C. f. alpha 1 fetoprotein in endoderm marker in shear stress and vitamin C.

5.2.10. Teratoma assay

For confirming pluripotency in vivo, samples of experimental groups injected under skin of mice. Teratoma did not form in any mice. This results supported differentiation experiment in vitro which reprogramming was not fully.

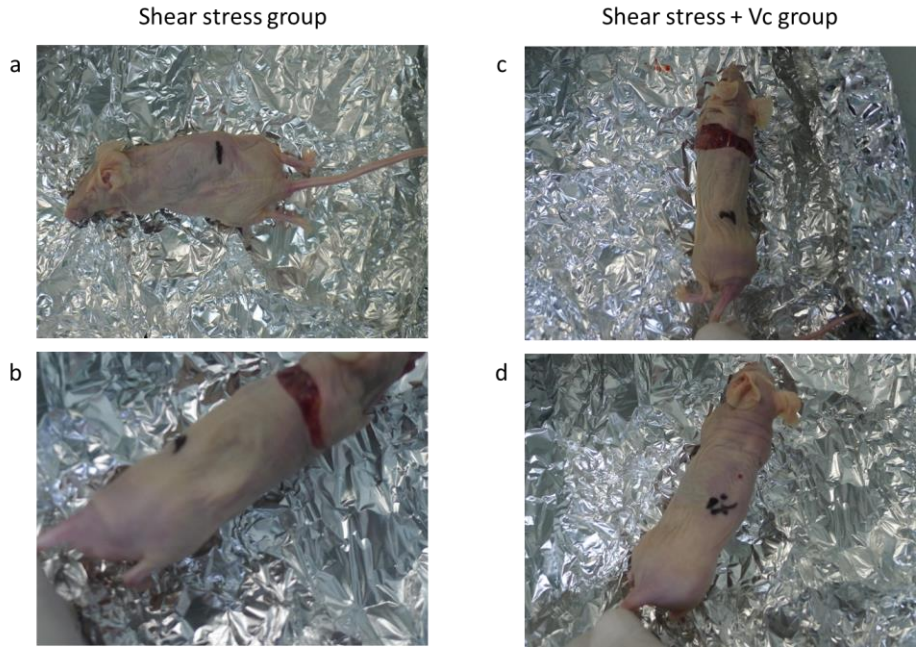

Figure 21. Teratoma formation. a, b. shear stress group. c, d. Group of combination of shear stress and vitamin C.

5.2.11. Nitric oxide (NO) assay

Figure 17 showed experimental groups released more nitric oxide compared to control group. Also, group of combination of shear stress and vitamin C more released than shear stress group. This data supported shear stress influenced low release of nitric oxide which affected reprogramming and vitamin C had an effect of releasing NO.

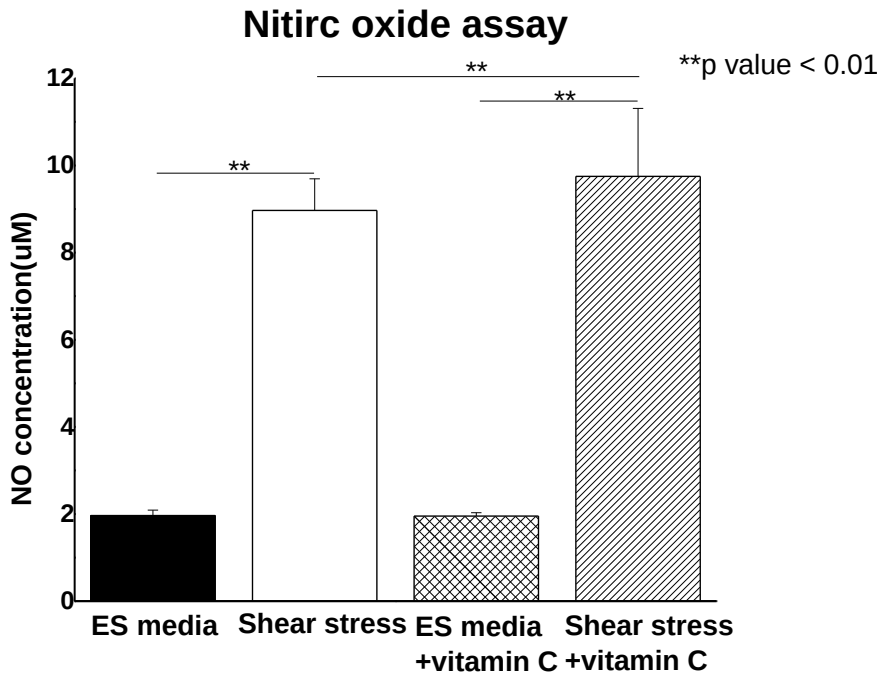

Figure 22. Nitric oxide assay of control and experimental groups. Error bars represent standard deviation, n=3, **P values < 0.01.

In summary, sphere formation, shear stress and vitamin C had an influence on reprogramming but it was impossible to reprogram pluripotent stem cell state. But, this method would help direct cell reprogramming. Further study need other external stimuli including increasing amount of vitamin C to full reprogram.

6. Conclusion

The objective of this research were a new method to obtain differentiation capacity of MEF using vitamin C or external stimuli such as acid and shear stress. Then, generating spheres under each condition were compared for confirming optimized reprogramming environment. In this research, the followings are the main results:

1) Acid helped that cells were formed spheres but it induced cell death. MEF spheres

2) Shear stress assisted to form spheres and reprogramming by releasing low concentration of nitric oxide.

3) Sphere formation through suspension culture is essential for reprogramming.

4) Vitamin C improved cell survival and may assist to reprogramming.

5) Reprogramming degree was affected how long spheres were formed.

6) Sphere formation, shear stress and vitamin C without transgene is not enough for perfect spheres. Their stimuli can reprogram partially.

This research showed possibility that methods using external stimuli and small molecule without transgenes can reprogram from somatic cells to differentiated cells. This method would be utilized in direct cell reprogramming field.

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Abstract

Reprogramming of Mouse Embryonic Fibroblast by External Stimuli and Vitamin C

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초록

리프로그래밍 개념이 확립된 후, 외부 유전자, 합성한 저분자 물질, 재조합된 단백질을 이용한 여러 리프로그래밍 방법들이 개발되었다. 유도만능줄기세포는 증식의 제한이 없고 3배엽층으로 분화될 수 있다. 유도만능줄기세포 같은 리프로그래밍된 세포들은 다양한 세포나 조직으로 분화할 수 있기 때문에 좋은 의학적 재료가 된다. 하지만 유도만능줄기세포를 만들기 위해 외부유전자나 바이러스를 쓰는 기존의 방식은 치료용으로 적합하지 않은데, 그 이유는 안전성 문제와 종양발생의 위험이 있기 때문이다.

이 연구에서는 외부 유전자 없이 저분화 물질과 외부 자극들을

이용하여 리프로그래밍하는 새로운 방법을 개발을 시도하였다. 먼저, 4개의 집단을 선택하였다; 산 (acid) 자극과 전단응력이 있는 집단, 산 자극만 있는 집단, 전단응력 자극만 있는 집단, 산과 전단응력 자극 모두 없는 집단. 산 실험에서는 세포를 pH 5.4 용액에 25분동안 담근다. 전단응력 실험에서는 파이펫팅을 하루에 2번씩 2분동안 부유배양상태에서 하였고 이를 7일간 지속하였다. 500ul의 배아줄기세포 배지를 60mm 페트리디쉬에 7일간 매일 넣어주었다. 7일 후, 구형의 세포덩어리들을 채취해서 전분화능 확인을 위한 alkaline phosphate (ALP) 염색과 면역세포화학 실험에 활용되었다. 모든 구형의 세포덩어리에서 ALP와 전분화능 단백질이 있는 것으로 보여주었지만 염색 정도는 완벽하지 않았다. 이 실험에서 산과 전단응력을 주지 않은 집단과 비교해봤을 때 산과 전단응력은 구를 형성하는데 도움을 주지만 산은 세포 사멸을 유도하였음을 보여주었다.

효율성을 증진시키기 위해, 자극 기간을 더 길게 바꾸고 산 대신 비타민 C를 선택하였다. 비타민 C는 리프로그래밍을 가속화시키고 유도만능줄기세포의 효율성을 증진시키는 것으로 보고되었다. 2개의 집단으로 설정하였다; 부유배양 상태에서의 전단응력, 부유배양 상태에서 전단응력과 비타민 C의 조합. 실험계획에 따라 배아줄기세포 배지 또는 10uM 비타민 C가 들어간 배아줄기세포 배지를 실험군에 매일 14일동안 주었다. 14일동안, 파이펫팅은 하루에 2번씩 2분동안

실행하였다. 14일 후, 모든 구형의 세포덩어리들은 채취해서 ALP 염색, 전분화능과 삼배엽 마커를 이용한 면역세포화학, 웨스턴 블롯, 유세포 분석기, 생체 내 기형종 형성 같은 전분화능과 분화능력을 확인하는 실험을 하였다.

두 집단 모두 정도의 차이는 있지만 ALP 염색, 전분화능과 삼배엽 마커를 이용한 면역세포화학, 웨스턴 블롯, 유세포 분석기, 실험관 내의 분화실험에서 긍정적 결과가 나왔다. 그러나 어떠한 집단에서도 생체 내 기형종은 형성되지 않았다. 유세포 분석기에서는 전단응력 집단과 전단응력과 비타민 C가 조합된 집단간의 유의미한 차이가 나지는 않았지만, 대부분의 데이터에서 전단응력과 10uM 비타민 C가 조합된 집단이 더 좋다고 나왔다. 모든 데이터를 종합해보았을 때. 부유배양 상태에서 전단응력과 10uM 비타민 C가 조합된 집단이 분화능을 가진 구형의 세포덩어리 형성에 적합했다.

이 연구는 외부 유전자 없이 외부 자극과 저분자 물질을 이용한 방법이 체세포에서 분화된 세포로 리프로그램을 할 수 있다는 가능성을 보여주었다. 이 연구는 직접교차분화 분야에 활용될 수 있다.

Keywords : 쥐의 배아섬유아세포, 리프로그래밍, 구 형성, 전단응력, 비타민 C, 일산화질소

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