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Clinical grade production of Mesenchymal Stem Cells derived from Embryonic Stem Cells (ESC-MSC) having Therapy Effect to a Rat Model of Myocardial Infarction

심근경색 동물 모델에 치료효과가 있는 임상 등급 배아줄기세포 유래 중간엽 줄기세포 생산

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Abstract

Clinical grade production of Mesenchymal Stem Cells derived from Embryonic Stem Cells (ESC-MSC) having Therapy Effect to a Rat Model of Myocardial Infarction

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Backgroud

Human embryonic stem cell (hESC) have great potentiality to stem cell therapy. However, treating embryonic stem cell itself to patient has the possibility of suffering harm because of tumorigenicity and immune rejection. In order to overcome these risks, hESCs were induced to other lineage usually for applying stem cell therapy to human. Mesenchymal Stem Cells induced

from ESC (hESC-MSCs) have the capability to self-renew and differentiate into various types of cell.

Methods and results

Working in cGMP facility was proved that hESC-MSC is culturing at aseptic condition. An efficient method of building up hESC-MSCs without any xeno factors during culture in cGMP facility was developed. we confirmed hESC with non xeno factor was growth well. Then via EB stage, MSCs was differentiated completely form ESC. Moreover, we documented and standardized the derivation and culture of MSCs for establishing stable cell line. we culture hESC on extracellular matrix instead of feeder cell and serum free media was used without xeno factors Our derived MSCs from ESCs were expressed CD44, CD73, CD90, CD29 and CD105 as MSCs' positive markers and not expressed MSCs' negative markers, CD34 and CD45.

We also studied therapeutic efficacy of hESC-MSCs in vivo. To reduce difference between entities, we use inbred rat (F344) and induce a rat model of Myocardial Infarction (MI). We injected cell to MI model at periinfarct zone. Through various analyses, we demonstrated that heart function of cell transplantation group in MI model was better condition than group with PBS.

Conclusion

our established method induced MSCs from ESCs in cGMP facility could overcome limitation of other derivation methods and get high purity of yield. Moreover, our method is easy, simple.

With animal experiment, we suggested hESC-MSCs is suitable to apply clinical trial about MI.

Keywords: human Embryonic Stem Cell, Mesenchymal Stem Cell, Differentiation, Myocardial Infarction

Student Number: 2013-24024

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Introduction

Recently, many people are endeavoring to apply cell therapy using stem cell for various diseases which is called stem cell therapy[1]. One of merits about stem cell therapy is unique properties of stem cells itself. Unlike other type of cells, stem cells have ability to self-renewal and it can induce any other mature cells. For this reason, stem cells would be a potential therapeutic substance for various human diseases.

Among stem cell therapy field, these days, mesenchymal stem cells (MSCs) especially has received a great popularity because of the several positive characteristics of MSCs[2]. One of characteristics is that MSCs, which come from another person, do not have immune reaction to recipients, and also MSCs have ability to proliferate like any other stem cell[3]. Nevertheless, using stem cell therapy to human has some limitations yet[4]. One is facility where the stem cell is culturing. Culturing cells should be on clean condition. However, generally, no one certify clean degree in normal facilities. Patient does not want to take therapy by using stem cells that is cultured on poor condition. Therefore, people have developed good manufacturing practice which is called GMP. GMP was documented all working procedure of making cell and aseptic condition. That is, culturing cells from GMP is guaranteed that cell is cultured aseptic condition by standardized procedures and facilities. another one is

xeno factors necessary to culture stem cell. Usually, feeder cells and media containing important nutrition from other species such as fetal bovine serum are needed to culture stem cell. Until now, these factors are utilized commonly to stem cell therapy, but it is not proved safety of stem cell from xeno factor. Developing new material instead of xeno factors has been actively studied[5-8]. Actually, serum free media and extra cellular matrix are used instead of FBS and feeder cell. Animal experiment was performed to evaluate the therapeutic efficacy of MSC culturing with non xeno factors by using a rat model of myocardial infarction.

Our study demonstrate we established the protocol to make embryonic stem cell(ESCs) derived MSCs(E-MSCs) through short embryoid body(EB) stage by using clinical grade human ESCs with non xeno factor[7,9-11]. And we did all experiment about culturing MSC in cGMP grade facility. Working in cGMP facility with standardized procedures of making hESCs-MSCs is guaranteed to aseptic condition to establish hESCs-MSCs[12,13]. In summary, clinical hESCs derived MSCs without any xeno factors by working in cGMP is the potential candidate for stem cell therapy in heart disease[14].

Material and Method

Clinical grade hESCs culture with non-feeder material

Clinical grade human embryonic stem cells(hESCs) (Lifemaps, Inc., USA) were cultivated on vitronectin (GIBCO, USA) coated dish. hESCs were maintained with serun free media, essential 8^{th} medium (GIBCO, USA) comprised of DMEM-F12 supplemented with 8 supplements. hESCs were cultured at 37℃ in 5% CO2 at 95% humidity and manually passaged every 5 days. Growth medium was changed every day.

Induction of clinical grade hESCs derived MSCs

Through the Embryonic body (EB) stage, hESCs were induced mesenchymal lineage differentiation, undifferentiated hESCs were harvested gently with 0.5M EDTA (invitrogen, USA) at 37℃ for 5 min and dissociated into a suspension of cell. Harvested cells were cultured in 60mm dish with essential 6th medium (GIBCO, USA), which is not containing xeno factors, for 14day to form EB. Culture media was changed every day. After 14days of forming EBs, the EBs, almost well-rounded shape, were selected

and attached with CTS CELLstart (GIBCO, USA) coated 30mm dish with StemPro® MSC SFM medium(A10332-01, GIBCO, USA). The culture media was changed every day during 8days. The outgrowth expended EBs were dissociated with TrypLE (GIBCO, USA) at 8days of culturing in 30mm dish, and it was seeded at CTSTM CELLstartTM coating 75T flask with StemPro® MSC SFM medium. The growth media, StemPro® MSC SFM medium, was changed every day. Completed hESCs derived MSCs (hESCs-MSCs) was started culture per 1x10^6 in one dish at every passage.

Flow cytometry analysis

To check the characterization of hESCs at passage 35 and hESCs-MSCs at passage13, the approximate 5×10^5 cells were washed with DPBS and stained with following these conjugated antibodies, CD44(Phycoerythrin (PE); BD Biosciences, USA), CD73(PE; BD Biosciences, USA), CD90(PE; BD Biosciences, USA), CD29(PE; BD Biosciences, USA), CD105(PE; BIO-RAD, USA), CD34, CD45(PE; DAKO, Denmark), TRA-1-60(PE; BD Biosciences, USA), SSEA1(PE; BD Biosciences, USA), SSEA3(Fluorescein isothiocyanate(FITC); BD Biosciences, USA). For evaluating characteristics of immune about hESCs-MSCs, immunogenic-related surface marker antibodies; human leukocyte antigen HLA-DR (FITC; BD Biosciences, USA), HLA-DQ (FITC;

BD Biosciences, USA) were used at 4℃ for 15 min, and as same protocol above. All cells were analyzed using a flow cytometer (FACS calibur; BD Biosciences, USA). and software(CellQuest; BD Biosciences, USA)

Differentiation analysis

Lineages differentiation of hESC-MCSs toward osteogenesis, adipogenesis and chondrogenesis were perform by using Human Mesenchymal Stem Cell Functional Identification Kit(SC006, R&D systems, USA). According to the manufacturer's protocol, chondrogenic differentiation capacity of MSCs was evaluated that hESC-MSCs were cultured with chondrogenic differentiation media containing D-MEM/F-12 99%, ITS supplement 1%, Penicillin100 U/mL, Streptomycin100 μg/mL and L-Glutamine 2 mM on 15ml cornical tube for 21 days. Differentiated chondrocyte from hESC-MSCs were analyzed through immunofluorescence by using primary antibody against aggrecan. For osteogenic differentiation, hESC-MSCs were incubated with differentiation media, a-MEM 99%, Fetal Bovine Serum 10%, Penicillin100 U/mL, Streptomycin100 μg/mL, L-Glutamine2 mM and osteogenesis inducible supplements for 21days. Osteogenic differentiation capacity of MSCs was performed through immunofluorescence, and the antibody against osteocalcin was used for evaluating osteogenic differentiation. The confocal

microscope (Carl Zeiss LSM710, Germany) was used to evaluate these lineages differentiation. Lastly, adipogenesis from hESC-MSCs were cultured with differentiation media, a-MEM 99%, Fetal Bovine Serum 10%, Penicillin100 U/mL, Streptomycin100 μg/mL, L-Glutamine2 mM and adipogenesis inducible supplements, for 21days. The oil red O was added during 5min in culture media before it was evaluated with the optical microscope(Olympus, Japan).

Growth curve assay

hESC-MSCs' growth curve was established during incubation period by multiplying the number of cells. Each point represents the number of cells by using COUNTESS (life technology, USA)

G-band staining

A karyotype analysis was commissioned a staining into GENDIX(Korea). It was performed by cytogenetic analysis on metaphase-cells of hESCs-MSCs at passages 13.

Animal preparation

All animal study protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital, Korea. The 7weeks old Fischer F344 inbred (orient bio inc, Korea) rats were bought. All rats were given free access to water and standard chow diet throughout the study, and were maintained in a temperature- and humidity-controlled room with a 12-h light/dark cycle. All rats should have about 1 week adaptation period before the study was started.

Surgical procedure for acute Myocardial Infarction(MI) model

At first, The 8 weeks old, 180~220g, Fischer F344 inbred rat(orient bio inc, Korea)were anesthetized using isoflurane(JW Pharmaceutical Co, Korea). to be shaved in chest. After shaving, he rats were anesthetized again using an intraperitoneal injection of 10mg/kg body weight zoletil(Virbac, USA). The rats were then ventilated mechanically with oxygen, and intubated with an 18-gauge cannula(BD Angiocath™, USA) by using a small animal ventilator (Harvard Apparatus, USA) at a rate of 60 - 70 breaths/min and a tidal volume of 2 - 3 mL. The heart was exposed by a left thoracotomy. The pericardium was resected

carefully avoiding damage to the heart, and the left anterior descending (LAD) artery was ligated permanently at 2 - 3 mm from the left atrium, using a 6 - 0 silk suture(AILEE co, Korea). When MI model was made completely, the MI zone was showed the presence of a clearly demarcated from tied point to apex. The movement on LV is also abnormal. After checking it, the chest and skin were closed using 4 - 0 silk sutures(AILEE co, Korea).

hESCs-MSCs transplantation for Injection in MI model

hESCs-MSCs cultured for 3~4days to inject MI model. Several trial of this experiment was cultured with CellTracker CM-DiI Dye(C-7000, Molecular Probes®, USA) for detecting MSC cell after sacrifice. 3 days after MI induction, Left thoracotomy in MI model was performed again to inject hESCs-MSCs. It anesthetized as described above. A total of 1.0 × 10^5 MSCs in 70 μL PBS(n=10) and only PBS(n=10), were injected into two site of Periinfarct area, using insulin syringes. After injection, the rats were followed up by using echocardiography until 8weeks.

Echocardiography

Echocardiography was performed on 4 times (baseline point(-3days), MI induction checking point(3days) and therapeutic efficiency check points (4weeks, 8weeks)). After anesthetization by isoflurane, the left-ventricular end-diastolic dimension (LVEDD), left-ventricular end-systolic dimension (LVESD) of rat was measured by a 9-MHz transducer connected to a Toshiba echocardiography machine (Nemio, Toshiba Co, Japan). Ejection Fraction (EF) was calculated using the following equation: EF = 100 × ([LVEDD^2 - LVESD^2] / LVEDD^2). All measurements were recorded over three times and the mean was calculated. A single operator who was blinded to the study groups performed all measurements.

Histological data

After the measurement of echocardiography at 8weeks, the heart in rats was excised. The extracted heart was perfused with PBS via coronary. And then, it fixed with 10% formalin at least 3days in room temperature. Before the fixed heart tissue was embedded in paraffin and made a block, it was cut approximately 2 mm under from ligation point. Using microtome, the paraffin block was sectioned into 4㎛ thickness for progressing histological

experiments. The paraffin section was stained with Masson's trichrome to evaluate infarct area and wall thickness in heart. For evaluating infarct area, the stained slides were scanned by scanner to get image. Then, the images were analyzed by SABIA(METOOSOFT, Korea). This software can make count percentage ratio collagen deposit part and normal tissue part. This software can represent a ratio of the normal part to the damage part in the heart tissue as a percentage. Wall thickness was used with an optical microscope to get specific damaged part of heart tissue. By using LAS 3.8(Leica, Germany), wall thickness was used with optical microscope to take image about specific damaged part of heart tissue. Wall thickness was measured 5 parts in collagen deposit area, and these measurements made average and were compared each group.

Immunofluorescence

Immunofluorescence was performed with conjugated primary antibodies to identify graft cells and how resident cell was affected by hESCs-MSCs. The paraffin heart sections were deparaffinized and dehydrated by using xylene(JUNSEI, Japan) and various concentration of absolute alcohol(HAYMAN, UK). After treating antigen retrieval to slide, it was incubated with blocking solution to cut off non-specific binding sites for 3hours. Before incubating fluorescence conjugated secondary antibody,

each slide was treated with primary antibodies against c-kit(FITC; Abcam, USA), WT1(Abcam, USA) and counterstained with 1 μg/mL DAPI as known cardiac stem cell marker. To take immunofluorescence image of all sections, the confocal microscope (Carl Zeiss, Germany) was used.

Statistical analysis

Analysis was performed by GraphPad Prism 6(GraphPad Software, USA). Probability value of <0.05 was considered statistically significant. Data are presented as mean ± standard deviation. The significant differences between the groups were analyzed by the unpaired t-test analysis of variance.

Result

New developmental method of clinical grade ESC-MSCs

The derivation protocol of MSCs from hESCs was established by several steps[2]. Following the figure1a, hVTN is a good coating material to culture clinical grade ESC, and it was formed enough packed colonies when it was ready to induce mesenchymal stem cell.(Figure 1) ESCs were dissociated through emzymatic methods, and it was cultured by suspension to make well around shaped embryonic body (EB) during 2days. EB were re-attached in CELLstart coating dish and it could come out cells for 8days Finally, outgrowing cells-EB complex was moved to 75T flask, and then outgrowth cells in a flask were begun to have characteristics of Mesenchymal lineage. After several times of subculture, it could be mesenchymal stem cell.(Figure 2) Well-differentiated mesenchymal stem cells had a jagged morphology and finger print pattern. mesenchymal stem cell line from clinical grade ESCs could proliferate well. (Figure 3 A,B)

These stable cells, could called hESC-MSCs, were expanded periodically and maintained proliferative capacity until 25th passages. Also, with G-band staining, hESCs-MSCs were shown normal karyotype at 13 passage that cell transplantation was used in MI model.(Figure 5 A)

Characterization of clinical grade ESCs-MSCs

After derivation, in FACS analysis, well known MSCs positive markers, CD44, CD73, CD90, CD29, CD105, were expressed on the surface of hESC-MSCs. However CD34, CD45 were not detected.(Figure 4) Moreover, expressions of HLA-DR and DQ, which are immunetolerant markers, were not detected on the surface of hESC-MSCs.(Figure 5 B) These data were proved that hESC-MSC were differentiated completely and had no immunological problem for applying stem cell therapy[5,7,8].

To ensure that MSCs could differentiate into adipocyte, chondrocyte and osteocyte, MSCs were cultured into each lineage specific culture conditions. After 21days of culture for inducing adipocyte, with oil red O staining, fat granules that were stained red and proved of adipocyte were detected by using optical microscope(Olympus, Japan). Also, chondrocyte were cultured to induce with suspension culture method from hESC-MCSs during 21days. After that, aggrecan, which is specific marker of chondrocyte, was detected in differentiated MSCs by immunofluorescence because hESCs-MSCs were induced completely to chondrogenic lineage[16,20,21].

At last, to make osteocyte, MSCs were cultured in osteogenic induced media for 21day, and it was checked by osteocalcin staining that is a maker for osteocyte through immunofluorescence. (Figure 4) Our data was proved hESC-MSC was well

differentiated through FACS, differentiated assay and various trials[22].

Echocardiography analysis

At baseline, two groups which are PBS group and hESC-MSCs treated group, left Ventricular End Diastolic Diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were shown a similar pattern until after 3days from MI induction. The reason that all groups were evaluated at 3days time point through echocardiography was confirmed whether the model was induced MI successfully. Therefore, ejection fraction of all group was decreased 20% as a rapid inclination percent because rats were induced MI properly[14]. (Figure 6)

After confirming, clinical hES-MCSc was transplanted periinfarct site into heart. Ejection Fraction of PBS Group was decreased sharply at 8weeks. On the other hand, Cell transplantation Group was shown slowly decreased pattern compared to PBS Group. On the other side of analysis, in delta value, control group that was not treated anything had small change, but PBS group had much lower value than cell transplantation group. It means that PBS group, compared to cell treated group, had severe loss of function in MI induced heart. (Figure 6 B, C)

In summary, through echocardiography, these results was proved

that rats induced MI were gotten minimal damage by injecting hESC-MSC, so hESC-MSC could be potential candidate material of stem cell therapy[14,19,22].

Wall Thickness, Fibrosis Area, Immunofluorescence

As time goes, the infarct zone of heart of MI model becomes thinner and wider. Wall thickness of infarct zone and fibrosis area of heart affects systolic motion in heart; contraction movement was important indicator to check function of heart, which is ejection fraction, fractional shortening.

By utilizing heart section slides with MT stained, we could evaluate histological function of heart. (Figure 7A) To evaluate wall thickness of infarction sites, five sides of infarct zone on heart section was measured equally; it was indicator evaluated average thickness of infarct zone between groups. By histological analysis program, wall thickness of infarct zone on cell treated group was much thicker than PBS group. With the software(metoosoft, Korea), section of heart can be analyzed in accordance with the color difference.(Figure 7 B, C)

Measured values, the percentage of fibrosis area against normal tissue, were obtained. The percentage of fibrosis area in PBS group is much higher than hESCs-MSCs treated group. These

results means that cell injected group had better heart function than PBS group histologically. To determine how hESC-MSCs affect positively in MI model, immunostaining was performed. Through immunofluorescence costaining, we could get a clue how hESCs-MSC worked in the MI heart.

As determined by staining with c-kit and WT1, cardiac stem cell marker, transplantation of clinical hESC-MSCs contaning DiI dye resulted as paracrine effect at border zones of the MI heart. C-kit and WT1 positive cells which were considered as cardiac stem cell were located nearby MSC with DiI at the border zone in heart tissue[23].(Figure 8)

Discussion

In this study, we clearly demonstrated that clinical grade hESC-MSC was induced through short EB stage, our own potential protocol to make a MCS is easy and it is also required economically short term till MSC derivation from ESC.

In the previous reports, various MSC derivation methods have been exposed many problems. One of methods is that specific genetic factors, which could induce mesechymal stem cell, were transfected to ESC or ASC. Transfecting factors method is available to establish mesenchymal stem cell, but it could be called GMO(Genetically Modified Organism) product. Another is a method making high purity of MSC population by using FACS or MACS sorting systems. Sorting MSC is also efficient and high purity. However, it could be damaged to MSC. For that reasons, our protocol, compared these above methods, were proved that needs not to use mechanical selection and separation to establish MSC. In typical way for growing MSCs, feeder cells were needed. Xeno or allo feeder cells were usually used to culture MSCs. However, having to make it grow MSC, both xeno feeder cell and allo feeder cell have involved pathogenic risks[2.3.6]. Nevertheless, our protocol to culture MSC used ECM instead of feeder cells, so our clinical grade ESC-MSC has risks of pathogen hardly.

Above our data about in vivo, transplantation with this cell in a rat model of MI dose not deteriorate anymore[24]. One of trial points in our experiment; we used inbred strain (F344) for making MI, not used outbred. The reason why we used inbred stain instead of outbred is that we wanted to reduce critically variation of each other. Outbred strain has various heart sizes, health condition and body weight. Moreover, using this animal couldn't represent efficient therapeutic effect exactly. In contrast, animal experiment with inbred strain could be minimalized variation among animals, and we could get more exact data from experiment[14,25,26].

Histologically, as time passes, in a rat model of MI model, wall thickness of heart was being thinner and fibrosis area also was becoming larger. In contrast, wall thickness and fibrosis area of group with cell transplantation don't be made worse. Our data showed that cell treated group was not more serious state, compared to PBS group, rather it seemed hESC-MSC has therapeutic efficacy in heart by treating to MI model. Ejection Fraction is one of indicators to analyze heart function by echocardiography. After inducing MI, naturally heart function was being worse. Thus, compared normal condition, ejection fraction could be reduced dramatically from baseline because of inducing MI. Even though ejection fraction of cell treatment group is decreased slowly from induction time point to 8 weeks, the degree of reduction, compared to PBS, is very small. By analyzing immunofluorescence to heart tissue section, cardiac stem cells were distributed significantly around MSC at infarct

area. The exact mechanism in in vivo is not clear , but we suggested hESC-MSCs have positive therapeutic efficacy [23].

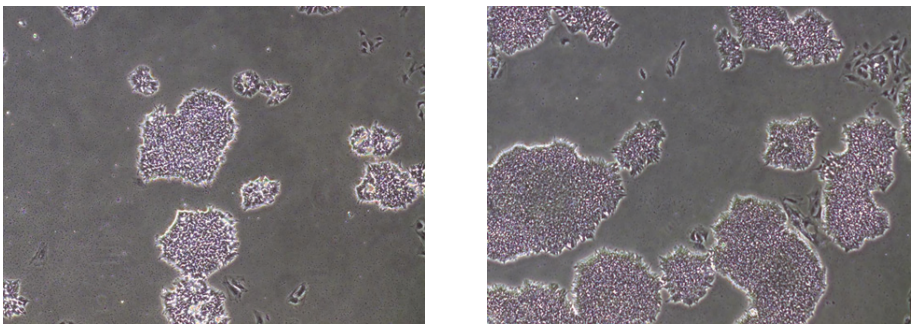
In this study, It is demonstrated we expected clinical embryonic stem cell derived mesenchymal stem cell without any xeno factors by working in cGMP could make cells for clinical trial stably and be the potential candidate for stem cell therapy in heart disease, and hESCs-MSCs in in vivo could affect to another resident cell with emitting growth factor as paracrine effects.

Conclusion

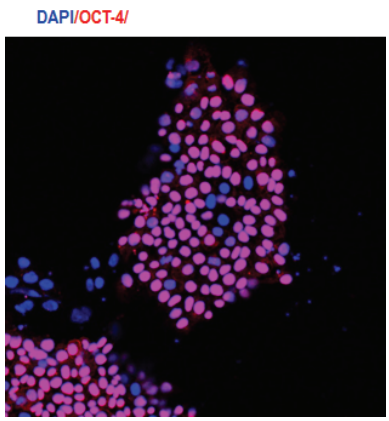
We build up a efficient method of making MSCs from hESC in cGMP facility. The mesenchymal stem cell surface markers are expressed well. and cell surface markers of immune reaction not detected. Moreover, with various experiments, hESC-MSCs were prove safety. we also checked that our hESC-MSC has thrapuetic efficacy though a rat model of MI. Thus, because our protocol overcame limitation of other methods to induce MSCs from ESCs, it would be a good standard for establishing MSCs from ESCs.

Figure

A.



B.



C.

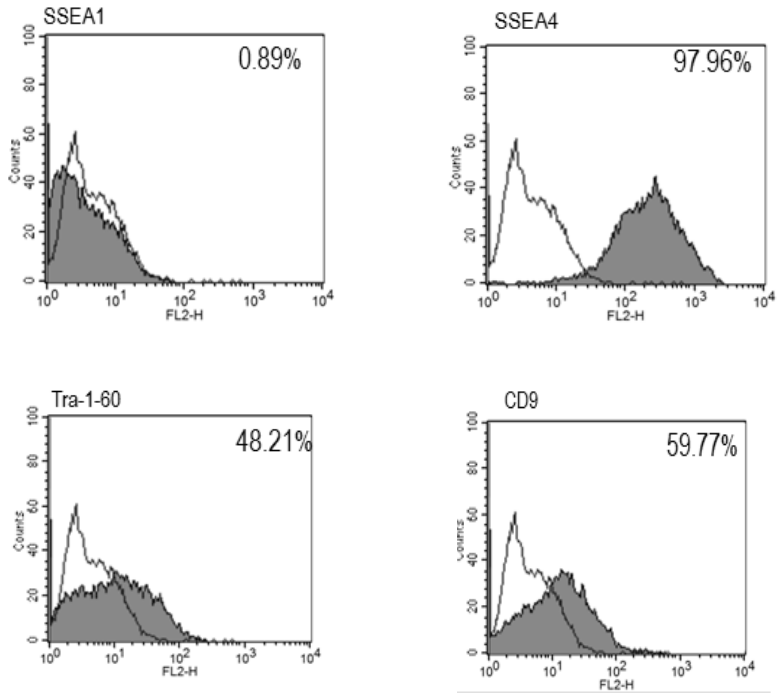


Figure 1. Culture and maintainance of embryonic stem cell with non xeno factors.

(A) The morphology of undifferentiated embryonic stem cell with non xeno factors; right side is 30% confluency and left side is 70% confluency. ESCs were observed by phase-contrast microscope. (B) Via Immunofluorescence, confirmation about pluripotency of ESC staining against Oct4. (C) Flow cytometry analysis of ESCs. Cells were trypsinized and stained with specific markers for hESCs

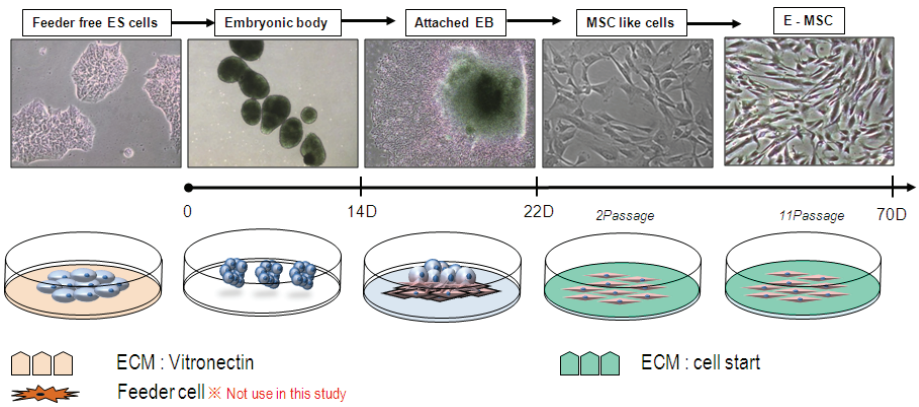


Figure 2. Clinical grade hESCs-MSCs derivation.

Well grown hESCs were harvested to form EB. After 2days of EB formation, EB was attached and cultured for 8 days. outgrowth expanded EB was induced mesenchymal lineage differentiation. Completed hESCs derived MSCs (hESCs-MSCs) was started culture

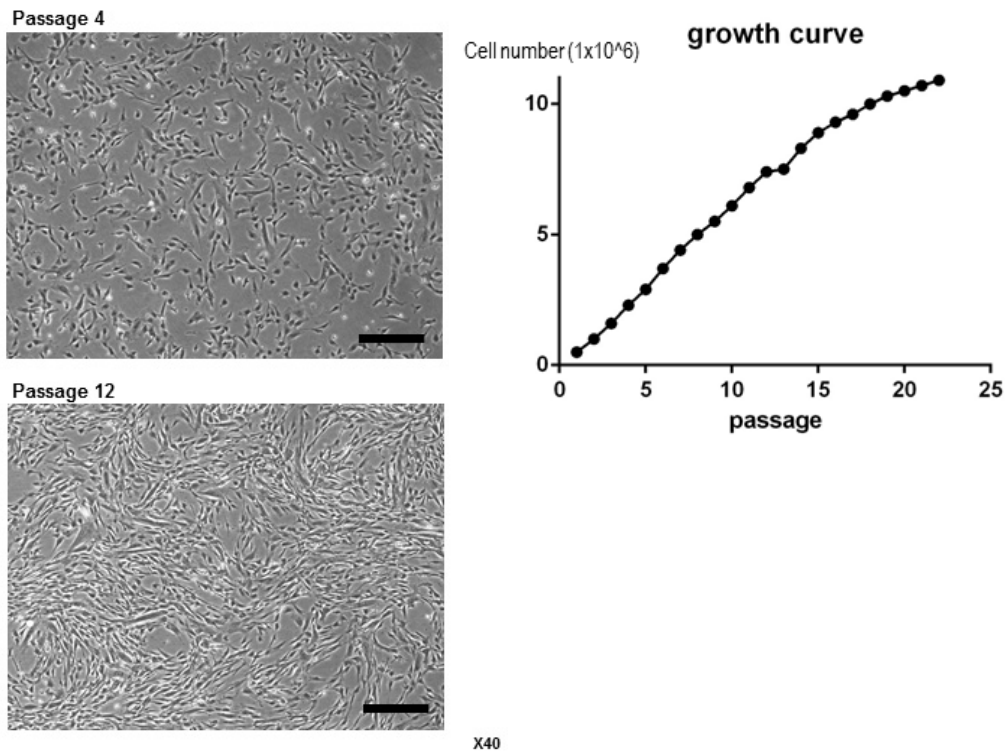
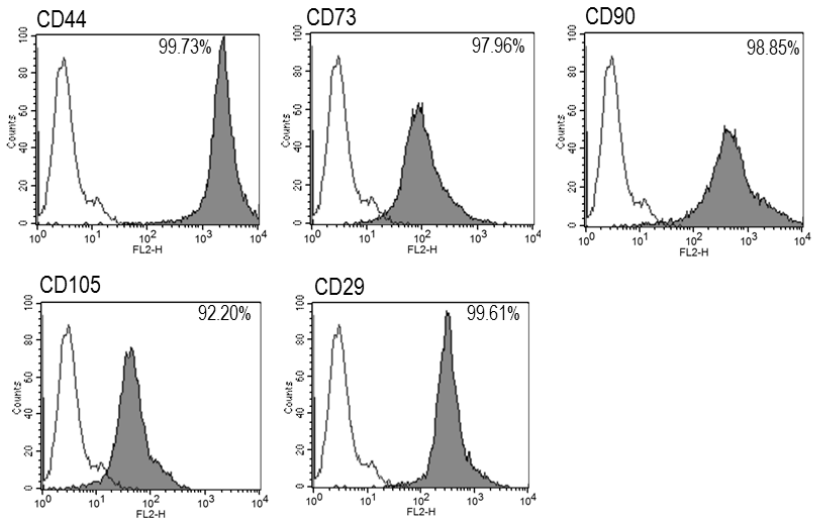


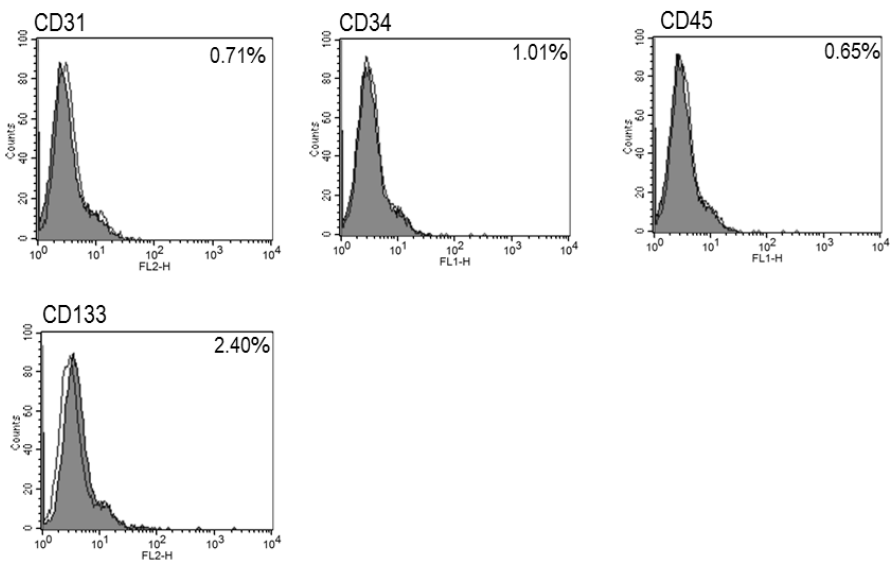
Figure 3. Morphology and growth curve of hESCs-MSCs.

(A) Morphology of hESCs-MSCs at confluency under optical microscopy have fingerprints-like pattern. (B) hESCs-MSCs subculured every 3~4 days

- **MSC positive marker**



- **MSC negative marker**



• **ESC positive marker**

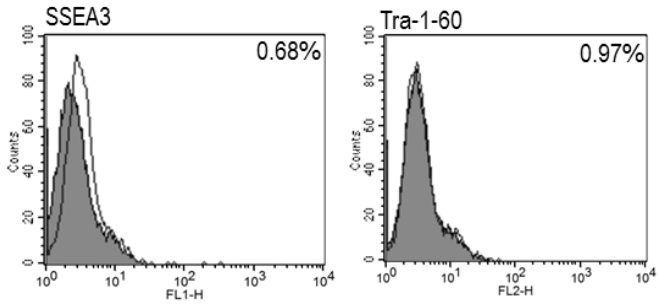


Figure 4. Flow cytometry analysis of hESCs-MSCs.

After expension at passage 11th of hESCs-MSCs, cells were stained with specific various markers for MSC, ESC and other lineages

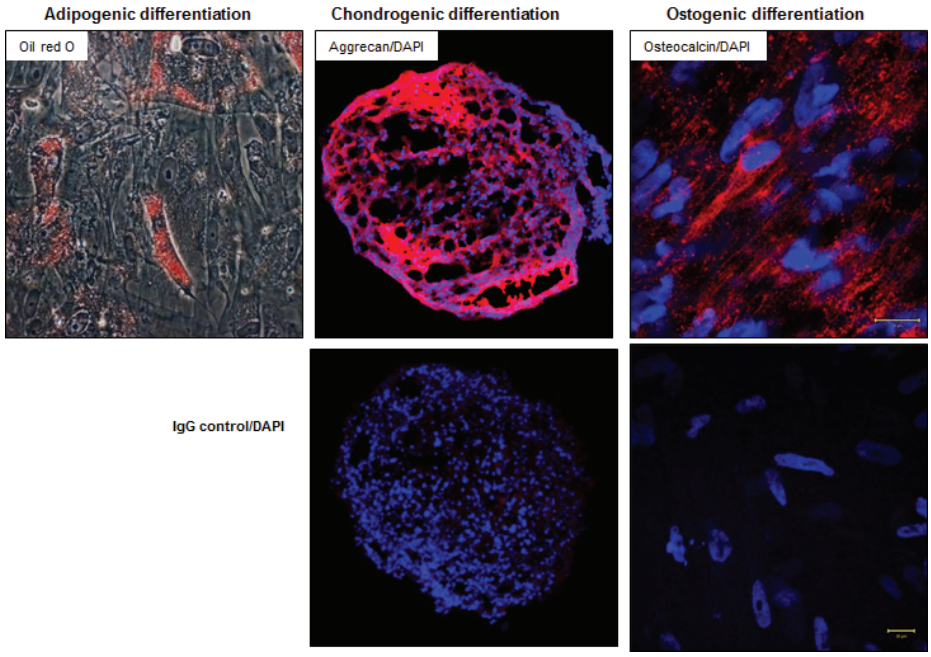
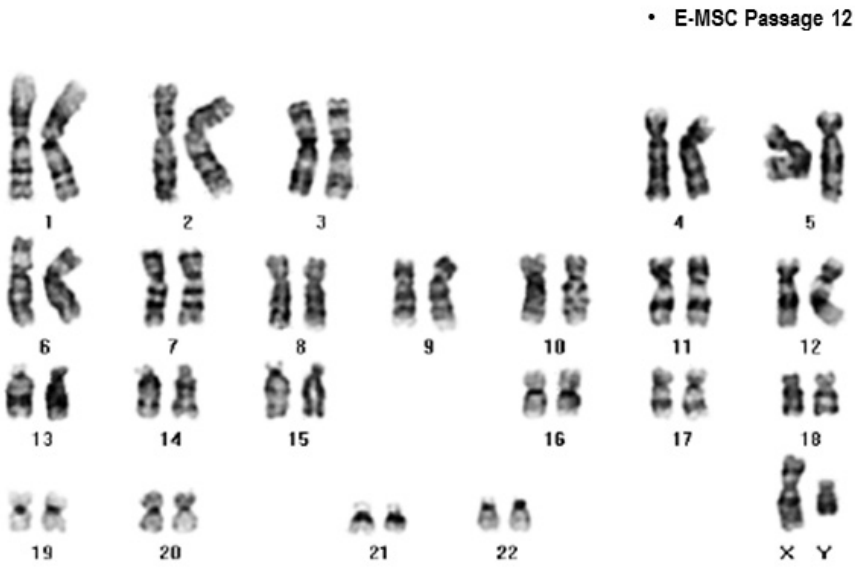


Figure 5. Functional differentiation of hESCs-MSCs

hESCs-MSCs differentiated into adipocyte, chondrocute, osteocyte under each differentiated conditions. Adipocytes was stained with Oil-red-O. Chondrocyte and osteocyte was performed against aggrecan and osteocalcin by immunofluorescence

A.



B.

• **Expression of human leukocyte antigen**

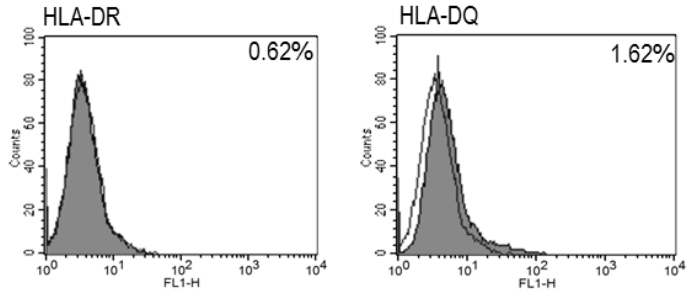
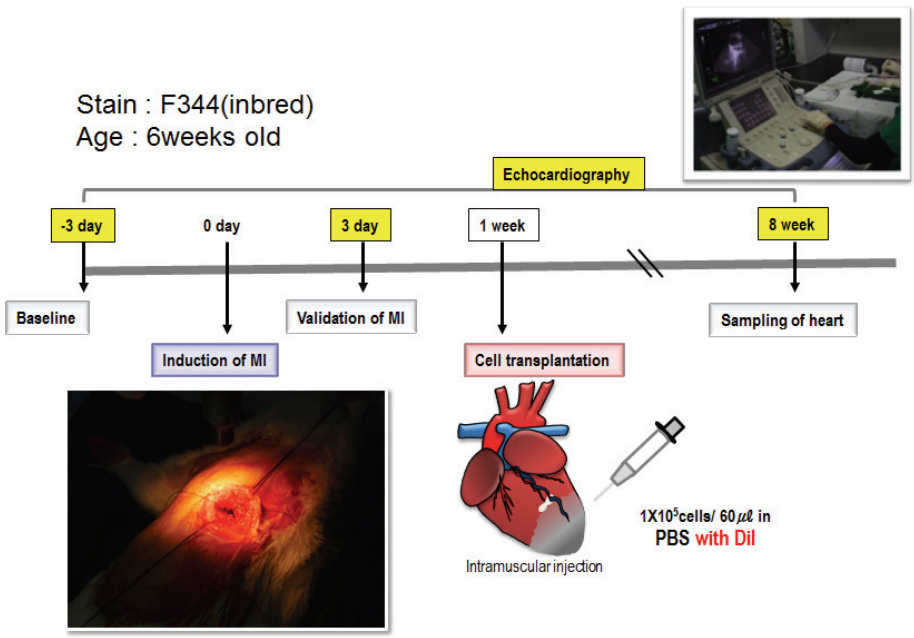


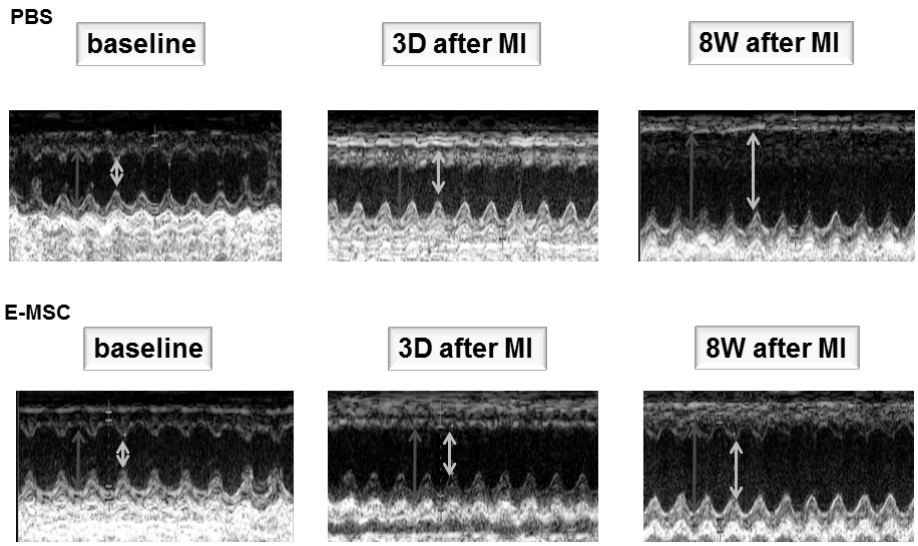
Figure 6. Biosafety of hESCs-MSCs for therapeutic application

(A) hESCs-MSCs after prolonged culture in expansion condition were karyotyped by G-band staining (B) Typical immune reaction markers, HLA-DR, HLA-DQ were not detected with FACS analysis

A.


Stain : F344(inbred)
Age : 6weeks old
Echocardiography
-3 day
0 day
3 day
1 week
8 week
Baseline
Induction of MI
Validation of MI
Cell transplantation
Sampling of heart
Intramuscular injection
1X10⁵cells/ 60㎕ in
PBS with Dil

B.


PBS
baseline
3D after MI
8W after MI
E-MSC
baseline
3D after MI
8W after MI

C.

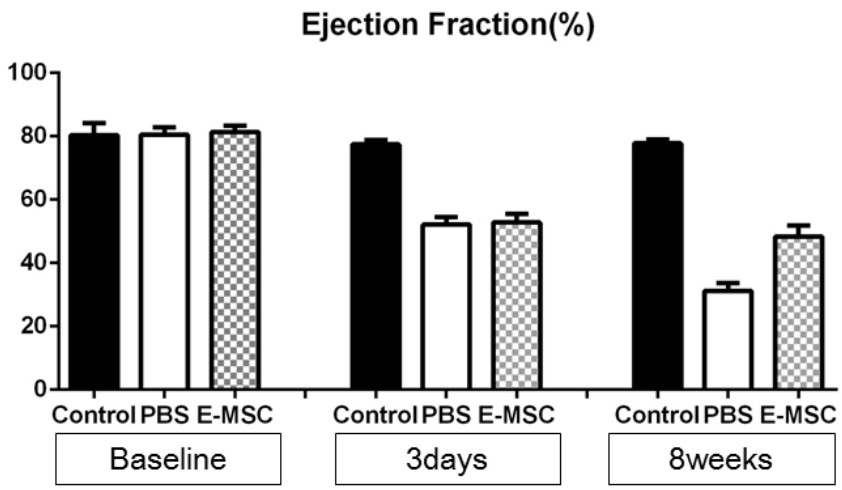
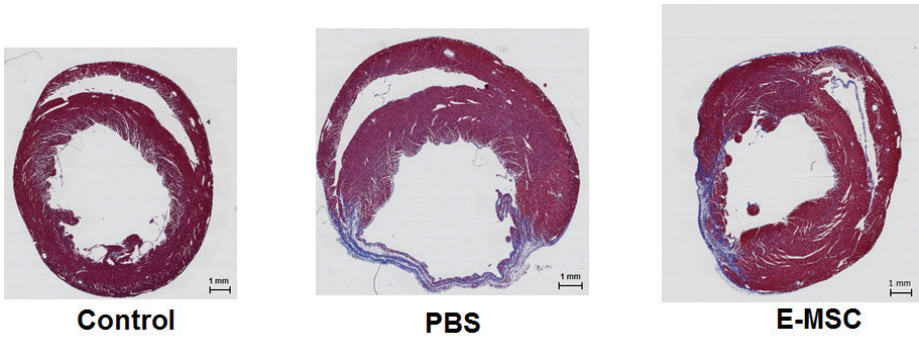

Ejection Fraction(%)
100
80
60
40
20
0
Control PBS E-MSC
Control PBS E-MSC
Control PBS E-MSC
Baseline
3days
8weeks

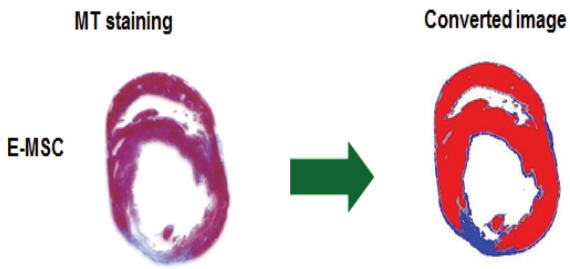
Figure 7. Animal experiment with a rat model of Myocardial infarction for evaluating therapeutic efficacy of hESCs-MSCs

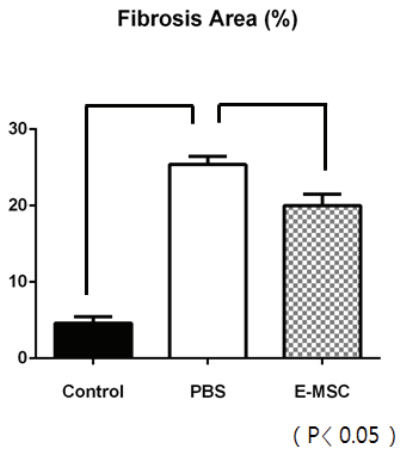
(A) scheme of animal experiment in this study. Inbred rats(F344) were used, and echocaridography was performed as indicator evaluating therapeutic efficacy (B) M-mode of echocardiography was measured to evaluate therapeutic efficacy of hESCs-MSCs (C) Ejection fraction analysis of animal experiment

A.


Control
PBS
E-MSC

B.


MT staining
Converted image
E-MSC


Fibrosis Area (%)
30
20
10
0
Control
PBS
E-MSC
(P< 0.05)

C.

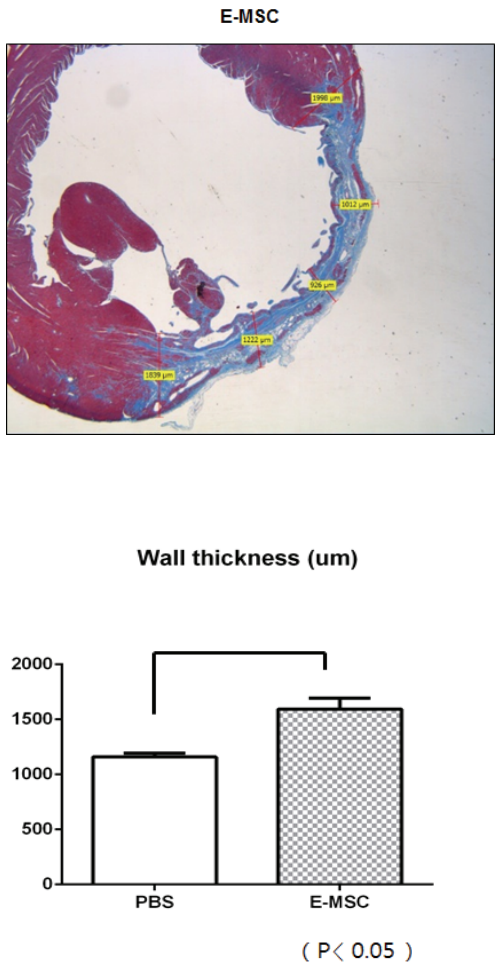
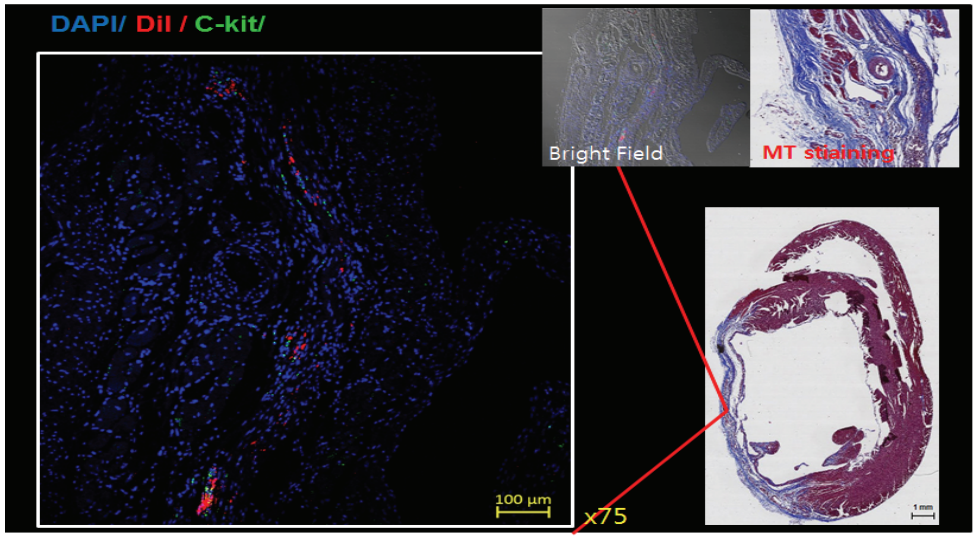


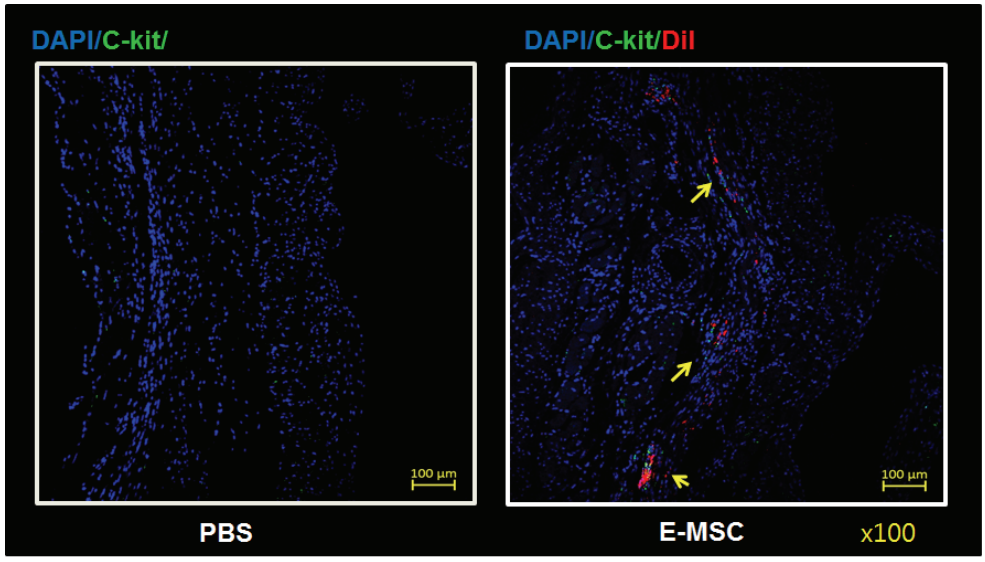
Figure 8. Histological analysis of MI heart in animal experiment via masson's trichrome(MT) staining

(A) Heart section with MT staining in animal experiment. (B) Measurement of Fibrosis area through color difference. (C) Measurement of wall thickness using software tool

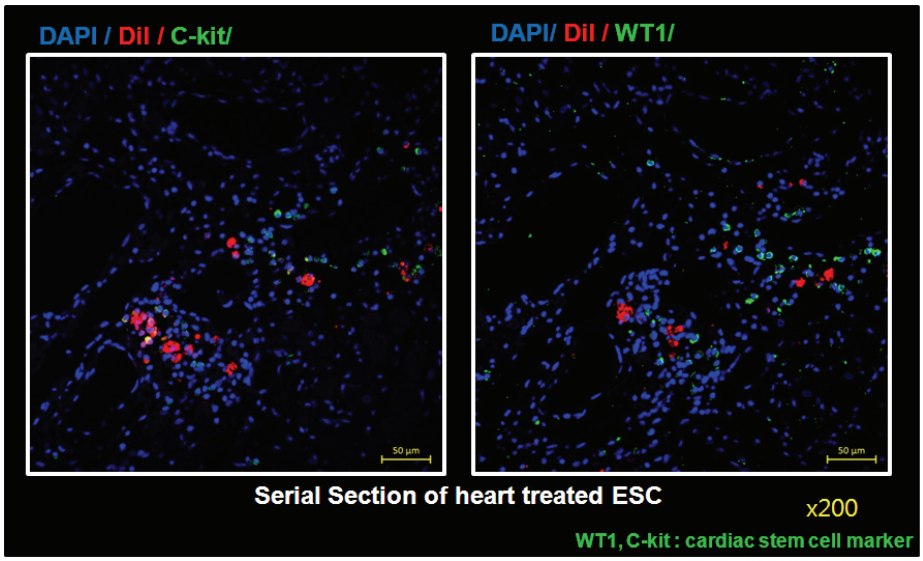
A.


DAPI/ DiI / C-kit/
Bright Field
MT staining
100 μm
x75
1 mm

B.


DAPI/C-kit/
DAPI/C-kit/DiI
100 μm
100 μm
PBS
E-MSC
x100

C.


DAPI / DiI / C-kit/
DAPI/ DiI / WT1/
50 μm
50 μm
Serial Section of heart treated ESC
x200
WT1, C-kit : cardiac stem cell marker

D.

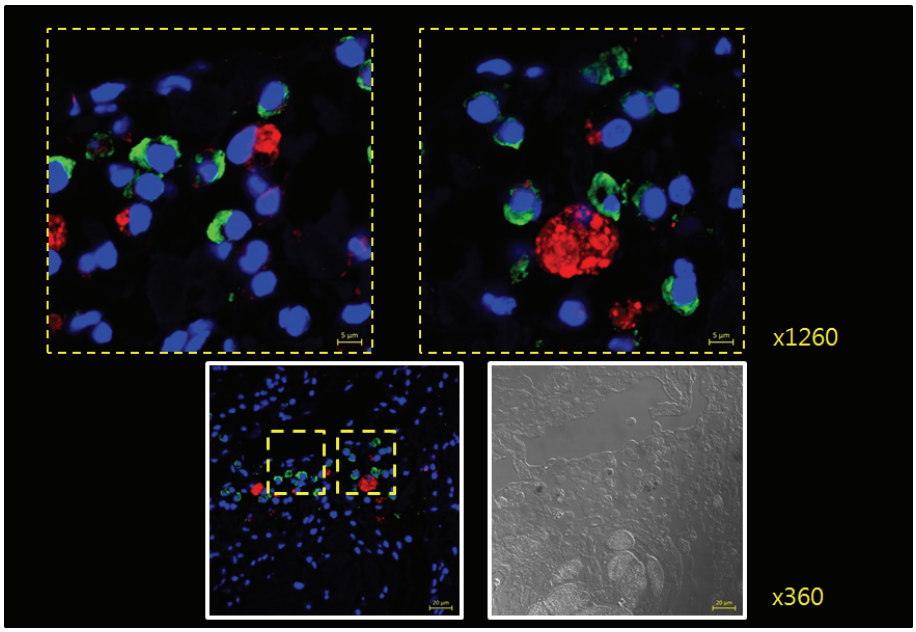

x1260
x360

Figure 9. Immunofluorescence analysis of heart section in animal experiment

(A) Injected hESCs-MSCs were located in border zone of MI heart (B) Each groups was performed staining with C-kit antibody via immunofluorescence (C) C-kit and WT-1 antibodies, known as cardiac stem cell markers, was stained Using serial section of same hESCs-MSCs treated group. (D) High magnification view of E-MSC treated heart staining with c-kit

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국문초록

심근경색 동물 모델에 치료효과가 있는 임상 등급 배아줄기세포 유래 중간엽 줄기세포 생산

서론

인간 배아 줄기 세포 (hESC)는 세포 치료법에서 큰 잠재력을 가지고 있다. 그러나, 환자에게 인간 배아 줄기 세포 자체를 투여하기에는 세포 자체의 발암원성과 면역 거부의 위험을 가지고 있다. 이러한 위험을 극복하기 위해, 일반적으로 인간 배아 줄기세포는 다양한 계통으로 분화된 세포를 세포치료법에 적용한다. 그 중 하나인 인간 배아 줄기 세포에서 유도 된 중간 엽 줄기 세포는 자가 분열이 가능하고 다양한 타입의 세포로도 분화가 가능한 세포이다.

여기, 우리는 cGMP 시설을 사용하여 배아 줄기 세포 유래 중간엽 줄기 세포를 유도 및 배양하는 효과적인 제작 방법을 제안한다. cGMP 시설은 배아 줄기 세포 유래 중간엽 줄기 세포가 무균 상태에서 배양되는 것을 증명할 수 있는 시설이다. 또한, 균일한 세포주 생산을 위해, 우리는 배아 줄기 세포 유래 중간엽 줄기 세포 제작방법을 문서화 와 표준화를 진행하였다. 우리가 유도한 배아 줄기 세포 유래 중간엽 줄기 세포는 중간엽 줄기세포 양성 마커인 CD44, CD73, CD90, CD29, CD105 발현되는 것을 확인했고, 중간엽 줄기

세포 음성 마커인, CD34, CD45가 발현되지 않는 것을 확인했다. 또한, 우리는 생체 내에서의 배아 줄기 세포 유래 중간엽 줄기 세포의 치료 효과를 연구 하였다. 개체간의 차이를 줄이기 위하여, 근교계통 랫드(F344)를 사용하여 심근 경색(MI) 모델을 유도하였고, MI 모델 심장의 경변 주위 지역에 세포를 주입하였다. 다양한 분석을 통해, 우리는 MI 모델에서 세포 이식 그룹의 심장 기능이 PBS 그룹보다 더 나은 심장 기능을 가지고 있다는 것을 보여주었다.

결론적으로, cGMP 시설에서 우리의 제작방법을 이용하여 얻은 배아 줄기 세포 유래 중간엽 줄기 세포는 다른 유도 방식의 단점을 극복할 수 있고 높은 생산율을 가진다. 또한, 우리의 제작방법은 쉽고 간단하다. 심근 경색 모델 동물 실험을 통해, 우리는 배아 줄기 세포 유래 중간엽 줄기 세포가 임상 시험을 적용하기에 적합하다고 사료된다.

키워드 : 인간 배아 줄기 세포, 중간엽 줄기 세포, 분화, 심근 경색

학번 : 2013-24024

이학석사 학위논문

Clinical grade production of Mesenchymal Stem Cells derived from Embryonic Stem Cells (ESC-MSC) having Therapy Effect to a Rat Model of Myocardial Infarction

심근경색 동물 모델에 치료효과가 있는 임상 등급 배아줄기세포 유래 중간엽 줄기세포 생산

2015년 8월

서울대학교 대학원

분자의학 및 바이오제약전공

김근천

Abstract

Clinical grade production of Mesenchymal Stem Cells derived from Embryonic Stem Cells (ESC-MSC) having Therapy Effect to a Rat Model of Myocardial Infarction

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Backgroud

Human embryonic stem cell (hESC) have great potentiality to stem cell therapy. However, treating embryonic stem cell itself to patient has the possibility of suffering harm because of tumorigenicity and immune rejection. In order to overcome these risks, hESCs were induced to other lineage usually for applying stem cell therapy to human. Mesenchymal Stem Cells induced

from ESC (hESC-MSCs) have the capability to self-renew and differentiate into various types of cell.

Methods and results

Working in cGMP facility was proved that hESC-MSC is culturing at aseptic condition. An efficient method of building up hESC-MSCs without any xeno factors during culture in cGMP facility was developed. we confirmed hESC with non xeno factor was growth well. Then via EB stage, MSCs was differentiated completely form ESC. Moreover, we documented and standardized the derivation and culture of MSCs for establishing stable cell line. we culture hESC on extracellular matrix instead of feeder cell and serum free media was used without xeno factors Our derived MSCs from ESCs were expressed CD44, CD73, CD90, CD29 and CD105 as MSCs' positive markers and not expressed MSCs' negative markers, CD34 and CD45.

We also studied therapeutic efficacy of hESC-MSCs in vivo. To reduce difference between entities, we use inbred rat (F344) and induce a rat model of Myocardial Infarction (MI). We injected cell to MI model at periinfarct zone. Through various analyses, we demonstrated that heart function of cell transplantation group in MI model was better condition than group with PBS.

Conclusion

our established method induced MSCs from ESCs in cGMP facility could overcome limitation of other derivation methods and get high purity of yield. Moreover, our method is easy, simple.

With animal experiment, we suggested hESC-MSCs is suitable to apply clinical trial about MI.

Keywords: human Embryonic Stem Cell, Mesenchymal Stem Cell, Differentiation, Myocardial Infarction

Student Number: 2013-24024

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Introduction

Recently, many people are endeavoring to apply cell therapy using stem cell for various diseases which is called stem cell therapy[1]. One of merits about stem cell therapy is unique properties of stem cells itself. Unlike other type of cells, stem cells have ability to self-renewal and it can induce any other mature cells. For this reason, stem cells would be a potential therapeutic substance for various human diseases.

Among stem cell therapy field, these days, mesenchymal stem cells (MSCs) especially has received a great popularity because of the several positive characteristics of MSCs[2]. One of characteristics is that MSCs, which come from another person, do not have immune reaction to recipients, and also MSCs have ability to proliferate like any other stem cell[3]. Nevertheless, using stem cell therapy to human has some limitations yet[4]. One is facility where the stem cell is culturing. Culturing cells should be on clean condition. However, generally, no one certify clean degree in normal facilities. Patient does not want to take therapy by using stem cells that is cultured on poor condition. Therefore, people have developed good manufacturing practice which is called GMP. GMP was documented all working procedure of making cell and aseptic condition. That is, culturing cells from GMP is guaranteed that cell is cultured aseptic condition by standardized procedures and facilities. another one is

xeno factors necessary to culture stem cell. Usually, feeder cells and media containing important nutrition from other species such as fetal bovine serum are needed to culture stem cell. Until now, these factors are utilized commonly to stem cell therapy, but it is not proved safety of stem cell from xeno factor. Developing new material instead of xeno factors has been actively studied[5-8]. Actually, serum free media and extra cellular matrix are used instead of FBS and feeder cell. Animal experiment was performed to evaluate the therapeutic efficacy of MSC culturing with non xeno factors by using a rat model of myocardial infarction.

Our study demonstrate we established the protocol to make embryonic stem cell(ESCs) derived MSCs(E-MSCs) through short embryoid body(EB) stage by using clinical grade human ESCs with non xeno factor[7,9-11]. And we did all experiment about culturing MSC in cGMP grade facility. Working in cGMP facility with standardized procedures of making hESCs-MSCs is guaranteed to aseptic condition to establish hESCs-MSCs[12,13]. In summary, clinical hESCs derived MSCs without any xeno factors by working in cGMP is the potential candidate for stem cell therapy in heart disease[14].

Material and Method

Clinical grade hESCs culture with non-feeder material

Clinical grade human embryonic stem cells(hESCs) (Lifemaps, Inc., USA) were cultivated on vitronectin (GIBCO, USA) coated dish. hESCs were maintained with serun free media, essential 8^{th} medium (GIBCO, USA) comprised of DMEM-F12 supplemented with 8 supplements. hESCs were cultured at 37℃ in 5% CO2 at 95% humidity and manually passaged every 5 days. Growth medium was changed every day.

Induction of clinical grade hESCs derived MSCs

Through the Embryonic body (EB) stage, hESCs were induced mesenchymal lineage differentiation, undifferentiated hESCs were harvested gently with 0.5M EDTA (invitrogen, USA) at 37℃ for 5 min and dissociated into a suspension of cell. Harvested cells were cultured in 60mm dish with essential 6th medium (GIBCO, USA), which is not containing xeno factors, for 14day to form EB. Culture media was changed every day. After 14days of forming EBs, the EBs, almost well-rounded shape, were selected

and attached with CTS CELLstart (GIBCO, USA) coated 30mm dish with StemPro® MSC SFM medium(A10332-01, GIBCO, USA). The culture media was changed every day during 8days. The outgrowth expended EBs were dissociated with TrypLE (GIBCO, USA) at 8days of culturing in 30mm dish, and it was seeded at CTSTM CELLstartTM coating 75T flask with StemPro® MSC SFM medium. The growth media, StemPro® MSC SFM medium, was changed every day. Completed hESCs derived MSCs (hESCs-MSCs) was started culture per 1x10^6 in one dish at every passage.

Flow cytometry analysis

To check the characterization of hESCs at passage 35 and hESCs-MSCs at passage13, the approximate 5×10^5 cells were washed with DPBS and stained with following these conjugated antibodies, CD44(Phycoerythrin (PE); BD Biosciences, USA), CD73(PE; BD Biosciences, USA), CD90(PE; BD Biosciences, USA), CD29(PE; BD Biosciences, USA), CD105(PE; BIO-RAD, USA), CD34, CD45(PE; DAKO, Denmark), TRA-1-60(PE; BD Biosciences, USA), SSEA1(PE; BD Biosciences, USA), SSEA3(Fluorescein isothiocyanate(FITC); BD Biosciences, USA). For evaluating characteristics of immune about hESCs-MSCs, immunogenic-related surface marker antibodies; human leukocyte antigen HLA-DR (FITC; BD Biosciences, USA), HLA-DQ (FITC;

BD Biosciences, USA) were used at 4℃ for 15 min, and as same protocol above. All cells were analyzed using a flow cytometer (FACS calibur; BD Biosciences, USA). and software(CellQuest; BD Biosciences, USA)

Differentiation analysis

Lineages differentiation of hESC-MCSs toward osteogenesis, adipogenesis and chondrogenesis were perform by using Human Mesenchymal Stem Cell Functional Identification Kit(SC006, R&D systems, USA). According to the manufacturer's protocol, chondrogenic differentiation capacity of MSCs was evaluated that hESC-MSCs were cultured with chondrogenic differentiation media containing D-MEM/F-12 99%, ITS supplement 1%, Penicillin100 U/mL, Streptomycin100 μg/mL and L-Glutamine 2 mM on 15ml cornical tube for 21 days. Differentiated chondrocyte from hESC-MSCs were analyzed through immunofluorescence by using primary antibody against aggrecan. For osteogenic differentiation, hESC-MSCs were incubated with differentiation media, a-MEM 99%, Fetal Bovine Serum 10%, Penicillin100 U/mL, Streptomycin100 μg/mL, L-Glutamine2 mM and osteogenesis inducible supplements for 21days. Osteogenic differentiation capacity of MSCs was performed through immunofluorescence, and the antibody against osteocalcin was used for evaluating osteogenic differentiation. The confocal

microscope (Carl Zeiss LSM710, Germany) was used to evaluate these lineages differentiation. Lastly, adipogenesis from hESC-MSCs were cultured with differentiation media, a-MEM 99%, Fetal Bovine Serum 10%, Penicillin100 U/mL, Streptomycin100 μg/mL, L-Glutamine2 mM and adipogenesis inducible supplements, for 21days. The oil red O was added during 5min in culture media before it was evaluated with the optical microscope(Olympus, Japan).

Growth curve assay

hESC-MSCs' growth curve was established during incubation period by multiplying the number of cells. Each point represents the number of cells by using COUNTESS (life technology, USA)

G-band staining

A karyotype analysis was commissioned a staining into GENDIX(Korea). It was performed by cytogenetic analysis on metaphase-cells of hESCs-MSCs at passages 13.

Animal preparation

All animal study protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital, Korea. The 7weeks old Fischer F344 inbred (orient bio inc, Korea) rats were bought. All rats were given free access to water and standard chow diet throughout the study, and were maintained in a temperature- and humidity-controlled room with a 12-h light/dark cycle. All rats should have about 1 week adaptation period before the study was started.

Surgical procedure for acute Myocardial Infarction(MI) model

At first, The 8 weeks old, 180~220g, Fischer F344 inbred rat(orient bio inc, Korea)were anesthetized using isoflurane(JW Pharmaceutical Co, Korea). to be shaved in chest. After shaving, he rats were anesthetized again using an intraperitoneal injection of 10mg/kg body weight zoletil(Virbac, USA). The rats were then ventilated mechanically with oxygen, and intubated with an 18-gauge cannula(BD Angiocath™, USA) by using a small animal ventilator (Harvard Apparatus, USA) at a rate of 60 - 70 breaths/min and a tidal volume of 2 - 3 mL. The heart was exposed by a left thoracotomy. The pericardium was resected

carefully avoiding damage to the heart, and the left anterior descending (LAD) artery was ligated permanently at 2 - 3 mm from the left atrium, using a 6 - 0 silk suture(AILEE co, Korea). When MI model was made completely, the MI zone was showed the presence of a clearly demarcated from tied point to apex. The movement on LV is also abnormal. After checking it, the chest and skin were closed using 4 - 0 silk sutures(AILEE co, Korea).

hESCs-MSCs transplantation for Injection in MI model

hESCs-MSCs cultured for 3~4days to inject MI model. Several trial of this experiment was cultured with CellTracker CM-DiI Dye(C-7000, Molecular Probes®, USA) for detecting MSC cell after sacrifice. 3 days after MI induction, Left thoracotomy in MI model was performed again to inject hESCs-MSCs. It anesthetized as described above. A total of 1.0 × 10^5 MSCs in 70 μL PBS(n=10) and only PBS(n=10), were injected into two site of Periinfarct area, using insulin syringes. After injection, the rats were followed up by using echocardiography until 8weeks.

Echocardiography

Echocardiography was performed on 4 times (baseline point(-3days), MI induction checking point(3days) and therapeutic efficiency check points (4weeks, 8weeks)). After anesthetization by isoflurane, the left-ventricular end-diastolic dimension (LVEDD), left-ventricular end-systolic dimension (LVESD) of rat was measured by a 9-MHz transducer connected to a Toshiba echocardiography machine (Nemio, Toshiba Co, Japan). Ejection Fraction (EF) was calculated using the following equation: EF = 100 × ([LVEDD^2 - LVESD^2] / LVEDD^2). All measurements were recorded over three times and the mean was calculated. A single operator who was blinded to the study groups performed all measurements.

Histological data

After the measurement of echocardiography at 8weeks, the heart in rats was excised. The extracted heart was perfused with PBS via coronary. And then, it fixed with 10% formalin at least 3days in room temperature. Before the fixed heart tissue was embedded in paraffin and made a block, it was cut approximately 2 mm under from ligation point. Using microtome, the paraffin block was sectioned into 4㎛ thickness for progressing histological

experiments. The paraffin section was stained with Masson's trichrome to evaluate infarct area and wall thickness in heart. For evaluating infarct area, the stained slides were scanned by scanner to get image. Then, the images were analyzed by SABIA(METOOSOFT, Korea). This software can make count percentage ratio collagen deposit part and normal tissue part. This software can represent a ratio of the normal part to the damage part in the heart tissue as a percentage. Wall thickness was used with an optical microscope to get specific damaged part of heart tissue. By using LAS 3.8(Leica, Germany), wall thickness was used with optical microscope to take image about specific damaged part of heart tissue. Wall thickness was measured 5 parts in collagen deposit area, and these measurements made average and were compared each group.

Immunofluorescence

Immunofluorescence was performed with conjugated primary antibodies to identify graft cells and how resident cell was affected by hESCs-MSCs. The paraffin heart sections were deparaffinized and dehydrated by using xylene(JUNSEI, Japan) and various concentration of absolute alcohol(HAYMAN, UK). After treating antigen retrieval to slide, it was incubated with blocking solution to cut off non-specific binding sites for 3hours. Before incubating fluorescence conjugated secondary antibody,

each slide was treated with primary antibodies against c-kit(FITC; Abcam, USA), WT1(Abcam, USA) and counterstained with 1 μg/mL DAPI as known cardiac stem cell marker. To take immunofluorescence image of all sections, the confocal microscope (Carl Zeiss, Germany) was used.

Statistical analysis

Analysis was performed by GraphPad Prism 6(GraphPad Software, USA). Probability value of <0.05 was considered statistically significant. Data are presented as mean ± standard deviation. The significant differences between the groups were analyzed by the unpaired t-test analysis of variance.

Result

New developmental method of clinical grade ESC-MSCs

The derivation protocol of MSCs from hESCs was established by several steps[2]. Following the figure1a, hVTN is a good coating material to culture clinical grade ESC, and it was formed enough packed colonies when it was ready to induce mesenchymal stem cell.(Figure 1) ESCs were dissociated through emzymatic methods, and it was cultured by suspension to make well around shaped embryonic body (EB) during 2days. EB were re-attached in CELLstart coating dish and it could come out cells for 8days Finally, outgrowing cells-EB complex was moved to 75T flask, and then outgrowth cells in a flask were begun to have characteristics of Mesenchymal lineage. After several times of subculture, it could be mesenchymal stem cell.(Figure 2) Well-differentiated mesenchymal stem cells had a jagged morphology and finger print pattern. mesenchymal stem cell line from clinical grade ESCs could proliferate well. (Figure 3 A,B)

These stable cells, could called hESC-MSCs, were expanded periodically and maintained proliferative capacity until 25th passages. Also, with G-band staining, hESCs-MSCs were shown normal karyotype at 13 passage that cell transplantation was used in MI model.(Figure 5 A)

Characterization of clinical grade ESCs-MSCs

After derivation, in FACS analysis, well known MSCs positive markers, CD44, CD73, CD90, CD29, CD105, were expressed on the surface of hESC-MSCs. However CD34, CD45 were not detected.(Figure 4) Moreover, expressions of HLA-DR and DQ, which are immunetolerant markers, were not detected on the surface of hESC-MSCs.(Figure 5 B) These data were proved that hESC-MSC were differentiated completely and had no immunological problem for applying stem cell therapy[5,7,8].

To ensure that MSCs could differentiate into adipocyte, chondrocyte and osteocyte, MSCs were cultured into each lineage specific culture conditions. After 21days of culture for inducing adipocyte, with oil red O staining, fat granules that were stained red and proved of adipocyte were detected by using optical microscope(Olympus, Japan). Also, chondrocyte were cultured to induce with suspension culture method from hESC-MCSs during 21days. After that, aggrecan, which is specific marker of chondrocyte, was detected in differentiated MSCs by immunofluorescence because hESCs-MSCs were induced completely to chondrogenic lineage[16,20,21].

At last, to make osteocyte, MSCs were cultured in osteogenic induced media for 21day, and it was checked by osteocalcin staining that is a maker for osteocyte through immunofluorescence. (Figure 4) Our data was proved hESC-MSC was well

differentiated through FACS, differentiated assay and various trials[22].

Echocardiography analysis

At baseline, two groups which are PBS group and hESC-MSCs treated group, left Ventricular End Diastolic Diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were shown a similar pattern until after 3days from MI induction. The reason that all groups were evaluated at 3days time point through echocardiography was confirmed whether the model was induced MI successfully. Therefore, ejection fraction of all group was decreased 20% as a rapid inclination percent because rats were induced MI properly[14]. (Figure 6)

After confirming, clinical hES-MCSc was transplanted periinfarct site into heart. Ejection Fraction of PBS Group was decreased sharply at 8weeks. On the other hand, Cell transplantation Group was shown slowly decreased pattern compared to PBS Group. On the other side of analysis, in delta value, control group that was not treated anything had small change, but PBS group had much lower value than cell transplantation group. It means that PBS group, compared to cell treated group, had severe loss of function in MI induced heart. (Figure 6 B, C)

In summary, through echocardiography, these results was proved

that rats induced MI were gotten minimal damage by injecting hESC-MSC, so hESC-MSC could be potential candidate material of stem cell therapy[14,19,22].

Wall Thickness, Fibrosis Area, Immunofluorescence

As time goes, the infarct zone of heart of MI model becomes thinner and wider. Wall thickness of infarct zone and fibrosis area of heart affects systolic motion in heart; contraction movement was important indicator to check function of heart, which is ejection fraction, fractional shortening.

By utilizing heart section slides with MT stained, we could evaluate histological function of heart. (Figure 7A) To evaluate wall thickness of infarction sites, five sides of infarct zone on heart section was measured equally; it was indicator evaluated average thickness of infarct zone between groups. By histological analysis program, wall thickness of infarct zone on cell treated group was much thicker than PBS group. With the software(metoosoft, Korea), section of heart can be analyzed in accordance with the color difference.(Figure 7 B, C)

Measured values, the percentage of fibrosis area against normal tissue, were obtained. The percentage of fibrosis area in PBS group is much higher than hESCs-MSCs treated group. These

results means that cell injected group had better heart function than PBS group histologically. To determine how hESC-MSCs affect positively in MI model, immunostaining was performed. Through immunofluorescence costaining, we could get a clue how hESCs-MSC worked in the MI heart.

As determined by staining with c-kit and WT1, cardiac stem cell marker, transplantation of clinical hESC-MSCs contaning DiI dye resulted as paracrine effect at border zones of the MI heart. C-kit and WT1 positive cells which were considered as cardiac stem cell were located nearby MSC with DiI at the border zone in heart tissue[23].(Figure 8)

Discussion

In this study, we clearly demonstrated that clinical grade hESC-MSC was induced through short EB stage, our own potential protocol to make a MCS is easy and it is also required economically short term till MSC derivation from ESC.

In the previous reports, various MSC derivation methods have been exposed many problems. One of methods is that specific genetic factors, which could induce mesechymal stem cell, were transfected to ESC or ASC. Transfecting factors method is available to establish mesenchymal stem cell, but it could be called GMO(Genetically Modified Organism) product. Another is a method making high purity of MSC population by using FACS or MACS sorting systems. Sorting MSC is also efficient and high purity. However, it could be damaged to MSC. For that reasons, our protocol, compared these above methods, were proved that needs not to use mechanical selection and separation to establish MSC. In typical way for growing MSCs, feeder cells were needed. Xeno or allo feeder cells were usually used to culture MSCs. However, having to make it grow MSC, both xeno feeder cell and allo feeder cell have involved pathogenic risks[2.3.6]. Nevertheless, our protocol to culture MSC used ECM instead of feeder cells, so our clinical grade ESC-MSC has risks of pathogen hardly.

Above our data about in vivo, transplantation with this cell in a rat model of MI dose not deteriorate anymore[24]. One of trial points in our experiment; we used inbred strain (F344) for making MI, not used outbred. The reason why we used inbred stain instead of outbred is that we wanted to reduce critically variation of each other. Outbred strain has various heart sizes, health condition and body weight. Moreover, using this animal couldn't represent efficient therapeutic effect exactly. In contrast, animal experiment with inbred strain could be minimalized variation among animals, and we could get more exact data from experiment[14,25,26].

Histologically, as time passes, in a rat model of MI model, wall thickness of heart was being thinner and fibrosis area also was becoming larger. In contrast, wall thickness and fibrosis area of group with cell transplantation don't be made worse. Our data showed that cell treated group was not more serious state, compared to PBS group, rather it seemed hESC-MSC has therapeutic efficacy in heart by treating to MI model. Ejection Fraction is one of indicators to analyze heart function by echocardiography. After inducing MI, naturally heart function was being worse. Thus, compared normal condition, ejection fraction could be reduced dramatically from baseline because of inducing MI. Even though ejection fraction of cell treatment group is decreased slowly from induction time point to 8 weeks, the degree of reduction, compared to PBS, is very small. By analyzing immunofluorescence to heart tissue section, cardiac stem cells were distributed significantly around MSC at infarct

area. The exact mechanism in in vivo is not clear , but we suggested hESC-MSCs have positive therapeutic efficacy [23].

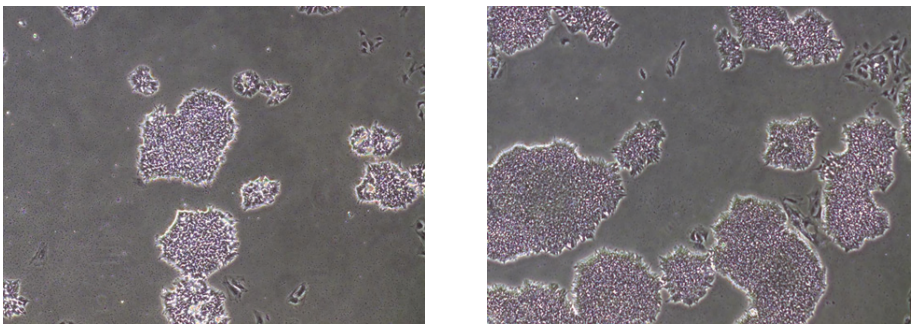
In this study, It is demonstrated we expected clinical embryonic stem cell derived mesenchymal stem cell without any xeno factors by working in cGMP could make cells for clinical trial stably and be the potential candidate for stem cell therapy in heart disease, and hESCs-MSCs in in vivo could affect to another resident cell with emitting growth factor as paracrine effects.

Conclusion

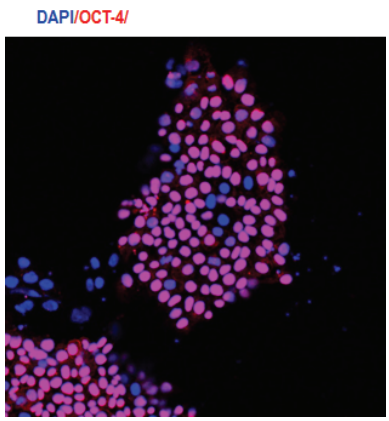
We build up a efficient method of making MSCs from hESC in cGMP facility. The mesenchymal stem cell surface markers are expressed well. and cell surface markers of immune reaction not detected. Moreover, with various experiments, hESC-MSCs were prove safety. we also checked that our hESC-MSC has thrapuetic efficacy though a rat model of MI. Thus, because our protocol overcame limitation of other methods to induce MSCs from ESCs, it would be a good standard for establishing MSCs from ESCs.

Figure

A.



B.



C.

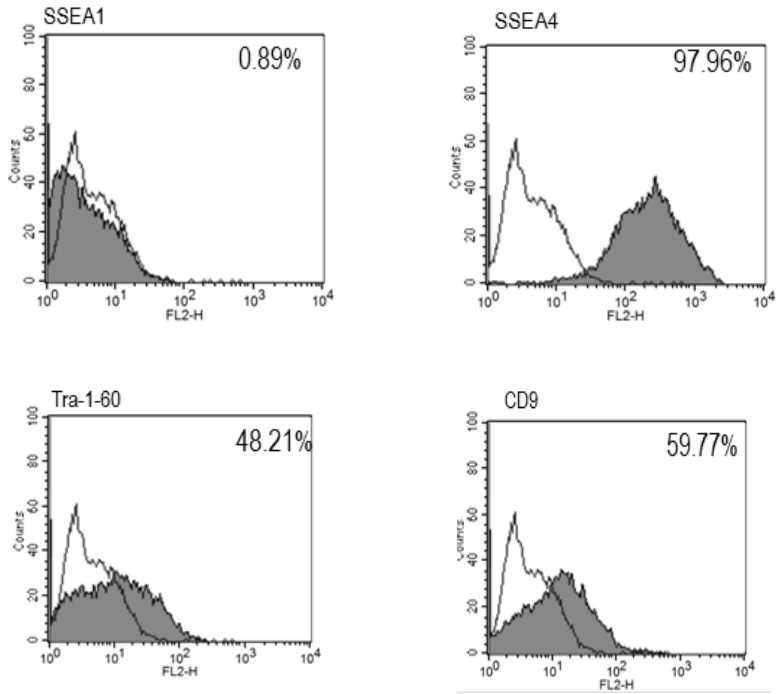


Figure 1. Culture and maintainance of embryonic stem cell with non xeno factors.

(A) The morphology of undifferentiated embryonic stem cell with non xeno factors; right side is 30% confluency and left side is 70% confluency. ESCs were observed by phase-contrast microscope. (B) Via Immunofluorescence, confirmation about pluripotency of ESC staining against Oct4. (C) Flow cytometry analysis of ESCs. Cells were trypsinized and stained with specific markers for hESCs

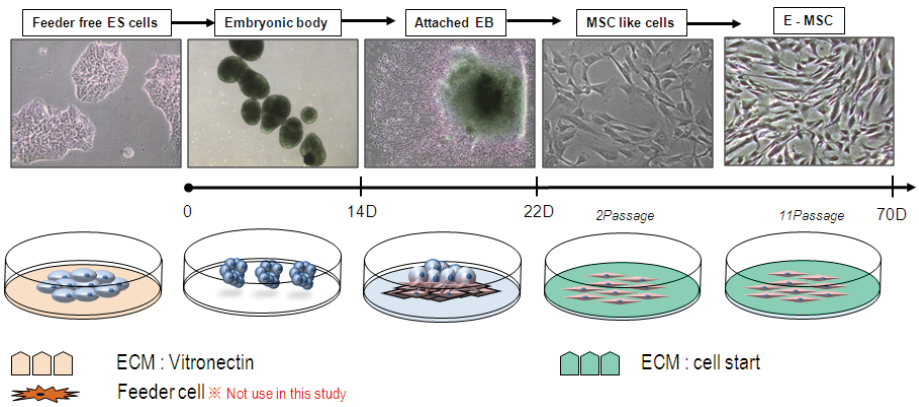


Figure 2. Clinical grade hESCs-MSCs derivation.

Well grown hESCs were harvested to form EB. After 2days of EB formation, EB was attached and cultured for 8 days. outgrowth expanded EB was induced mesenchymal lineage differentiation. Completed hESCs derived MSCs (hESCs-MSCs) was started culture

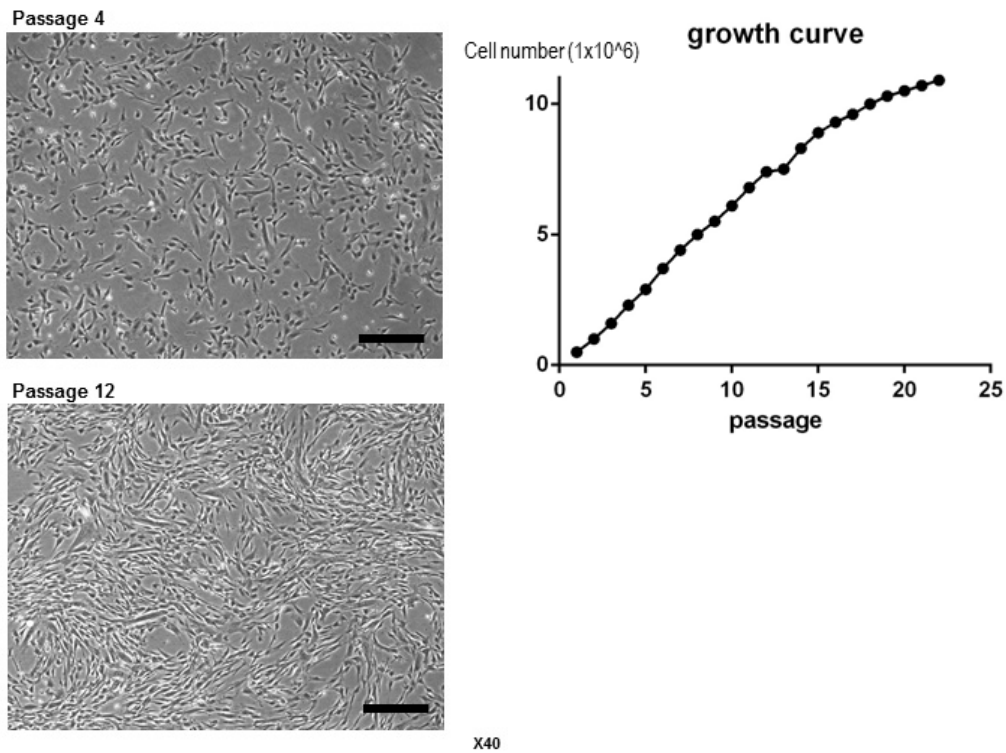
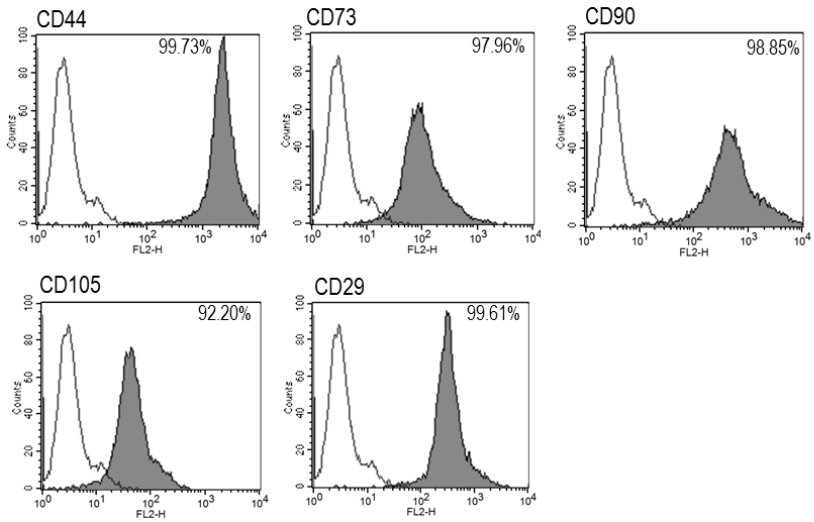


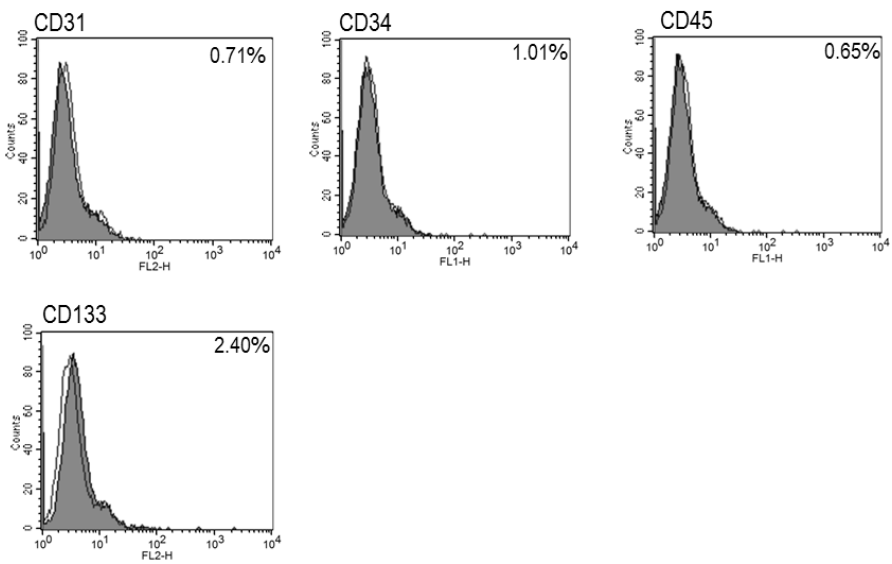
Figure 3. Morphology and growth curve of hESCs-MSCs.

(A) Morphology of hESCs-MSCs at confluency under optical microscopy have fingerprints-like pattern. (B) hESCs-MSCs subculured every 3~4 days

- **MSC positive marker**



- **MSC negative marker**



• **ESC positive marker**

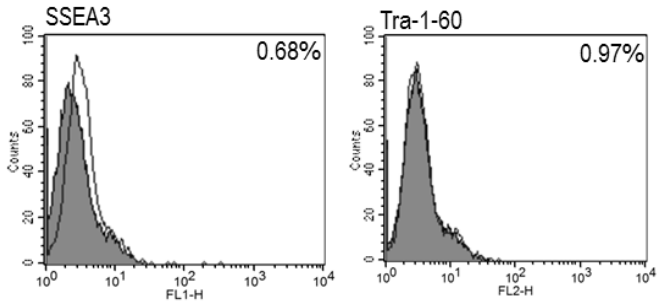


Figure 4. Flow cytometry analysis of hESCs-MSCs.

After expension at passage 11th of hESCs-MSCs, cells were stained with specific various markers for MSC, ESC and other lineages

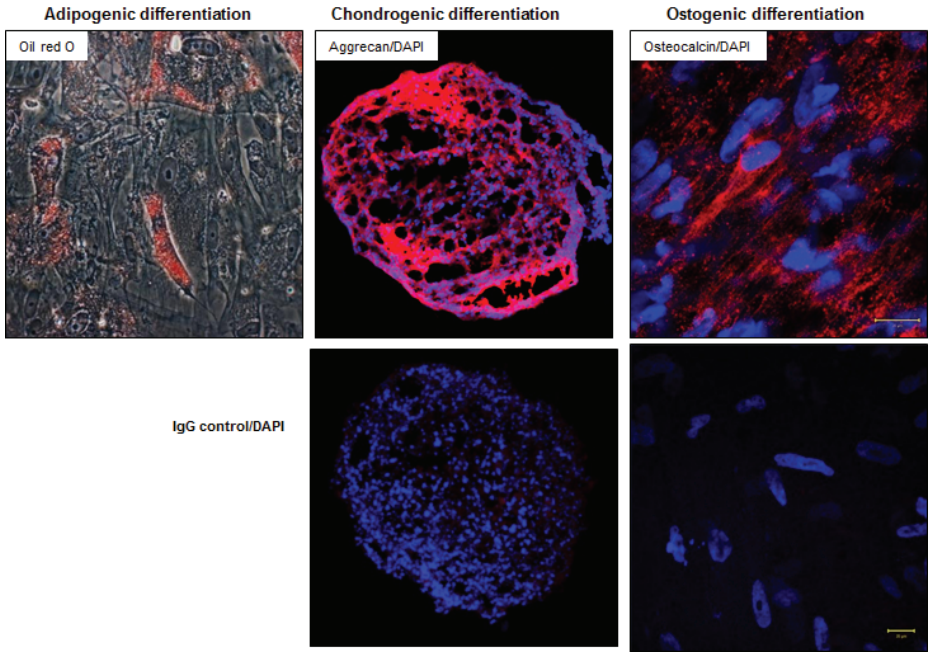
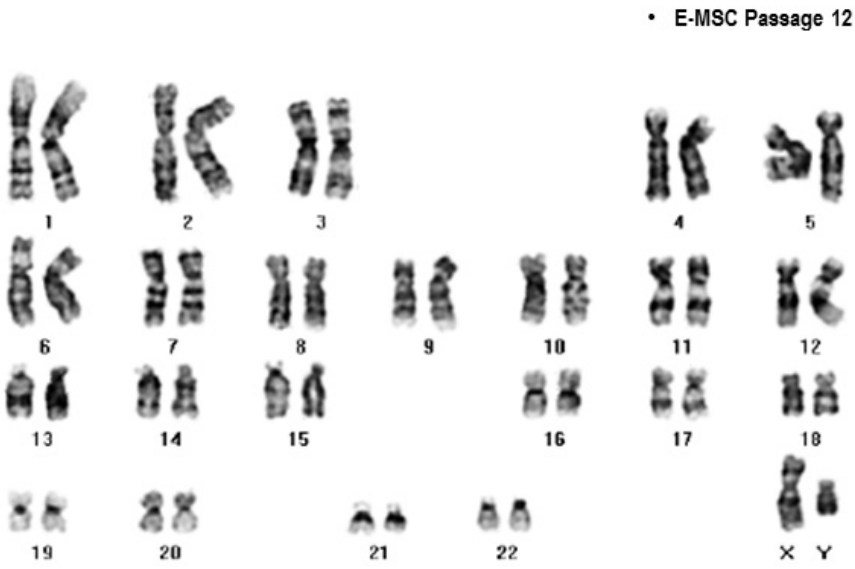


Figure 5. Functional differentiation of hESCs-MSCs

hESCs-MSCs differentiated into adipocyte, chondrocute, osteocyte under each differentiated conditions. Adipocytes was stained with Oil-red-O. Chondrocyte and osteocyte was performed against aggrecan and osteocalcin by immunofluorescence

A.



B.

• **Expression of human leukocyte antigen**

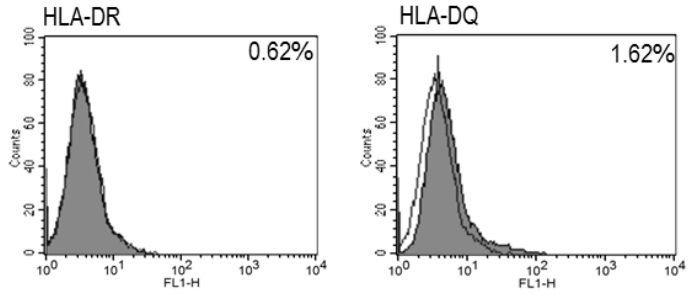
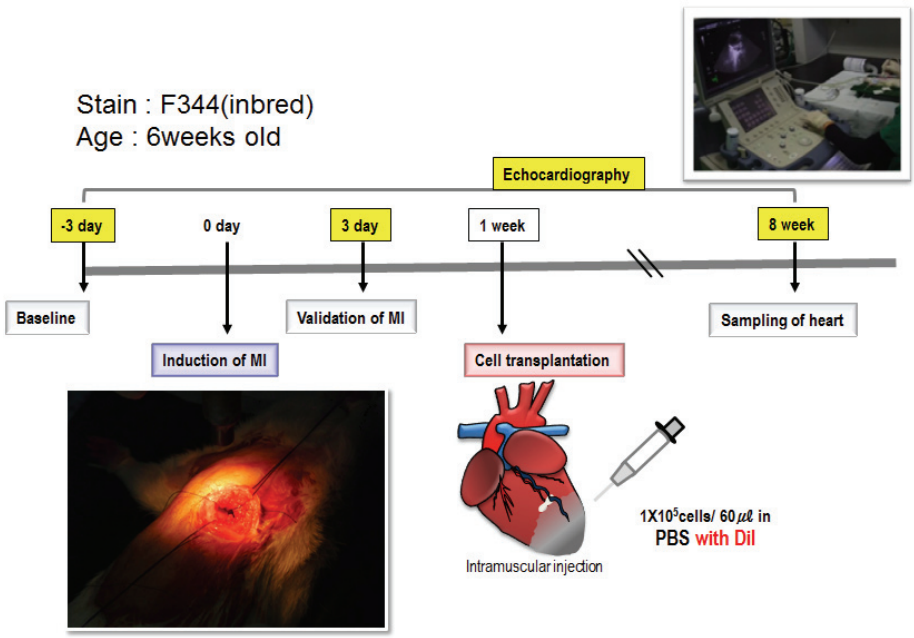


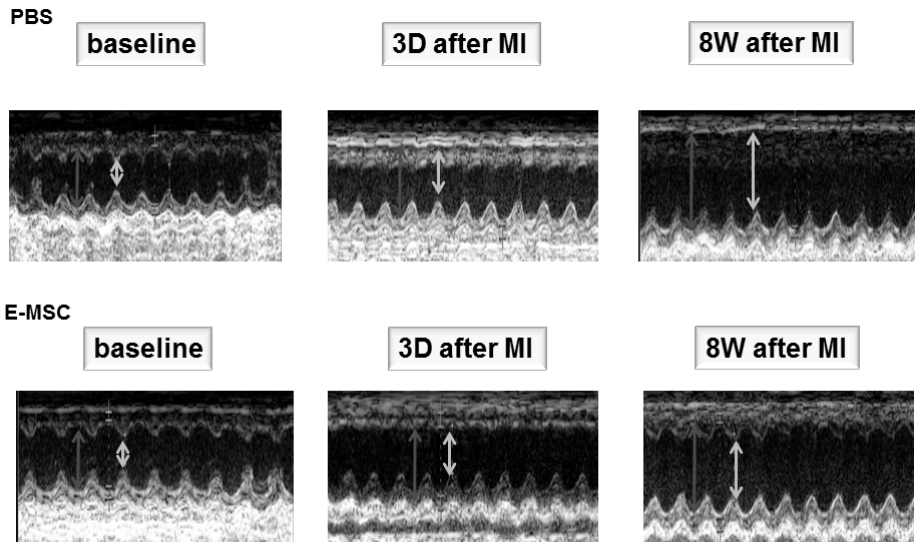
Figure 6. Biosafety of hESCs-MSCs for therapeutic application

(A) hESCs-MSCs after prolonged culture in expansion condition were karyotyped by G-band staining (B) Typical immune reaction markers, HLA-DR, HLA-DQ were not detected with FACS analysis

A.


Stain : F344(inbred)
Age : 6weeks old
Echocardiography
-3 day
0 day
3 day
1 week
8 week
Baseline
Induction of MI
Validation of MI
Cell transplantation
Sampling of heart
Intramuscular injection
1X10⁵cells/ 60㎕ in
PBS with Dil

B.


PBS
baseline
3D after MI
8W after MI
E-MSC
baseline
3D after MI
8W after MI

C.

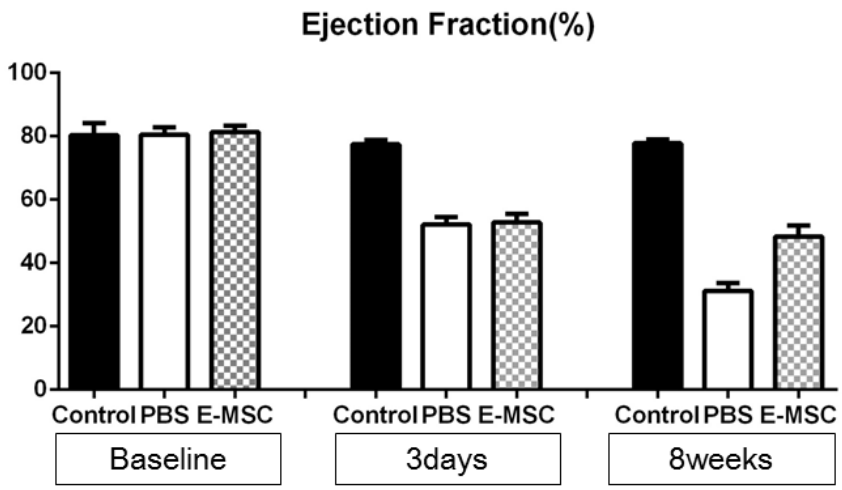
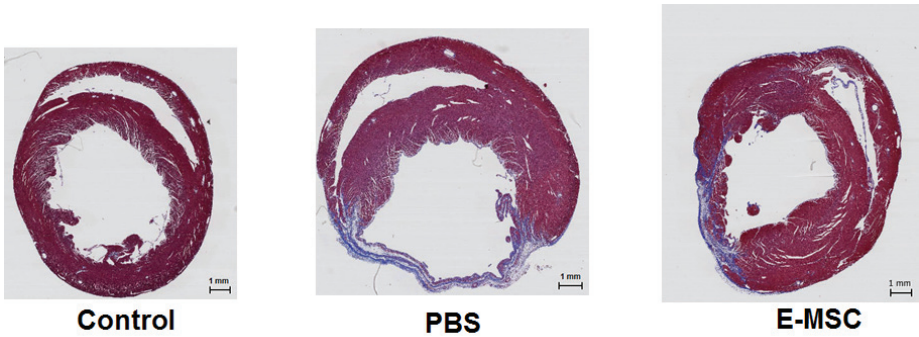

Ejection Fraction(%)
100
80
60
40
20
0
Control PBS E-MSC
Control PBS E-MSC
Control PBS E-MSC
Baseline
3days
8weeks

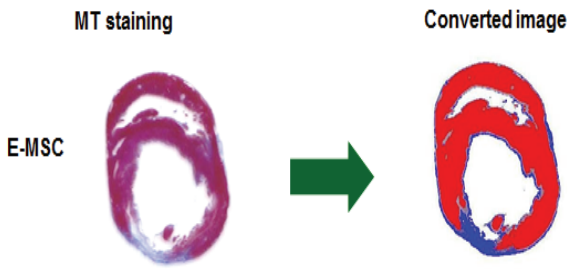
Figure 7. Animal experiment with a rat model of Myocardial infarction for evaluating therapeutic efficacy of hESCs-MSCs

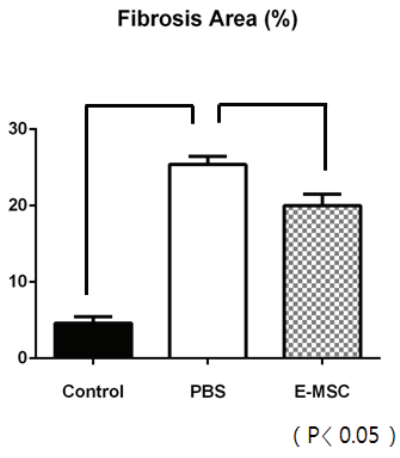
(A) scheme of animal experiment in this study. Inbred rats(F344) were used, and echocaridography was performed as indicator evaluating therapeutic efficacy (B) M-mode of echocardiography was measured to evaluate therapeutic efficacy of hESCs-MSCs (C) Ejection fraction analysis of animal experiment

A.


Control
PBS
E-MSC

B.


MT staining
Converted image
E-MSC


Fibrosis Area (%)
30
20
10
0
Control
PBS
E-MSC
(P< 0.05)

C.

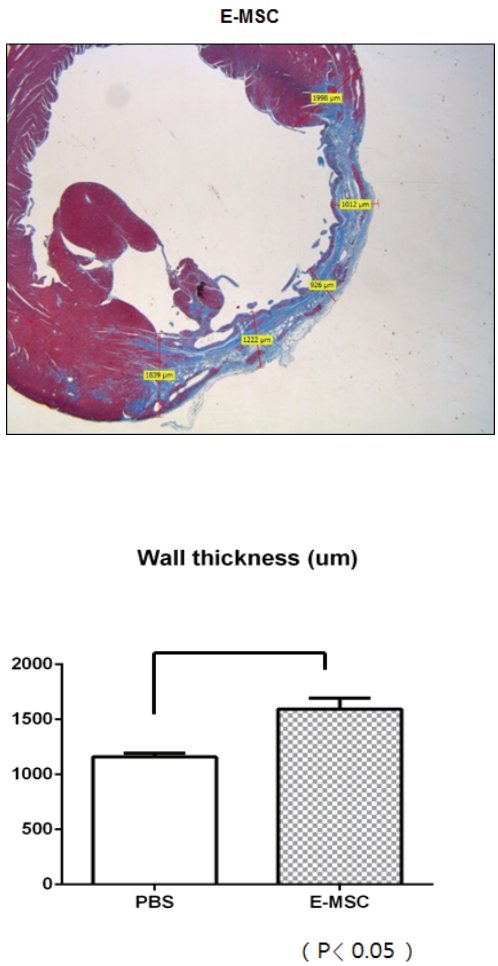
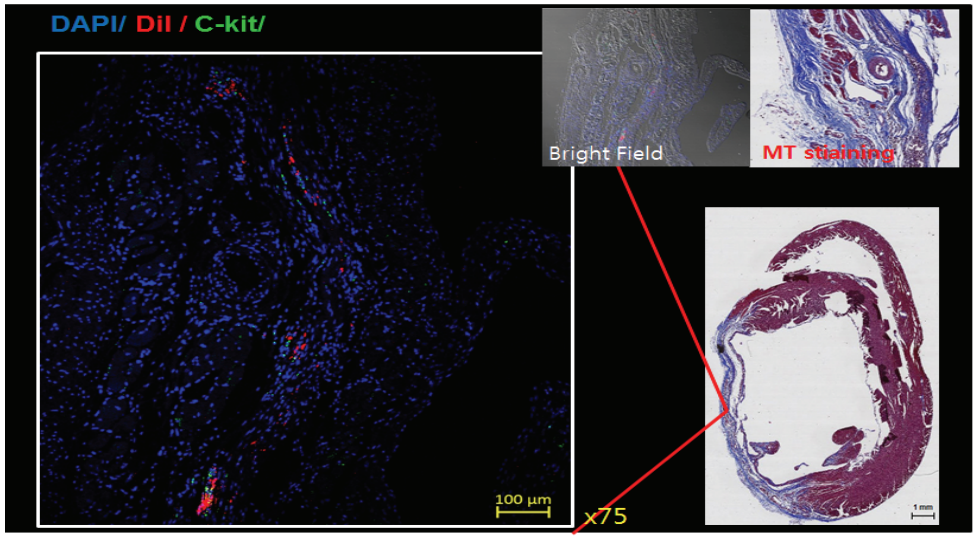


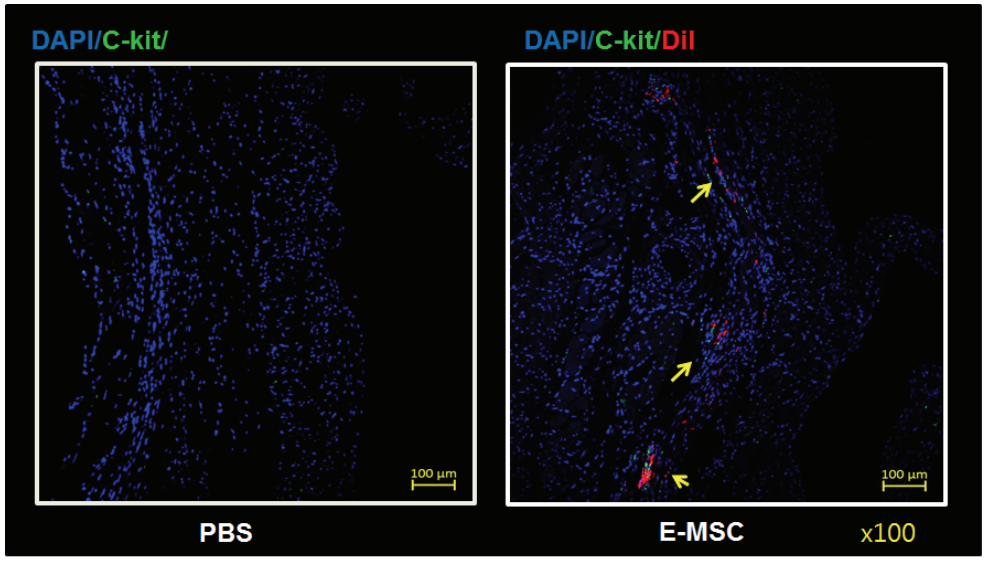
Figure 8. Histological analysis of MI heart in animal experiment via masson's trichrome(MT) staining

(A) Heart section with MT staining in animal experiment. (B) Measurement of Fibrosis area through color difference. (C) Measurement of wall thickness using software tool

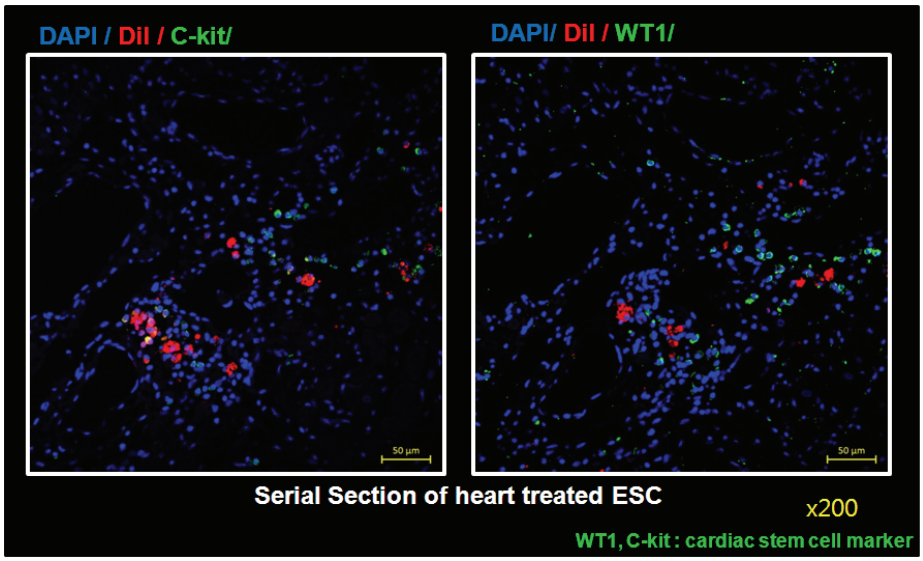
A.


DAPI/ DiI / C-kit/
Bright Field
MT staining
100 μm
x75
1 mm

B.


DAPI/C-kit/
DAPI/C-kit/DiI
100 μm
100 μm
PBS
E-MSC
x100

C.


DAPI / DiI / C-kit/
DAPI/ DiI / WT1/
50 μm
50 μm
Serial Section of heart treated ESC
x200
WT1, C-kit : cardiac stem cell marker

D.

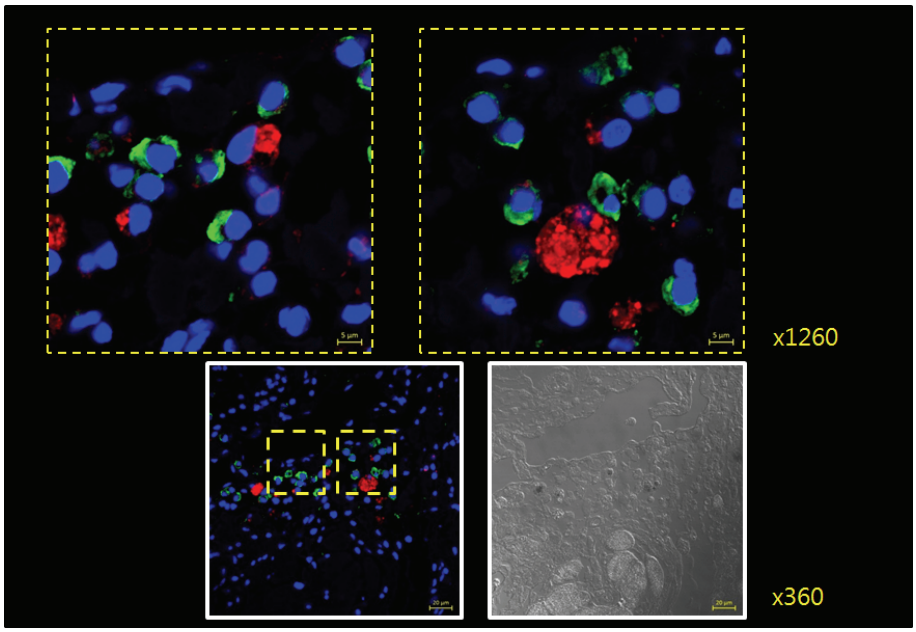

x1260
x360

Figure 9. Immunofluorescence analysis of heart section in animal experiment

(A) Injected hESCs-MSCs were located in border zone of MI heart (B) Each groups was performed staining with C-kit antibody via immunofluorescence (C) C-kit and WT-1 antibodies, known as cardiac stem cell markers, was stained Using serial section of same hESCs-MSCs treated group. (D) High magnification view of E-MSC treated heart staining with c-kit

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국문초록

심근경색 동물 모델에 치료효과가 있는 임상 등급 배아줄기세포 유래 중간엽 줄기세포 생산

서론

인간 배아 줄기 세포 (hESC)는 세포 치료법에서 큰 잠재력을 가지고 있다. 그러나, 환자에게 인간 배아 줄기 세포 자체를 투여하기에는 세포 자체의 발암원성과 면역 거부의 위험을 가지고 있다. 이러한 위험을 극복하기 위해, 일반적으로 인간 배아 줄기세포는 다양한 계통으로 분화된 세포를 세포치료법에 적용한다. 그 중 하나인 인간 배아 줄기 세포에서 유도 된 중간 엽 줄기 세포는 자가 분열이 가능하고 다양한 타입의 세포로도 분화가 가능한 세포이다.

여기, 우리는 cGMP 시설을 사용하여 배아 줄기 세포 유래 중간엽 줄기 세포를 유도 및 배양하는 효과적인 제작 방법을 제안한다. cGMP 시설은 배아 줄기 세포 유래 중간엽 줄기 세포가 무균 상태에서 배양되는 것을 증명할 수 있는 시설이다. 또한, 균일한 세포주 생산을 위해, 우리는 배아 줄기 세포 유래 중간엽 줄기 세포 제작방법을 문서화 와 표준화를 진행하였다. 우리가 유도한 배아 줄기 세포 유래 중간엽 줄기 세포는 중간엽 줄기세포 양성 마커인 CD44, CD73, CD90, CD29, CD105 발현되는 것을 확인했고, 중간엽 줄기

세포 음성 마커인, CD34, CD45가 발현되지 않는 것을 확인했다. 또한, 우리는 생체 내에서의 배아 줄기 세포 유래 중간엽 줄기 세포의 치료 효과를 연구 하였다. 개체간의 차이를 줄이기 위하여, 근교계통 랫드(F344)를 사용하여 심근 경색(MI) 모델을 유도하였고, MI 모델 심장의 경변 주위 지역에 세포를 주입하였다. 다양한 분석을 통해, 우리는 MI 모델에서 세포 이식 그룹의 심장 기능이 PBS 그룹보다 더 나은 심장 기능을 가지고 있다는 것을 보여주었다.

결론적으로, cGMP 시설에서 우리의 제작방법을 이용하여 얻은 배아 줄기 세포 유래 중간엽 줄기 세포는 다른 유도 방식의 단점을 극복할 수 있고 높은 생산율을 가진다. 또한, 우리의 제작방법은 쉽고 간단하다. 심근 경색 모델 동물 실험을 통해, 우리는 배아 줄기 세포 유래 중간엽 줄기 세포가 임상 시험을 적용하기에 적합하다고 사료된다.

키워드 : 인간 배아 줄기 세포, 중간엽 줄기 세포, 분화, 심근 경색

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