Hepatocyte Growth Factor/Scatter Factor Can Induce Angiogenesis Independently of Vascular Endothelial Growth Factor

Shiladitya Sengupta, Ermanno Gherardi, Lynda A. Sellers, Jeanette M. Wood, Ram Sasisekharan, Tai-Ping D. Fan

Objective—Hepatocyte growth factor/scatter factor (HGF/SF) promotes vascular endothelial growth factor (VEGF) expression and induces angiogenesis in multiple pathological conditions. The present study was designed to delineate the HGF/SF and VEGF signaling cascades during angiogenesis by using PTK787, a selective VEGF receptor antagonist.

Methods and Results—PTK787 produced a concentration-dependent (10⁻⁸ to 10⁻⁶ mol/L) inhibition of VEGF-induced angiogenesis, without altering the basal or HGF/SF-induced response in vitro. In contrast, the nonspecific kinase inhibitor genistein blocked the HGF/SF-induced effect. Both VEGF and HGF/SF induced a rapid phosphorylation of extracellular receptor kinases-1 and -2 (ERKs) and Akt. PTK787 inhibited the VEGF-induced activation of Akt and ERKs, without affecting the HGF/SF-induced phosphorylation. Treatment with VEGF and HGF/SF increased total neovascularization in a murine scaffold granuloma model, but no additive or synergistic interactions were observed. PTK787 (50 mg/kg) blocked the VEGF-induced response without altering the basal or HGF/SF-induced neovascularization.

Conclusions—We demonstrate that HGF/SF can induce angiogenesis independently of VEGF, possibly through the direct activation of the Akt and ERKs. These results demonstrate the necessity of a multitargeted approach for the rational design of newer therapies to inhibit pathophysiologival angiogenesis. (Arterioscler Thromb Vasc Biol. 2003;23:69-75.)

Key Words: hepatocyte growth factor/scatter factor • vascular endothelial growth factor • angiogenesis • PTK787

Angiogenesis is defined as the formation of new blood vessels from an existing vascular bed.¹ It is a key step in physiological processes such as wound healing and the menstrual cycle. In contrast, multiple pathological conditions such as cancer, atherosclerosis, arthritis, diabetic retinopathy, and psoriasis are characterized by overt angiogenesis.² The fine balance between physiological and pathological angiogenesis is mediated by the interplay of multiple endogenous angiogenic and antiangiogenic modulators.

Vascular endothelial growth factor (VEGF) is a potent and endothelium-specific factor, with a key role in pathological angiogenesis. Its specificity arises from the preferential expression of its tyrosine kinase receptors on the endothelial cell (EC) surface, resulting in the development of new therapeutic approaches that target the VEGF–VEGFR system.³ Recently, an orally active inhibitor, PTK787, was described as a highly selective antagonist of the VEGF tyrosine kinases.⁴ Interestingly, VEGF was reported to mediate the neovascularization induced by multiple factors. Indeed, synergistic interactions have been observed between VEGF and fibroblast growth factor⁵ or with placental growth factor.⁶ Therefore, Tille et al⁷ projected an optimistic view that VEGFR antagonists could have a broad-spectrum inhibitory effect on pathological angiogenesis.

Hepatocyte growth factor/scatter factor (HGF/SF) was discovered independently as a mitogen for hepatocytes and as a fibroblast-derived factor that induced scattering in polarized epithelial cells.⁸,⁹ It binds to the met-tyrosine kinase receptor and has been implicated in angiogenesis.¹⁰ Lamszus et al¹¹ demonstrated that HGF/SF conferred a growth advantage to human breast cancer xenotransplants, linked with a higher microvessel density. HGF/SF was also shown to be an angiogenic factor in proliferative diabetic retinopathy¹² and in rheumatoid and osteo arthritis.¹³ Interestingly, HGF/SF was shown to act in synergy with VEGF for the induction/amplification of angiogenesis.¹⁴,¹⁵ Furthermore, Wojta et al¹⁶ reported that HGF/SF increases the expression of VEGF and thereby initiates angiogenesis. The current study was aimed at ascertaining whether the angiogenic effects of HGF/SF are mediated through VEGF. PTK787 was used to “knock out”

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VEGFR downstream signaling and thereby delineate the signaling pathways of HGF/SF and VEGF.

**Methods**

**Growth Factors and Chemicals**

Murine and human recombinant HGF/SFs were generated using an NSO mouse melanoma cell line transfected by electroporation with mouse or human HGF/SF cDNA. The proteins were purified by elution from a heparin-Sepharose column and then a Mono-S column. Murine and human recombinant VEGF506 were procured from RELIATech. The tyrosine kinase inhibitor genistein was procured from Sigma. The VEGFR inhibitor, PTK787/ZK222584, was synthesized by Schering AG, Berlin, Germany, and supplied by Novartis Pharma AG (Basel, Switzerland). Hypnorsin (fentanyl fluanisone) and Hympovel (midazolam) were obtained from Janssen Pharmaceutica Roche, respectively. Radioactive $^{133}$Xenon ($^{133}$Xe) was purchased from Dupont Pharma. Matrigel was obtained from Becton Dickinson.

**In Vitro Tube Formation Assay**

Human umbilical vein endothelial cells (HUVECs), between passages 2 and 6, were plated on 6-well plates and grown to confluence. Synchronized cells were plated in 24-well plates (Costar, Corning Ltd), and each well was coated with 200 $\mu$L Matrigel (diluted, 1:3, vol/vol, in phosphate-buffered saline [PBS]). The drugs and growth factors were added to the media, and the cells were allowed to incubate for 16 hours. Where appropriate, the cells underwent pretreatment with the inhibitors, which was maintained for the entire duration of the experiments. At the end of 16 hours, the cells were fixed in 10% formalin and visualized with a 20× objective on an Olympus inverted microscope.

**In Vitro Scratch Model**

A confluent monolayer of synchronized HUVECs was scraped with a multichannel wounder, thereby producing 11 parallel lesions, each 400 $\mu$m wide, on the monolayer. Coverslips were rinsed in PBS to dislodge any cellular debris and placed into a well containing the appropriate treatment. Cells were pretreated with the enzyme inhibitors for 1 hour before wounding of the monolayer. At 24 hours after injury, the trypan blue exclusion method.

**Cell Proliferation**

ECs were injured as previously and cultured for 24 hours in media supplemented with 1% fetal calf serum and the appropriate treatment. At the end of this period, they were washed in ice-cold PBS and trypsinized, and cell growth was expressed as counts by using the trypsin blue exclusion method.

**Western Blot Detection of Phosphorylation Status of ERK1/2 and Akt**

A confluent monolayer of cells was wounded as described earlier, and the cellular proteins were solubilized by rapid mixing with sample buffer (3×) under reducing conditions. Equivalent amounts of protein per sample were electrophoretically resolved on 10% polyacrylamide gels and transferred onto a nitrocellulose (0.22-μm) membrane. Anti-phospho extracellular receptor kinase (ERK)1/2 and anti-phospho Akt antibodies (1:800 dilution, New England Biolabs and Cell Signaling Technologies, respectively) and anti-ERK1/ERK2 and Akt total-protein antibodies (1:500 dilution, Santa Cruz Biotechnology) were used to probe the membrane. The signal was amplified with a 1:2000 dilution of the appropriate horseradish peroxidase–conjugated secondary antibody (Bio-Rad), and the immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Life Science).

**In Vivo Angiogenesis Assay**

**Surgical Protocol**

Male BALB/c mice (Tucks, UK) were anesthetized with 4% isoflurane and maintained on 2% isoflurane in a mixture of oxygen (0.8 L/min) and nitrous oxide (0.6 L/min). An incision was made 0.5 cm caudal from the base of the tail, and 2 bilateral subcutaneous air pockets were created up to the dorsal subsacral region. A sterile polyether polyurethane scaffold (160 mm3) was inserted into each pocket, and the incision was closed with silk sutures (Mersilk).

**Assay for Functional Status of Neovasculature**

On day 15, the animals were anesthetized with a combination of fentanyl citrate–fluanisone and midazolam (diluted 1:1 in 20 in saline). Vascularization was assessed as a function of blood flow through the implants by direct injection of $^{133}$Xe-containing saline into the scaffold and monitoring of its clearance for a 6-minute period. Radioactivity was measured by using a microprocessor scalar rate meter (Nuclear Enterprise) linked to a collimated, low-energy x-ray/gamma-ray sodium iodide crystal with an aluminum entrance window, on an HG-type mount coupled to an NE 5289C preamplifier. The data were expressed as the percentage of $^{133}$Xe cleared at every 40 seconds, calculated according to the formula: (initial count−count at t(seconds))/100%/initial count.

**Cellular Proliferation in Cultures**

All experiments were repeated at least 3 times with replicates, and the data were expressed as mean SEM. Data were tested by ANOVA, followed by Newman-Keuls or Bonferroni’s post hoc test, with the level of significance set at $P<0.05$. All experiments were repeated at least 3 times with replicates, and the data were expressed as mean SEM. Data were tested by ANOVA, followed by Newman-Keuls or Bonferroni’s post hoc test, with the level of significance set at $P<0.05$. All experiments were repeated at least 3 times with replicates, and the data were expressed as mean SEM. Data were tested by ANOVA, followed by Newman-Keuls or Bonferroni’s post hoc test, with the level of significance set at $P<0.05$.
alter the basal or HGF/SF-induced tubulogenesis, but it abolished the VEGF (500 pmol/L)-induced response in a concentration-dependent manner. Interestingly, the inhibition by PTK787 was partially overcome at a higher concentration of VEGF (1 nmol/L), demonstrating the competitive nature of antagonism between the ligand and the antagonist.4 As shown in Figure 1, HGF/SF was capable of driving tubulogenesis despite the presence of VEGF-blockade by PTK787.

**Effect of PTK787 on VEGF and HGF/SF**

**Reendothelialization of the Wounded Monolayer**

The growth factors were evaluated in a multichannel wounding assay, which was extensively validated by Lauder et al, and allows the amplification of signals over a single scratch model. We observed that a serum concentration of 1% was essential for cell survival after injury but did not exert any growth advantage. This allowed a clear delineation of the growth factor–induced recovery and proliferation from the basal response, and HGF/SF was found to induce complete recovery after 72 hours (data not shown). However, at this magnitude of injury, treatment with PTK787 resulted in a loss of cells beyond 24 hours, posing a technical limitation for image analysis in the groups not treated with HGF/SF.

As shown in Figure 2, the addition of VEGF (5×10^-10 mol/L) or HGF/SF (10^-9 to 2×10^-9 mol/L) induced a significant regeneration of the lesion. The coadministration of VEGF (5×10^-10 mol/L) with HGF/SF (10^-9 mol/L) increased total recovery, compared with the recovery observed with either of the growth factors at identical concentrations. The administration of PTK787 induced a concentration-dependent (10^-8 to 10^-6 mol/L) inhibition of the monolayer regeneration induced by VEGF. Even at 10^-6 mol/L, PTK787 did not inhibit the basal and HGF/SF-induced regeneration.

Both HGF/SF and VEGF induced cell proliferation in the current study. Coadministration of HGF/SF (9×10^-9 mol/L) with VEGF (5×10^-10 mol/L) increased the total cell proliferation effect that was greater than the effects of either of the growth factors alone, but which was less than a synergistic effect. Furthermore, PTK787 (10^-6 mol/L) blocked VEGF-induced cell proliferation but did not alter the HGF/SF-induced response, and it reversed the HGF/SF plus VEGF–induced response to a level comparable to that of the treatments. The data represent mean ± SEM from n=3. ***P<0.001 vs vehicle-treated control; ##P<0.01 vs VEGF (10^-9 mol/L); +P<0.01 vs VEGF+PTK (10^-7 mol/L). h.p.f. indicates high-power field.

**Figure 1.** Effect of PTK787 on the VEGF- and HGF/SF-induced cordlike structure/tube formation by HUVECs. Cells were visualized with a 20× objective on an Olympus inverted microscope. Each photomicrograph depicts a random field of view and shows the alignment of HUVECs under different treatment conditions. The effect of the treatments was quantified using (J) the percentage density of tubes per cell cluster in a view field. The data represent mean ± SEM from n=3. ***P<0.001 vs vehicle-treated control; ##P<0.01 vs VEGF (10^-9 mol/L); +P<0.01 vs VEGF+PTK (10^-7 mol/L). h.p.f. indicates high-power field.
Effect of PTK787 on the VEGF- and HGF/SF-Induced Phosphorylation of ERK1/2 and Akt

Incubation of injured cells with VEGF or HGF/SF resulted in rapid phosphorylation of ERK1/2 within 15 minutes, as shown in Figure 3. A negligible additive effect was evident when both of the growth factors were added together. Incubating the cells with PTK787 (10⁻⁷ mol/L) abolished the VEGF-induced phosphorylation but did not affect the HGF/SF-induced effect. PTK787 (10⁻⁷ mol/L) also blocked the phosphorylation of Akt induced by VEGF (1 nmol/L) but failed to inhibit the HGF/SF-induced effect. Treatment with the growth factors or the drug did not alter the total protein levels of ERK1/2 or Akt.

Angiogenic Effects of HGF/SF and VEGF In Vivo

The in vivo effects of HGF/SF and VEGF were studied by using the murine scaffold implant model. The neovascular response was quantified as a function of the total number of vessels entering the implant and the t₁/₂ of ¹³³Xe clearance from the scaffold along with the total clearance of ¹³³Xe during a period of 6 minutes, a method validated by Hu et al.¹⁸ As shown in Figure 4, VEGF induced a dose-dependent neovascularization into the implant. The administration of HGF/SF (30 ng per scaffold) also induced significant neovascularization of the scaffold implant, greater than that induced by VEGF. PTK787 (50 mg/kg by mouth) inhibited the angiogenic response to VEGF (100 ng per scaffold) but failed to alter the basal or HGF/SF-induced angiogenesis (Figure 3). A good correlation was observed between the vessel counts, the total ¹³³Xe cleared, and the changes in the t₁/₂ of clearance (Table 1).

To test the hypothesis that HGF/SF and VEGF may act synergistically, the growth factors were coadministered at submaximal concentrations of 3 and 20 ng per scaffold, respectively. However, no synergism or additive interaction was observed in the angiogenic outcome, and PTK787 (50 ng per scaffold) did not alter the neovascularization induced by...
the coadministration of the factors (supplementary Figure I; please see http://atvb.ahajournals.org).

As shown in Figure 4C, there was no difference in the body weight of mice between the treatment groups, and no overt toxicity was evident in any of the animals. To conclusively prove that the clearance of $^{133}$Xe was dependent on the functional vasculature, some of the animals were killed before injection of $^{133}$Xe. As shown in Figure 4D, there was no clearance of $^{133}$Xe from the scaffolds after cessation of circulation.

### Discussion

Recent studies have suggested that HGF/SF increases the expression of VEGF, thereby initiating angiogenesis in a paracrine manner.\(^{14,16}\) However, in the current study, although both HGF/SF and VEGF induced angiogenesis in vitro and in vivo, no synergistic effect was observed between the 2 growth factors. Furthermore, the selective VEGFR inhibitor, PTK787, did not alter the neovascularization induced by HGF/SF, but it blocked the VEGF-induced angiogenesis. These findings suggest that HGF/SF can induce angiogenesis through a VEGF-independent pathway, possibly through the activation of the mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3 kinase (PI3K) cascades. HGF/SF is a heparin-binding glycoprotein of mesenchymal origin that acts as a potent mitogen and motogen,\(^{19}\) leading to organogenesis and tissue regeneration.\(^{20}\) HGF/SF-induced receptor phosphorylation was demonstrated to trigger the migration and proliferation of ECs.\(^{10}\) Indeed, in the present study, we observed a similar HGF/SF-induced EC phenotype.

In normal physiology, the ECs are quiescent and migrate and proliferate only in the presence of an angiogenic cue. The present model of studying the behavior of ECs, after mechanical injury to a confluent, synchronized monolayer, therefore most closely resembles a clinicopathological situation. VEGF was found to induce a concentration-dependent proliferation of ECs in this assay and also to promote regeneration of the denuded area. Interestingly, the combination of HGF/SF and VEGF produced a monolayer regeneration lesser than an additive effect, although cell proliferation was additive but not synergistic. This suggests that although VEGF and HGF/SF may cross-talk for the induction of proliferation of HUVECs, the pathways leading to the migratory components could be distinct.

To delineate the possibility that HGF/SF induces angiogenesis through VEGF, we used PTK787, a selective inhibitor of the VEGFR. PTK787 was demonstrated to inhibit the phosphorylation of VEGFR at a nanomolar range but failed to inhibit HGF/SF-induced c-met phosphorylation, even at $10^{-5}$ mol/L.\(^4\) Consistent with the findings of that earlier study, PTK787 completely inhibited the VEGF-induced regeneration and cell proliferation but did not alter the basal or HGF/SF-induced effects. Furthermore, it knocked out the VEGF component, without affecting the HGF/SF-induced response, from the additive effect observed on cell proliferation. In contrast, genistein, a nonspecific tyrosine kinase inhibitor, could block the HGF/SF-induced monolayer regeneration and cell proliferation (supplementary Figure II; please see http://atvb.ahajournals.org). This suggested that HGF/SF could signal independently of VEGF and is sufficient to induce angiogenesis.

The MAPK (ERKs) and the PI3K (Akt) pathways have been implicated in HGF/SF- and VEGF-induced cell proliferation.\(^{21}\) The activation of ERK and Akt has also been demonstrated in the initiation of angiogenesis.\(^{22,23}\) We observed a rapid phosphorylation of ERK1/2 and Akt after exposure to HGF/SF and VEGF. A negligible increase in the phosphorylation of ERK1/2 was observed after coincubation with HGF/SF and VEGF, which could possibly explain the additive effects on cell proliferation. Interestingly, a recent study has reported that HGF/SF induces VEGF through the activation of the MAPK pathways,\(^{24}\) raising the possibility of VEGF feeding back to MAPK signaling. However, in the current study, treatment with PTK787 did not alter the HGF/SF-induced phosphorylation of either ERK1/2 or Akt.

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### Table: The Effect of Growth Factor and Drug Treatments on the T1/2 Clearance of $^{133}$Xe From the Scaffold Implants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$T_{1/2}$ (seconds)</th>
<th>SEM</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>447</td>
<td>51</td>
<td>0.97</td>
</tr>
<tr>
<td>VEGF (20 ng/scaffold)</td>
<td>237</td>
<td>40</td>
<td>0.81</td>
</tr>
<tr>
<td>VEGF (100 ng/scaffold)</td>
<td>160</td>
<td>8</td>
<td>0.97</td>
</tr>
<tr>
<td>VEGF (100 ng/scaffold) + PTK787 (50 mg/kg)</td>
<td>318</td>
<td>55</td>
<td>0.90</td>
</tr>
<tr>
<td>PTK787 (50 mg/kg)</td>
<td>491</td>
<td>64</td>
<td>0.97</td>
</tr>
<tr>
<td>HGF/SF (30 ng/scaffold)</td>
<td>84</td>
<td>4</td>
<td>0.94</td>
</tr>
<tr>
<td>HGF/SF (30 ng/scaffold) + PTK787 (50 mg/kg)</td>
<td>78</td>
<td>4</td>
<td>0.94</td>
</tr>
</tbody>
</table>

The functionality of the neovasculature was quantified by measuring the clearance of injected $^{133}$Xe from the scaffold. The $T_{1/2}$ clearance was calculated by nonlinear regression curve fitting in a one-phase exponential decay equation, with Prism 3 software (Graphpad). A faster clearance indicates greater vascularization.

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**Figure 3.** The concentration-effect response of the VEGFR inhibitor, PTK787, on VEGF- and HGF/SF-induced MAPK (ERK1 and ERK2) and Akt activation, as determined by Western blot analysis with anti-phospho ERK and anti-phospho Akt antibodies. A confluent monolayer of cells was injured with a multichannel wounder. Cells were harvested in sample buffer at 15 minutes after injury. PTK787 inhibited the phosphorylation of Akt and ERK by VEGF (10$^{-9}$ mol/L) to basal levels at a concentration of 10$^{-7}$ mol/L. There was no change in the HGF/SF-induced phosphorylation of ERK1/2 and Akt. The total levels of ERK1/2 and Akt proteins were consistent in all of the treatment groups. C indicates vehicle-treated controls; K, PTK787 (10$^{-9}$ mol/L); H, HGF/SF (10$^{-9}$ mol/L); and V, VEGF (10$^{-9}$ mol/L).
but inhibited the VEGF-induced phosphorylation. This further demonstrates that HGF/SF can signal independently of VEGF in this model system, possibly through the MAPK and the PI3K pathways. Indeed, in a separate study, we have demonstrated that PD98059 and LY294002, inhibitors of MAPK and PI3K, could abolish the angiogenic effects of HGF/SF in vitro and in vivo.

HGF/SF was shown to induce a strong angiogenic response in the Matrigel plug and in the cornea model. In the current study, a similar angiogenic effect was observed in the murine scaffold granuloma. Interestingly, HGF/SF was found to be more potent in inducing neovascularization than was VEGF. Furthermore, the angiogenic effect of VEGF was inhibited by PTK787, which did not affect the HGF/SF-induced neovascularization. This was an interesting finding, because HGF/SF was demonstrated to act in a paracrine manner through VEGF for inducing angiogenesis in the rabbit hindlimb ischemic model.

To test for synergism, we studied the angiogenic outcome after the coadministration of the 2 growth factors at submaximal doses. In a recent study, Xin et al. had reported the existence of such an interaction. However, in the present study, we observed only a nominal increase in the angiogenic response, which was less than an obvious additive or synergistic effect. Toyoda et al. had demonstrated that the level of VEGF was elevated in HGF/SF-overexpressing mice and inferred that the enhanced angiogenesis observed during wound healing in this transgenic model was due to the induction of VEGF. However, our present findings indicate that although both HGF/SF and VEGF are proangiogenic, HGF/SF is sufficient and can signal independently of VEGF for the induction of neovascularization. This finding was consistent with that reported by Schmidt et al. They found that although fibroblast growth factor-2, VEGF, and HGF/SF were implicated in the induction of angiogenesis in high-grade tumors, HGF/SF was an independent angiogenic factor.

The conclusion of this study that potent angiogenic factors such as HGF/SF and VEGF can signal independently raises interesting questions. Would it be possible to inhibit patho-

Figure 4. Effect of a VEGFR-2 inhibitor, PTK787, on the VEGF- and HGF/SF-induced angiogenesis in a mouse scaffold granuloma model. Sterile polyether polyurethane scaffolds (160 mm3) were aseptically implanted in mice, which were treated for 10 days. On day 15, (A) the neovascularization into the scaffold was quantified by counting the number of vessels entering the implant. B, The functionality of the vessels was quantified by measuring the clearance of 133Xe from the scaffold. Graph in C shows the effect of the treatments on body weight. Data are expressed as percentage of vehicle-treated control values unless otherwise stated. **P<0.01, ***P<0.001 vs vehicle; +P<0.05 vs VEGF (100 ng per scaffold). Data shown are mean±SEM of n=4 to 6. Graph D shows the total clearance of 133Xe from the scaffold when administered after cessation of circulation.
logical angiogenesis by using a single, specific antagonist or inhibitor? Recent studies with PTK787 have reported the inhibition of tumor angiogenesis in monotherapy.3,4 The ability of this compound to inhibit VEGF-induced neovascularization, coupled with its oral activity and tolerability as observed in the present study, suggests that it may be the drug of choice for the treatment of tumors that are dependent on VEGF or in pathological conditions where VEGF plays a key role. However, in pathological conditions such as arthritis13 and proliferative diabetic retinopathy,12 wherein HGF/SF is a major angiogenic factor, or in tumors expressing HGF/SF, a rational approach targeting both HGF/SF and VEGF needs to be designed for a successful therapeutic outcome.

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References
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Supplemental data

Suppl. Fig.1. Effect of co-administration of a sub-maximal dose of VEGF and HGF/SF on the neovascularisation in a mouse-granuloma model. Sterile polyether polyurethane scaffolds (160 mm^3) were aseptically implanted in mouse. On day 15, (A) neovascularisation into the scaffold was quantified by counting the vessels entering the implant. (B) The functionality of the vessels was assessed by measuring the clearance of $^{133}$Xe over a period of 6 min post-administration into the scaffold. Data are expressed as % of vehicle-treated control values and represent mean ± SEM of n=4-8 per data point. **P<0.01, ***P<0.001 vs veh.

Suppl. Fig.2. Effect of Genistein, a tyrosine kinase inhibitor, on the HUVECs monolayer-regeneration induced by HGF/SF in the multichannel-wounding model. Photomicrographs depict Nomarsky images of (A) the total recovery at 24 h following vehicle treatment, (B) total monolayer regeneration following HGF/SF-treatment, and (C) effect of genistein (5 µM) on HGF/SF-induced regeneration of the wounded monolayer. The graphs, (D) shows the total monolayer regeneration at 24 h post-injury, following incubation with HGF/SF in the absence/presence of genistein, and (E) show the number of cells (as a % of basal value) at 24 hours following vehicle or HGF/SF-treatment in the presence or absence of genistein. Data are mean ± SEM of 3-4 independent experiments with duplicate wells in each. ***P<0.001 vs basal (untreated controls). #p<0.01, ##p<0.001 vs basal, + P<0.001 vs HGF/SF. Effect of genistein on HGF/SF-induced re-endothelialisation process.