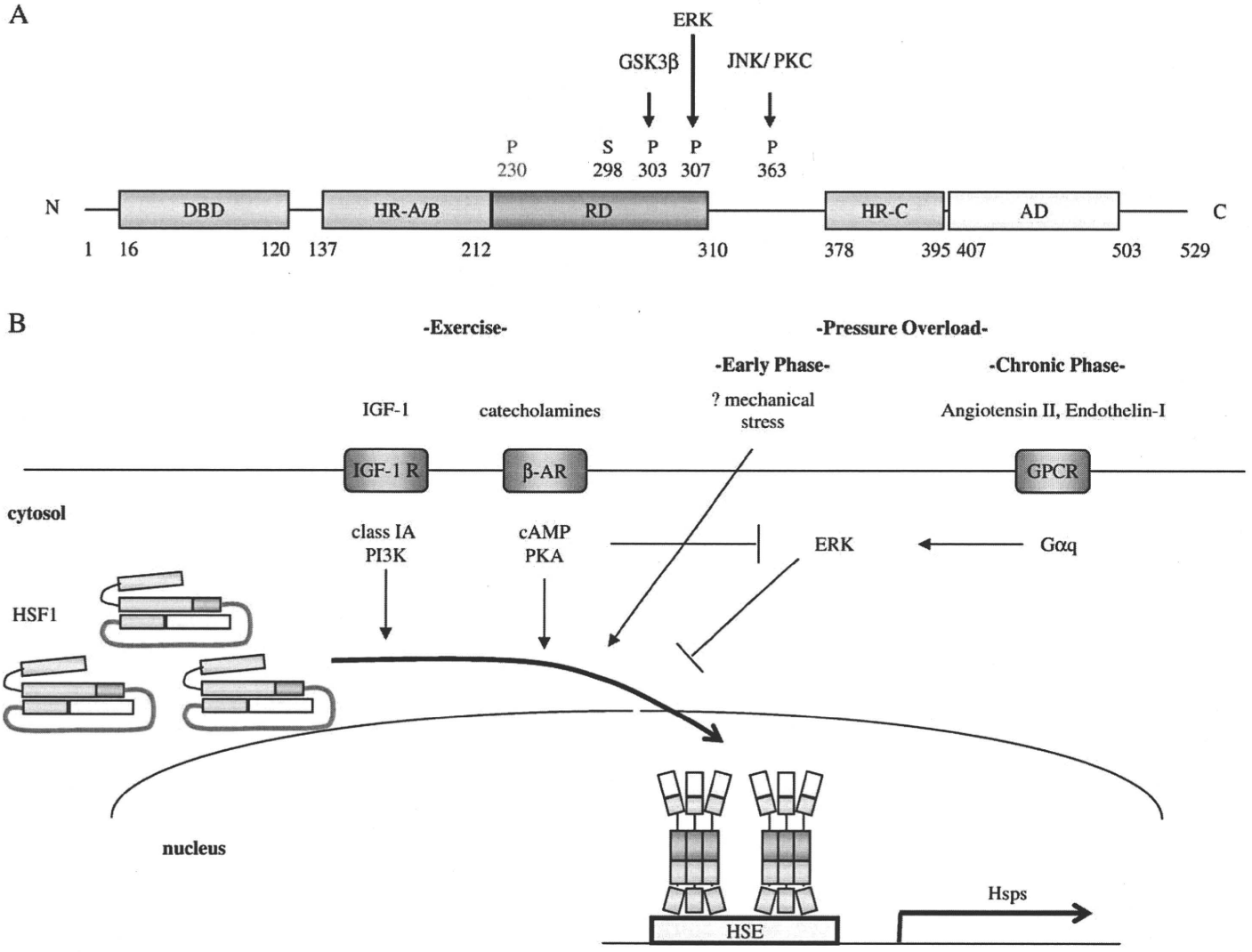


**Figure 1.** Potential regulators of HSF1 in cardiac hypertrophy. (**A**) Structure of HSF1. DBD indicates DNA-binding domain; HR, hydrophobic repeat; RD, regulatory domain: AD, transcriptional activation domain; P, phosphorylated site (the activating site is indicated in red); S, sumoylated site. (**B**) 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-3$\beta$ (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-3$\beta$ (Shiojima and Walsh 2006), which is a negative regulator of HSF1. It could be assumed that IGF-1-induced inhibition of GSK-3$\beta$ 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 $\beta$-adrenergic agonist) increases cardiac expression of HSP70 (White and White 1986), whereas inhibition of protein kinase A (PKA), a downstream kinase of the $\beta$-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 $\beta$-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 G$\alpha$q 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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**PII S1050-1738(08)00019-4** **TCM**

# 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

nature
cell biology

# A crucial role of a high mobility group protein HMGA2 in cardiogenesis

Koshiro Monzen[1,9], Yuzuru Ito[2,9], Atsuhiko T. Naito[3,9], Hiroki Kasai[1], Yukio Hiroi[1], Doubun Hayashi[1,4], Ichiro Shiojima[3], Tsutomu Yamazaki[5], Kohei Miyazono[6], Makoto Asashima[2,7,8], Ryozo Nagai[1] and Issei Komuro[3,10]

**The high mobility group (HMG) of nuclear proteins regulates expression of many genes through architectural remodelling of the chromatin structure, and formation of multiprotein complexes on promoter/enhancer regions. This leads to the active transcription of their target genes[1–3]. Here we show that HMGA2, a member of the HMGA sub-family of HMG proteins, has a critical function in cardiogenesis. Overexpression of *HMGA2* enhanced, whereas siRNA-mediated knockdown of *HMGA2* blocked, cardiomyocyte differentiation of the embryonal carcinoma cell line P19CL6. Moreover, overexpression of a dominant-negative HMGA2 or morpholino-mediated knockdown of HMGA2 expression blocked normal heart formation in *Xenopus laevis* embryos, suggesting that HMGA2 has an important role in cardiogenesis both *in vitro* and *in vivo*. Mechanistically, HMGA2 associated with Smad1/4 and showed synergistic trans-activation of the gene for a cardiac transcription factor Nkx2.5; a conserved HMGA2 binding site was required for the promoter activity of *Nkx2.5* gene, both in P19CL6 cells and in transgenic *Xenopus* embryos. Thus, HMGA2 is a positive regulator of *Nkx2.5* gene expression and is essential for normal cardiac development.**

The process of vertebrate heart development is regulated by a network of multiple transcription factors and signalling proteins[4,5]. Congenital heart malformations occur in approximately 1% of the population[6]; this high susceptibility of the developing heart to diseases may be in part due to the complexity of the molecular framework that controls cardiogenesis. A precise understanding of the causes of heart malformations is therefore imperative for establishing therapeutic strategies for congenital heart diseases. Molecules involved in the early stage of cardiac development are of particular interest because they may also function in repair or regeneration of the injured heart[7]. In this regard, cardiac transcription factors such as Nkx2.5 (**A001667**), GATA-4 (**A001029**) and MEF2C (**A001503**) have been investigated extensively, because they are essential for normal heart development and they are also encoded by early cardiac marker genes expressed in the heart-forming region, when cardiac precursors are specified in the anterior lateral mesoderm[5]. Signalling molecules such as bone morphogenetic proteins (BMPs), fibroblast growth factors, Wnts and soluble Wnt inhibitors have also been characterized as inducers or inhibitors of cardiac mesoderm specification[5,7]. However, it is not clear how these signalling molecules and transcription factors regulate the commitment of undifferentiated mesodermal cells into cardiac precursors.

To further investigate the regulatory mechanisms that control the early stage of cardiomyocyte differentiation, we used P19CL6 cells, which are derived from P19 murine embryonal carcinoma cell lines and differentiate into beating cardiomyocytes in the presence of 1% dimethyl sulphoxide (DMSO)[8]. Differential mRNA display was performed to isolate mRNA species whose expression was upregulated in the early stage (day 6) of P19CL6 differentiation into cardiomyocytes, when early cardiac marker genes, such as *Nkx2.5*, start to be expressed. Among the 50 clones isolated, five were confirmed by northern blotting to be upregulated at day 6 of differentiation and one of these five clones was found to be a cDNA encoding the HMG protein HMGA2. Northern blot analysis revealed that the expression of *HMGA2* mRNA was detected from day 2 and peaked at day 6 after DMSO treatment (Fig. 1a), indicating that the expression of *HMGA2* precedes those of early cardiac marker genes during cardiomyocyte differentiation.

HMGA2 is a member of the HMG superfamily of non-histone chromatin proteins, which consists of three sub-families, HMGA, HMGB and HMGN[1,3]. HMG proteins modulate the expression of many genes by remodelling the chromatin architecture and promoting the formation of multiprotein complexes on the promoter/enhancer region, leading to the active transcription of their target genes. The HMGA sub-family consists of four members, HMGA1a–c,

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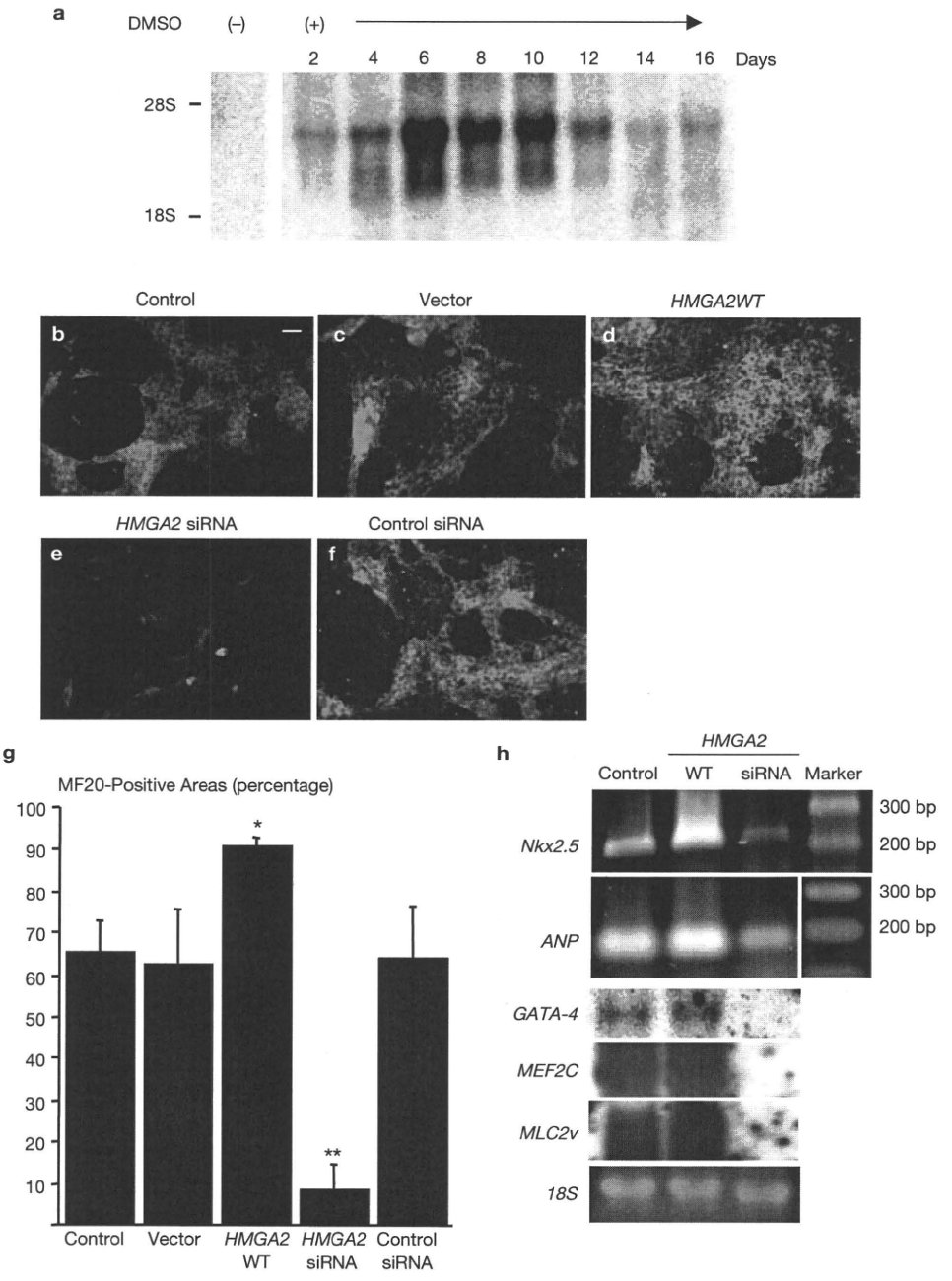


**Figure 1** HMGA2 is an essential positive regulator of cardiomyocyte differentiation. (**a**) Expression of *HMGA2* mRNA during P19CL6 differentiation into cardiomyocytes was assessed by northern blot analysis. (**b–f**) The effect of *HMGA2* overexpression or *HMGA2* knockdown on cadiomyocyte differentiation was assessed with MF20 staining in control (**b**), empty vector-transfected (**c**), *HMGA2* overexpressing (**d**), *HMGA2* siRNA-transfected (**e**) and control siRNA-transfected (**f**) P19CL6 cells. Scale bar is 100 μm. (**g**) Quantification of MF20-positive area. The results are shown as mean ± s. d. $*P < 0.05$, compared with control or vector-transfected cells, $**P < 0.01$ compared with control or irrelevant siRNA-transfected cells (one-way ANOVA, followed by Fisher's PLSD test; $n = 6$) (**h**) The effect of *HMGA2* overexpression or *HMGA2* knockdown on cardiac marker gene expression was assessed by RT-PCR (for *Nkx2.5* and *atrial natriuretic peptide* (*ANP*)) and northern blot (for *GATA-4*, *MEF2C* and *myosin light chain 2v* (*MLC2v*)).

generated by alternative splicing of *HMGA1* gene transcripts, and HMGA2, encoded by the *HMGA2* gene. All HMGA proteins except HMGA1c contain three short basic repeats called AT-hooks, which bind to the minor groove of AT-rich DNA stretches[1]. HMGA proteins are also able to associate with multiple transcription factors and regulate the expression of their target genes. For example, expression of the interferon-β gene is regulated by a multiprotein complex containing NF-κB, interferon regulatory factor, activating transcription factor-2/c-Jun and HMGA1a[9]. HMGA proteins also participate in the regulation of the genes for interleukin-2 receptor α and the insulin receptor[10,11]. HMGA proteins are expressed ubiquitously and abundantly during embryogenesis, whereas their expression is low or undetectable in fully differentiated adult tissues[1,2]. This suggests that HMGA proteins regulate normal cell growth and differentiation.

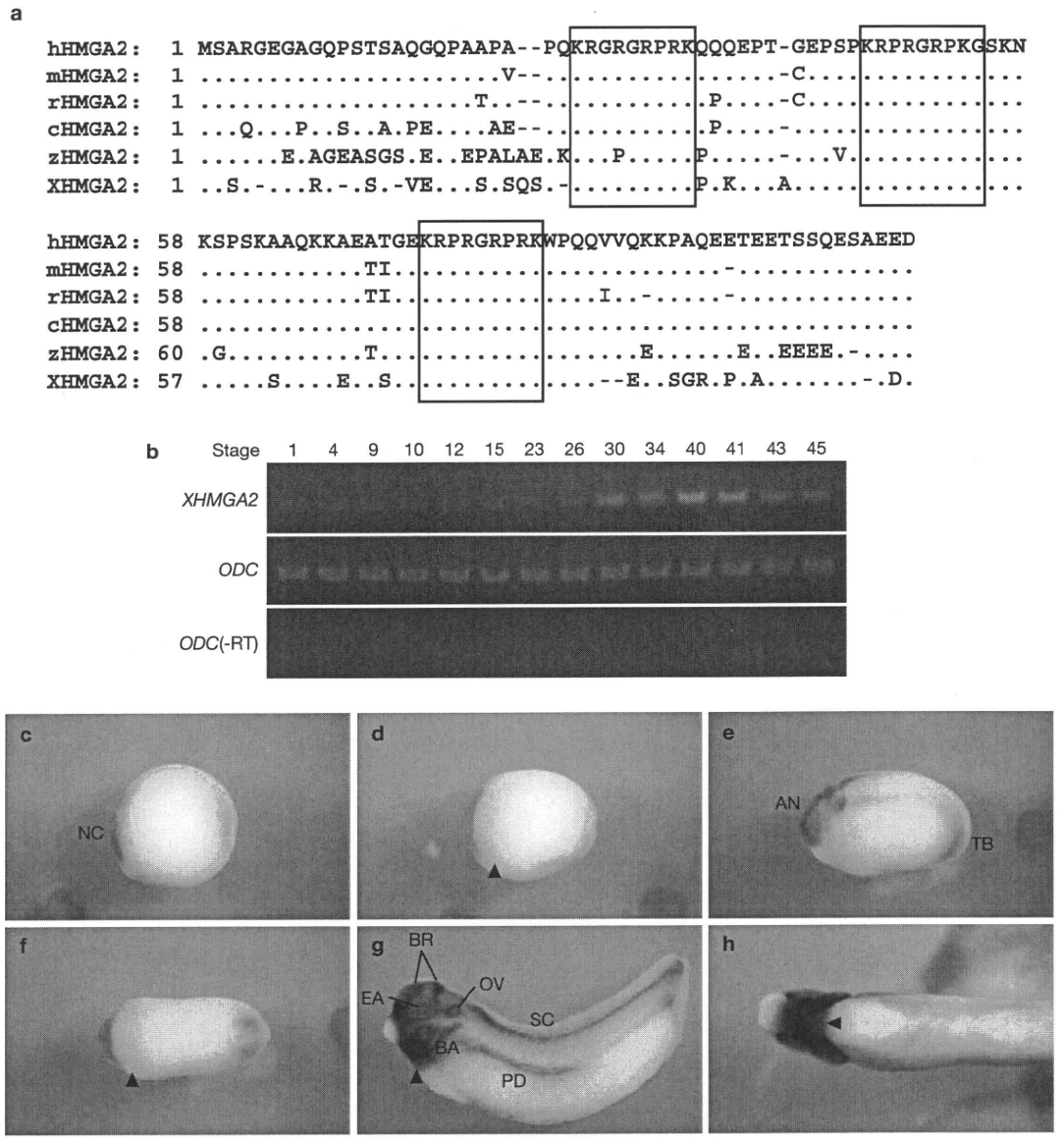


**Figure 2** cDNA sequence and expression pattern of *XHMGA2*. (**a**) Amino-acid sequences of XHMGA2 protein were compared with those of human (NM_001094371), mouse (NM_010441), rat (NM_032070), chicken (NM_205001) and zebrafish (NM_212680) HMGA2. Dots indicate identities and dashed lines are introduced to maximize the alignment. Boxes represent the three AT-hooks. (**b**) Expression of *XHMGA2* mRNA during embryogenesis analysed by RT-PCR. *Ornithine decarboxylase* (*ODC*) represents internal controls. (**c–h**) Expression of *XHMGA2* mRNA during embryogenesis analysed by *in situ* hybridization. Lateral (**c**) and ventral (**d**) view of stage-15 embryo. *XHMGA2* expression was detected in neural crest (NC) and weakly in precardiac region (arrowhead). Lateral (**e**) and ventral (**f**) view of stage-23 embryo. *XHMGA2* was expressed in anterior neural tissue (AN), tailbud (TB) and weakly in precardiac region (arrowhead). Lateral (**g**) and ventral (**h**) view of stage-32 embryo. *XHMGA2* was detected in brain (BR), spinal cord (SC), eye anlage (EA), otic vesicle (OV), branchial arch (BA), pronephric duct (PD) and heart anlage (arrowhead). Anterior is left and dorsal is top for **c**, **e**, **g**, and anterior is left for **d**, **f**, **h**.

To examine whether HMGA2 regulates cardiomyocyte differentiation, we analysed the ability of P19CL6 clones stably overexpressing wild-type *HMGA2* (P19CL6–HMGA2) to differentiate into cardiomyocytes. The extent of cardiomyocyte differentiation was assessed by the area positive for an anti-α-myosin heavy chain antibody (MF20). In response to DMSO, P19CL6–HMGA2 differentiated into beating cardiomyocytes more efficiently than the parental P19CL6 cells (Fig. 1b–d, g), and there was a positive correlation between the extent of cardiomyocyte differentiation and the level of *HMGA2* expression in multiple P19CL6–HMGA2 clones (Supplementary Information, Fig. S1a). On the other hand, siRNA-mediated knockdown of *HMGA2* blocked cardiomyocyte differentiation of P19CL6 cells without altering the expression of mesodermal marker genes such as *Brachyury* and *Flk-1*, whose expressions precede those of early cardiac marker genes such as *Nkx2.5* (Fig. 1e–g; Supplementary Information, Fig. S1b). The expression levels of cardiac marker genes was upregulated in response to HMGA2 overexpression, whereas they were downregulated by knockdown of *HMGA2* expression (Fig. 1h). Together, these results suggest that HMGA2 is an essential positive regulator of cardiomyocyte differentiation.

To investigate the role of HMGA2 in cardiogenesis *in vivo*, we performed experiments using *Xenopus* embryos. We first cloned *XHMGA2*, a *Xenopus* orthologue of *HMGA2*. *XHMGA2* encodes a protein of 105

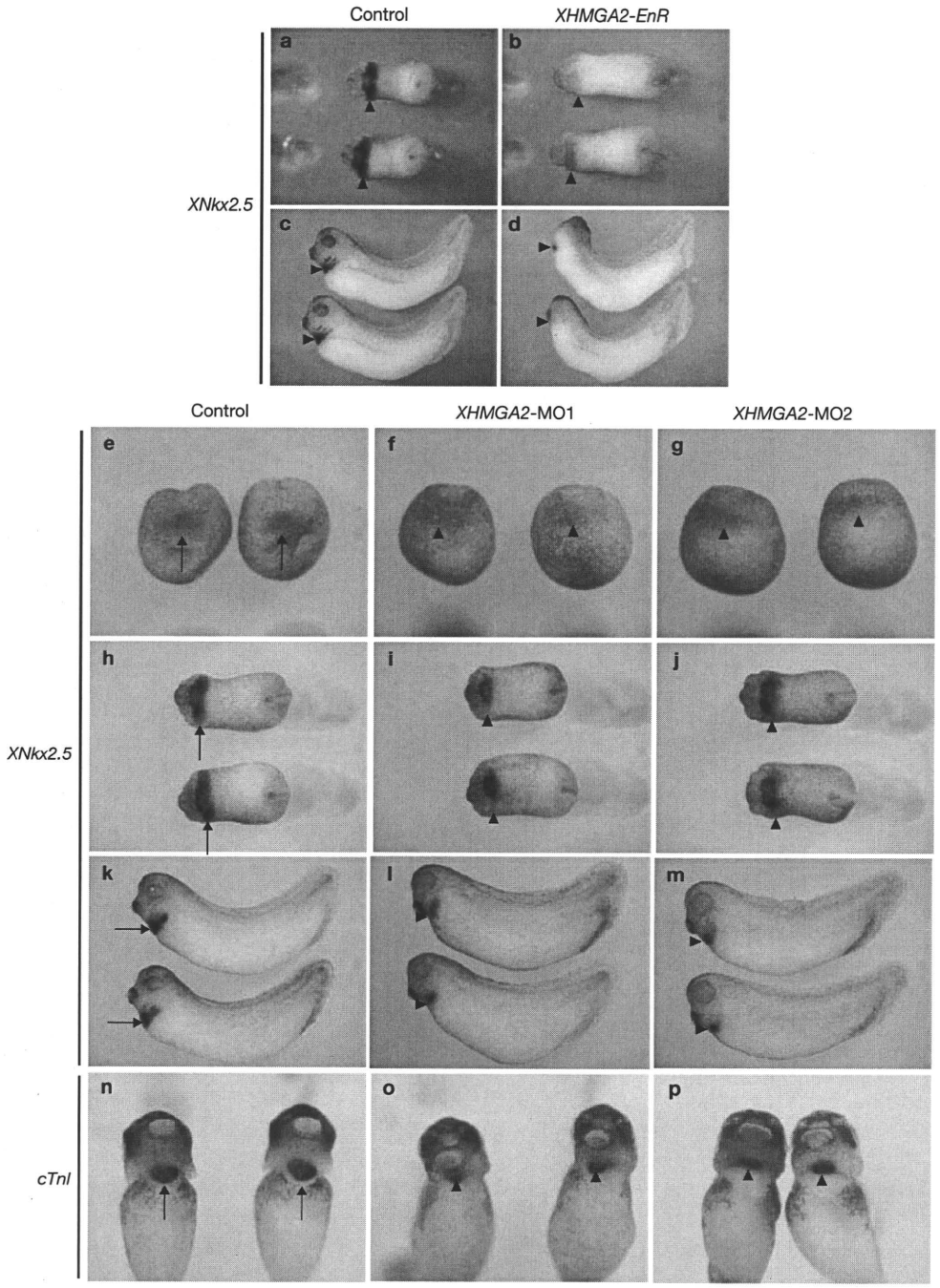


**Figure 3** XHMGA2 is essential for cardiogenesis. (**a–d**) Overexpression of a dominant-negative *XHMGA2* mutant in *Xenopus* embryos blocked *XNkx2.5* expression. Ventral view of stage-23 (**a**, **b**) and lateral view of stage-34 (**c**, **d**) embryos. Expression of *XNkx2.5* mRNA was decreased in *XHMGA2–EnR* mRNA-injected embryos (**b**, **d**, arrowheads), compared with that of control embryos (**a**, **c**, arrowheads). (**e–p**) MO-mediated *XHMGA2* knockdown results in impaired cardiogenesis. Whole-mount *in situ* hybridization analysis was performed sequentially for *Xenopus* embryos at stage 15 (**e–g**), stage 23 (**h–j**) and stage 34 (**k–m**) for *XNkx2.5*, and at stage 41 for *cardiac troponin I* (*cTnI*) (**n–p**). Expression of *XNkx2.5* mRNA was detected in uninjected control embryos (**e**, **h**, **k**, arrows), whereas it was attenuated in both XHMGA2–MO1 (**f**, **i**, **l**) and XHMGA2–MO2 (**g**, **j**, **m**)-injected embryos (arrowheads). *In situ* hybridization analysis for *cardiac troponin I* revealed that the heart size in MO-injected embryos (**o**, **p**, arrowheads) was smaller than that of control embryos (**n**, arrows).

amino acids that contains three highly conserved AT-hooks and is 60–70% homologous with HMGA2 proteins in other species (Fig. 2a). Reverse transcriptase-polymerase chain reaction (RT–PCR) analysis revealed that *XHMGA2* was maternally transcribed in the early stage of embryogenesis and its expression levels increased at stage 30 (Fig. 2b). *In situ* hybridization analysis revealed that *XHMGA2* was expressed in neural crest cells and the precardiac region at the neurula stage (Fig. 2c, d), and in anterior neural tissue, tailbud and precardiac region at the early tailbud stage (Fig. 2e, f). At the late tailbud stage, a strong signal was detected in brain, spinal code, eye anlage, otic vesicle, branchial arch, pronephric duct and heart anlage (Fig. 2g, h). To examine whether XHMGA2 regulates cardiogenesis *in vivo*, we injected

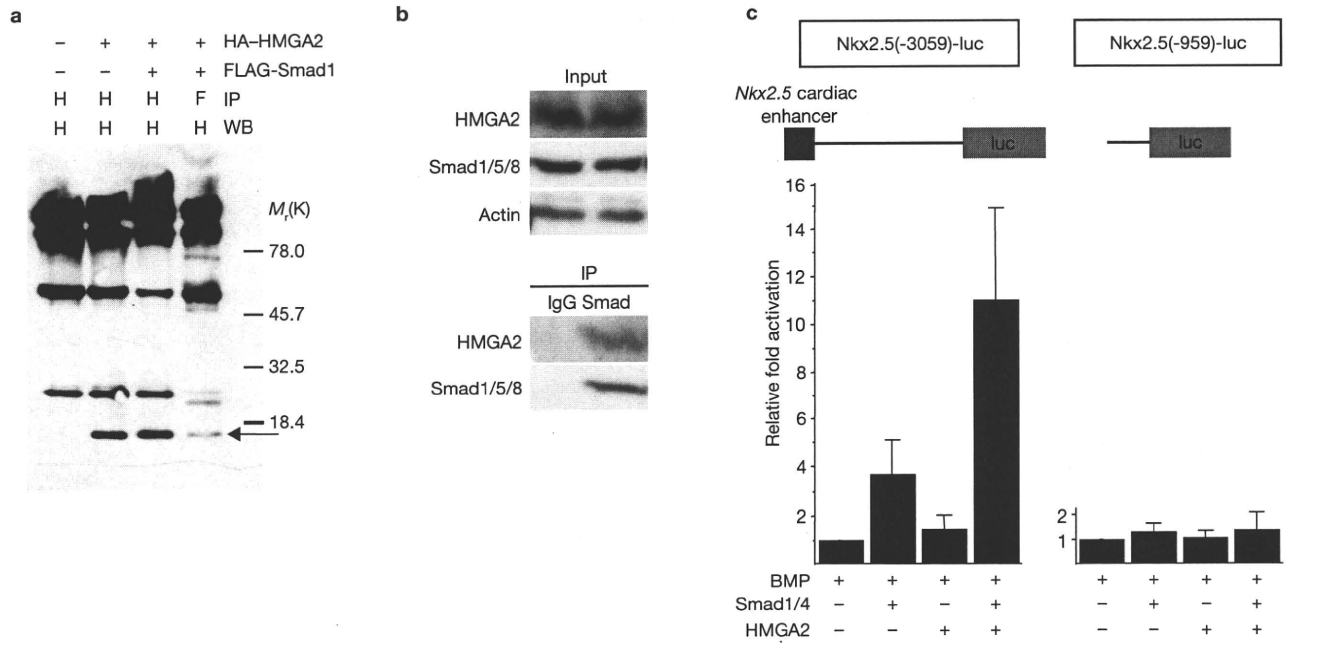


**Figure 4** HMGA2 upregulates *Nkx2.5* promoter activity in collaboration with Smads. (**a**) HA–HMGA2 interacted with FLAG–Smad1 in COS7 cells, as revealed by IP-western blot (WB) analysis. HA–HMGA2 was immunoprecipitated with an anti-FLAG antibody (arrow). H, anti-HA antibody; F, anti-FLAG antibody. (**b**) Endogenous HMGA2 interacted with endogenous Smad1/5/8 in P19CL6 cells. IP-western blot analysis revealed that a positive HMGA2 signal was detected in anti-Smad1/5/8 immunoprecipitates but not in control rabbit-IgG immunoprecipitates. (**c**) Luciferase reporter gene assay using Nkx2.5(–3059)-luc and Nkx2.5(–959)-luc. HMGA2 and Smad1/4 showed synergistic transactivation of –3059 bp *Nkx2.5* promoter but not –959bp *Nkx2.5* promoter. *Nkx2.5* cardiac enhancer at –3059/–2554 is shown as a blue box. The results are expressed as mean ± s. d. ($n = 6$).

mRNA encoding a dominant-negative mutant of XHMGA2 at the 8-cell stage into the dorsal region of two dorsal-vegetal blastomeres fated to be heart and liver anlage. Dominant-negative XHMGA2 was constructed as a fusion protein of full-length XHMGA2 and a transcriptional repressor domain of *Drosophila melanogaster* engrailed (XHMGA2–EnR)[12]. *In situ* hybridization analysis revealed that the expression of *Xenopus Nkx2.5* (*XNkx2.5*) was markedly downregulated in embryos injected with *XHMGA2–EnR* mRNA (Fig. 3a–d). We also used a morpholino (MO)-mediated knockdown strategy to downregulate XHMGA2 expression. Two different non-overlapping MOs (*XHMGA2*–MO1 and *XHMGA2*–MO2), which were confirmed to specifically recognize the target sequences of *XHMGA2* mRNA (Supplementary Information, Fig. S2), were injected into the dorsal region of two dorsal-vegetal blastomeres at the 8-cell stage. *In situ* hybridization analysis showed downregulation of *XNkx2.5* expression in MO-injected embryos and this effect of *XHMGA2* knockdown was observed from the neurula stage (Fig. 3e–m). *In situ* hybridization analysis of *cardiac troponin I* at the tadpole stage showed that the hearts of MO-injected embryos were reduced in size, compared with control embryos (Fig. 3n–p), and rhythmic contraction of the heart, normally observed at this stage, was attenuated or completely absent in MO-injected embryos (Supplementary Information, Movie 1). These phenotypes of morphants were rescued by co-injection of MO-resistant *XHMGA2* plasmid DNA (Supplementary Information, Fig. S3). These results strongly suggest that HMGA2 is essential for normal cardiac development *in vivo*.

Marked upregulation of *Nkx2.5* in P19CL6 cells by HMGA2 overexpression, compared with other early cardiac-marker genes such as *GATA-4* or *MEF2C* (Fig. 1h) suggests that *Nkx2.5* may be a direct target gene of HMGA2. BMP is a potent positive regulator of *Nkx2.5* expression during cardiogenesis[13]. BMP treatment causes the formation of heterooligomers containing BMP-specific Smad1/5/8 and the common-mediator Smad4, which translocate to the nucleus and activate transcription[14]. It has been shown that conserved binding sites for Smad proteins are required for cardiac-specific *Nkx2.5* enhancer activity[15,16]. We therefore tested the hypothesis that HMGA2 mediates BMP-responsive expression of *Nkx2.5* gene in collaboration with the Smad family of transcription factors. We first examined whether HMGA2 interacts with BMP-responsive Smads. Immunoprecipitation (IP)-western blot analysis of COS7 cell lysate cotransfected with HA-tagged HMGA2 and FLAG-tagged Smad1 showed that HMGA2 and Smad1 interacted with each other (Fig. 4a). Similar IP-western analysis demonstrated that endogenous HMGA2 interacted with endogenous Smad1/5/8 in P19CL6 cells treated with DMSO for 6 days (Fig. 4b). We also tested whether HMGA2 and Smads cooperatively regulate *Nkx2.5* promoter activity. In the presence of BMP stimulation, Smad1/4, but not HMGA2, transactivated the –3059 bp murine *Nkx2.5* promoter, and simultaneous expression of Smad1/4 and HMGA2 showed synergistic promoter activation (Fig. 4c, left panel). Deletion of the promoter region between –3059 and –959 resulted in a loss of transactivation by Smad1/4 and HMGA2 (Fig. 4c, right panel). These observations suggest that HMGA2 and Smad1/4 form a protein complex and synergistically upregulate *Nkx2.5* promoter activity, and that the HMGA2/Smad-responsive element(s) is located at –3059/–959 of the *Nkx2.5* regulatory region.

Previously, a cardiac enhancer at –3059/–2554 in *Nkx2.5* 5′-flanking region was characterized[17], which contains four Smad binding elements (SBEs) at –3038 (SBE-1), –3027 (SBE-2), –2774 (SBE-3) and –2758 (SBE-4; refs 15, 16). These SBEs are highly conserved among chick, mouse and human, and SBE-3 and SBE-4 are also conserved

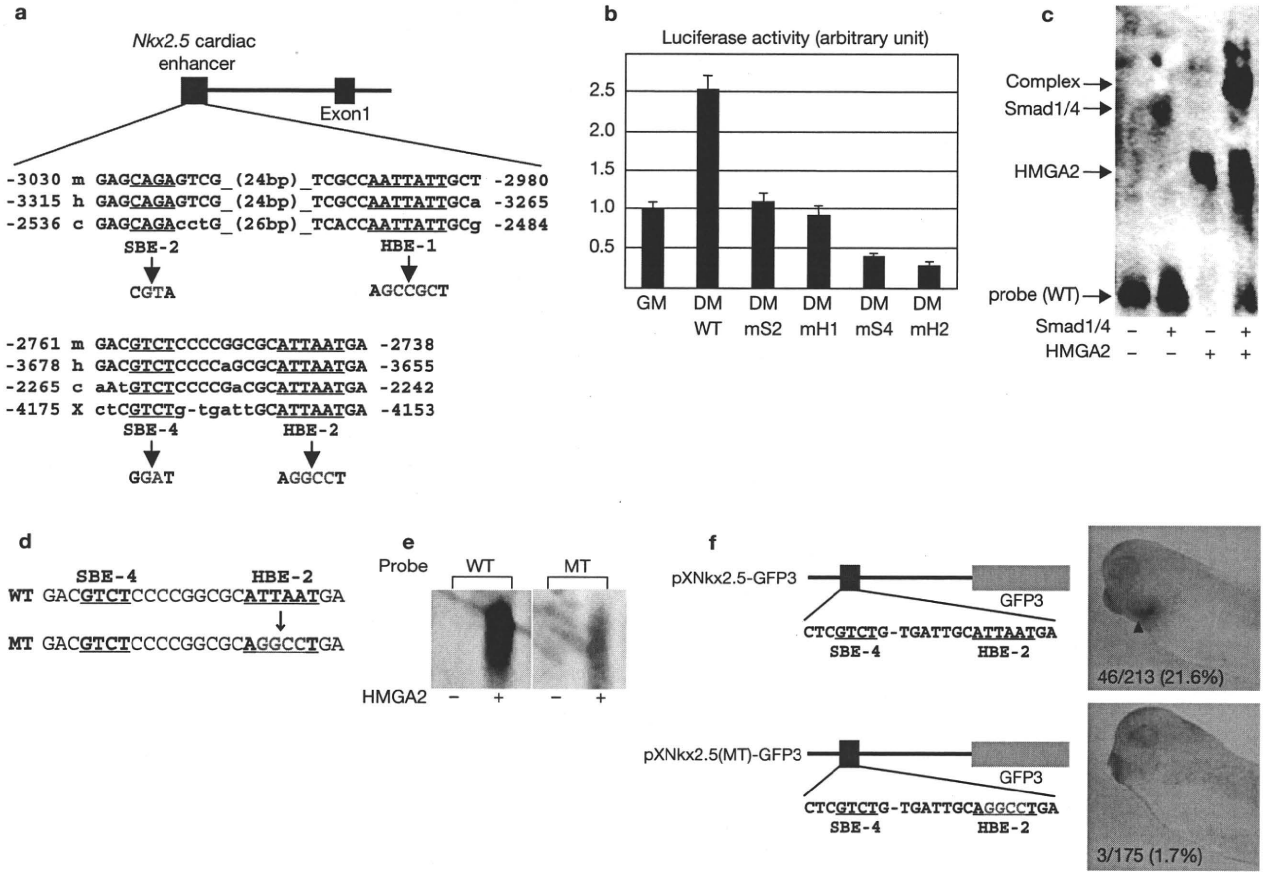


**Figure 5** HMGA2 binding is required for full activation of *Nkx2.5* promoter. (**a**) Sequence comparison of SBE-2/HBE-1 and SBE-4/HBE-2 elements in the –3059/–2554 *Nkx2.5* cardiac enhancer. These elements are evolutionarily conserved in human, mouse and chick, and SBE-4/HBE-2 sites are also conserved in *Xenopus*. Nucleotides that are not conserved are shown by small letters: m, mouse; h, human; c, chick; x, *Xenopus*. (**b**) Mutations introduced into conserved SBE-2/HBE-1 and SBE-4/HBE-2 elements attenuated *Nkx2.5* promoter activity during cardiomyocyte differentiation of P19CL6 cells. *Nkx2.5* promoter activity was assessed in P19CL6 cells at day 6 of differentiation. GM, growth medium (without DMSO); DM, differentiation medium (containing 1% DMSO); WT, wild-type *Nkx2.5* promoter; mS2, mH1, mS4 and mH2, *Nkx2.5* promoter containing mutations at SBE-2, HBE-1, SBE-4 and HBE-2, respectively ($n = 3$). (**c**) Interaction between Smad1/4 and HMGA2 on the SBE-4/HBE-2 element, as revealed by EMSA. Smad1/4 and HMGA2, either alone or in combination, bound to a 24-bp probe containing the SBE-4/HBE-2 element. (**d**) Sequences used for the EMSA probe. Mutations introduced into the HBE-2 site are shown in red. WT, wild-type probe; MT, mutated probe. (**e**) Binding of HMGA2 to SBE-4/HBE-2 element was reduced by mutations introduced into the HBE-2 site. (**f**) *GFP* reporter genes used to generate transgenic *Xenopus* embryos are shown on the left. pXNkx2.5(mt)–GFP plasmid contains mutations in the conserved HBE-2 site, which are indicated by red. GFP expression as assessed by *in situ* hybridization is shown on the right panels. The number of embryos showing *GFP* expression (arrowhead) per total number of surviving embryos for both plasmids are indicated.

in *Xenopus*[18], suggesting that this cardiac enhancer may be a target of HMGA2 and Smads. Indeed, there were two potential AT-rich HMGA2 binding elements (HBEs) adjacent to SBE-2 and SBE-4 (Fig. 5a). These HBEs were conserved in chick, mouse and human, and HBE-2 was also conserved in *Xenopus*[15,16,18]. Mutations introduced into SBE-2 or HBE-1 reduced the promoter activity of *Nkx2.5* by 50–60%, and more than 80% reduction in the promoter activity was observed by mutations introduced into SBE-4 or HBE-2 (Fig. 5b), suggesting that HMGA2 and Smads regulate *Nkx2.5* promoter activity cooperatively through these elements. As mutations introduced into the proximal element containing SBE-4/HBE-2 had a profound effect on *Nkx2.5* promoter activity, a 24-bp sequence containing SBE-4/HBE-2 (Fig. 5a, d) was used as a probe to examine protein–DNA interactions. Electrophoretic mobility shift assay (EMSA) showed that Smad1/4, HMGA2 and a complex containing Smad1/4 and HMGA2 bound to the 24-bp sequence (Fig. 5c). Mutation of the A/T-rich HBE-2 markedly attenuated the ability of HMGA2 to bind to this sequence (Fig. 5d, e). We also analysed *XNkx2.5* promoter activity *in vivo* in transgenic *Xenopus* embryos. Transgenic embryos expressing *green fluorescent protein* (*GFP*) gene under the control of the –4295 bp *XNkx2.5* promoter, which contains conserved SBE-4 and HBE-2, were assayed for their *GFP* mRNA expression by *in situ* hybridization. The *GFP* transgene containing the –4295 bp *XNkx2.5* promoter induced detectable *GFP* expression in the heart, and mutations introduced into HBE-2 abolished it (Fig. 5f). These findings suggest that HMGA2 and BMP-responsive Smads upregulate *Nkx2.5* promoter activity cooperatively through Smad- and HMGA2-binding elements, and that the conserved HMGA2 binding site is essential for *Nkx2.5* expression both *in vitro* and *in vivo*.

In this study we have demonstrated that HMGA2 promotes cardiomyocyte differentiation *in vitro* and is essential for cardiogenesis both *in vitro* and *in vivo*. Expression of HMGA2 is high during embryogenesis but low or undetectable in the adult tissues, suggesting that HMGA2 regulates normal cell growth and differentiation in general. Of note, transient expression of HMGA2 was observed in the heart after myocardial

infarction (Supplementary Information, Fig. S4), suggesting that HMGA2 is one of the fetal genes re-expressed in the myocardium in response to biomechanical stress. HMGA2 has been implicated in both benign and malignant tumours. Rearrangement of *HMGA2*, which results in the generation of a chimaeric or a truncated HMGA2 protein that contains three AT-hooks but lacks its carboxy-terminus, is frequently observed in benign human tumours of mesenchymal origin[19]. Overexpression of wild-type HMGA2 has also been reported in several malignant tumours[19]. Moreover, transgenic mice overexpressing the C-terminal-truncated form of HMGA2 are large and obese with lipomas[20,21]. Targeted disruption of the *HMGA2* gene in mice causes general growth retardation and impaired adipocyte differentiation[22,23], consistent with the suggestion that HMGA2 regulates cell growth and differentiation. In the experiments shown in Fig. 3, MOs were injected into the dorsal region of two dorsal-vegetal blastomeres fated to be heart and liver anlage. Examination of other lineage markers under these experimental conditions revealed that the expression of *XHex*, a marker gene for liver and thyroid gland, was attenuated in the liver and absent in the thyroid gland (Supplementary Information, Fig. S5d–f). Similarly, *Xmsr*, an endothelial-cell marker gene, showed a perturbed pattern of expression (Supplementary Information, Fig. S5g–i). Furthermore, when *XHMGA2*–MOs were injected into other regions at the 8-cell stage (Supplementary Information, Fig. S6a), different phenotypes of morphants were observed, depending on the site of MO injection and the area of MO distribution (Supplementary Information, Fig. S6b–f). Thus, the phenotype of *XHMGA2* knockdown is not necessarily restricted to the heart.

In this study we have also demonstrated that HMGA2 forms a protein complex with BMP-responsive Smad transcription factors that coordinately upregulate the promoter activity of *Nkx2.5* through evolutionarily conserved Smad- and HMGA2-binding elements. As HMGA2 has been shown to regulate the proliferation and/or differentiation of multiple cell types, there is a possibility that the regulation of cardiogenesis by HMGA2 is indirect and mediated by its effects on other cell types. However, we favour the idea that HMGA2 regulates cardiomyocyte differentiation directly, as our data suggest that HMGA2 promotes cardiogenesis through the transcriptional activation of *Nkx2.5*. Specifically, reduced expression of *Nkx2.5* by inhibition of HMGA2 and downregulation of *Nkx2.5* promoter activity by mutations in the HBE-2 site both *in vitro* and *in vivo* strongly suggest that *Nkx2.5* is a direct target of HMGA2. In this regard it is noteworthy that HMGA2 regulates the expression of an organ-specific transcription factor in collaboration with a growth factor-mediated signalling system and consequently contributes to organogenesis. In contrast to the *Nkx2.5* promoter, there was no cooperative upregulation of the BMP-responsive reporter BRE-luc or the TGF-β-responsive reporter p3TP-luc by the co-expression of Smad1/4/HMGA2 or Smad2/4/HMGA2, (Supplementary Information, Fig. S7). Thus, HMGA2 is not generally involved in transcriptional regulation mediated by Smads, but rather, is required for a specific subset of Smad-responsive transcriptional regulation in a context-dependent manner. Simultaneous interaction of HMGA2 with DNA and Smads with DNA may be necessary for synergistic transactivation by HMGA2 and Smads.

We speculate that the defect in cardiogenesis induced by HMGA2 inactivation is caused by downregulation of BMP-mediated *Nkx2.5*, as inactivation of *Nkx2.5* is sufficient to disrupt normal heart formation *in vivo*[4]. However, the possibility that HMGA2 is required for the proliferation of embryonic cardiomyocytes cannot be excluded. *HMGA2* knockdown in *Xenopus* embryos resulted in cardiac defects, whereas no cardiac abnormalities have been reported in *HMGA2* knockout mice. Although the exact reason for this apparent discrepancy is not clear at this time, the lack of cardiac phenotype in *HMGA2* mutant mice may be due to the genetic redundancies between *HMGA1* and *HMGA2* or among other HMG family members. As HMGA2 seems to regulate the early stage of cardiac development, further studies on this molecule may provide insights into myocardial regenerative medicine and the pathophysiology of congenital heart diseases. □

## METHODS

**Plasmids and reagents.** pcDNA3–HMGA2 was provided by G. Manfioletti (University of Trieste, Italy). FLAG-tagged Smad1/2/4, GST–Smad1/4 and p3TP–luc have been described previously[24]. BRE–luc was provided by P. ten Dijke (Leiden University Medical Center, Netherlands)[25]. Nkx2.5(–3059)-luc and Nkx2.5(–959)-luc were provided by K. E. Yutzey (Cincinnati Children's Medical Center, Cincinnati, OH)[15]. pCS-Fast-EnR was provided by M. Whitman (Harvard Medical School, Boston, MA)[12]. XCarGFP3 was provided by E. Amaya (University of Manchester, UK)[26]. Natural bovine BMP cocktail was purchased from Sangi.

**Differential mRNA display.** Differential mRNA display and subcloning of re-amplified cDNA fragments were performed as described previously[27].

**P19CL6 cell culture and stable transformants.** P19CL6 cells were cultured and induced to differentiate into cardiomyocytes as described previously[8]. To obtain P19CL6 clones stably overexpressing *HMGA2*, pcDNA3–HMGA2 was transfected into P19CL6 cells and neomycin-resistant clones were selected.

**siRNA-mediated knockdown in P19CL6 cells.** For *HMGA2* knockdown, 2-For-Silencing siRNA kit (Qiagen) was used. siRNAs were transfected at day 2 of differentiation. Sequences of *HMGA2* siRNA were as follows. Duplex 1: r(AGU AUA AGU UAA UAC UGA A)dTdT for sense, r(UUC AGU AUU AAC UUA UAC U)dGdA for antisense. Duplex 2: r(GGA AAU CUA CAC AGC CAA A)dTdT for sense, r(UUU GGC UGU GUA GAU UUC C)dCdG for antisense. MARK1 siRNA included in the kit was used as an irrelevant control siRNA.

**Immunocytochemisry.** Immunostaining with an anti-MF20 antibody was performed as described previously[8]. MF20-positive areas were measured at day 14 of differentiation.

**RNA analysis.** Northern blot, RT-PCR and quantitative real-time PCR for RNA analysis in P19CL6 cells were performed as described previously[8,28]. In *Xenopus* embryos, RT-PCR was performed as described previously[29]. PCR primers and PCR conditions are available in the Supplementary Information.

**IP-western blot analysis.** Total cell lysate was prepared from COS7 cells transfected with expression vectors for HA–HMGA2 and FLAG–Smad1 from P19CL6 cells induced to differentiate for 6 days. IP-western blot analysis was performed essentially as described previoulsy[30] using anti-HA monoclonal antibody 12CA5 (Roche) and anti-FLAG monoclonal antibody M2 (Kodak) for COS7 cell lysate, and anti-Smad1/5/8 and anti-HMGA2 (HMGI-C) rabbit polyclonal antibodies (Santa Cruz) for P19CL6 cell lysate.

**Luciferase reporter gene assay.** Nkx2.5–luc, pRL–CMV (an internal control) and effector constructs were transfected into COS7 cells or P19CL6 cells, and luciferase activity was measured with a luminometer (Berthold Lumat LB9507) 48 h after transfection. BMP cocktail (100 ng $ml^{-1}$) was added to COS7 medium. Mutations were introduced into Nkx2.5–luc plasmid using QuikChange II Site-Directed Mutagenesis Kit (Stratagene).

**EMSA.** Probes for EMSA were labelled using Biotin 3′ End DNA Labeling Kit (Pierce Biotechnology) and EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology). The HMGA2 protein

(HMGIC(48–109)-$NH_2$) was purchased from Phoenix Pharmaceuticals. GST–Smad1 and GST–Smad4 fusion proteins were purified with B-PER GST Spin Purification Kit (Pierce Biotechnology).

**Isolation of *XHMGA2* cDNA.** The entire coding region of *XHMGA2* was amplified by RT-PCR using mRNA obtained from embryos at the early tailbud stage, based on the sequence of a *Xenopus* EST (NM_001094371), which encodes a full-length protein similar to human HMGA2. The following primers were used: XHMGA2-U (5′-ATG AGC TCA AGG GAA GGA GCC-3′), XHMGA2-D (5′-CTA GTC GTC TTC AGA TTC CTG GG-3′). We cloned the PCR product into a pCS2+ vector (pCS2+XHMGA2).

**Microinjection of *XHMGA2–EnR* mRNA.** An expression vector for *XHMGA2–EnR* was constructed by a PCR-based cloning strategy. A cDNA fragment encoding Engrailed repressor domain (EnR) was amplified from *FAST–EnR* plasmid by PCR. mRNA for microinjection was synthesized with mMESSAGE mMACHINE kit (Ambion) from plasmids encoding *XHMGA2–EnR*. Synthesized mRNAs were microinjected as described previously[29].

**MO experiments.** The sequences of *XHMGA2* MOs were: *XHMGA2*–MO1, 5′-AGC TCA TGG TAG AGA GTG TGT GTG C-3′; XHMGA2-MO2, 5′-GCC CGG CGA TCC TGG AGC ACC TTA A-3′. MO activities and specificities were checked by co-injection of *5′XHMGA2–EGFP* or *XHMGA2–EGFP* mRNA (Supplementary Information, Fig. S2). For this, the *XHMGA2* coding region with 73 bp 5′ untranslated region (UTR) and *XHMGA2* coding region without 5′UTR (Supplementary Information, Fig. S2a) were inserted into the *ClaI* site of the EGFP–CS2 vector[29] to construct expression vectors for 5′XHMGA2–EGFP and XHMGA2–EGFP, respectively. We injected the MOs into two dorsal-vegetal blastomeres at the 8-cell stage. Rescue experiments were performed by injecting optimal-effect doses of XHMGA2–MO1 or XHMGA2–MO2 in conjunction with pCS2+*XHMGA2* plasmid DNA, which lacks 5′UTR and therefore is MO-resistant (100 pg per embryo). For tracing of injected MOs, β-*gal* mRNA was co-injected and embryos were pre-stained with Red-gal (Research Organics) before whole-mount *in situ* hybridization.

**Whole-mount *in situ* hybridization.** The following plasmid templates were linearized, and digoxigenin-substituted antisense RNA probes were transcribed with T7 or SP6 RNA polymerase: *XNkx2.5, XHex, Xmsr* and *XHMGA2* (a PCR-amplified coding region subcloned into pBluescript II SK+); *Xenopus cardiac troponin I* (a PCR amplified coding region subcloned into pGEM-T Easy). Embryos were processed for whole-mount *in situ* hybridization using BM purple substrate (Roche) and then the processed pigmented embryos were bleached by 9% $H_2O_2$, 21% $H_2O$ and 70% methanol.

**Generation and analysis of transgenic *Xenopus* embryos.** pXNkx2.5–GFP3 was generated by replacing the *cardiac actin* promoter in XCarGFP3 (ref. 18) with 4295 bp promoter sequence of *XNkx2.5*. This fragment was amplified by PCR from *Xenopus* tailbud genomic DNA using the XNkx2.5p-U primer (5′-ACC TGA GCT CGG GGG AAA TAT ACA CAA GGC C-3′) and XNkx2.5p-D primer (5′-GCA CCG GTG ACG GTA TCA GGT AAA CCC CAC A-3′). pXNkx2.5(mt)–GFP3 was created by site-directed mutagenesis. Both plasmids were digested by *SacI* and injected. Generation of transgenic *Xenopus* embryos was carried out as described previously[31].

Accession codes. USCD-Nature Signaling Gateway (**http://www.signaling-gateway.org**): **A001667, A001029** and **A001503**

*Note: Supplementary Information is available on the Nature Cell Biology website.*

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AUTHOR CONTRIBUTIONS

K. Monzen, Y. I. and A. T. M. contributed equally to this work; I. K. designed and supervised the research; K. Monzen, Y. I., A. T. M., H. K., Y. H. and D. H. performed experiments; I. S., T. Y., K. Miyazono, M. A. and R. N. contributed new reagents/analytical tools; K. Monzen, Y. I. and A. T. M. analysed the data; K. Monzen, Y. I., I. S. and I. K. prepared the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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

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*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 $2\times10^5$ 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

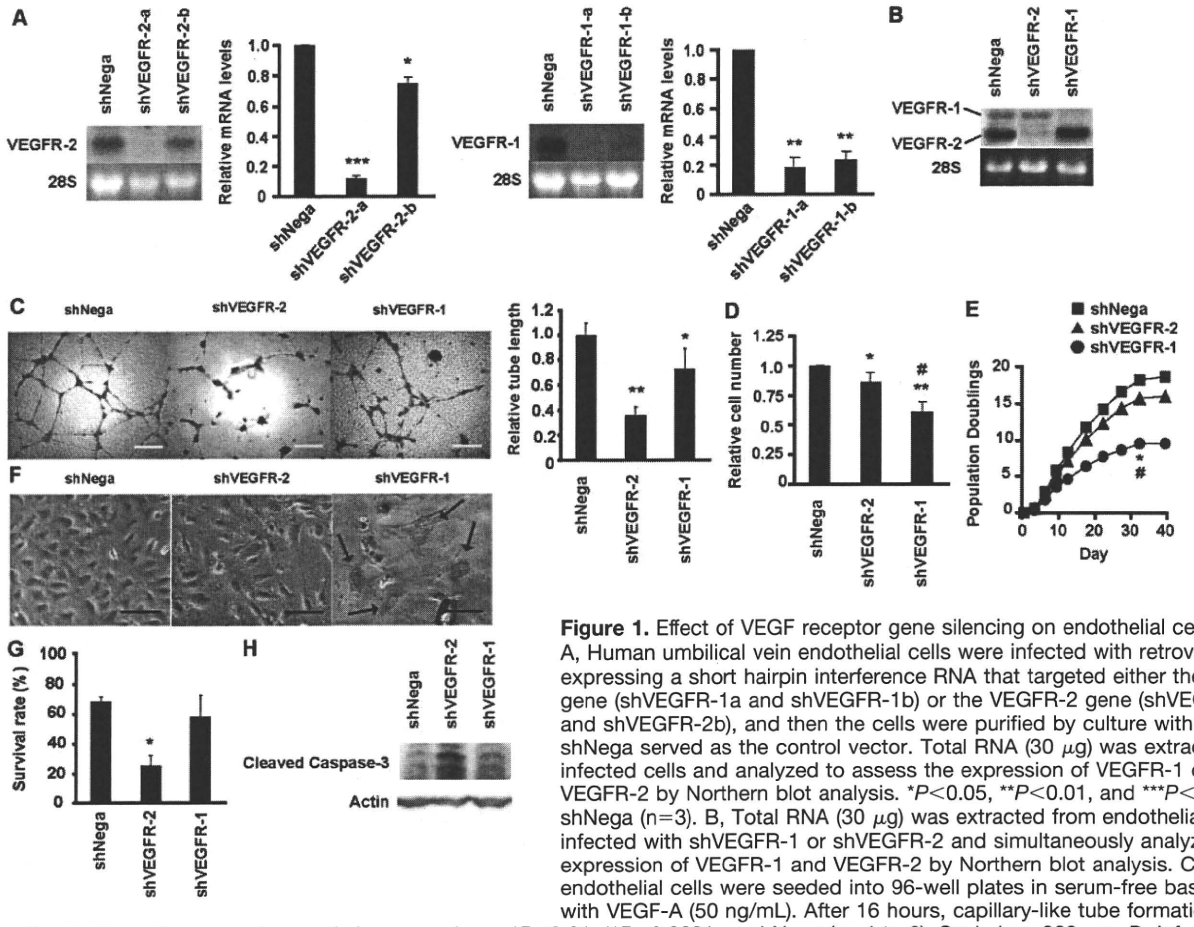


**Figure 1.** Effect of VEGF receptor gene silencing on endothelial cell function. A, Human umbilical vein endothelial cells were infected with retroviral vectors expressing a short hairpin interference RNA that targeted either the VEGFR-1 gene (shVEGFR-1a and shVEGFR-1b) or the VEGFR-2 gene (shVEGFR-2a and shVEGFR-2b), and then the cells were purified by culture with antibiotics. shNega served as the control vector. Total RNA (30 $\mu$g) 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 $\mu$g) 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 $\mu$m. D, Infected endothelial cells were seeded at a density of $2\times10^5$ 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 $\beta$-galactosidase staining (arrow) of endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Scale bar: 100 $\mu$m. G, Infected endothelial cells were seeded at the density of $1\times10^5$ 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).

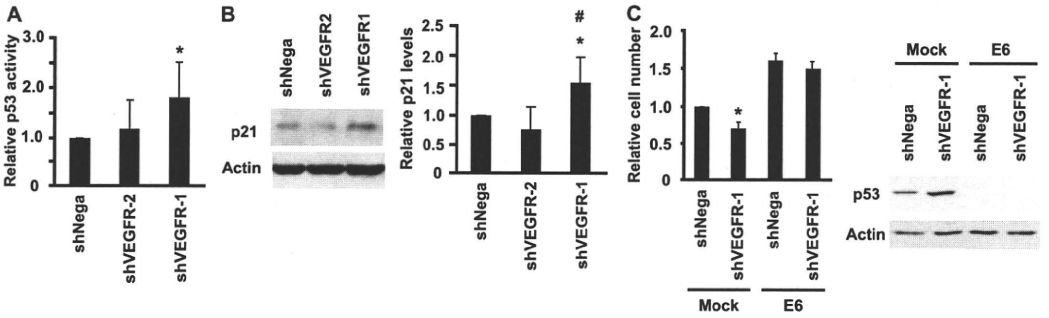


**Figure 2.** VEGFR-1 deletion induces activation of the p53/p21 signal pathway. A, A luciferase reporter gene plasmid (pPG13-Luc) containing the p53-binding sequence was transfected into endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Luciferase activity was measured at 48 hours after 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 $\mu$g) 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 $2\times10^5$ 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-

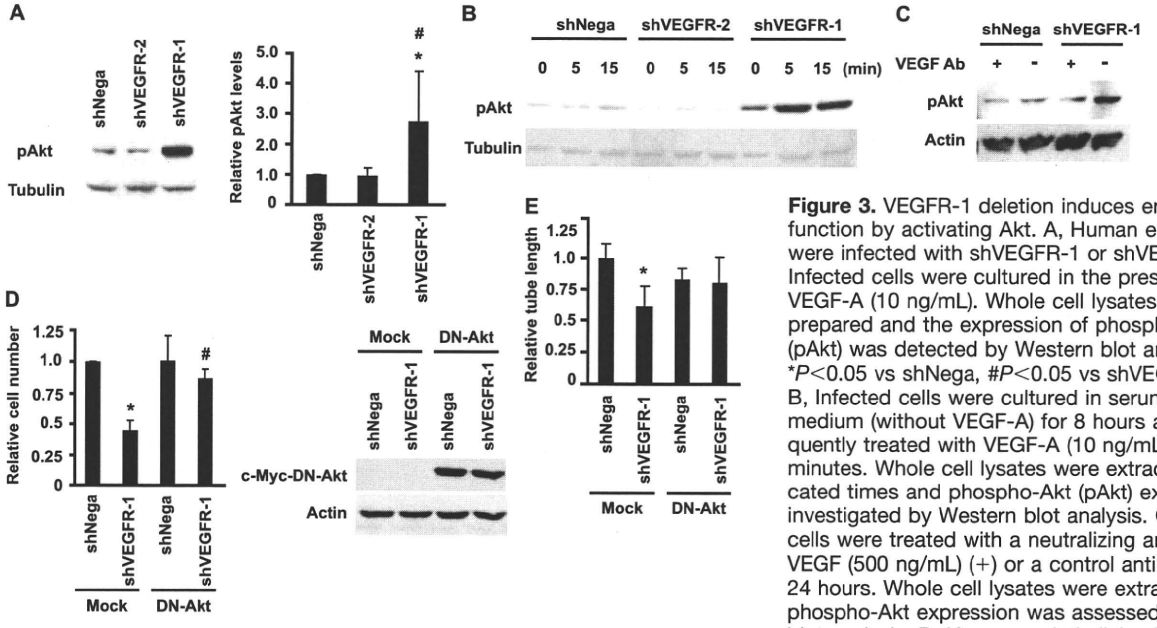


**Figure 3.** VEGFR-1 deletion induces endothelial dysfunction by activating Akt. A, Human endothelial cells were infected with shVEGFR-1 or shVEGFR-2. Infected cells were cultured in the presence of VEGF-A (10 ng/mL). Whole cell lysates (30 $\mu$g) were prepared and the expression of phosphorylated 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).

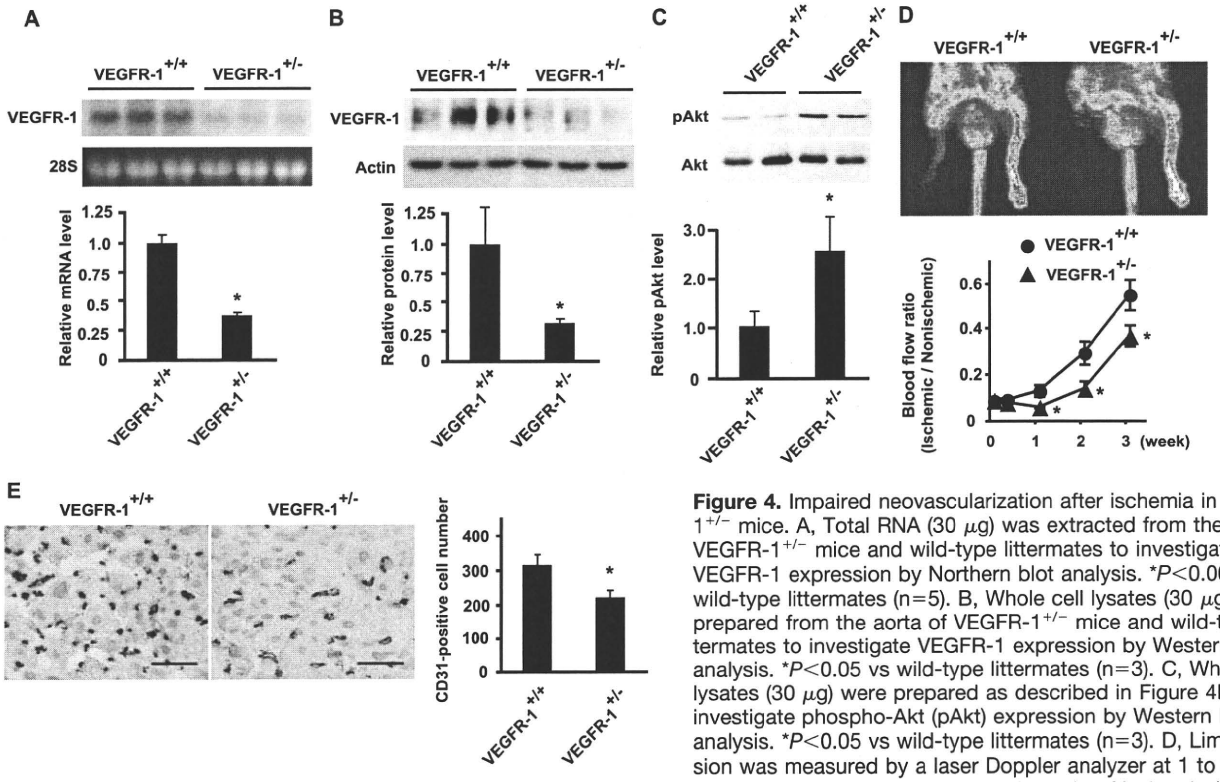


**Figure 4.** Impaired neovascularization after ischemia in VEGFR-$1^{+/-}$ mice. A, Total RNA (30 $\mu$g) was extracted from the lung of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Northern blot analysis. *$P<0.001$ vs wild-type littermates (n=5). B, Whole cell lysates (30 $\mu$g) 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 $\mu$g) 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 $\mu$m. 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,

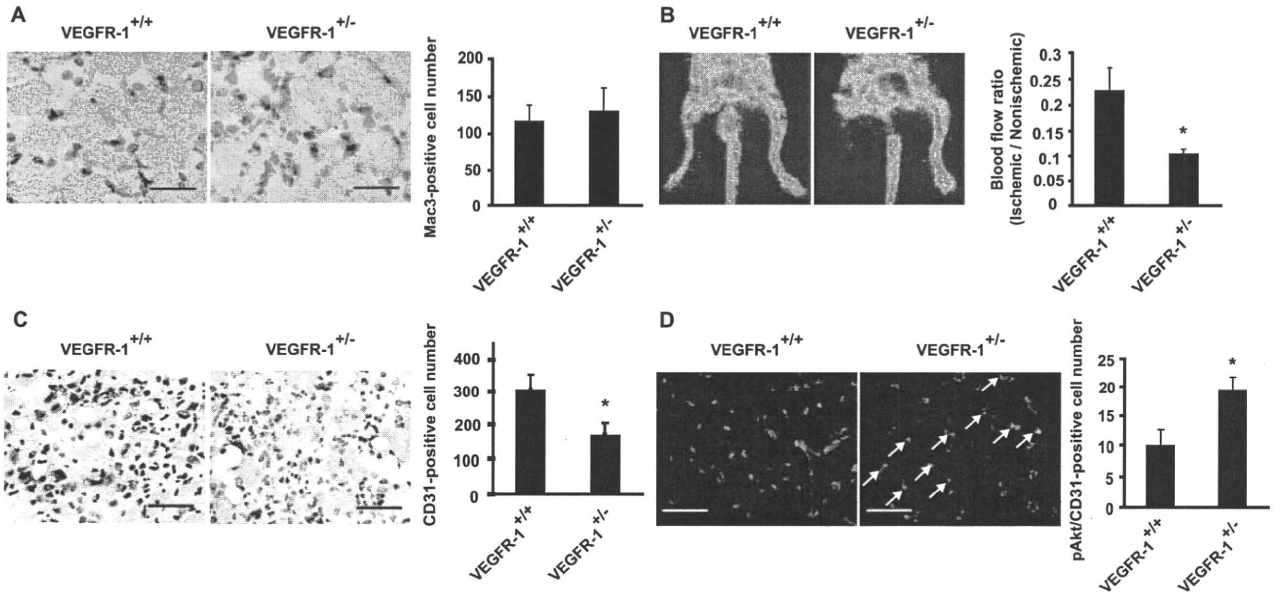


**Figure 5.** Role of bone marrow–derived cells in impaired neovascularization in VEGFR-1$^{+/-}$ mice. A, Immunohistochemistry for Mac3 (brown) in ischemic limbs. Scale bar: 50 $\mu$m. The number of Mac3-positive cells per square millimeter is shown (n=4). B, Wild-type bone marrow cells were transplanted 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 $\mu$m. *$P$<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 $\mu$m. 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

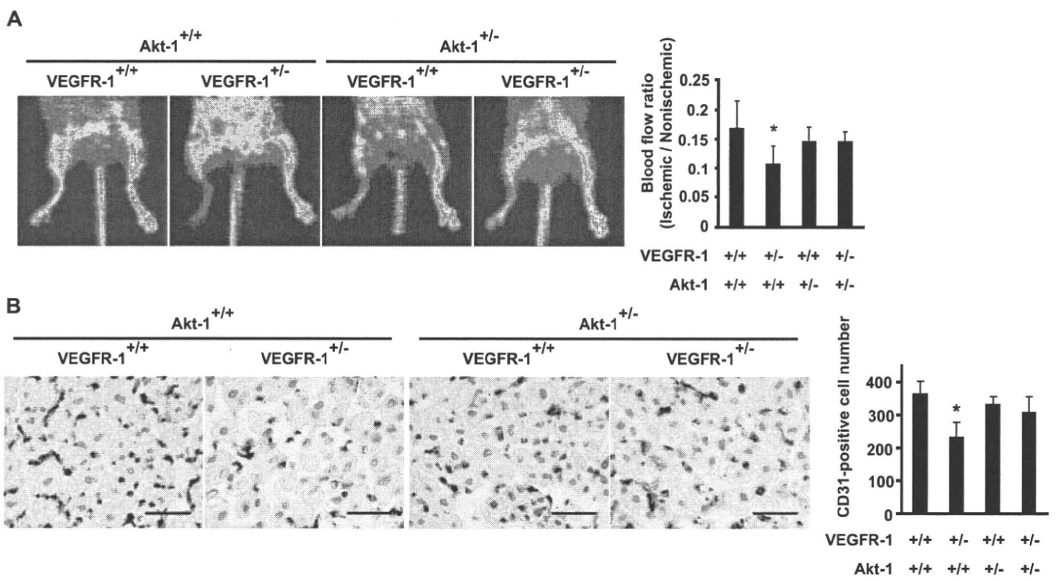


**Figure 6.** Inhibition of Akt signaling ameliorates the impairment of neovascularization in VEGFR-1$^{+/-}$ mice. A, Limb perfusion was measured by a laser Doppler analyzer at 1 week after creation of ischemia. *$P$<0.01 vs wild-type littermates (n=14 to 18). B, Immunohistochemistry for CD31 (brown)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