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Rationale: Neointimal hyperplasia contributes to atherosclerosis and restenosis after percutaneous coronary intervention. Vascular injury in each of these conditions results in the release of mitogenic growth factors and hormones that contribute to pathological vascular smooth muscle cell growth and inflammation. Hepatocyte growth factor (HGF) is known as an antiinflammatory growth factor, although it is downregulated in injured tissue. However, the precise mechanism how HGF reduces inflammation is unclear.

Objective: To elucidate the mechanism how HGF and its receptor c-Met reduces angiotensin II (Ang II)–induced inflammation.

Methods and Results: HGF reduced Ang II–induced vascular smooth muscle cell growth and inflammation by controlling translocation of SHIP2 (Src homology domain 2–containing inositol 5’-phosphatase 2), which led to Ang II–dependent degradation of epithelial growth factor receptor. Moreover, the present study also revealed a preventive effect of HGF on atherosclerotic change in an Ang II infusion and cuff HGF transgenic mouse model.

Conclusions: These data suggest that the HGF/c-Met system might regulate extrinsic factor signaling that maintains the homeostasis of organs. (Circ Res. 2009;105:667-675.)

Key Words: hepatocyte growth factor ■ c-Met ■ SHIP2 ■ epithelial growth factor receptor ■ neointimal formation

Neointimal hyperplasia contributes to atherosclerosis, restenosis after percutaneous coronary intervention, and venous bypass graft disease.1 Vascular injury in each of these conditions results in the release of mitogenic growth factors and hormones, which contribute to pathological vascular growth. Many of these molecules contribute to neointimal hyperplasia by activating phosphoinositol (PI)3-kinase in vascular smooth muscle cells (VSMCs). Recently, sirolimus (rapamycin), which inhibits a downstream effector of PI3-kinase, a mammalian target of rapamycin (mTOR), has recently shown promising results in the prevention of in-stent restenosis.2 Additionally, a previous report showed that both local and systemic treatment with wortmannin (a selective phosphoinositide 3-kinase inhibitor) before carotid arterial injury blocked the rise in arterial Akt activation and cyclin D1 expression, and these effects correlated with the inhibition of medial VSMC proliferation.3

In contrast, angiotensin II (Ang II) type I receptor blockers (ARBs), potent antihypertensive drugs, are well known to prevent atherosclerotic change including restenosis after percutaneous coronary intervention,4 where the local tissue renin-angiotensin system is activated. Moreover, Ang II type I receptor knockout mice showed significant inhibition of neointimal formation in a cuff replacement model.5 These clinical and experimental studies suggest that arterial phosphoinositide 3-kinase inhibition, especially that induced by Ang II, might be an effective therapeutic strategy to target pathological VSMC growth and inflammation after vascular injury. Recently, we showed that Ang II–induced endothelial progenitor cell senescence was prevented through a reduction of reactive oxygen species (ROS) in transgenic (Tg) mice with cardiac-specific overexpression of HGF with high levels of serum HGF. In addition, HGF attenuated Ang II–induced phosphatidylinositol trisphosphate (PIP3)/rac1 activity in vitro.6 However, the link between HGF and Ang II signaling is an enigma. Here we showed that HGF and its receptor, c-Met, regulate Ang II signaling by Ang II–dependent
epithelial growth factor receptor (EGFR) downregulation via the ubiquitin proteasome system.

Methods

Western Blotting
Western blotting was performed as previously described. Information about the antibodies, GTP-rac1 pull-down assay kit, and reagents used in the study is available in the Online Data Supplement.

In Vitro Experiments of Plasmid and Small Interfering RNA Transfection
Human aortic VSMCs (passage 4 to 9), purchased from Lonza Walkersville Inc (Portsmouth, NH), were cultured in smooth muscle basal medium with supplements and 5% FBS. All stimuli were performed after 24-hour serum starvation. Please see the online supplemental information about the plasmid and small interfering (si)RNA using in the study.

MTS Assay
VSMCs were harvested and reseeded at a concentration of 0.5×10^4 cells per well (96-well plate). After 24-hour starvation, VSMCs were stimulated with Ang II or EGF with or without HGF, losartan potassium, superoxide dismutase (SOD) and the inhibitors. Mitogenic activity was assessed by MTS assay (Promega, Madison, Wis). Cell numbers were counted with a hemocytometer. Briefly, 1×10^4 VSMCs seeded into 24-well plates were treated with Ang II for 24 hours with or without HGF pretreatment. After stimulation, the cell monolayers were resuspended in 100 μL of trypsin-EDTA, and the cell numbers were counted with a hemocytometer.

Purification of Secreted Heparin-Binding EGF
Secreted soluble heparin-binding EGF (HB-EGF) was purified by heparin affinity chromatography (GE Healthcare Bio-Sciences Corp, Tokyo, Japan), according to the instructions of the manufacturer. Information about the protocol of purification of secreted HB-EGF is available in the Online Data Supplement.

HGF Tg Mouse Models
Mice (C57BL6 background) with cardiac-specific (α-myosin heavy chain–driven) overexpression of human HGF with high levels of heart-derived serum HGF were generated. There are no significant differences in cardiac function between wild-type (WT) and HGF Tg mice in the basal condition (Online Table I, a). The characteristics of HGF Tg mice are detailed by Sanada et al. Information about the HGF Tg mice and animal models is available in the Online Data Supplement.

All procedures were performed in accordance with the guidelines of the Institutional Animal Committee of Osaka University School of Medicine.

Statistical Analysis
Values are expressed as means±SE. ANOVA and t test, followed by Bonferroni adjustment for multiple comparison, were used for comparisons of more than two groups. A probability value of less than <0.05 was considered to indicate significance of mean differences.

Results

Molecular Mechanism of Inhibitory Effects of HGF on Ang II–Induced VSMC Growth
First, the effect of HGF on Ang II– or EGF–induced VSMC growth was examined. Although HGF alone did not affect VSMC growth, consistent with a previous report, HGF significantly attenuated the growth of VSMCs induced by Ang II or EGF. This effect was also observed with a specific Ang II type 1–specific blocker, losartan potassium (100 μmol/L) (Figure 1a). Similarly, as shown in Figure 1c, HGF significantly attenuated nuclear factor κB activation induced by Ang II as assessed by promoter analysis. Consistent with previous reports that Ang II–induced VSMC growth depends on Akt and extracellular signal-regulated kinase (ERK)1/2, PD98059 (a specific MEK inhibitor), as well as LY294002 (a specific PI-3 kinase inhibitor), inhibited the growth of VSMCs induced by Ang II (Figure 1b). Because VSMC hypertrophy, as well as proliferation, also enhances the MTS level, we counted cell number. In agreement with a previous report, Ang II did not affect proliferation (Online Figure I, a). These data suggest that the reduction in the Ang II–induced MTS signal by HGF might be attributable to inhibition of Ang II–induced VSMC hypertrophy.

As shown in Figure 1a and 1c, SOD, an oxidative stress inhibitor, also inhibited Ang II–induced growth of VSMCs and nuclear factor κB activation. Moreover, our previous report demonstrated the unique antioxidative action of HGF by inhibiting rac1 activity in endothelial progenitor cells. Therefore, we hypothesized that HGF might prevent Ang II–induced inflammation and growth of VSMCs by inhibiting the PI-3 kinase–ROS axis in VSMCs. To explore the molecular mechanism further, we examined the effect of HGF on Ang II–induced O2− production in VSMCs. As shown in Figure 2a and 2b, dihydroethidium (DHE) staining clearly demonstrated that HGF pretreatment, as well as SOD (50 U/mL), significantly attenuated Ang II–induced O2− production, although in the basal condition, HGF itself acted as a prooxidant. Because the SOD treatment, as well as the dominant negative rac1 (N17 rac1), also significantly inhibited Ang II–induced O2− production, we focused on rac1 activity. Although Ang II stimulated GTP-rac1 with 2 peaks
HGF attenuated Ang II–induced O$_2^-$ production by inhibiting rac1 activation even in VSMCs.

To elucidate the possible link between Ang II and HGF signaling, we focused on the role of EGFR in Ang II signaling, because the production of ROS following rac1 activation by Ang II is reported to depend mainly on EGFR transactivation.11 Our data confirmed these findings, because transfection of siRNA against EGFR inhibited Ang II–induced O$_2^-$ production (Figure 2a and 2b). Using EGFR siRNA, Ang II–induced phosphorylation of Akt and mTOR, but not ERK1/2, was mediated via EGFR in VSMCs (Online Figure 1, b), although inhibition of Akt phosphorylation was significant but not complete. Previous reports show that Ang II induces Akt phosphorylation through the transactivation of the insulin-like growth factor I receptor or platelet-derived growth factor receptor, as well as EGFR.13 These reports and our data suggested that Akt regulation by Ang II might occur mainly through EGFR-dependent mechanisms.

Akt is known to be involved in not only antiapoptotic signaling but also proliferative signaling through regulation of glycogen synthase kinase 3β, mTOR, and p70S6K phosphorylation.13 Also, it has become clearer that constitutively active Akt, including Ang II, promotes cell senescence in the vasculature,14,15 which leads to atherosclerosis.16 Thus, inhibition of excess Akt activity is important to prevent atherosclerotic change. Interestingly, following Ang II stimulation, EGFR degradation was enhanced by HGF pretreatment, whereas Ang II alone slightly reduced the expression of EGFR (Figure 3a). Because Ang II–induced EGFR transactivation depends on the production of HB-EGF, secreted soluble HB-EGF was examined using heparin affinity chromatography. As shown in Figure 3b, HGF did not reduce Ang II-induced GTP-rac1 at 3 hours (Figure 1c) and also inhibited translocation of rac1 from the cytosol to the plasma membrane (Figure 1d). These results indicate that HGF attenuated Ang II–induced O$_2^-$ production by inhibiting rac1 activation even in VSMCs.

Figure 1. HGF prevents Ang II–induced VSMC growth and inflammation. a, VSMC growth assessed by MTS assay. VSMCs were stimulated with Ang II (10$^{-7}$ mol/L) or EGF (20 ng/mL) for 24 hours with or without HGF (50 ng/mL), losartan potassium (100 μmol/L), or SOD (50 U/mL) (n=5). *P<0.01 vs control, †P<0.01 vs Ang II, ‡P<0.01 vs EGF. b, VSMC growth assessed by MTS assay. VSMCs were stimulated with Ang II (10$^{-6}$, 10$^{-7}$, or 10$^{-8}$ mol/L) for 24 hours with or without pretreatment with PD98059 (10 μmol/L) and/or LY294002 (10 μmol/L) (n=5). *P<0.01 vs control, †P<0.01 vs Ang II (10$^{-7}$, 10$^{-6}$ mol/L). c, NFκB-Luc promoter assay. VSMCs were stimulated with Ang II for 6 hours with or without HGF pretreatment for 12 hours, or LY294002 (10 μmol/L), PD98059 (10 μmol/L), or SOD (50 U/mL) for 3 hours (n=5). *P<0.01 vs control, †P<0.01 vs Ang II. Ang II indicates Ang II 10$^{-7}$ mol/L; HGF, HGF 50 ng/mL; SOD, SOD 50 U/mL.

Figure 2. HGF prevents Ang II–induced O$_2^-$ production by inhibiting rac1 activation. a, DHE staining of VSMCs after 3 hours of Ang II (10$^{-7}$ mol/L) stimulation with or without HGF (50 ng/mL) or SOD (50 U/mL). VSMCs were pretreated with HGF for 12 hours or SOD for 3 hours before Ang II stimulation (n=5). b, Measurement of fluorescence intensity of DHE staining visualized by confocal microscopy. *P<0.01 vs control, †P<0.01 vs Ang II, ‡P<0.05 vs control. c, Effect of HGF on Ang II–induced rac1 activation (n=3). *P<0.01 vs control. d, Effect of HGF on rac1 translocation (n=5). HGF indicates HGF 50 ng/mL; SOD, SOD 50 U/mL. VSMCs were pretreated with HGF for 12 hours or SOD for 3 hours before Ang II stimulation.
II–induced HB-EGF secretion as compared to Ang II alone. In addition, following EGF stimulation, EGFR degradation was also enhanced by HGF pretreatment (Figure 3c). Moreover, a specific Ang II type 1 receptor specific blocker, losartan potassium, did not alter EGFR expression following Ang II stimulation, whereas it significantly inhibited Ang II–induced phosphorylation of Akt and ERK1/2 (Online Figure I, c). These studies indicate the possibility that HGF prevented Ang II signaling mainly through EGFR downregulation. These effects might be mediated by the presence of HGF, because overexpression of c-Met without HGF did not change Ang II–induced EGFR expression and Akt phosphorylation (data not shown). Thus, the HGF/c-Met system is important in its interaction with EGFR. More importantly, downregulation of EGFR by HGF was not limited in the case of Ang II and EGF stimulation. As shown in Online Figure I (d and e), HGF reduced EGFR expression after endothelin-1 and transforming growth factor-β stimulation, which are both known to induce EGFR transactivation. Ligand-dependent downregulation of EGFR by HGF would be a common mechanism under growth factor stimulation that induces EGFR transactivation.

**HGF Receptor, c-Met, Regulated SHIP2 Translocation**

It is noteworthy how HGF downregulated EGFR following Ang II stimulation in VSMCs. Recent studies reveal that Src homology domain 2 (SH2)-containing inositol 5′-phosphatase 2 (SHIP2) binds to EGFR, which contributes to PIP3/Akt activation. SHIP2 also binds to c-Met directly and is involved in HGF-induced cell scattering. In addition, SHIP2 regulates ligand-dependent downregulation of EGFR in HeLa cells. Consistent with a previous report, transfection of SHIP2 siRNA also resulted in Ang II–dependent EGFR degradation in VSMCs, although Ang II slightly reduced EGFR expression in VSMCs transfected with control

**Figure 3.** HGF induced Ang II–dependent EGFR degradation. a, HGF induced EGFR degradation immediately after Ang II stimulation (n=5). *P<0.01 vs control, #P<0.01 vs identical Ang II time course. b, Secreted soluble HB-EGF examined by heparin affinity chromatography (n=3). *P<0.01 vs control. Ang II indicates Ang II 10⁻⁷ mol/L; HGF, HGF 50 ng/mL. c, HGF induced EGFR degradation immediately after EGF stimulation (n=3). *P<0.01 vs control. #P<0.01 vs identical EGF time course. VSMCs were pretreated with HGF (50 ng/mL) for 12 hours before EGF (20 ng/mL) stimulation.

**Figure 4.** HGF prevents Ang II–induced SHIP2 translocation to EGFR. a, Ang II significantly degraded EGFR expression in SHIP2 siRNA-transfected VSMCs (n=3). Human EGF receptor and control siRNA were transfected 48 hours before Ang II stimulation. *P<0.01 vs control, #P<0.01 vs identical Ang II time course. b and c, Ang II induced binding of SHIP2 to EGFR for a short time (b), whereas HGF bound SHIP2 to c-Met for a long time (c) (n=4). *P<0.01 vs 0 hour control. d, Pretreatment with HGF decreased binding of SHIP2 to EGFR and SHIP2 phosphorylation induced by Ang II (n=5). *P<0.01 vs identical control, #P<0.01 vs identical Ang II time course.
Expected, HGF significantly increased the binding of Cbl to EGFR after Ang II stimulation in SHIP2 siRNA-transfected VSMCs (Figure 5a). As expected, HGF significantly increased the binding of Cbl to EGFR by sequestering Cbl in Hep2 cells.21 It was confirmed because SHIP2 regulates ligand-dependent downregulation of EGFR after Ang II stimulation as compared to Ang II alone (Figure 5b). Increased ubiquitin activity induced by HGF was confirmed by the observation that a proteasome inhibitor, MG132, significantly inhibited HGF-induced EGFR degradation (Figure 5c). To further verify the changes in localization of EGFR and Cbl, immunocytochemical staining was performed (Figure 5d). In control cells, EGFR was localized mainly in the plasma membrane (Figure 5d, image A; white arrowheads). After 30 minutes of Ang II stimulation, EGFR was distributed to the cytoplasm with or without HGF pretreatment (D and G; white asterisk). Scale bar=10 μm. VSMCs were pretreated with HGF (50 ng/mL) for 12 hours before Ang II (10−7 mol/L) stimulation.

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Reduction of ROS Production and Inflammation by Ang II Infusion in HGF Tg Mice

To confirm the inhibitory effect of HGF on Ang II–induced vascular inflammation and ROS production in vivo, an Ang II infusion model (0.7 mg/kg per day) in HGF Tg mice and WT mice (littermates) was used. In WT mice, Ang II infusion increased systolic and diastolic blood pressure with a maximum blood pressure at 7 days (Online Table I, b). However, in HGF Tg mice, the Ang II–induced increase of systolic and diastolic blood pressure were reduced as compared to those of
WT. After a 3-week Ang II infusion, HGF Tg mice showed a smaller medial area than that in WT (Online Table I, c). As shown in Figure 6a, after Ang II infusion, EGFR expression in the abdominal aorta was significantly decreased in HGF Tg mice, whereas it was modestly increased in WT mice. In contrast, c-Met expression was significantly increased in HGF Tg mice, whereas it was significantly decreased in WT mice. To elucidate the role of SHIP2 in vivo, Western immunoblotting was carried out. Although SHIP2 translocated to EGFR in WT mice after 1 week of Ang II infusion, this translocation was significantly inhibited in HGF Tg mice by the binding of SHIP2 to c-Met (Figure 6b). In addition, after 1 week of Ang II infusion, circulating serum mouse HGF levels was significantly higher in HGF Tg mice than in WT mice (Figure 6c). Local HGF levels in the abdominal aorta was maintained in HGF Tg mice as compared to that in WT mice (Figure 6d). These in vivo data indicate the possibility that after Ang II infusion, the local HGF/c-Met system is negatively regulated, but the addition of HGF maintains c-Met expression, which might cause a reduction of EGFR expression.

Next, ROS production and inflammation were examined by immunohistochemical staining. As shown in Figure 7a, DHE staining clearly demonstrated a marked reduction in ROS production mainly in the media of the abdominal aorta in HGF Tg mice as compared to WT mice. Additionally, one of the NADPH oxidase components of VSMCs, p22 phox, expression, was reduced in HGF Tg mice as compared to WT mice (Figure 7b). The expression of inflammatory cytokines (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, monocyte chemotactrant protein-1) was also significantly reduced in HGF Tg mice as compared to WT mice (Figure 7c). These observations were also confirmed by Western blotting (Figure 7d).

**Reduction of Neointimal Formation Induced by Cuff Placement in HGF Tg Mice**

A previous report revealed that EGFR-targeted therapy inhibits neointimal hyperplasia in balloon-injured rat carotid artery. Moreover, Ang II type I receptor knockout mice show significant inhibition of neointimal formation in a cuff placement model. Clinically, potent antihypertensive drugs, ARBs, are well known to prevent atherosclerotic change including restenosis after percutaneous coronary intervention, where the local tissue renin-angiotensin system is activated. To confirm the inhibitory effect of HGF on neointimal hyperplasia in vivo, a femoral artery cuff placement model in HGF Tg mice and WT mice was used. As shown in Online Figure II (a and b), HGF Tg mice showed significant inhibition of neointimal formation as compared to WT mice. Inhibition of neointimal formation in HGF Tg mice was accompanied by a significant increase in serum HGF level (Online Figure II, c) and c-Met expression but decreased EGFR expression as compared to WT mice (Online Figure III, a and b). Consistently, a reduction of iKB and Akt phosphorylation was also observed in HGF Tg mice as compared to WT mice (Online Figure III, a and c). Migration of macrophages, stained with MOMA2 antibody, was observed mainly in the adventitia after cuff replacement in WT mice. However, there were fewer migrated macrophages in HGF Tg mice as compared to WT mice (Online Figure IV, a and b). DHE staining clearly demonstrated that in HGF Tg mice, ROS production was reduced mainly in the media of the femoral artery as compared to WT mice (Online Figure IV, c and d).

**Discussion**

The present study showed the inhibitory effect of the HGF/c-Met system on the growth of VSMCs and inflammation both in vitro and in vivo. The present data prove that the growth of VSMCs and inflammation are inhibited by HGF, only in the presence of growth factors such as Ang II. Importantly, the inhibitory effect of HGF on VSMC growth is attributable to the unique cross talk between Ang II and HGF signaling. In the present study, SHIP2 was identified as a key mediator main between Ang II and HGF. SHIP2 is known to be expressed ubiquitously and possesses 5'-phosphatase.
activity and an SH2 domain. Recently, SHIP2 was reported to interact with Cbl and Cbl-associated protein in COS-7 cells. Prasad et al showed that SHIP2 sequesters Cbl from EGFR, thereby regulating the kinetics of the EGFR-Cbl association and subsequent degradation of EGFR. They also reported a role of SHIP2 in EGFR endocytic uptake. More recently, some reports have shown that CIN85 is a multidomain adaptor protein involved in Cbl-mediated downregulation of EGFR. CIN85 src homology 3 domains specifically bind to a proline–arginine (PxxxPR) motif in Cbl, and this association seems to be important for EGFR endocytosis. SHIP is one of the CIN85 effectors with PxxxPR motifs. Acting as a molecular scaffold, CIN85 clusters its effectors and recruits them to their site of action. These reports suggest that the SHIP2-CIN85 complex might recruit to EGFR and sequester Cbl from EGFR.

Our in vitro results revealed that HGF downregulated EGFR via the inhibition of SHIP2 binding to EGFR, causing an increase in the binding of Cbl to EGFR and activation of ubiquitin (the proposed mechanism is shown in Online Figure V). A key factor in the interaction between HGF and Ang II signaling is translocation of SHIP2 from EGFR to c-Met (see Figure 4a through 4d). The HGF/c-Met system inhibits the translocation of SHIP2 to EGFR after Ang II stimulation, through SHIP2 binding to its own receptor, c-Met. Under these conditions, a reasonable amount of SHIP2 might not be recruited to EGFR after Ang II stimulation, which would maintain the binding of Cbl with EGFR. Additionally, as shown in Figure 2a through 2d, HGF acts as a prooxidant under basal conditions. SHIP2 is also essential for PIP3 activity. If translocation of SHIP2 between c-Met and EGFR controls each PIP3 activity, Ang II and HGF might reduce ROS production each other. Further investigations are needed to elucidate this interaction.

Although HGF is known to be an angiogenic growth factor, it also has antiinflammatory activity against vascular endothelial growth factor and tumor necrosis factor-α in endothelial cells, and antifibrotic activity against transforming growth factor-β. Our earlier report showed that HGF prevented Ang II–induced endothelial progenitor cell senescence by inhibiting rac1 activation. Although the detailed mechanism is still uncertain, interestingly, it is known that these molecules cause inflammation mainly through EGFR transactivation. Ang II and transforming growth factor-β are strong negative regulators of local HGF production in human VSMCs and cardiomyocytes. In addition, high d-glucose–induced endothelial cell death stimulated VSMC growth accompanied by decreased local HGF production. These reports and our experiment suggest, however, that expression of c-Met is thought to be different depending on pathological conditions, local HGF level is thought to be commonly decreased in several pathological conditions. More interestingly, our previous report also showed that an ARB and angiotensin-converting enzyme inhibitor significantly increased local HGF expression in the heart of cardiomyopathic hamster. Our present study
indicates the possibility that the HGF/c-Met system might contribute to the pleiotropic effects of ARBs and ACE inhibitors, which prevent the progression of atherosclerosis. However, we should take account of the difference between animal experiments and clinical trials in humans using ARB or ACE inhibitor, because there are some negative clinical data that single ARB or ACE inhibitor is not effective on atherosclerosis. 

Overall, the present study demonstrated that the HGF/c-Met system prevents Ang II–induced atherosclerotic change including Ang II–induced ROS production and inflammation through EGFR degradation by an E3 ligase, the Cbl-mediated ubiquitin system. The HGF/c-Met system might regulate extrinsic factor signaling that maintains the homeostasis of many organs, although further studies are necessary to clarify the role of this unique system.

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Disclosures
None.

References


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Supplemental Information

Negative Action of HGF /c-Met System on Angiotensin II Signaling
Via Ligand-dependent EGF Receptor Degradation Mechanism in VSMC

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Running title; Met regulates EGF receptor expression
Supplemental Materials and Methods

Western blotting

Antibodies to human Akt, p-Akt (ser 473), ERK1/2, p-ERK1/2, mTOR, p-mTOR, EGF receptor, anti-human SHIP2 and c-Met were purchased from Cell Signaling, Boston, USA. Antibody to mouse SHIP2 was purchased from Santa Cruz, CA, USA. Antibody to human Cbl was purchased from BD Pharmingen, Franklin Lake, NJ, USA. MOMA2 antibody was purchased from CHEMICON International, Temecula, CA, USA. Human recombinant HGF was purchased from PeproTech EC, London, UK. Human recombinant EGF was purchased from R&D Systems Inc. Minneapolis, MN, USA. Endothelin-1 acetate salt was purchased from BACHEM Inc., California, USA. Losartan potassium was purchased from LTK Laboratories, St. Paul, MN, USA. GTP-rac1 pull-down assay kit was purchased from Stressgen, Ann Arbor, MI, USA. All other chemicals and reagents were purchased from Sigma, St. Louis, MO, USA.

In vitro experiments of plasmid and siRNA transfection

Human full-length wild type c-Met plasmid (pUC-Sra/c-methu) was donated by Dr. Toshikazu Nakamura, Department of Molecular Regenerative Medicine, Osaka University Graduate School of Medicine, Japan. Human EGF receptor, cMet, and control siRNA were
purchased from Santa Cruz, CA, USA. The NF-κB-Luc promoter, the c-Met plasmid DNA and each siRNA was transfected using an electroporation system (Amaxa, Gaithersburg, MD, USA). Around 60% of VSMC were transfected (data not shown).

**Purification of secreted HB-EGF**

Secreted soluble heparin binding - EGF (HB-EGF) was purified by heparin affinity chromatography (GE Healthcare Bio-Sciences Corp., Tokyo, Japan) as manufacturer’s instruction. Briefly, before exposure to the indicated agents in serum-free media, 80-90% confluent cells were rendered quiescent and washed twice with PBS. After stimulation for the indicate time, the conditioned medium was centrifuged and filtered through a 0.45-mm filter. Each sample of conditioned medium was applied immediately to a heparin-Sepharose column, preequilibrated with binding buffer (10 mM sodium phosphate, pH 7). After extensive washing with binding buffer, bound proteins were eluted with elution buffer (10mM sodium phosphate, 1M NaCl, pH 7). The eluent was concentrated by precipitation with TCA (at a final concentration of 10% TCA). After incubation on for 10 min at room temperature, the eluent was pelleted by centrifugation at 12,000 g for 10min. The pellet was subjected to analysis by Western blotting with anti-HB-EGF antibody (Cell Signaling, Boston, USA).
HGF Tg mouse models

To generate a mouse which serum HGF level is increased, we generated cardiac-specific over-expression of human HGF transgenic mice (hetero HGF Tg mice, C57BL7 background). Because, kidney and vasculature specific over-expression of HGF result in kidney cystic disease and placental malformation, the heart in which it is known that a cystic disease hardly occurred was targeted. There is no pathological change in heart in HGF Tg mice (Supplemental Table. 1a). Angiotensin II infusion models were generated in 8-week-old HGF Tg mice and wild type mice (littersmates). Briefly, an incision was made in the midscapular resion and an osmotic minipump (Alzet model 2004, Alze Corp., Palo Alto, CA) containing angiotensin II (infusion rate 0.7 mg/kg/day) was implanted. Blood pressure was measured by tail cuff plethysmography. Mouse serum and abdominal aorta HGF level were measured using a mouse HGF EIA kit (Institute of Immunology Co., Ltd., Tokyo, Japan). Cuff-mediated vascular injury was induced by placing a polyethylene tube around the right femoral artery. After isolating the right femoral artery from the surrounding tissue, a tube (3-mm, PE-50; Becton-Dickinson) was opened longitudinally, loosely placed around the artery and then closed with sutures. All procedures were performed in accordance with the guidelines of the Institutional Animal Committee of Osaka University School of Medicine.
Supplemental Table and Figure Legend

Supplemental Table 1

Characteristics of HGF Tg mice and WT (littermate) mice

(a) Cardiac status under basal condition

(b) Blood pressure following Ang II infusion

n=4, †P<0.05 vs. identical WT mice time course.

(c) Cross sectional area of media of abdominal aorta after Ang II infusion for 3 weeks, n=4,
†P<0.05 vs. identical WT mice.

Supplemental Fig. 1

(a) VSMC number. VSMC were stimulated with Ang II or 5% FBS for 24 hr with or without HGF (50 ng/ml). n=4. *P<0.01 vs. control. (b) EGFR-dependent pathway in Ang II signaling.

n=3. Human EGF receptor and control siRNA were transfected 48 hr before Ang II stimulation.

(c) EGFR expression, Akt and ERK1/2 phosphorylation after Ang II (10^{-7} M) stimulation with or without losartan potassium (100 μM) pretreatment for 12 hr. Pretreatment with losartan potassium almost completely inhibited Ang II-induced Akt and ERK1/2 phosphorylation. n=3.

(d)(e) EGFR expression after EGF (20 ng/ml) or TGF-β (5 ng/ml) stimulation with or without
HGF (50 ng/ml) pretreatment for 12 hr. n=3. (f) Effect of SHIP2 siRNA on SMC growth assessed by MTS. Human SHIP2 and control siRNA were transfected 48 hr before Ang II (10^{-7} M), ET-1 (10^{-7} M) or EGF (20 ng/ml) stimulation. n=5. *P<0.01 vs. control and SHIP2 siRNA-transfected control. #P<0.01 vs. control siRNA-transfected VSMC with same stimulation.

**Supplemental Fig. 2**

Cuff-induced neointimal formation and EGFR expression in HGF Tg mice and WT mice.

(a) Neointimal formation after cuff placement for 28 days. (b) Quantification of intima/media ratio. n=5. *P<0.01 vs. no injury, †P<0.05 vs. WT mice. (c) Mouse serum HGF level after cuff placement for 1 week. n=4. *P<0.01 vs. non-injury WT. #P<0.01 vs. WT mice+ cuff (1W).

**Supplemental Fig. 3**

(a) Western blots from femoral artery with cuff placement for 1 week in HGF Tg and WT mice. n=5. (b) (c) Quantification of western blotting. *P<0.01 vs. WT mice, □P <0.01 vs. WT mice+ cuff (1W), †P<0.05 vs. WT mice, ††P<0.05 vs. WT mice + cuff (1W).

**Supplemental Fig. 4**
Macrophage accumulation and O$_2^-$ production after cuff placement for 1 week.

(a) HGF Tg mice showed reduced cuff-induced macrophage accumulation. MOMA2 staining showed macrophages (green) in tissue sections retrieved from cuff-placed femoral artery, n=5. *P<0.01 vs. WT mice, #P<0.01 vs. WT mice+ cuff (1W). Scale bar = 200µm. (b) Quantification of % of MOMA2-stained area. (c) DHE staining in tissue sections retrieved from cuff-placed femoral artery. n=5. (d) Measurement of fluorescence intensity of DHE staining visualized by confocal microscopy. *P<0.01 vs. WT mice, #P<0.01 vs. WT mice+ cuff (1W).

Supplemental Fig. 5

Proposed mechanisms of inhibition of Ang II signaling by HGF/c-Met system.

(a) Under basal condition, SHIP2 prevents excess ubiquitination by inhibiting Cb1 binding to EGFR after Ang II stimulation. (b) The HGF/c-Met system inhibits SHIP2 translocation to EGFR after Ang II stimulation through binding of SHIP2 to its own receptor, c-Met. Under these conditions, Cbl binds to EGFR significantly, which might degrade EGFR and prevent Ang II signaling.
(a) Cardiac status  

<table>
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<tr>
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<th>EF (%)</th>
<th>FS (%)</th>
<th>IVS (mm)</th>
<th>PW (mm)</th>
<th>LVDd (mm)</th>
<th>LVDs (mm)</th>
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<tr>
<td>WT</td>
<td>88.4 ± 0.81</td>
<td>48.8 ± 0.34</td>
<td>0.74 ± 0.029</td>
<td>0.69 ± 0.056</td>
<td>2.58 ± 0.075</td>
<td>1.32 ± 0.028</td>
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<tr>
<td>HGF Tg</td>
<td>89.8 ± 1.57</td>
<td>51.4 ± 0.93</td>
<td>0.73 ± 0.056</td>
<td>0.73 ± 0.032</td>
<td>2.69 ± 0.046</td>
<td>1.32 ± 0.09</td>
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(b) Blood pressure  

Systolic blood pressure (mmHg)  

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<th>3W</th>
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<tbody>
<tr>
<td>WT</td>
<td>110.7 ± 2.9</td>
<td>113.0 ± 3.54</td>
<td>112.3 ± 1.89</td>
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<tr>
<td>HGF Tg</td>
<td>106.3 ± 0.63</td>
<td>105.8 ± 1.84 †</td>
<td>106.8 ± 1.11</td>
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Diastolic blood pressure (mmHg)  

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<tr>
<td>WT</td>
<td>62.5 ± 3.202</td>
<td>78.7 ± 3.497</td>
<td>66.0 ± 3.162</td>
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<tr>
<td>HGF Tg</td>
<td>64.0 ± 3.719</td>
<td>69.5 ± 2.754 †</td>
<td>65.5 ± 3.069</td>
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(c) Medial area (mm²)  

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<tbody>
<tr>
<td>WT</td>
<td>0.082 ± 0.002</td>
<td>0.104 ± 0.004</td>
</tr>
<tr>
<td>HGF Tg</td>
<td>0.081 ± 0.001</td>
<td>0.096 ± 0.002 †</td>
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Supplemental Table 1  
Characteristics of HGF Tg mice and WT (littermate) mice  

(a) Cardiac status under basal condition. (b) Blood pressure following Ang II infusion. n=4, †P<0.05 vs. identical WT mice time course.  
(c) Cross sectional area of media of abdominal aorta after Ang II infusion for 3 weeks, n=4, †P<0.05 vs. identical WT mice.
Supplemental Fig. 1

(a) VSMC number. VSMC were stimulated with Ang II or 5% FBS for 24 hr with or without HGF (50 ng/ml). n=4. *P<0.01 vs. control. (b) EGFR-dependent pathway in Ang II signaling. n=3. Human EGF receptor and control siRNA were transfected 48 hr before Ang II stimulation. (c) EGFR expression, Akt and ERK1/2 phosphorylation after Ang II (10^{-7} M) stimulation with or without losartan potassium (100 µM) pretreatment for 12 hr. Pretreatment with losartan potassium almost completely inhibited Ang II-induced Akt and ERK1/2 phosphorylation. n=3. (d,e) EGFR expression after EGF (20 ng/ml) or TGF-β (5 ng/ml) stimulation with or without HGF (50 ng/ml) pretreatment for 12 hr. n=3. (f) Effect of SHIP2 siRNA on SMC growth assessed by MTS. Human SHIP2 and control siRNA were transfected 48 hr before Ang II (10^{-7} M), ET-1 (10^{-7} M) or EGF (20 ng/ml) stimulation. n=5. *P<0.01 vs. control and SHIP2 siRNA-transfected control. #P<0.01 vs. control siRNA-transfected VSMC with same stimulation.
Supplemental Fig. 2

Cuff-induced neointimal formation and EGFR expression in HGF Tg mice and WT mice.

(a) Neointimal formation after cuff placement for 28 days. (b) Quantification of intima/media ratio. n=5. *P<0.01 vs. no injury, †P<0.05 vs. WT mice.
(c) Mouse serum HGF level after cuff placement for 1 week. n=4. *P<0.01 vs. non-injury WT. #P<0.01 vs. WT mice+ cuff (1W).
Supplemental Fig. 3

(a) Western blots from femoral artery with cuff placement for 1 week in HGF Tg and WT mice. n=5. (b) (c) Quantification of western blotting. *P<0.01 vs. WT mice, □ P<0.01 vs. WT mice + cuff (1W), †P<0.05 vs. WT mice, ††P<0.05 vs. WT mice + cuff (1W).
Supplemental Fig. 4
Macrophage accumulation and $O_2^*$ production after cuff placement for 1 week.

(a) HGF Tg mice showed reduced cuff-induced macrophage accumulation. MOMA2 staining showed macrophages (green) in tissue sections retrieved from cuff-placed femoral artery, n=5. *P<0.01 vs. WT mice, #P<0.01 vs. WT mice+ cuff (1W). Scale bar = 200µm. (b) Quantification of % of MOMA2-stained area. (c) DHE staining in tissue sections retrieved from cuff-placed femoral artery. n=5. (d) Measurement of fluorescence intensity of DHE staining visualized by confocal microscopy. *P<0.01 vs. WT mice, #P<0.01 vs. WT mice+ cuff (1W).
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