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Role of Heat Shock Transcriptional Factor 1 and Heat Shock Proteins in Cardiac Hypertrophy

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Cardiac hypertrophy is an independent risk factor for cardiovascular disease. Initially, cardiac hypertrophy is an adaptive response to increased wall stress, but sustained stress leads to heart failure. It remains unclear how the transition from adaptive cardiac hypertrophy to maladaptive cardiac hypertrophy occurs. It has been postulated that there are two forms of cardiac hypertrophy, which are physiologic and pathologic cardiac hypertrophy. Unlike pathologic cardiac hypertrophy caused by chronic pressure or volume overload, cardiac hypertrophy induced by exercise is associated with less fibrosis and better systolic function, suggesting that adaptive mechanisms may be involved in exercise-induced cardiac hypertrophy. Therefore, elucidation of the molecular differences between these two types of cardiac hypertrophy may provide insights into the mechanisms underlying the transition from adaptive cardiac hypertrophy to heart failure. By comparing the two types of cardiac hypertrophy, we have identified heat shock transcription factor 1 and its target heat shock proteins as key factors involved in the adaptive mechanism of cardiac hypertrophy. In this review, we summarize the protective role of heat shock transcription factor 1 and heat shock proteins in cardiovascular disease.
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• Introduction

Heart failure is the final outcome of various heart diseases, and cardiac hypertrophy is one of the main causes of heart failure. The Framingham Heart Study revealed that there is a relationship between the severity of cardiac hypertrophy and the incidence of cardiovascular events, and that cardiac hypertrophy is an independent risk factor for heart failure, arrhythmia, myocardial infarction, and sudden death (Levy et al. 1990, Behar et al. 1992, Haider et al. 1998, Verdecchia et al. 2001). Therefore, it is important to develop therapeutic strategies for this condition, but the precise mechanisms underlying the transition from cardiac hypertrophy to heart failure are still largely unknown.

Cardiac hypertrophy is induced by various pathologic or physiologic stimuli. For example, acute pressure overload initially induces adaptive cardiac hypertrophy that is associated with normal cardiac function, but systolic and diastolic dysfunction occur in the setting of chronic pressure overload, resulting in heart failure. Thus, chronic pressure overload is thought to cause pathologic or maladaptive cardiac hypertrophy. On the other hand, regular exercise can induce cardiac hypertrophy without causing systolic or diastolic dysfunction (Pluim et al. 2000). Because exercise-induced cardiac hypertrophy does not progress to heart failure, it is thought to be physiologic or adaptive cardiac hypertrophy. Although it has been reported that these two types of cardiac hypertrophy are morphologically (Richey and Brown 1998, Iemitsu et al. 2001, McMullen and Jennings 2007), functionally, and molecularly distinct from each other, the precise mechanism underlying these differences remains unclear. What are the exact differences between pathologic and physiologic cardiac hypertrophy? Why is cardiac function preserved in physiologic cardiac hypertrophy? Why does sustained pressure overload cause heart failure? Answering these questions will provide insights into novel therapeutic options for both cardiac hypertrophy and heart failure.

• Pathologic and Physiologic Cardiac Hypertrophy

The differences between these two conditions include the stimuli inducing cardiac hypertrophy, their duration of action, and the signaling pathways involved. Pathologic cardiac hypertrophy is induced by persistent stress, such as pressure overload and volume overload caused by hypertension or valvular heart disease. On the other hand, physiologic cardiac hypertrophy is induced by intermittent stress such as exercise. Thus, the manifestations of cardiac hypertrophy caused by various stimuli may depend on their duration and intensity. In a recent study, Perrino et al. (2006) applied intermittent pressure overload to the heart and investigated the role of the duration of stress in the development of cardiac failure. Despite only developing mild cardiac hypertrophy, the hearts exposed to intermittent pressure overload displayed various pathologic changes, including diastolic dysfunction and histologic abnormalities.

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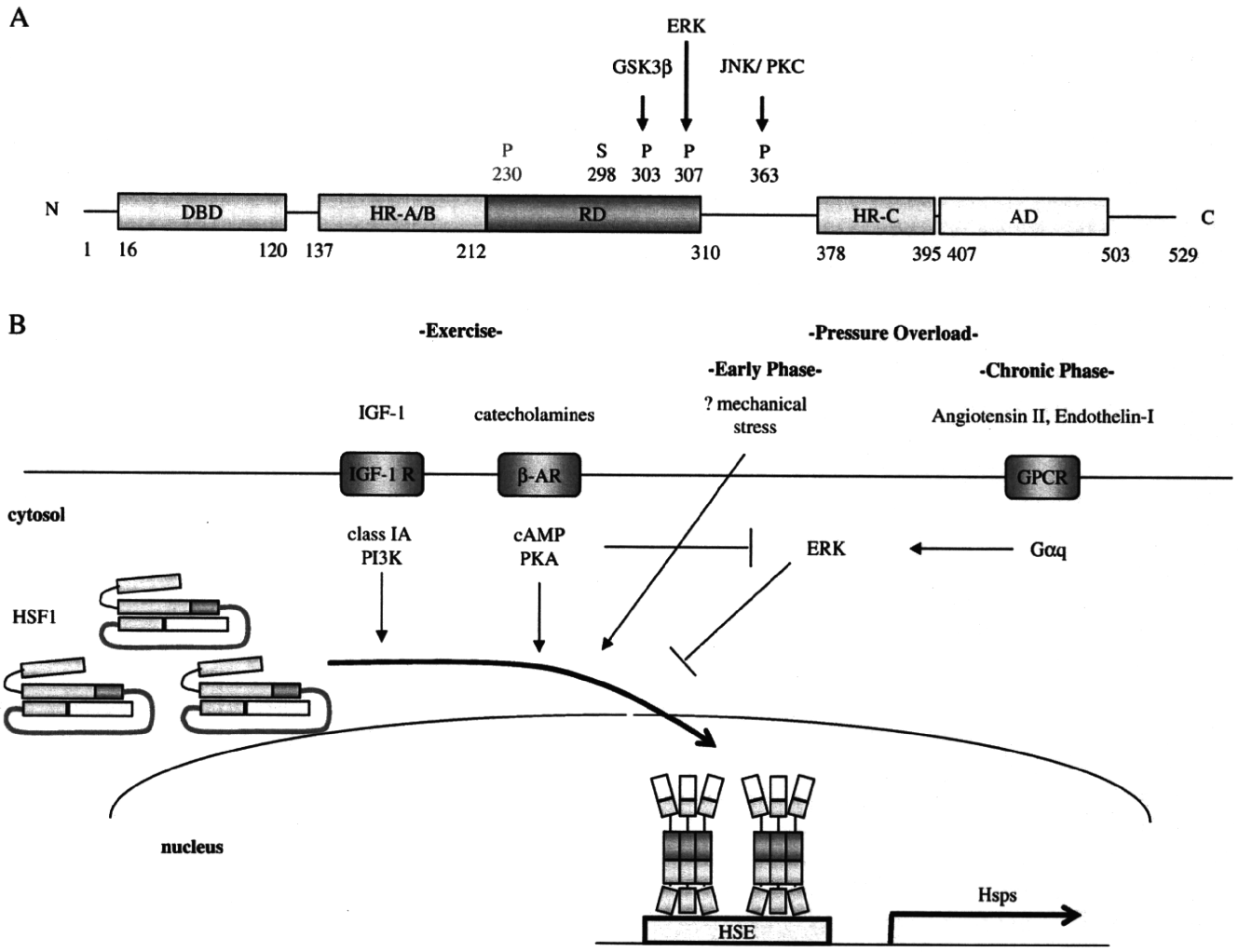

Figure 1. Potential regulators of HSF1 in cardiac hypertrophy. (**A**) Structure of HSF1. DBD indicates DNA-binding domain; HR, hydrophobic repeat; RD, regulatory domain: AD, transcriptional activation domain; P, phosphorylated site (the activating site is indicated in red); S, sumoylated site. (**B**) Potential regulatory mechanism of HSF1. Under nonstressful conditions, HSF1 exists as a monomer whose transcriptional activity is repressed by phosphorylation of the repressing sites (Ser303, Ser307, and Ser363). Upon stress, phosphorylation of the activating site (Ser230) is enhanced, thereby promoting the transcriptional activity of the trimerized and DNA-bound HSF1. The ratio of phosphorylation between the activating and repressing sites may be influenced by various stimuli, such as IGF-1, catecholamine, and angiotensin II, and determine the magnitude of the transcriptional activity. IGF-1R indicates IGF-1 receptor; β-AR, β adrenergic receptor; GPCR, G-protein-coupled receptor.

Thus, the nature of the stress acting on the heart, rather than its duration, may be a key determinant of the maladaptive phenotype.

A number of studies have shown that various signaling pathways contribute to the development of pathologic and physiologic cardiac hypertrophy by using mice that overexpress or lack specific genes (Richey and Brown 1998, Selvetella et al. 2004, Heineke and Molkentin 2006, Shiojima and Walsh 2006, McMullen and Jennings 2007). Endocrine factors such as angiotensin II and endothelin 1 induce pathologic cardiac hypertrophy (Yamazaki et al. 1995, Yamazaki et al. 1996), whereas inhibition of angiotensin II by angiotensin-converting enzyme inhibitors or angiotensin II receptor type 1 blockers can lead to regression of cardiac hypertrophy (Okin et al. 2003). Overexpression of Gαq in the heart, which is activated by these factors, also induces cardiac hypertrophy associated with cardiac dysfunction (D'Angelo et al. 1997), whereas overexpression of an inhibitory peptide that interferes with Gαq coupling prevents the onset of maladaptive cardiac hypertrophy (Akhter et al. 1998). These findings suggest that the Gαq-mediated pathway is important for the development of pathologic cardiac hypertrophy.

The calcium/calmodulin-dependent phosphatase calcineurin has also been suggested to have a role in pathologic cardiac hypertrophy. Transgenic mice that overexpress active forms of calcineurin or its downstream transcription factor (NFAT3) develop cardiac hypertrophy and heart failure (Molkentin et al. 1998). Calcineurin inhibitors, such as cyclosporin A and FK506, suppress angiotensin II-induced cardiomyocyte hypertrophy in vitro and inhibit pressure overload-induced cardiac hypertrophy in vivo (Molkentin et al. 1998, Shimoyama et al. 2000). Overexpression of a dominant-negative mutant of calcineurin in

the heart also suppresses the induction of pathologic cardiac hypertrophy by pressure overload (Zou et al. 2001).

On the other hand, it has been reported that the insulin-like growth factor-1 (IGF-1)/class I_A phosphoinositide 3-kinase (PI3K) pathway is activated in physiologic cardiac hypertrophy. Cardiac production of IGF-1 is significantly higher in athletes than in control subjects (Neri Serneri et al. 2001, Melling et al. 2006), and serum levels of IGF-1 increase in response to training (Koziris et al. 1999). Transgenic mice overexpressing the IGF-1 receptor or a constitutively active form of class I_A PI3K in the heart develop cardiac hypertrophy without cardiac dysfunction or an increase of fibrosis (Shioi et al. 2000, McMullen et al. 2004). In contrast, transgenic mice with reduced cardiac class I_A PI3K activity have smaller hearts and show a blunted hypertrophic response to exercise training, but not to pressure overload (McMullen et al. 2003, Luo et al. 2005). These results suggest that the IGF-1/class I_A PI3K pathway is involved in the regulation of cardiac growth during postnatal development, and that this pathway plays a crucial role in inducing physiologic cardiac hypertrophy.

Although there have been a number of previous reports about the stimuli and signaling pathways involved in the regulation of physiologic or pathologic cardiac hypertrophy, the target genes and molecules of these pathways remain unclear. To answer these questions, various research groups have compared the pattern of cardiac gene expression between physiologic and pathologic cardiac hypertrophy (Richey and Brown 1998, Iemitsu et al. 2001, McMullen and Jennings 2007). These studies have shown that an array of genes display differential expression, suggesting that such differences might be involved in producing the two distinct phenotypes of cardiac hypertrophy. However, it remains to be determined whether these gene products actually promote different types of cardiac hypertrophy. Recently, we examined gene expression patterns in the heart and found differences in the expression of about 100 genes between physiologic and pathologic cardiac hypertrophy. Among them, we examined the role of heat shock proteins (HSPs) and heat shock transcription factor 1 (HSF1) in cardiac hypertrophy because the expression of *Hsp70* and *Hsp27* was only elevated in physiologic cardiac hypertrophy.

- **Role of Heat Shock Transcriptional Factor 1/HSPs in Cardiovascular Disease**

Heat shock proteins are ubiquitously expressed, and their expression is enhanced by various acute and chronic stimuli, such as heat shock, heavy metals, low molecular weight toxins, infection, and oxidative stress (Li and Laszlo 1985, Benjamin and McMillan 1998, Morimoto 1998, Pockley 2002, Westerheide and Morimoto 2005). Heat shock proteins act to ensure the proper protein folding, as well as to prevent protein misfolding and assist in protein refolding to the correct state. Expression of HSPs is mainly regulated by HSF1 at the transcriptional level. In the unstressed state, HSF1 exists as a latent monomer, with repressed DNA binding and transcriptional activity. Upon activation, HSF1 undergoes multiple processes that include a monomer-to-trimer transition, nuclear accumulation, binding to the heat shock element located in the promoter region of each HSP gene, and transcriptional activation (Figure 1). Heat shock transcription factor 1–heat shock element DNA binding is not sufficient to elicit maximal transcription of the HSP genes, and it is necessary for HSF1 to be modified by phosphorylation and sumoylation to increase its transcriptional activity (Holmberg et al. 2002, Westerheide and Morimoto 2005). It has been suggested that HSF1 is repressed by GSK-3β (Ser303), ERK (Ser307), and JNK (Ser363) under normal conditions, whereas it is activated by hyperphosphorylation (Ser-230) upon exposure to various stresses (Figure 1A) (Chu et al. 1996, Chu et al. 1998, Morimoto 1998, Holmberg et al. 2002). However, the mechanisms underlying the activation of HSF1, particularly its regulation by phosphorylation, remain unclear.

A number of studies have shown that HSF1 and HSPs confer protection against cardiovascular disease. Induction of HSF1 and HSP expression by various stimuli, such as heat shock, reduces the size of infarcts after ischemia/reperfusion (Donnelly et al. 1992, Marber et al. 1993, Bennani et al. 1998). Transgenic mice overexpressing a constitutively active form of HSF1 or inducible Hsp70 in the heart show more resistance to ischemia/reperfusion injury compared with wild-type mice (Marber et al. 1995, Plumier et al. 1995, Zou et al. 2003). In contrast, the cardiac function of inducible Hsp70 knockout mice is markedly impaired by ischemia/reperfusion injury (Kim et al. 2006). In addition to a protective effect against ischemia/reperfusion injury, it has been reported that HSPs have a beneficial role in myocardial infarction, doxorubicin-induced cardiomyopathy, and atrial fibrillation (Baljinnyam et al. 2006, Brundel et al. 2006, Venkatakrishnan et al. 2006, Liu et al. 2007, Wakisaka et al. 2007).

Our recent study identified HSF1 as a critical transcription factor that regulates cardiac hypertrophy (Sakamoto et al. 2006). Heat shock transcription factor 1 was only activated in exercise-induced cardiac hypertrophy, but not in chronic pressure overload-induced cardiac hypertrophy. When heterozygous $HSF1^{+/-}$ mice (Inouye et al. 2004) were forced to exercise (which is thought to induce physiologic cardiac hypertrophy), significant systolic dysfunction occurred. In contrast, when transgenic mice that expressed a constitutively active form of HSF1 (Nakai et al. 2000) were exposed to chronic pressure overload (which is thought to induce pathologic cardiac hypertrophy), their systolic function was preserved. These results indicate that HSF1 is a key molecule for preservation of systolic function during the development of cardiac hypertrophy under both pathologic and physiologic conditions. Accumulation and aggregation of unfolded proteins are associated with an increase of protein synthesis in hypertrophied hearts and induce cardiomyocyte death that eventually leads to systolic dysfunction (Okada et al. 2005). Thus, the protective effects of HSF1 may be attributable to the functions of HSPs in protein folding and degradation. In addition to such well-known functions, accumulating evidence indicates that different HSPs directly act on the cell death machinery and inhibit the signaling pathway for cell death at various points (Sreedhar and Csermely 2004). For example, Hsp27 binds to cytochrome c and prevents it from binding to Apaf-1 (Bruey et al. 2000), whereas Hsp70 prevents Apaf-1 from recruiting procaspase-9 (Beere et al. 2000), thereby inhibiting apoptotic cell death. It is

conceivable that sustained activation of HSF1 prevents the onset of cardiac dysfunction in hypertrophic hearts through the mechanisms involving a direct action of HSPs on the cell death machinery as well as their functions in protein degradation.

• Potential Regulators of HSF1 in Cardiac Hypertrophy

Heat shock transcription factor 1 and HSPs are upregulated by exercise (Taylor et al. 1999, Hamilton et al. 2001, Sakamoto et al. 2006), but the mechanisms involved are not fully understood. As mentioned above, the IGF-1/class I_A PI3K pathway is thought to play an important role in inducing physiologic cardiac hypertrophy (McMullen et al. 2004). Interestingly, expression of HSPs is increased in the hearts of transgenic mice, with enhancement of cardiac IGF-1 or class I_A PI3K, suggesting a potential relationship between this signaling pathway and HSF1 activity. Consistent with this notion, the IGF-1/class I_A PI3K pathway is known to inhibit GSK-3β (Shiojima and Walsh 2006), which is a negative regulator of HSF1. It could be assumed that IGF-1-induced inhibition of GSK-3β contributes to the activation of HSF1 in exercise-induced cardiac hypertrophy (Figure 1B).

Another possibility is that catecholamines may upregulate HSF1 and HSPs after exercise, because circulating levels of catecholamines are increased by exercise. Isoproterenol (a β-adrenergic agonist) increases cardiac expression of HSP70 (White and White 1986), whereas inhibition of protein kinase A (PKA), a downstream kinase of the β-adrenergic receptor, suppresses exercise-induced upregulation of *Hsp70* (Melling et al. 2004). Moreover, exercise-induced activation of PKA attenuates the phosphorylation of ERK, which is a negative regulator of HSF1 (Melling et al. 2006). Taken together, these findings suggest that exercise may upregulate HSF1 by activating the β-adrenergic signaling pathway that induces PKA-mediated inactivation of ERK (Figure 1B). Although activation of protein kinase C in the heart during exercise is thought to have a protective role, it remains unclear whether this pathway is involved in the upregulation of HSF1 and HSPs after exercise (Yamashita et al. 2001, Melling et al. 2004). Moreover, posttranslational modifications rather than phosphorylation may regulate the transcriptional activity of HSF1 during exercise.

Our findings showed that HSF1 was only activated in the early phase of pressure overload (the adaptive phase), but not in the chronic phase (the maladaptive phase) (Sakamoto et al. 2006). Other groups have also demonstrated that acute pressure overload activates HSF1 and increases the expression of HSPs (Delcayre et al. 1988, Izumo et al. 1988, Nishizawa et al. 2002). Why is HSF1 downregulated during the chronic phase of pressure overload? Production of autocrine/paracrine factors such as angiotensin II and endothelin 1 is increased by pathologic stimuli and plays a critical role in inducing pathologic cardiac hypertrophy. These factors bind to G-protein-coupled receptors, leading to dissociation of the Gαq subunit and activation of downstream signaling molecules, which include negative regulators of HSF1 such as ERK and JNK. Accordingly, this signaling pathway may induce pathologic cardiac hypertrophy partly via the inactivation of HSF1 (Figure 1B), although there is a conflicting report that angiotensin II does not influence the activity of HSF1 (Nishizawa et al. 2002). Further studies are necessary to elucidate precisely how HSF1 activity is regulated as cardiac hypertrophy develops.

• Conclusion and Future Prospects

Because there have been many reports that induction of HSF1 and HSPs has a beneficial effect in animal models of cardiovascular disease, activation of HSF1 and HSPs could be a novel therapeutic strategy for various cardiovascular diseases. Geranylgeranylacetone, an anti-ulcer agent, has been reported to upregulate HSF1 and HSPs, and shows a protective effect against ischemia/reperfusion injury and atrial fibrillation (Yamanaka et al. 2003, Brundel et al. 2006, Wakisaka et al. 2007). Exercise also upregulates HSF1 and HSPs, and it ameliorates cardiac dysfunction in hypertensive animals (Scheuer et al. 1982, Schaible et al. 1986, Moreno Junior et al. 1995, Emter et al. 2005). Moreover, recent studies have further demonstrated the protective effect of exercise on cardiac function in animal models of myocardial infarction and ischemia/reperfusion injury (Hoshida et al. 2002). However, conflicting data also suggest that any increase of HSPs in the heart after exercise is not necessary for protection against ischemia/reperfusion injury and that moderate exercise does not improve cardiac dysfunction in hypertensive rats (Taylor et al. 1999, Hamilton et al. 2001). Moreover, excessive exercise accelerates the rate of progression from cardiac hypertrophy to heart failure in untreated hypertensive rats (Sarma and Schulze 2007). To develop a novel therapeutic strategy targeting the HSF1/HSP system for patients with cardiovascular disease, one is required to perform further studies of elucidating the protective mechanisms involved.

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Understanding Proteasome Assembly and Regulation: Importance to Cardiovascular Medicine

Glen W. Young, Yueju Wang, and Peipei Ping*

The cardiac proteasome is increasingly recognized as a complex, heterogeneous, and dynamic organelle contributing to the modulation of cardiac function in health and diseases. The emerging picture of the proteasome system reveals a highly regulated and organized molecular machine integrated into multiple biologic processes of the cell. Full appreciation of its cardiovascular relevance requires an understanding of its proteolytic function as well as its underlying regulatory mechanisms, of which assembly, stoichiometry, posttranslational modification, and the role of the associating partners are increasingly poignant. (Trends Cardiovasc Med 2008;18:93–98) *Published by Elsevier Inc.*

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• Introduction

The mammalian protein degradation machinery is dominated by the proteasome, as it endoproteolytically cleaves more than 70% of intracellular proteins (Rock et al. 1994). The core of this multimeric protease is a duplex of two sets of 14 subunits, housing duplicate sites of trypsin-like, caspase-like, and chymotrypsin-like peptidase activities. Termed the *20S proteasome*, its gated pores maintain the complex in a latently active state, enabling only limited

Vascular Endothelial Growth Factor Receptor-1 Regulates Postnatal Angiogenesis Through Inhibition of the Excessive Activation of Akt

Jun-ichiro Nishi,* Tohru Minamino,* Hideyuki Miyauchi, Aika Nojima, Kaoru Tateno, Sho Okada, Masayuki Orimo, Junji Moriya, Guo-Hua Fong, Kenji Sunagawa, Masabumi Shibuya, Issei Komuro

Abstract—Vascular endothelial growth factor (VEGF) binds both VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). Activation of VEGFR-2 is thought to play a major role in the regulation of endothelial function by VEGF. Recently, specific ligands for VEGFR-1 have been reported to have beneficial effects when used to treat ischemic diseases. However, the role of VEGFR-1 in angiogenesis is not fully understood. In this study, we showed that VEGFR-1 performs "fine tuning" of VEGF signaling to induce neovascularization. We examined the effects of retroviral vectors expressing a small interference RNA that targeted either the VEGFR-1 gene or the VEGFR-2 gene. Deletion of either VEGFR-1 or VEGFR-2 reduced the ability of endothelial cells to form capillaries. Deletion of VEGFR-1 markedly reduced endothelial cell proliferation and induced premature senescence of endothelial cells. In contrast, deletion of VEGFR-2 significantly impaired endothelial cell survival. When VEGFR-1 expression was blocked, VEGF constitutively activated Akt signals and thus induced endothelial cell senescence via a p53-dependent pathway. VEGFR-$1^{+/-}$ mice exhibited an increase of endothelial Akt activity and showed an impaired neovascularization in response to ischemia, and this impairment was ameliorated in VEGFR-$1^{+/-}$ Akt$1^{+/-}$ mice. These results suggest that VEGFR-1 plays a critical role in the maintenance of endothelial integrity by modulating the VEGF/Akt signaling pathway. **(*Circ Res*. 2008;103:261-268.)**

Key Words: VEGF ■ Akt ■ senescence ■ p53

Angiogenesis involves the differentiation, proliferation, and migration of endothelial cells, leading to tubulogenesis and the formation of vessels.[1] One of the most important receptors for angiogenesis is the vascular endothelial growth factor (VEGF) receptor, which is a member of the receptor tyrosine kinase family.[2,3] VEGF receptor (VEGFR)-1 and VEGFR-2 are closely related receptor tyrosine kinases and have both common and specific ligands. VEGFR-1 has weaker kinase activity, whereas VEGFR-2 is a highly active kinase that stimulates a variety of signaling pathways and induces a broad range of biological responses. Despite its weak kinase activity, VEGFR-1 is essential for normal development and angiogenesis.[4] VEGFR-1 null mutant mice die in utero because of the overgrowth of endothelial cells and vascular disorganization.[5,6] In contrast, mice expressing the VEGFR-1 that lacks the tyrosine kinase domain develop a normal cardiovascular system,[7] suggesting that VEGFR-1 kinase activity might not be required for vascular development during embryogenesis and that VEGFR-1 may act as a decoy receptor. Consistent with this concept, selective activation of chimeric VEGFR-1 (in the absence of chimeric VEGFR-2)[8] or a VEGF mutant that binds to VEGFR-1 does not influence cell proliferation, migration, or survival in vitro.[9–11]

However, recent studies have demonstrated that the role of VEGFR-1 in postnatal angiogenesis is more complicated than was initially recognized. For example, treatment with placenta growth factor (PlGF), a specific ligand for VEGFR-1, was reported to promote angiogenesis in vitro[11,12] and in vivo.[13] Overexpression of PlGF also induced angiogenesis in tumors[14] and the skin.[15] It has been suggested that stimulation by PlGF induces the heterodimerization of VEGFR-1 with VEGFR-2, leading to transactivation of VEGFR-2 and the promotion of angiogenesis.[8,16,17] Another possible explanation for the positive effect of PlGF on angiogenesis is that it prevents VEGF from binding to VEGFR-1, thereby

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increasing the binding and activation of VEGFR-2. In other studies, PlGF was shown to protect against hyperoxic vascular damage in the retina without provoking retinal neovascularization.[18] These results suggest that VEGFR-1 can either positively or negatively regulate angiogenesis depending on the circumstances, but further studies are required to better understand the role of this receptor in postnatal angiogenesis.

In the present study, we examined the effects of VEGFR-1 deletion on angiogenesis by using the retroviral vector expressing a small interference RNA that targeted the VEGFR-1 gene. Deletion of VEGFR-1 markedly reduced endothelial cell proliferation and thus impaired angiogenesis. Likewise, VEGFR-$1^{+/-}$ mice exhibited an impaired neovascularization in response to ischemia. This impairment was restored by inhibiting the excessive activation of Akt by VEGF. These results suggest that VEGFR-1 plays a critical role in the maintenance of endothelial integrity by modulating the VEGF/Akt signaling pathway.

Materials and Methods

Short Hairpin Interference RNA Vectors

The mammalian retrovirus expression vector pSIREN-RetroQ (Clontech) was used to achieve the expression of short hairpin interference RNA (shRNA) in human endothelial cells.

Statistical Analysis

Data are shown as mean±SEM. Differences between groups were examined by Student *t* test or ANOVA followed by the Bonferroni procedure for comparison of means. Values of $P<0.05$ were considered statistically significant.

Results

Effect of VEGF Receptor Gene Silencing on Endothelial Cell Function

To elucidate the role of VEGFR-1 in angiogenesis, we constructed mammalian retroviral vectors expressing a short hairpin interference RNA that targeted either the VEGFR-1 gene (shVEGFR-1) or the VEGFR-2 gene (shVEGFR-2). Northern blot and Western blot analyses revealed that introduction of each construct into human umbilical vein endothelial cells caused effective and stable downregulation of the expression of the target molecule (Figure 1A and 1B, and supplemental Figure IA [available online at http://circres.ahajournals.org]). It is noted that either shVEGFR-1 or shVEGFR-2 did not affect VEGFR-2 or VEGFR-1 expression, respectively (Figure 1B, and supplemental Figure IA). We used two kinds of constructs for the following experiments and both of them achieved similar results. The nonsilencing control vector (shNega) was used as a control. After infected endothelial cells were purified by incubation with antibiotics, we performed the tube formation assay. Deletion of VEGFR-1 or VEGFR-2 significantly impaired tube formation compared with control cells (Figure 1C). We next examined the proliferative activity of infected cells. We seeded 2×10^5 infected cells into 100-mm dishes with VEGF-A on day 0 and counted cell number on day 3. Compared with shNega-infected control endothelial cells, both shVEGFR-1– and shVEGFR-2–infected cells showed significantly lower proliferation (Figure 1D). Deletion of VEGFR-1 caused more marked impairment of cell proliferation than deletion of VEGFR-2 (Figure 1D). This inhibitory effect of VEGFR-1 deletion was more evident when infected endothelial cells were subjected to long-term culture. Although VEGFR-2 deletion slightly reduced the lifespan of cells compared with that of control cells, VEGFR-1 deletion significantly shortened the lifespan of endothelial cells (Figure 1E). As a result, shVEGFR-1–infected cells underwent irreversible growth arrest earlier than shVEGFR-2-infected cells (Figure 1E). After growth arrest, the cells exhibited characteristics of senescence, becoming flatter and larger and showing an increase of senescence-associated β-galactosidase activity (Figure 1F). These findings suggest that VEGFR-1 deletion induces premature endothelial cell senescence. We next examined the effect of VEGFR-1 deletion on endothelial survival. We cultured infected cells in regular growth medium for 24 hours and subsequently cultured the cells under serum-free conditions with VEGF-A. After 24 hours, the number of viable cells was counted. As compared with the viability of control cells, deletion of VEGFR-2, but not VEGFR-1, markedly decreased cell viability (Figure 1G). Consistent with these findings, activation of caspase 3 was detected in cells with VEGFR-2 deletion, but not VEGFR-1 deletion (Figure 1H). These results suggest that VEGFR-1 is involved in the regulation of angiogenesis by regulating endothelial cell proliferation and senescence, whereas VEGFR-2 may be crucial for endothelial survival as well as cell proliferation.

VEGFR-1 Deletion Induces Endothelial Dysfunction by Activating Akt

To investigate the molecular mechanisms of premature senescence induced by VEGFR-1 deletion, we examined the transcriptional activity of p53 and its target gene p21. We transfected VEGFR-1–deleted endothelial cells with the luciferase reporter gene containing 13 copies of the p53-binding consensus sequence (pPG13-Luc). Deletion of VEGFR-1 significantly induced p53 transcriptional activity compared with that in shNega-infected cells, whereas VEGFR-2 deletion had no effect (Figure 2A). Likewise, p21 expression was significantly higher in VEGFR-1–deleted endothelial cells than in control cells or VEGFR-2–deleted cells (Figure 2B). However, expression of bax, another target molecule regulated by p53, was not altered in VEGFR-1–deleted endothelial cells compared with control cells (supplemental Figure IB). Ablation of p53 by the introduction of HPV16 E6 oncoprotein abolished the inhibitory effect of VEGFR-1 deletion on cell proliferation (Figure 2C). These results suggest that VEGFR-1 deletion induces endothelial cell senescence via a p53-dependent pathway.

We have previously demonstrated that Akt negatively regulates the endothelial cell lifespan by activating the p53/p21 pathway.[19] It has also been shown that Akt plays a central role in the regulation of angiogenesis by VEGF.[20] Thus, we examined the level of phosphorylated Akt in VEGFR-1-deleted endothelial cells. Western blot analysis

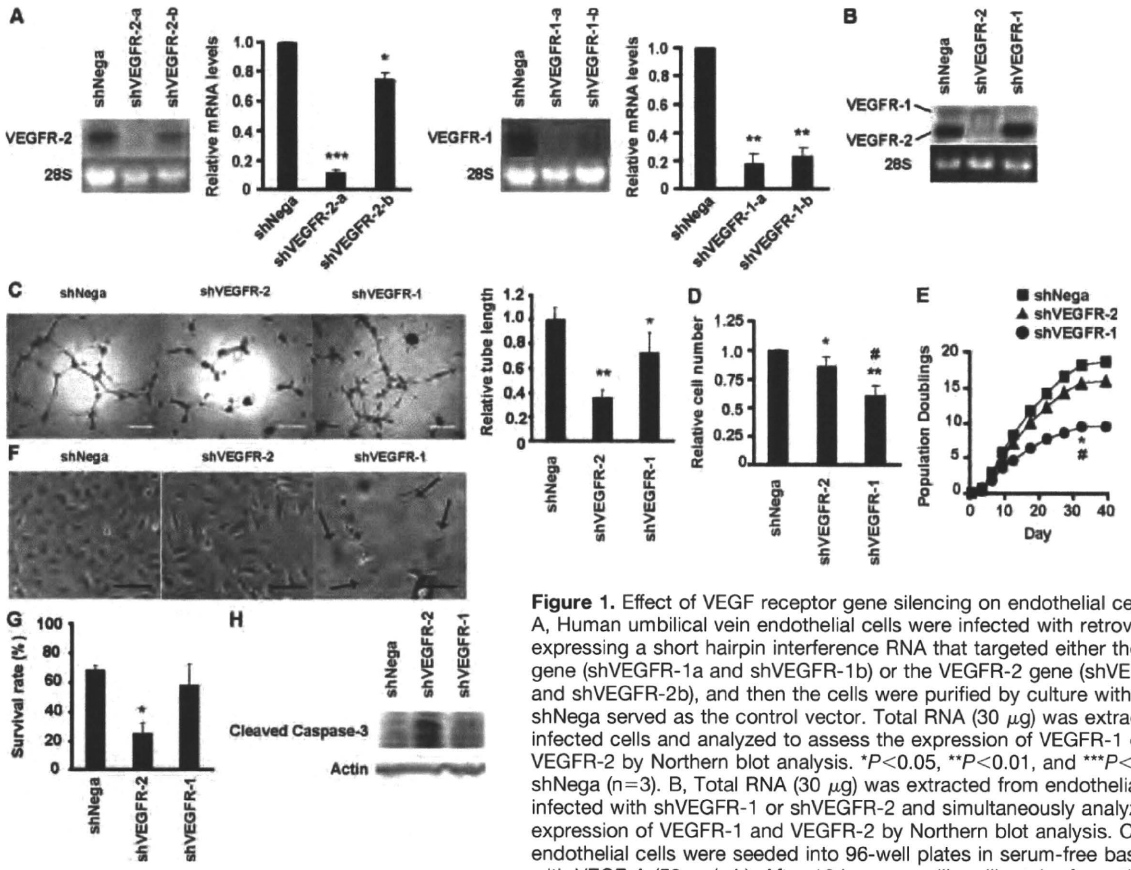

Figure 1. Effect of VEGF receptor gene silencing on endothelial cell function. A, Human umbilical vein endothelial cells were infected with retroviral vectors expressing a short hairpin interference RNA that targeted either the VEGFR-1 gene (shVEGFR-1a and shVEGFR-1b) or the VEGFR-2 gene (shVEGFR-2a and shVEGFR-2b), and then the cells were purified by culture with antibiotics. shNega served as the control vector. Total RNA (30 μg) was extracted from infected cells and analyzed to assess the expression of VEGFR-1 or VEGFR-2 by Northern blot analysis. *P<0.05, **P<0.01, and ***P<0.001 vs shNega (n=3). B, Total RNA (30 μg) was extracted from endothelial cells infected with shVEGFR-1 or shVEGFR-2 and simultaneously analyzed the expression of VEGFR-1 and VEGFR-2 by Northern blot analysis. C, Infected endothelial cells were seeded into 96-well plates in serum-free basic medium with VEGF-A (50 ng/mL). After 16 hours, capillary-like tube formation was estimated by using an angiogenesis image analyzer. *P<0.01, **P<0.0001 vs shNega (n=4 to 6). Scale bar: 300 μm. D, Infected endothelial cells were seeded at a density of 2×10^5 cells per 100-mm dish and cultured with VEGF-A (day 0). Then cell number was counted on day 3. *P<0.001, **P<0.0001 vs shNega, #P<0.001 vs shVEGFR-2 (n=13 to 14). E, Infected cell populations were passaged until cells underwent senescence, and the total number of population doublings was determined. *P<0.01 vs shNega, #P<0.05 vs shVEGFR-2 (n=4 to 6). F, Morphology and senescence-associated β-galactosidase staining (arrow) of endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Scale bar: 100 μm. G, Infected endothelial cells were seeded at the density of 1×10^5 cells per 60-mm dish and cultured for 24 hours in growth medium. After washing twice with PBS, the cells were cultured in serum-free DMEM with VEGF-A (10 ng/mL). After 24 hours of serum starvation, the number of viable cells and the total number of cells were counted by a hemocytometer. *P<0.0001 vs shNega (n=4 to 6). H, The lysates were extracted from cells, which are prepared as described in legend for G, and analyzed for cleaved caspase-3 expression by Western blotting.

showed that VEGFR-1 deletion led to a marked increase of the phosphorylated Akt level compared with that in control cells or cells with VEGFR-2 deletion, even under serum-free conditions (Figure 3A). VEGFR-1 deletion increased pAkt levels even in the absence of VEGF, presumably attributable to autocrine VEGF signaling (Figure 3B). Treatment with VEGF markedly increased pAkt levels within 5 to 15 minutes in VEGFR-1–deleted cells but not in VEGFR-2–deleted cells (Figure 3B). Treatment with a neutralizing anti-VEGF antibody reduced the phosphorylated Akt level in VEGFR-1-deleted cells (Figure 3C), suggesting that VEGFR-1 inhibits the activation of Akt by VEGF. To further investigate the relationship between constitutive Akt activation and endothelial cell dysfunction induced by VEGFR-1 deletion, we examined the effect of inhibition of Akt. We infected human endothelial cells with a retroviral vector encoding a dominant-negative form of Akt (DN-Akt)[19] or the empty vector encoding resistance to neomycin alone (Mock). Both cell populations were then infected with shNega or shVEGFR-1. We found that VEGFR-1 deletion markedly inhibited the proliferation of mock-infected endothelial cells (Figure 3D, Mock), whereas this inhibitory effect was significantly ameliorated in DN-Akt–infected cells (Figure 3D, DN-Akt). Consequently, VEGFR-1 deletion significantly impaired tube formation by mock-infected cells, but not DN-Akt-infected cells (Figure 3E). Likewise, inhibition of Akt activation prevented the induction of p21 expression by VEGFR-1 deletion (supplemental Figure II). These results suggest that VEGFR-1 deletion causes dysregulation of activation of the VEGFR-2/Akt signaling pathway by VEGF-A, and that constitutive activation of Akt is related to the impaired ability of VEGFR-1–deleted endothelial cells to proliferate and form capillary-like structures. VEGF-induced phosphorylation of eNOS was enhanced, but production of cGMP was significantly reducued by VEGFR-1 deletion, presumably because constitutive activation of Akt increases cellular reactive oxygen species[19] that inactivate this enzyme (supplemental Figure IC and ID).

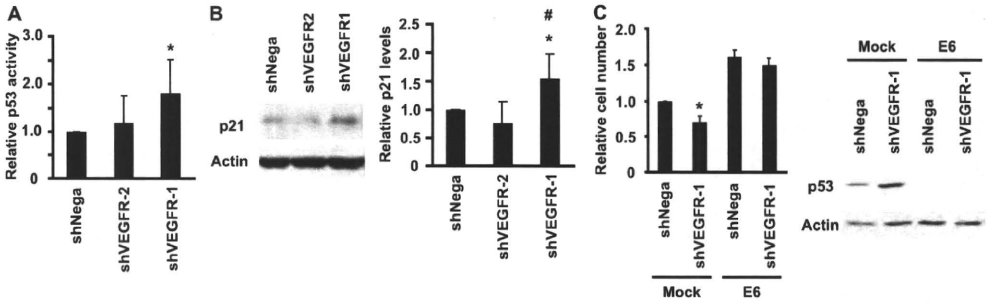

Figure 2. VEGFR-1 deletion induces activation of the p53/p21 signal pathway. A, A luciferase reporter gene plasmid (pPG13-Luc) containing the p53-binding sequence was transfected into endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Luciferase activity was measured at 48 hours after transfection in the presence of VEGF-A (10 ng/mL) as described in Methods. *$P<0.05$ vs shNega (n=5). B, Whole cell lysates (30 μg) were prepared from infected endothelial cells and p21 expression was assessed by Western blot analysis. *$P<0.05$ vs shNega, #$P<0.01$ vs shVEGFR-2 (n=4). C, Human endothelial cells were infected with pLNCX (Mock) or pLNCX E6 (E6). Infected cell populations were then transduced with shNega or shVEGFR-1. After purification, double-infected cells were seeded at a density of 2×10^5 cells per 100-mm dish in the presence of VEGF-A (day 0), and cell number was counted on day 3. *$P<0.05$ vs Mock/shNega (n=3). Western blot analysis revealed that introduction of E6 effectively ablated p53 expression (right panel).

Influence of VEGFR-1 Deletion on Neovascularization In Vivo

To examine the influence of VEGFR-1 deletion on neovascularization in vivo, we produced a hindlimb ischemia model in VEGFR-$1^{+/-}$ mice and assessed blood flow recovery and the capillary density of ischemic tissue. VEGFR-1 mRNA levels were significantly lower in VEGFR-$1^{+/-}$ mice than in wild-type mice (Figure 4A). Aortic expression of VEGFR-1 protein was decreased in VEGFR-$1^{+/-}$ mice compared with wild-type mice (Figure 4B). Consistent with the in vitro data, phospho-Akt levels were significantly higher in VEGFR-$1^{+/-}$ mice than in wild-type mice (Figure 4C and supplemental Figure III). There was no significant difference in plasma VEGF levels between the two groups (data not shown). Laser Doppler image analysis revealed that blood flow recovery was significantly impaired in VEGFR-$1^{+/-}$ mice compared with their wild-type littermates (Figure 4D). Likewise, VEGFR-$1^{+/-}$ mice exhibited significantly fewer CD31-positive cells in the ischemic tissues than their wild-type littermates (Figure 4E), suggesting that decreased expression of VEGFR-1 led to reduced neovascularization of ischemic tissue.

There are several reports indicating that VEGFR-1 kinase activity is required for VEGF-induced migration of hematopoietic cells including macrophages,[21–26] and it was reported that infiltration of macrophages plays a critical role in pathological angiogenesis during ischemia, inflammation, and tumor development.[27–29] Therefore, we examined the number of infiltrating macrophages in ischemic tissue, but we found no significant difference in the number of Mac3-

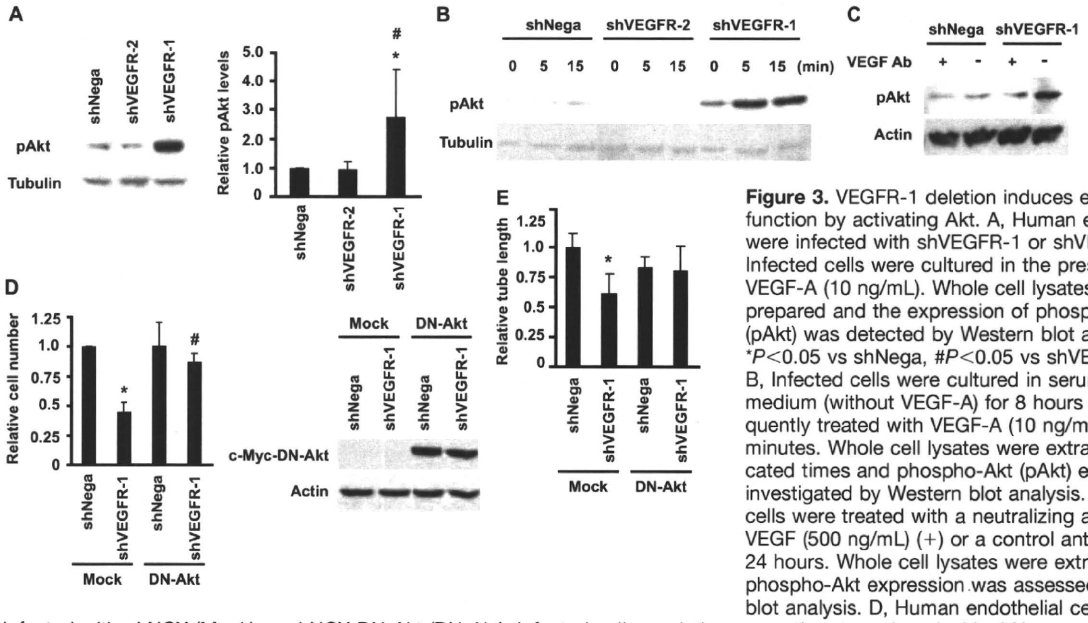

Figure 3. VEGFR-1 deletion induces endothelial dysfunction by activating Akt. A, Human endothelial cells were infected with shVEGFR-1 or shVEGFR-2. Infected cells were cultured in the presence of VEGF-A (10 ng/mL). Whole cell lysates (30 μg) were prepared and the expression of phosphorylated Akt (pAkt) was detected by Western blot analysis. *$P<0.05$ vs shNega, #$P<0.05$ vs shVEGFR-2 (n=5). B, Infected cells were cultured in serum-free basal medium (without VEGF-A) for 8 hours and subsequently treated with VEGF-A (10 ng/mL) for 5 to 15 minutes. Whole cell lysates were extracted at indicated times and phospho-Akt (pAkt) expression was investigated by Western blot analysis. C, Infected cells were treated with a neutralizing antibody for VEGF (500 ng/mL) (+) or a control antibody (−) for 24 hours. Whole cell lysates were extracted and phospho-Akt expression was assessed by Western blot analysis. D, Human endothelial cells were infected with pLNCX (Mock) or pLNCX DN-Akt (DN-Akt). Infected cell populations were then transduced with shNega or shVEGFR-1 and were subjected to the proliferation assay as described in legend for Figure 2C. *$P<0.005$ vs Mock/shNega, #$P<0.005$ vs Mock/shVEGFR-1 (n=6 to 8). Expression of c-Myc-tagged DN-Akt was confirmed by Western blot analysis (right panel). E, Double-infected endothelial cells (prepared as in Figure 3C) were subjected to the tube-forming assay. *$P<0.05$ vs Mock/shNega (n=3).

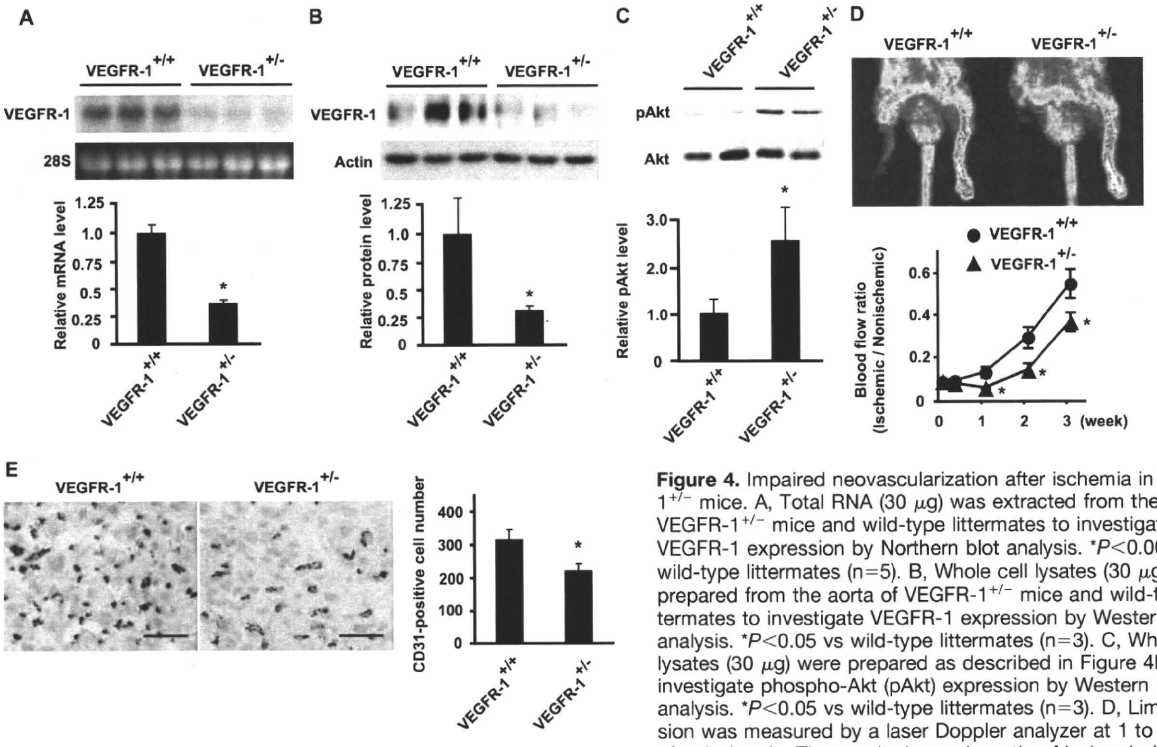

Figure 4. Impaired neovascularization after ischemia in VEGFR-$1^{+/-}$ mice. A, Total RNA (30 μg) was extracted from the lung of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Northern blot analysis. *$P<0.001$ vs wild-type littermates (n=5). B, Whole cell lysates (30 μg) were prepared from the aorta of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Western blot analysis. *$P<0.05$ vs wild-type littermates (n=3). C, Whole cell lysates (30 μg) were prepared as described in Figure 4B to investigate phospho-Akt (pAkt) expression by Western blot analysis. *$P<0.05$ vs wild-type littermates (n=3). D, Limb perfusion was measured by a laser Doppler analyzer at 1 to 3 weeks after ischemia. The graph shows the ratio of ischemic (right) to nonischemic limb (left) blood flow. *$P<0.05$ vs wild-type littermates (n=16). E, Immunohistochemistry for CD31 (brown) in ischemic limbs. Scale bar: 50 μm. The number of CD31-positive cells per square millimeter is shown in the graph. *$P<0.05$ vs wild-type littermates (n=4).

positive cells between VEGFR-$1^{+/-}$ mice and their wild-type littermates (Figure 5A). To further test the possible involvement of bone marrow–derived cells, we transplanted wild-type bone marrow cells into VEGFR-$1^{+/-}$ mice or their wild-type littermates. We then produced a hindlimb ischemia model and assessed blood flow recovery and the capillary density of ischemic tissue. Despite the transplantation of wild-type bone marrow, blood flow recovery was still significantly impaired in VEGFR-$1^{+/-}$ mice (Figure 5B). The number of CD31-positive cells was also lower in VEGFR-$1^{+/-}$ mice than in their wild-type littermates (Figure 5C). Thus, it is unlikely that impaired neovascularization in VEGFR-$1^{+/-}$ mice is attributed to reduced migration of bone marrow–derived cells. We could not detect VEGFR-1 expression in muscle cells (supplemental Figure IV). It was noted that the number of endothelial cells double positive for phospho-Akt and CD31 was significantly higher in VEGFR-$1^{+/-}$ mice than in their wild-type littermates (Figure 5D).

Inhibition of Akt Signaling Ameliorates the Impairment of Neovascularization in VEGFR-$1^{+/-}$ Mice

Next, we examined whether an increase of endothelial Akt activity contributed to impaired neovascularization in VEGFR-$1^{+/-}$ mice. Akt1 is the predominant isoform of Akt in endothelial cells and is thought to play an important role in postnatal angiogenesis.[30] It has been reported that the angiogenic response of $Akt1^{-/-}$ mice was enhanced in a tumor angiogenesis model, but was decreased in a hindlimb ischemia model,[30,31] so we thus used $Akt1^{+/-}$ mice for our in vivo experiments. Consistent with the previous reports,[32] phospho-Akt levels were lower in the aorta of $Akt1^{+/-}$ mice compared with wild-type littermates (supplemental Figure V). After creating hindlimb ischemia in VEGFR-$1^{+/-}$ $Akt1^{+/-}$ mice, we examined the extent of blood flow recovery and the capillary density 1 week later. We found that there were no significant differences of blood flow recovery and capillary density between $Akt1^{+/-}$ mice and $Akt1^{+/+}$ mice (Figure 6A and 6B). Decreased VEGFR-1 expression significantly reduced blood flow recovery in $Akt1^{+/+}$ mice, but not in $Akt1^{+/-}$ mice (Figure 6A). Likewise, the capillary density of ischemic tissue was significantly reduced in VEGFR-$1^{+/-}$ $Akt1^{+/+}$ mice compared with wild-type mice, but VEGFR-$1^{+/-}$ $Akt1^{+/-}$ mice had a similar capillary density to that of VEGFR-$1^{+/+}$ $Akt1^{+/-}$ mice (Figure 6B). These results suggest that an increase of endothelial Akt activity may be responsible for impaired neovascularization in VEGFR-$1^{+/-}$ mice.

Discussion

In the present study, we demonstrated that VEGFR-1 modulates postnatal angiogenesis through inhibition of the excessive activation of Akt by VEGF. It has been reported that VEGF and VEGFR-1 can be simultaneously induced by various stimuli, including hypoxia.[33] Thus, the role of VEGFR-1 may vary, depending on the extent of activation of Akt. For example, when overproduction of growth factors such as VEGF and insulin leads to excessive activation of Akt and impairs normal regulation of endothelial proliferation,

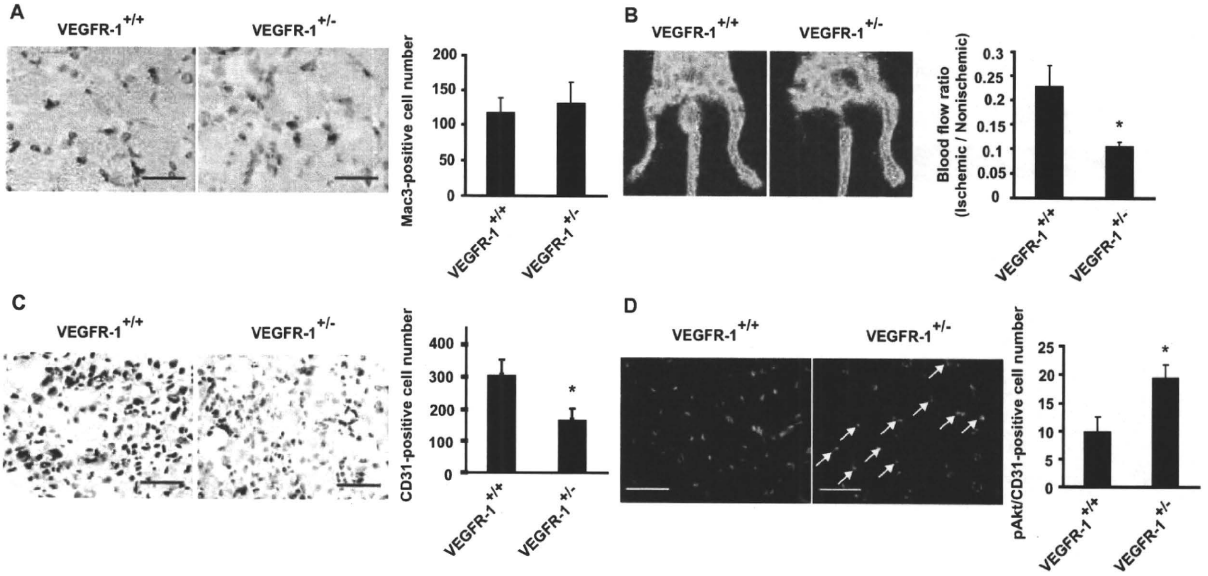

Figure 5. Role of bone marrow–derived cells in impaired neovascularization in VEGFR-1$^{+/-}$ mice. A, Immunohistochemistry for Mac3 (brown) in ischemic limbs. Scale bar: 50 μm. The number of Mac3-positive cells per square millimeter is shown (n=4). B, Wild-type bone marrow cells were transplanted into VEGFR-1$^{+/-}$ mice or their wild-type littermates. Limb perfusion was measured by a laser Doppler analyzer at 1 week after ischemia. *P<0.05 vs wild-type littermates (n=6). C, Immunohistochemistry for CD31 (brown) in ischemic limbs of bone marrow–transplanted mice. Scale bar: 50 μm. *P<0.05 s wild-type littermates (n=6). D, Activation of Akt in endothelial cells of ischemic limbs from VEGFR-1$^{+/-}$ mice. Representative immunostainings for phospho-Akt (red) and CD31 (green) were shown. Arrows indicate phospho-Akt/CD31-positive cells (yellow). Scale bar: 50 μm. The graph shows the ratio of phospho-Akt/CD31-positive cell number to all CD31-positive cell number. *P<0.05 vs wild-type littermates (n=5).

VEGFR-1 may act as a positive regulator of angiogenesis by inhibiting activation of VEGFR-2. Conversely, VEGFR-1 may exert a negative effect on angiogenesis when growth factors appropriately activate the Akt signaling pathway to induce endothelial cell proliferation. These mechanisms may provide an explanation as to why the effects of PlGF on angiogenesis were reported to differ.

Although there is evidence to suggest that VEGFR-1 interacts with the p85 subunit of phosphatidylinositol-3 kinase (PI3K) to regulate its activity,[34–36] VEGFR-1 appears to exert its inhibitory effect on angiogenesis mainly by blocking the activation of Akt mediated by VEGF via VEGFR-2 for the following reasons. First, treatment with VEGF-A increased Akt activity in VEGFR-1–deleted cells, but not in VEGFR-2–deleted cells (Figure 3A and 3B). Second, treatment with a neutralizing anti-VEGF antibody reduced the enhanced activation of Akt in VEGFR-1-deleted cells (Figure 3C). Finally, treatment with PlGF did not provoke any

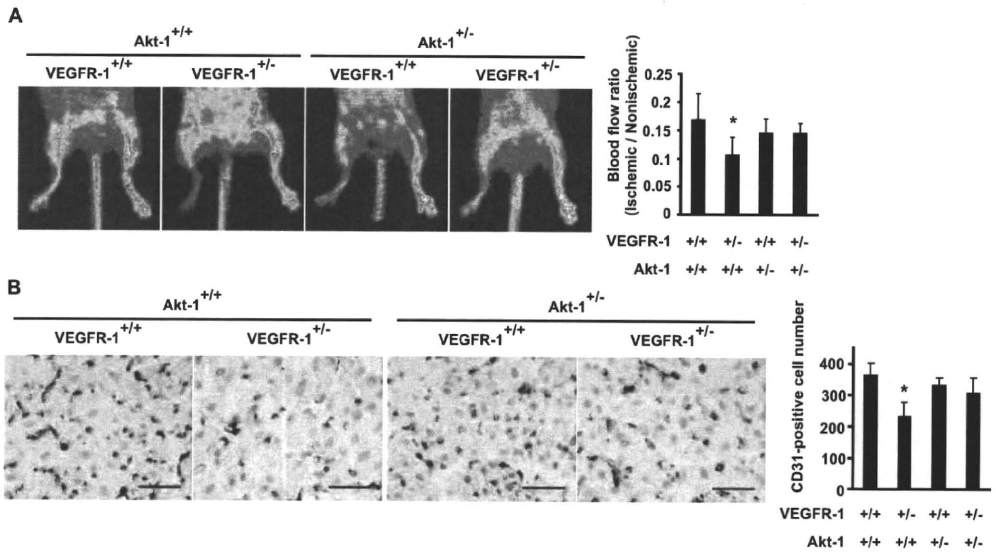

Figure 6. Inhibition of Akt signaling ameliorates the impairment of neovascularization in VEGFR-1$^{+/-}$ mice. A, Limb perfusion was measured by a laser Doppler analyzer at 1 week after creation of ischemia. *P<0.01 vs wild-type littermates (n=14 to 18). B, Immunohistochemistry for CD31 (brown) in ischemic limbs. Scale bar: 50 μm. *P<0.05 vs wild-type littermates (n=6 to 7).

biological response in the presence of anti-VEGF antibody (J. Nishi, T. Minamino, unpublished data, 2007). Our results are consistent with previous studies[37,38] demonstrating that tyrosine phosphorylation of VEGFR-2 was elevated in VEGFR-1–deficient embryonic stem cells, whereas loss of VEGFR-1 led to decreased sprout formation and migration, which resulted in reduced vascular branching. This reduction was restored by blockade of the VEGFR-2 signaling pathway as well as by treatment with soluble VEGFR-1. Although Bussolati et al demonstrated that VEGFR-1 but not VEGFR-2 increases endothelial production of NO, thereby promoting tube formation,[39] cGMP production was significantly decreased in VEGFR-1–deleted endothelial cells (supplemental Figure ID). Moreover, VEGF treatment failed to activate Akt in VEGFR-2-deleted endothelial cells (Figure 3B) and introduction of mutant VEGFR-1 lacking the sites for interaction with PI3K did not mimic the effects of shVEGFR-1 (J. Nishi, T. Minamino, unpublished data, 2007). Taken together, these results suggest that VEGFR-1 acts to provide "fine tuning" of VEGF signaling to achieve the proper formation of blood vessels. The biological consequences of VEGFR-1 deletion appears to be related to loss of its decoy effect, but other mechanisms might be involved such as "cross talk" between VEGFR-1 and VEGFR-2,[8,16,17] direct regulation of the VEGFR-2 signaling pathway by VEGFR-1,[39,40] and some undefined effect of the extracellular domain of membrane-bound VEGFR-1.[41]

We have previously demonstrated that constitutive activation of Akt induced by insulin promotes senescence-like arrest of endothelial cell growth via a p53/p21-dependent pathway.[19] Moreover, tube formation was significantly reduced by overactivation of Akt. Likewise, constitutive activation of Akt has been reported to promote the senescence in other types of cells such as endothelial progenitors and mouse embryonic fibroblasts.[42,43] The study using conditional transgenic mice has demonstrated that sustained activation of Akt in endothelial cells causes increased blood vessel size and generalized edema within 2 weeks and that these changes are reversible.[44] Using the same mouse model, it has been reported that chronic activation of Akt over 8 weeks leads to endothelial cell senescence and loss of endothelium-dependent stroke protection.[45] Recent studies by several groups demonstrated that diabetic state induces activation of the Akt pathway, thereby contributing to the pathology of diabetic complications.[42,46–48] We also detected increased Akt activity in endothelial cells on the surface of coronary atherosclerotic lesions in patients with diabetes.[19] Moreover, accumulating evidence suggests that vascular cell senescence contributes to the pathogenesis of age-associated vascular diseases including diabetic vasculopathy.[49] Thus, these results suggest the potential of the treatment for vascular dysfunction associated with diabetes and aging by modulating Akt activity with a soluble form of VEGFR-1.

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Disclosures

None.

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Gremlin Enhances the Determined Path to Cardiomyogenesis

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Abstract

Background: The critical event in heart formation is commitment of mesodermal cells to a cardiomyogenic fate, and cardiac fate determination is regulated by a series of cytokines. Bone morphogenetic proteins (BMPs) and fibroblast growth factors have been shown to be involved in this process, however additional factors needs to be identified for the fate determination, especially at the early stage of cardiomyogenic development.

Methodology/Principal Findings: Global gene expression analysis using a series of human cells with a cardiomyogenic potential suggested *Gremlin* (*Grem1*) is a candidate gene responsible for *in vitro* cardiomyogenic differentiation. Grem1, a known BMP antagonist, enhanced DMSO-induced cardiomyogenesis of P19CL6 embryonal carcinoma cells (CL6 cells) 10–35 fold in an area of beating differentiated cardiomyocytes. The Grem1 action was most effective at the early differentiation stage when CL6 cells were destined to cardiomyogenesis, and was mediated through inhibition of BMP2. Furthermore, BMP2 inhibited Wnt/β-catenin signaling that promoted CL6 cardiomyogenesis.

Conclusions/Significance: Grem1 enhances the determined path to cardiomyogenesis in a stage-specific manner, and inhibition of the BMP signaling pathway is involved in initial determination of Grem1-promoted cardiomyogenesis. Our results shed new light on renewal of the cardiovascular system using Grem1 in human.

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Introduction

The critical event in heart formation is commitment of mesodermal cells to a cardiomyogenic fate and their migration into anterolateral regions of the embryo during late gastrulation. In this process, morphogenic movements and cardiac fate determination are regulated by cytokines such as bone morphogenetic proteins (BMPs) [1–3], and fibroblast growth factors (FGFs) [4–7]. These secreted proteins from neighboring endoderm, ectoderm, and the mesoderm itself, play important roles in induction of cardiac transcription factors [8] and differentiation of cardiomyocytes in amphibians [9] and avians [4]. Cardiomyogenic signals, such as BMPs and FGFs, indeed activate expression of cardiac specific transcriptional factors (Csx/Nkx2.5, Gata4, Mef2c), and these transcriptional factors activate expression of circulating hormones (atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP)), and cardiac specific proteins (myosin heavy chain (MyHC), myosin light chain (MyLC)). Wnt family proteins, cysteine-rich, and secreted glycoproteins, have also been implicated in embryonic development [10,11], and cardiomyogenesis [12,13]. In *Drosophila*, '*wingless*', a homologue of vertebrate Wnt is involved in expression of '*tinman*', a *Drosophila* homologue of Csx/Nkx2.5, through '*armadillo*', a *Drosophila* ortholog of β-catenin, and drives heart development [14]. In vertebrates, however, Wnt1/3a, which activates the canonical Wnt/β-catenin signaling pathway leading to stabilization of β-catenin as a downstream molecule through inactivation of glycogen synthase kinase-3β, inhibits cardiomyocytic differentiation from cardiac mesoderm [15–18]. Wnt11 promotes cardiac differentiation via the non-canonical pathway in *Xenopus* [12] and murine embryonic cell lines [19]. The secretion of Wnt inhibitors such as 'Cerberus', 'Dickkopf' and 'Crescent' by the anterior endoderm prevents Wnt3a secreted by the neural tube from inhibiting heart formation [15–17].

In this study, we performed GeneChip analysis to identify multiple extracellular determinants, such as cytokines, cell

membrane-bound molecules and matrix responsible for cardiomyogenic differentiation, and evaluated the statistical significance of differential gene expression by NIA array analysis (http://lgsun.grc.nia.nih.gov/ANOVA/) [20], a web-based tool for microarray data analysis. We found that Grem1 enhances the determined path to cardiomyogenesis in a stage-specific manner, and that inhibition of the BMP signaling pathway is, at least in part, involved in initial determination of Grem1-promoted cardiomyogenesis.

Results

GeneChip and statistical analysis

To identify cytokines and transcription factors responsible for cardiomyogenic differentiation, 69 human cells were analyzed, depending on gene expression levels, by GeneSpringGX software, and clustered into 30 groups (Fig. 1A, Table 1). Among the 30 groups, 21 groups included cells with a cardiomyogenic potential (Fig. 1B: red numbers). To identify genes specific for these groups, hierarchical clustering was employed, using the average distance method. Genes with the lowest average expression E(G1) within the cluster that can differentiate into cardiomyocytes and genes with the highest average expression E(G2) outside the cluster were identified, as previously described [20–22]. Genes which have E(G1)>E(G2) were estimated, using the False Discovery Rate (FDR<0.05). Grem1 was nominated as a cluster-specific cardiomyocyte-promoting gene in cells that could differentiate into cardiomyocytes following NIA array analysis (Fig. 1B). The gene expression profile reported in this paper has been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo: accession no. GSE8481, GSM41342-GSM41344, and GSM201137- GSM201145).

Cardiomyogenic differentiation of CL6 cells with Grem1 and DMSO

To investigate cardiomyogenic activity of Grem1, P19CL6 embryonal carcinoma cells (CL6 cells) were used for assessment of *in vitro* cardiomyogenic differentiation, since CL6 cells are reproducibly and stably induced into beating cardiomyocytes by DMSO (Fig. 2Aa) [23]. CL6 cells did not differentiate following exposure to Grem1 alone at concentrations of 63 or 125 ng/ml for 14 days (Fig. 2B). However, Grem1 dramatically promotes DMSO-induced cardiomyogenic differentiation at a concentration of 63 and 125 ng/ml; Grem1 (125 ng/ml) especially increased DMSO-induced cardiomyogenic differentiation of CL6 cells as assessed by beating area (Fig. 2Ab and B) (Movie S1 and S2, http://1954.jukuin.keio.ac.jp/umezawa/kami/index.html).

RT-PCR of differentiated or undifferentiated CL6 cells

To investigate gene expression as well as morphological analysis, i.e. beating, during cardiomyogenic differentiation, RT-PCR analysis was performed to detect expression of cardiomyocyte-specific/associate transcription factors, and structural genes (Fig. 2C). Genes encoding *Csx/Nkx2.5*, *Gata4*, *Hand2*, *Mef2c*, *ANP*, *BNP*, *MyLC-2a*, *MyLC-2v*, and *β-MyHC* were up-regulated during cardiomyogenic differentiation of CL6 cells treated with Grem1 and DMSO (Fig. 2C: lanes 6, 7 versus lane 3). Triplicate independent experiments confirmed the concentration-dependent Grem1 action on cardiomyogenic differentiation. The cardiomyocyte-specific genes (*Csx/Nkx2.5*, *Gata4*, *MyLC-2a*, *MyLC-2v*) expression level of CL6 cells treated with DMSO and Grem1 (63 and 125 ng/ml) were also the same as or higher than that of DMSO-induced CL6 cells by semi-quantitative RT-PCR (Figure S1).

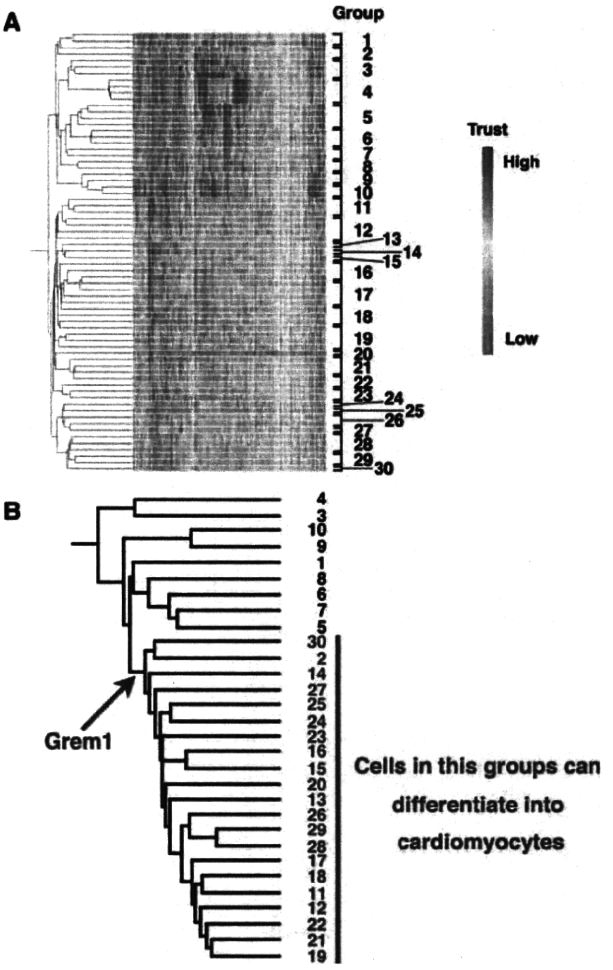

Figure 1. Hierarchical clustering analysis on cultured human cells. (A) Hierarchical clustering analyzed by GeneSpring. Based on gene expression pattern, 69 human cells were clustered into 30 sub-groups. The raw data from the GeneChip analysis are available at the GEO database with accession number GSE8481, GSM41342- GSM41344, and GSM201137- GSM201145. **(B)** Hierarchical clustering analysis was performed by NIA array (http://lgsun.grc.nia.nih.gov/ANOVA/), using averaged values of 30 sub-groups. Among the 30 groups, 21 groups included cells with a cardiomyogenic potential. To identify genes specific for these groups, hierarchical clustering was employed. *Grem1* was nominated as a cluster-specific cardiomyocyte-promoting gene in cells that could differentiate into cardiomyocytes.
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Immunocytochemistry of differentiated or undifferentiated CL6 cells

To examine CL6 cells for expression of cardiomyocytic protein, immunocytochemical analysis was performed. CL6 treated with Grem1 (125 ng/ml) and DMSO exhibited clear striation with immunostain using anti-cTnT or anti-α-actinin (Fig. 2Da and b). The MF20- and cTnT-positive cells after exposure to Grem1 and DMSO formed clusters (Fig. 2Ea), compared with the cells after exposure to DMSO alone (Fig. 2Eb). CL6 cells treated with Grem1 alone were negative for MF20 and cTnT, but became positive for both markers following exposure to Grem1 (63 and 125 ng/ml) and DMSO (Fig. 2F). The beating area (Fig. 2B) showed a tendency similar to the MF20- and cTnT-positive area (Fig. 2F), thus there were positive correlations between them.

Table 1. 69 human cells clustered into 30 groups

Group		Title	Description	GSM
1	Normal epithelial cell,primary	NHEK-Neo1	Normal epidermal keratinocyte, neonate, primary	GSM210361
		NHBE-1	Normal bronchial epithelial cell, primary	GSM210362
2	Pulmonary epithelial cell line	A549	Pulmonary epithelial cell line	GSM210363
		BEAS-2B control (6hr)	Bronchial epithelial cell line	GSM210364
3	Lymphocyte	RPMI8226control (6hr)	B cell line	GSM210365
		Raji-1	B cell line	GSM210366
		NK92	NK cell line	GSM210367
4	Myelomonocytic leukemia	U937c	U937 control	GSM210368
		U937h	U937+HRF	GSM210369
		U937ha	U937+HRF+antibody	GSM210370
		U937a	U937+antibody	GSM210371
5	Embryonal carcinoma, cancer	NCR-G3	Embryonal carcinoma, NCR-G3, non-adherent	GSM201141
		NCR-G2NAd	Embryonal carcinoma, NCR-G2, non-adherent	GSM210373
		NCR-G4Ad	Embryonal carcinoma, NCR-G4, adherent	GSM201142
		NCR-G3Ad	Embryonal carcinoma, NCR-G3, adherent	GSM210375
6	ES cell	H1_P43	Undifferentiated hES	GSM41342
		H1-P46	Undifferentiated hES	GSM41343
		H1-P41	Undifferentiated hES	GSM41344
7	Embryonal carcinoma, cancer	NCR-G2Ad	Embryonal carcinoma, NCR-G2, adherent	GSM201140
		NCR-G1	Embryonal carcinoma, NCR-G3, non-adherent	GSM201139
8	Ewing, cancer	NCR-EW2	Ewing, cancer	GSM210378
		NCR-EW3	Ewing, ETV4, cancer	GSM210379
9	Ewing, cancer	GST6	Ewing, POU5F1, cancer	GSM201137
		GST6-extra	Ewing, POU5F1, cancer	GSM210381
10	Ewing, cancer	GST6-5az	Ewing, POU5F1, 5azaC, cancer	GSM201138
		GST6-5az-extra	Ewing, POU5F1, 5azaC, cancer	GSM210383
11	Bone marrow cell, primary	H4-1	Bone marrow cell, primary	GSM201143
		UBT5	Bmi-1, hTERT, bone marrow cell	GSM210385
		UBET7	Bmi-1, E6, hTERT, bone marrow cell	GSM210386
12	Ligament-derived cells	#10	Ligament, primary	GSM210387
	Marrow stromal cells	H10-2Vec	Vector, bone marrow cell	GSM210388
		H10-2TERT	hTERT, bone marrow cell	GSM210389
		H10-2Bmi1	Bmi-1, bone marrow cell	GSM210390
13	Placenta, primary	PL90	Placenta, primary	GSM210391
14	De-differentiated chondrocyte	TdHC1	E6, E7, hTERT, de-differentiated chondrocyte	GSM210392
15	Neural differentiated marrow stromal cell	UET13 Neural differentiation	E7, hTERT, neural differentiation, bone marrow cell	GSM210393
16	Neural differentiated marrow stromal cell	UET13 Neural differentiation1	E7, hTERT, neural differentiation, bone marrow cell	GSM210394
		UET13 Neural differentiation4	E7, hTERT, neural differentiation, bone marrow cell	GSM210395
		UET13 Neural differentiation5	E7, hTERT, neural differentiation, bone marrow cell	GSM210396
17	Cord blood-derived cells	UET13	E7, hTERT, bone marrow cell	GSM210397
		UCB408	Cord blood, primary	GSM210398
		UCB408E6E7-31	E6, E7, umbilical cord blood	GSM210399
	Adipocyte cell, primary	HAdPC1(5/21)	HAdpc1E6E7TERT28	GSM210400
18	Marrow mesenchymal cell, primary	UEET12	E6, E7, hTERT, bone marrow cell	GSM210401
		UEE16	E6, E7, bone marrow cell	GSM210402
		EPC hTERT+1	E6, E7, hTERT, endometrial cell	GSM201144
19	Cord blood, primary	UCB302	Cord blood, primary	GSM210382
		UCB302-D7	Cord blood, primary	GSM210405
		UCB302TERT	hTERT, cord blood	GSM210406
		UET9	E7, hTERT, bone marrow cell	GSM210407

Table 1. cont.

Group		Title	Description	GSM
20	Cord blood, primary	UCB408E7-32	E7, hTERT, cord blood	GSM210408
21	Fetal fibroblast, primary	HFDPC cont.	Normal follicular dermal papillar cell, primary	GSM210409
		PL112	Placenta, primary	GSM210410
		HF7-3	Fetal fibroblast, primary	GSM210411
22	Bone marrow cell, primary	3F0664	Bone marrow cell (commercial item), primary	GSM201145
		BM-MSC	Bone marrow-derived mesenchymal stem cells	GSM38627
23	ES cell-derived mesenchymal cell	H1 clone 2	ES cell-derived mesenchymal precursor	GSM38628
		H9 clone 1	ES cell-derived mesenchymal precursor	GSM38629
24	Endometrial cell	EPC100	E6, E7, hTERT, endometrial cell	GSM210413
25	Bone marrow cell, primary	Yub10F	Bone marrow cell, primary	GSM210414
26	Endometrial cell	EPC hTERT+2	E6, E7, hTERT, endometrial cell	GSM210415
		EPC Control	E6, E7, hTERT, endometrial cell	GSM210416
27	Endometrial cell	EPC214	E6, E7, hTERT, endometrial cell	GSM210417
28	Menstruation blood-derived mesenchymal cell, primary	#E4	Menstruation blood, primary	GSM210418
		#E4HRF	Menstruation blood, HRF treatment, primary	GSM210419
		#E5HRF	Menstruation blood, HRF treatment, primary	GSM210420
29	Menstruation blood-derived mesenchymal cell, primary	#E6	Menstruation blood, primary	GSM210421
		#E6HRF	Menstruation blood, HRF treatment, primary	GSM210422
30	Menstruation blood-derived mesenchymal cell, primary	#E5	Menstruation blood, primary	GSM210423

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Grem1 and DMSO were most effective at the early stage (days 1–3) of CL6 differentiation

To determine if Grem1 (125 ng/ml) functions during the early or the late stage of differentiation, CL6 cells were treated with Grem1 for different time periods (Fig. 3A). Grem1 and DMSO were most effective on CL6 differentiation at 1–3 days (Fig. 3B, C) as assessed by percentages of MF20-positive area and beating area. Since Grem1 inhibits BMPs through direct binding [24], we hypothesized that BMP signaling is inhibitory to CL6 cardiomyogenesis during days 1–3. To confirm this hypothesis, RT-PCR analysis was performed to determine expression of the early mesodermal marker (*BrachyuryT* and *Tbx6*), cardiomyocyte-specific transcription factors (*Csx/Nkx2.5*), structural genes (*β-MyHC*), and *Gapdh* (Fig. 4A). DMSO induced the *BrachyuryT* and *Tbx6* genes, and their expressions peaked at 3 days and then decreased; BMP2 down-regulated expression of these genes at 3–7 days. The *Csx/Nkx2.5* and *β-MyHC* genes started to be expressed at days 3 and 5, respectively, and their expression increased up to 14 days, at which time the timeframe analysis was terminated. BMP2 clearly inhibited expression of the *Csx/Nkx2.5* and *β-MyHC* genes (Fig. 4A, lanes 1–7 versus lanes 8–14).

To examine cardiomyogenic differentiation, immunocytochemical analysis was performed on CL6 cells treated with the inducers. CL6 cells treated with DMSO and BMP2 for the first 3 days were negative for sarcomeric myosin (MF20) at 14 days, but became positive for sarcomeric myosin, following exposure to DMSO alone during days 1–3 (Fig. 4B). To determine if DMSO induces BMP production in CL6 cells, expression levels of *Bmp2* and *Bmp4* were determined by quantitative real-time RT-PCR analysis (Fig. 4C). DMSO clearly induced the *Bmp2* and *Bmp4* genes, and DMSO-induction was inhibited by BMP2 protein. The expression level of *Bmp2* was highest during days 7–10 (Fig. 4C: *Bmp2*) in DMSO-induced CL6 cells, and that of *Bmp4* was highest during days 5–7 (Fig. 4C: *Bmp4*).

To investigate BMP signaling on cardiomyogenic differentiation, we used the *Id1* promoter-Lux plasmid that includes the luciferase gene driven by the *Id1* promoter, known as a BMP target promoter (Fig. 4D). DMSO increased BMP signaling activity that peaked at 5 days (Fig. 4D, open square). BMP2 protein increased BMP signaling activity at 3 days (Fig. 4D, closed square), but lost BMP signaling activity at 5 days and later, implying that this loss of BMP signaling leads to lack of cardiomyogenic induction.

Since Wnt/β-catenin signaling is involved in CL6 cardiomyogenesis [23,25], we hypothesized that the BMP effect on CL6 cardiomyogenesis is mediated through Wnt/β-catenin signaling. Expression of Wnt3a, an activator of canonical Wnt signaling, was indeed detected in CL6 cells exposed to DMSO, and BMP2 significantly down-regulated *Wnt3a* expression at day 3 (Fig. 4E). By using the TOPflash plasmid [23] which includes the luciferase gene driven by two sets of three copies of the TCF recognition site, Wnt/β-catenin signaling was assessed to investigate the effect of BMP2. Wnt/β-catenin signaling activity increased at 48 h after treatment with DMSO. Activity was increased by DMSO treatment but decreased by BMP2 (Fig. 4F). Time course analysis revealed that Wnt/β-catenin activity peaked at 5 days after DMSO treatment, and decreased thereafter (Fig. 4G). BMP2 inhibited DMSO-induced Wnt/β-catenin activity throughout the experimental period (up to 14 days). These results imply that BMP signaling inhibits CL6 cardiomyogenesis at the early stage through inhibition of Wnt/β-catenin signaling.