HGF-induced capillary morphogenesis of endothelial cells is regulated by Src

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\textit{Abbreviations:} HGF, hepatocyte growth factor; HGFR, HGF receptor; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; FBS, fetal bovine serum; EBM-2, endothelial basal medium-2; DMSO, dimethyl sulfoxide.
Abstract

The signal transduction pathway involved in hepatocyte growth factor (HGF)-induced capillary morphogenesis of endothelial cells was investigated. HGF-induced capillary morphogenesis of the murine spleen endothelial cell line MSS31 was inhibited by a Src family kinase inhibitor, PP2. Stable expression of kinase-inactive Src in MSS31 cells inhibited HGF-induced activation of Src as well as capillary morphogenesis. The HGF-induced capillary morphogenesis of human umbilical vein endothelial cells was also inhibited by PP2, and was reduced by the downregulation of Src by small interfering RNA. These results suggest that HGF induces capillary morphogenesis of endothelial cells through Src.

Key words: hepatocyte growth factor, endothelial cells, capillary morphogenesis, Src
Hepatocyte growth factor (HGF) induces angiogenesis. HGF stimulates the proliferation, secretion of proteases, migration, survival, and differentiation (capillary morphogenesis or tube formation) of endothelial cells through its specific receptor tyrosine kinase, c-Met/hepatocyte growth factor receptor (HGFR) [1-5]. In addition, HGF mobilizes endothelial progenitor cells from bone marrow, which contributes to vasculogenesis [6]. HGF also indirectly induces angiogenesis through the upregulation of platelet-activating factor, vascular endothelial growth factor, interleukin 8, and ets-1, and downregulation of thrombospondin 1 [7-11]. The Ras/mitogen-activated protein kinase pathway and phosphoinositide 3-kinase/Akt pathway are implicated in cell survival, matrix metalloproteinase production, proliferation, and cell migration [4, 12-14]. However, signal transduction pathways leading to the differentiation of HGF-treated endothelial cells are largely unknown.

The Src family protein tyrosine kinases play crucial roles in a variety of cellular responses, embryonic development, and pathological conditions such as tumor progression and angiogenesis [15, 16]. Blockade of common signaling molecules downstream of a panel of growth factor receptors such as the Src family of kinases is an attractive strategy to inhibit tumor angiogenesis because the angiogenic signals via several proangiogenic growth factor receptors could be inhibited simultaneously. Since signals via HGFR are involved in tumor progression and angiogenesis in many human cancers, it is important to determine whether the Src family kinases are required for HGF-induced angiogenesis. While a Src family kinase inhibitor PP1 inhibited HGF-mediated nitric oxide production along with proliferation of human umbilical vein
endothelial cells (HUVECs) [17], the role of Src family kinases in HGF-induced cellular responses of endothelial cells is still poorly understood.

In the present study, we show for the first time that Src activity is required for the HGF-induced capillary morphogenesis of endothelial cells.

**Materials and methods**

*Materials* Recombinant human HGF was obtained from R & D Systems, Minneapolis, MN. Type I collagen gel was purchased from Cohesion Technologies Inc., Palo Alto, CA. Growth factor-reduced Matrigel® matrix was purchased from BD Bioscience, Bedford, MA. Anti-Src antibodies (N-16 for immunoprecipitation and SRC2 for immunoblotting) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FuGENE® 6 transfection reagent was obtained from Roche Diagnostics Corporation, Indianapolis, IN. The cDNA encoding kinase-inactive Src in the pUSE plasmid was purchased from Upstate Cell Signaling Solutions, Lake Placid, NY. HiPerFect® transfect reagent, Alexa Fluor 555-labeled negative control siRNA (5’-UUCUUCGAACGUGUCACGU-3’), and human Src siRNA (Hs_SRC_5_HP Validated siRNA) were purchased from Qiagen K.K., Tokyo, Japan. The Src inhibitor PP2 was obtained from Merck Company, Tokyo, Japan. PP2 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM as stock solutions and stored at -20°C before use. A working solution of PP2 was made by further
dilution of the stock solution in DMSO before being dissolved in culture medium. The final concentration of DMSO in the culture medium was always 0.1% (V₀/V₀).

**Cell culture** A murine spleen endothelial cell line, MSS31 cells [18], were the kind gifts of Drs. M. Abe and Y. Sato (Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) and were cultured in alpha-modification of minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). HUVECs and their culture medium were obtained from Cambrex, Walkersville, MD, and the cells were cultured in endothelial cell basal medium-2 (EBM-2) supplemented with 2% FBS, 10 ng/ml VEGF-A, 20 ng/ml fibroblast growth factor-2, 10 ng/ml endothelial growth factor, 10 ng/ml insulin-like growth factor-I, 50 μg/ml ascorbic acid, 100 ng/ml heparin, and 10 pM dexamethasone.

**Capillary morphogenesis assay** MSS31 cells suspended in serum-free α-MEM with or without indicated treatments were cultured between two layers of type I collagen gels for 18-20 h, as described previously [19]. Briefly, MSS31 cells were seeded onto the first collagen gel layer in the presence or absence of the indicated treatments. After 4 h the medium was removed, and the cells were overlaid with a second collagen gel layer, and the culture was continued for 16 h. HUVECs suspended in EBM-2 medium containing 0.5% FBS with or without the indicated treatments were seeded onto growth factor-reduced Matrigel and cultured for up to 24 h [20]. To quantify the length of capillaries, 3 different phase-contrast photomicrographs (x 4 objectives) per well were
taken, and the length of each capillary was measured using NIH image software (version 1.64). The capillary length of HGF-stimulated parental or empty vector-transfected cells was set to 1.0.

Transfection of kinase-inactive Src into MSS31 cells MSS31 cells were transfected with a mixture of plasmid containing the cDNA encoding kinase-inactive Src, and FuGENE 6 reagent. After 48 h, stable transformants were selected with G418. After 2 weeks, colonies from a single G418 resistant cell were picked up, and the overexpression of kinase inactive Src was determined by immunoblotting. To identify stable cell lines expressing the kinase inactive, dominant negative Src, cells were either stimulated with HGF or left untreated and the activity of the dominant negative was estimated by *in vitro* kinase assays.

Transfection of siRNA in HUVECs HUVECs were transfected with siRNA using HiPerFect® reagent. To examine the transfection efficiency, Alexa Fluor 555-labeled negative control siRNA was transfected into HUVECs. The following day, the cells were washed, fixed with paraformaldehyde, and examined with a fluorescent microscope. To downregulate Src protein in cells, Src validated siRNA was transfected into HUVECs cultured in 6-well plates. After 2 days, cells were detached from the wells and the capillary morphogenesis assay was performed. Four thousand cells from each treatment were lysed, and the expression of Src was examined by immunoblotting.
**In vitro kinase assay** The *in vitro* kinase assay for Src was described previously [21]. Briefly, cells were serum-starved for 2 h then were either left untreated or stimulated with HGF for 10 min. The Src was immunoprecipitated, and the kinase assay was performed using acid-denatured enolase as the substrate. The incorporation of \([\gamma-^{32}P]\) ATP into enolase was examined by SDS-PAGE, followed by autoradiography.

**Immunoblotting** Ten percent of the total cell lysate for the *in vitro* kinase assay or the cell lysate from 4,000 siRNA-treated cells was separated on SDS-polyacrylamide gels. Proteins were electrotransferred onto polyvinylidene difluoride membranes. Membranes were incubated with the indicated antibodies followed by incubation with peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL) and exposed to X-ray film.

**Statistical analysis** Values are presented as the mean capillary length ± SD. Differences between two groups were determined by Mann-Whitney’s U test. Differences were considered significant when the *P* value was less than 0.05.
Results

**HGF induces capillary morphogenesis in MSS31 cells and is dependent on Src activity**

We examined the effect of HGF on capillary morphogenesis in MSS31 cells. As shown in Fig. 1, HGF stimulated capillary morphogenesis, and the Src inhibitor PP2 dose-dependently inhibited this effect in MSS31 cells. HGF also activated Src in MSS31 cells (not shown). We then established MSS31 cell lines stably expressing kinase-inactive Src and examined the dominant-negative effect on endogenous Src. As shown in Fig. 2A, HGF activated endogenous Src in empty vector-transfected MSS31 cells (Mock cells). However, in two stable cell lines expressing kinase-inactive Src (denoted SrcKD-10 and -14 cells, respectively), HGF failed to increase Src activity. We next examined the effect of HGF on capillary morphogenesis in these cells. As shown in Fig. 2B, Mock cells formed capillary structures in response to HGF-treatment. However, HGF failed to induce capillary morphogenesis in the SrcKD-10 and -14 cell lines, suggesting that HGF-induced capillary morphogenesis requires Src activation in MSS31 cells.

**Src activity is required for HGF-induced capillary morphogenesis in HUVECs**

We next examined the effect of HGF on capillary morphogenesis in HUVECs. As shown in Fig. 3A, HGF induced capillary morphogenesis in HUVECs and this effect was attenuated by treatment of the cells with PP2. As shown in Fig. 3B, HGF activated Src in HUVECs. To examine the transfection efficiency, Alexa Fluor 555-labeled negative control siRNA was transfected into HUVECs and we observed that more than 95% of cells
were labeled with Alexa Fluor 555 (submitted for publication). We then transfected Src siRNA into HUVECs and HGF-induced capillary morphogenesis was examined. As shown in Fig. 4A, siRNA efficiently downregulated Src in HUVECs. HUVECs treated with Src siRNA, but not with control siRNA, exhibited impaired capillary morphogenesis in the presence of HGF (Fig. 4B) suggesting that Src is required for HGF-induced capillary morphogenesis in HUVECs.
Discussion

In the present study, we show that HGF activated Src in two different endothelial cells, MSS31 cells and HUVECs. A Src family kinase inhibitor, PP2 inhibited HGF-induced capillary morphogenesis in these cells (Fig. 1 and 3A). Inhibition of endogenous Src via stable expression of kinase-inactive Src (MSS31 cells; Fig. 2B) or downregulation of Src with siRNA (HUVECs; Fig. 4B) blocked HGF-induced capillary morphogenesis. These results indicate that Src is a downstream signaling target of c-Met/HGFR in endothelial cells, and that HGF-induced capillary morphogenesis of endothelial cells requires Src activity.

Src was involved in the fibroblast growth factor-2 (FGF-2) induced migration of endothelial cells [22]. The Src family inhibitor PP2 inhibited FGF-2-induced capillary morphogenesis, tube formation, and \textit{in vivo} angiogenesis [21, 23, 24]. Expression of dominant negative Fyn blocked FGF-2 and angiopoietin 2 (Ang2) induced tube formation [21, 25]. Inhibition of Src attenuated VEGF-induced \textit{in vivo} angiogenesis by inducing apoptosis of endothelial cells [26]. Src is also involved in VEGF-induced migration [27, 28]. Selective downregulation of Src family kinases by small interfering RNA (siRNA) demonstrated that Yes was important for VEGF-mediated migration, whereas Fyn was required for VEGF-promoted tube formation [29]. It has been shown that the anti-VEGF-A monoclonal antibody bevacizumab (Avastin) showed antivascular effect in human rectal cancer [30]. However, targeting solely VEGF signaling failed to demonstrate potent antitumor activity in clinical trials. Recent study using a preclinical
model has shown that blocking VEGF signaling changed the VEGF-dependent angiogenesis to other proangiogenic factor-dependent angiogenesis, such as FGF-2 [31]. Thus, the inhibition of signaling molecules commonly working downstream of several proangiogenic growth factor receptors, such as Src family kinases, by small molecule protein kinase inhibitors could represent a broad range antiangiogenic strategy.
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References


**Figure legends**

**Fig. 1.** HGF induces capillary morphogenesis by MSS31 cells, which is inhibited by PP2. MSS31 cells were cultured between two collagen gel layers in the presence or absence of HGF (50 ng/ml) with either 0.1% DMSO or PP2 at the indicated concentrations. To quantify the length of capillaries, 3 different phase-contrast photomicrographs (x 4 objectives) per well were taken, and the length of each capillary was measured using NIH image software. Values are expressed as the mean ± SD of 3 pictures. Capillary length of HGF-stimulated cells was set at 1.0. Bar; 100 μm. Reproducible data were obtained from two independent experiments.

**Fig. 2.** Stable expression of kinase inactive Src in MSS31 cells inhibits HGF-induced Src activation and capillary morphogenesis. **A.** MSS31 cells transfected with empty vector (denoted Mock cells) or stable cell lines expressing kinase-inactive Src (denoted SrcKD-10 and -14 cells, respectively) were cultured in 6 cm dishes. After serum-starvation, cells were either stimulated or left unstimulated with 100 ng/ml HGF for 8 min. Cells were lysed and Src was immunoprecipitated from 90% of cell extracts and the kinase activity was determined by in vitro kinase assays. Acid-denatured enolase was used as the substrate. The remaining 10% of the cell extract was used to assess the loaded amount of Src. Reproducible data were obtained from two independent experiments. **B.** HGF-induced capillary morphogenesis was impaired in SrcKD-10 and -14 cells. Cells were cultured between two collagen gel layers in the absence or presence of HGF at 50 ng/ml. Values are expressed as the mean ± SD of 3 pictures. Capillary length
of HGF-stimulated Mock cells was set to 1.0. Bar; 100 μm. Reproducible data were obtained from two independent experiments.

**Fig. 3. A.** HGF promotes capillary morphogenesis of HUVECs, and this effect is inhibited by PP2. HUVECs were cultured on the surface of growth factor-reduced Matrigel in the presence or absence of HGF (20 ng/ml) with either 0.1% DMSO or PP2. Values are expressed as the mean ± SD of 3 pictures. Capillary length of HGF-stimulated cells was set to 1.0. Bar; 100 μm. Reproducible data were obtained from two independent experiments. **B.** HGF activates Src in HUVECs. HUVECs were serum-starved, untreated or treated with 100 ng/ml of HGF for 8 min. Afterward, in vitro kinase assays and immunoblotting were carried out as described in the legend of Fig. 2 A. Reproducible data were obtained from two independent experiments.

**Fig. 4. A.** Src is downregulated by treatment with Src siRNA in HUVECs. Equal numbers of HUVECs treated with either control siRNA (10 nM) or Src siRNA (10 nM) were lysed and proteins were separated by SDS-PAGE followed by the immunoblotting. **B.** HGF-promoted capillary morphogenesis is impaired in HUVECs treated with Src siRNA. HUVECs treated with either control siRNA (10 nM) or Src siRNA (10 nM) were cultured on growth factor-reduced Matrigel, and capillary morphogenesis assays were carried out as described in the legend of Fig. 3B. Values are expressed as the mean ± SD of 3 pictures. Capillary length of HGF-stimulated cells was set to 1.0. Bar; 100 μm. Reproducible data were obtained from two independent experiments.
Fig. 1. S. Kanda et al.
Fig. 2. S. Kanda et al.
**A**

- DMSO
- HGF + DMSO
- HGF + PP2 (1 μM)

**B**

- **HGF**
  - -
  - +
- **DMSO**
- **HGF**
  - -
  - +
  - +
  - +

**Capillary length**

- 0
- 1
- 2

**PP2**

- 0
- 0.2
- 1

**IB : anti-Src**

**In vitro kinase assay**

**enolase**

*Fig. 3. S. Kanda et al.*
A

Cont. siRNA : + -
 Src siRNA : - +

IB : anti-Src

IB : anti-GAPDH

B

Cont. SI RNA  Src siRNA

HGF (-)

HGF (+)

Capillary length

HGF : - + - +

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