의학석사 학위논문

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심근경색에서 KLF2 를 통한 아토바스타틴의 심장 외막 전구세포에서 혈관 내피 세포로의 분화 촉진

2013 년 2 월

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의과학과 전공

양 혜 림

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이 논문을 의학 석사 학위논문으로 제출함

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Atorvastatin promotes adult epicardial progenitor cells differentiation into endothelial cells under the myocardial infarction condition through Klf2

by
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A thesis submitted in partial fulfillment of the requirements for the degree of
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ABSTRACT

Atorvastatin promotes adult epicardial progenitor cells differentiation into endothelial cells under the myocardial infarction condition through KLF2

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Objective

Myocardial infarction(MI) is related to inflammatory response, leading to the fibrosis that occurs in response to injured stimuli inducing cardiomyocyte death. Epicardial progenitor cells (EpiPCs) have been identified from adult epicardium as well as during development and may be differentiated for renewal of vessels at low basal level or neovascularization following cardiac injury.

Here, we discovered that atorvastatin promotes KLF2 activation via ERK5, which induces the differentiation of adult epicardial cells into endothelial

cells (ECs) and also promotes vessel formation, not fibroblast causing fibrosis, which suppressed fibroblast factor such as Snail.

Methods and Results

First of all, we established new culture method using a different attaching character of EpiPCs and confirmed adult EpiPCs marker wt-1 positivity. Treating atorvastatin in hypoxic conditioned adult EpiPCs, we found out that adult EpiPCs were differentiated into endothelial cells and vessels were formed in the long time culture. Also, we identified atorvastatin working mechanism that facilitates KLF2 activation, which regulates the differentiation of adult epicardial cells to ECs via ERK5 as response in the hypoxia condition. Hypoxia condition inactivates KLF2, which activates CD31expression at the cell surface to differentiate endothelial cell (EC). On the other hand, atorvastatin treatment in the hypoxic condition activated KLF2 via ERK5 and increased CD31 and suppressed fibroblast factor such as Snail.

Conclusions

We demonstrate that atorvastatin promotes KLF2 activation, which induces the differentiation of adult epicardial cells into endothelial cells, not fibroblast causing fibrosis via ERK5 and also facilitates vessel formation in the myocardial infarction condition. Moreover, we found out that most of adult EPDCs are determined cell type, nearly fibroblast, in the adult epicardial progenitor cells and only some cells have a

differentiation potentiality that is target of atorvastatin to regenerate cardiac damage such as MI.

Keywords: myocardial infarction, statin, adult epicardial progenitor cell, Klf2, Snail

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INTRODUCTION

Myocardial infarction (MI) is related to inflammatory response, leading to the replacement of damaged cardiomyocytes with granulation tissue and scar.[1] It causes the fibrosis that occurs in response to injured stimuli inducing cardiomyocyte death by MI that activates a reparative response. [2] Myocardial regeneration appears to be mediated by multipotent cardiac stem cells (CSCs) in the heart that appear to new vascular structure and myocyte. These CSCs exist in the mouse, rat, dog, and human adult heart. [3,4]

Epicardium plays an important role in the coronary vasculature during embryogenesis and some epicardial cells delaminate from the epicardium and migrate into the subepicardium where they generate epicardially derived cells (EpiPCs) via epithelial-to-mesenchymal transition (EMT) process. EpiPCs are pluripotent stem cells that are crucial in cardiac development. [5] Recently, It is reported that EpiPCs have been identified from mammal adult epicardium as well as during development and EpiPCs derived from adult mouse may be induced to differentiate into smooth muscle cells and endothelial cells (ECs) for renewal of vessels at low basal level or neovascularization following cardiac injury. [6,7] Also, It is known that Zebrafish within vertebrates has the ability to regenerate

the heart following ventricular apex resection; under these conditions, epicardial cells undergo epithelial-to-mesenchymal transformation, migrate into the wound and participate to myocardial regeneration and blood vessel formation. [8] Also, Smart et al reported that a novel genetic label of the activated adult progenitors via re-expression of a key embryonic epicardial gene, Wilm's tumor 1(*Wt1*), a peptide previously shown to restore vascular potential to adult epicardium derived progenitor cells with injury. [9] They found out that the adult heart contains a resident stem or progenitor cell population, which has the potential to contribute terminally differentiated cardiomyocytes after myocardial infarction.[6,9] Based on these finding, it is already known that EpiPCs exist in the adult mouse heart, which they retain the ability to give rise to myocardial precursors and vascular cells. However, it is mainly focused these cells can differentiate cardiomyocyte rather than supporting cardiomyocyte survival after MI and it is unclear how adult EpiPCs are differentiated into EC and what is facilitating the change to regenerate cardiac damage such as MI. We assumed that EpiPCs from adult mouse may play a key role for vessels formation to enhance cardiomyocyte survival and contribute cardio-protection in the adult mouse. It was known that adult epicardium and EpiPCs derived from mouse may be induced to differentiate into smooth muscle cells and endothelial cells at low basal level and we supposed that the proportion of vascular cells may change by

specific drug, atorvastatin to repair heart injury condition such as myocardial infarction. Statin induces a robust mobilization of murine progenitor cells and endothelial progenitor cells via PI3K/Akt pathways and enhance a endothelialization of injured vessels. [10,11] It is reported that several transcription factors regulate endothelial cell development and there are several major regulators of endothelial transcription, including Kruppel-like family (KLF), MEF2C etc.[12]

However, it is unknown whether statin is effective to repair heart injury by regulating adult EpiPCs differentiation and which transcription factor is key regulator in the adult mouse heart repair rather than development phase. Based on these finding, the aim of the study was to establish whether atorvastin promotes adult epicardial cells into ECs with a certain mechanism to repair damaged heart after myocardial infarction.

In this study, we provide evidence that atorvastatin facilitates KLF2 mediated differentiation of adult EpiPCs and also induces vessel formation in the myocardial infarction condition.

METHODS

Materials

Atorvastatin was provided from Pfizer (New York, NY). Antibodies used in this study were purchased from the following sources: anti-WT1 (SantaCruz), anti-PECAM1 (SantaCruz), anti-α-SMA (Abcam), anti-Tie2 (SantaCruz), anti-CD31 APC (eBioscience) and anti-Klf2 (SantaCruz). The specific ERK5 Blocker was purchased from XMD 8-92 triflate (SantaCruz).

Animals and surgical methods

Myocardial infarction was induced by coronary artery ligation in FUB-Tie2 male mouse at 6 weeks age for 10 days.

Cell Culture & Isolation of WT-1 positive cells

We established new primary culture from C57 mouse hearts to acquire epicardial progenitor cells (EpiPCs). After a harvest of mouse hearts, Each heart was incubated in 2ml DMEM with 0.1% collagenase type I (Invitrogen) in 37℃ waterbath for 100min to separate EPC from a mouse heart. And then vortex was done briefly to seperate well EPC from hearts. Cells downed by centrifuge were seeded on 1.5% gelatin-coated (Sigma) culture plates and incubated in DMEM, High Glucose,

GlutaMAX™ (Gibco) with 15% fetal bovine serum (Lonza) and antibiotic-antimycotic (Gibco). After 1day culture, unattached cells were transferred to another dish and cultured for 2days. For hypoxic condition, we used hypoxia chamer for 1 day, 4 days, 7 days.

After primary culture, we needed to isolate only WT-1 positive cells. So, specific culture method was used to isolate only WT-1 positive cell. Cells attached early are thought to have lower hierarchy than cells attached later. Culture was conducted for 1days and then that supenants were transferred to other new dish. To confirm these cells WT-1 positive, IHC was done in transferred cells and untransferred cells using R.T.U VECTASTATIN® Kit (VECTOR) and VECTOT® DAB (VECTOR). As our anticipation, transferred cells were all stained WT-1 positive. Pictures were taken by Olympus IX71.

MT-Staning

Myocardial sections were stained with Masson's Trichrome staining (MT) for estimation of infarct size with Sigma-Aldrich protocol. Pictures were taken by Olympus BX50.

Immunofluorescence

Immunofluorescent staining for α-SMA, CD31 (PECAM1) or Klf2 was performed using confocal miscroscopy, Zeiss LSM 710 (Carl Zeiss, Jena, Germany). Nuclear staining was done by DAPI (Sigma).

Immunohistochemistry

Immumohistochemistry (IHC) was performed in mice after myocardial infarction for 10 days using R.T.U VECTASTATIN® Kit (VECTOR). Hearts were harvested and fixed into the 10% buffed formalin for 24h. After fixation, hearts were dehydrated and made into paraffin blocks. Each of samples was sectioned of 6μm thick. For IHC staining, samples were removed paraffin in the 65℃ oven and dehydrated with xylen and ethanol. Sections were retrieved using 10x Target Retrieval Solution, Citrate pH6.0 (DAKO) and quenched with 0.3% hydrogen peroxide. Blocking was done with Biotin and Streptavidin Blocking Kit (VECTOR) after incubation with 2.5% normal horse serum. Samples were incubated overnight at 4°C with the primary antibodies and done by incubating the sections for 30 min with biotinylated pan-specific antibody anti-Mouse /Rabbit/Goat IgG. Sections were washed, incubated streptavidin/peroxidase complex for 30min. VECTOT® SG (VECTOR) and AEC plus High Sensitivity Substrate Chromogen Ready to Use (DAKO) was used to visualize immunostaining . Pictures were taken by Olympus BX50.

siRNA Transfection

For the blockage of Klf2, Klf2 siRNA oligomers were used (Snatacruz). Also, a negative control siRNA was purchased from Dhrmacon and Transfection concentration of siRNA was performed at 20μM. To transfect siRNA, we used chemical agent METAFECTIN PRO(Biontex, CO).

Real Time-Polymerase Chain Reaction

Real Time-PCR was done in mouse EpiPCs cultured in a normoxia, only hypoxia condition (1day, 4days and 7days) and hypoxia condition added atorvastatin (1μM). Smaples (1 μg) of each total mRNA extract were used to perform first strand cDNA synthesis using a kit (Takara Bio Inc., Japan) in a total volume of 20 μl, following manufacturer's instructions. One-twentieth of each cDNA generated was used as a template for real time-PCR using a FS Universal SYBR Green Master (Rox) (Roche). Reaction conditions were as follows using 7500 Real-Time PCR System (Applied Biosystems). Primer sets for amplification were as follows: WT-1, CD-31, Tie-2, α-SMA, collagen type-1, TGF-ß, VEGF, Snail1, Snail2, Klf2, Klf4, Klf6 and Mef2c.

Statistical Analysis

The unpaired 2-tailed t test was used to compare continuous variables. $p<0.05$ was considered statistically significant.

RESULTS

Myocardial infarction led the heart muscle fibrosis and this defect is decreased by atorvastatin.

It is known that Myocardial infarction (MI) leads the heart muscle fibrosis.[13,20,21] I conducted permanent Myocardial infarction for 10day as described previously and stained hear section with MT-staining methods to confirm whether Atorvastatin occurred to suppress MI. As a result, I confirmed fibrosis area of blue colored region and found out that atorvastatin reduces the fibrosis area occurred in the mouse heart compared with only MI condition (Figure 1A).

TO observe this appearance more detail, I took pictures with a high-power microscope and appeared hard fibrosis by MI compared to non-MI control. Also I found that vessel like shapes significantly appeared at the board zone starting fibrosis in the MI condition added atorvastatin (Figure 1B).

Atorvastatin induced the vessel formation in the adult mouse heart of MI condition from EpiPCs

To investigate whether atorvastatin carry out key role in the myocardial infarction condition to recovery this defect appeared at the heart, I performed the MI surgery for 10 days at the Tie-2 GFP mouse because evaluated ECs differentiation from EpiPCs in MI. The adult heart contains a resident stem or progenitor cell population in mice and they reveal a novel genetic label of the activated adult progenitors, Wilm's tumor 1 (*Wt1*).[9] I could observe the epicardium region stained WT-1 positive in the MI condition compared the non-MI control (Figure 2A, 2B). Notably, epicardium region consisted of WT-1 positive cells increased when I gave a MI conditioned mouse atorvastatin and I found out vessels expressing the endothelial cell marker Tie-2 or the epicedial progenitor cell marker WT-1 (Figure 2C-a). It was figures of magnified vessels formed region. I found out different two vessels, one was contained only tie-2 positive cells appearing already existing vessel and another vessel consisted of tie-2/wt-1 double positive cells meaning newly formed nascent vessels with cells derived from epicardium (Figure2C-b).

Epicedial progenitor cells of adult mouse heart were cultured by new methods.

I wondered whether Atorvastatin stimulates EpiPCs into formation of new vessels to improve the pathological condition by MI. To this question, I needed the EpiPCs culture method from adult mouse heart. Indeed, it is reported the method using Thymosin β4, which stimulates significant

outgrowth from quiescent adult epicardial explants. [6] But, I did set up new method using a different attaching character of EpiPCs (Figure 3A) and examined IHC to evaluate EpiPCs marker wt-1 positivity. As a result, 1day attached cells were stained weakly wt-1 with partially WT-1 strong stains but second day attached cell appeared mostly strong WT-1 stain(Figure 3B). Next, I counted the cell number according to the WT-1 stain grade considering that hierarchy of WT-1 strong stained EpiPCs was higher than weak stained cells and a group of second day attached cells contained higher hierarchy cells compared to a group of 1 day attached cells (Figure 3C). The result suggested that the culture method was effective to isolate EpiPCs from the epicardium.

Atorvastatin stimulated endothelial cell differentiation from EpiPCs of adult mouse heart and triggered formation of new vessels in the hypoxia condition.

I found out that atorvastatin could induce the new vessels formation to rescue the defect condition of myocardial infarction through some in vivo experiments. So, I investigated whether atorvastatin could promote to differentiate EpiPCs into endothelial cells and induce new vessels in the cell level. For this, I mimicked the MI condition to hypoxia condition on the cell level using a hypoxia chamber (Figure 4A). To assess whether cultured EpiPCs are differentiated into endothelial cells, I performed

Immuno-fluorescence analysis and the result revealed that there were clustered cells of small size surrounded by mature fibroblast expressing strongly fibroblast marker a-SMA and weakly endothelial cell marker CD31 in the normoxia condition. In the hypoxia condition, there were also gathered small sized cells some expressing strongly α-SMA/weakly CD31 and some cells expressing strongly CD31/ weakly α-SMA. But, there were notable change that the number of cells expressing very strongly CD31 in the clustered cells population increased when atorvastatin was treated at the hypoxia condition compared to only the hypoxia condition (Figure 4B). Together, these results appeared that there existed the population of clustered cells in the EpiPCs and that population wasn't completely differentiated condition. Cells of the population potentially possessed a differentiation possibility into cells of another character. Interestingly, the number of cells strongly expressing endothelial cell marker CD31 increaseed in atorvastatin treated hypoxia condition and those cells could differentiate into endothelial cells. Also, I counted cell numbers in three groups following by degree of each maker expression to evaluate distinct group representing cells expressing differently ECs and Smooth Muscle Cells (SMCs) / Fibroblasts (FB) markers. Also, I determined that ECs and SMCs/ Fibroblasts markers double positive cells were named to vasculoblasts. The number of fibroblastic cell expressing α-SMA strongly was higher in the normoxia than other conditions and both vasculoblatstic

cells expressing a-SMA/CD31 weakly and endothelial cells expressing CD31 strongly were more in atorvastatin treated hypoxia condition than other conditions (Figure 4C). By real-time PCR, the expression of CD31, a-SMA and collagen-1 were identified in cultured EpiPCs to find out changes of total cell level. The result revealed that endothelial cell markers CD31/Tie-2 also increased by atorvastatin and SMC/Fibroblast marker α-SMA/collagen-1 didn't almost change or decreased by atorvastatin (Figure 4D).

I wondered whether vessels were formed after a increase of CD-31 positive cells and performed long time culture more than previous culture to test this hypothesis. By immunofluoresence(IF), it was found that after adding atorvastatin, the number of vessel consisted of CD31positive cells was significantly higher compared with the only hypoxia condition(Figure 5A). I assessed the CD31 positive endothelial cell (EC)number per each vessel obtained by IF and indicated the number of EC was increased by atorvastatin (Figure 5B).

KLF2 was a regulator for EpiPCs differentiation into ECs.

It was reported that several transcription factors regulate EC development and there are several major regulators of endothelial transcription, including Kruppel-like family (KLF), MEF2C etc.[12] To find out which

factor plays important role for adult EpiPCs differentiation into ECs by atorvastatin, real time-PCR was done in each condition.RNA level of KLF2 notably increased compared to other factors, such as KLF4, KLF6, Mef2c (Figure 6A). It was reported that The MEK5/Erk5 MAPK cascade was implicated in the regulation of endothelial integrity and vasoprotective statins potently induce KLF4 and KLF4-dependent gene expression via activation of Erk5 in the human endothelial cell. [17] I wondered which factor was regulated via ERK5 in response of atorvastatin and examined ERK5 block by XMD 8-92.

It was appeared that KLF2 and Mef2c reversely decreased by ERK5 blocker in the atorvastatin added hypoxia condition compared to other factors (Figure 6B). It was well known that the Kruppel-like factor (KLF) family was transcriptional regulators implicated in the regulation of cellular growth and differentiation.[18] One member of this family, KLF2 was strongly expressed in endothelial cells and is required for normal vessel formation. [19] Therefore, I performed to assess whether the KLF2 played a crucial role in response to atorvastatin and some change of KLF2 as transcriptional factor is triggered by atorvastatin. In the normoxia condition, there existed gathered cells of small size which indicated vasculoblast expressing KLF2 and weakly CD31 in the cell (Figure 7A). In the hypoxia condition, vasculoblasts size got bigger a little than the size of the normoxia condition and KLF2 was scattered over cells meaning

KLF2 was inactive condition because KLF2 was a transcription factor. Furthermore, vasculoblasts expressed CD31 weakly and some vasculoblasts expressed CD31 at the cell surface (Figure 7B). However, there was some change in the atorvastatin treated hypoxia condition. KLF2 moved into a nucleus for the role as a transcriptional factor, saturated in the nuclear and that cells expressed more clearly CD31 at vasculoblasts surface that cells and the number of that cells increased. (Figure 7C).High magnification of each marker expression change in vasculoblast was shown (Figure 7D). Taken together, the result showed that KLF2 is the regulator responding by atorvastatin in the hypoxia condition and induces the differentiation of adult EpiPCs into CD31 positive EC. Next, I wondered how KLF2 regulated differentiation of adult EpiPCs into ECs in the response of atorvastatin treated hypoxia condition. It was reported that Snail was mainly activated in the infarct area and it was involved in *de novo* cardiac fibrosis by activating fibroblasts after myocardial infarction in mice.[16] I then investigated the expression pattern at mRNA level of Snail family, Snail1 Slug in KLF2 knock down condition by real time-PCR analysis. Snail1/Slug decreased in the atorvatatin treated hypoxia condition compared to only hypoxia condition, whereas it was reversely increase in the KLF2 knock down condition (Figure 8A). Also, it was identified that the endothelial cell marker CD31 decreased and SMC/Fibroblast marker α-SMA/collagen-1

increased by the KLF2 knock down (Figure 8B). These results suggested that KLF2 was a critical regulator of adult EpiPCs differentiation into ECs rather than into SMCs/Fibroblasts by atorvastatin.

DISCUSSION

The present study has shown that the heart is not a terminally differentiated organ and that resident cardiac stem cells contribute to cardiac repair following injury [14] as well as physiological cardiac tissue homeostasis replacing dead cells during the life. [15] Epicardium plays an important role in the coronary vasculature during embryogenesis and some epicardial cells migrate into the subepicardium where they generate epicardiall progenitor cells (EpiPCs) after EMT. Here in, We established economical new method using a different attaching character of EpiPCs instead of costly Thymosin β-4 and this culture method is easier to handle because we use the enzyme such as collagenase to isolated adult WT1 positive epicardial cells from mouse heart instead of epicardial explants method to culture from a heart tissue directly.

Moreover, I found out that EpiPCs from adult mouse play a key role for vessels formation following MI to enhance cardiomyocyte survival and contribute cardioprotection in the adult mouse. I showed that cultured EpiPCs were differentiated into endothelial cells in the hypoxia condition

mimicked myocardial infarction and vessels were formed in the long time culture. Statin was only reported that it induces a mobilization of murine progenitor cells and endothelial progenitor cells via PI3K/Akt pathways and enhance a endothelialization of injured vessels. [10,11] I applied this fact to the heat injury condition, MI to regenerate cardiac damage. As a result, it was more facilitated when atorvastatin was treated in the hypoxia condition than only hypoxia condition. Not only did the number of differentiated endothelial cells increase, but newly formed vessel's number also increased in atorvatatin treated hypoxia condition.

It reported that several transcription factors regulate endothelial cell development and there are several major regulators of endothelial transcription, including Kruppel-like family (KLF), MEF2C etc.[12] However, it was unknown which transcription factor regulates differentiation of EpiPCs in the adult mouse heart repair, not embryo development phase. We found out that RNA level of KLF2 notably increased compared to other factors by real time-PCR.

The MEK5/Erk5 MAPK cascade was implicated in the regulation of endothelial integrity and vasoprotective statins potently induced KLF4 and KLF4-dependent gene expression via activation of Erk5 in the human endothelial cell.[17] Based on this study, I wondered which factor was regulated via ERK5 in response of atorvastatin and examined ERK5 block by XMD 8-92. It was appeared that KLF2 reversely decreased by ERK5

blocker in the atorvastatin added hypoxia condition compared to other factors. I identified that KLF2 regulates the differentiation of adult EpiPCs into ECs via ERK5 as response by atorvastatin in the hypoxia condition. Hypoxia condition inactivates KLF2, which activates CD31 and CD31express at the cell surface to differentiate EC. On the other hand, after atorvastatin treatment in the hypoxia condition, activated KLF2, strongly localized nuclear and increase CD31 activation and expression. Unfortunately, there was a little cell population possessing differentiation potentiality in the cultured adult EpiPCs so that experiment result like RT-PCR didn't dramatic with big change.

Considering the consequences of our experimentation, there were three populations; First group was the population of fibroblast α-SMA positive CD31 negative, Second group was that fibroblast marker, α-SMA is expressed strongly and endothelial marker, CD31 is weakly. Last group is that α-SMA was weakly expressed and CD31 is strongly expressed, which is named vasculoblast. We found out that α-SMA strongly expressed cell population is already decided to be fibroblast. However, I also figured out that vasculoblasts possessed a potential possibility of the differentiation to bidirectional form.

Followed phenomena can be implicated in hypoxia condition (Figure 9). Hypoxia condition inactivated KLF2 and might induce vasculoblasts to differentiate SMC/FB. On the other hand, after atorvastatin treatment in

hypoxia condition, it activated KLF2 via ERK5 and KLF2 increased CD31, which promoted the differentiation of adult EpiPCs into ECs and suppressed to SMC/FB via Snail inhibition by KLF2.

CONCLUSION

In this study, I demonstrated that atorvastatin facilitates KLF2 activation, which induced the differentiation of adult EpiPCs into ECs, not fibroblast causing fibrosis, and inhibited Snail via KLF2 and also promoted vessel formation in the myocardial infarction condition. In addition to, I found out that most of adult EpiPCs were determined cell type, nearly fibroblast, in the adult epicardial progenitor cells and only some cells have a differentiation potentiality that was vasculoblasts. This study was consistent with our observation that atorvastatin reduced the fibrosis area occurred in the mouse heart compared to only myocardial infarction condition. A better understanding of the mechanisms could help in the development of therapeutic strategies to regenerate cardiac damage.

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FIGURES

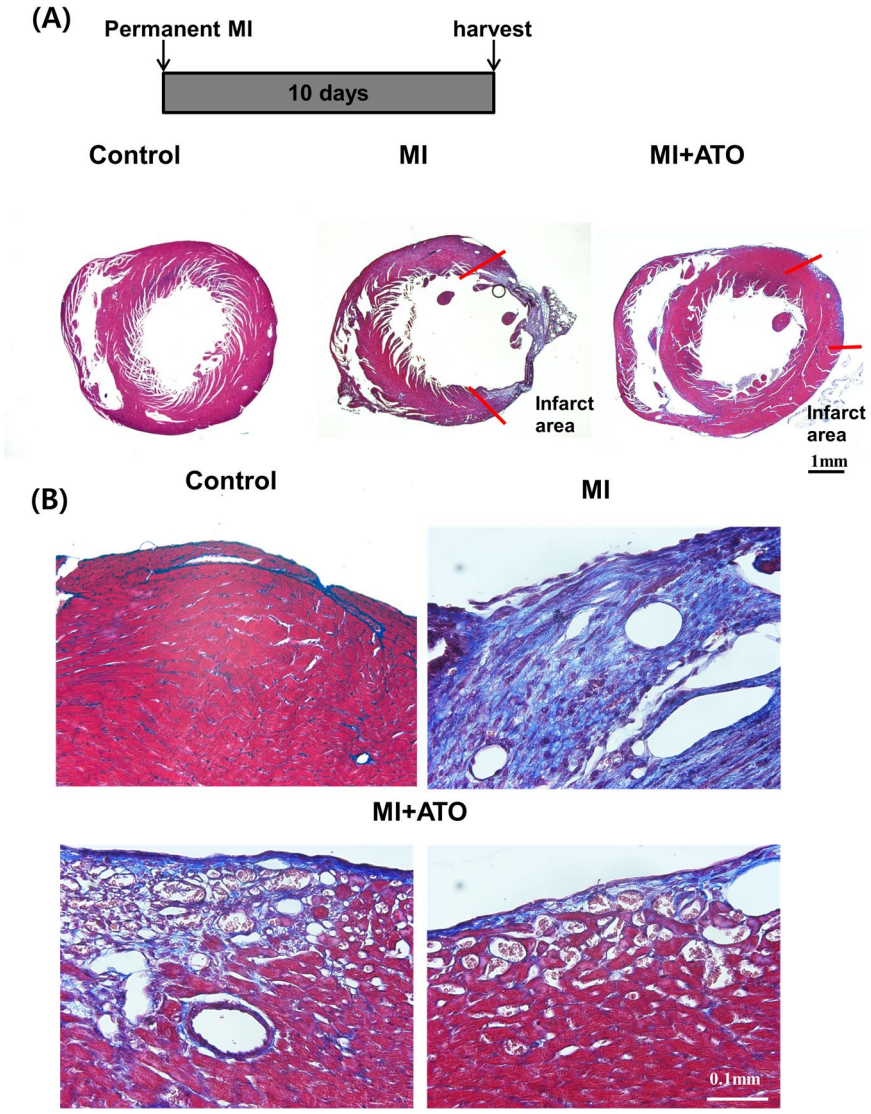


Figure 1. Myocardial infarction (MI) induced the heart muscle fibrosis and this defect was decreased by atorvastatin. (A) After 10days permanent MI, fibrosis area (blue colored) was appeared in the only myocardial infarction condition and the fibrosis area was reduced in atorvastatin

treated MI mouse heart compared with only MI. (12.5x) (B) These pictures were taken with a high-power microscope and the hard fibrosis by MI compared to non-MI control was observed. Vessel like shapes significantly were appeared at the board zone starting fibrosis in the myocardial infarction condition added atorvastatin. (200x)

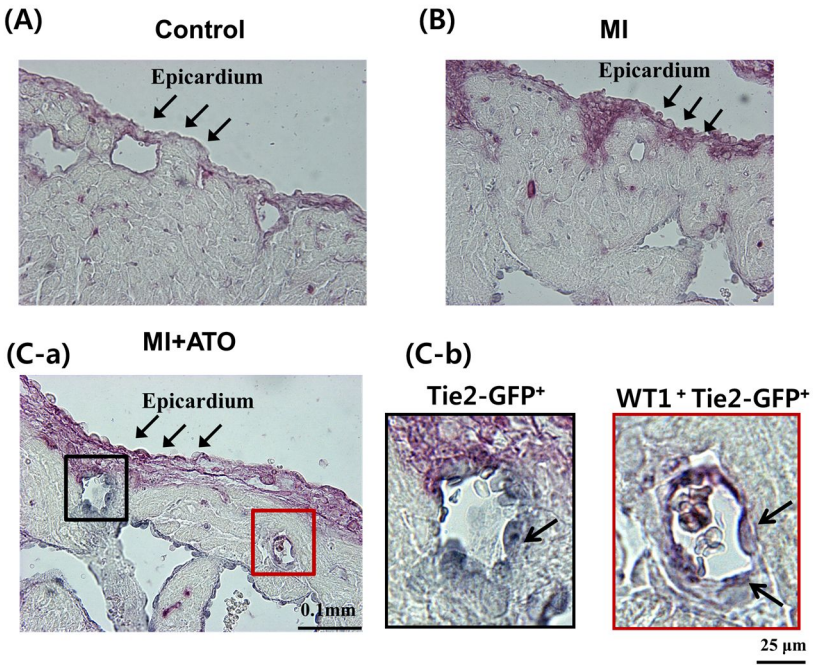


Figure 2. Atorvastatin facilitated the vessel formation in the adult mouse heart of MI condition from Epicardium. (A),(B) By IHC, the epicardium region was stained WT-1 positive in the MI condition compared the non-MI control.(200x) (C-a) Epicardium area consisted of wt-1 positive cells increased in atorvastatin treated MI condition and vessels were found out.(200x) (C-b) These figures were magnified vessels and one vessel was expressing only mature endothelial cell markerTie-2. However, another vessel was composed of epicedial progenitor cell marker wt-1/ tie-2GFP double positive cells or only GFP positive cells.

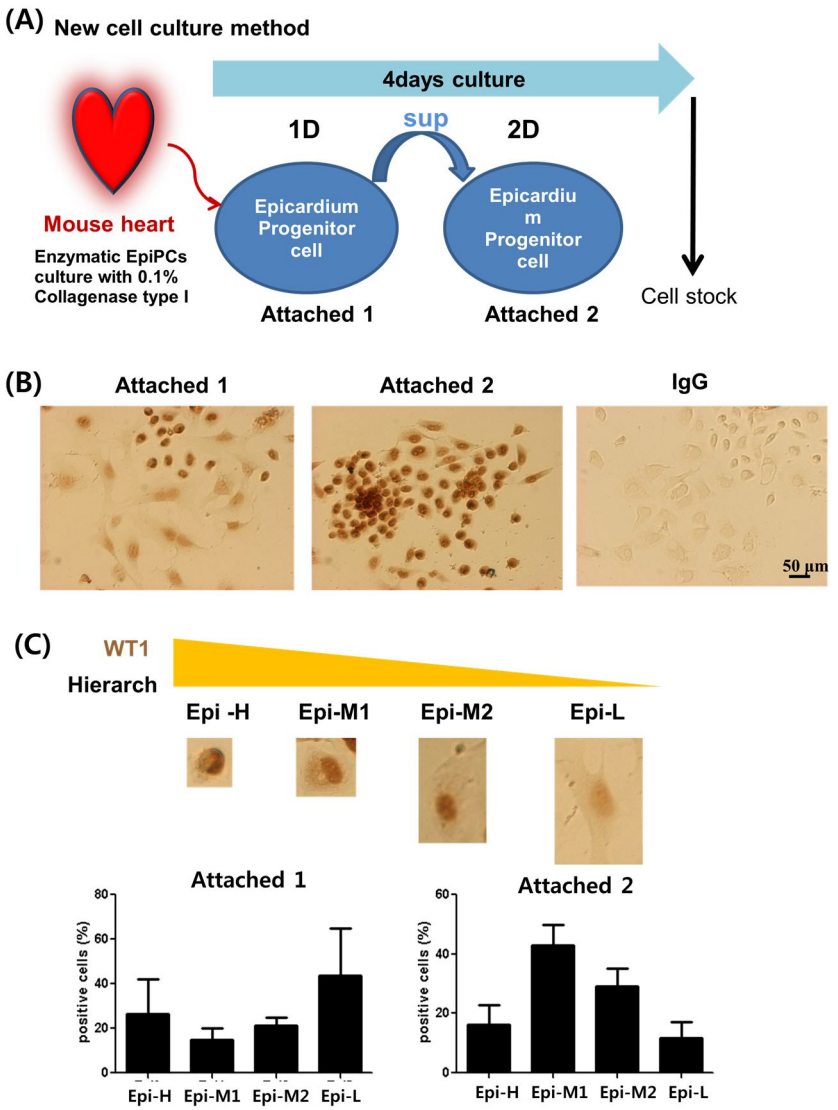
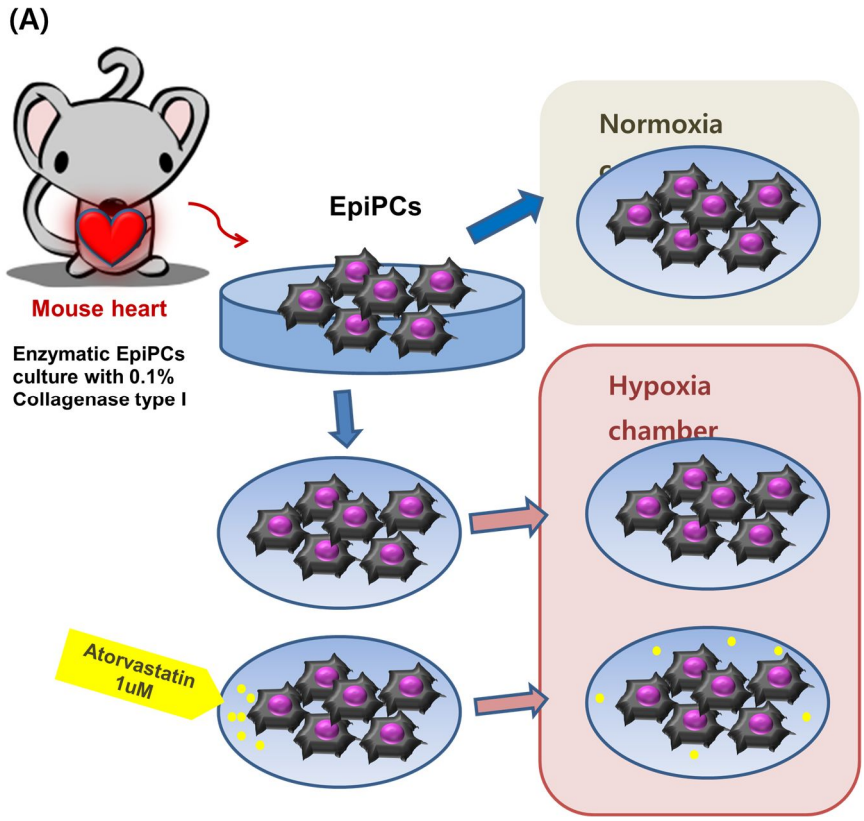
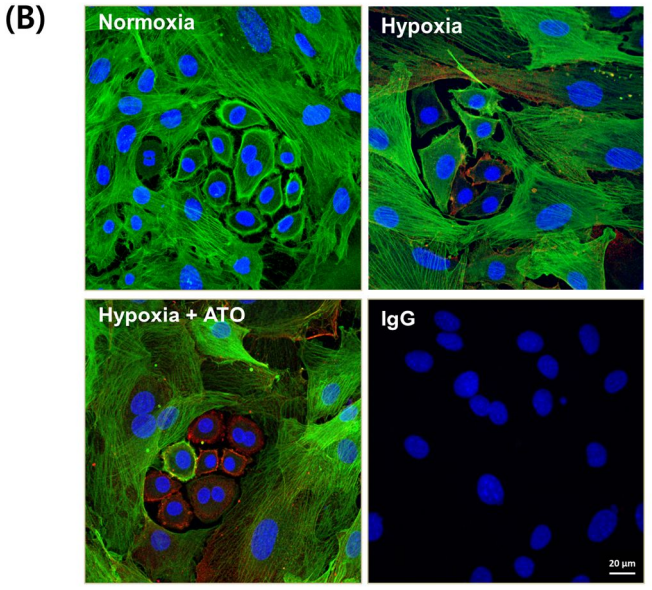
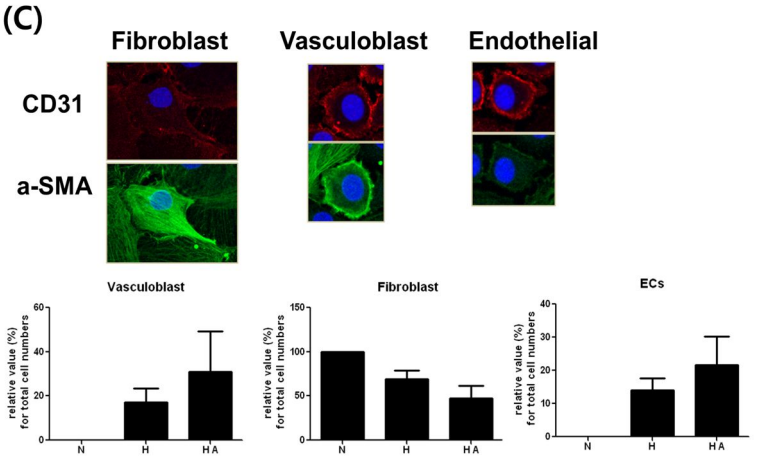


Figure 3. EpiPCs of adult mouse heart were cultured by new culture methods. (A) By new method using a different attaching character of EpiPCs, epicardium was separated from mouse heart with collogenase type1and floating cells were transferred to other dish every 1 day. (B) IHC to evaluate EpiPCs marker WT-1 positivity was done and 1day attached

cells(Attached 1) were stained weakly WT -1 but second day attached cell (Attached 2) appeared mostly strong WT -1 stain. (C) The hierarchy of WT -1 strong stained EpiPCs was higher than weak stained cells and the cell number according to the WT -1 strongly stained was more in Attached2.(Epi-High : high hierarchy, WT-1 strongly stained cell/ Epi-Middle :middle hierarchy, WT-1 partially strong stained cell/ Epi-Low: low hierarchy, WT-1 weakly stained cell)


(A)
Mouse heart
Enzymatic EpiPCs culture with 0.1% Collagenase type I
EpiPCs
Normoxia
Hypoxia chamber
Atorvastatin 1uM


(B)
Normoxia
Hypoxia
Hypoxia + ATO
IgG
20 μm


(C)
Fibroblast
Vasculoblast
Endothelial
CD31
a-SMA
Vasculoblast
Fibroblast
ECs
relative value (%) for total cell numbers
N
H
H A

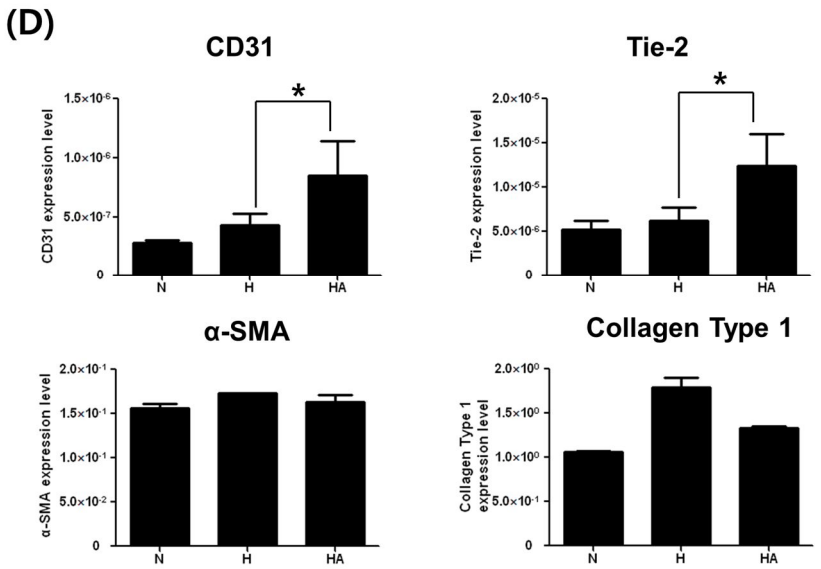


Figure 4. Atorvastatin stimulated endothelial cell differentiation from adult EpiPCs in the hypoxia condition. (A) The MI condition was mimicked to hypoxia condition on the cell level using a hypoxia chamber. After isolate of adult EpiPCs with our culture method, cells were treated with three conditions; normoxia(N), hypoxia(H), hypoxia added atorvastatin(1μM) (HA) (B) IF analysis revealed that there were clustered cells of small size surrounded by mature fibroblast expressing a-SMA and weakly CD31 in the normoxia condition. In the hypoxia condition, there were also gathered cells some expressing strongly α-SMA/weakly CD31 and some cells expressing strongly CD31/ weakly α-SMA. But, the number of cells expressing very strongly CD31 in the clustered cells population increased in atorvastatin treated hypoxia condition compared

to the only hypoxia condition(400x) (C) Cells wrer counted as distinct group of cells expressing differently EC and SMC/Fibroblast markers. The number of fibroblastic cell expressing α-SMA was higher in the N than other conditions(H, HA) Both vasculoblatstic cells expressing weakly α-SMA/CD31 and endothelial cells expressing strongly CD31 were more in atorvastatin treated hypoxia condition (HA)than other conditions.(D) Real time analysis revealed that ECmarker CD31/Tie-2 increased in atorvastatin treated hypoxia condition and SMC/Fibroblast marker α-SMA/collagen-1 didn't almost change or decreased in atorvastatin treated hypoxia condition. *P<0.05

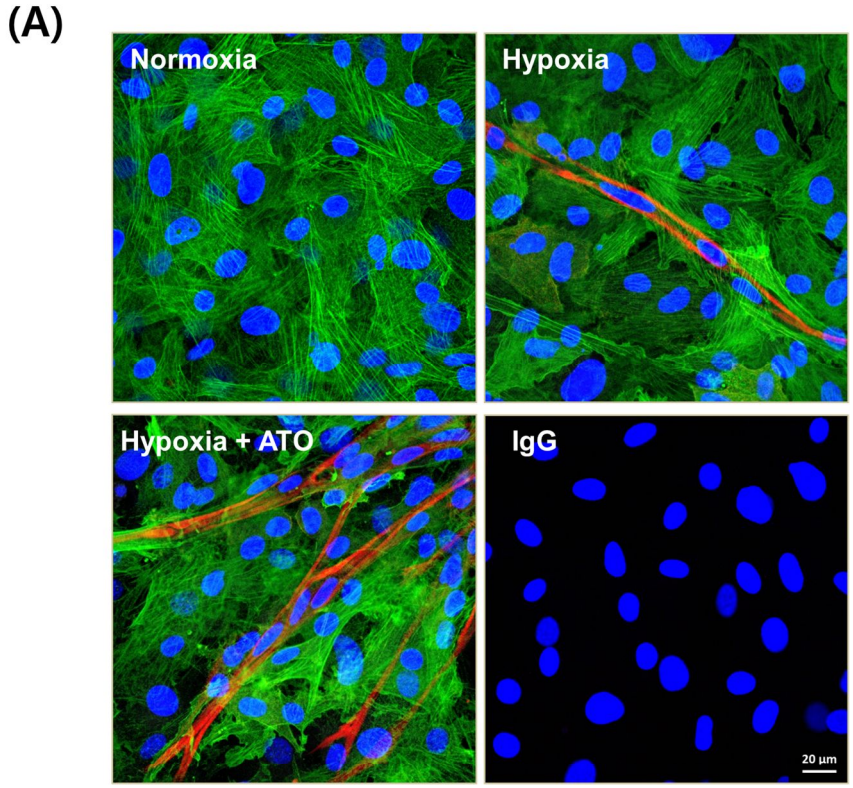


Figure 5. Atorvastatin facilitated new vessels formation in the hypoxia condition. (A) After long time culture (7days), IF result reveled that the number of vessel consisted of CD31positive cells was significantly higher

in atorvastatin treated hypoxia condition. (B) Bargraph of percentages of ECs obtained by IF. The number of CD31 positive ECs increased in HA.

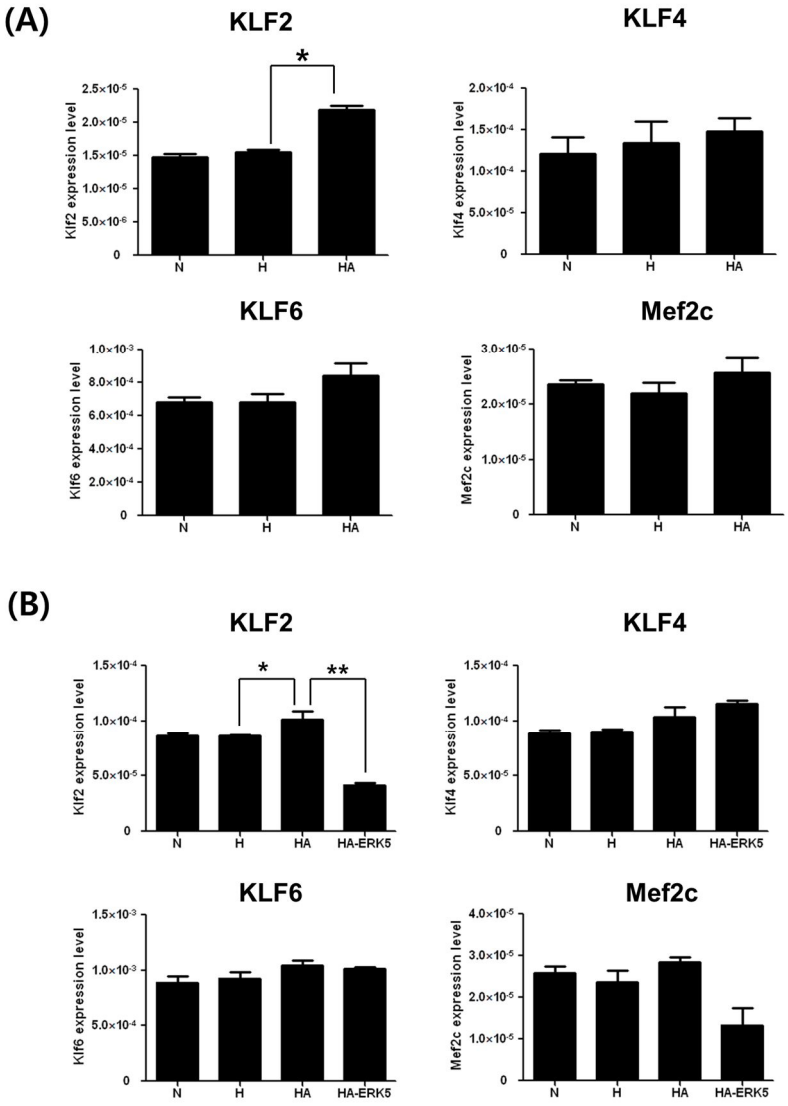
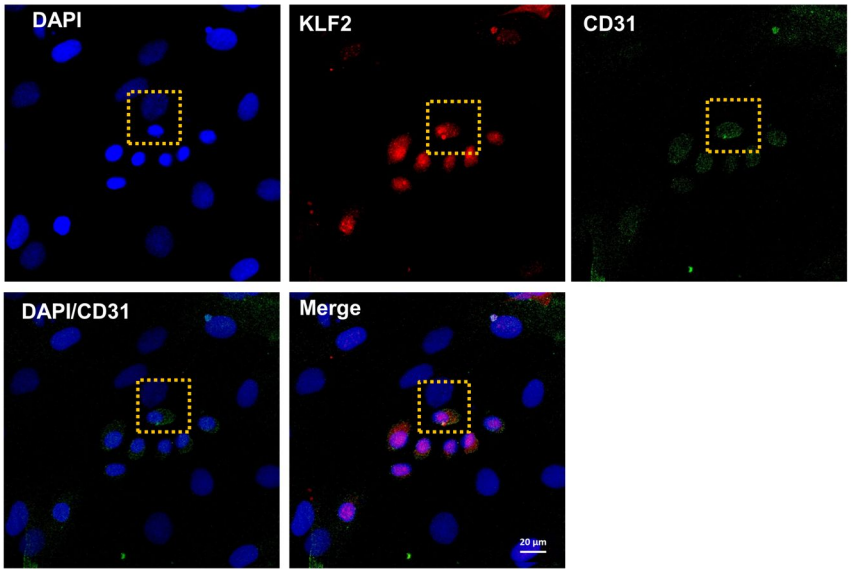
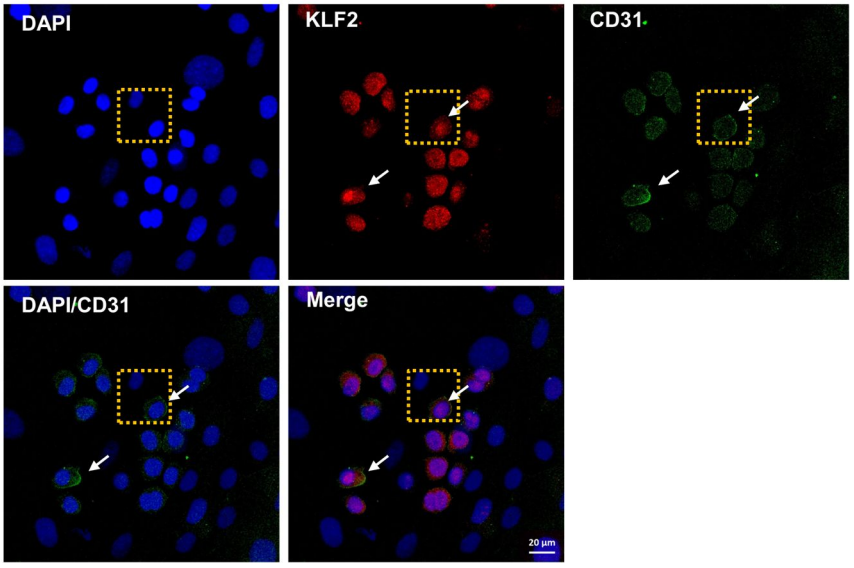


Figure 6. Regulator for EpiPCs differentiation into ECs: KLF2 (A) RNA level of KLF2 notably increased compared to other factors. $P<0.01$ (B) when ERK5 block XMD 8-92 was treated, KLF2 reversely was decreased by ERK5 blocker in the atorvastatin added hypoxia condition compared to other factors. *$P<0.05$ **$P<0.02$

(A) Normoxia



(B) Hypoxia



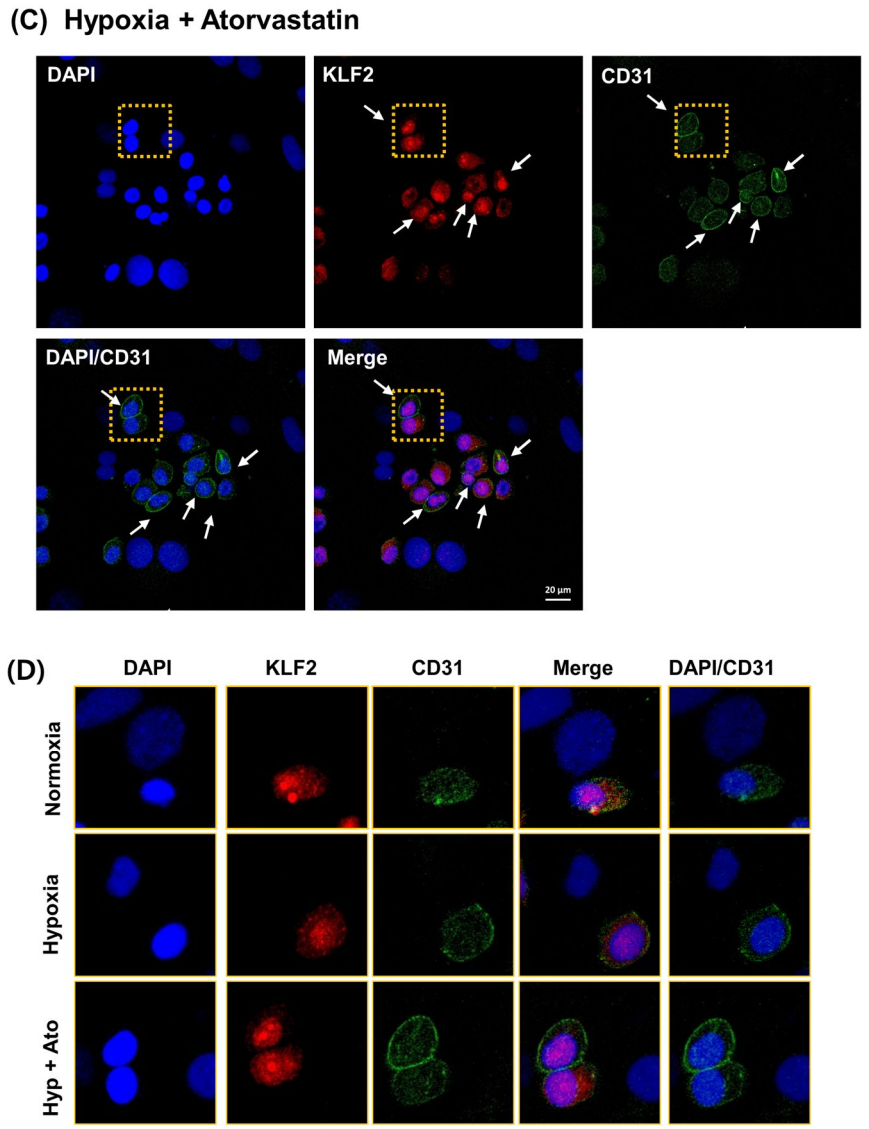


Figure 7. Atorvastatin regulated KLF2 expression in vasculoblast for EpiPCs differentiation into ECs (A) In the normoxia condition, KLF2 was weakly expressed at nuclear in vasculoblasts and CD31 was expressed very weakly in the cell (400x). (B) In the hypoxia condition, vasculoblasts got bigger a little and KLF2 was scattered over cells. Also, most these

cells expressed CD31 very weakly and some vasculobalst expressed CD31 at the cell surface. (C) In the atorvastatin treated hypoxia condition, KLF2 moved into a nucleus in vasculoblasts and that cells expressed CD31more clearly at the cell surface and the number of that cells increased. (D) High magnification of each marker expression change in vasculoblast (A through C)

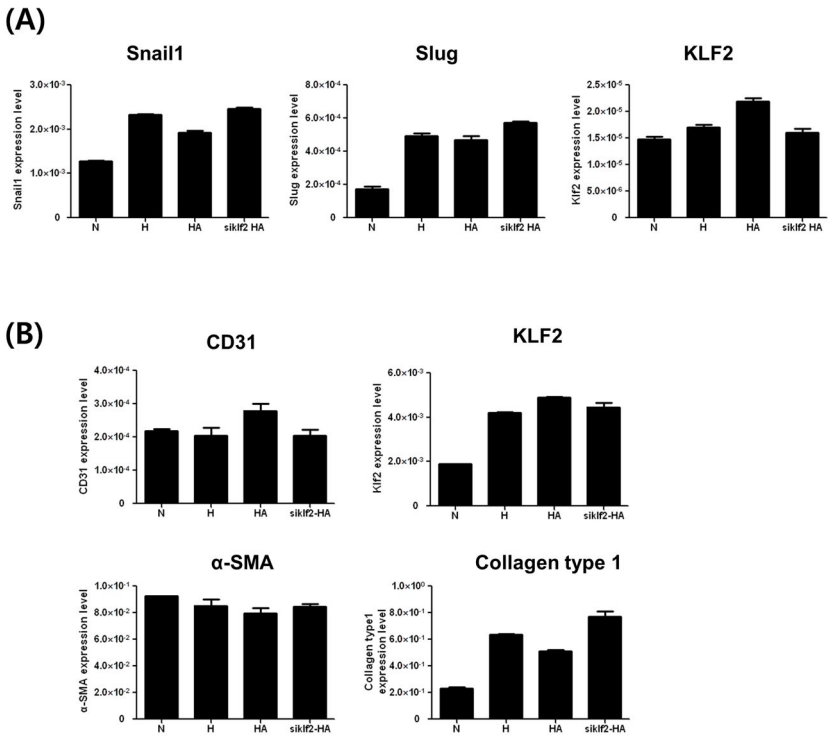


Figure 8. atorvastatin increased KLF2 expression that inhibited fibrosis marker Snail1,Slug and regulated EC maker, SMC/FB marker. (A)Snail1/Slug decreased in the atorvatatin treated hypoxia condition compared to only hypoxia condition, whereas it was reversely increase in the KLF2 knock down condition.(B) When KLF2 was knocked down, the Endothelial cell marker CD31 decreased and SMC/FB marker a-SMA/collagen-1 increased in atorvastatin treated hypoxia condition.

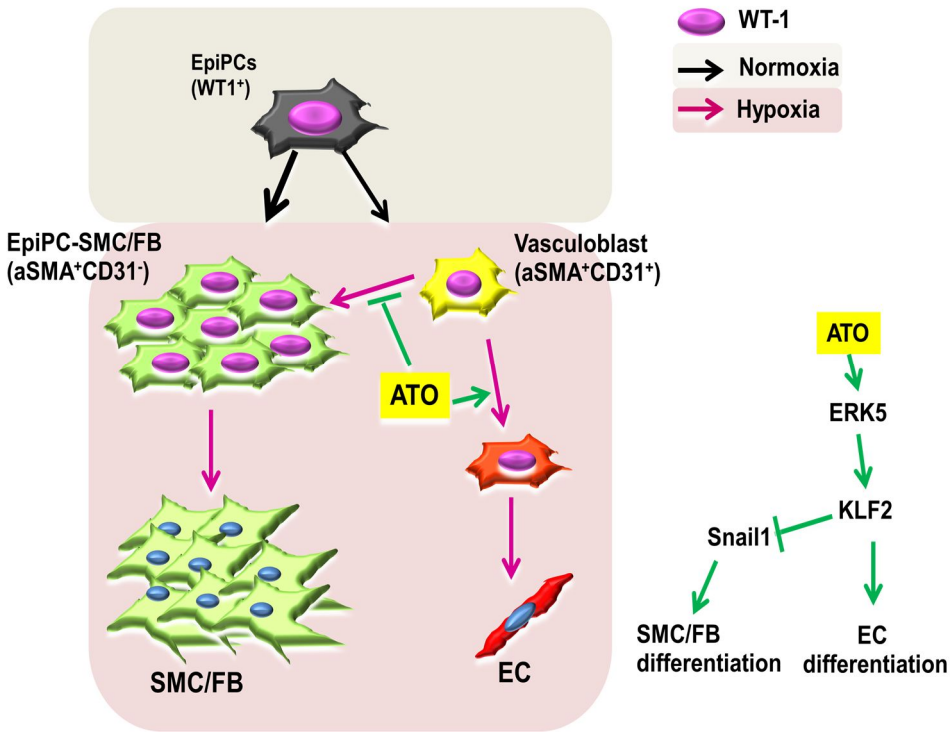


Figure 9. Schematic figure of EpiPCs to ECs differentiation by atorvastatin in hypoxia condition through KLF2.

TABLES

Table 1. Real-Time PCR Primer Sequences

Primer name		Sequence	Size
18S RNA	F	GTAACCCGTTGAACCCCATT	151bp
	R	CCATCCAATCGGTAGTAGCG	
CD31	F	TGCAGGAGTCCTTCTCCACT	180bp
	R	ACGGTTTGATTCCACTTTGC	
Tie2	F	GAGGACAGGCTATAAGGATACG	182bp
	R	GGTGAACAGGTTTCCTCCTAT	
a-SMA	F	CTGACAGAGGCACCACTGAA	160bp
	R	CATCTCCAGAGTCCAGCACA	
Collagen	F	GAGCGGAGAGTACTGGATCG	172bp
	R	GCTTCTTTTCCTTGGGGTTC	
Klf2	F	ACCAAGAGCTCGCACCTAAA	116bp
	R	GTGGCACTGAAAGGGTCTG	
Klf4	F	GTGGACCTGACCTGCCGTCT	170bp
	R	GGAGGAGTGGGTGTCGCTGT	
Klf6	F	TCCACACCTCCATCTTCTCC	173bp
	R	TGCTTTCAAGTGGGAGCTTT	
Mef2c	F	TGATCAGCAGGCAAAGATTG	161bp
	R	ATCAGACCGCCTGTGTTACC	
Snail	F	CTTGTGTCTGCACGACCTGT	177bp
	R	CAGGAGAATGGCTTCTCACC	
Slug	F	CATTGCCTTGTGTCTGCAAG	189bp
	R	CAGTGAGGGCAAGAGAAAGG	

국문초록

심근경색에서 Klf2 를 통한 아토바스타틴의 심장 외막 전구세포에서 혈관 내피 세포로의 분화 촉진

목적

심근경색은 면역반응과 관련 있어 심근의 섬유화를 유발해 심근세포의 사멸을 일으켜 심장 기능의 저하를 가져온다. 심장 외막에는 분화전의 전구세포가 존재하는데 배아의 발달 단계만 아니라 성인의 심장에서도 존재하여 심근경색과 같은 병적 상황에서 심근기능의 회복을 위해 활성화되지만 혈관재생을 위한 내피세포로의 분화는 낮은 수준으로 이뤄진다. 본 연구에서는 아토바스타틴이라는 특정 약물을 사용해 심근경색 상황에서 미비한 수준으로 이뤄지는 혈관형성을 ERK5 를 통한 KLF2 발현을 조절하여 혈관 내피 세로로의 분화를 촉진하여 심근 기능의 회복을 도와줄 수 있는 치료 가능성을 제시하는 연구를 수행하였다.

방법과 결과

우선 성인 쥐의 심장에서 심장 외막의 전구세포를 분리하기 위한 방법을 세포가 붙는 시간이 다르다는 것을 이용해 고안하였고 심장 외막 전구세포의 표지 마커인 WT-1 을 이용해 분리한 세포들이 심장 외막의 전구세포임 확인하였다. 이렇게 분리한 세포들을 심근경색

상황을 세포 수준에서 적용하기 위해 하이폭시아 챔버를 사용한 저산소증 조건을 만들어 주었고 아토바스타틴을 투여했을 때 내피세포로 분화되는 것이 촉진 되는 것을 내피세포 마커인 CD31 과 Tie-2 로 확인 하였고 시간이 더 지나자 혈관이 형성되고 아토바스타틴에 의해 더 촉진 됨을 알아 내였다. 이러한 아토바스타틴의 효과가 ERK 를 통해 KLF2 의 발현을 촉진하여 심장 외막의 전구 세포들이 혈관 내피세포로 분화하도록 한다는 것을 밝혀 내었다. 또한 혈관 내피세포로의 분화 시 아토바스타틴에 의해 심근의 섬유화를 일으키는 Snail family(Snail1, Slug)를 억제하고 그런 현상이 KLF2 를 통해 일어남을 밝혀냈다.

결론

아토바스타틴은 저산소증 상태에서 KLF2 의 발현을 촉진하여 심장 외막의 전구세포들이 혈관 내피세포 분화하는 것을 촉진하면서 Snail 을 억제해 심근의 섬유화되는 것을 막아준다. 더욱이 이렇게 분화된 혈관 내피 세포들이 혈관을 형성하는 것을 더욱 촉진하여 준다. 따라서 아토바스타틴으로 인한 KLF2 의 작용은 심근경색과 같은 심장 기능의 손상을 회복할 수 있는 치료 타켓으로 작용할 가능성을 제공한다.

주요어: 심근경색, 심장 외막 전구세포, 스타틴, Klf2, Snail

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