Signals Through Glycoprotein 130 Regulate the Endothelial Differentiation of Cardiac Stem Cells

Tomomi Mohri, Yasushi Fujio, Masanori Obana, Tomohiko Iwakura, Koichi Matsuda, Makiko Maeda, Junichi Azuma

Objective—Cardiac Sca-1+ cells were originally identified as multipotent stem cells. To address the regulation of their differentiation, we investigated the effects of the proinflammatory cytokines on their endothelial differentiation.

Methods and Results—We examined the effects of the proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-11, and cardiotrophin-1 (CT-1) on the cardiac Sca-1+ cell differentiation. IL-11 and CT-1, whose receptor systems use glycoprotein 130 (gp130), induced endothelial-specific genes in Sca-1+ cells, but not TNF-α, IL-1β, or IL-6, analyzed by RT-PCR and by immunocytochemistry. Immunoblot analyses showed that IL-11 and CT-1 activated signal transducer and activator of transcription 3 (STAT3), a downstream target of gp130, but not other cytokines. Though IL-6 receptor is not endogenously expressed in Sca-1+ cells, IL-6 exhibited the activity to induce the endothelial markers in the presence of soluble IL-6 receptor, an agonistic receptor, associated with STAT3 phosphorylation. Moreover, the inhibition of STAT3, by its dominant-negative form or siRNA, suppressed the induction of endothelial specific genes by IL-11 and CT-1. Finally, LIF and IL-11 transcripts were upregulated in postinfarct myocardium, accompanied by the induction of Sca-1+/VE-cadherin+ cells.

Conclusions—Gp130/STAT3 pathway plays critical roles in the regulation of endothelial differentiation of cardiac Sca-1+ cells. (Arterioscler Thromb Vasc Biol. 2009;29:754-760.)

Key Words: cytokines ■ gp130 ■ Sca-1 ■ endothelial cells ■ neovascularization

Cardiac functions are regulated by various kinds of neurohumoral factors through paracrine/autocrine systems. Cardiac cells produce a wide range of the proinflammatory cytokines and their family, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), and IL-11, under pathological conditions. These cytokines transduce their signals in cardiac myocytes, vascular cells, or infiltrating inflammatory cells, modulating cardiac remodeling, repair, and regeneration after myocardial injury as a result.1-4 Based on the signaling pathways, these cytokines can be classified into 3 families; TNF-α family, IL-1β family, and IL-6 family. TNF-α exerts its cellular effects through 2 distinct receptors, TNF receptor type 1 (TNFR1) and type 2 (TNFR2), and shares its signaling pathways with death signals such as Fas.5 IL-1β signaling is exclusively transduced through IL-1 receptor type 1 (IL-1RI), a member of Toll-like receptor/IL-1 receptor superfamily. Toll-like receptor/IL-1 receptor superfamily members are essential for innate immune and inflammatory responses.5,6 IL-6, LIF, CT-1, and IL-11 belong to IL-6 family. IL-6 family cytokines bind to their specific receptor subunit (α subunit), such as IL-6 receptor (IL-6R), LIF receptor (LIFR), and IL-11 receptor (IL-11R). The IL-6 family cytokine-its receptor α subunit complex makes a dimer with glycoprotein 130 (gp130), a common receptor subunit, followed by the activation of signal transducer and activator of transcription 3 (STAT3).8 Recent studies have revealed that one of the most important roles of cardiac cytokines is the regulation of cardiomyocyte–endothelial cell interaction. LIF and CT-1 upregulate the expression of vascular endothelial growth factor (VEGF) in cardiac myocytes.9 Importantly, VEGF, secreted from cardiomyocytes, is required to maintain endothelial cell survival, which is critical for preserving myocardium.10 These findings strongly propose the importance of the cytokine network between cardiac myocytes and endothelial cells; however, the effects of these cytokines on the tissue resident stem or progenitor cells, which have the potential to differentiate into endothelial cells, remain to be fully addressed. So far, several kinds of cardiac stem cells have been identified in the adult hearts. These cardiac stem cells differentiate into 3 lines of cardiac cells including cardiomyocytes, smooth muscle cells, and endothelial cells.11,12 Interestingly, the injection of cardiac stem cells into injured hearts promotes cardiac repair and regeneration,11,12 with the increase in capillary density, though the signals responsible for

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the differentiation of cardiac stem cells are unknown. Recently, we have revealed that LIF regulates the endothelial differentiation of cardiac Sca-1+ cells, proposing that the activation of LIF signaling in Sca-1+ cells might promote vessel formation.

In the present study, we investigated the effects of the cardiac proinflammatory cytokines and their family on endothelial differentiation of cardiac Sca-1+ cells and demonstrated that signals through gp130 play critical roles in regulating the endothelial differentiation of Sca-1+ cells. Moreover, IL-6 family cytokines are upregulated in postinfarct myocardium, associated with the induction of Sca-1+/VE-cadherin+ cells, suggesting pathophysiological significances of the IL-6 family cytokines in the regulation of cardiac stem cell differentiation. The understanding of the biological effects of cardiac cytokines on stem cell functions might provide the insights into the development for novel targeted therapies to tissue resident stem cells.

Materials and Methods
An expanded Materials and Methods section is available in the online Data Supplement at http://atvb.ahajournals.org.

Animal Experiments
The care of all animals was in compliance with the Osaka University animal care guideline. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996).

Preparation of Cardiac Sca-1+ Cells
Cardiac Sca-1+ cells were isolated by magnetic cell sorting (MACS) from C57Bl/6 mice (10- to 12-week-old) with about 98% purity, as described previously.

RT-PCR Analyses
RT-PCR was performed as previously described. Gene-specific primers used for PCR amplification were shown in supplemental Table I.

Immunoprecipitation Analysis
Cell lysates were prepared in RIPA buffer, and incubated with anti–p-Tyr antibody (Santa Cruz Biotechnology) and Protein A agarose (Santa Cruz Biotechnology) overnight at 4°C. After washing with RIPA buffer, immunoprecipitates were resuspended in SDS-PAGE buffer, and immunoblotted with anti-gp130 antibody (Santa Cruz Biotechnology).

Immunoblot Analyses
Immunoblotting was performed as previously described.

Immunocytochemical Examination
Immunocytochemical analyses were performed as previously described.

Construction and Infection of Adenoviral Vectors
Adenoviral vectors expressing dominant negative STAT3 (dnSTAT3) and β-galactosidase were previously described. Adenoviral infection was performed at a multiplicity of infection of 100 for 24 hours. Forty-eight hours after infection, cells were cultured in the medium containing each cytokine.

Gene Silencing of STAT3 With siRNA
Cells were transfected with control or STAT3 siRNA using Lipofectamine RNAi MAX (Invitrogen). Forty-eight hours after transfection, cells were cultured in the medium containing each cytokine.

Generation of Myocardial Infarction
Myocardial infarction (MI) was generated by coronary artery ligation according to previous report with minor modification. In immunohistological analyses, the hearts were excised 14 days after surgery and embedded in OCT compound. In experiments for the estimation of cytokine expression, total RNA was prepared from hearts 0, 1, 4, 7, and 14 days after surgery.

Histological Examination
Frozen sections (5 μm thick) embedded in OCT compound were prepared from normal or MI hearts (n=3 mice), and fixed with 4% paraformaldehyde. After blocking with 3% BSA/0.1% Triton X-100 in TBS, sections were incubated with anti–Sca-1 (BD Bioscience) and anti–VE-cadherin antibodies (Santa Cruz Biotechnology) followed by the incubation with Alexa Fluor 488–conjugated anti-rat IgG (Molecular Probes) and Alexa Fluor 546–conjugated anti-goat IgG (Molecular Probes).

Statistical Analysis
Statistical significance was determined by paired t test or Student t test. Data were presented as mean±SE or mean±SD. Probability value <0.05 was considered to be statistically significant.

Results
IL-11 and CT-1 Induced the Expression of Endothelial Cell Specific Markers in Cardiac Sca-1+ Cells
To address the effects of the proinflammatory cytokines and their families on the differentiation of cardiac Sca-1+ cells, we first examined the expression of the receptors for these cytokines in cardiac Sca-1+ cells by RT-PCR. The transcripts of the receptors for TNF-α, IL-1β, and IL-11 were expressed in cardiac Sca-1+ cells (supplemental Figure I). LIFR, which functions as the ligand-specific receptor for both CT-1 and LIF, was also expressed. In contrast, IL-6R was not detected in cardiac Sca-1+ cells. We also confirmed that gp130, common receptor subunit for IL-6 family cytokines, was expressed in cardiac Sca-1+ cells.

Next, we examined whether these proinflammatory cytokines induce the endothelial differentiation in cardiac Sca-1+ cells. Cardiac Sca-1+ cells were cultured with TNF-α, IL-1β, IL-6, IL-11, or CT-1 for 14 days, and the expression of the marker genes for endothelial cells was analyzed by RT-PCR (Figure 1A and 1B). Cells, cultured with LIF, were used as positive control. Though both TNF-α and IL-1β activated their downstream signaling pathways (supplemental Figure II), they did not induce the endothelial specific marker genes in cardiac Sca-1+ cells. In contrast, endothelial cell-specific markers, VE-cadherin, CD31, and Flk-1, were upregulated in response to IL-11 (VE-cadherin: 14.3-fold, CD31: 13.5-fold, Flk-1: 11.0-fold compared with control, n=4, analyzed by real time RT-PCR) and CT-1 (VE-cadherin: 8.1-fold, CD31: 7.5-fold, Flk-1: 7.9-fold compared with control, n=4, by real time RT-PCR), but not in response to IL-6 (Figure 1A and 1B and supplemental Figure III). Although cardiac Sca-1+ cells have also been demonstrated to be differentiated into other cardiac cell lineages, neither IL-11 nor CT-1 induced the
differentiation into cardiomyocytes or smooth muscle cells (supplemental Figure IV). These findings suggest that IL-11 receptor and LIF receptor can functionally transduce their signals and induce endothelial markers in cardiac Sca-1+ cells. Indeed, the stimulation of IL-11 receptor or LIF receptor, with IL-11 or LIF, respectively, resulted in the rapid phosphorylation of gp130 (Figure 1C).

Recently, c-kit+ cells have also been reported to be another population of cardiac stem cells. Therefore, we prepared c-kit+ cells from adult murine hearts by MACS and examined the effects of LIF on their differentiation. RT-PCR analyses demonstrated that c-kit+ cells also differentiated into endothelial cells (supplemental Figure V). However, the repeated experiments using MACS methods achieved the preparation of lower number of c-kit+ cells than Sca-1+ cells. Thus further experiments focused on Sca-1+ cells in this study.

Next, to examine the expression of endothelial marker proteins in cytokines-treated cardiac Sca-1+ cells, immunocytochemical analyses were performed. Cells were cultured with cytokines for 14 days and stained with anti-VE-cadherin antibody (Figure 1D and supplemental Figure VI). The cells cultured with IL-11 or CT-1 showed the expression of VE-cadherin at higher frequency than those with TNF-α, IL-1β, or IL-6. Consistently, the frequency of CD31 positive cells was also increased in the cells cultured with IL-11 or CT-1 (data not shown). The uptake of acetylated low-density lipoprotein (Ac-LDL) is characteristic of endothelial cell lineage. To investigate the functional relevance of the newly-differentiated endothelial cells derived from cardiac Sca-1+ cells, we examined whether the CD31-positive cells incorporated Dil-labeled Ac-LDL and confirmed that the endothelial marker-positive cells from cardiac Sca-1+ cells have a functional property of endothelial lineage cells (supplemental Figure VII).

**IL-11 and CT-1 Activated STAT3 in Cardiac Sca-1+ Cells**

To address the signaling pathways of IL-11 and CT-1, we examined whether IL-11 or CT-1 activates STAT3 and ERK1/2 in cardiac Sca-1+ cells. Cardiac Sca-1+ cells were stimulated with IL-11 or CT-1 for the indicated time, and then cell lysates were immunoblotted with antiphospho-specific antibodies (Figure 2). STAT3 was prominently phosphorylated by IL-11 or CT-1, reaching a peak within 15 minutes of stimulation, whereas the phosphorylation of ERK1/2 was not remarkable. In contrast, IL-6, TNF-α and IL-1β, which did not show the capacity of induction of endothelial markers, did not activate STAT3. Because much attention has been paid to Akt-mediated vessel formation, we also examined the effects of IL-6 family cytokines on the activation of Akt. IL-6 family cytokines did not lead to the activation of Akt in cardiac Sca-1+ cells (data not shown).

**Soluble Form of IL-6R Conferred the Potential to Induce the Sca-1+ Cell Commitment to Endothelial Cell Lineage on IL-6**

Because the data presented above demonstrated the importance of gp130 signals in the endothelial differentiation, we examined the effects of sIL-6R on the Sca-1+ cells differentiation, because sIL-6R has the potential for activating gp130 in an IL-6-dependent manner. The cells were cultured in medium containing IL-6 in the presence or absence of sIL-6R for 14 days and stained with anti-VE-cadherin antibody (Figure 3A). The cells cultured with IL-6 in the presence of sIL-6R expressed VE-cadherin, confirming the critical roles of signals through gp130 activation in the endothelial lineage commitment. Consistently, IL-6 remarkably activated STAT3 in the presence of sIL-6R (Figure 3B and 3C). Thus, these data suggest that activation of gp130/
STAT3 signals is a critical event for the induction of cardiac Sca-1+ cell differentiation to endothelial cells.

**STAT3 Was Required for IL-6 Family–Mediated Commitment of Cardiac Sca-1+ Cells to Endothelial Cell Lineage**

To examine whether STAT3 activation is essential for the endothelial differentiation of cardiac Sca-1+ cells by IL-6 family cytokines, we analyzed the effects of the inhibition of STAT3 by adenoviral vectors expressing dnSTAT3 (Figure 4A). IL-11 and CT-1 did not induce the endothelial differentiation in dnSTAT3-expressing cells, whereas endothelial-specific genes were upregulated by IL-11 and CT-1 in the cells expressing β-galactosidase, a control. Similarly, gene silencing experiments using siRNA for STAT3 demonstrated that the inhibition of STAT3 pathways also suppressed the induction of endothelial-specific genes by IL-6 family cytokines (Figure 4B and 4C). Further, to characterize STAT3-knockdown cardiac Sca-1+ cells, Dil-labeled Ac-LDL uptake was examined (Figure 4D). As shown in supplemental Figure VII, LIF treatment increased the frequency of Ac-LDL-positive cells. Importantly, compared with cells transfected with control siRNA, Ac-LDL uptake was reduced in cells transfected with siRNA for STAT3. These data propose the additional evidence for the requirement of STAT3 for gp130-mediated endothelial differentiation in Sca-1+ cells. We have also noticed that control siRNA have a tendency to inhibit Ac-LDL uptake in comparison with nontreated cells, though not significantly. The precise mechanisms remain to be known; however, the transfection procedure might have affected Ac-LDL uptake.

**The Upregulation of IL-6 Family Cytokines Was Associated by the Commitment of Sca-1+ Cells to Endothelial Lineage After Myocardial Infarction**

To explore the pathophysiological significances of the IL-6 family cytokines in cardiac stem cell fate in vivo, we generated MI model and, first, we examined the gene expression profiles of IL-6 family cytokines in the hearts (Figure 5A). Real time RT-PCR analyses have demonstrated that the transcripts of IL-11 and LIF were elevated 1 day after MI (IL-11: 32.9-fold, LIF: 19.9-fold compared with no operation) and the expression levels were gradually reduced, whereas CT-1 was transiently decreased and recovered to the basal level. IL-6R was also immediately upregulated, and the induction was sustained up to 14 days after infarction. Because these data suggest that IL-6 family signals are activated in the postinfarct myocardium, we investigated the dynamics of cardiac Sca-1+ cells by immunofluorescent microscopy using anti–Sca-1 and anti-VE-cadherin antibodies (Figure 5B). Consistent with the previous report,24 a significant number of Sca-1+/VE-cadherin+ cells were detected in myocardium. Importantly, the double positive cells

![Figure 2](http://atvb.ahajournals.org)  
*Figure 2.* IL-11 and CT-1 activate STAT3 in cardiac Sca-1+ cells. A, Sca-1+ cells were stimulated with IL-11 or CT-1 for the indicated time, and phosphorylation of STAT3 and ERK1/2 was analyzed by immunoblotting. B, Quantification of the time course of STAT3 phosphorylation. C, Quantification of STAT3 phosphorylation by each cytokine. *P<0.05, **P<0.01 vs 0 minutes (control).

![Figure 3](http://atvb.ahajournals.org)  
*Figure 3.* Soluble IL-6 receptor confers the potential to induce VE-cadherin on IL-6. A, Sca-1+ cells cultured with IL-6 in the presence or absence of sIL-6R for 14 days, stained with anti-VE-cadherin antibody and the frequency of VE-cadherin–positive cells was calculated. **P<0.01 vs control. B and C, After stimulation with IL-6 in the presence (closed column) or absence (open column) of sIL-6R, STAT3 phosphorylation was examined. *P<0.05, **P<0.01 vs 0 minutes. #P<0.05 vs IL-6 treatment at each time point.
were increased in number at the border zones of postinfarct myocardium (normal: 4.9±1.9 cells/high power field [HPF], MI: 7.9±1.8 cells/HPF, P<0.01, n=9 fields from 3 mice). Moreover, Sca-1+ cells expressed VE-cadherin more abundantly at border zone in the infarct hearts than in normal hearts.

**Discussion**

In the present study, we examined the effects of the proinflammatory cytokines and their family on cardiac Sca-1+ cell commitment to endothelial cell lineage. CT-1, IL-11, and LIF induced the endothelial cell lineage commitment of the Sca-1+ cells, although not TNF-α, IL-1β, or IL-6. CT-1 and IL-11 activated STAT3 in cardiac Sca-1 cells, as is the case with LIF. IL-6, whose receptor is not expressed in cardiac Sca-1+ cells, gained the potential to induce the endothelial markers in the presence of sIL-6R. Moreover, we demonstrated that the inhibition of STAT3 abrogated the endothelial commitment of cardiac Sca-1+ cells. Finally, by generating

**Figure 4.** STAT3 is required for the gp130-mediated endothelial lineage commitment of cardiac Sca-1+ cells. A, Sca-1+ cells infected with adenoviral vectors expressing dnSTAT3 (dn) or β-galactosidase (β) were cultured with each cytokine for 14 days. Expression of endothelial specific genes was analyzed. B, Cells transfected with control or STAT3 siRNA were cultured with LIF for 5 minutes and STAT3 phosphorylation was analyzed. C and D, Cells transfected with siRNA were cultured with each cytokine for 14 days. RT-PCR for endothelial markers (C) and Ac-LDL uptake assay (D) were performed. **P<0.01 vs control. #P<0.05, ##P<0.01 vs LIF+control siRNA.

**Figure 5.** The upregulation of IL-6 family cytokines is accompanied by the commitment of Sca-1+ cells to endothelial lineage after MI. A, The expression of IL-6 family cytokines in infarct hearts was analyzed by RT-PCR. *P<0.05, **P<0.01 vs no operation (day 0). B, Representative images from normal and infarct hearts stained with anti-Sca-1 and anti-VE-cadherin antibodies (bar, 25 μm). Arrows indicate Sca-1+/VE-cadherin+ cells.
MI model, we proposed the association between the upregulation of LIF and IL-11 and the induction of Sca-1+/VE-cadherin+ cells in postinfarct myocardium.

The inflammation is closely associated with neovascularization. Accumulating evidence has demonstrated that proinflammatory cytokines mediate angiogenic signals in vascular cells. In skeletal muscles, IL-1β is required for neovascularization through the mononuclear cells-skeletal muscle interaction.25 Although TNF-α has reported to have both proangiogenic and antiangiogenic properties, TNFR2-deficient mice showed impaired capacity of blood flow recovery after hindlimb ischemia surgery, accompanied with reduction of endothelial cell/endothelial progenitor cell survival and migration.26,27 Similarly, IL-6 upregulates VEGF expression in rheumatoid arthritis, leading to the promotion of vessel formation in the inflamed joints.28 In cardiovascular system, we previously demonstrated that LIF and CT-1 increased VEGF expression in cardiac myocytes through STAT3 activation, which enhanced the capillary density in the heart.29,29 In addition to these cytokine-mediated angiogenic effects, here we have demonstrated that not only LIF30 but also a wide range of IL-6 family cytokines, which are produced from cardiac cells in response to cardiac stress, can induce the endothelial cell lineage commitment of cardiac stem cells, suggesting that cardiac stem cells may function as endothelial progenitors under diverse pathological conditions. Interestingly, Flk-1, one of VEGF receptors, was induced by IL-6 family cytokines in cardiac Sca-1+ cells. Moreover we have confirmed that VEGF activated its downstream signaling pathway in LIF-treated Sca-1+ cells, but not in non-treated Sca-1+ cells (data not shown). Therefore, it is possible that IL-6 family-induced VEGF in myocardium stimulates not only preexisting endothelial cells but also Sca-1+ cell-derived differentiating endothelial cells through paracrine systems.

In this study, we characterized the differentiating Sca-1+ cells by RT-PCR, because RT-PCR is much reliable to analyze the stem cell fate than immunofluorescence.30 To estimate the maturity of newly-differentiating endothelial cells from Sca-1+ cells, we compared their expression level of CD31 with those in matured mouse microvascular cell line by FACS (data not shown). Compared with the fully-differentiated endothelial cell line, only less than 20% of CD31 protein was expressed in newly-differentiating endothelial cells induced by LIF, suggesting that IL-6 family cytokines promote the commitment of Sca-1+ cells to endothelial cells, but that these cytokines might not achieve full differentiation. In this context, further analyses on the expression profile of cytokine receptors, such as flk-1, might provide more information on molecular mechanisms of Sca-1+ cell-derived endothelial differentiation.

IL-6 family cytokines stimulate gp130, a common receptor subunit, leading to activation of STAT3. Therefore, the redundancy among LIF, CT-1, IL-11, and IL-6/sIL-6R in Sca-1+ cell commitment to endothelial cells should be derived from the common signaling pathways through gp130/STAT3. Pathologically it should be emphasized that IL-6 exhibited the potential to induce the endothelial differentiation in the presence of sIL-6R, because the expression of IL-6R was elevated after MI, as reported previously.31 Unexpectedly, CT-1 exhibited a lower potency than LIF, though LIF and CT-1 share LIFR as their receptor α subunit. The relative potency of CT-1 to LIF may depend on cell lineage. LIF and CT-1 have similar relative potencies in inhibiting the growth of the mouse MI cells and in inducing the hypertrophy in cultured cardiomyocytes.32 Considering the previous reports that CT-1 requires the third receptor component in neuronal cells,33 the expression level of the third receptor may be low in Sca-1+ cells, though the third receptor has not been identified.

IL-6 family cytokines, such as LIF, CT-1, and IL-11, activate the downstream signaling pathways in cardiac myocytes and contribute to cytoprotection and vessel formation in the heart.4 Based on these beneficial effects of gp130/STAT3 activation in cardiomyocytes, the molecular target therapies toward gp130 are being explored as a novel therapeutic strategy against heart disease.35,36 The data presented here propose the possibility that the therapeutic strategy toward gp130 could achieve additional outcomes by inducing neovascularization through differentiation of cardiac stem cells into endothelium.

In summary, activation of gp130/STAT3 is a critical event for the endothelial lineage commitment of cardiac Sca-1+ cells. Our findings suggest that cardiac cytokine network, especially IL-6 family, could regulate the cell fate of cardiac stem cells.

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Disclosures
None.

References


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Supplementary Figure I

cardiac Sca-1 + cells

heart

liver

TNFR1

TNFR2

IL-1RI

IL-6R

IL-11R

LIFR

gp130

GAPDH
Supplementary Figure II

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Supplementary Figure V

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Supplementary Figure VI

control  IL-11  CT-1  LIF
Supplementary Figure VII

CD31  Dil-Ac-LDL  Hoechst  Merge
Supplement Material

Supplementary Materials and Methods

Animal experiments

The care of all animals was in compliance with the Osaka University animal care guideline. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Preparation of cardiac Sca-1+ cells

Cardiac Sca-1+ cells were isolated by magnetic cell sorting (MACS) from C57Bl/6 mice (10- to 12-week-old, Japan SLC) with about 98% purity, as described previously. Newly isolated cardiac Sca-1+ cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FBS overnight. To maintain multipotency of the differentiation, Sca-1+ cells were cultured in mNSCM (modified neural stem cell medium) consisting of Dulbecco’s MEM and Ham’s F12 (ratio 1:1, DMEM/F12) supplemented with 5 mM HEPES, 10 µg/mL of ITS (5 µg/mL of insulin, 5 µg/mL of transferrin, and 5 ng/mL of sodium selenite), 10 ng/mL of bFGF, 20 ng/mL of EGF, and 1000 units/mL of LIF.

Human IL-11, murine IL-6, murine CT-1, murine IL-1β, human bFGF and human EGF were purchased from Peprotech EC (London, UK). Murine LIF and murine TNF-α were from Chemicon International (CA). Murine soluble IL-6R (sIL-6R) was the product from R&D Systems, MN. Soluble IL-6R contains an extracellular ligand binding domain of murine IL-6R but not a transmembrane region. Soluble IL-6R/IL-6 complex is capable of acting as an agonist of
gp130.

**RT-PCR analyses**

RT-PCR was performed as previously described\(^3\). Briefly, total RNA was prepared with acid guanidinium thiocyanate-phenol-chloroform method. Total RNA (1 µg) was subjected for first strand cDNA synthesis by using the oligo(dT) first strand primer (Invitrogen, CA). Gene-specific primers used for PCR amplification were shown in Supplementary Table I. The PCR products were size-fractionated by 2% agarose gel electrophoresis and detected by staining with ethidium bromide.

For the quantification of PCR products, RT-PCR was carried out on ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers.

**Immunoprecipitation analysis**

Cells were stimulated with cytokines for 5 min and then washed with ice-cold PBS twice. Cell lysates were prepared by adding ice-cold RIPA buffer containing 150 mM NaCl, 0.2% Triton X-100, 0.1% SDS, 50 mM Tris-HCl (pH 8.0) and 0.5 M Na\(_3\)VO\(_4\), and protease inhibitor cocktail (0.022% 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.0005% aprotinin, 0.001% bestatin, 0.0005% E-64, 0.0009% leupeptin and 0.0006% pepstatin A), and centrifuged at 15000 rpm for 10 min. Cell lysates were incubated with anti-p-Tyr antibody (PY99, Santa Cruz Biotechnology, CA) and Protein A agarose (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitates were washed 3 times with ice-cold RIPA buffer, and the pellets were resuspended in 2× SDS-PAGE sample buffer containing 0.1 M Tris-HCl, 4% SDS, 12% β-mercaptoethanol, 20% glycerol and
bromophenol blue. The immunoprecipitates were separated by SDS-PAGE and immunoblotting was performed with anti-gp130 antibody (Santa Cruz Biotechnology).

**Immunoblot analyses**

Immunoblotting was performed as previously described. Briefly, cells were stimulated with cytokines for the indicated time. After washed with ice-cold PBS twice, cell lysates were prepared by adding SDS-PAGE sample buffer containing 0.05 M Tris-HCl, 2% SDS, 6% β-mercaptoethanol, 10% glycerol and bromophenol blue, and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, MA). The membrane was blocked with 2% skim milk and incubated with anti-phospho-STAT3 (Cell Signaling Technology, MA), anti-phospho-ERK1/2 (Cell Signaling Technology), anti-phospho-JNK (Cell Signaling Technology) or anti-phospho-p38 MAPK (Cell Signaling Technology) antibody as a first antibody. ECL system was used for detection. To quantify the extent of phosphorylation, the membranes were reprobed with anti-STAT3 (Santa Cruz Biotechnology), anti-ERK1/2 (Cell Signaling Technology), anti-JNK (Cell Signaling Technology) or anti-p38 MAPK (Santa Cruz Biotechnology) antibody. The band intensities of phospho-proteins were normalized with those of total proteins. Anti-GAPDH antibody was purchased from Chemicon international.

**Immunofluorescent examination**

Cells were fixed with 3.7% formaldehyde in TBS for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 3 min. After washed with PBS, cells were incubated with anti-NFκB p65 antibody (Santa Cruz Biotechnology) followed by the incubation with Alexa Fluor 546-conjugated secondary antibody (Molecular Probes, CA). Nuclei were
stained with Hoechst dye. The stained cells were examined by fluorescence microscopy (Olympus IX70).

**Preparation of cardiac c-kit+ cells**

Isolation of cardiac c-kit+ cells from 57Bl/6 mice (10- to 12-week-old) was performed by MACS with anti-c-kit antibody (BD Biosciences).

**Cellular uptake of Dil-labeled Ac-LDL**

For uptake of Dil-labeled Ac-LDL (DiI-Ac-LDL, Molecular Probes), cells were incubated with DiI-Ac-LDL (5 µg/mL) for 5 hours. After washed with PBS, cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Then, cells were incubated with anti-CD31 antibody (BD Biosciences) followed by the incubation with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Nuclei were stained with Hoechst dye. The stained cells were examined by fluorescence microscopy (Olympus IX70).

**Immunocytochemical examination**

Immunocytochemical analyses were performed as previously described. Cells were fixed with 3.7% formaldehyde in TBS for 20 min at room temperature, and incubated with anti-CD31 (BD Biosciences, CA) or anti-VE-cadherin antibody (BD Biosciences), followed by the incubation with alkaline phosphatase-conjugated secondary antibody (Santa Cruz biotechnology). Cells were washed with TBS and incubated in BCIP/NBT solution (SIGMA, MO). After staining, positively-stained cells were counted in number by a person who was blinded to the culture conditions.
**Construction and infection of adenoviral vectors**

Adenoviral vectors expressing dominant negative STAT3 (dnSTAT3) and control adenoviral vectors expressing β-galactosidase were previously described. Cells infected with the adenoviral vectors at a multiplicity of infection (MOI) of 100 for 24 hours. After removal of adenoviral vectors, cells were incubated with DMEM/F12 supplemented with ITS (10 µg/mL) for 24 hours, and then cultured in the medium containing each cytokine.

**Gene silencing of STAT3 with siRNA**

Stealth RNAi targeted to mouse STAT3 (#1: MSS209600, #2: MSS209602) and control Stealth RNAi were purchased from Invitrogen. Cells were transfected with siRNA using Lipofectamine RNAi MAX reagent (Invitrogen) according to the manufacturer’s protocol. Forty-eight hours after transfection of siRNA, cells were cultured in the medium containing each cytokine.

**Generation of myocardial infarction**

Myocardial infarction (MI) was generated by coronary artery ligation according to previous report with minor modification. Briefly, C57Bl/6 mice (8- to 10-week-old) were anesthetized and mechanically ventilated with 80% oxygen containing 1.5% isoflurane. After left thoracotomy, the left coronary artery was ligated with a 7-0 silk suture. Infarction was confirmed by discoloration of the ventricle and by ST-T changes in electrocardiographic monitor. The chest and the skin were closed with 5-0 silk sutures.

In immunohistological analyses, the hearts were excised 14 days after surgery and embedded in OCT compound. In experiments for the estimation of cytokine expression, total RNA was
prepared from hearts 0, 1, 4, 7 and 14 days after surgery.

**Histological examination**

Frozen sections (5 µm thick) embedded in OCT compound were prepared from normal or MI hearts (n=3 mice), and fixed with 4% paraformaldehyde in PBS for 20 min. After blocked with 3% BSA/0.1% Triton X-100 in TBS for 30 min, sections were incubated with rat anti-Sca-1 (BD Bioscience) and goat anti-VE-cadherin antibodies (Santa Cruz Biotechnology) followed by the incubation with Alexa Fluor 488-conjugated anti-rat IgG (Molecular Probes) and Alexa Fluor 546-conjugated anti-goat IgG (Molecular Probes). Nuclei were stained with Hoechst dye. Sections were examined by fluorescence microscopy (Olympus IX70) and the number of Sca-1+/VE-cadherin+ cells was counted in 3 fields per heart under 400× magnification.

**Statistical analysis**

Statistical significance was determined by paired t-test or student’s t-test. Data were presented at mean ± S.E. or mean ± S.D. $p$ value < 0.05 was considered to be statistically significant.
Supplementary Figure Legends: details for figure legends in manuscript

Figure 1. IL-11 and CT-1 induce the expression of endothelial specific markers in cardiac Sca-1+ cells. (A) Cardiac Sca-1+ cells were cultured with TNF-α (1 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-11 (20 ng/mL) or CT-1 (20 ng/mL) for 14 days. Total RNA was prepared and RT-PCR was performed for endothelial cell specific genes, such as VE-cadherin, CD31 and Flk-1. Cells cultured in the medium containing LIF (1000 units/mL) were used as the positive control. GAPDH was used as the internal control. Representative data are shown. Experiments were repeated three times with similar results. (B) Cardiac Sca-1+ cells were cultured with IL-11 (20 ng/mL), CT-1 (20 ng/mL) or LIF (1000 units/mL) for 14 days. Total RNA was prepared and real time RT-PCR was performed for endothelial cell specific genes. The expression levels of endothelial cell specific genes were normalized with that of GAPDH. Data are shown as mean ± S.E. from four independent cultures. *p<0.05, **p<0.01 versus control. (C) Cardiac Sca-1+ cells were stimulated with IL-11 (20 ng/mL) or LIF (1000 units/mL) for 5 minutes. Cell lysates were immunoprecipitated with anti-p-Tyr antibody, and then the immunoprecipitates were immunoblotted with anti-gp130 antibody (phospho-gp130). Cell lysates were used for analysis of the expression of total gp130 (gp130). (D) Cardiac Sca-1+ cells were cultured in the medium containing TNF-α (1 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-11 (20 ng/mL) or CT-1 (20 ng/mL) for 14 days and stained with anti-VE-cadherin antibody. Cells cultured in the medium containing LIF (1000 units/mL) for 14 days were used as the positive control. Representative microscopic images cultured with or without IL-11, CT-1 or LIF were shown in Supplementary Figure VI. The frequency of VE-cadherin-positive cells was quantified. Data are shown as mean ± S.E. from five fields. *p<0.05, **p<0.01 versus control. Experiments were repeated three
times with similar results.

**Figure 2. IL-11 and CT-1 activate STAT3 in cardiac Sca-1+ cells.** (A) Cardiac Sca-1+ cells were stimulated with IL-11 (20 ng/mL) or CT-1 (20 ng/mL) for the indicated time. Cell lysates were immunoblotted with anti-phospho-STAT3 or anti-phospho-ERK1/2 antibody. The membranes were reprobed with anti-STAT3 or anti-ERK1/2 antibody. Representative data are shown. (B) Quantification of the time course of the phosphorylation of STAT3 by IL-11 or CT-1. Data are shown as mean ± S.E. from three independent immunoblot assays. *p<0.05, **p<0.01 versus 0 min. (C) Quantification of the phosphorylation of STAT3 by TNF-α, IL-1β, IL-6, IL-11, CT-1 or LIF. Cardiac Sca-1+ cells were stimulated with TNF-α (1 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-11 (20 ng/mL), CT-1 (20 ng/mL) or LIF (1000 units/mL) for 15 min. Cell lysates were immunoblotted with anti-phospho-STAT3 antibody. The membrane was reprobed with anti-STAT3 antibody. Data are shown as mean ± S.E. from three independent immunoblot assays. *p<0.05, **p<0.01 versus control (0 min).

**Figure 3. Soluble IL-6 receptor confers the potential to induce VE-cadherin, an endothelial marker protein, on IL-6.** (A) Cardiac Sca-1+ cells were cultured in the medium containing IL-6 (20 ng/mL) in the presence or absence of indicated concentration of sIL-6R for 14 days. Cells were stained with anti-VE-cadherin antibody and the frequency of VE-cadherin-positive cells was calculated. Data are shown as mean ± S.E. from five fields. **p<0.01 versus control. Experiments were repeated three times with similar results. (B and C) Cardiac Sca-1+ cells were stimulated with IL-6 (20 ng/mL) in the presence or absence of sIL-6R (100 ng/mL) for the indicated time. Cell lysates were immunoblotted with anti-phospho-STAT3. The membranes were
reprobed with anti-STAT3 antibody. (B) Representative data are shown. (C) The phosphorylation of STAT3 by IL-6 alone (open column) or by combination of IL-6 and sIL-6R (closed column) was quantified. Data are shown as mean ± S.E. from three independent immunoblot assays. *p<0.05, **p<0.01 versus 0 min. #p<0.05 versus IL-6 treatment at each time point.

Figure 4. STAT3 is required for the gp130-mediated endothelial lineage commitment of cardiac Sca-1+ cells. (A) Cardiac Sca-1+ cells were transfected with adenoviral vectors expressing dnSTAT3 (dn) or β-galactosidase (β), and then cultured with IL-11 (20 ng/mL), CT-1 (20 ng/mL) or LIF (1000 units/mL) for 14 days. Total RNA was prepared and RT-PCR was performed for endothelial cell specific genes. Representative data are shown. Experiments were repeated three times with similar results. (B) Cells were transfected with control siRNA (control) or with two independent siRNAs targeting different sequences of STAT3 (STAT3#1 and STAT3#2). Forty eight hours later, cells were stimulated with LIF (1000 units/mL) for 5 min. Cell lysates were immunoblotted with anti-phospho-STAT3 antibody. The membranes were reprobed with anti-STAT3 and anti-GAPDH antibodies. Representative data are shown. (C) Cells were transfected with control siRNA (10 nM) or STAT3 siRNA (10 nM) and then cultured with IL-11 (20 ng/mL), CT-1 (20 ng/mL) or LIF (1000 units/mL) for 14 days. Total RNA was prepared and RT-PCR was performed for VE-cadherin and CD31. Representative data are shown. Experiments were repeated three times with similar results. (D) Cells transfected with siRNA (10 nM) were cultured in the presence of LIF (1000 units/mL) for 14 days and then treated with DiI-labeled Ac-LDL (5 µg/mL) for 5 hours. The percentage of cells positive for Ac-LDL was calculated. Data are shown as mean ± S.E. from five fields. **p<0.01 versus control. #p<0.05,
##p<0.01 versus LIF + control siRNA. Experiments were repeated three times with similar results.

Figure 5. The upregulation of IL-6 family cytokines is accompanied by the commitment of Sca-1+ cells to endothelial lineage after MI. (A) IL-6 family cytokines are upregulated in post-infarct myocardium. Total RNA was prepared from hearts after MI and real time RT-PCR was performed for IL-11, CT-1, LIF and IL-6R. The expression levels of IL-6 family cytokine genes were normalized with that of GAPDH. Data are shown as mean ± S.D. from 3-5 mice. *p<0.05, **p<0.01 versus no operation (day 0). (B) Identification of Sca-1+ cells expressing VE-cadherin after MI. Frozen sections were prepared from hearts 14 days after MI and co-stained with anti-Sca-1 and anti-VE-cadherin antibodies. Representative microscopic images were shown (scale bar, 25 µm). Arrows indicate Sca-1+/VE-cadherin+ cells.
Legends for Supplementary Figures

Supplementary Figure I. Expression profiles of cytokine receptors in cardiac Sca-1+ cells.
Total RNA was isolated from cardiac Sca-1+ cells and murine hearts, and RT-PCR was performed for cytokine receptors. Total RNA isolated from murine liver was used as the positive control. GAPDH was used as the internal control. Representative data are shown.

Supplementary Figure II. TNF-α and IL-1β treatment activate their downstream signaling pathways, such as JNK, p38 MAPK and NFκB, in cardiac Sca-1+ cells. (A) Cardiac Sca-1+ cells were cultured in the medium containing TNF-α (1 ng/mL) or IL-1β (10 ng/mL) for the indicated time. Cell lysates were immunoblotted with anti-phospho-JNK or anti-phospho-p38 MAPK antibody. The membranes were reprobed with anti-JNK or anti-p38 MAPK antibody. Representative data are shown. (B) Cardiac Sca-1+ cells were cultured in the medium containing TNF-α (1 ng/mL) or IL-1β (10 ng/mL) for 30 min and stained with anti-NFκB p65 antibody. Representative microscopic images were shown (scale bar, 50 μm). NFκB p65 were translocated from the cytoplasm to the nucleus by TNF-α and IL-1β.

Supplementary Figure III. IL-11 and CT-1 induce the endothelial cell lineage commitment of cardiac Sca-1+ cells in a dose- and time course-dependent manner. (A and B) Cardiac Sca-1+ cells were cultured in the medium containing indicated concentration of IL-11 (A) or CT-1 (B) for 14 days and RT-PCR was performed for endothelial cell specific genes, such as VE-cadherin, CD31 and Flk-1. Cells cultured in the medium containing LIF (1000 units/mL) were used as the positive control. GAPDH was used as the internal control. Representative data are shown. Experiments
were repeated three times with similar results. (C and D) Cardiac Sca-1+ cells were cultured in the medium containing IL-11 (20 ng/mL, C) and CT-1 (20 ng/mL, D) for indicated time. RT-PCR was performed for endothelial cell specific genes. Representative data are shown. Experiments were repeated three times with similar results.

Supplementary Figure IV. IL-11 and CT-1 do not induce the differentiation of cardiac Sca-1+ cells into cardiomyocytes or smooth muscle cells. Cardiac Sca-1+ cells were cultured with TNF-α (1 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-11 (20 ng/mL), CT-1 (20 ng/mL) or LIF (1000 units/mL) for 14 days. Total RNA was prepared and RT-PCR was performed for endothelial cell, cardiomyocyte (α-MHC, Nkx-2.5 and GATA4) and smooth muscle cell (calponin and GATA6) specific genes. Total RNA prepared from heart was used as the positive control. GAPDH was used as the internal control. Representative data are shown. Experiments were repeated three times with similar results.

Supplementary Figure V. LIF induces the expression of endothelial cell marker genes in cardiac c-kit+ cells. Cardiac c-kit+ cells and cardiac Sca-1+ cells were cultured with or without LIF (1000 units/mL) for 14 days. Total RNA was prepared and RT-PCR was performed for endothelial cell specific genes. Representative data were shown.

Supplementary Figure VI. IL-11 and CT-1 induce the commitment of cardiac Sca-1+ cells to the endothelial cell lineage. Cardiac Sca-1+ cells were cultured in the medium containing TNF-α (1 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-11 (20 ng/mL) or CT-1 (20 ng/mL) for 14 days and stained with anti-VE-cadherin antibody. Cells cultured in the medium containing LIF (1000
units/mL) for 14 days were used as the positive control. (A) Representative microscopic images cultured with or without IL-11, CT-1 or LIF were shown (scale bar, 100 µm).

Supplementary Figure VII. Endothelial marker-positive cells, differentiated from cardiac Sca-1+ cells, incorporate Dil-Ac-LDL. To induce the differentiation, cardiac Sca-1+ cells were cultured in the medium containing LIF (1000 units/mL) for 14 days. Cells were treated with Dil-Ac-LDL for 5 hours and stained with anti-CD31 antibody. Representative microscopic images were shown (scale bar, 100 µm). CD31-positive/Dil-Ac-LDL-positive cells were observed in three independent cultures. Of note, more than 90% of CD31-positive cells incorporated Dil-Ac-LDL.
## Supplementary Table

### Supplementary Table I  PCR primers used in the present study

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