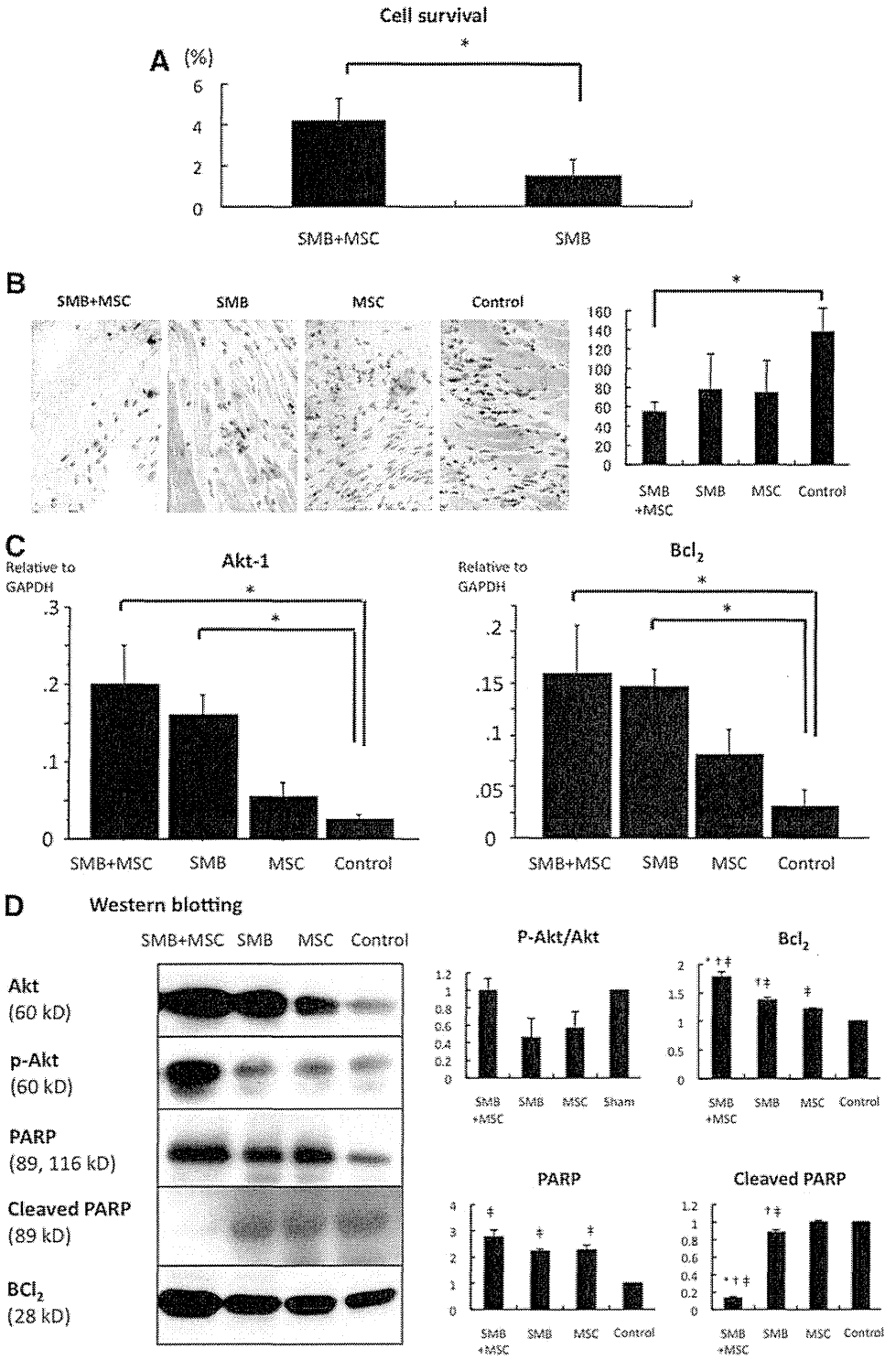


**FIG. 6.** Cell survival. **(A)** Survival of transplanted cells of rat origin was significantly greater in the SMB+MSC sheet group than in the SMB sheet group. $N = 4$ in each group. $^*p < 0.05$. **(B)** The number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive myocytes was significantly lower in SMB+MSC group than in control. $N = 4$ in each group. $^*p < 0.05$. **(C)** Expressions of mRNA in the transplanted infarct area of hearts were determined by real-time PCR using rat-specific primers. The expressions of Akt-1 and $Bcl_2$ mRNA were significantly increased in the SMB+MSC sheet group compared with the other groups. $N = 4$ in each group. $^*p < 0.05$. **(D)** Western blotting showed that $Bcl_2$ was much more enhanced, and cleaved PARP was significantly downregulated in the SMB+MSC group. There was no significant difference in the ratio of phosphorylation of Akt over Akt. $N = 3$ in each group. $^*p < 0.05$ versus SMB. $^\dagger p < 0.05$ versus MSC. $^\ddagger p < 0.05$ versus control. Error bars = SD. Color images available online at www.liebertpub.com/tea

sheets' release of HGF and VEGF but not of IGF-1, bFGF, or SDF-1, *in vitro*. The transplantation of SMB-only cell sheets into the chronically ischemic failing rat heart resulted in reversed LV remodeling, including increased capillaries, attenuated collagen accumulation, and prolonged cell survival, which increased global functional recovery, mediated by the paracrine effects of upregulated HGF and VEGF in the myocardium.

Recent studies, including ours,[3–9] have suggested that a paracrine effect mediated by cytokines secreted from the transplanted cell sheets is a likely mechanism for the therapeutic effects on the myocardium, which was a focus of the present study. Here, we added h-MSCs to the cell sheets to enhance the potential performance of the transplanted r-SMB sheets. Our *in vitro* findings, that h-MSCs enhanced rat mRNA levels and the secretion of cytokines such as r-HGF

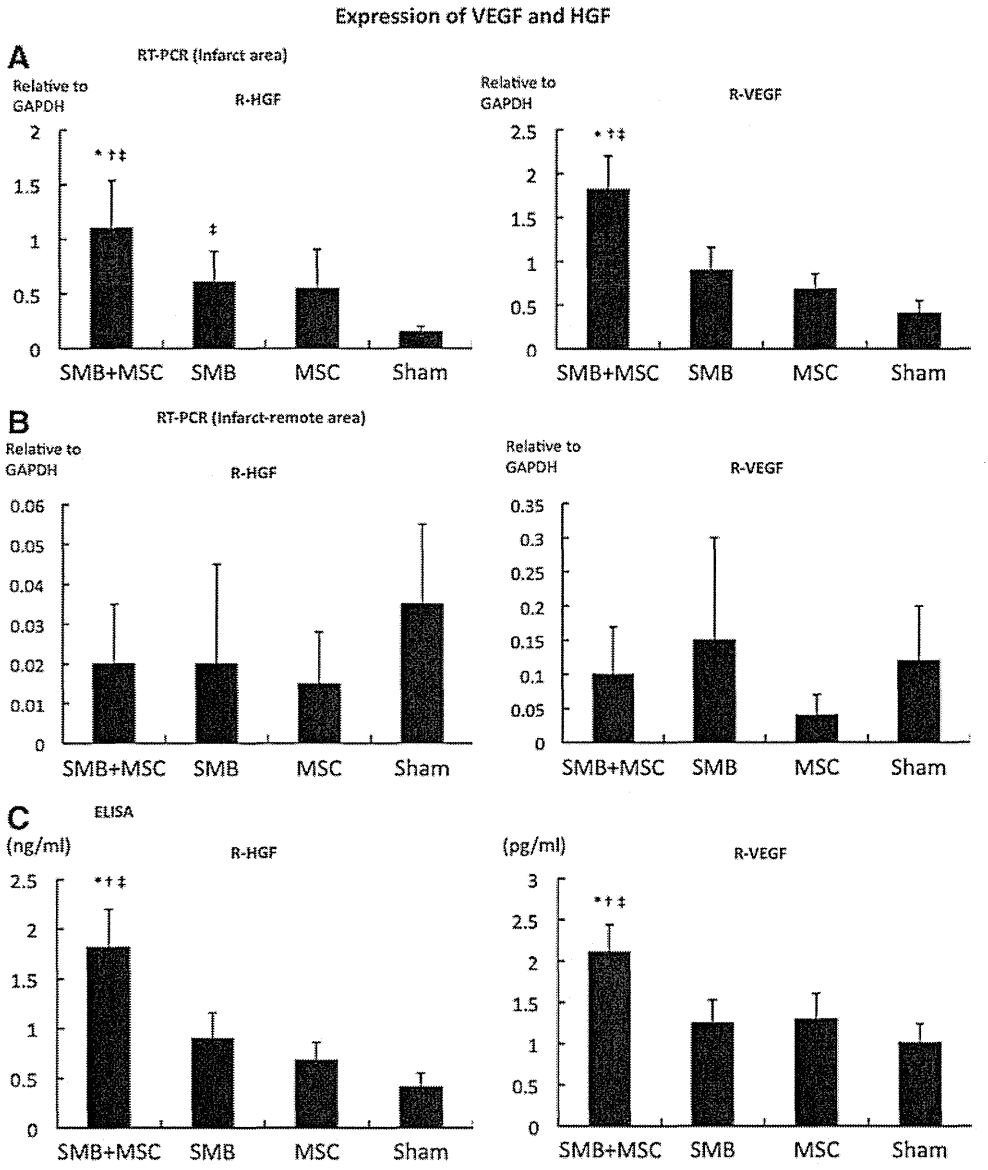


**FIG. 7.** Expression of VEGF and HGF is higher at the infarct area. **(A, B)** Levels of mRNA in the transplanted infarct and infarct-remote heart areas by real-time PCR using rat-specific primers. The HGF and VEGF mRNA expressions within the transplanted infarct area of the hearts were significantly increased in the SMB+MSC sheet group compared with the other groups. $N=4$ in each group. $^{*}p<0.05$ versus SMB. $^{\dagger}p<0.05$ versus MSC. $^{\ddagger}p<0.05$ versus sham. **(C)** Intramyocardial protein levels of HGF and VEGF, analyzed by ELISA, were significantly greater in the heart in the SMB+MSC sheet group compared with the other groups. $^{*}p<0.05$ versus SMB. $^{\dagger}p<0.05$ versus MSC. $^{\ddagger}p<0.05$ versus control. Error bars = SD.

and r-VEGF from r-SMBs, suggested that transplanted co-cultured cell sheets would secrete r-HGF and r-VEGF *in vivo*. Although the exact mechanisms by which "feeder layers" support cell growth have not been elucidated, it is possible that h-MSCs enhance the r-SMBs directly (via cellular interaction) or indirectly (via secreted cytokines from the h-MSCs).[16] A more comprehensive examination aimed at differentiating these effects might help reveal how feeder layers work.

HGF and VEGF participate in many complex molecular and cellular mechanisms, and their signaling pathways have been intensively investigated *in vivo*.[3,9] SMBs or MSCs act as the natural supplier of both HGF and VEGF and provide feasible and safe sources for cell therapy in clinical applications. Indeed, SMBs and bone marrow-derived mesenchymal stem cell sheets can secrete growth factors (e.g., HGF and VEGF) into the myocardium and accelerate neovascularization in the damaged area.[5–8] More recent reports have revealed that angiogenesis induced by HGF or VEGF, an antifibrotic effect promoted by HGF, or the migration and survival of SMBs supported by VEGF,[17] could be beneficial to an impaired heart.[7,8] In addition, our data from a cytokine/chemokine multiplex immunology assay indicate that leptin may also be beneficial (e.g., by inducing angiogenesis though the Jak/STAT pathway).[18] Other cytokines may also contribute to the improvement of cardiac function by single-cell-type cell sheets in as-yet-undiscovered ways.

The mechanism by which the implanted cell sheet attenuates ventricular remodeling and improves cardiac function seems to depend on the cell sheet being placed over the scarred area of the myocardium and leads to repair of the anterior wall thickness, reduction of LV wall stress, and the improvement of ejection performance.[3] Previous studies indicated that the surviving myocardium and implanted cell sheet attenuate complex cellular and molecular events, including hypertrophy, fibrosis, apoptosis of the myocardium, and the pathological accumulation of extracellular matrix.[9] Similarly, the greater cellularity observed after cell-sheet

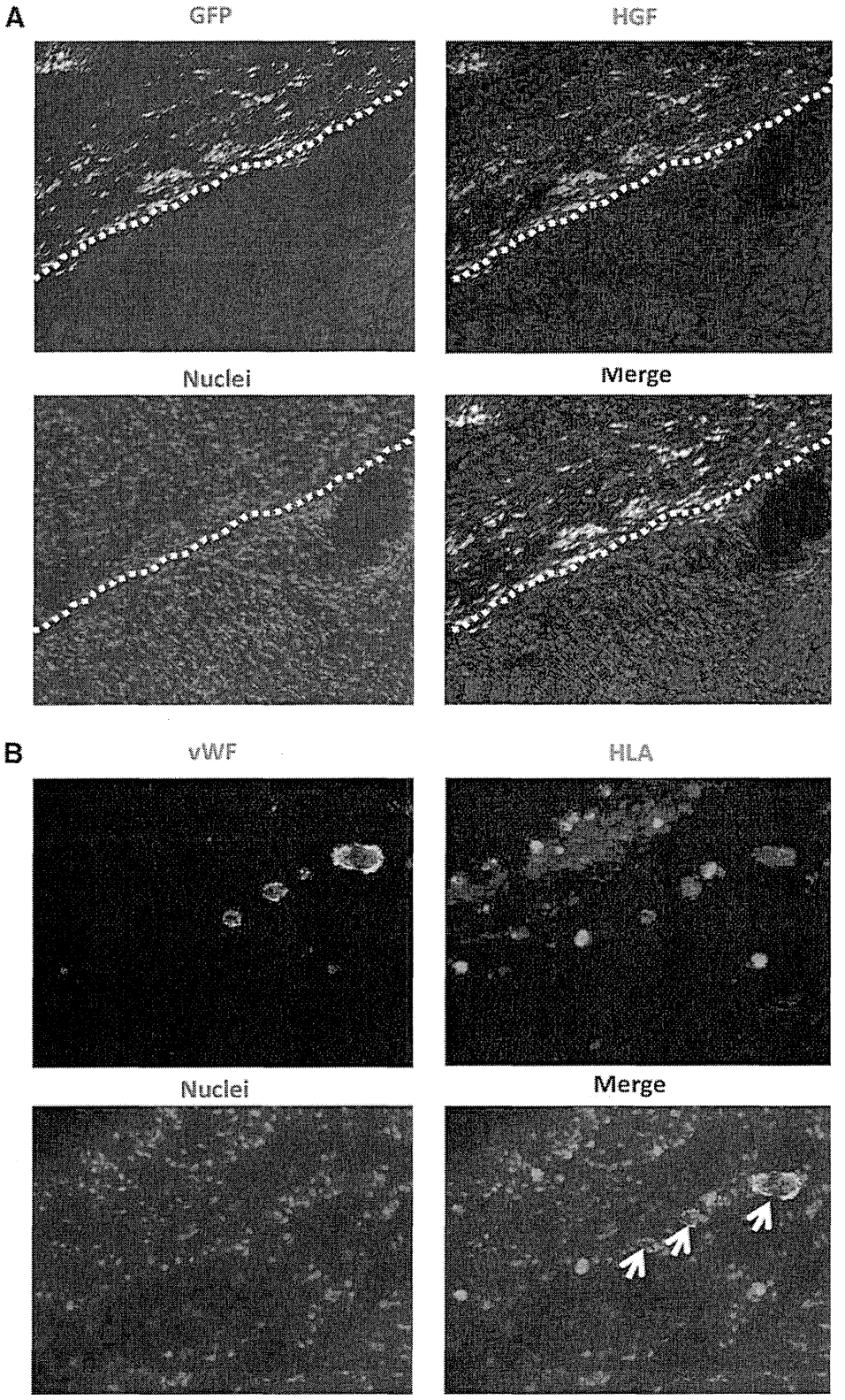


**FIG. 8.** Characterization of transplanted cells. **(A)** Cryosections were stained with an antibody to HGF to detect the distribution of SMB and HGF in the heart. HGF expressions and GFP-positive cells were found in the myocardium after transplantation of the SMB+MSC sheet. White broken line shows the border between the transplanted cell sheet and the host heart. Green indicates GFP; red, HGF; blue, nuclei. **(B)** Cryosections were stained with antibodies to human leukocyte antigen (HLA) and to von Willebrand factor (vWF). Human vWF-positive (white arrows) staining was observed in the host vessels in the h-MSC-transplanted group. Green indicates vWF; red, HLA; blue, nuclei. Color images available online at www.liebertpub.com/tea

treatment might have resulted from released SDF-1, which is related to cell migration, adhesion, and proliferation, by the transplanted cell sheet[19,20]

In this study, we performed additional investigations on the paracrine mechanism from a new perspective, by analyzing signaling pathways within the myocardium following cell-sheet transplantation because the signals induced by released paracrine mediators presumably activate phosphorylation cascades of signaling molecules. We found that STAT3 and Akt phosphorylations were significantly increased, and cleaved PARP was significantly downregulated, 24 h after the co-cultured cell-sheet implantation. Together

with our findings that vascular density was significantly enhanced, and myocardial apoptosis and fibrosis was significantly attenuated in the co-cultured group, it is possible that the co-cultured cell-sheet transplantation induced angiogenesis partially through the Jak/STAT signaling pathway[18] and that it prolonged cell survival by preventing apoptosis through PI-3K/Akt-mediated signaling, which is partially modulated by HGF.[21]

Although we emphasized combining SMBs with h-MSCs, some investigators have focused on different combinations of various cell sources. Sekine *et al.*[22] reported that cardiomyocytes co-cultured with endothelial cells induce greater numbers of capillaries, due to increased secretion of angiogenic growth factors.[22] Another report showed that a dermal fibroblast sheet co-cultured with endothelial progenitor cells was more effective than either single cell-type sheet for improving damaged heart function, accompanied by the inhibition of fibrotic tissue formation and the acceleration of neovascularization in the infarcted myocardium.[23] Thus, the paracrine effect may be improved by combining different cell sources; however, further investigation focused on determining the optimal combinations of cell sources is needed.

Regarding h-MSCs as a cell source, bone marrow-derived or adipose tissue-derived stem cells are reported to differentiate into mature endothelial cells and participate in blood vessel formation in the recipient heart.[24] The presence of endothelial capillary networks improves the survival and organization of implanted cells by maintaining a minimum intercapillary distance to provide oxygen and nutrients. Therefore, the presence of endothelial capillary networks may be partially correlated with cardiac function.

For future tissue engineering for cardiac therapy, the creation of thick cell-dense constructs with functional vessels may be essential. Capillary formation occurs via two basic vessel-constructing processes: angiogenesis, that is, the formation of new capillaries via sprouting or intussusception from pre-existing vessels, and vasculogenesis, which occurs in the developing embryo.[25] Here, the morphology of the vessel formation within myocardial tissues, including the diameter, composition, and fragility of vessel walls, suggested that improper vascularization may occur under pathological conditions. It is likely that not only biological factors but also physical stimuli such as flow and shear stress are required to mimic the *in vivo* environment and enable the formation of mature vascular networks.

A potential limitation of this study is that the exact number of transplanted cells was different in each group *in vivo*. Clinically, open-chest surgery is unlikely to gain easy acceptance except in certain situations; however, less invasive methods (e.g., intracoronary catheter-based procedures) might be technically difficult for carefully placing the cell sheets. Additionally, further studies that include longer timeframe than 8 weeks are needed to examine a longer term restoration of heart function post-MI. It is likely that the source of HGF is the transplanted SMB; however, it is unclear whether the source of other therapeutic cytokines is the transplanted cells, such as SMBs, MSCs, or both, or native cardiac cells.

In conclusion, we found that h-MSCs enhanced the paracrine effects of r-SMB sheets, thus enhancing angiogenesis, lowering fibrosis, inhibiting cellular hypertrophy, improving cardiac function, and prolonging cell survival in MI model rats. These observations of improved effects from this co-cultured cell sheet may lead to new regeneration therapies for heart failure following advanced cardiomyopathy that are superior to the conventional SMB-only cell-sheet technique.

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## Disclosure Statement

T.S. is a consultant for CellSeed, Inc. T.O. is an Advisory Board Member in CellSeed, Inc., and the inventor/developer designated on the patent for temperature-responsive culture surfaces.

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ORIGINAL ARTICLE

# Impact of cardiac stem cell sheet transplantation on myocardial infarction

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**Abstract**
*Purpose* Myocardial infarction (MI) remains a major cause of mortality because of the limited regenerative capacity of the myocardium. Transplantation of somatic tissue-derived cells into the heart has been shown to enhance the endogenous healing process, but the magnitude of its therapeutic effects is dependent upon the cell-source or cell-delivery method. We investigated the therapeutic effects of C-Kit positive cardiac cell (CSC) cell-sheet transplantation therapy in a rat model of MI.
*Methods and results* CSCs of human origin were sorted and cultured to generate scaffold-free CSC cell-sheets. One-layered or 3-layered cell-sheets were transplanted into nude rats 1 h after left coronary artery ligation. We observed a significant increase in the left ventricular ejection fraction and a significant decrease in left ventricular systolic dimension at 2 and 4 weeks in the 3-layer group, but not in the 1-layer or sham groups. Consistently, there was less accumulation of interstitial fibrosis in the 3-layer group than in the 1-layer or sham groups. Moreover, capillary density was significantly greater in the 3-layer group than in the 1-layer or sham groups.
*Conclusions* The 3-layered cell-sheet improved cardiac function associated with angiogenic and anti-fibrotic effects. Thus, CSC is a promising cell-source to use with the cell-sheet method for the treatment of cardiac failure, as long as a sufficient number of cells are delivered.

**Keywords** Cardiac · Stem cell · Myocardial infarction

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## Introduction

The limited regenerative capacity of the myocardium accounts for the fact that cardiac failure related to myocardial infarction (MI) remains a major cause of morbidity and mortality worldwide, despite major advances in medical and/or interventional treatments [1]. The treatment of cardiac failure relies on strategies that are designed to target and/or limit residual or persistent myocardial ischemia, additional myocardial damage, pathological cardiac remodeling, and hemodynamic impairment, including cardiac dyssynchrony [2]. On the other hand, the transplantation of somatic tissue-derived stem/progenitor cells into the heart has been shown to enhance the endogenous healing process of the damaged myocardium, while the magnitude of the therapeutic effects are dependent on the cell-source, cell-number, cell-delivery method, and target cardiac pathology [3–5]. It has been shown that the transplantation of C-kit-positive heart-derived cells into the MI heart yields functional recovery, mediated by proliferation and differentiation into the heart-composing cells in situ, and by releasing cardioprotective factors that activate native healing processes [6]. However, the optimal preparation and delivery method of CSCs into the heart has not been established.

The cell-sheet method, in which aggregated cells in a sheet shape cultured under a thermoresponsive dish are attached to the epicardial surface [7], has been shown to deliver a large scale of cultured cells with minimal damage to the cells or native cardiac tissues [8]. This enhances its therapeutic effects and minimizes inflammation-related

complications, representing a promising cell-delivery method in CSC transplantation therapy [9]. However, there are concerns about potential ischemia of the implanted cell-sheet, which would limit cellular function, survival, and therapeutic potential. According to a previous study, a 3-layered cell-sheet generated by skeletal myoblasts showed greater therapeutic effects than a 1-layered cell-sheet, while a 5-layered cell-sheet did not enhance the effects, possibly because of ischemia in the implanted cell-sheet [10]. Based on the hypothesis that the therapeutic potential of CSC cell-sheet treatment might be dependent on the number of layers of the cell-sheet, we investigated the therapeutic effects of CSC cell-sheet transplantation therapy on MI hearts using a rat model.

## Methods

All studies using human tissues and experimental animals were carried out under approval of the institutional ethical committee. Human tissues were collected only after obtaining written informed consent. This investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85-23, revised 1996). All experimental procedures and evaluations were carried out in a blinded manner.

### Isolation and culture of C-Kit-positive human cardiac cells and cell-sheet generation

Discarded cardiac tissue samples were taken from the left ventricular apex of a 31-year-old man with dilated cardiomyopathy, requiring daily cardiovascular procedures in Osaka University Hospital. Cardiac cells were dissociated from the tissues, cultured, and then sorted for C-kit using FACSAria (BD Biosciences) to yield C-Kit positive cardiac cells, which were then cultured for expansion with multiple passages. The cells were then incubated in thermoresponsive dishes (35 mm UpCell, CellSeed, Tokyo, Japan) at 37 °C for 2 days prior to transplantation, when the cells were incubated at 25 °C to induce their spontaneous detachment, to yield a mono-layered scaffold-free CSC cell-sheet that included $1.5 \times 10^6$ cells (Fig. 1a). The 3-layered cell-sheet was generated by filling up the mono-layered cell-sheet, as described previously [10].

### Generation of AMI model and CSC cell-sheet transplantation

Thirty-nine athymic female nude rats, 8 weeks of age, were subjected to permanent ligation of the left coronary artery (LCA) under general anesthesia with endotracheal intubation and isoflurane inhalation, as previously described [10]. LCA ligation-related death occurred prior to treatment in 16 %. The rats that survived for 50 min after the ligation were randomly assigned to the following three treatment groups: transplantation of a 3-layered cell-sheet ($n = 12$), transplantation of a l-layered cell-sheet ($n = 10$), or a sham operation ($n = 11$). In the two transplantation groups, the cell-sheet was attached directly to the epicardial surface of the ischemic/infarct area (Fig. 1b) [10]. The cell-sheet was large enough to cover all of the ischemic or infarcted area. By 20 min after the transplantation, when the cell-sheets were properly fixed to the cardiac surface, the chest was closed and the rats were allowed to recover in individual temperature-controlled cages until they were killed 28 days after the treatment.

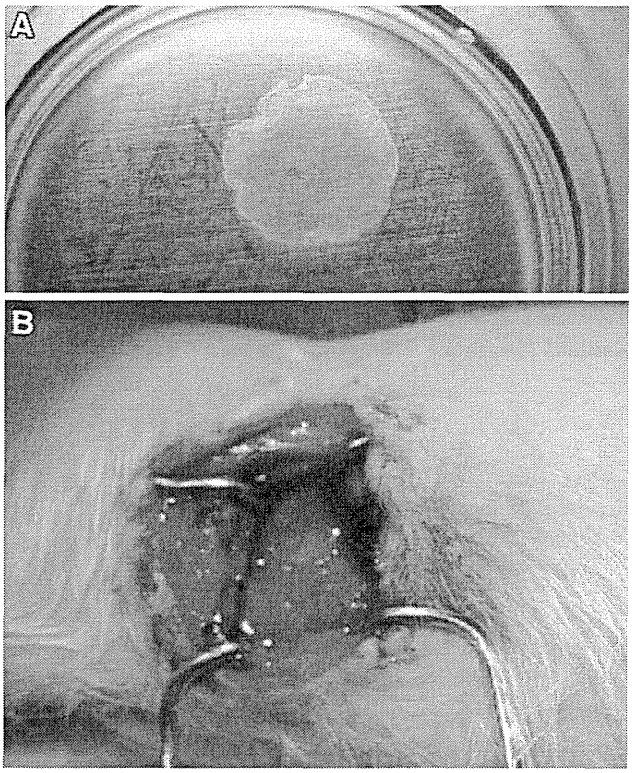


**Fig. 1** A mono-layered cell-sheet was generated by c-kit positive cardiac cells of human origin on thermoresponsive dishes in vitro (**a**). A mono-layered or 3-layered cell-sheet was transplanted over the left ventricular free wall of the rat heart, which had been subjected to ischemia by permanent ligation of the corresponding coronary artery, 1 h prior to the treatment (**b**)

### Transthoracic echocardiography

Transthoracic echocardiography was performed under isoflurane inhalation, using a system equipped with a 12 MHZ transducer (GE Healthcare). Diastolic and systolic dimensions of the left ventricular diastolic and

systolic dimensions (LVDD and LVDS, respectively) were measured at the papillary muscle level by the M-mode, while the LV ejection fraction (LVEF) was calculated by the following formula: $(LVDD^3–LVDS^3)/LVDd^3 \times 100$ [10, 11].

### Histology

The ventricles were immerse-fixed in 4 % paraformaldehyde, embedded in paraffin, and cut into 5 micrometres using a microtome for histological studies. The sections were stained by hematoxylin–eosin (HE) or Masson trichrome (MT) and assessed by optical microscopy (Olympus, Tokyo, Japan). Metamorph software was used to separate stained and non-stained myocardium by MT staining and to quantitatively calculate each area. The sections were labeled immunohistologically by polyclonal anti-von Willebrand factor antibody (vWF, DAKO, Glostrup, Denmark), and visualized by the LSABTM kit (DAKO), which is an automated immunostaining system based on the LSAB Lepto strept avidin–biotin-peroxidase method. The sections were labeled immunohistologically by the anti-human-specific HLA antibody or anti-cardiac troponin (cTn) I antibody, visualized by corresponding secondary antibodies that were counterstained by DAPI, and assessed by confocal microscopy (Olympus).

### Statistics

Values are expressed as mean ± SEM. The three groups were compared with 1-way or 2-way ANOVA as appropriate, followed by the Fisher protected least-significant difference test, or the Kruskal–Wallis test, followed by the post hoc pairwise Wilcoxon-Mann–Whitney $U$ test, as appropriate. Differences were considered significant at $P < 0.05$. All analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA).

## Results

### Functional recovery following CSC cell-sheet transplantation

Scaffold-free CSC cell-sheet was prepared from primary C-kit positive cardiac cells of human origin, cultivated in thermoresponsive dishes. We transplanted the 1-layered or 3-layered cell-sheets onto the epicardial surface of the nude rat 1 h after the permanent LCA ligation. A sham operation was performed for the control group. Cardiac performance was serially assessed by transthoracic echocardiography just after the treatment (baseline), and then 1, 2, and 4 weeks after the treatment.

Before any intervention, the LVEF, LVDD, and LVDS did not differ significantly among the groups (Fig. 2). However, for 4 weeks after treatment, the LVEF showed a significantly progressive reduction, while the LVDD and LVDS showed a significantly progressive increase in the sham group and the 1-layer group. Conversely, in the 3-layer group, the LVEF showed a significant increase, and the LVDS showed a significant decrease 2 and 4 weeks following the transplantation, while the LVDD did not change significantly in this group over the 4 weeks. Notably, the LVEF in the 3-layer group was significantly greater than that in the 1-layer group or sham group, while the LVDS in the 3-layer group was significantly lower than that in the 1-layer group or sham group. The LVDD did not differ significantly among the groups at any time.

### Histological reverse LV remodeling following CSC cell-sheet transplantation

We assessed gross structure, interstitial fibrosis and capillary distribution in the myocardium 4 weeks after the CSC cell-sheet transplantation to qualitatively and semi-quantitatively explore the degree of LV remodeling in each group by HE staining, Masson Trichrome staining, and immunohistolabelling for von Willebrand factor, respectively. The infarcted area, in which the cell-sheet was transplanted, was clearly thicker in the 3-layer group than in the 1-layer or sham groups, as assessed by the HE staining (Fig. 3a–c). In addition, the myocardial structure in the peri-infarcted area was better preserved in the 3-layer group than in the 1-layer group or the sham group. There seemed to be less accumulation of interstitial fibrosis in the peri-infarcted and infarct-remote myocardium of the 3-layer group than in the 1-layer group or sham groups (Fig. 3d–f). In fact, computer-based morphometry confirmed significantly less fibrosis in the 3-layer groups than in the 1-layer group or sham group (Fig. 4a). Capillary density in the peri-infarcted myocardium was significantly greater in the 3-layer group than in the 1-layer group or sham group (Fig. 4b).

### Phenotypic fate of the transplanted CSCs in the heart

The transplanted CSCs in the heart were phenotypically assessed by immunohistolabelling for human-specific HLA, which clearly dissected the transplanted cells in the native cardiac tissue. While the transplanted cells were rarely present in the 1-layer group 4 weeks after transplantation, the 3-layer group showed abundant human-specific HLA-positive transplanted cells in the tissues epicardially attached to the native cardiac tissue, which were assumed to consist of the remaining transplanted cell sheet and accumulated cells of native origin (Fig. 5a).

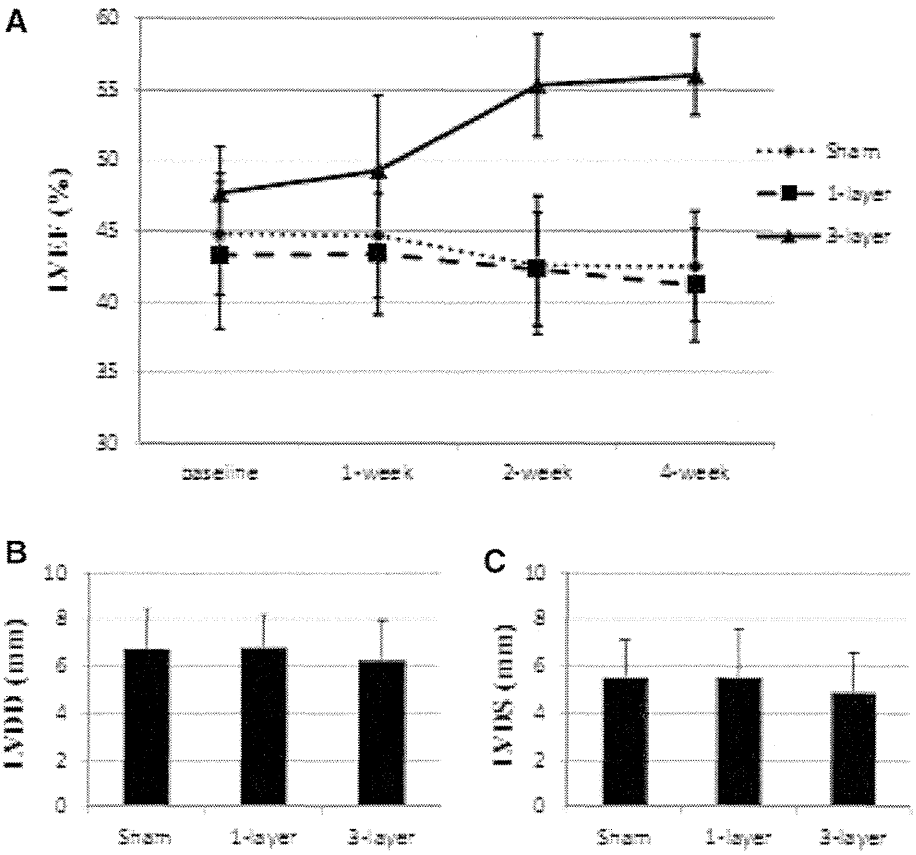


**Fig. 2** Cardiac performance measures, such as left ventricular ejection fraction (LVEF) (**a**), LV diastolic dimension (LVDD, **b**), and LV systolic dimension (LVDS, **c**), were assessed echocardiographically immediately after treatment and then 1, 2, and 4 weeks after treatment (sham operation vs. 1-layer cell-sheet transplantation vs. 3-layer cell-sheet transplantation)

Notably, some human-specific HLA-positive transplanted cells were present in the native myocardium, suggesting the migration of transplanted cells into the native cardiac tissue (Fig. 5b–d).

## Discussion

This study demonstrated clearly that the transplantation of CSC cell-sheets to treat the MI heart yielded significant recovery of cardiac performance in a cell-sheet layer dependent manner. Consistently, the hearts transplanted with the multi-layered cell-sheet showed significantly more preserved gross myocardial structure, reduced interstitial fibrosis, and increased capillary density than the hearts transplanted with a mono-layered cell-sheet. Moreover, the differentiation of heart-composing cells, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells, was greater in the hearts transplanted with the multi-layered cell-sheet than in those transplanted with the mono-layered cell-sheet.

The transplanted cell-source is known to be a major determinant of the therapeutic effects of cell transplantation therapy for cardiac failure [10–12]. The transplantation of skeletal myoblast transplantation predominates anti-fibrotic effects, whereas that of bone marrow-derived cell transplantation predominates neoangiogenesis in the ischemic/infarcted myocardium. These effects are mediated by indirect effects, in which cell transplantation upregulates a variety of cardioprotective factors to enhance the native healing process, although differentiation of the transplanted cells into the functional heart-composing cells, such as cardiomyocytes or vascular cells rarely occur following the transplantation of skeletal myoblasts or bone marrow-derived cells [13, 14]. In contrast, the transplantation of CSCs has been shown to yield therapeutic effects both directly and indirectly [15, 16]. This study showed that the transplantation of CSCs induced both anti-fibrotic and neoangiogenic effects in a transplanted cell number-dependent manner, indicating that CSCs might have released soluble factors to activate the anti-fibrotic and angiogenic process of the native myocardium following the transplantation. Moreover, the differentiation into the cardiomyocytes and vascular cells, shown in this study, suggests potential direct contribution of these cells to functional recovery, although the magnitude of these direct effects on the global cardiac function remains unclear.

The number of transplanted cells is also an important contributor to the therapeutic effects. Although the cell-sheet method has been shown to deliver more cells into the heart than other delivery methods, such as intramyocardial or intracoronary injection [10], ischemia in the transplanted

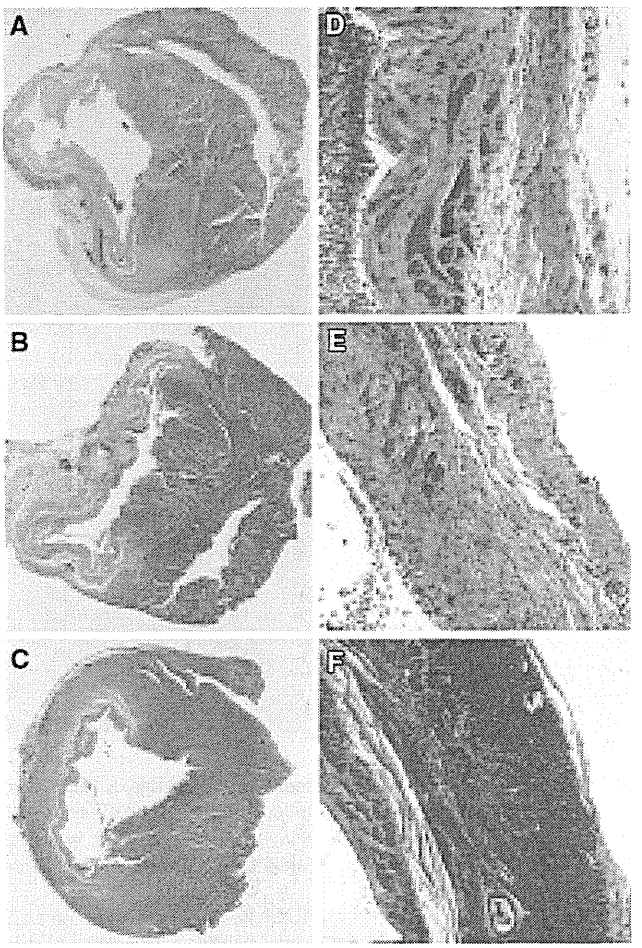


**Fig. 3** The gross structure of the heart 4 weeks after treatment was assessed by H&E staining. The sham group (**a**) and the 1-layer group (**b**) showed a large infarcted area in the left ventricular (LV) free wall, but the 3-layer group (**c**) showed a better preserved LV free wall. Interstitial fibrosis 4 weeks after the treatment was assessed by Masson Trichrome staining, which showed more accumulated fibrosis in the sham group (**d**) and the 1-layer group (**e**) than in the 3-layer group (**f**)

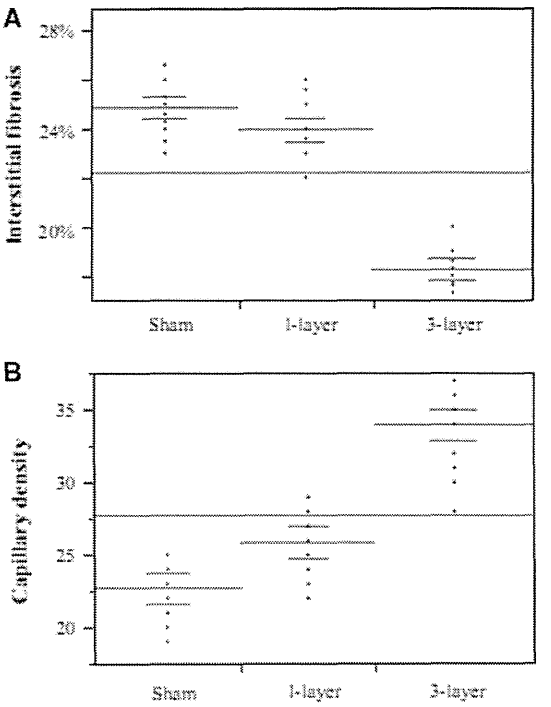


**Fig. 4** Masson Trichrome staining revealed a significantly lower percentage of fibrosis 4 weeks after the treatment in the 3-layer group than in the sham or 1-layer groups ($P < 0.05$ vs. the sham and the 1-layer group). Capillary density 4 weeks after treatment, assessed by immunohistochemistry for von Willebrand factor, was significantly greater in the 3-layer group than in the sham or 1-layer groups ($P < 0.05$ vs. the sham and the 1-layer group)

cell-sheet might be a critical limiting factor to the effects. In fact, it was reported that ischemia-related cell-necrosis occurs in the transplanted cells in accordance with the number of cell-sheets filled up [10, 17]. Furthermore, our researchers reported previously that the therapeutic effects of skeletal myoblast cell-sheets increased with the number of layers, but plateaued at five layers, possibly because of ischemia-related functional impairment of the transplanted cell-sheet, although skeletal myoblasts are known to be highly resistant to ischemic stimuli [10, 18, 19]. This study showed that the therapeutic effects of the CSC cell-sheet increased up until three layers, despite poor vascular support after acute infarction of the cell-sheet transplanted area, warranting 3-layered CSC cell-sheet transplantation for treating ischemia-related cardiac failure. Integration of the transplanted CSC cell-sheet into the native myocardium is also a concern of this treatment, as the cell-sheet was simply attached to the epicardial surface. However, this study unveiled that the transplanted cells migrated into the native myocardium and differentiated to heart-composing cells, although the biological mechanisms of this migration process remain unclear.

This study is limited by fact that we used a rodent model transplanted with cells of human origin. The difference in factors related to biological actions between the rat and the human might have modulated the therapeutic effects of this treatment, although a number of previous reports would justify using this model to mimic the clinical scenario [17, 20]. Moreover, using the cells from one patient in the in vivo study might not be appropriate to investigate the effects of CSC of human origin in general, although the cellular behavior did not seem to differ among more than five patients in vitro (data not shown), in accordance with previous reports [21].

In conclusions, the 3-layered cell-sheet improved cardiac function associated with angiogenic and anti-fibrotic effects in a rat model. Thus, the delivery of a sufficient number of CSCs by a cell-sheet method represents a promising treatment for cardiac failure, although further optimization is essential.

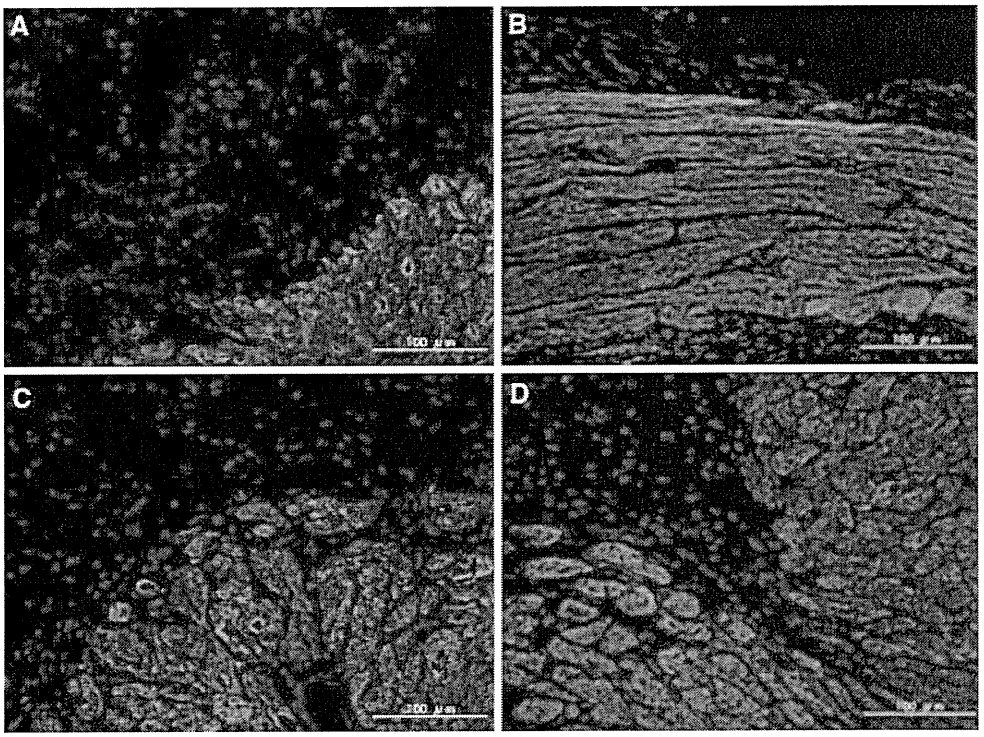


**Fig. 5** The presence and distribution of transplanted CSCs of human origin were immunohistologically assessed using human-specific anti-HLA antibody. By 4 weeks after transplantation, the 3-layer group showed abundant human-specific HLA-positive transplanted cells in tissues that were epicardially attached to the native cardiac tissue (**a**). Some human-specific HLA-positive transplanted cells were present in the interstitium of the native myocardium (**b–d**)

**Conflict of interest** There are no relationships or conflicts of interest related to this manuscript.

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ORIGINAL ARTICLE
Regenerative Medicine

# Human Cardiac Stem Cells With Reduced Notch Signaling Show Enhanced Therapeutic Potential in a Rat Acute Infarction Model

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*Background:* Because human cardiac stem cells (CSC) have regeneration potential in damaged cardiac tissue, there is increasing interest in using them in cell-based therapies for cardiac failure. However, culture conditions, by which CSCs are expanded while maintaining their therapeutic potential, have not been optimized. We hypothesized that the plating cell-density would affect proliferation activity, differentiation and therapeutic potential of CSCs through the Notch signaling pathway.

*Methods and Results:* Human CSCs were plated at 4 different densities. The population doubling time, C-KIT positivity, and dexamethasone-induced multidifferentiation potential were examined in vitro. The therapeutic potential of CSCs was assessed by transplanting them into a rat acute myocardial infarction (AMI) model. The low plating density (340 cells/cm$^2$) maintained the multidifferentiation potential with greater proliferation activity and C-KIT positivity in vitro. On the other hand, the high plating density (5,500 cells/cm$^2$) induced autonomous differentiation into endothelial cells by activating Notch signaling in vitro. CSCs cultured at low or high density with Notch signal inhibitor showed significantly greater therapeutic potential in vivo compared with those cultured at high density.

*Conclusions:* CSCs cultured with reduced Notch signaling showed better cardiomyogenic differentiation and therapeutic potentials in a rat AMI model. Thus, reducing Notch signaling is important when culturing CSCs for clinical applications. (*Circ J* 2014; **78:** 222–231)

Key Words: Cardiac stem cells; Cell culture; Notch signaling

Cardiac failure is a major cause of reduced quality of life and mortality.[1,2] Although cardiac tissue is known to have limited regeneration capacity, it has been shown that damaged cardiac tissue is regenerated by cardiac stem cells (CSCs), which are identified as C-KIT-positive cells in the heart,[3] through their proliferation and differentiation into functional cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells (ECs), and through release of a variety of factors that activate native healing processes.[4] Although transplantation of autologous stem cells into the heart has been proven to enhance this regenerative capacity of the damaged heart,[5] transplantation of CSCs that have been expanded in vitro may have promise in maximizing the regeneration process.[6] The magnitude of the therapeutic effect of CSC transplantation is determined by the cell preparation and delivery method, though the protocol for preparing CSCs has not been fully established.[7] Determination of the culturing protocol will be critical for the clinical application of patient-derived CSCs that may be isolated from limited biopsy samples.

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The cell preparation protocol of CSCs involves multiple steps, including enzymatic digestion of tissues, cell isolation, and cultivation, that will affect the fundamental behavior and therapeutic potential of CSCs.[8,9] Although the cell isolation protocol has been intensively studied,[3,10–13] the cell cultivation protocol has not. Among the many parameters of the culture

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conditions, it has been suggested that cell density at plating modulates fundamental behavior of stem cells such as proliferation and differentiation, indicating that the plating density may affect the therapeutic potential of CSCs.[14,15] In addition, it was reported that Notch signaling in direct cell-cell communication may be associated with fundamental behavior of cells under cultivation, including the lineage specification of cardiac progenitor cells.[16–18] We hypothesized that the plating cell-density would affect proliferation activity, differentiation and therapeutic potential of human CSCs by regulating Notch signaling, with the aim of exploring the optimal CSC cultivation protocol for treating cardiac failure.

## Methods

### Isolation and Cultivation of CSCs

All procedures were in accordance with the ethical standards of the institutional committee on human experimentation (control number: 729-4). CSCs were isolated from the right atrium of 3 patients with dilated cardiomyopathy (12–55 years old; data from the 12-year-old patient are mainly used in this report). In short, the cells were separated from the tissue by enzymatic digestion [37°C with 1 mg/ml of collagenase (17454, Serva Electrophoresis, Heidelberg, Germany) in Ham's F12 medium] after dissecting fat and fibrous tissues and mincing. The digestion was performed for a total of 5 reactions (60 rpm×20 min/reaction, 8 ml/reaction in 50 ml tube) then overnight digestion (for 12 h) was performed for the remaining debris with 0.1 mg/ml of collagenase solution (37°C, 60 rpm, 10 ml in 50 ml tube). After each reaction, the supernatant was collected and then the cells were collected by centrifugation (4°C, 500 g, 5 min) and plated on a normal 10-cm culture dish (353003, BD Biosciences, Franklin Lakes, NJ, USA) with complete medium [Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; SH30406.02, Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 5 mU/ml human erythropoietin (E5627-10UN, Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml basic fibroblast growth factor (100-18B, PeproTech, Rocky Hill, USA), 0.2 mmol/L L-glutathione (G6013, Sigma-Aldrich) with antibiotics].[6] The medium was changed on day 2. On day 5, the cells were collected with trypsin (T3924, Sigma-Aldrich) and replated on 10-cm dishes at 170 cells/cm$^2$. At the second passage (P1), the cells were collected with non-enzymatic solution (C5914, Sigma-Aldrich), labeled with anti-C-KIT antibody (130-091-735, Miltenyi Biotec, Bergisch Gladbach, Germany) followed by FcR blocking reagent (130-059-901, Miltenyi Biotec) in 3% FBS/PBS, and subjected to FACS (FACS Aria, BD Biosciences) to isolate the CSCs. As the negative control sample, mouse IgG1-phycoerythrin (130-092-212, Miltenyi Biotec) was used. The dead cells were excluded from the sample by using 7AAD (559925, BD Biosciences). After sorting, the CSCs were passaged (340 cells/cm$^2$) every 5 days. At P5, CSCs were sorted again as described. The purified CSCs were plated on normal culture dishes at different densities with and without 100 nmol/L of gamma secretase inhibitor XXI (GSI; Merck, Darmstadt, Germany)[16] and passaged every 5 days.

### qPCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase digestion (RNase-Free DNase set, Qiagen). The extracted RNA was subjected to reverse transcription (Omniscript reverse transcriptase, Qiagen) with random primers (Invitrogen-Life Technologies, Carlsbad, CA, USA). qPCR (quantitative real-time PCR) was performed using predesigned TaqMan primers/probes [assay ID; GAPDH: Hs99999905_m1, P21: Hs00355782_m1, P53: Hs01034249_m1, ETS1: Hs00901425_m1, TIE2: Hs00945155_m1, HES1: Hs00172878_m1, C-KIT: Hs00174029_m1, IL8: Hs99999034_m1, VEGFA: Hs00900055_m1, cTnT: Hs00165960_m1, PDGFRB: Hs01019589_m1, HGF: Hs00900070_m1, Applied Biosystems-Life Technologies, Carlsbad, CA, USA] and a 7500 Fast real-time PCR system (Applied Biosystems). GAPDH was used as the internal control.

### Tube Formation Assay

CSCs in EBM2 (CC-3156, Lonza, Basel, Switzerland) supplemented with 0.2% FBS were plated onto a Matrigel (BD Biosciences) -coated 96-well plates (7.5×10$^3$ cells per well) and incubated for 16 h.[19] Subsequently, the total tube length per well was measured[20] by analytic software (BZII, Keyence, Osaka, Japan).

### Immunocytofluorescence Analysis of Cell Differentiation

αMEM supplemented with 10% FBS and 10$^{-8}$ mol/L dexamethasone was used to induce differentiation of CSCs.[3] In short, CSCs were replated (3,400 cells/cm$^2$) onto a cover slip coated with 0.1% gelatin and incubated under 5% $CO_2$ at 37°C for 7 days. The cells were fixed with 4% paraformaldehyde and labeled with primary antibodies against αSA (alpha sarcomeric actin, A2172, Sigma-Aldrich), anti-αSMA (alpha smooth muscle actin, A2574, Sigma-Aldrich), or anti-TIE2 (T6577, Sigma-Aldrich). The samples were visualized with appropriate secondary antibodies and counterstained with DAPI.

### Rat Acute Myocardial Infarction (AMI) Model and Cell Transplantation

The animal study protocols were approved by the Animal Care and Use Committee of the Osaka University (21-030-2). The left coronary artery (LCA) was permanently ligated in nude rats (F344/NJcl-rnu/rnu, 8-week-old females, CLEA Japan, Tokyo, Japan) under inhalation anesthesia with 2.0% isoflurane through endotracheal intubation.[21] Immediately after the ligation, 8×10$^4$ cells[3] or 3×10$^6$ cells (for immunostaining against HNA/MLC and qPCR for VEGFA/HGF (Hepatocyte growth factor)) per rat were transplanted by intramuscular injection into the infarct's border zone.

### Transthoracic Echocardiography (TTE)

TTE was performed under inhalation anesthesia with 1.5% isoflurane as described previously.[21]

### Histological Examination

Rat hearts were collected after retrograde infusion of phosphate-buffered saline (PBS) supplemented with 50 mmol/L potassium chloride and 100 units/ml heparin. The hearts were embedded in OCT (Sakura Finetek Japan, Tokyo, Japan), cut into 5-μm sections, and fixed with 4% paraformaldehyde.[22] The sections were then stained with Masson's trichrome or immunohistologically against von Willebrand factor (vWF; A0082, Dako, Glostrup, Denmark), HNA (human nuclear antigen, MAB1281, Millipore, MA, USA), or MLC (myosin light chain, ab79935, Abcam, MA, USA) by similar methods to those mentioned earlier.

To calculate the percentage of left ventricle (LV) that was fibrotic, the total LV and fibrotic areas (blue-colored) were traced and measured using analytical software (BZII, Keyence). The percentage of MLC-positive cells was calculated as the number of HNA and MLC double-positive cells divided by the number of HNA-positive cells in a high-power magnification area (×200).

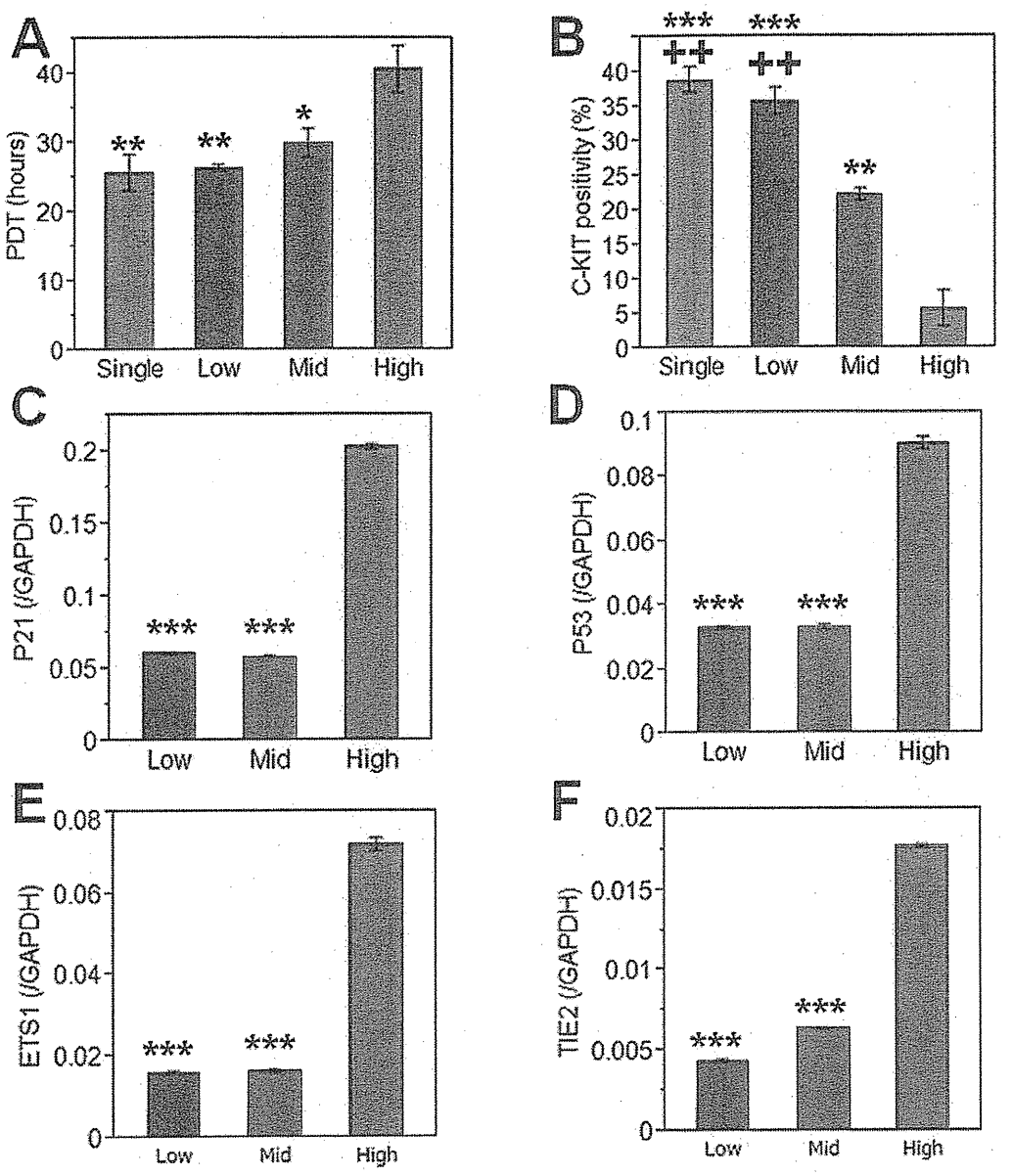


**Figure 1.** Population doubling time (PDT) and C-KIT positivity in cardiac stem cells. (A) PDT was averaged for 3 passages. (B) C-KIT positivity was assessed by FACS and averaged for 3 passages. (C–F) qPCR analysis. The values are the average for triplicate in a representative experiment. *P<0.05, **P<0.01, ***P<0.0001 vs. "High" group. ++P<0.01 vs. "Mid" group.

### Statistical Analysis

All data represent the mean±SEM. Statistical analyses were performed using software (JMP9, SAS Institute Japan, Tokyo, Japan or Prism 5, GraphPad Software, La Jolla, CA, USA). For multiple comparisons, ANOVA with Tukey's HSD post-hoc test was used. For the qPCR analysis of C-KIT, interleukin (IL)-8, and VEGF (Vascular endothelial growth factor) A, the raw data (normalized by GAPDH) of the "High" or "High+GSI" group were normalized to the data of the "Low" group from the same patient, because samples from 3 different patients were used for this experiment, and 1-sample t-test (vs. Low group) or unpaired t-test (High vs. High+GSI group) was used. For echocardiography, the delta values (the difference between before and 3 weeks after the transplantation) were used. If the P-value was <0.05, the difference was considered significant.

## Results

### Plating Density-Dependent Proliferation Activity and Purity of CSCs

Based on previous reports, we hypothesized that the plating density would affect the proliferation activity and purity of CSCs. We expected that higher plating density would increase Notch signaling, induce differentiation and therefore decrease the proliferation activity and purity of CSCs. To examine this, we isolated CSCs from samples (eg, $5.6\times10^5$ cells/g muscle at P1). After several passages of culture, we purified C-KIT pos-

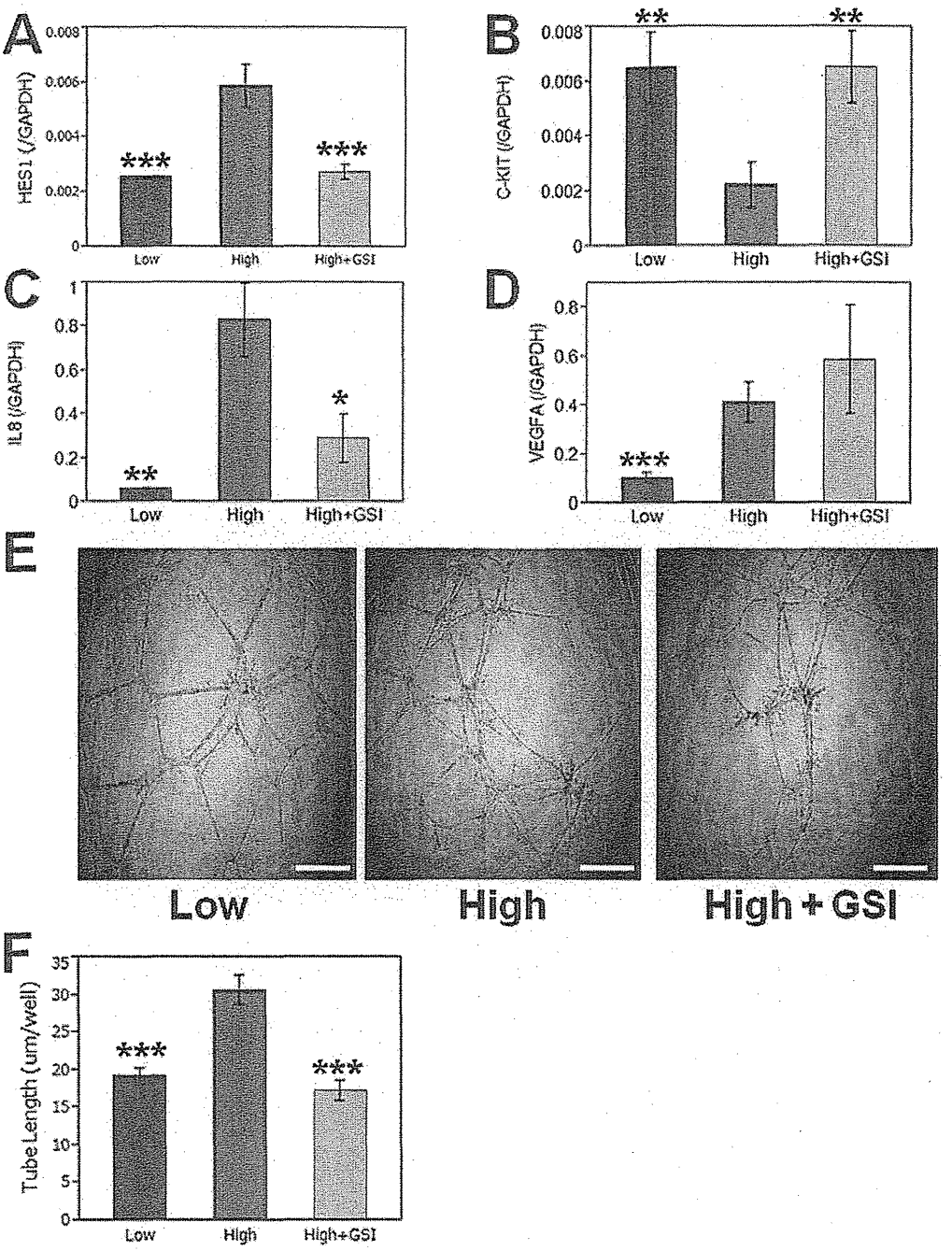


**Figure 2.** Vascular endothelial cell (EC)differentiation of cardiac stem cells resulting from high-density culture through Notch signaling activation. (**A–D**) qPCR analysis was performed after 3 passages under each plating density condition for the target gene of Notch signaling (*HES1*), C-KIT, and EC-related genes (IL-8 and VEGFA). (**E**) Representative morphologies of tube formation assay are shown. Original magnification, ×20. Scale bars=500$\mu$m. (**F**) Quantitative analysis of the tube formation assay. The total length of tubes per well was measured. The results from 12–14 wells per group were analyzed. *P<0.05, **P<0.01, ***P<0.0001 vs. "High" group.

itive cells by FACS (93.0±0.3%; mean±SEM) and plated at different densities (cells/cm$^2$): 86 (Single), 340 (Low), 1,400 (Mid), or 5,500 (High).

The proliferation activity of CSCs in relation to plating density was assessed by population doubling time (PDT), which was measured at each passage until the 3rd passage and then averaged (**Figure 1A**). The Single, Low and Mid groups showed a significantly shorter PDT than the High group. Purity of CSCs in relation to plating density was assessed by C-KIT positivity, which was measured at each passage until the 3rd passage and then averaged (**Figure 1B**). The Single and Low groups showed significantly greater C-KIT positivity than

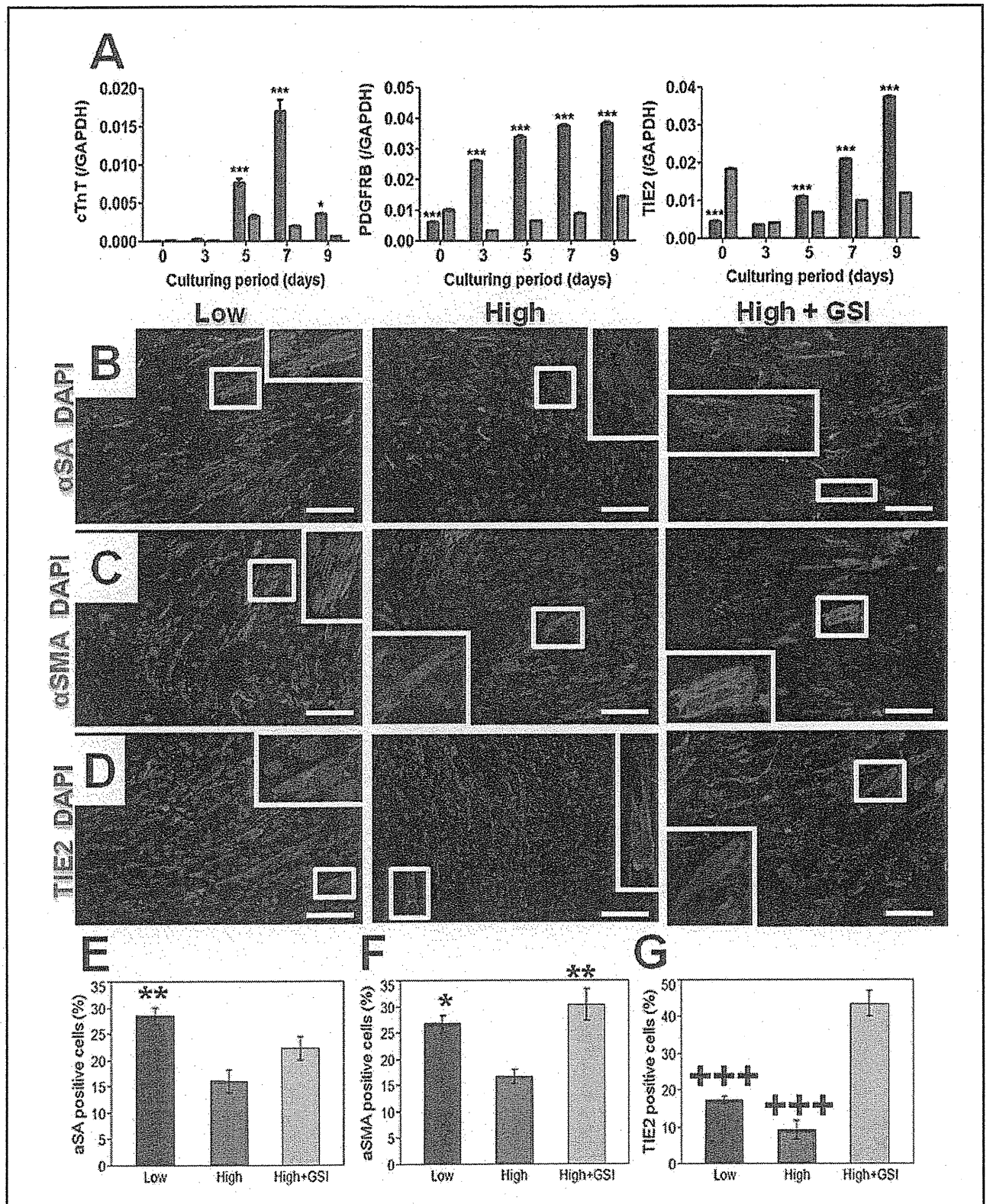


**Figure 3.** Differentiation potentials of cardiac stem cells (CSC) in vitro. (**A**) CSCs cultured at a low (blue) or high (red) plating density were incubated with dexamethasone for 9 days. The expression levels of cardiomyocyte (cTnT), smooth muscle cell (PDGFRB), and endothelial cell (TIE2) markers were analyzed by qPCR during the culture period. (**B–D**) Immunostaining for differentiated cells on day 7. Scale bars=100 $\mu$m. (**E–G**) Quantitative analysis of the immunostaining. The results from 11–20 fields per group were examined. *P<0.05, **P<0.01 vs. "High" group. +++P<0.0001 vs. "High+GSI" group.

the Mid and High groups, and the Mid group showed significantly greater C-KIT positivity than the High group. These findings suggested that lower plating cell densities might contribute to preservation of the proliferation activity and purity of CSCs in vitro.

### Plating Density-Dependent Transcriptional Profiles of CSCs

Cellular function of CSCs in relation to the plating cell-density and Notch signaling was further assessed by qPCR for cell-cycle regulating genes (P21 and P53), EC markers (ETS1, TIE2, IL8, and VEGFA), Notch-signaling target gene (*HES1*)[23] and C-KIT. Expression of these genes in CSCs was analyzed in the Low, Mid and High groups. In addition, GSI, which is a known Notch signal inhibitor,[16,17,24,25] was added to the culture medium of the High group to investigate the influence of Notch signaling on the gene expression.

The Low and Mid groups showed significantly lower levels of expression of P21, P53, ETS1, and TIE2, compared with the High group (**Figures 1C–F**). In addition, the Low group showed lower expressions of HES1, IL8 and VEGFA than the High group, and C-KIT expression was significantly greater in the Low group than in the High group (**Figures 2A–D**). Of note, the addition of GSI in the High group diminished the expression of HES1 and IL-8, but not of VEGFA. In addition, C-KIT expression in the High group was restored to that of the Low group by GSI treatment (**Figure 2B**). These findings suggested that higher plating densities might induce endothelial differentiation followed by termination of cell cycles, which slows PDT and diminishes the level of expression of C-KIT through Notch signal activation.

Plating density-dependent EC differentiation of CSCs in relation to Notch signaling was further assessed by the tube formation assay on Matrigel in vitro. Consistent with EC marker expression, the High group generated longer tubes than the Low group. Notably, GSI treatment diminished the tube formation activity in the High group to the level of the Low group, indicating active involvement of Notch signaling in the plating density-dependent EC differentiation of CSCs in vitro (**Figures 2E–F**).

### Retained Multipotency in CSCs Under Low-Density Culture

Because we confirmed that a higher plating density might induce EC differentiation of CSCs, we next examined the multipotency of CSCs in relation to the plating density by inducing differentiation in vitro. We expected CSCs at a lower plating density to maintain multipotency, and conversely, lose multipotency at a higher plating density. CSCs that were cultured in Low and High conditions were replated with dexamethasone and incubated for 9 days.[3,26,27] Expressions of cTnT (cardiomyocyte marker), PDGFRB (SMC marker), and TIE2 in each group were serially assessed by qPCR. cTnT, PDGFRB, and TIE2 were all upregulated in Low group over the culture period compared with the High group (**Figure 3A**), suggesting that CSCs cultured at low density may retain greater multipotency than those cultured under High conditions.

The differentiation potential of CSCs was further assessed by immunostaining for αSA (cardiomyocyte marker), αSMA (SMC marker) and TIE2 on day 7 (**Figures 3B–D**). The number of αSA- or αSMA-positive cells was significantly greater in the Low group than in the High group, and the TIE2-positive cell number tended to be greater in the Low group than in the High group (**Figures 3E–G**). Of note, the cells cultured under High conditions with GSI showed a significantly greater positivity for αSMA and TIE2 than those under pure High culture conditions, though GSI treatment did not affect significantly αSA positivity. These findings suggested that the multipotency of CSCs was hampered by higher plating density through Notch signaling-mediated EC differentiation.

### Therapeutic Effects of CSCs in a Rat AMI Model

Our in vitro data suggested that CSCs at a lower plating density maintained multipotency. Because multipotency may be required for their therapeutic potential, we next examined this in relation to the plating density and Notch signaling by transplanting CSCs into a rat AMI model.[3] We expected CSCs cultured under Low and High+GSI conditions to have greater therapeutic potential than those under High conditions. CSCs that were prepared in Low, High, or High+GSI culture conditions suspended with PBS or PBS only were injected into the infarct-border zone just after permanent ligation of the LCA. Effects of the CSC-transplantation therapy were assessed by standard TTE.

TTE revealed that all groups consistently showed progressive enlargement of the end-diastolic volume of the LV. However, the Low group showed significantly less progressive enlargement in the end-systolic volume of the LV and significantly less progressive reduction in LV ejection fraction compared with the High group, which showed a similar trend to the PBS-only group (**Figures 4A–C**). Notably, the enlarged LV end-systolic volume and reduced LV ejection fraction in the High group were restored to the levels of the Low group by culturing intact CSCs under High+GSI conditions for 3 passages before transplantation. These findings suggested the reduced therapeutic potential of the High group was mediated by Notch signaling activation during cultivation, which compromised the multipotency of CSCs through EC differentiation.

### LV Remodeling and Angiogenesis After CSC Transplantation

Because the therapeutic potential of CSCs is dependent not only on multipotency but also paracrine effects, the latter (LV remodeling and angiogenesis) were further assessed histologically at 3 weeks after transplantation. We expected CSCs cultured under Low and High+GSI conditions to show greater paracrine effects than those under High conditions.

The Low and High+GSI groups had reduced area of scarring and preserved structure of LV compared with the High group, which showed a similar scar size and structure to the PBS-only group, as assessed by Masson's trichrome staining (**Figure 4D**). The percentage of fibrosis in the LV was significantly less in the Low and High+GSI groups compared with the PBS-only and High groups (**Figure 4F**). vWF-positive arterioles and capillaries were more prominent in the infarct-border zone of the Low, High and High+GSI groups compared with the PBS-only group (**Figures 4E,G**). These results indicated that not only direct differentiation potential (multipotency) but also the paracrine effect (antifibrotic effect) of the Low and High+GSI groups might be greater than those of the High group.

### Differentiation Potential of CSCs in Vivo

Finally, we examined whether the greater multipotency of the Low and High+GSI groups in vitro reflected greater therapeutic potential in vivo as compared with the High group. The phenotypic fate of the transplanted CSCs, in relation to their plating cell-density and Notch signaling, was assessed in excised rat hearts at 3 weeks after transplantation.

MLC and HNA-double-positive cells were present in the infarct-border zone of the Low and High+GSI groups, but were rarely detected in the High group (**Figure 5A**). Quantitative assessment showed that 60% of the HNA-positive transplanted

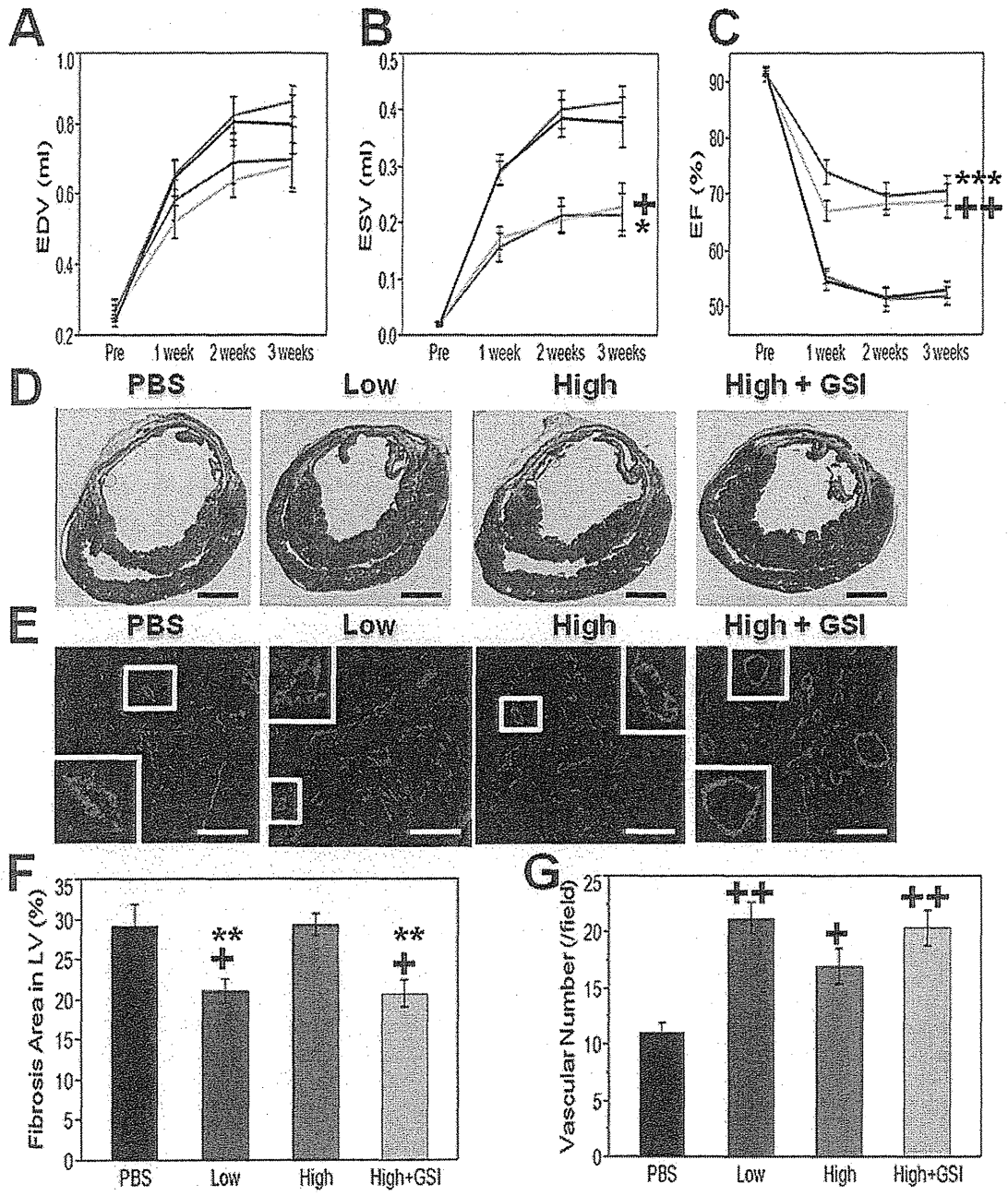


**Figure 4.** Therapeutic potential of cardiac stem cells in a rat model of acute myocardial infarction. (**A–C**) Echocardiography before (Pre) and after transplantation (1, 2, or 3 weeks). (**A**) End-diastolic volume (EDV, ml), (**B**) end-systolic volume (ESV, ml), (**C**) ejection fraction (EF, %) of rat left ventricle. Black lines indicate PBS treatment, blue lines "Low", red lines "High", and yellow lines "High+GSI" group. The results from 9–11 rats per group were examined. *P<0.05, ***P<0.0001 Low vs. "PBS" and "High" groups. +P<0.05, ++P<0.01 High+GSI vs. "PBS" and "High" groups. (**D–E**) Masson's trichrome staining and immunostaining against vWF. The insets show the enlarged image of the indicated area. Scale bars=2 mm in (**D**) and 200 $\mu$m in (**E**). (**F–G**) Quantitative analysis of D and E. (**F**) The results from 9–11 sections at the mid-ventricle level (transplanted site) from 9–11 rats per group were analyzed. (**G**) The results from 54–66 fields from 9–11 rats per group were analyzed. **P<0.01 vs. "High" group. +P<0.05, ++P<0.01 vs. "PBS" group. PBS, phosphate-buffered saline; vWF, von Willebrand factor.

cells in the Low group and 70% in the High plus GSI group were positive for MLC, compared with the High group in which only 20% of the HNA-positive cells were MLC-positive (**Figure 5B**). In addition, the human-derived cardiomyocytes survived at least for 3 weeks after transplantation and resided mainly in the infarct-border zone. These results indicated that the lower plating density with lower Notch signaling maintained the multipotency of CSCs in vitro and their cardiomyogenic differentiation potential in vivo, which resulted in a greater therapeutic potential in the rat model of AMI.

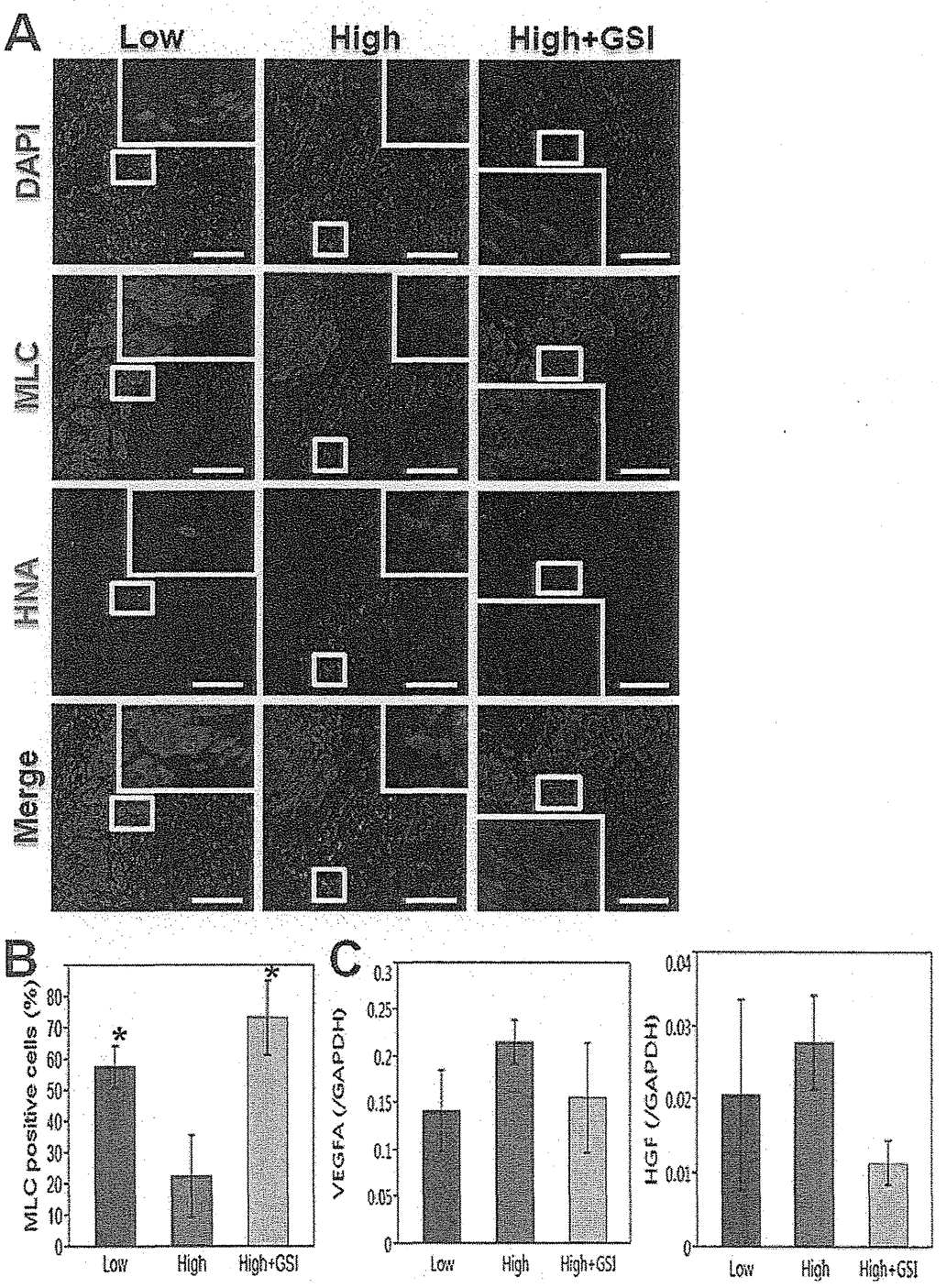


**Figure 5.** Differentiation potential of cardiac stem cells into cardiomyocytes in vivo. (**A**) Representative images for immunostaining against myosin light chain (MLC, red) and human nuclear antigen (HNA, green). DAPI (blue) was used for nuclear staining. Scale bars=100 $\mu$m. (**B**) Quantitative analysis of HNA-positive cells that were MLC-positive in vivo. The results from 10–31 fields per group (from 2–4 rats per group) were analyzed. *P<0.05 vs. "High" group. (**C**) qPCR analysis for VEGFA and HGF. Rat hearts were collected at 2 days after transplantation. GAPDH was used as internal control; 3–4 rats per group were used.

Regarding the paracrine effects, we examined by qPCR some paracrine factors (VEGFA and HGF)[4] at 2 days after transplantation. These experiments showed no significant difference in the expression levels of the paracrine factors among the groups (**Figure 5C**).

## Discussion

We discovered that Notch signaling was activated in CSCs under high cell-plating conditions and induced EC differentiation. Notch signaling is implicated as a key regulator of arte-