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Efficient generation of Induced pluripotent stem cells from human circulating multipotent stem cells (CiMS)

혈중 순환 다분화능 줄기세포의 효과적인 유도 만능 줄기세포로의 역분화능에 대한 연구

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Abstract

Efficient generation of Induced pluripotent stem cells from human circulating multipotent stem cells (CiMS)

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The discovery of induced pluripotent stem cells (iPS) has shown the new possibility of molecular understandings for development and therapeutic applications for patient-specific disease. To achieve these objectives in clinical situations such as patient-specific cell therapy, choosing appropriate cell sources that make methods easy to isolate

from corresponding patient is important. Human Dermal Fibroblasts (HDFs) are used for reprogramming. But, due to the invasive skin biopsy and the need for prolonged culture, more proper cell line for reprogramming is needed. In this context, human peripheral blood has been suggested for reprogramming cause of their good accessibility, but they need to be isolated into the specific type of cells such as G-CSF mobilized CD34 positive cells or T cells using MACS or FACS to improve the reprogramming efficiency. Even after sorting, they show low efficiency of gene transfection.

Here, we propose novel cells for reprogramming into iPS cells. These cells are named circulating multipotent adult stem cell (CiMS) gained from human peripheral blood mononuclear cells with high efficiency. CiMS shows enhanced gene transduction efficiency compared to human dermal fibroblast. They can be isolated from only a 10ml of blood sample and proliferate actively over 10 passages so that can be stocked for cell banking. For these reasons, we

suggest CiMS for the appropriate cell source of reprogramming. Also iPS reprogramed from CiMS is an outstanding progenitor cells compared to other pluripotent cells (PSCs) in cardiovascular cell differentiation.

Finally, we obtained more than 40 iPS cell lines from blood sample of patients. In this study we propose a new optimal cell line for making iPS and further making iPS cell bank. Taken together, these cells can be applied for the useful regenerative medicine tool.

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Key words: Cell reprogramming; Induced Pluripotent Cell (iPS); Cardiovascular cell differentiation; Regenerative medicine

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LIST OF ABBREVIATION

CiMS: Circulating multipotent adult stem cell

MSC: Mesenchymal stem cell

iPS Cell: Induced pluripotent stem cell

ES Cell: Embryonic stem cell

HDF: Human Dermal Fibroblast

CiMS-iPS cells: iPS cells derived CiMS

HDF-iPS cells: iPS cells derived HDF

EC: Endothelial cell

VSMC: Vascular smooth muscle cell

PBMC: Peripheral blood mononuclear cell

OCT 3/4: Octamer-binding transcription factor 3/4

SOX2: SRY (sex determining region Y)-box 2

Klf4: Kruppel-like factor 4

EB: Embryoid bodies

ALP: Alkaline Phosphatase

ChIP assay: Chromatin immunoprecipitation assay

INTRODUCTION

In 2006 Yamanaka's group found that ectopic expression of Oct3/4, Sox2, Klf4 and cMyc using viral (retroviral) gene transfer can transform murine somatic cells into iPS (induced pluripotent stem) cells.[1] An year later, human iPS cells from fibroblasts has also been generated by two research groups independently.[2,3]

iPS cells have pluripotent potential like embryonic stem (ES) cells. Because of their features that can differentiate into almost every somatic cell type, can be made from autologous source, and avoid ethical concerns and immune rejection which cell therapy with ES cell had, they are nominated as the ideal source for patient and disease-specific regenerative therapy.

To make it feasible, it has to pay close attention for determining the original cell for making iPS cells. First, the donor cells need to be accessed easily with relatively non-invasive method. Secondly, they need to proliferate actively for gaining large quantities of cells. Third, it has to be easily transfected and express exogenous gene actively. Lastly, cells should be obtained regardless of age, sex, ethnic group, health state of the

individual.

In human, fibroblast is usually used for reprogramming. But because of its invasive preparation, and the need for a long period of culture to apply at reprogramming[4], new candidates are constantly proposed.

Cells isolated from peripheral blood has been focused as source of reprogramming because they can be gained easily together with high accessibility and non-invasiveness. In previous studies, human terminally differentiated circulating T cells (hTDCTCs) and granulocyte colony stimulating factor (G-CSF)-mobilized CD34 positive cells have been isolated and used for reprogramming. However, these cells showed low virus transduction efficiency and T cell receptor gene-rearrangement pattern shown from original T cell clone of the patient. And these cells required the subcutaneous injection of G-CSF and selection using FACS.[5]

Here we suggest a new type of adult stem cells isolated from the human peripheral blood sample for convincing donor cells of reprogramming. These cells are multipotent stem cell from heart endocardium. We named these cells as the circulating multipotent stem cell, CiMS. CiMSs can replicate actively over 10 passage so

can be stocked for other experiment. CiMSs are the optimum cell for making iPS cells because CiMSs can be obtained from the small volume (10 ml) of blood sample and have the short culture period, and high transduction efficiency (twice more efficient compared to fibroblast). Indeed, it took 8 days to obtain reprogramed colonies after 4 factor virus transduction without feeder cells and the colonies sustained appearance of embryonic stem (ES) cells like colony on the feeder after manual transfer. Their gene expression, epigenetic state and ability to differentiate into the three germ layers also had similar features to ES cells.

Selecting donor cell for reprogramming is also important because iPS cell lines derived from different origin lineages has molecular and functional difference each other.[6,7] Recent studies described that some epigenetic status of original cell can be remained and make the different characteristics of multiple PSCs. Furthermore, this can make distinguishable differentiation potentials among PSCs.[6,7] CiMS is a multipotent stem cell derived from heart endocardium. And recently we observed that expression of GATA4, early cardiomyocyte differentiation marker and SOX17 which is involved in heart development were highly detected in

CiMS. Thus, we expected that iPS cells from CiMS (CiMS-iPS cells) is more potent to differentiate into cardiovascular cells. As expected, we confirmed that CiMS iPS cells possess a higher differentiation ability into each endothelial cell (ECs), vascular smooth muscle cells (VSMCs), and cardiomyocyte (CMCs) than others.

Loosely compacted chromatin is epigenetically more accessible state.[8] Typically DNA methylation and histone modification can alter chromatin compaction level and genetical activity. DNA methylation at CpG island makes chromatin more compact.[9,10] But, effects of histone modification are different by where and how modification occurs.[11] In general, H3K4me3 observed at gene promoters and it role as genetically active signal[12] on the other hand H3K27me3 and H3K9me3 act as genetically repressive signal.[13] We expected CiMS can remain euchromatic state at the mesodermal gene. [14,15] As expected, we found out that this superior ability in differentiation into cardiovascular cells is due to their different epigenetics, histone modification status, but not DNA methylation status.

CiMSs are feasible for efficient iPS generation. Furthermore their superior differentiation ability into cardiovascular cells will facilitate the advance of the regenerative medicine.

MATERIALS AND METHODS

Cell culture - CiMS, 293T, STO feeder, CiMS-iPS

This study was approved by the Institutional Review Board of the Seoul National University Hospital.

Human peripheral blood samples (50 cc) were obtained from donors with informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples with Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) according to the manufacturer's recommendations. Freshly isolated mononuclear cells were suspended in EGM-2MV BulletKit system (Lonza, Basel, Switzerland) containing of endothelial basal medium-2, 5% fetal bovine serum, human epidermal groth factor (hEGF), vascular endothelial growth factor (VEGF), human fibroblast growth factor-basic (hFGF-B), Insulin growth factor-1 (R3-IGF-1) and ascorbic acid. Mononuclear cells were seeded on the fibronectin-coated (Sigma, St. Louis, MO) six-well plate at 6 X 10^6 cells per well and incubated in a 5% CO_2 incubator at 37° C. The medium was changed every single day for up to 2 weeks after plating. Adherent CiMS cells were observed from as early as 5 days after the start of culture and

gradually formed colonies. CiMS colonies grown in culture were maintained with EGM-2MV media and sub-cultured using 0.05% Trypsin-EDTA solution. CiMS cells were split every 3 to 4 days. For growth curve, CiMS cells were plated at a density of 2 X 10^5 cells/well into 6-well plates and counted in triplicates on 5 or 6 days during one month.

293T cells were maintained in Dulbecco's modified eagle medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 0.5% penicillin and streptomycin (Invitrogen).

STO as feeder cells were maintained in high glucose DMEM containing 10% FBS and 0.5% penicillin and streptomycin on the 0.1% gelatin-coated dish.

Human iPS cells and ES cells were maintained on STO feeder cells in human ES medium supplemented with 10 ng/ml bFGF (Invitrogen)(Ref Yasuhiro Kazuki, complete genetic correctio..). For sub-culture, every 7 days, human iPS cells were mechanically picked and disaggregated using pipette tip and pipetting. Then, the dissociated colonies were transferred to the STO feeder with 5 μM Y27632 (Sigma) in human ES medium, DMEM/F12 containing 20% KnockOut serum replacement (Invitrogen), 2 mM L-glutamine, 1X10-4 M nonessential amino

acids, 1X10−4 M 2−mercaptoethanol (Invitrogen), 50 units and 50 mg/ml penicillin and streptomycin, and , 100㎍/ml bFGF.

Retrovirus production and Reprogramming CiMS

293T cells were plated at $3X10^6$ cells per 100 mm dish and grown in high glucose DMEM containing 10% FBS. The cultures were maintained at 37° C in 5% CO_2 incubator throughout the virus production period. The next day, when confluency of cells reached 90%, cells were respectively transfected with retroviral vectors containing human OCT3/4, SOX2, KLF4, c−Myc vectors and packaging vectors with PEI solution (Sigma). Twenty hours after transfection, the transfection medium was washed out and replaced with a fresh growth medium. The following day, the first virus−containing supernatant was gathered and filtered through a 0.22 ㎛ pore−size filter. The filtered supernatant was concentrated using ultracentrifugation with 25,000 rpm for 1 hour 30min in 4° C.

Human CiMS cells were seeded at $2X10^5$ cells per well in 6 well plate before transduction. CiMS cells within three and four passage were used for transduction of retrovirus containing Yamanaka's 4 factors after primary culture. After concentration

by ultracentrifugation, the four retroviruses were added to CiMS cells with the 10 μg/ml Polybrene (Sigma). Twenty-four hours after transduction, the medium was changed with new fresh growth medium. Eight to ten days after transduction, iPS cell-like colonies were shown in transduced CiMS. iPS cell-like colonies were mechanically picked up and transferred into the new STO feeder. Two days after colony attachment, the medium was changed with human ES cell medium supplemented with 10 ng/ml bFGF(R&D) and the medium was changed every second day.

Immunofluorescence

Cells were seeded into cover glass-bottom dish (SPL). For immunofluorescence staining, cells were washed twice with PBS, and fixed with methanol for 10 minutes in -20℃. After washing with 0.05% TBS-T 3 times, blocking process was executed with solution consisting of 1% BSA, 0.05% TritonX-100 in PBS for 30 minutes at room temperature. Primary antibodies included human Oct4 (1:100, Santacruz biotechnology), Nanog (1:100, Abcam), Tra-1-81 (1:100, Cell signaling technology), α-fetoprotein (1:100, Satacruz biotechnology), α-sarcomeric

actin (1:100, Abcam), Smooth muscle actin (1:100, Abcam), nestin (1:100, Santacruz biotechnology), GFAP (1:100, Sigma) and β-III tubulin (1:100, Chemicon). Used secondary antibodies were Anti-mouse IgG Alexa 488, anti-rabbit IgG Alexa 488, anti-goat IgG Alexa 488, anti-mouse IgG Alexa 555, anti-rabbit IgG Alexa 555 and anti-goat IgG Alexa 555 (1:100 respectively). Nuclei were stained with SYTOX blue (invitrogen) and DAPI (Sigma). Specimens were observed using a confocal microscope (Carl Zeiss).

Alkaline peroxidase staining and karyotyping

For alkaline phosphatase staining, the BCIP/NBT substrate system (DAKO) was used. Cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. After washing out paraformaldehyde with PBS, the cells were incubated with BCIP/NBT solution for 30 minutes at room temperature. Karyotype analysis was performed by standard protocol for the chromosomal G-band analyses at the Nihon Gene Research Laboratories, Japan. And data was analyzed by Karyotype Analysis program, ChIPS-Karyo(Chromosome Image Processing System)(GenDix, Inc., Korea)

RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). For obtaining cDNA, 1 microgram of total RNA is used for the reverse transcription with Power cDNA synthesis kit (INTRON, Korea) and Oligo dT primer. PCR was performed with AmpliTaqGold (Applied Biosystems). Information of primers used in PCR are identical with those of Yamanaka's primers.[2]

Microarray

Total RNA from human ES cells, CIMS iPS Cells and CIMS, as using negative control, were isolated using RNeasy Mini Kit columns (Qiagen). Each RNA quality was verified by Agilent 2100 bioanalyser using the RNA 6000 Nano Chip (Agilent Technologies), and quantity was compulsive using the ND-1000 Spectrophotometer (NanoDrop Technologies). As input into the Affymetrix procedure, 300 nanograms RNA samples were used as described at the protocol (http://www.affymetrix.com). Prepared each RNA was converted to double stranded cDNA. Then fragmentation and end-labeling was going on. Generated end-labeled cDNA fragments were hybridized using GeneChip Human Gene 1.0 ST arrays and the outputs were stained and

scanned using a Genechip Array scanner 3000 7G (Affymetrix). Using Affymetrix microarray suite 5 (MAS5) algorithm, the detection (Present/Absent) call was made.

In vitro spontaneous differentiation

For Embryoid bodies(EB) formation, human CiMS-iPS cells were harvested using treatment with 10 units/ml dispase. The cell clumps were plated to petri-dish and growing in hES medium. The medium was changed every other day. After 8 days of EB culture, EBs were plated on 0.5% gelatin-coated plate in the same medium and grow them for another two weeks.

Teratoma formation

The CiMS-iPS Cells from two confluent 100 mm dish were harvested by 10 units/ml dispase (Roche) treatment for twenty minutes at 37° C and collected into 15 ml tubes, and centrifuged, and the pellets were suspended in DMEM/F12 mixed with Matrigel (Roche). These harvested cells were injected subcutaneously into dorsal flank of a SCID mouse (CREA, Japan). Nine weeks after injection, the mouse was sacrificed. And we

excise the tumor as a whole then weighted, and fixed with 4% paraformaldehyde in PBS. And using liquid paraffin, harden the teratoma. The paraffin-embedded teratomas was sectioned and stained with hematoxylin and eosin.

Real-time PCR

Real-time PCR was performed using FastStart SYBR Green (Roche) and analyzed with Applied Biosystems 7500 Real Time PCR System. Gene expression level were normalized to the level of 18s rRNA and quantified using $2^{(-\Delta Ct)}$ method. Primers were listed in Table1.

Bisulfite PCR

1 milligram of genomic DNA was isolated from human iPS cells and ES cells. Each DNA was treated with bisulfite using EpiTect Bisulfite Kit (Qiagen). To amplify the promoter regions, such as human Oct3/4 and NANOG or Brachury T, we used PCR. The PCR products were purified using Zymoclean gel DNA recovery kit (Zymo). The insert was subcloned into pCR2.1-TOPO vector (invitrogen), and sequenced with M13 forward and reverse primers.

In vitro differentiation into each 3 different lineage

Each PSCs harvested by 100ug/ml dispase (Invitrogen) treatment for 90 minutes at 37° C and collected into 15 ml tubes, and centrifuged, and were passaged on DMEM/F12 mixed with Matrigel (Corning) –coated 35mm dishes (Corning) at a density of $2X10^6$ cells in mTesR1 (Stemcell Technologies) medium with 10mM ROCKi without feeders. When each PSCs were grown to near full confluency, differentiation induction was started. During induction, the above-mentioned cells were treated with proper factors for differentiation into each lineage.

For early ectoderm differentiation, PSCs were cultured in KSR medium supplemented with 10 μM SB431542 (TOCRIS) and 500nM LDN193189 (Sigma) for 3 days. Every 24 hour, medium was changed. KSR medium are composed of KnockOut DMEM (1x) containing 15% KnockOut serum replacement, 1% Pen Strep, 1% L-Glutamine 200mM, 1% MEM Non-Essential Amino Acids 100x, and 1% 2-mercaptoethanol 1000x. All materials were purchased from Life Technologies.

For early mesoderm differentiation, PSCs were cultured in RPMI1640 containing 1% B27 without insulin (All purchased from Life Technologies) supplemented with 10 mM CHIR99021

(Cayman) for 2 days. Every 24 hour, medium was changed.

For early endoderm differentiation, PSCs were treated with 100 ng/ml Activin A (ROCHE), 3 μM CHIR99021 (Cayman) in RPMI 1640 containing 1x Glutamax I (Invitrogen) and 1% Pen Strep, 1% L-Glutamine 200mM for three successive days with concentrations of 0%, 0.2% and 2% Fetal bovine serum (Invitrogen).

In vitro differentiation into cardiovascular cells

Differentiation into cardiomyocyte was started when confluency of PSC nearly full as well. For cardiomyocyte differentiation, PSCs were treated with 10 μM CHIR 99021 in RPMI1640 containing 1% B27 without insulin (cardiomyocyte differentiation media). The next day, cells were stimulated 20ng/ml Activin A (R&D systems) in cardiomyocyte differentiation media for 2 days. And subsequently medium changed cardiomyocyte differentiation media. The next day, cells were stimulated 5 μM IWR1 (Sigma) in cardiomyocyte differentiation media for 48 hours without media change. And then cells maintained at cardiomyocyte medium for 2 days. And after 2 days cells maintained in RPMI 1640 containing 1% B27.

For differentiation into endothelial cell and vascular smooth muscle cell, dispase-detached 2.5 X 10^5 PSCs were seeded in one well of a 6-well plate (Eppendorf) in mTesR1. The next day, each induction was started.

For endothelial cell differentiation, PSCs were treated with RPMI16400 containing 2% B27 and CHIR 99021. The next day, cells were stimulated with RPMI16400 containing 2% B27 medium with 100ng/ml BMP4 (R&D systems) and 10 μM PD98059 (Sigma). Subsequently, the cells were cultured in RPMI16400 containing 2% B27 medium with 10uM VEGF (Sigma) and 100ng/ml bFGF 50ng/ml for 20 days.

For Vascular smooth muscle cell differentiation, PSCs were cultured in RPMI 1640 containing 1% B27 and 10 μM CHIR99021 for 1 day. The next day, medium was changed with RPMI 1640 containing 1% B27 supplemented with 100ng/ml BMP4 and 10 μM LY290002 (Peprotech). And the third day of differentiation, medium was changed with Endothelial Cell Growth Medium (Lonza) supplemented 20ng/ml PDGF BB (R&D systems) and 2ng/ml TGF-β1(Peprotech).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using Chromatin Immunoprecipitation (ChIP) Assay Kit (Merck) and followed manufacturer's recommendation. 1 X 10^6 cells were used for each immunoprecipitation reaction. 2 ㎍ of Histone H3 tri methyl K4-ChIP grade, 3 ㎍ Histone H3 tri methyl K9-ChIP grade, 2 ㎍ Histone H3 tri methyl K27-ChIP grade antibodies from Abcam were used. For negative control of ChIP reaction, 2 ㎍ of Rabbit IgG (Invitrogen) was used also included to allow for normalization. PCR of the ChIP products was carried out using AmpliTaq Gold™ DNA Polymerase. Primer information weas listed in Table1.

Statistical Analysis

All data were calculated as mean ± SD. Group comparisons were performed by one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), and number of asterisks on top of each graphs mean statistical significance, "*" ," **" ," ***" means that p-value range is 0.01 to 0.05, 0.001 to 0.01, 0.001 to 0.01, respectively.

RESULT

High efficiency of reprogramming of Circulating pluripotent stem cells from human peripheral blood

We isolated PBMC from blood and obtained the adult stem cell using EGM-2MV with 95.6% efficiency which is named CiMS. When we cultured PBMC in EGM-2MV medium on fibronectin coated culture plate, adherent CiMS cells were emerged after 5 days of culture and gradually formed colony. CiMS cells showed features between mesenchymal stem cell (MSC) and endothelial cell. CiMS grew in a disperse pattern or big colony (Figure 1A) and we used disperse type for experiment. It had surface markers SH2, SH3, CD13, CD29 positive, CD14, CD45 negative (Figure 1B). When we tested transduction efficiency with GFP retrovirus using 1 $\mu\ell$ of $9.4x10^4$ TU virus, transduction efficiency of CiMS was 93.75%, more than double compare to that of human fibroblast, 49.44%. And it showed even higher transduction efficiency than that of 293T (76.44%) (Figure 1C).

$2X10^5$ CiMS cells were transduced with Yamanaka's retroviral viruses (Oct4, Sox2, KLF4, c-Myc) for 18 hours. Five days after transduction, morphologically transformed cells were started to

form colonies. Eight days after transduction, these transformed colonies were mechanically picked up and re-seeded on STO feeder layer (Figure 2A). We compared the number of ALP positive cells when introduced 4 factors into CiMSs and fibroblast, after re-seed on STO. There were much more ALP positive cells when introduced with 4 factors into CiMSs than when introduced into CiMSs fibroblast (Figure 1D). This result means CiMSs have more potential of reprogramming into pluripotent stem cells than fibroblasts.[16]

When the colonies were squeezed through a gap in the STO cells, human ES media was supplied daily. These colonies formed typical cells with a ES morphology at approximately one week after human ES media (Figure 2B). CiMS-iPS cells expressed the typical pluripotency-related transcription factors and surface markers like OCT4, NANOG, Tra-1-81 and ALP as determined by immunocytochemistry and ALP staining (Figure 2C).

Expression of hES cell marker genes in CiMS-iPS

RT-PCR data showed the expression of pluripotent ES marker of CiMS-iPS cells. Various markers of pluripotent ES cells were

detected in all CiMS-iPS cell clones at the similar levels compared with those of the hES cell. Meanwhile they did not appear in parental CiMS cells (Figure 3A).

To compare the global transcript profiles, cDNA of human CiMS, human ES cells, and human CiMS-iPS cells was examined using DNA microarrays. Genes patterns upregulated in human ES cells and in human CiMS-iPS cells appeared similar but not with exactly same pattern and gene expression patterns in human CiMS cells were very different from previous two patterns. 764,885 genes were analyzed among three different cell lines by microarray. Expression differences between human CiMSs and human CiMS-iPS cells, and between CiMSs and human ES cells showed big gap, as detected respectively with 3,665 genes and 4,025 genes shown more than 2-fold expression difference. In contrast, differences of the expressions between human CiMS-iPS cells and human ES cells appeared very few, only 427 genes show >2-fold difference (Figure 3B).

The methylation status of CpG regions of the promoters of pluripotent gene marker, Nanog and Oct3/4 was estimated by bisulfite genomic PCR (Fig. 3C). The methylation percentage of CpGs in the Nanog promoter regions of CiMSs, CiMS-iPS cells

and ES cells were evaluated as 50%, 5%, and 18.3%, respectively. The respective percentages for Oct3/4 in CiMSs , CiMS-iPS cells and ES cells were 78%, 2.8%, and 10%. These results show that transcription of Nanog and Oct3/4 turn out to be more active after transformed into iPS cells.[8] Karyotyping analyses of the three CiMS-iPS cell lines demonstrated that two of the iPS clone showed a normal karyotype of 46XY and 46XX, while one line showed an chromosomal abnormality, 46, XY, t(2;14)(p24;q11.2). (Figure 3D)

In vitro spontaneous differentiation of CiMS-iPS cells into three germ layers

In order to form embryoid bodies, CiMS-iPS cells were floated in non-coated plastic dishes for 10 days. After 10days, EBs were transferred to gelatin coated culture dishes, then EBs derived from CiMS-iPS cells were attached onto the bottom of dish and initiated differentiation. After 3 weeks, the cells were detected with α-fetoprotein (endoderm marker), α-sarcomeric actin, α-smooth muscle actin (mesoderm marker), and nestin, GFAP, β-III tubulin (ectoderm marker)by immunofluorescence (Figure 4A). RT-PCR was also performed

to detect marker gene expression from three germ layers and showed expression of endoderm (AFP, GATA4), mesoderm (TnTc, Brachyury) and ectoderm (BMP4, Ncam1) (Figure 4B) in differentiated iPSs. As expected, these markers were not detected in undifferentiated cells from the same colonies.

Teratoma derived from CiMS-iPS cells

To confirm the pluripotency of iPS cells in vivo, teratoma formation was examined.[17] Human CiMS-iPS colonies were harvested and injected into nude mice subcutaneously. Two months after injection, encapsulated cystic teratomas were formed and histological examination revealed that differentiated tissues from all three germ layers were positive, endoderm (stratified squamous epithelium, mucus-producing epithelium and ciliated epithelium), mesoderm (cartilage and skeletal muscle) and ectoderm (pigmented retinal epithelium and neuroepithelial rosette) (Figure 5)

Efficient differentiation ability of CiMS -iPS cells into cardiovascular cells.

There are reports that differentiation efficiency is different by their origin.[6] Because CiMSs are cells from heart endocardium, we expect that differentiation of CiMS –iPS cells into cardiovascular cells is superior than that of others. Because differentiation into mesodermal cells have to preceed, before differentiated into cardiovascular cells, we first determined whether mesenchymal differentiation of CiMS–iPS cells is better than HDF–iPS cells and ES cells. We treated each iPS cells and ES cells with CHIR 99021, GSK3β inhibitor which is important for differentiation into mesoderm lineage[18] and estimated that the expression of mesodermal genes by RT–Real time PCR and RT–PCR. Pluripotency markers, OCT4 and NANOG were down regulated at all the iPS type and human ES by the time. And as expected, CiMS–iPS cells expressed markers of the early mesoderm earlier or greater than HDF–iPSs and hES cells. (Fig. 6 A, C). On the other hand, the same tendency was not observed in the endodermal differentiation or endodermal differentiation. (Fig. 6 B)

Next we differentiated multiple pluripotent stem cell clones into endothelial cell (EC)[19,20], vascular smooth muscle cells (VSMC)[21,22] and cardiomyocyte (CMC)[23] which are cells

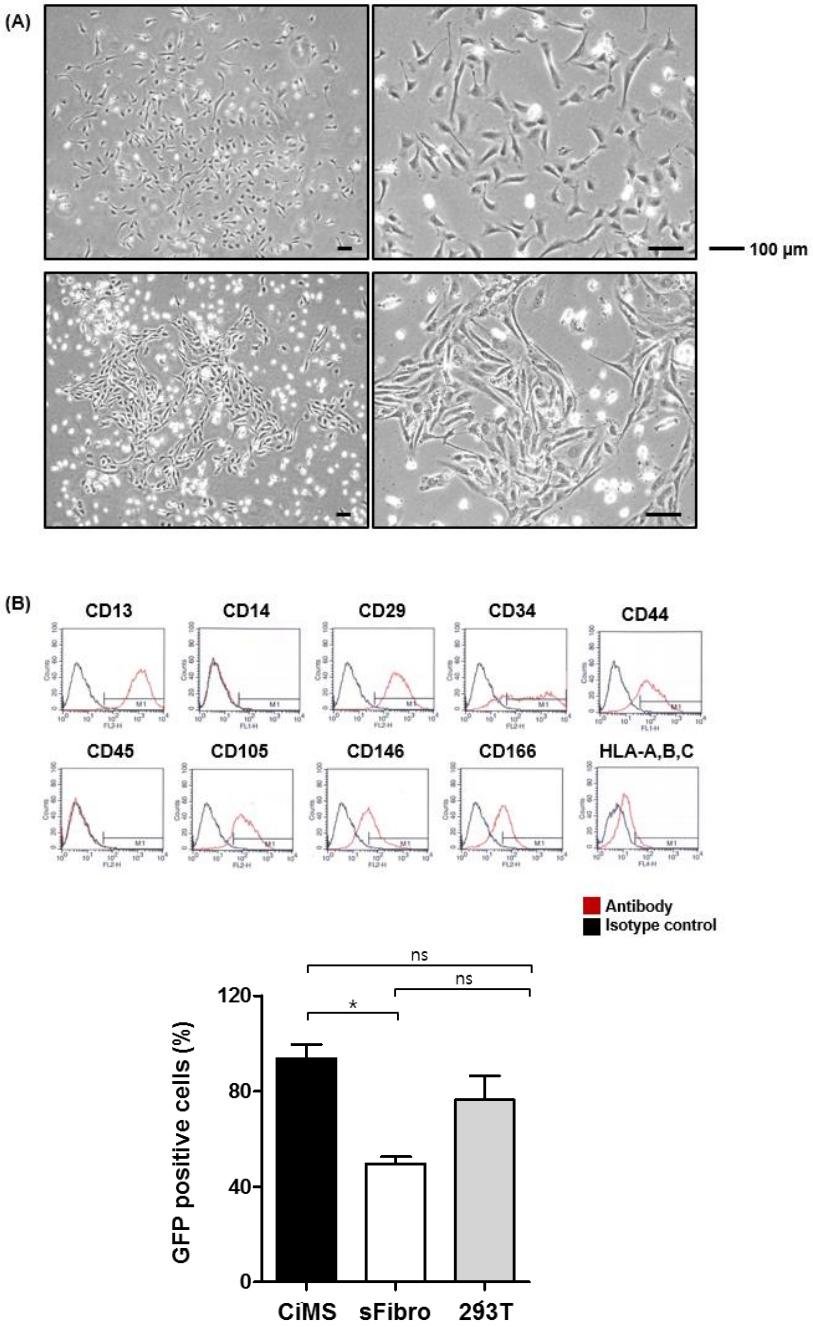
representing cardiovascular cells. When we differentiated iPS cells into EC, VSMC, CMC as described in figure 6D. RT-real time PCR showed that expression of each markers were faster or greater at differentiation of CiMS-iPS cells than that of HDF-iPS cells or ESs (Figure 6 D).

High mesodermal differentiation efficiency cause of different epigenetic status

In order to clarify whether different epigenetic states make these differences of differentiation efficiency among the multiple PSCs[6], we first compared patterns of genomic DNA methylation at CpG sites of Brachyury T of three different PSCs by bisulfite PCR. We observed that there are few differences within these cells (Figure 7A).

Next, the changes of the histone mutation level in the differentiation of each lineage were compared using chromatin immunoprecipitation (ChIP) assay. Interestingly, when CiMS iPS differentiate into mesoderm, H3K4 tri-methylated level showed most up-regulated level compared to other PSCs. And the H3K4 tri-methylated level of differentiated CiMS iPS cells was highest than others. And during differentiation, the level of H3K9 and

H3K27 tri-methylated level was significantly down regulated while other two PSCs showed no significant change (Figure 7 B). However, when PSCs were differentiated into endoderm and ectoderm lineage, there are no significant difference of histone modification level between them (Figure 7 C, D).



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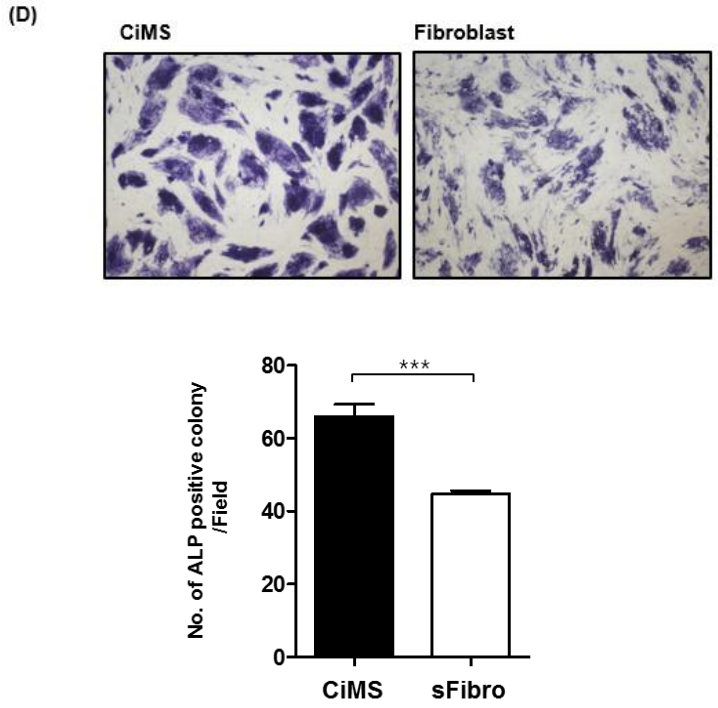


Figure 1. Characteristics of human circulating multipotent adult stem cells (CiMSs)

(A) Cell morphology of CiMS appeared as disperse type and colony type

(B) FACS analysis of surface markers expressed by CiMS

(C) Transduction efficiency was estimated using GFP retroviral transduction. Retroviral transduction efficiency was higher at CiMS (99.75%) than Fibroblast (49.44%) and even 293T (76.44%). (N=2)

(D) ALP staining of 4 factors transduced CiMS and 4 factors transduced fibroblast after re-seeded on STO.

Quantification of ALP positive colonies is also shown. (N=4 in each group) Mean number of ALP positive cells in transduced CiMSs was 66.0, that is many more than that of transduced fibroblast (n=44.75).

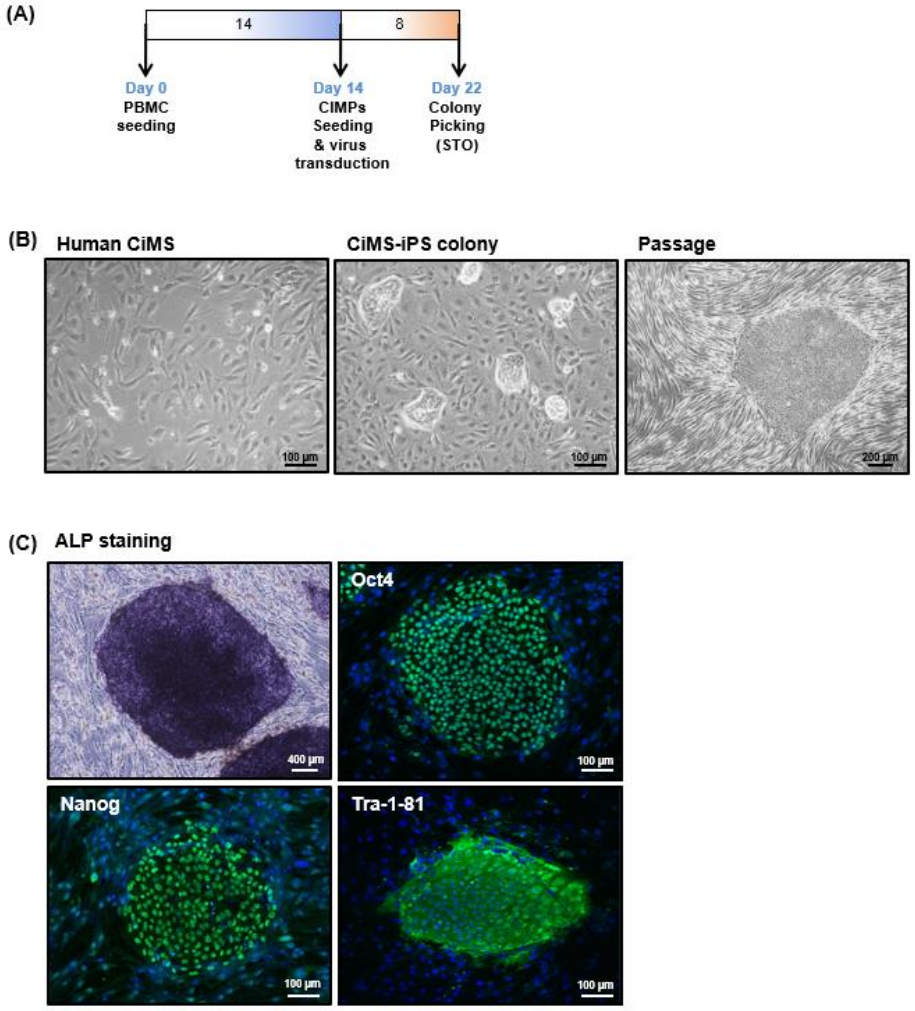


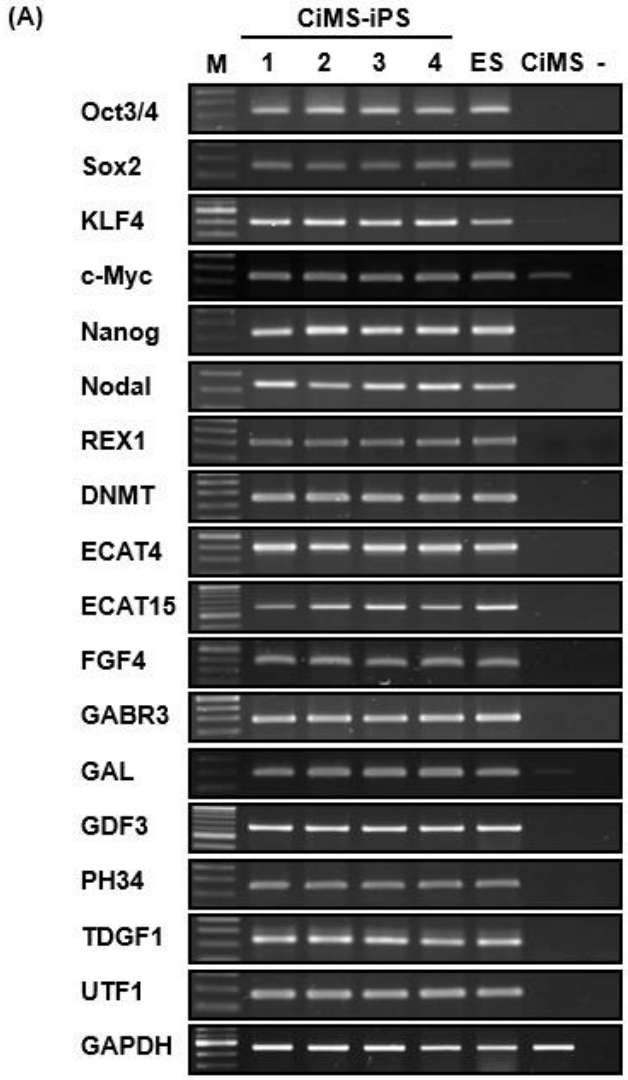
Figure 2. Generation of induced pluripotent stem cells (iPS Cells) from human circulating multipotent adult stem cells (CiMSs)

(A) A scheme of the generation of CiMS iPS cells . Eight days after transduction of CiMS with Yamanaka's

retroviral viruses (Oct4, Sox2, KLF4, c-Myc), the transformed colonies were picked up and re-seeded on STO feeder cells.

(B) ES-like colonies were appeared approximately one week after reseeding on STO feeder cells.

(C) Expression levels of pluripotency markers, OCT4, NANOG, Tra-1-81 were monitored by immunocytochemistry. ALP, which is highly detected in PSCs of colonies were also detected by ALP staining at CiMS-iPS colonies.



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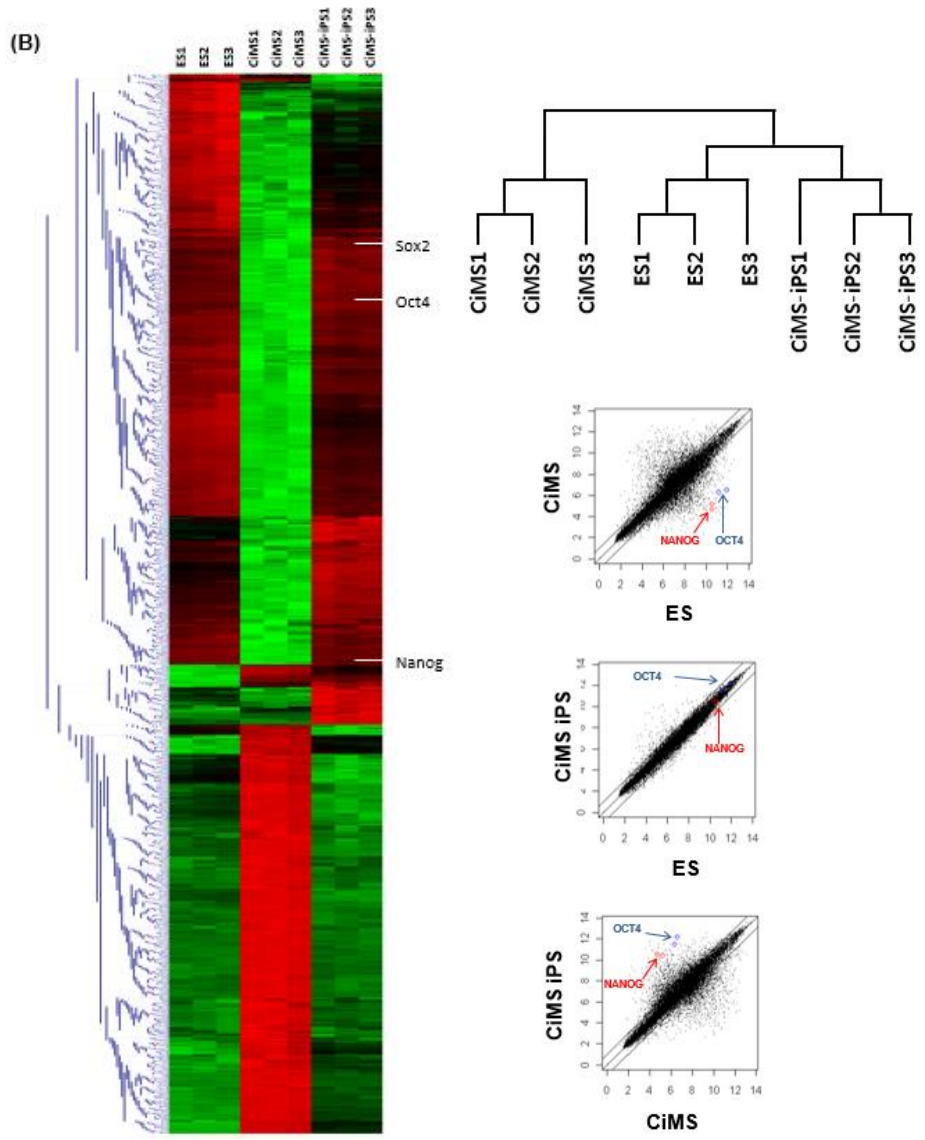

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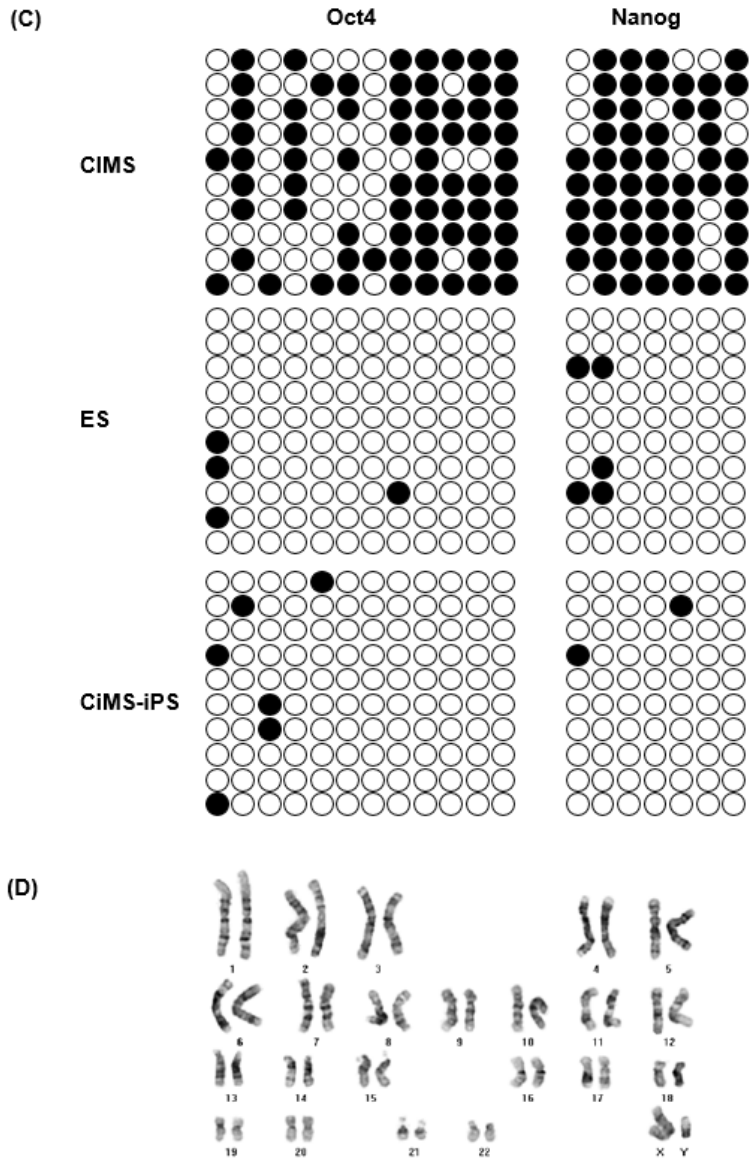


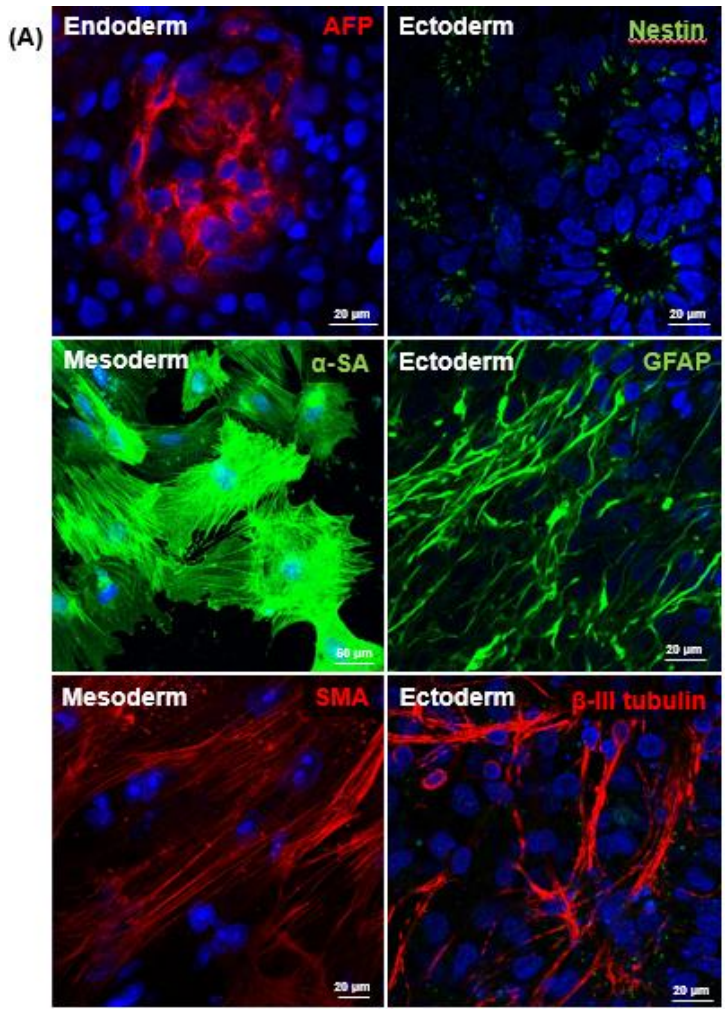
Figure 3. Expression of hES Cell marker genes and analyses of promoter region in CiMS-iPS

(A) Expression level of pluripotency gene of CiMS-iPS cells were similar with that of ES cells confirmed by RT PCR

(B) Global gene expression profiles were compared among human CiMS, human ES cells, and human CiMS-iPS cells by DNA microarrays. They show the profiles of human ES cells, and human CiMS-iPS being similar while profiles of human CiMS and profiles of human ES cells or human CiMS-iPS cells were quite different.

(C) Methylation status of CpG sites of the pluripotency-associated genes, Nanog and Oct3/4 were confirmed by Bisulfite genomic PCR. Methylation status of CiMS-iPS cells was similar with that of ES cells. But status of human CiMS either appeared quite far from the status of human ES cells or human CiMS-iPS cells.

(D) Karyotyping analyses of the three CiMS-iPS cell lines


(A)
Endoderm
AFP
Ectoderm
Nestin
Mesoderm
α-SA
Ectoderm
GFAP
Mesoderm
SMA
Ectoderm
β-III tubulin
20 μm
60 μm

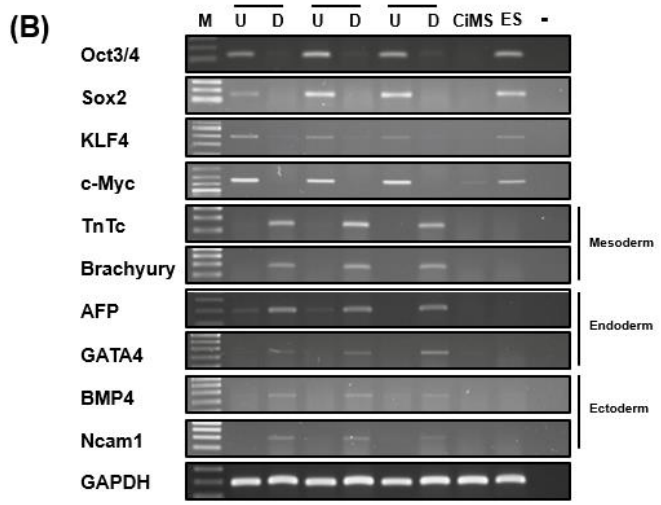

(B)
M
U
D
U
D
U
D
CiMS
ES
-
Oct3/4
Sox2
KLF4
c-Myc
TnTc
Brachyury
AFP
GATA4
BMP4
Ncam1
GAPDH
Mesoderm
Endoderm
Ectoderm

Figure 4. Three germ layers in vitro induced by CiMS-iPS cells

(A) Spontaneous differentiation of CiMS-iPS cells in vitro. Differentiated three germ layers were shown by immunocytochemistry. Gene markers of each germ line were stained as described. DAPI (Blue) indicate the nucleus of the cells.

(B) RT PCR showed each three germ line marker of spontaneously differentiated CiMS iPS cells.

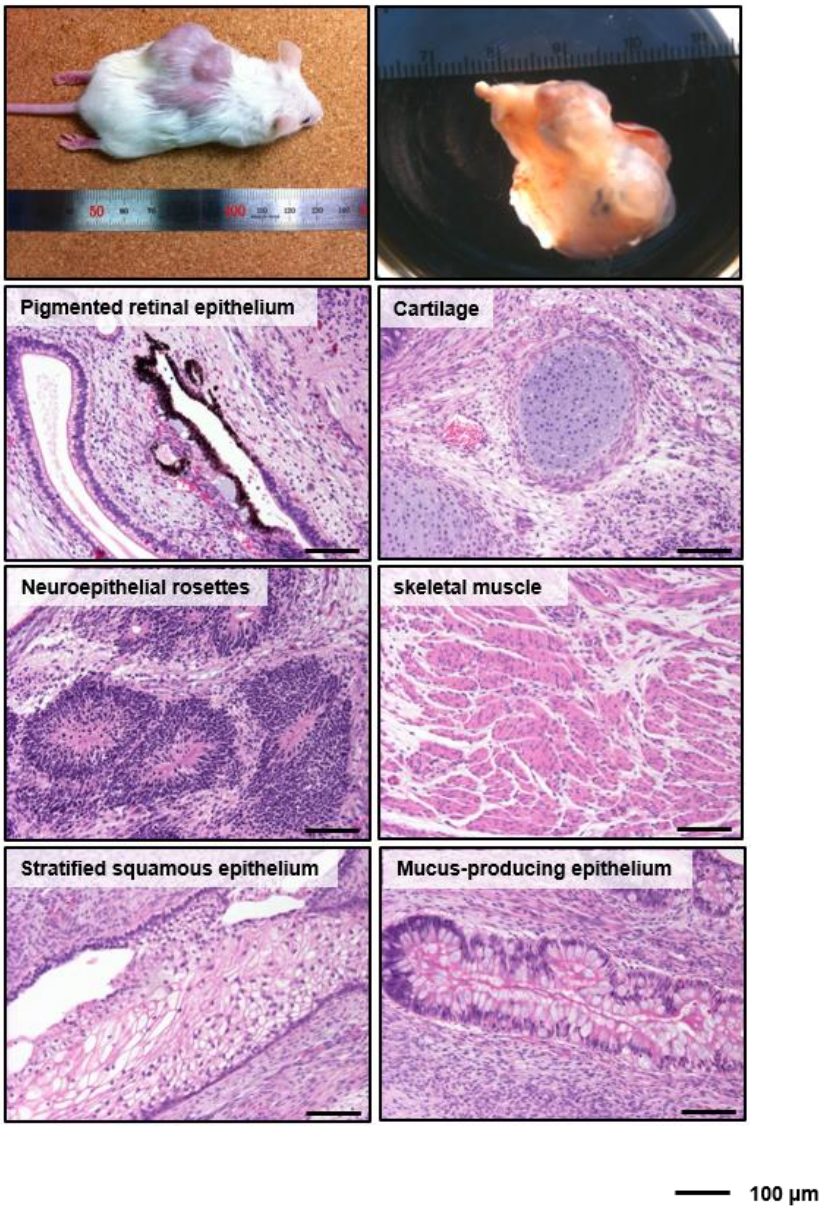
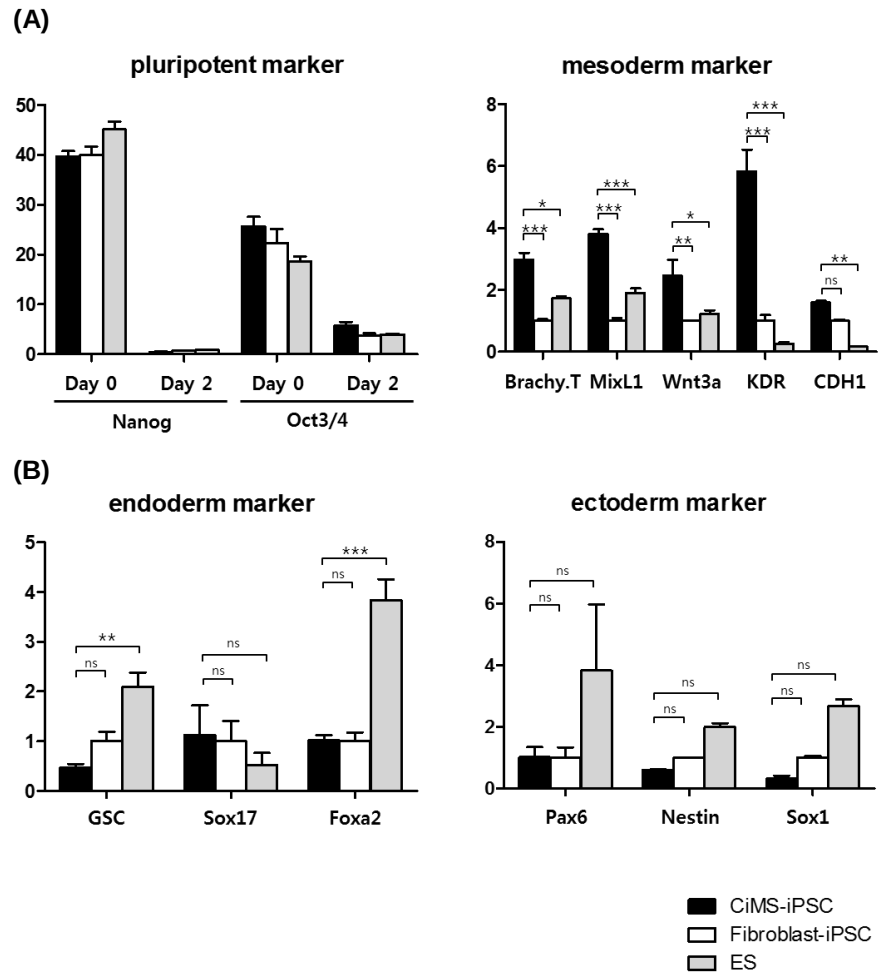
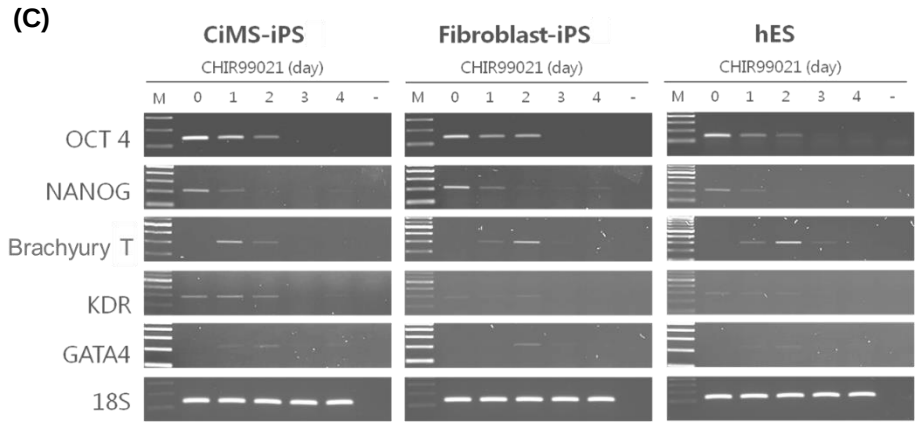


Figure 5. Teratoma derived from CiMS-iPS cells

Teratoma formation was confirmed by staining differentiated tissues from each three germ line with hematoxylin and eosin





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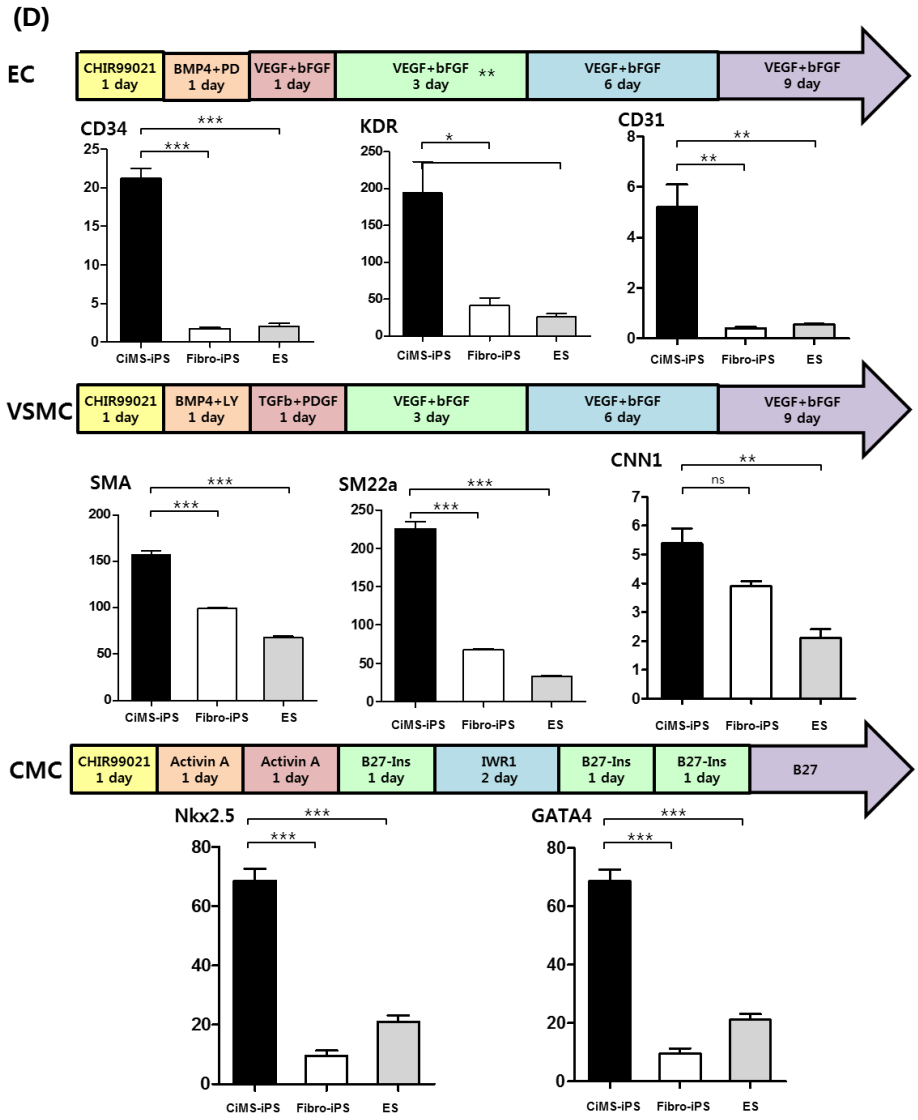


Figure 6. The mesoderm differentiation preference in the differentiation process of CiMS-iPSC to three germ layer

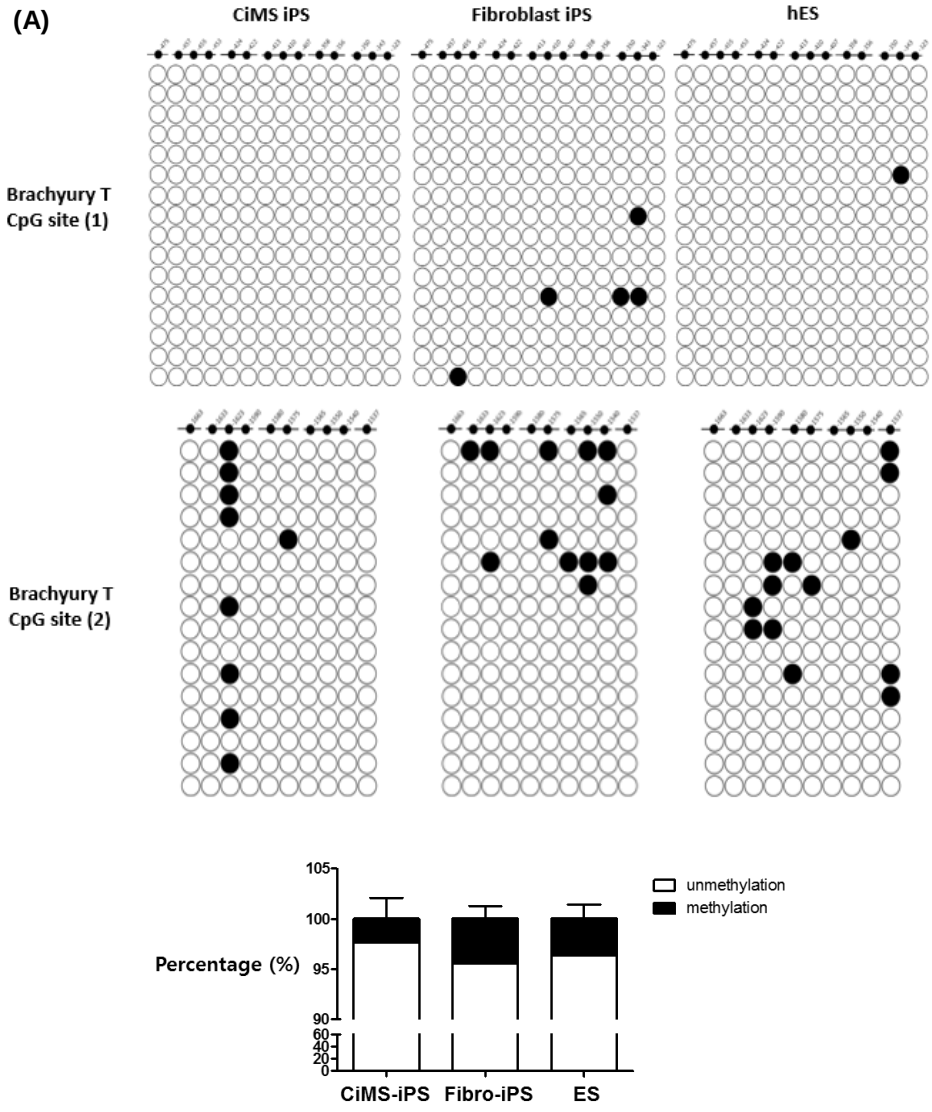
(A) Expression of pluripotent genes of D0 and D2 of mesodermal differentiation were estimated by RT-Real time PCR. And Expression of mesodermal genes

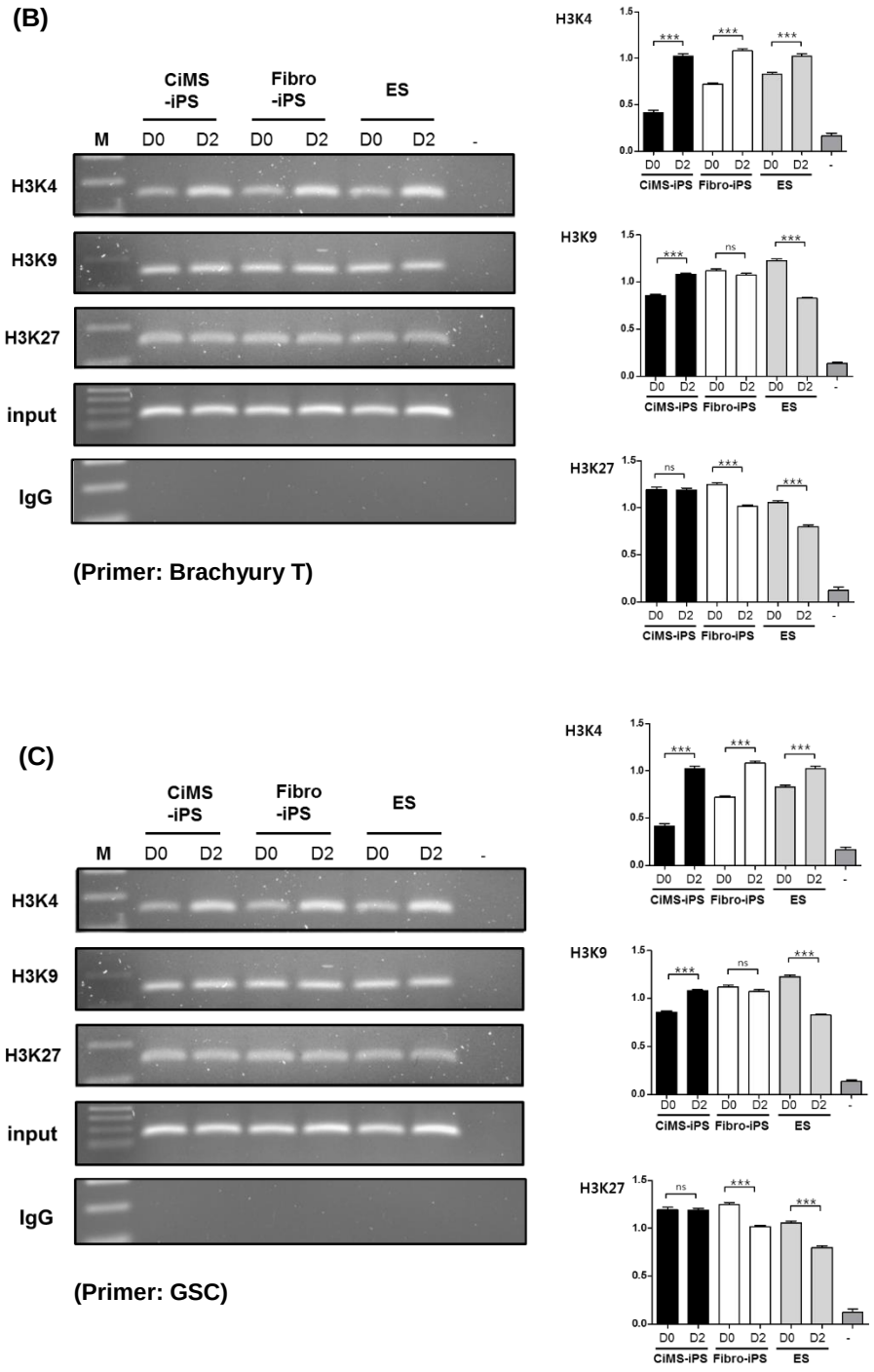
of D2 of mesodermal differentiation were compared with each other.

(B) Expression of early endodermal genes of D4 of endodermal differentiation and early ectodermal genes of D5 of ectodermal differentiation were compared with each other.

(C) The expression of mesodermal genes and pluripotent genes were estimated by RT-PCR. Genes associated pluripotency were down-regulated with same speed among CiMS-iPS cells and HDF-iPS cells and ES cells. But the markers of differentiation into mesoderm were detected earliest or greatest in differentiation of CiMS-iPS cells.

(D) A scheme of differentiation of CiMS iPS into EC, VSMC, CMC and markers of each gene expression during differentiation. And expression of each markers of D5 of EC differentiation, D6 of VSMC differentiation, D12 of CMC differentiation were compared with each differentiation of the other pluripotent cells.


(A)
CiMS iPS
Fibroblast iPS
hES
Brachyury T
CpG site (1)
Brachyury T
CpG site (2)
Percentage (%)
105
100
95
90
60
40
20
0
unmethylation
methylation
CiMS-iPS
Fibro-iPS
ES


(B)
CiMS -iPS
Fibro -iPS
ES
M
D0
D2
D0
D2
D0
D2
-
H3K4
H3K9
H3K27
input
IgG
(Primer: Brachyury T)
H3K4
H3K9
H3K27
***
ns
CiMS-iPS
Fibro-iPS
ES
(C)
CiMS -iPS
Fibro -iPS
ES
(Primer: GSC)

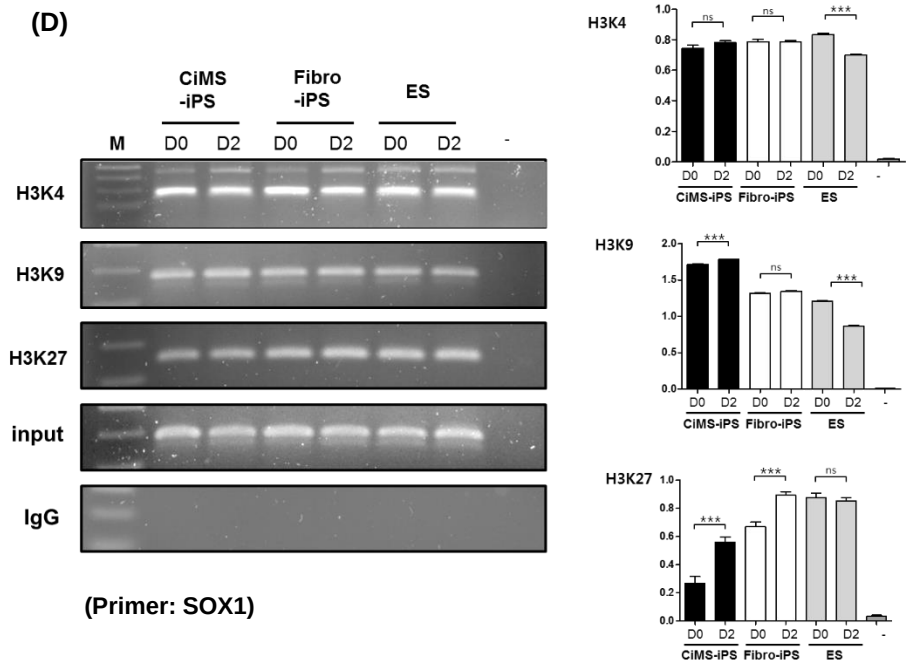


Figure 7. Differentially emerged epigenetic status among pluripotent cells

(A) Patterns of genomic DNA methylation at CpG site of T (Brachyury) were confirmed by bisulfite PCR among three different PSCs. There were little differences with three different PSCs.

(B) Histone modification levels of transcription site of Brachyury T during mesodermal differentiation of the three different PSCs were compared. The level of H3K4 tri-methylation of CiMS iPS cells was highly increased compared to others, while the levels of

H3K9, H3K27 were decreased significantly compared to others.

(C) Histone modification levels of transcription site of GSC during endodermal differentiation of the three different PSCs were compared. The level of H3K4 tri-methylation of CiMS iPS cells was highly increased compared to others, while the levels of H3K9, H3K27 were decreased significantly compared to others

(D) Histone modification levels of transcription site of Sox1 during ectodermal differentiation of the three different PSCs were compared. The level of H3K4 tri-methylation of CiMS iPS cells was highly increased compared to others, while the levels of H3K9, H3K27 were decreased significantly compared to others

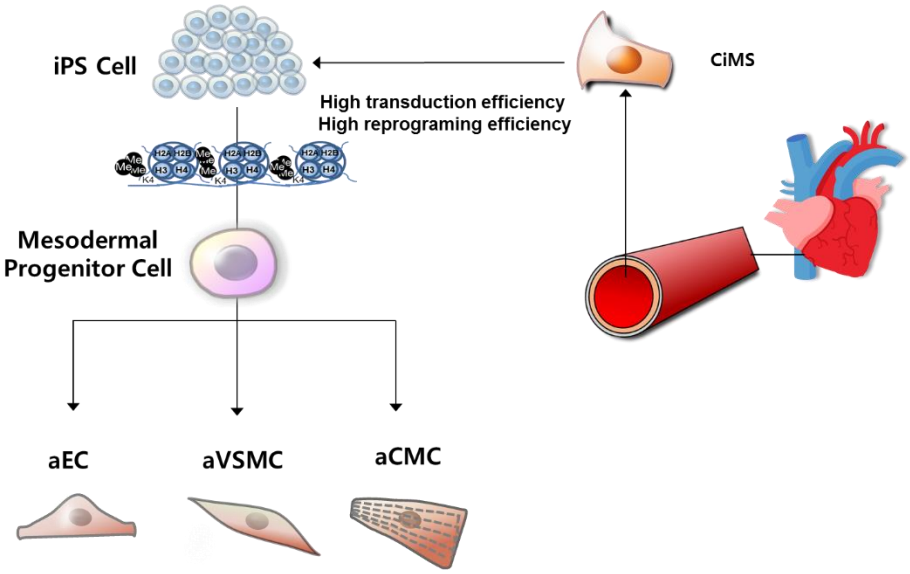


Figure 8. Schematic figure of reprograming CiMS into iPS and differentiation into cardiovascular cells of CiMS iPS

CiMSs which are isolated in heart endocardium, reprogrammed into iPS with high transduction efficiency and reprograming efficiency. CiMS iPS can differentiate with high efficiency cause of advantageous epigenetic status.

Table 1. List of primers

Gene name	F, R	Sequence (5’ →3’)	Tm (℃)
Real time PCR−Mesoderm differentiation			
Mixl1	F	TACCCCGACATCCACTTGCG	60
	R	ATCTCCGGCCTAGCCAAAGG	59
Brachyury T	F	ACTGGATGAAGGCTCCCGTCTCCTT	63
	R	CCAAGGCTGGACCAATTGTCATGGG	63
Wnt3a	F	ACTCCTCTGCAGCCTGAAGC	56
	R	GAACTGGTGCTGGCACTCCT	56
CDH1	F	GCATTGCCACATACACTCTC	56
	R	AATCTCCATTGGATCCTCAA	56
Real time PCR−Ectoderm differentiation			
Nestin	F	AAGCCCTGAACCCTCTTTGC	58
	R	GGGAAGAGGTGATGGAACCA	58
Sox1	F	TCCCCCGCGTGAACTG	54
	R	CAAGGCATTTTGCGTTCACA	53
Real time PCR−Endoderm differentiation			
GSC	F	GAGGAGAAAGTGGAGGTCTGGTT	60
	R	CTCTGATGAGGACCGCTTCTG	60
Foxa2	F	GGGAGCGGTGAAGATGGA	57
	R	TCATGTTGCTCACGGAGGAGTA	58
Real time PCR−EC differentiation			
CD31	F	TTCCTGACAGTCTCTTGAGTGGGT	58
	R	TTTGGCTAGGCGTGGTTCTCATCT	62
KDR	F	GCGATGGCCTCTTCTGTAAG	54
	R	ACACGACTCCATGTTGGTCA	53
CD34	F	CCAGAGTTACCTACCCAGGG	52
	R	TGGGGTGGTGAACACTGTGC	59
Real time PCR−VSMC differentiation			
SMA	F	CCAGCTATGTGTGAAGAAGAGG	58
	R	GTGATCTCCTTCTGCATTCGGT	58
SM22a	F	CGCGAAGTGCAGTCCAAAATCG	60

	R	GGGCTGGTTCTTCTTCAATGGGC	62
CNN1	F	GAGTGTGCAGACGGAACTTCAGCC	64
	R	GTCTGTGCCCAGCTTGGGGTC	64
Real time PCR-CMC differentiation			
Nkx2.5	F	TTTGCATTCACTCCTGCGGA	65
	R	ACTCATTGCACGCGTCATAATCGC	64
GATA4	F	TCCAAACCAGAAAACGGAAG	54
	R	TCGCACTGACTGAGAACGTC	53
Real time PCR-Housekeeping gene			
18s rRNA	F	CCTGCGGCTTAATTTGACTC	56
	R	ACCAACTAAGAAGAACGGCATG	57
ChIP assay			
Brachyrury T	F	GAAAGCAATGACACAGCAGA	56
	R	AGGGAAATGGACGGAAATAA	55
SOX1	F	GCGAGGAGACAGCACACC	59
	R	CCTGATGCACAAACCACTTG	56
GSC	F	GAGCTACAGGCAGAGGAAATCGCA	62
	R	CTGGGCGGGCGGCCTAATTG	62

Discussion

In this study, we presented new candidate cell, CiMS, adult stem cell from heart endocardium, for establishing iPS cells.

CiMS can be easily obtained with a small volume of peripheral blood (10ml). This approach can escape from invasive preparation for obtaining sample in which preparation from fibroblast requires. CiMS do not need a long period of maintenance in culture before reprogramming while approach with fibroblast is needed. CiMS have a twice higher transduction efficiency compared to fibroblast (Figure 1D). These advantages make CiMS, an ideal cell line for establishing iPS Cells.

After 8 days of culture, we introduce Yamanaka factors into CiMS, we can identify ES-like colonies without feeder cells. (Figure 2)

This CiMS-iPS cells show the essential features iPS cells must have, including expression of pluripotent genes (Fig. 3 A,B), epigenetic status like ES(Fig. 3 C,D), in vitro differentiation ability into three germ layers (Fig. 4) and derivation teratoma (Fig. 5).

CiMS-iPS cells differentiate more easily into mesoderm than fibroblast-iPS cells and hES (Figure 6A). We have also shown

that terminal differentiation into the typical cardiovascular cell, EC, VSMC, and CMC [19,20,21,22] are also observed with the more remarkable result in CiMS iPS cells compared to fibroblast iPS cells and ESs (Figure 6 D).

There are reports that epigenetic features of the original cell can remain after reprogramming and that can affect their differentiation potential even iPS cells acquired the molecular and functional characteristic of ES.[6,7] And recently, we confirmed that CiMS highly expressed early cardiomyocyte marker GATA4 and SOX17 which is involved in hematopoietic development. Based on previous reports and our observation, we thought that CiMS-iPS cells might be more potent in differentiation into cardiovascular cells than fibroblast- derived iPS cells and ES cells and this is due to more accessible epigenetic state than other PSCs.[8] Genomic DNA methylation level at CpG site of Brachyury T did not have big differences among the PSCs[12] (Figure 7 A). However, we observed that H3K4 tri-methylation level of CiMS iPS cells was the greatest increased and H3K9 tri-methylation level and H3K27 tri-methylation level were decreased significantly during mesodermal differentiation, whereas the differentiation into ectoderm and endoderm showed

no significant difference between the PSCs (Figure 7 B,C,D).

In conclusion, our study provides a more pragmatic strategy for the establishment of patient and disease –specific pluripotent stem cells. Moreover, this approach provides a great benefit for application in the regenerative medicine.

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국문초록

혈중 순환 다분화능 줄기세포의 효과적인 유도 만능 줄기세포로의 역분화능과 그의 유리한 중배엽 분화능에 대한 연구

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전 미 카

유도 만능 줄기 세포의 발견은 발생학 연구의 분자생물학적 접근에 큰 발돋움을 일으켰으며 또한 환자 맞춤 질병 치료를 향한 새로운 창을 제시하였다. 환자 맞춤형 유도 만능 줄기 세포 치료를 위해서는 자가 세포를 이용하여 유도 만능 줄기 세포를 생성해야 한다. 이에 접근이 용이하고 배양이 비교적 쉬운 인간 섬유아세포가 유도 만능 줄기 세포 제작에 가장 광범위하게 사용되었다. 하지만 침습적 세포 획득 방법과 역분화에 사용하기 위해 오랜 기간 세포를 배양해야 하는 단점(한 달 가량), 그리고 제한된 계대 수 때문에 역분화에 사용할 더 적합한 세포에 대한 필요성이 대두되어

왔다. 이에 자연스레 접근성이 높고 비침습적 방법으로 획득이 가능한 혈액 세포에 대한 관심이 높아졌다. 하지만 현재까지 보고된 바에 의하면 역분화 유도에 혈액 세포를 이용하기 위해서는 혈액 샘플 체취 전 체내 화학약품 주입과 유세포분석을 통한 샘플 분리를 필요로 하는 불편함이 있었다. 또한 이 단계들을 거침에도 역분화 실험 시 바이러스 도입 효율이 상당히 낮게 나타나는 결과를 보였다.

이에 본 연구에서 유도 만능 줄기세포 형성에 적합한 새로운 근원 세포를 제시한다. 우리는 인간 말초 혈액 샘플에서 심내막에 존재하는 중배엽 유래 성체 줄기 세포를 분리하였고 이 세포를 혈중 순환 다분화능 줄기 세포 (CiMS)로 명명하였다. 이 세포는 말초 혈액 샘플 10ml 만으로도 충분히 분리될 수 있으며 10 계대 이상 활발히 분열하여 추후 실험을 위해 동결 보존되는 것이 가능하다. 또한 짧은 세포 배양기간만 거쳐도 바로 역분화 실험에 사용될 수 있으며 인간 섬유아세포보다 향상된 유전자 도입능력을 보였다. 이러한 이유들로 우리는 이 혈중 순환 다분화능 줄기 세포 (CiMS)가 유도 만능 줄기 세포 유도를 위한 근원 세포로서

적격하다고 판단하였다. 더 나아가 우리는 혈중 순환 다분화능 줄기세포 유래 유도 만능 줄기세포의 심혈관계 세포로의 분화능이 인간 섬유아 세포 유래 유도 만능 줄기세포나 인간 배아줄기세포의 분화능보다 우수함을 증명하였다.

이에 우리는 본 연구로써 유도 만능 줄기세포의 임상적 적용에의 한계를 극복하고 재생 의학 발전에 기여할 수 있을 것이라 기대한다.

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주요어: 혈중 순환 다분화능 줄기 세포 (CiMS); 역분화; 유도 만능 줄기 세포; 심혈관계 세포 분화; 환자 맞춤 질병 치료; 재생 의학

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