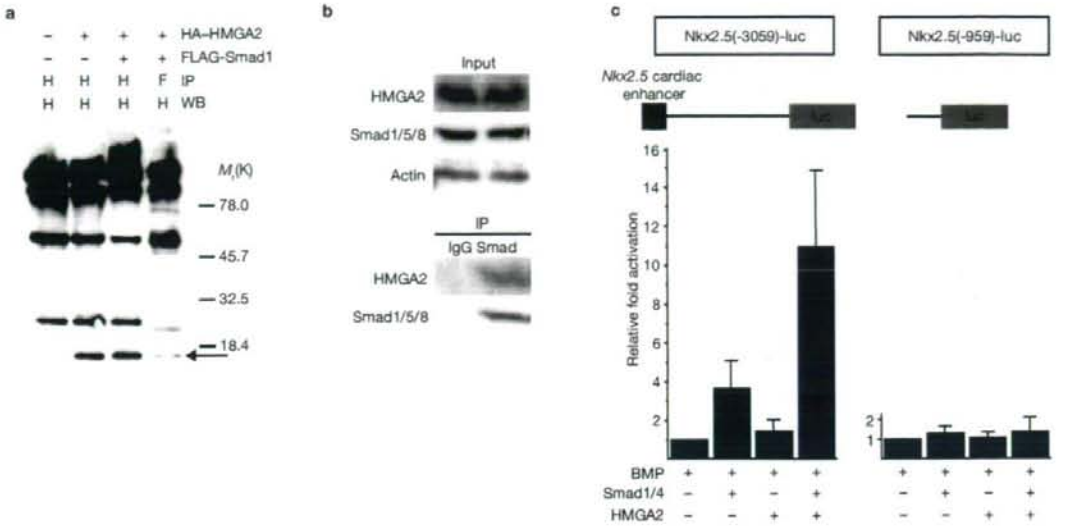

Figure 4 HMGA2 upregulates *Nkx2.5* promoter activity in collaboration with Smads. (**a**) HA–HMGA2 interacted with FLAG–Smad1 in COS7 cells, as revealed by IP-western blot (WB) analysis. HA–HMGA2 was immunoprecipitated with an anti-FLAG antibody (arrow). H, anti-HA antibody; F, anti-FLAG antibody. (**b**) 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

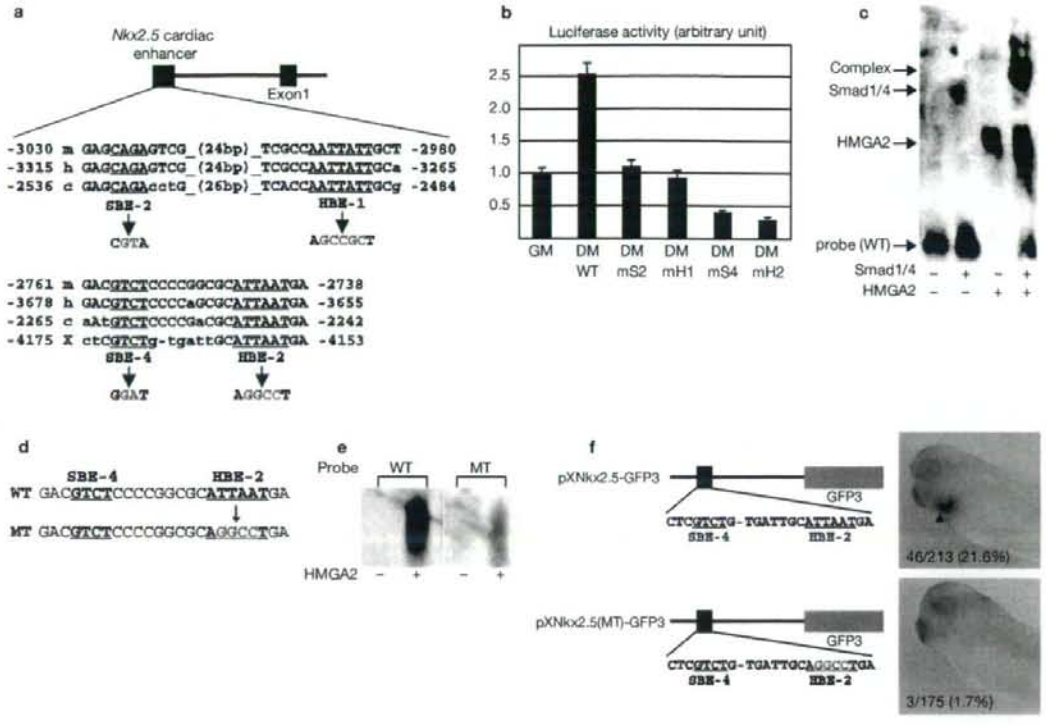

Figure 5 HMGA2 binding is required for full activation of *Nkx2.5* promoter. (**a**) Sequence comparison of SBE-2/HBE-1 and SBE-4/HBE-2 elements in the –3059/–2554 *Nkx2.5* cardiac enhancer. These elements are evolutionarily conserved in human, mouse and chick, and SBE-4/HBE-2 sites are also conserved 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)[18]. 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 previously[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[28] 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 GAA 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$^{+/-}$ Akt1$^{+/-}$ 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

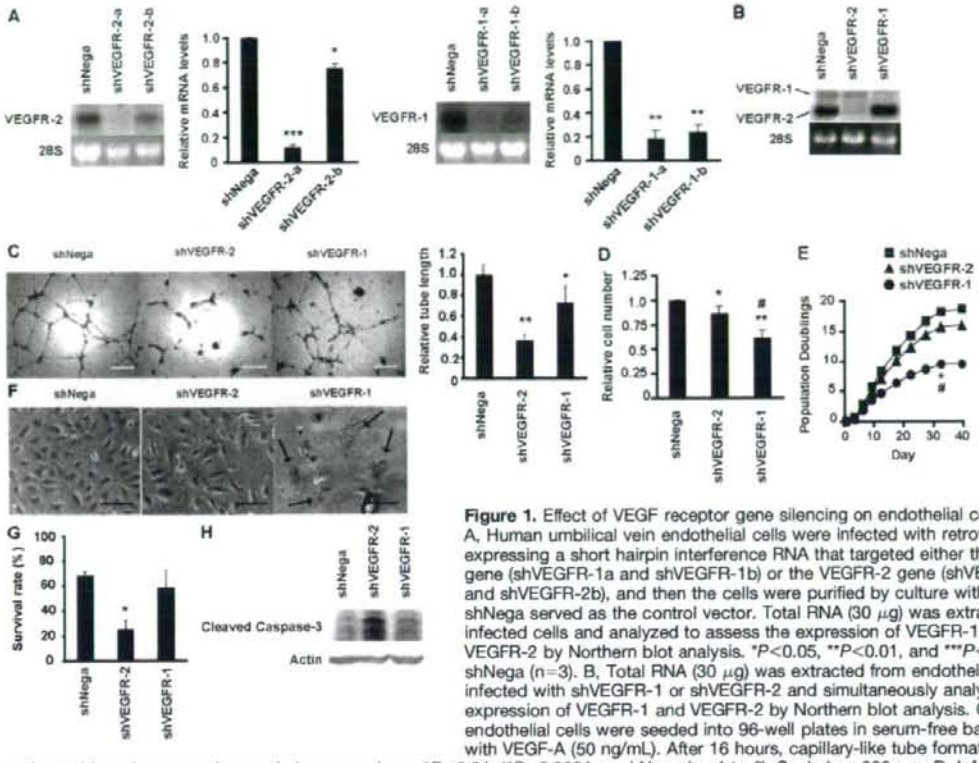

Figure 1. Effect of VEGF receptor gene silencing on endothelial cell function. A, Human umbilical vein endothelial cells were infected with retroviral vectors expressing a short hairpin interference RNA that targeted either the VEGFR-1 gene (shVEGFR-1a and shVEGFR-1b) or the VEGFR-2 gene (shVEGFR-2a 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 reduced 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).

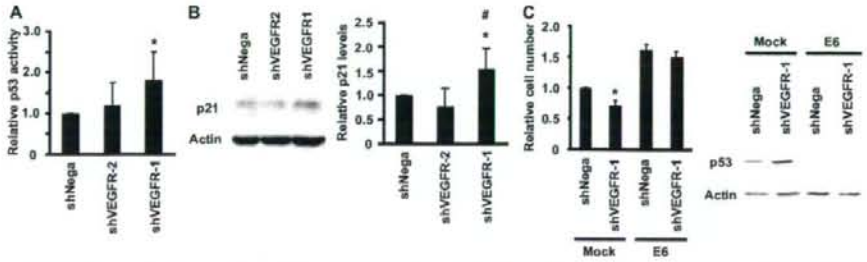

Figure 2. VEGFR-1 deletion induces activation of the p53/p21 signal pathway. A, A luciferase reporter gene plasmid (pPG13-Luc) containing the p53-binding sequence was transfected into endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Luciferase activity was measured at 48 hours after transfection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

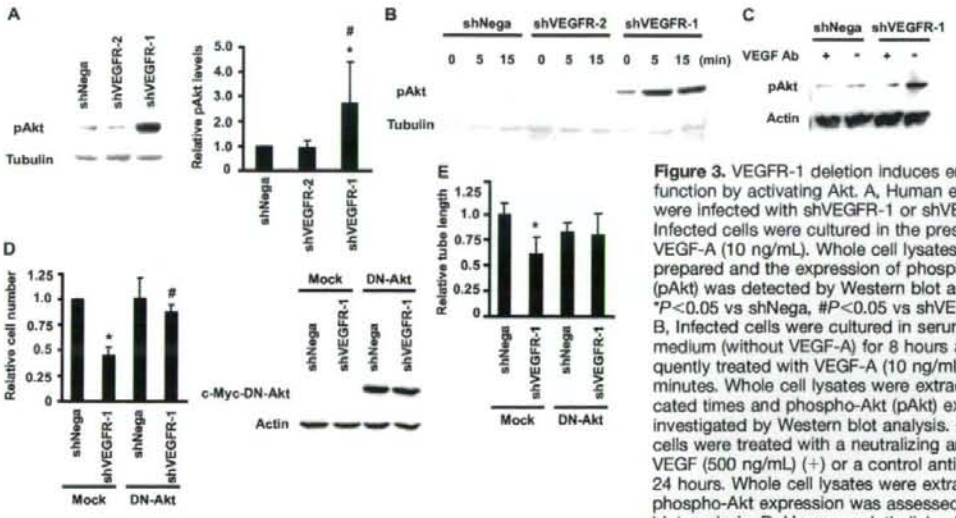

Figure 3. VEGFR-1 deletion induces endothelial dysfunction by activating Akt. A, Human endothelial cells were infected with shVEGFR-1 or shVEGFR-2. Infected cells were cultured in the presence of VEGF-A (10 ng/mL). Whole cell lysates (30 μg) were prepared and the expression of phosphorylated Akt (pAkt)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

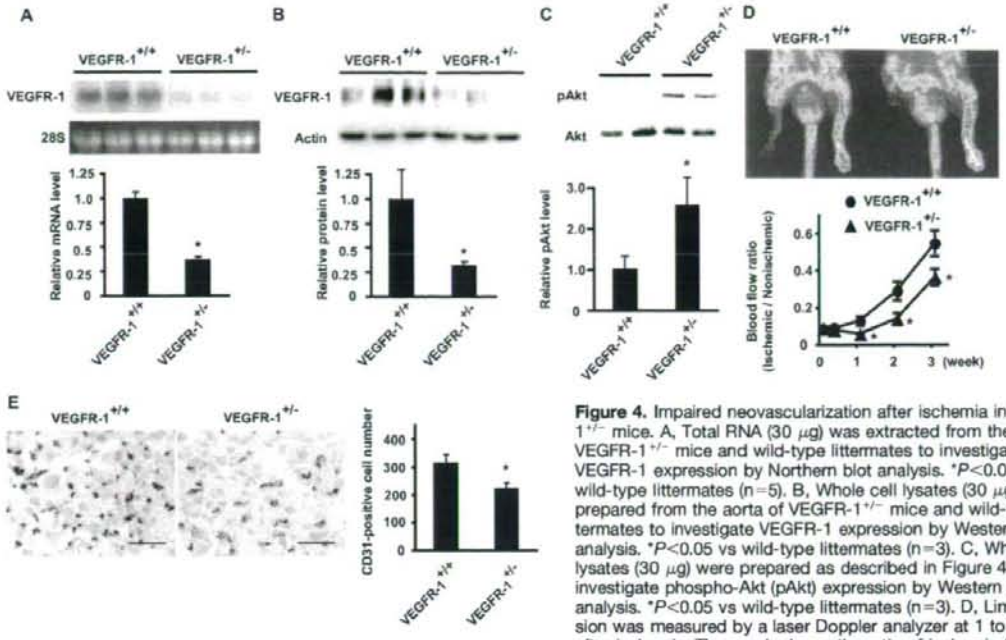

Figure 4. Impaired neovascularization after ischemia in VEGFR-$1^{+/-}$ mice. A, Total RNA (30 μg) was extracted from the lung of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Northern blot analysis. *$P<0.001$ vs wild-type littermates (n=5). B, Whole cell lysates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

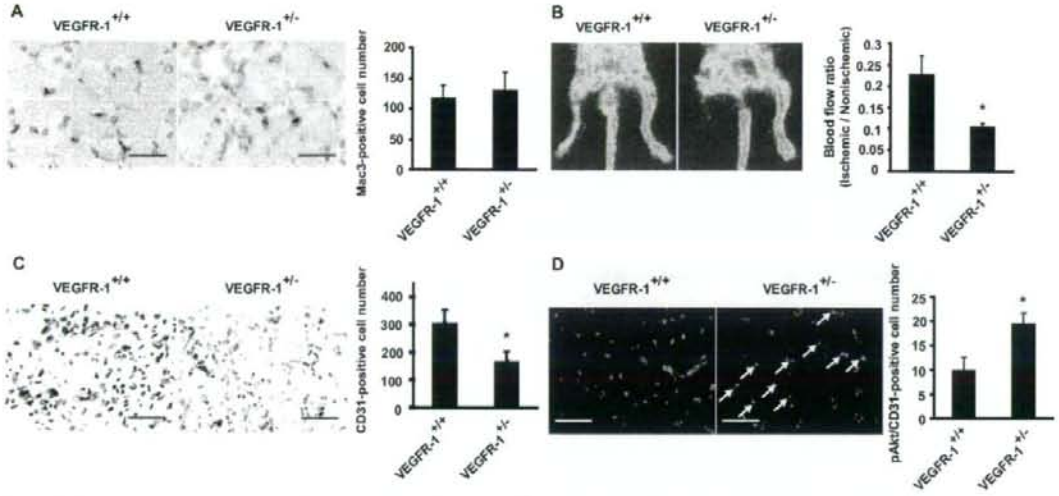

Figure 5. Role of bone marrow–derived cells in impaired neovascularization in VEGFR-1 $^{+/-}$ mice. A, Immunohistochemistry for Mac3 (brown) in ischemic limbs. Scale bar: 50 μm. The number of Mac3-positive cells per square millimeter is shown (n=4). B, Wild-type bone marrow cells were transplanted into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

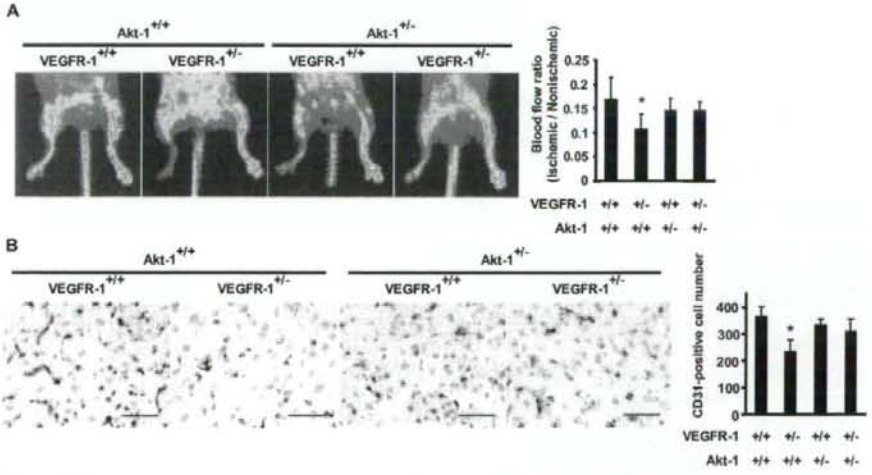

Figure 6. Inhibition of Akt signaling ameliorates the impairment of neovascularization in VEGFR-1 $^{+/-}$ mice. A, Limb perfusion was measured by a laser Doppler analyzer at 1 week after creation of ischemia. *P<0.01 vs wild-type littermates (n=14 to 18). B, Immunohistochemistry for CD31 (brown) in 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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Peroxisome Proliferator-Activated Receptor γ and Cardiovascular Diseases

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Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and form heterodimers with retinoid X receptor. Three PPAR isoforms have been isolated and termed α, β (or δ) and γ. Although PPARγ is expressed predominantly in adipose tissue and associated with adipocyte differentiation and glucose homeostasis, PPARγ is also present in a variety of cell types. Synthetic antidiabetic thiazolidinediones (TZDs) are well known as ligands and activators for PPARγ. After it was reported that activation of PPARγ suppressed production of pro-inflammatory cytokines in activated macrophages, medical interest in PPARγ has grown and there has been a huge research effort. PPARγ is currently known to be implicated in various human chronic diseases such as diabetes mellitus, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and Alzheimer's disease. Many studies suggest that TZDs not only ameliorate insulin sensitivity, but also have pleiotropic effects on many tissues and cell types. Although activation of PPARγ seems to have beneficial effects on cardiovascular diseases, the mechanisms by which PPARγ ligands prevent their development are not fully understood. Recent data about the actions and its mechanisms of PPARγ-dependent pathway in cardiovascular diseases are discussed here. (*Circ J* 2009; **73:** 214–220)

Key Words: Atherosclerosis; Cardiac hypertrophy; Heart failure; PPARγ; Thiazolidinedione

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily that heterodimerize with the retinoid X receptor (RXR) and bind to specific response elements termed PPAR responsive elements (PPREs) in target gene promoters. The PPREs are direct repeats of the hexameric consensus sequence AGGTCA, separated by 1 nucleotide. These nuclear receptors are ligand-dependent transcription factors, and activation of target gene transcription depends on the binding of the ligand to the receptor. PPARs have 3 isoforms, α, β (or δ) and γ. PPAR α regulates genes involved in fatty acid oxidation, whereas PPARγ promotes adipocyte differentiation and glucose homeostasis. The main function of PPARβ/δ has yet to be ascertained, but involvement in the regulation of fatty acid oxidation seems likely. PPAR α is present mainly in the liver, kidney, and muscle, whereas PPARγ is expressed predominantly in adipose tissue. PPARβ/δ is almost ubiquitously expressed. It was recently demonstrated that PPARγ is also expressed in a variety of cell types. After it was reported that activation of PPARγ suppresses production of inflammatory cytokines in activated macrophages, medical interest in PPARγ has grown, along with a huge research effort.

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PPARγ

Peroxisome is a subcellular organelle that plays a crucial role in cellular metabolism. Peroxisome enzymes are implicated in a broad range of catabolic and anabolic enzymatic pathways, such as fatty acid oxidation, biosynthesis of both glycerolipids and cholesterol, and metabolism of reactive oxygen species. Peroxisome proliferation induced in rodents is associated with cellular responses to a range of chemical compounds. In 1990, Issemann and Green reported that peroxisome proliferators activate a member of the steroid hormone receptor superfamily in mouse liver! This nuclear receptor was named PPAR. Soon after, 3 major types of PPAR (α, β/δ, and γ) were recognized. PPARγ is associated with adipocyte differentiation and glucose homeostasis. PPARγ is expressed in a variety of cell types, including adipocytes, macrophages, vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and cardiomyocytes[2–7] Several lines of evidence have demonstrated the functional significance of PPARγ in atherosclerotic lesions[8,9]

Activity of PPARγ is depressed by phosphorylation of a serine residue (Ser^{112}) in the N-terminal domain, mediated by a member of the mitogen-activated protein (MAP) kinase family, extracellular signal-regulated protein kinase (ERK). In addition, another member of MAP kinase family, c-Jun N-terminal kinase (JNK) also phosphorylates PPARγ at Ser^{82} and reduces the transcriptional activity of PPARγ. The association of PPARγ polymorphism with metabolic syndrome has also been examined![10,11] In the presence of ligand, PPARγ binds to coactivator complexes, resulting in the activation of target genes. In the absence of ligand, PPARγ binds to the promoters of several target genes and associates with a corepressor complex, leading to active repression of target genes. This process is referred to as active repression (**Fig 1**). The corepressor complex constitutes corepressor proteins, such as nuclear receptor corepressor

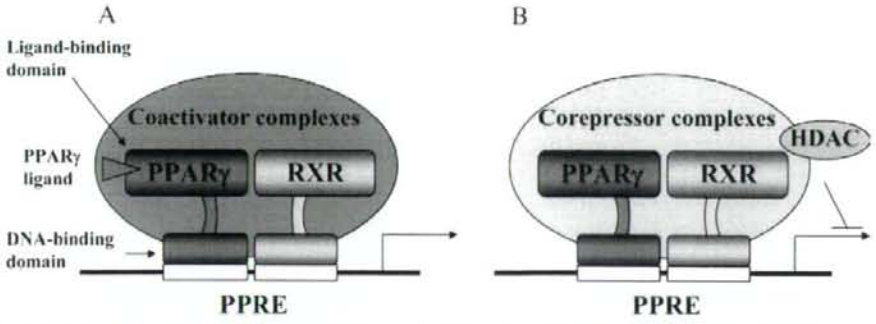

Fig 1. Transactivation and active repression. PPARγ functions as a heterodimer with RXR. (**A**) In the presence of ligand, PPARγ binds to coactivator complexes, resulting in the activation of target genes. (**B**) In the absence of ligand, PPARγ binds to the promoters of several target genes and associates with corepressor complexes, leading to active repression of target genes. HDAC, histone deacetylase; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR responsive element; RXR, retinoid X receptor.

(NCoR) and silencing mediator of retinoid and thyroid hormone receptors, histone deacetylases (HDACs) and transducin β-like protein 1 (TBL1). HDACs are essential in maintaining repressed chromatin structure and TBL1 exchanges a corepressor complex for a coactivator complex in the presence of ligand.[12]

Many nuclear receptors are proposed to sequester inflammatory transcription factors, such as nuclear factor-κB (NF-κB) and AP-1, by inhibiting their DNA-binding activities, resulting in inhibition of inflammatory target genes. In the presence of ligand, PPARγ also interacts with inflammatory transcription factors and inhibits their DNA-binding activities. PPARγ blocks clearance of the corepressor complex in a ligand-dependent manner, and PPARγ stabilizes the corepressor complex bound to the promoter of inflammatory genes.[13] It was demonstrated that PPARγ associates with the protein inhibitor of activated STAT1 (PIAS1), which is a small ubiquitin-like modifier (SUMO)-E3 ligase, in a ligand-dependent manner. PIAS1-induced SUMOylation of the ligand-binding domain of PPARγ enables the receptor to maintain NCoR on the promoter of inflammatory genes.[14] These are the suggested mechanisms of PPARγ transrepression.

PPARγ Ligands

Natural and synthetic ligands bind to PPARγ, resulting in conformational change and activation of PPARγ. The PGD_2 metabolite, 15d-PGJ_2, was the first endogenous ligand for PPARγ to be discovered. Although 15d-PGJ_2 is the most potent natural ligand of PPARγ, the extent to which its effects are mediated through PPARγ in vivo remains to be determined. Two components of oxidized low density lipoprotein (ox-LDL), the 9-hydroxy and 13-hydroxy octadecadienoic acids (HODE), are also potent endogenous activators of PPARγ.[15,16] Activation of 12/15-lipoxygenase induced by interleukin (IL)-4 also produced endogenous ligands for PPARγ;[17] however, whether these natural ligands act as physiological PPARγ ligands in vivo remains unknown. The antidiabetic thiazolidinediones (TZDs), such as troglitazone, pioglitazone, ciglitazone and rosiglitazone, which are used to control glucose concentration in patients with diabetes mellitus (DM), are pharmacological ligands of PPARγ. They bind PPARγ with various affinities and it is conceivable that their insulin-sensitizing and hypoglycemic effects are exerted by activating PPARγ. However, the molecular mechanisms by which TZDs affect insulin resistance and glucose homeostasis are not fully understood. They seem to mediate their effects primarily through adipose tissue, because TZDs alter the expression level of genes that are involved in lipid uptake, lipid metabolism and insulin action in adipocytes. TZDs enhance adipocyte insulin signaling and reduce the release of free fatty acids. TZDs also decrease the inflammation of adipose tissue that is induced by obesity and contributes to increased insulin resistance. There is a possibility that TZDs improve insulin sensitivity in skeletal muscle and liver, the main insulin-sensitive organs, through these multiple adipocentric actions. PPARγ has been demonstrated to have an antiinflammatory effect, leading to initiation of treatment trials for patients with inflammatory diseases. RXR, which interacts with the PPARs, is activated by 9-cis retinoic acid. When combined as a PPAR:RXR heterodimer, the PPAR ligands and 9-cis retinoic acid act synergistically on PPAR responses.

PPARγ and Atherosclerosis

Atherosclerosis is a complex process to which many different factors contribute. Injury of the endothelium, proliferation of VSMCs, migration of monocytes/macrophages, and the regulatory network of growth factors and cytokines are important in the development of atherosclerosis. In addition, chronic inflammation of the vascular wall is also involved. As mentioned earlier, PPARγ has antiinflammatory effect. PPARγ ligands have been shown to reduce production of inflammatory cytokines, such as IL-1β, IL-6, inducible nitric oxide synthase and tumor necrosis factor-α (TNF-α), by inhibiting the activity of transcription factors such as activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), and NF-κB in monocytes/macrophages.[2,3] Those findings suggest that PPARγ activation may have beneficial effects in modulating inflammatory responses in atherosclerosis. Interestingly, expression of PPARγ has been demonstrated in atherosclerotic plaques.[8] Macrophages affect the vulnerability of plaque to rupture and they are implicated in the secretion of matrix metalloproteinases (MMPs), enzymes that are important in the degradation of extracellular matrix. In macrophages and VSMCs, PPARγ

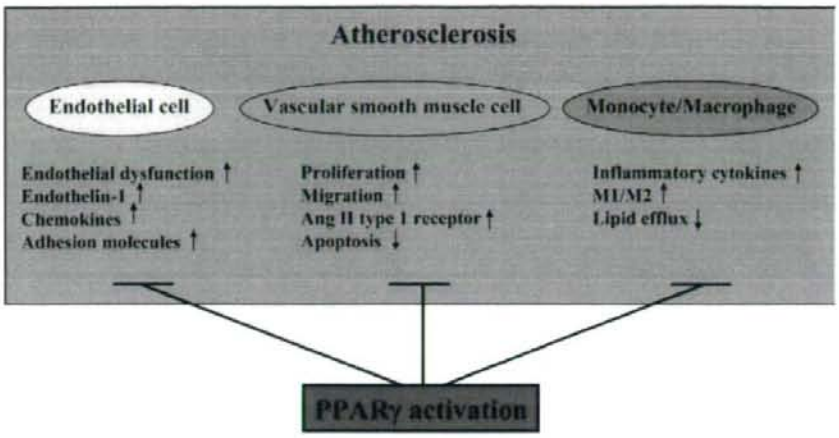

Fig 2. In atherosclerosis, PPARγ inhibits progression of the atherosclerotic lesion. PPAR, peroxisome proliferator-activated receptor.

ligands have been shown to reduce the expression of MMP-9, resulting in the inhibition of migration of VSMCs, and plaque destabilization.[3,4] Although activation of T lymphocytes represents a critical step in atherosclerosis, PPARγ ligands also reduce the activation T lymphocytes.[18] Recently, it was reported that PPARγ is a key regulator of M1/M2 polarization.[19] Classically activated macrophages (M1) express a high level of pro-inflammatory cytokines and reactive oxygen species, whereas alternatively activated macrophages (M2) play an antiinflammatory role in atherosclerosis. PPARγ agonists prime monocytes into M2 and PPARγ expression is enhanced by M2 differentiation.[20]

VSMC proliferation and migration are also critical events in atherosclerosis and vascular-intervention-induced restenosis. TZDs inhibit both these changes in the VSMCs and neointimal thickening after vascular injury.[21–24] Furthermore, TZDs induce apoptosis of VSMCs via p53 and Gadd45.[25,26] Angiotensin II (AngII) plays an important role in vascular remodeling via the AngII type 1 receptor (AT1R) and accelerates atherosclerosis. Although AngII induces transcriptional suppression of PPARγ, activation of PPARγ inhibits AT1R gene expression at a transcriptional level in VSMCs.[27–29] Expression of adhesion molecule by ECs, leading to adhesion of leukocytes, is a critical early step in atherosclerosis. PPARγ ligands inhibit the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 and decreased production of chemokines, such as IL-8 and monocyte chemotactic protein-1 (MCP-1) via suppressions of AP-1 and NF-κB activities in ECs.[30–32] PPARγ ligands also inhibit MCP-1-induced monocytes migration.[33] Endothelin-1 (ET-1) is involved in the regulation of vascular tone and endothelial functions, and induces proliferation of VSMCs. In bovine aortic ECs, PPARγ ligands suppressed transcription of the ET-1 promoter by interfering with AP-1.[34]

PPARγ activation by major oxidized lipid components of ox-LDL, 9-HODE and 13-HODE has an important role in the development of lipid-accumulating macrophages through transcriptional induction of CD36, a scavenger receptor.[35] These findings suggest that atherogenic ox-LDL particles could induce their own uptake through activation of PPARγ and expression of CD36, leading to atherosclerosis. However, several studies have demonstrated that activation of PPARγ does not promote lipid accumulation in either mouse or human macrophages.[36–38] Liver X receptor α (LXRα) is an oxysterol receptor that promotes cholesterol excretion and efflux by modulating expression of ATP-binding cassette transporter 1 (ABCA1).[37,38] LXRα was recently identified as a direct target of PPARγ in mouse and human macrophages.[39,40] Although the PPARγ-induced increase in CD36 expression might accelerate lipid uptake in macrophages, subsequent activation of LXRα and upregulation of ABCA1 appear to induce lipid efflux.

Diep et al have demonstrated that rosiglitazone and pioglitazone attenuate the development of hypertension and structural abnormalities, and improve endothelial dysfunction in AngII-infused rats.[41] These TZDs also prevented upregulation of AT1R, cell cycle proteins, and inflammatory mediators. Rosiglitazone, but not the PPARα ligand fenofibrate, prevented hypertension and endothelial dysfunction in DOCA-salt hypertensive rats.[42] It has been reported that serum levels of the soluble CD40 ligand are elevated in acute coronary syndrome and associated with increased cardiovascular risk. Treatment with rosiglitazone decreased the serum levels of soluble CD40 and MMP-9 in type 2 diabetic patients with coronary artery disease.[43] Taking all the evidence together, PPARγ ligands may prevent the progression of atherosclerotic lesions, particularly in patients with DM (**Fig 2**).

PPARγ and Ischemic Heart Disease

As the effects of PPARγ on the heart are not fully understood, we and others have examined whether PPARγ is involved in various heart diseases. Although the expression of PPARγ in cardiac myocytes is low compared with adipocytes, PPARγ ligands seem to act on cardiac myocytes.[7,44] We demonstrated that PPARγ ligands inhibited the cardiac expression of TNF-α at the transcriptional level, in part by antagonizing NF-κB activity.[7] Because TNF-α expression is elevated in the failing heart and has a negative inotropic effect on cardiac myocytes, treatment with PPARγ ligands may prevent the development of congestive heart failure. Diabetic cardiomyopathy, which is characterized by

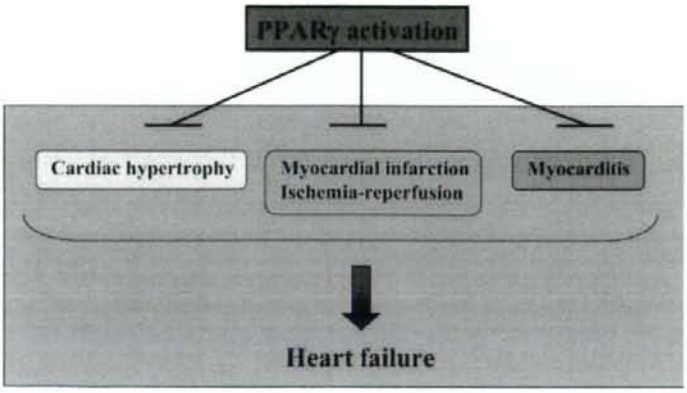

Fig 3. Actions of PPARγ in heart diseases. PPARγ inhibits the progression of heart failure following cardiac hypertrophy, myocardial infarction, ischemia–reperfusion injury, and myocarditis. PPAR, peroxisome proliferator-activated receptor.

systolic and diastolic dysfunction, is a major complication of DM, and therefore TZDs seem to be beneficial for the impaired cardiac function in patients with DM. Following our study, the role of PPARγ in myocardial ischemia–reperfusion (IR) injury has been elucidated[45–48] In animal models, PPARγ ligands reduced the size of the myocardial infarct and improved contractile dysfunction after IR through inhibition of the inflammatory response. IR injury activates JNK, and subsequently JNK induces increases in both AP-1 DNA-binding activity and apoptotic cells. It has been shown in rats that rosiglitazone inhibits the activation of JNK and AP-1 after myocardial IR.[46] Furthermore, pioglitazone has been reported to attenuate left ventricular remodeling and heart failure after myocardial infarction (MI) in mice.[49] Both of these effects of TZDs ligands were associated with decreases in inflammatory cytokines and chemokines.[49,50]

PPARγ and Cardiac Hypertrophy

The PPARγ ligands, troglitazone, pioglitazone and rosiglitazone, inhibited AngII-induced hypertrophy of neonatal rat cardiac myocytes.[51–53] Because generalized PPARγ gene deletion causes embryonic lethality, we examined the role of PPARγ in the development of cardiac hypertrophy in vivo using heterozygous PPARγ-deficient ($PPAR\gamma^{+/-}$) mice.[53] Pressure overload-induced cardiac hypertrophy was more prominent in heterozygous $PPAR\gamma^{+/-}$ mice than in wild-type (WT) mice. Treatment with pioglitazone strongly inhibited the pressure overload-induced cardiac hypertrophy in WT mice and moderately in $PPAR\gamma^{+/-}$ mice.[53] Thereafter, 2 other groups examined the role of PPARγ in the heart by using cardiomyocyte-specific PPARγ knockout mice.[54,55] Duan et al reported that these mice develop cardiac hypertrophy through elevated NF-κB activity;[54] and unexpectedly, rosiglitazone induced cardiac hypertrophy in both the WT mice and cardiomyocyte-specific PPARγ knockout mice through activation of p38 MAP kinase independent of PPARγ. Ding et al reported that cardiomyocyte-specific PPARγ knockout mice displayed cardiac hypertrophy from approximately 3 months of age and then progress to dilated cardiomyopathy;[55] most mice died from heart failure within 1 year after birth. Mitochondrial oxidative damage and reduced expression of manganese superoxide dismutase were recognized in the cardiomyocyte-specific PPARγ knockout mice.[55] These mice models demonstrate that PPARγ is essential for protecting cardiomyocytes from stress and oxidative damage, although the expression level of PPARγ in cardiomyocytes is low. On the other hand, Son et al demonstrated that cardiomyocyte-specific PPARγ transgenic mice develop dilated cardiomyopathy associated with increased uptake of both fatty acid and glucose.[56] Rosiglitazone increased this glucolipotoxicity in cardiomyocyte-specific PPARγ transgenic mice. If PPARγ in the heart is expressed at a high level, rosiglitazone may cause cardiotoxic effects; however, as noted earlier the expression level of PPARγ in the heart is quite low.

PPARγ and Myocarditis

Experimental autoimmune myocarditis (EAM) is a T-cell-mediated disease characterized by infiltration of T cells and macrophages, leading to massive myocarditis necrosis, which develops into heart failure in the chronic phase.[57] The onset of EAM in rats occurs approximately 2 weeks after the first immunization with porcine cardiac myosin. At this time, small numbers of $CD4^+$ T cells and macrophages start to infiltrate into the myocardium and various cytokines are expressed. Macrophage inflammatory protein-1α (MIP-1α) is a C-C chemokine that induces leukocyte accumulation in tissue sites of inflammation. We previously demonstrated that MIP-1α mRNA and protein are highly expressed in the hearts of rats with EAM from day 11 after first immunization.[57] Th1 cells produce interferon-γ (IFN-γ), which is mainly involved in cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which participate in humoral responses. Immune dysfunction associated with autoimmune disease is known to involve an imbalance between Th1 and Th2 cells.

It has been reported that pioglitazone treatment markedly reduces the severity of myocarditis in a rat model of EAM.[58,59] Pioglitazone suppressed expression of inflammatory cytokines and activation of myocardiogenic T cells in the myocardium of EAM rats.[58] The mRNA levels of MIP-1γ were upregulated in the hearts of EAM rats, but not in the hearts of those in the pioglitazone group. Furthermore, treatment with pioglitazone decreased the expression levels of pro-inflamatory cytokine (TNF-α and IL-1β) genes and Th1 cytokine (IFN-γ) genes, and increased the expression levels of Th2 cytokine (IL-4) gene.[59] These results suggest that PPARγ ligands may have beneficial effects on myocarditis by inhibiting MIP-1α expression and modulating the Th1/Th2 balance (**Fig 3**).

Efficacy and Safety of TZD Treatment in the Clinical Setting

Despite the beneficial effects of TZDs in the basic experiments, their propensity to cause fluid retention is a serious side-effect. Clinical studies report TZD-induced peripheral fluid retention, and an increase in plasma volume in 2–5% of patients on monotherapy[60] Fluid retention was more likely to occur with concomitant insulin use, and in patients with underlying cardiac dysfunction or renal insufficiency. The exact mechanisms for TZD-induced fluid retention are not well understood, and it remains unclear whether TZDs directly cause the development of de novo congestive heart failure. It is known that the level of vascular endothelial growth factor is increased in the patients who develop fluid retention with TZD therapy[61] and this may lead to peripheral edema through increased vascular permeability. The insulin-sensitizing action of TZDs also induces water and salt retention. PPARγ is highly expressed in the kidney and collecting-duct-specific PPARγ knockout mice demonstrated no effects of TZD on fluid retention or the expression level of sodium channel ENaC-γ[62,63] These findings suggest that activation of the sodium channel in the collecting duct cells expressing PPARγ may be a mechanism of fluid retention. In patients without evidence of heart failure, careful examination did not reveal any worsening of left ventricular function by TZDs[64] There are very few studies investigating the safety of TZDs in patients with preexisting heart failure. Although a recent study demonstrated that there is not a direct association between the risk of fluid retention and the baseline degree of severity of heart failure in diabetic patients treated with TZDs, the prescription of TZDs for patients with established heart failure should be avoided at present[60,65]

The PROactive (Prospective Pioglitazone Clinical Trial in Macrovascular Events) study has shown that pioglitazone significantly decreases the occurrence of all-cause mortality, nonfatal MI, and nonfatal stroke in patients with type 2 DM and macrovascular diseases[66] Pioglitazone significantly reduced the occurrence of fatal and nonfatal MI by 28% in the PROactive study[66] Although there was a 1.6% absolute increase in heart failure hospitalizations in the pioglitazone group compared with the placebo group, the number of heart-failure-related deaths was almost identical. In contrast to the PROactive study, it has been recently reported that rosiglitazone treatment is associated with increased incidence of MI by meta-analysis[67,68] Although meta-analysis has a number of limitations and the increased risk in MI is still controversial, those results attracted the attention of many clinicians. There are some differences in the actions of pioglitazone and rosiglitazone. Pioglitazone has more beneficial effects on the lipid profile than rosiglitazone[69] As mentioned earlier, rosiglitazone, but not pioglitazone, induced cardiac hypertrophy by a non-PPARγ-mediated pathway[54] Pioglitazone represses NF-κB activation and VCAM-1 expression in a PPARα-dependent manner[70] Pioglitazone was recently reported to increase the number and function of endothelial progenitor cells (EPCs) in patients with stable coronary artery disease and normal glucose tolerance[71] Pioglitazone may induce angiogenesis by modulating EPC mobilization and function. In the future, more mechanistic studies are required to investigate the differences in action between pioglitazone and rosiglitazone.

Conclusions

The American Heart Association (AHA) and American Diabetes Association (ADA) have released a consensus statement that advises caution regarding the use of TZDs in patients with known or suspected heart failure[72] Because there is a possibility that TZDs may unmask asymptomatic cardiac dysfunction by increasing plasma volume, they should be avoided in patients with congestive heart failure of New York Heart Association (NYHA) class III or IV. The data from in vitro studies suggest that TZDs exert direct actions on vascular cells and cardiomyocytes, independent of their glucose-mediated mechanisms. Further studies using tissue-specific gene targeting mice are necessary to address in vivo the pleiotropic effects of PPARγ on the cardiovascular system. If the beneficial roles of PPARγ can be solved, modulation of PPARγ may become a promising therapeutic strategy for cardiovascular diseases. Because cardiac hypertrophy can be seen even in normotensive diabetic patients, and diabetic cardiomyopathy is a major complication of DM, antidiabetic agents such as the TZDs would be expected to have beneficial effects on cardiac hypertrophy and dysfunction in patients with DM. It has been already clarified that 3-hydroxy-3-methyglutaryl-coenzyme A reductase inhibitors, statins, have pleiotropic effects in cardiovascular diseases. The effects of PPARγ ligands are similar to those of statins in many respects. A recent study demonstrated that statins activate PPARγ through ERK and p38 MAP-kinase-dependent cyclooxygenase-2 expression in macrophages[73] Further studies are needed to elucidate the molecular mechanisms of the pleiotropic effects of PPARγ ligands in cardiovascular disease.

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