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Leukemia Inhibitory Factor Induces Endothelial Differentiation in Cardiac Stem Cells*

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Tomomi Mohri[‡], Yasushi Fujio[‡1], Makiko Maeda[‡], Takashi Ito[‡], Tomohiko Iwakura[‡], Yuichi Oshima[‡], Yoriko Uozumi[‡], Masashi Segawa[§], Hiroshi Yamamoto[§], Tadamitsu Kishimoto[¶], and Junichi Azuma[‡]

From the [‡]Department of Clinical Evaluation of Medicines and Therapeutics and [§]Department of Immunology, Graduate School of Pharmaceutical Sciences, and the [¶]Laboratory of Immune Regulation, Graduate School of Frontier Bioscience, Osaka University, 1-6 Yamadaoka, Suita City, Osaka 565-0871, Japan

The importance of interleukin 6 (IL-6)-related cytokines in cardiac homeostasis has been studied extensively; however, little is known about their biological significance in cardiac stem cells. Here we describe that leukemia inhibitory factor (LIF), a member of IL-6-related cytokines, activated STAT3 and ERK1/2 in cardiac Sca-1+ stem cells. LIF stimulation resulted in the induction of endothelial cell-specific genes, including VE-cadherin, *Flk-1*, and *CD31*, whereas neither smooth muscle nor cardiac muscle marker genes such as *GATA4*, *GATA6*, *Nkx-2.5*, and calponin were up-regulated. Immunocytochemical examination showed that about 25% of total cells were positively stained with anti-CD31 antibody 14 days after LIF stimulation. Immunofluorescent microscopic analyses identified the Sca-1+ cells that were also positively stained with anti-von Willebrand factor antibody, indicating the differentiating process of Sca-1+ cells into the endothelial cells. IL-6, which did not activate STAT3 and ERK1/2, failed to induce the differentiation of cardiac stem cells into the endothelial cells. In cardiac stem cells, the transduction with dominant negative STAT3 abrogated the LIF-induced endothelial differentiation. And the inhibition of ERK1/2 with the MEK1/2 inhibitor U0126 also prevented the differentiation of Sca-1+ cells into endothelial cells. Thus, both STAT3 and ERK1/2 are required for LIF-mediated endothelial differentiation in cardiac stem cells. Collectively, it is proposed that LIF regulates the commitment of cardiac stem cells into the endothelial cell lineage, contributing to neovascularization in the process of tissue remodeling and/or regeneration.

Cardiac homeostasis is maintained by various kinds of extracellular signals through paracrine factors. Among these signals, interleukin 6 (IL-6)[2]-related cytokines have been demonstrated to play important roles in cardioprotection (1), vessel formation (2, 3), and cell-cell adhesion (4) in the heart. IL-6-related cytokines utilize glycoprotein 130 (gp130) as a common receptor. Signals through gp130 activate the signal transducer and activator of transcription (STAT) proteins and extracellular signal-regulated kinases 1/2 (ERK1/2) (5). Activated STAT proteins function as latent transcription factors and up-regulate a wide range of target genes, including *bcl-xL* (6), *VEGF* (7), metallothionein (1), *MnSOD* (manganese superoxide dismutase) (8), and *Wnt5a* (4). Recently it has been reported that cardiomyocyte-restricted ablation of the *STAT3* gene results in heart failure, accompanied by impairment of vessel growth and high sensitivity to cardiac injury (9, 10). Thus the importance of IL-6-related cytokines/gp130/STAT pathway has been established in cardiac myocytes; however, the possibility remains to be fully addressed that IL-6-related cytokines stimulate the non-myocyte population in the heart and contribute to cardiac homeostasis.

Previously it was believed that cardiac myocytes exited from the cell cycle immediately after birth and regenerated only to a lesser extent. However, recently, cardiac stem cells have been identified in the myocardium and demonstrated to differentiate into cardiomyocytes. Thus far, two kinds of cardiac stem cells, c-*kit*+ cells (11) and Sca-1+ cells (12, 13), have been reported. It is demonstrated that c-*kit*+ cells differentiate into vascular smooth muscle cells and endothelial cells, as well as cardiac myocytes, whereas Sca-1+ cells differentiate into osteoblasts or adipocytes. Despite the potential importance in the clinical application, the physiological signals responsible for the differentiation of the stem cells remain to be fully elucidated.

In the present study, we examined the regulatory mechanisms for the endothelial differentiation of cardiac Sca-1+ cells. We then have demonstrated that leukemia inhibitory factor (LIF), an IL-6-related cytokine, promotes endothelial differentiation. Inhibition of STAT3 activity or ERK1/2 activity prevents endothelial differentiation, suggesting that both STAT3 and ERK play important roles in endothelial differentiation of cardiac stem cells. These data indicate that signals through gp130 could induce endothelial differentiation of cardiac stem cells as a potential source of endothelial cells. This study proposes a novel mechanism of gp130-mediated neovascularization.

MATERIALS AND METHODS

Preparation of Cardiac Sca-1+ Cells—Sca-1+ cardiac stem cells were prepared according to a previous report (13) with minor modification. Briefly, hearts from adult C57Bl/6 mice (10–12 weeks old; Japan SLC) were treated with 0.1% collagenase for 30 min (12). Cells were filtered through 80-μm mesh and suspended in PBS supplemented with 3% FBS. To separate Sca-1+ cells, cells were incubated with biotinylated anti-Sca-1 antibody (BD Biosciences) for 15 min on ice and washed with IMag buffer (consisting of PBS with 0.5% bovine serum albumin and 2 mM EDTA) followed by incubation with streptavidin-conjugated particles for 30 min on ice. The labeled cells were resuspended in IMag buffer, and the Sca-1+ cells were separated from the cell suspension by using IMagnet (BD IMag Cell Separation System, BD Biosciences)

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[1] To whom correspondence should be addressed: Dept. of Clinical Evaluation of Medicines and Therapeutics, 1-6 Yamadaoka, Suita City, Osaka, 565-0871, Japan. Tel.: 81-6-6879-8252; Fax: 81-6-6879-8253; E-mail: fujio@phs.osaka-u.ac.jp.

[2] The abbreviations used are: IL-6, interleukin-6; LIF, leukemia inhibitory factor; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; Sca-1, stem cell antigen-1; gp130, glycoprotein 130; vWF, von Willebrand factor; mNSCM, modified neural stem cell medium; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; RT, reverse transcriptase; dnSTAT3, dominant negative STAT3;PBS, phosphate-buffered saline; FBS, fetal bovine serum.

according to the manufacturer's protocol. Flow cytometric analysis confirmed that the 97.6 ± 1.1% of the cells were Sca-1 positive cells, which is consistent with previous studies (12, 13).

Newly isolated cardiac Sca-1+ cells were incubated in Iscove's modified Dulbecco's medium 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 modified Eagle's medium and Ham's F12 (ratio 1:1) supplemented with 5 mM HEPES, ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite), 10 ng/ml bFGF, 20 ng/ml EGF, and 1000 units/ml LIF (11) as described under "Results." In some experiments, Sca-1+ cells were amplified in mNSCM and used for the assay.

TABLE 1
PCR primers used in the present study

Genes	Direction	Sequence
VE-cadherin	Forward	5′-ATCTTCCTCTGCATCCTCAC-3′
	Reverse	5′-GTAAGTGACCAACTGCTCGT-3′
Flk-1	Forward	5′-TGCCGGCATGGTCTTCTGTGAGG-3′
	Reverse	5′-CATTGAGCTCTGTTCTCGCTGTAC-3′
CD31	Forward	5′-GAGCCCAATCACGTTTCAGTTT-3′
	Reverse	5′-TCCTTCCTGCTTCTTGCTAGCT-3′
Nkx-2.5	Forward	5′-CAGTGGAGCTGGACAAAGCC-3′
	Reverse	5′-TTGTAGCGACGGTTCTGGAA-3′
GATA4	Forward	5′-CTGTCATCTCACTATGGGCA-3′
	Reverse	5′-CCAAGTCCGAGCAGGAATTT-3′
Calponin	Forward	5′-GCACATTTTAACCGAGGTCC-3′
	Reverse	5′-TGACCTTCTTCACAGAACCC-3′
GATA6	Forward	5′-AAAGCTTGCTCCGGTAACAG-3′
	Reverse	5′-GGACAGACTGACACCTATGT-3′
GAPDH	Forward	5′-CATCACCATCTTCCAGGAGCG-3′
	Reverse	5′-GAGGGGCCATCCACAGTCTTC-3′

LIF was purchased from Chemicon International. IL-6, bFGF, and EGF were purchased from Peprotech EC (London, UK). U0126 (Cell signaling, MA), a highly selective inhibitor for MEK1/2, was used for the inhibition of ERK1/2.

Immunoblot Analyses—Immunoblotting was performed as described previously (14). Briefly, cells were stimulated with IL-6-related cytokines for the indicated time. After being washed with ice-cold PBS twice, cell lysates were prepared by the addition of SDS-PAGE sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, MA). The membrane was blocked with 2% skim milk and incubated with anti-phospho-STAT3, anti-phospho-ERK1/2, or anti-phospho-Akt (all from Cell Signaling) 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, Santa Cruz, CA), anti-ERK1/2 (Cell Signaling), or anti-Akt (Cell Signaling) antibody. The band intensities of phospho-proteins were normalized with those of total proteins. The activities of phospho-specific antibodies were confirmed by using the extract from cardiomyocytes stimulated LIF as a positive control.

RT-PCR Analyses—RT-PCR was performed as described previously (6). Briefly, total RNA was prepared using the acid guanidinium thiocyanate-phenol-chloroform method (15). Total RNA (1 μg) was subjected to first strand cDNA synthesis by using the oligo(dT) first strand primer. Gene-specific primers used for PCR amplification are shown in Table 1. The PCR products were size-fractionated by 2% agarose gel electrophoresis and detected by staining with ethidium bromide.

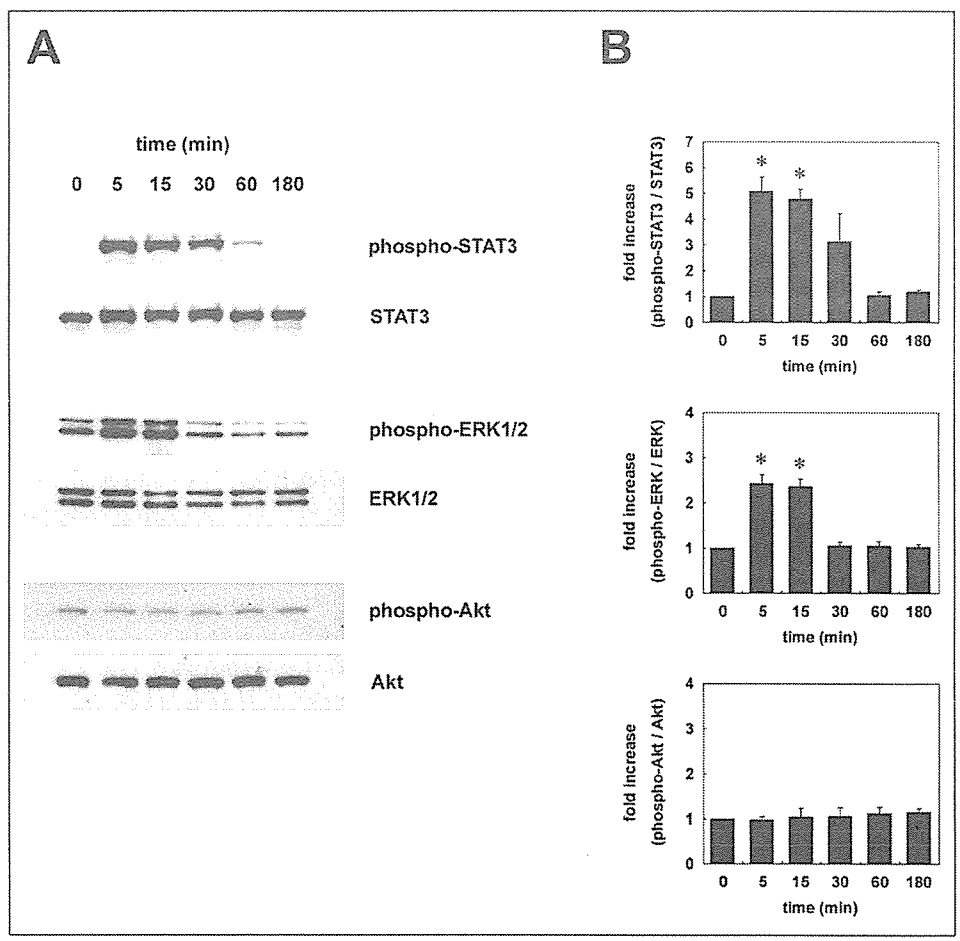

FIGURE 1. **LIF activates STAT3 and ERK, but not Akt, in cardiac Sca-1+ cells.** Cardiac Sca-1+ cells were stimulated by 1000 units/ml LIF for the indicated time. Cell lysates were immunoblotted with anti-phospho-STAT3, anti-phospho-ERK1/2, or anti-phospho-Akt antibody. Blots were reprobed with anti-STAT3, anti-ERK1/2, or anti-Akt antibody. *A*, representative data are shown. *B*, quantitative analysis of tyrosine phosphorylation of STAT3, ERK1/2, and Akt. The band intensities of phospho-proteins were normalized with that of total proteins. Data are presented as means ± S.E. ($n = 3$). *, $p < 0.05$ *versus* control (paired *t* test).

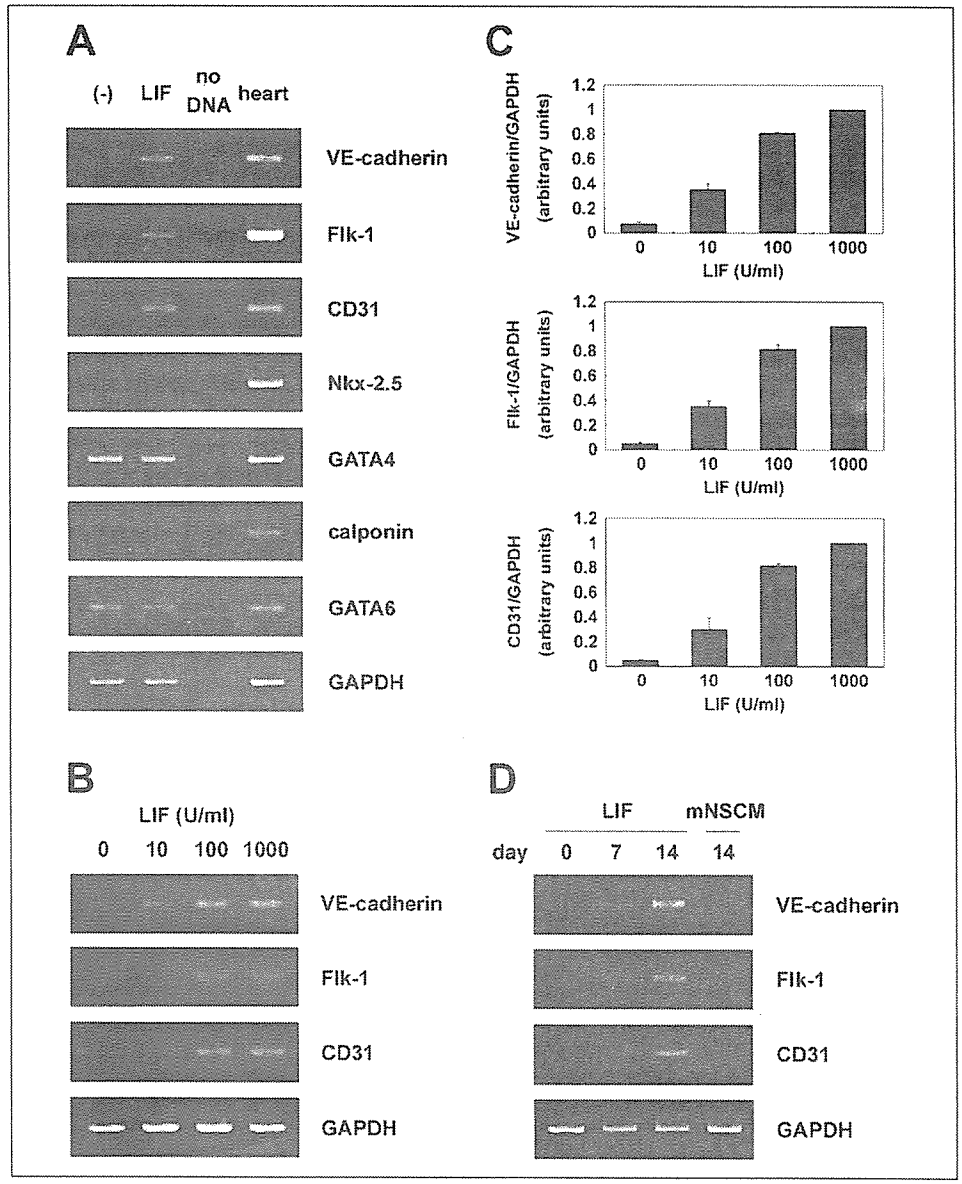

FIGURE 2. **Signals through gp130 up-regulate endothelial cell-specific marker genes in cardiac Sca-1+ cells.** *A*, cardiac Sca-1+ cells were stimulated with or without LIF (1000 units/ml) for 14 days. Total RNA was prepared, and RT-PCR was performed for as described under "Materials and Methods." Total RNA prepared from heart was used as positive control. GAPDH was used as an internal control. Experiments were repeated three times with similar results. *B* and *C*, Sca-1+ cells were cultured with the indicated concentrations of LIF for 14 days. Total RNA was prepared, and RT-PCR was performed for the endothelial cell-specific genes. GAPDH was used as an internal control. Experiments were repeated three times with similar results. Representative data are shown in *B*. The band intensities of endothelial markers were normalized with that of GAPDH. Data are presented as means ± S.E. ($n = 3$) (C). *D*, Sca-1+ cells were maintained in mNSCM containing EGF, bFGF, and LIF for 10 days followed by cultivation in medium containing only LIF (1000 units/ml) or in mNSCM for the indicated time. Total RNA was prepared, and RT-PCR was performed. GAPDH was used as an internal control. Experiments were repeated three times with similar results.

Immunocytochemical Examination—Cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature and incubated with anti-CD31 antibody (BD Biosciences) followed by the incubation with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotechnology). Cells were washed with Tris-buffered saline and incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (BCIP/NBT, Sigma). After staining, positively stained cells were counted by a person who was blinded to the culture conditions.

Immunofluorescent Examination—Immunofluorescent microscopic analyses were performed as described previously (16). Briefly, cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature and stained with anti-Sca-1 (R&D Systems) and anti-von Willebrand factor antibodies (Santa Cruz Biotechnology). Alexa Fluor 488- or 546-conjugated secondary antibody (Molecular Probes) was used for detection. Nuclei were stained with Hoechst dye. Cells were examined by Olympus IX70.

Adenovirus Vectors—Generation of the adenovirus vector expressing dominant negative STAT3 (dnSTAT3) was described previously (17). Adenovirus vector expressing β-galactosidase was used as a control.

Adenovirus vectors were amplified in HEK293 cells and purified by CsCl ultracentrifugation (18). Sca-1+ cells were infected at a multiplicity of infection of 100 for 12 h and cultured under the indicated conditions. By this method, more than 90% of Sca-1+ cells were transfected with adenovirus vectors (data not shown).

Statistical Analysis—Statistical significance was determined by paired *t* test or Student's *t* test. Data were presented as mean ± S.E., and $p < 0.05$ was considered statistically significant.

RESULTS

LIF Activates STAT3 and ERK1/2 in Cardiac Stem Cells—Signals through gp130 play important roles in cardiac homeostasis. Recently, cardiac stem cells have been reported to contribute to cardiac repair and regeneration (11, 12). Thus, we examined whether signals through gp130 are transduced in cardiac stem cells. Cardiac Sca-1+ cells were stimulated with LIF for the indicated time. Cell lysates were prepared, and activation of STAT3, ERK1/2, and Akt was analyzed by immunoblotting with phospho-specific antibodies (Fig. 1*A*). STAT3 and ERKs,

but not Akt, were phosphorylated by LIF stimulation. STAT3 and ERKs were activated within 5 min of LIF stimulation (Fig. 1*B*).

LIF Induces Endothelial Differentiation in Cardiac Stem Cells—We examined the effects of LIF on the differentiation of Sca-1+ cells. By RT-PCR (Fig. 2*A*), it was revealed that LIF induced VE-cadherin, *Flk-1*, and *CD31*, marker genes for endothelial cells, whereas *Nkx-2.5* and calponin was not up-regulated by LIF. GATA4 and GATA6 were expressed in nonstimulated Sca-1+ cells, and their expression was not affected by LIF. LIF induced the expression of endothelial marker genes in a dose-dependent manner (Fig. 2, *B* and *C*). The endothelial markers were submaximally induced by 100units/ml LIF.

Previously it was reported that cardiac c-*kit*+ stem cells could be maintained as undifferentiated cells in mNSCM, Dulbecco's modified Eagle's medium, and Ham's F12 (ratio 1:1) containing bFGF, EGF, and LIF (11). Thus we analyzed the expression of the endothelial markers in Sca-1+ cells cultured in mNSCM. In mNSCM, cardiac Sca-1+ cell population did not express endothelial marker genes; however, cells were differentiated into endothelial cells when moved to the medium containing only LIF (Fig. 2*D*). The induction of endothelial marker genes was detected 7 days after stimulation, and afterward their expression increased. These data suggest that Sca-1+ cells can be maintained in the presence of bFGF, EGF, and LIF without impairing the potential for endothelial differentiation. In preliminary studies, we noticed that cardiac Sca-1+ cells occasionally showed expression of CD31, an endothelial marker, as described previously (12), when cultured in the presence of FBS more than 5 days and that FBS-mediated differentiation depended on the batches of FBS (data not shown). Therefore, in the present study, Sca-1+ cells were amplified in mNSCM. Sca-1+ cells cultured in mNSCM without LIF showed reduced potential for differentiation into endothelial cells, suggesting that LIF could contribute to maintenance of the potential for endothelial differentiation from Sca-1+ cells in the presence of bFGF and EGF (data not shown).

To estimate the frequency of endothelial differentiation from Sca-1+ cell culture, cells were cultured in the presence or absence of LIF for the indicated time and stained with anti-CD31 antibody (Fig. 3*A*). At day 3, CD31+ cells were not observed either in the presence or absence of LIF. Cells positively stained with anti-CD31 antibody were detected 7 days after LIF stimulation. Thereafter, the frequency of CD31+ cells increased up to about 25% of total cells 14 days after LIF stimulation (Fig. 3*B*). In contrast, CD31+ cells were not significantly detected when the cells were cultured in the absence of LIF. We analyzed the effects of bFGF on LIF-induced endothelial differentiation because bFGF is also known to be a potent angiogenic growth factor, and it was found that co-stimulation with bFGF and LIF did not increase the number of CD31 positive cells, as compared with LIF stimulation (data not shown).

The cardiac stem cells were cultured in the presence of LIF for 14 days and co-stained with anti-Sca-1 and anti-von Willebrand factor (vWF) antibodies (Fig. 4). Immunofluorescent microscopic analyses detected Sca-1+ cells that also showed positive staining for vWF, an endothelial marker, thus suggesting the transition from Sca-1+ cells to endothelial lineage.

Both STAT3 and ERK1/2 Are Required for LIF-mediated Endothelial Differentiation—To analyze the signaling pathways involved in endothelial differentiation, we tested the effects of IL-6, which utilizes gp130 as one subunit of its receptor system. In contrast to LIF, IL-6 did not activate STAT3 or ERK1/2 in cardiac stem cells (Fig. 5*A*). Consistently, the endothelial cell-specific genes, including VE-cadherin, *Flk-1*, and *CD31*, were not increased in response to IL-6 (Fig. 5*B*). We also addressed the additional effects of IL-6 to LIF-mediated endothelial differentiation; however, endothelial differentiation, induced by LIF, was not enhanced by co-stimulation with IL-6 (Fig. 5*C*). Thus, further efforts were made to address the functional significance of STAT3 and ERK1/2.

To examine whether STAT3 activity is involved in the endothelial differentiation of Sca-1+ cells by LIF, we analyzed the effects of the inhibition of STAT3 by using adenovirus vectors expressing dnSTAT3 (Fig. 6*A*). LIF failed to induce the endothelial markers in dnSTAT3-expressing cells, whereas endothelial marker genes were up-regulated in

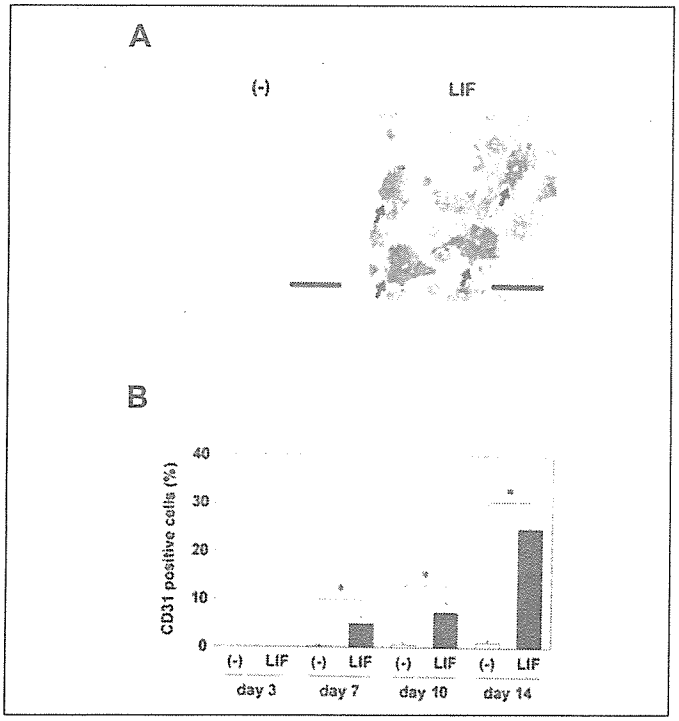

FIGURE 3. **Quantitative estimation of the frequency of the differentiation of Sca-1+ cells into endothelial cells.** Cardiac Sca-1+ cells were cultured in medium containing 0 or 1000 units/ml LIF for the indicated time and then stained with anti-CD31 antibody. *A*, cells were cultured in the presence or absence of LIF for 14 days. Representative immunocytochemical microscopic images were shown (magnification ×100, *scale bar* = 100 μm). *Arrows* indicate CD31-positive cells. *B*, cells were cultured in the presence or absence of LIF for the indicated time. Cells were stained with anti-CD31 antibody. The frequency of CD31-positive cells was calculated in five fields. Each field contains 80–100 cells. Data are shown as mean ± S.E. *, $p < 0.05$ (Student's *t* test). Experiments were repeated three times with similar results.

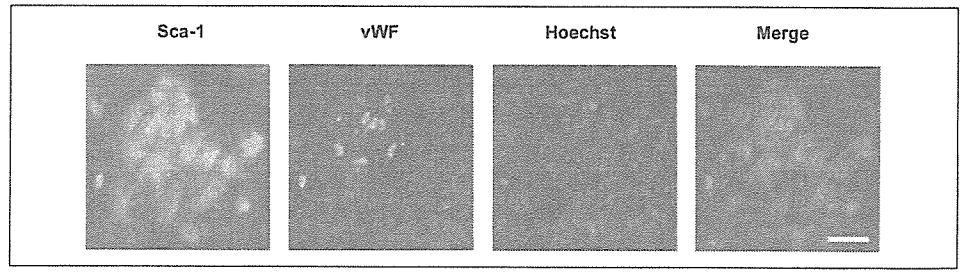

FIGURE 4. **LIF induces the expression of vWF, an endothelial marker, in cardiac Sca-1+ cells.** Cardiac Sca-1+ cells were cultured in the medium containing LIF (1000 units/ml) for 14 days and co-stained with anti-Sca-1 and anti-vWF antibodies. Representative immunofluorescent microscopy was shown (magnification ×100, *scale bar* = 100 μm). Sca-1+/vWF+ cells were observed in three independent cultures.

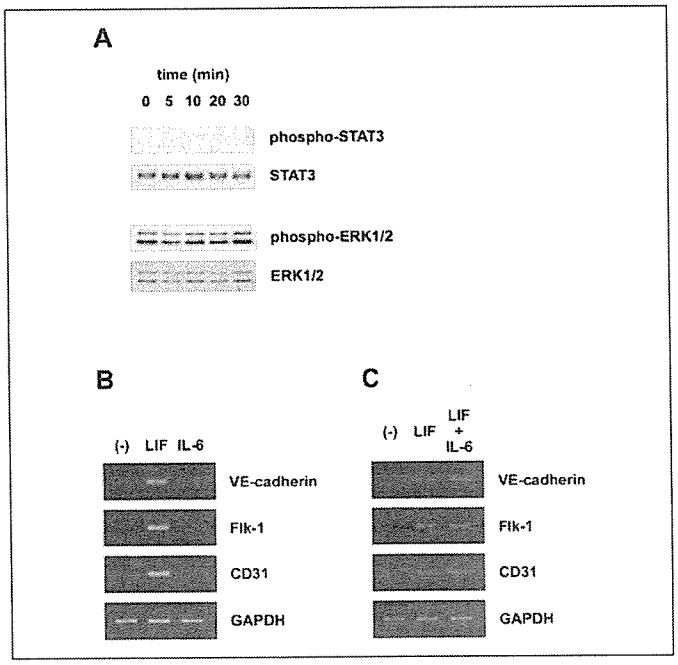

FIGURE 5. **IL-6 does not induce the expression of endothelial marker genes in cardiac Sca-1+ cells.** *A*, cardiac Sca-1+ cells were stimulated by IL-6 (20 ng/ml) for the indicated time. Cell lysates were prepared and immunoblotted with anti-phospho-STAT3 or anti-phospho-ERK1/2 antibody. Blots were reprobed with anti-STAT3 or anti-ERK1/2 antibody. Experiments were repeated three times with similar results. *B*, cardiac Sca-1+ cells were cultured in the presence of IL-6 (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. GAPDH was used as an internal control. Experiments were repeated three times with similar results. *C*, to examine the additional effects of IL-6 on LIF-mediated differentiation, Sca-1+ cells were cultured in the presence of LIF or LIF plus IL-6 for 14 days. Total RNA was prepared, and RT-PCR was performed for endothelial cell-specific genes. GAPDH was used as an internal control. Experiments were repeated three times with similar results.

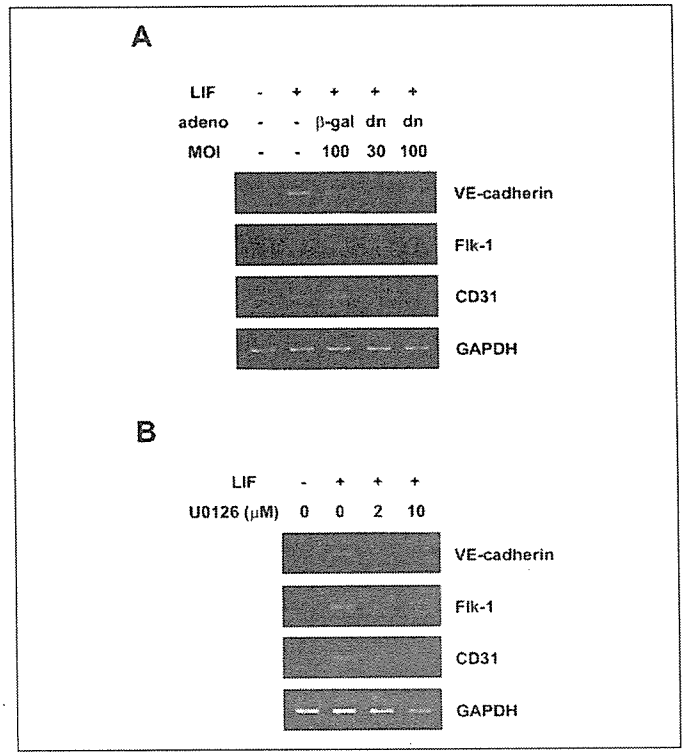

FIGURE 6. **LIF induces endothelial differentiation of cardiac Sca-1+ cells through STAT3 and ERK1/2.** *A*, cardiac Sca-1+ cells were transduced with adenovirus vectors (*adeno*) expressing dominant negative STAT3 (*dn*) or β-galactosidase (*β-gal*) as a control at the indicated multiplicity of infection (*MOI*) and cultured with (+) or without (−) LIF (100 units/ml) for 14 days. Total RNA was prepared, and expression of endothelial marker genes was examined by RT-PCR. GAPDH was used as an internal control. Experiments were repeated three times with similar results. *B*, cardiac Sca-1+ cells were cultured in the presence or absence of LIF (100 units/ml) with or without U0126, an ERK inhibitor. Total RNA was prepared, and expression of endothelial marker genes was examined by RT-PCR. GAPDH was used as an internal control. Experiments were repeated three times with similar results.

the cells adenovirally transfected with β-galactosidase, a control. Next, we tested the effects of U0126, an MEK inhibitor, on the endothelial differentiation of cardiac stem cells (Fig. 6*B*). The inhibition of ERK1/2 with U0126 inhibited the induction of endothelial marker genes by LIF. These data indicate that both STAT3 activity and ERK activity are required for the LIF-mediated differentiation of Sca-1+ cells into endothelial cells. We confirmed that neither transfection of dnSTAT3 nor treatment with U0126 increased the frequency of pyknotic nuclei as analyzed by Hoechst staining, suggesting that neither inhibition of STAT3 nor of ERK affected cell viability (data not shown).

DISCUSSION

In the present study, we have demonstrated that LIF induced endothelial differentiation in cardiac stem cells. LIF stimulation rapidly activated STAT3 and ERK1/2 in cardiac stem cells. LIF up-regulated endothelial cell-specific genes without inducing either smooth muscle- or cardiac muscle-specific markers. The inhibition of STAT3 or ERK pathways abrogated endothelial differentiation, indicating that both STAT3 and ERKs are required for LIF-induced endothelial differentiation.

Initially, it was believed that postnatal neovascularization results exclusively from fully differentiated endothelial cells. However, recent studies have established that bone marrow-derived endothelial progenitor cells contribute to vessel formation (19). Endothelial progenitor cells circulate in the peripheral blood, home to the target organs, and differentiate into endothelial cells (20). In the progenitor cells, VE-cadherin and Flk-1 are highly expressed, like fully differentiated endothelial cells. In contrast, by RT-PCR we have demonstrated that neither VE-cadherin nor *Flk-1* is expressed in cardiac undifferentiated Sca-1+ cells, as reported previously (12, 13). Thus, cardiac Sca-1+ cells, used in the present study, are a population that is distinct from endothelial progenitors. Therefore it could be proposed that the cardiac Sca-1+ population is a novel source of endothelial cells in the heart. This proposal is supported by the recent findings that skeletal muscle-derived Sca-1+ cells are also differentiated into endothelial cells (21), suggesting the possible involvement of tissue-resident stem cells in vessel formation.

Cardiac Sca-1+ cells are known to be differentiated into cardiomyocytes (12, 13). In the present study, we examined whether LIF induced cardiac differentiation. However, it was revealed that LIF stimulation up-regulated endothelial cell-specific genes without inducing smooth muscle or cardiomyocyte markers. In the process of *in vivo* neovascularization, differentiated endothelial cells migrate and form the capillary tubes. Therefore, we analyzed the migratory activities of LIF in cardiac Sca-1+ cells; however, we could not detect an LIF-mediated increase in cell motility (data not shown). Other factors might promote the cell motility, resulting in capillary tube formation *in vivo*. LIF-induced endothelial differentiation is reminiscent of the abnormality in the placenta of LIF receptor-null mice; LIF receptor-null mice exhibit a reduction of the fetal blood vessel component in the placenta (22). It would be interesting to examine whether the tissue-resident stem cells from the peripheral organs, especially from the placenta, can be differentiated into endothelial cells by signals through gp130.

Activation of gp130 results in the rapid induction of the target genes via STAT and ERK proteins after stimulation with IL-6-related cytokines (23). Consistently, LIF rapidly activated STAT and ERK, whereas

the up-regulation of endothelial cell-specific genes such as VE-cadherin, *Flk-1*, and *CD31* was detected more than 24 h after LIF stimulation. Therefore it is unlikely that these endothelial cell-specific genes are direct targets of gp130-mediated transcriptional proteins, including STAT proteins. We examined the effects of LIF on the expression of *HoxA9* gene as an early-stage endothelial marker (24), because HoxA9 is rapidly induced by angiogenic stimuli such as shear stress and plays important roles in the expression of the endothelial genes in endothelial progenitor cells (25). However, HoxA9 was not up-regulated immediately after LIF stimulation (data not shown). Thus it is suggested that cardiac stem cells are differentiated into endothelial cells by LIF through differential mechanisms from the endothelial progenitor cells. Further studies will be required to elucidate the direct target genes of the signals through gp130 in cardiac stem cells.

The Gp130/STAT signaling pathway is activated under pathophysiological conditions in cardiac myocytes. IL-6-related cytokines are produced in cultured cardiomyocytes exposed to pathological stresses such as catecholamine, (26), mechanical stretch (27), and hypoxia (28), leading to activation of the gp130/STAT3 pathway in the myocardium. *In vivo* studies also demonstrated that LIF is up-regulated in pathological hearts, including hypertrophied hearts (29) and failing hearts (30). Moreover, LIF is produced in atrial and ventricular myocytes in failing hearts (31), suggesting the importance of the paracrine system of IL-6-related cytokines. Previously we demonstrated that activation of STAT3 promotes vessel formation through paracrine of angiogenic factors in the heart (2, 7), proposing a cardioprotective machinery via cardiomyocyte-endothelial cell interaction. Consistently, accumulating evidence has confirmed the importance of STAT3 in controlling the paracrine circuits, resulting in neovascularization in pathophysiological situations such as tumor growth (32, 33), psoriasis (34), and diabetic retinopathy (35). Here, in addition to the promotion of vessel growth through paracrine system, we propose that LIF-mediated activation of gp130 directly induces the endothelial differentiation of tissue-resident stem cells, suggesting a novel role of gp130 signaling in angiogenesis. These combined angiogenic properties may explain the prominent neovascularization induced by signals through gp130 during myocardial remodeling (3), although further efforts should be made to elucidate how much cardiac stem cells contribute to angiogenesis *in vivo*.

In summary, the present study has revealed that signals through gp130 transduce endothelial differentiation in cardiac stem cells and that IL-6-related cytokines are the paracrine factors determining the commitment of cardiac stem cells into the endothelial cell lineage. These findings propose a novel mechanism by which signals through gp130 contribute to neovascularization in the process of tissue remodeling and/or regeneration.

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REFERENCES

1. Oshima, Y., Fujio, Y., Nakanishi, T., Itoh, N., Yamamoto, Y., Negoro, S., Tanaka, K., Kishimoto, T., Kawase, I., and Azuma, J. (2005) *Cardiovasc. Res.* **65,** 428–435
2. Osugi, T., Oshima, Y., Fujio, Y., Funamoto, M., Yamashita, A., Negoro, S., Kunisada, K., Izumi, M., Nakaoka, Y., Hirota, H., Okabe, M., Yamauchi-Takihara, K., Kawase, I., and Kishimoto, T. (2002) *J. Biol. Chem.* **277,** 6676–6681
3. Zou, Y., Takano, H., Mizukami, M., Akazawa, H., Qin, Y., Tako, H., Sakamoto, M., Minamino, T., Nagai, T., and Komuro, I. (2003) *Circulation* **108,** 748–753
4. Fujio, Y., Matsuda, T., Oshima, Y., Maeda, M., Mohri, T., Ito, T., Takatani, T., Hirata, M., Nakaoka, Y., Kimura, R., Kishimoto, T., and Azuma, J. (2004) *FEBS letters* **573,** 202–206
5. Kunisada, K., Hirota, H., Fujio, Y., Matsui, H., Tani, K., Yamauchi-Takihara, K., and Kishimoto, T. (1996) *Circulation* **94,** 2626–2632
6. Fujio, Y., Kunisada, K., Hirota, H., Yamauchi-Takihara, K., and Kishimoto, T. (1997) *J. Clin. Investig.* **99,** 2898–2905
7. Funamoto, M., Fujio, Y., Kunisada, K., Negoro, S., Tone, E., Osugi, T., Hirota, H., Izumi, M., Yoshizaki, K., Walsh, K., Kishimoto, T., and Yamauchi-Takihara, K. (2000) *J. Biol. Chem.* **275,** 10561–10566
8. Negoro, S., Kunisada, K., Fujio, Y., Darville, M. I., Eizirik, D. L., Funamoto, M., Osugi, T., Izumi, M., Oshima, Y., Nakaoka, Y., Hirota, H., Kishimoto, T., and Yamauchi-Takihara, K. (2001) *Circulation* **104,** 979–981
9. Jacoby, J. J., Kalinowski, A., Liu, M.-G., Zhang, S. S.-M., Gao, Q., Chai, G.-X., Ji, L., Iwamoto, Y., Li, E., Schneider, M., Russell, K. S., and Fu, X.-Y. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 12929–12934
10. Hilfiker-Kleiner, D., Hilfiker, A., Fuchs, M., Kaminski, K., Schaefer, A., Schieffer, B., Hillmer, A., Schmiedl, A., Ding, Z., Podewski, E., Podewski, E., Poli, V., Schneider, M. D., Schulz, R., Park, J.-K., Wollert, K. C., and Drexler, H. (2004) *Circ. Res.* **95,** 187–195
11. Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., Leri, A., Kajstura, J., Nadal-Ginard, B., and Anversa, P. (2003) *Cell* **114,** 763–776
12. Oh, H., Bradfute, S. B., Gallardo, T., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L. H., Behringer, R. R., Garry, D. J., Entman, M. L., and Schneider, M. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 12313–12318
13. Matsuura, K., Nagai, T., Nishigaki, N., Oyama, T., Nishi, J., Wada, H., Sano, M., Toko, H., Akazawa, H., Sato, T., Nakaya, H., Kasanuki, H., and Komuro, I. (2004) *J. Biol. Chem.* **279,** 11384–11391
14. Fujio, Y., and Walsh, K. (1999) *J. Biol. Chem.* **274,** 16349–16354
15. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
16. Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N., and Walsh, K. (2000) *Circulation* **101,** 660–667
17. Kunisada, K., Tone, E., Fujio, Y., Matsui, H., Yamauchi-Takihara, K., and Kishimoto, T. (1998) *Circulation* **98,** 346–352
18. Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D., and Newgard, C. B. (1994) in *Methods in Cell Biology*, Vol. 43, pp. 161–189, Academic Press, New York
19. Asahara, T., Murohara, T., Sullivan, A., Silver, M., Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J. M. (1997) *Science* **275,** 964–967
20. Kalka, C., Masuda, H., Takahashi, T., Gordon, R., Tepper, O., Gravereaux, E., Pieczek, A., Iwaguro, H., Hayashi, S., Isner, J. M., and Asahara, T. (2000) *Circ. Res.* **86,** 1198–1202
21. Tamaki, T., Akatsuka, A., Ando, K., Nakamura, Y., Matsuzawa, H., Hotta, T., Roy, R. R., and Edgerton, V. R. (2002) *J. Cell Biol.* **157,** 571–577
22. Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S. A., Gliniak, B. C., McKenna, H. J., Papayannopoulou, T., Thoma, B., Cheng, L., Donovan, P. J., Peschon, J. J., Bartlett, P. F., Willis, C. R., Wright, B. D., Carpenter, M. K., Davison, B. L., and Gearing, D. P. (1995) *Development (Camb.)* **121,** 1283–1299
23. Kishimoto, T., Taga, T., and Akira, S. (1994) *Cell* **76,** 253–262
24. Gorski, D. H., and Walsh, K. (2000) *Circ. Res.* **87,** 865–872
25. Rossig, L., Urbich, C., Bruhl, T., Dernbach, E., Heeschen, C., Chavakis, E., Sasaki, K., Aicher, D., Diehl, F., Seeger, F., Potente, M., Aicher, A., Zanetta, L., Dejana, E., Zeiher, A. M., and Dimmeler, S. (2005) *J. Exp. Med.* **201,** 1825–1835
26. Funamoto, M., Hishinuma, S., Fujio, Y., Matsuda, Y., Kunisada, K., Oh, H., Negoro, S., Tone, E., Kishimoto, T., and Yamauchi-Takihara, K. (2000) *J. Mol. Cell. Cardiol.* **32,** 1275–1284
27. Pan, J., Fukuda, K., Saito, M., Matsuzaki, J., Kodama, H., Sano, M., Takahashi, T., Kato, T., and Ogawa, S. (1999) *Circ. Res.* **84,** 1127–1136
28. Hishinuma, S., Funamoto, M., Fujio, Y., Kunisada, K., and Yamauchi-Takihara, K. (1999) *Biochem. Biophys. Res. Commun.* **264,** 436–440
29. Kurdi, M., Randon, J., Cerutti, C., and Bricca, G. (2005) *Mol. Cell. Biochem.* **269,** 95–101
30. Podewski, E. K., Hilfiker-Kleiner, D., Hilfiker, A., Morawietz, H., Lichtenberg, A., Wollert, K. C., and Drexler, H. (2003) *Circulation* **107,** 798–802
31. Jougasaki, M., Leskinen, H., Larsen, A. M., Cataliotti, A., Chen, H. H., and Burnett, J. C. J. (2003) *Eur. J. Heart Fail.* **5,** 137–145
32. Niu, G., Wright, K. L., Huang, M., Song, L., Haura, E., Turkson, J., Zhang, S., Wang, T., Sinibaldi, D., Coppola, D., Heller, R., Ellis, L. M., Karras, J., Bromberg, J., Pardoll, D., Jove, R., and Yu, H. (2002) *Oncogene* **21,** 2000–2008
33. Bromberg, J. (2002) *J. Clin. Investig.* **109,** 1139–1142
34. Sano, S., Chan, K. S., Carbajal, S., Clifford, J., Peavey, M., Kiguchi, K., Itami, S., and Nickoloff, B. J. (2005) *Nat. Med.* **11,** 43–49
35. Suganami, E., Takagi, H., Ohashi, H., Suzuma, K., Suzuma, I., Oh, H., Watanabe, D., Ojima, T., Suganami, T., Fujio, Y., Nakao, K., Ogawa, Y., and Yoshimura, N. (2004) *Diabetes* **53,** 2443–2448