Mechanisms of Hepatocyte Growth Factor–Induced Retinal Endothelial Cell Migration and Growth

Wenyi Cai,1 Susan L. Rook,1 Zhen Y. Jiang,1 Noriko Takabara,1 and Lloyd Paul Aiello1,2,3

PURPOSE. Hepatocyte growth factor (HGF), also called scatter factor, stimulates growth and motility in nonocular endothelial cells and smooth muscle cells through its receptor c-Met. Recent reports suggest that HGF is increased in the serum and vitreous of patients with proliferative diabetic retinopathy and that smooth muscle cells and retinal pigment epithelial cells secrete HGF in the eye. However, little is known about HGF’s action in the retina. In this study, the activity, expression, and signaling pathways of HGF were investigated in bovine retinal microvascular endothelial cells (BRECs).

METHODS. Mitogenic and motogenic effects of HGF on BRECs were examined using cell counts, thymidine uptake, and migration assays. MAP kinase (MAPK) phosphorylation was examined by Western blot analysis. Protein kinase C (PKC), MAPK, and PI3 kinase involvement were evaluated using selective inhibitors and activity assays. Expression of HGF and c-Met was evaluated by reverse transcription-polymerase chain reaction.

RESULTS. HGF and c-Met were both expressed in BRECs. HGF stimulated BREC growth in a time- and dose-dependent manner, observed at HGF concentrations of 5 ng/ml or more and maximal (410%) at 100 ng/ml (P < 0.001). HGF increased BREC migration in a dose-dependent manner with a maximal 3.4-fold increase at 50 ng/ml after 5 hours. HGF induced time- and dose-dependent MAPK phosphorylation, initially evident at 5 minutes (P < 0.001) or 5 ng/ml (P < 0.050) and maximal after 15 minutes (>80-fold, P < 0.001) or 50 ng/ml (>20-fold, P < 0.001), respectively. MAPK phosphorylation was maintained for more than 2 hours. This response was inhibited 31% by 0.1 μm wortmannin and 76% by 30 μm LY294002, another PI3 kinase inhibitor. The non–isoform-selective PKC inhibitor GFX inhibited HGF-induced MAPK phosphorylation by only 15% at 5 μm. Combined PKC and PI3 kinase inhibition was additive (P < 0.05). Cell migration was inhibited 30% by wortmannin (P < 0.01) and 32% by GFX (P < 0.05), and again the effect was additive (P < 0.001). HGF-induced BREC growth was suppressed by PI3 kinase, PKC, or MAPK inhibition (all P < 0.01). HGF (50 ng/ml) stimulated PI3 kinase activity 347% (P < 0.001) and PKC activity 37% (P < 0.05). HGF-induced MAPK phosphorylation and mitogenesis were not inhibited by vascular endothelial growth factor (VEGF)-neutralizing antibody.

CONCLUSIONS. HGF and its receptor are expressed in BREC, and HGF stimulates both BREC growth and migration at concentrations observed in the human eye with diabetic retinopathy. HGF signaling appears to involve activation of both PKC and PI3 kinase, inducing MAPK phosphorylation that is critical for migration and growth. However, VEGF does not appear to mediate these initial HGF effects. These results indicate that HGF could have a significant role in mediating retinal endothelial cell proliferation and migration in diabetic retinopathy, and they begin to elucidate the signal transduction pathway by which this action may occur. (Invest Ophthalmol Vis Sci. 2000;41:1885–1893)

Intraocular neovascularization is responsible for many of the complications characteristic of such diverse disorders as proliferative diabetic retinopathy (PDR), retinopathy of prematurity, central retinal vein occlusion, sickle cell retinopathy, and numerous others. This angiogenic response involves the activation, proliferation, and migration of endothelial and is regulated by a variety of humoral factors, including basic fibroblast growth factor (bFGF),1 growth hormone,2 insulin-like growth factor-1 (IGF-1),3 and vascular endothelial growth factor (VEGF).4,5 Numerous studies suggest that VEGF plays a major role in mediating the retinal neovascularization characteristic of these disorders. This evidence is particularly strong for PDR6–10; however, other growth factors are also likely to be important in these processes.

Hepatocyte growth factor (HGF) is a mesenchyme-derived pleiotropic protein composed of a 69-kDa α-chain and 34-kDa β-chain.11 HGF receptor is the c-Met proto-oncogene product, a transmembrane tyrosine kinase that is autophosphorylated in response to HGF binding.12 HGF acts as a mitogen, motogen, and morphogen in many cells and tissues, including nonocular endothelial cells.11,12 HGF corneal pellet assays also suggest that HGF is a potent angiogenic factor in vivo.13 The expression of HGF and c-Met have been detected in endothelial and

From the 1Research Division and 2Beetham Eye Institute, Joslin Diabetes Center, Boston, Massachusetts; and the 3Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: Lloyd Paul Aiello, Joslin Diabetes Center, One Joslin Place, Boston, MA 02115. lpaiello@joslin.harvard.edu
smooth muscles cells of the aorta.\textsuperscript{14} HGF and c-Met have only recently been reported in the eye and only in endothelial and epithelial cells of the cornea,\textsuperscript{15} trabecular meshwork,\textsuperscript{16} and pigment epithelial cells of the retina.\textsuperscript{17} Recent studies have demonstrated that both serum and vitreous concentrations of HGF are significantly elevated in diabetic patients with PDR, compared with levels in nondiabetic control subjects. HGF concentrations are also higher in patients with active PDR than in those with quiescent PDR.\textsuperscript{18–20}

Although HGF has been fairly extensively evaluated in nonocular mesenchymal-derived cells, the understanding of its role in the eye is largely incomplete. Particularly little is known regarding HGF’s activity and signal transduction mechanism in the retina. Thus, we investigated the biologic effects, expression, and intracellular signaling pathways for HGF in retinal microvascular endothelial cells. Our findings that HGF and its receptor are expressed in retinal endothelial cells (RECs); that RECs are sensitive to the mitogenic and motogenic effects of HGF at physiologically relevant concentrations; and that PI3 kinase, protein kinase C (PKC), and MAP kinase pathways are involved in this response, strongly suggest that HGF plays an important role in mediating intraocular neovascularization in conditions such as PDR.

**METHODS**

**Cell Culture**

Bovine RECs (BRECs) were isolated from bovine eyes, as previously described,\textsuperscript{21} and cultured in endothelial basal medium (Clonetics, San Diego, CA) with 10% plasma-derived horse serum (PDHS; Wheaton, Millville, NJ), 50 mg/ml heparin, and 50 micrograms/ml endothelial cell growth factor (ECGF; Boehringer-Mannheim, Chicago, IL) in fibronectin-coated dishes. Cells at passages 3 through 9 were used for experiments. Studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Migration Assay**

Migration was performed using modified Boyden chambers containing polycarbonate membrane (Transwell, 8.0 µm pore size; Costar, Cambridge, MA) with slight modification from the previously described method.\textsuperscript{22} Cells were starved in 2% calf serum overnight and then seeded at 3 \times 10^5 cells/well on Transwell plates coated with collagen (10 µg/ml). Media containing HGF with or without inhibitors were added to the lower chamber. After incubation at 37°C for 5 hours, determined by initial time course experiments, the upper surface of the filter was scraped with a cotton-tipped stick to remove nonmigrated cells, and membranes were then fixed with 70% ethanol. Migrated cells were counted using automated computer software (Phase 3 imaging system; Media Cybernetics, Silver Spring, MD) under an inverted microscope (AX70TRF; Olympus, Tokyo, Japan) using a \times 40 objective after nuclear staining with green nucleic acid stain (Molecular Probes, Leiden, The Netherlands).

**Growth Assay**

Cells were seeded into 12-well plates at a density of 1 \times 10^{6} cells/well, and PDHS in the media was reduced to 1% the following day. Cells were then exposed to HGF at the concentra-

**MAPK Phosphorylation**

MAP kinase phosphorylation was evaluated by Western blot analysis. After overnight starvation in 2% PDHS, cells were stimulated with HGF at the indicated dose and time. Cells were lysed in \times 1 Laemmli buffer (50 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], and 10% glycerol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 2 micrograms/ml aprotonin, 10 micrograms/ml leupeptin, 1 mM NaF, 0.5 mM Na3VO4). Phospho-MAPK and total MAPK were detected using anti-phospho-specific antibodies (ERK1 and ERK2; New England Bio-Labs, Beverly, MA), anti-total MAPK antibodies (Santa Cruz Biotech, Santa Cruz, CA) and a chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL), according to the manufacturer’s instructions, on identical aliquots from identical cell lysates on tandem-run gels. All inhibitors were added to cells for 30 minutes before HGF treatment.

**PKC and PI3 Kinase Activity Assays**

In situ PKC and PI3 kinase activity were assayed as previously described.\textsuperscript{23} For in situ PKC activity, starved cells were stimulated with HGF (50 ng/ml) for 10 minutes before addition of PKC-specific peptide substrate (RKRTLRLR). After 15 minutes’ incubation, the reaction was stopped with 20% trichloroacetic acid. The PKC-dependent phosphorylated peptide substrate bound to the filter was quantified by scintillation counting. PI3 kinase activity was measured on silica gel thin-layer chromatography (TLC) plates. After HGF stimulation (50 ng/ml) for 5 minutes, BREC was lysed and immunoprecipitated with anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). The PI3 kinase reaction was initiated by the addition of 5 micrograms adenosine triphosphate (ATP; 0.5 mM) containing 30 uCi [γ32P]ATP for 10 minutes and stopped by addition of 20 micrograms HCl (8 N) and 160 micrograms chloroform-methanol (1:1). Activity was measured as generation of PI3-phosphate (PIP) from phosphatidylinositol.

**[3H]-Thymidine Uptake**

BREC was seeded into 24-well plates at a density of 1 \times 10^{4} cells/well. The media were replaced with Dulbecco’s modified Eagle’s medium (DMEM) with 2% PDHS the next day. After 24 hours, cells were stimulated by HGF, with or without inhibitors, for 18 hours. [3H]-thymidine (NEN, Boston, MA) was then added (0.25 uCi/well) for an additional 6 hours,\textsuperscript{24} after which cells were washed, fixed, and lysed, and [H]-thymidine uptake was determined by scintillation counting, as previously described.\textsuperscript{25}

**RT-PCR for HGF and c-Met**

Reverse transcription–polymerase chain reaction (RT-PCR) for HGF and c-Met was performed according to the method reported by Parrott and Skinner.\textsuperscript{26} The cDNA was produced by RT (Perkin–Elmer, Foster City, CA) at 42°C for 15 minutes. The forward and reverse primers used for HGF were 5’-ACA GCT TTI TGC CTT CGA GCA GCT ATC GGG GTA AAG ACC TAC AGG-3’ and 5’-CAT CAA ACC CCT TGT CGG GAT A-3’, which generate a 292-bp PCR product. The c-Met primers were 5’-GTA AGT GCC CCA AGT GTA AGG-3’ and 5’-GCC CTC TTC CTA TGA CTT C-3’, which generate a 313-bp PCR product. PCR products
were gel purified, subcloned using a kit (Topo TA; Invitrogen, Carlsbad, CA), and sequenced in both directions to confirm their identities.

RESULTS

HGF and c-Met Expression
To determine whether HGF and its receptor c-Met were expressed in BRECs, RT-PCR was performed on RNA isolated from confluent BRECs. Primers were expected to yield PCR products of 292 and 313 bp for HGF and c-Met, respectively. PCR products of the expected size for HGF and c-Met were readily detected in BRECs (Fig. 1). The PCR fragments were subcloned and sequenced in both directions, confirming their identity. An additional higher molecular weight PCR product (~390 bp) of unknown origin was also detected for HGF. These findings are consistent with results previously reported in bovine ovarian follicles and could represent a splicing variant, a higher molecular weight precursor, or PCR primer cross reactivity with another molecule.

HGF-Stimulated Growth and Migration
To determine whether physiologically relevant doses of HGF could induce BREC growth, cells were subjected to HGF dose-response (Fig. 2a) and time-course evaluation (Fig. 2b). Cell number was significantly increased by HGF concentrations as low as 5 ng/ml (45%, P < 0.05) after 4 days and was maximally stimulated 4.1-fold at 100 ng/ml (P < 0.001). Cell growth was significantly increased by HGF (25 ng/ml) as early as 2 days after stimulation (38%, P < 0.05) and was increased 2.8-fold after 4 days (P < 0.001). HGF-stimulated cell number continued to increase, even after 4 days of stimulation.

The effect of HGF on BREC migration was evaluated using Transwell plates (Fig. 3). HGF increased BREC migration in a dose-dependent manner by 1.1-, 1.7- (P < 0.001), and 3.4-fold (P < 0.001) after 5 hours in 5, 25, and 50 ng/ml HGF, respectively.

HGF-Stimulated MAPK Phosphorylation
Previous studies have shown that activation of MAPK activity (p44 and p42) is required for cell proliferation and migration and that HGF can stimulate MAPK in human corneal epithelial cells, mouse keratinocytes, and rat hepatocytes. To examine whether HGF activates MAPK in BRECs, we evaluated HGF-induced MAPK phosphorylation using ERK1 and ERK2 phospho-specific Western blot analysis. As shown in Figure 4, rapid phosphorylation of p44 and p42 was observed within 5 minutes after HGF (25 ng/ml) stimulation (P < 0.01), reaching a maximal 80- to 110-fold increase after 15 minutes (P < 0.001). MAPK phosphorylation (p42 and p44) remained significantly elevated for more than 30 minutes (Fig. 4), and was 9- to 42-fold and 6- to 14-fold elevated after 45 minutes and 2 hours, respectively, only returning to baseline levels after 4 hours (data not shown). Phosphorylation of both p42 and p44 after 10 minutes (Fig. 5) was significantly elevated at HGF concentrations as low as 5 ng/ml (6.7-fold, P < 0.05) and was maximally stimulated 21- to 42-fold at an HGF concentration of 50 ng/ml (P < 0.001).

FIGURE 1. BRECs expressed HGF and its receptor c-Met. Total RNA was isolated from REC, and RT-PCR was performed using specific primer pairs for HGF (lane 2) and its receptor c-Met (lane 3). A 100-bp DNA ladder marker is shown in lane 1. The predicted and observed size of HGF and c-Met PCR fragments were 292 bp and 313 bp, respectively. A representative experiment is shown; similar results were observed in more than three independent experiments.

FIGURE 2. HGF-stimulated BREC growth. BREC (1 × 10³ cells/well) were seeded into 12-well plates. HGF was added at the indicated concentration (A) or at 25 ng/ml (B) the following day. Cells were counted using a hemocytometer after 4 days (A) or at the indicated times after HGF addition (B). Values are expressed as mean ± SE and P is in comparison with baseline (A) or the same day (B). Results of three independent experiments, each performed with quadruplicate wells per point.
PI3 Kinase, PKC, and MAPK-Mediated HGF Action

To begin to elucidate the mechanisms by which HGF stimulates BREC migration and growth, we investigated whether PI3 kinase, PKC, and/or MAPK pathways were involved in HGF action using selective inhibitors at previously characterized appropriate concentrations: PI3 kinase inhibitors (wortmannin and LY294002), PKC inhibitors (GFX and LY333531), and MAPK inhibitor (PD98059). HGF-induced MAPK phosphorylation was slightly inhibited by GFX at 5 and 10 μM, but this did not reach statistical significance (Fig. 6a). LY333531 at a high, non–isoform-selective dose (200 nM) showed a similar result. There was no effect with LY333531 at 20 nM, a PKC-β isoform-selective concentration. PI3 kinase inhibitors were more efficient in blocking HGF-induced MAPK phosphorylation. Wortmannin inhibited MAPK phosphorylation by 30% and 66% at 0.1 μM and 1 μM, respectively (P < 0.01). LY294002 suppressed MAPK phosphorylation by 74% at 30 μM (P < 0.05) and 90% at 50 μM (P < 0.01). Wortmannin degrades more rapidly than LY294002 in culture, and that may account for the different extent of inhibition. Additive inhibition of MAPK phosphorylation (66%, P < 0.05) was observed when 5 μM GFX and 0.1 μM wortmannin were combined. Higher inhibitor concentrations resulted in substantially greater inhibition of migration (1 μM wortmannin: 57%, P < 0.001; 10 μM GFX: 74%, P < 0.001). We also confirmed the critical role the MAPK pathway in REC migration using MAPK inhibitor PD98059, which completely inhibited cell migration at 25 nM.

The role of PI3 kinase and PKC in HGF-induced BREC proliferation was evaluated using [H]-thymidine uptake (Fig. 6C). Because long-term exposure of GFX and LY294002 are not tolerated by RECs, those compounds were not evaluated. LY333531 at 200 nM is a non–isoform-selective PKC inhibitor similar to GFX but with less toxicity than GFX, presumably because of greater PKC specificity. HGF at 25 ng/ml increased [H]-thymidine uptake 96% (P < 0.001), a response inhibited 17% by wortmannin (0.1 μM) and 18% by the non–isoform-selective concentration (200 nM) of LY333531 (P < 0.01). Combination treatment resulted in additive inhibition of 30% (P < 0.01). MAPK inhibitor PD98059 (25 μM) completely inhibited HGF-induced [H]-thymidine uptake (P < 0.001). Control cells treated with combined wortmannin and LY333531 had no effect, whereas PD98059 treatment of control cells reduced baseline growth by 30% (P < 0.001).

HGF-Stimulated PI3 Kinase and PKC Activity

To further evaluate the role of PI3 kinase and PKC pathways in mediating HGF action on BREC, we measured the ability of...
HGF to increase PI3 kinase and PKC activity. PI3 kinase activity increased 4.5-fold within 5 minutes of HGF (50 ng/ml) stimulation (Fig. 7, *P*, 0.001). In situ PKC activity increased 37% (1.24 ± 0.13 to 1.70 ± 0.14 picomoles/mg protein per minute) after 10 minutes of similar treatment (*P*, 0.05, data not shown).

HGF-Induced VEGF-Independent MAPK Phosphorylation and Mitogenesis

Because HGF stimulates VEGF expression in several cell types,37–41 we evaluated whether HGF-induced MAPK phosphorylation (Fig. 8a) or mitogenesis (Fig. 8b) was mediated by VEGF. Although VEGF-neutralizing antibody inhibited VEGF-induced MAPK phosphorylation 61% and 88% (p42 and p44, respectively), it did not suppress HGF-induced MAPK phosphorylation (Fig. 8a). VEGF-neutralizing antibody had little effect on unstimulated cells. Similarly, VEGF-neutralizing antibody completely inhibited VEGF-induced thymidine uptake (108%, *P*, 0.01) and slightly increased HGF-induced thymidine uptake (24%, *P* = NS, Fig. 8b). VEGF-neutralizing antibody did not have a statistically significant effect on unstimulated cells.

**DISCUSSION**

Numerous ocular diseases result in the growth of blood vessels within the eye. The development of this intracocular neovascularization is a complex process thought to involve modulation of numerous factors with stimulatory, permissive, synergistic, or inhibitory activities toward angiogenesis. Several growth factors have been extensively evaluated to determine their roles in mediating intraocular angiogenesis, particularly with regard to the ischemic retinopathies such as PDR. Currently, VEGF is thought to be a principle stimulator of angiogenesis in these conditions,5 with growth hormone and IGF-1 being permissive of neovascularization, bFGF being highly synergistic with VEGF,24 and pigment epithelial-derived factor possibly being inhibitory.43 However, because the initiation and control of angiogenesis is such a complex process, it is likely that there are other factors that may also contribute significantly to this response.

Hepatocyte growth factor (HGF), also known as scatter factor, is a well described mitogen, motogen, and morphogen in many nonocular cells and tissues.11–14 In the eye, HGF and its receptor c-Met have been reported only in endothelial and epithelial cells of the cornea,15,43 human trabecular meshwork,16 and retinal pigment epithelial cells.17 HGF is also found in the aqueous humor,44 lacrimal gland, and tears.45 However, recent studies have identified HGF in the vitreous.
HGF and its receptor c-Met are expressed in RECs. Furthermore, HGF was very effective at stimulating REC growth and migration (two critical components of the angiogenic response) in a dose- and time-dependent manner. Growth was induced by HGF concentrations as low as 5 ng/ml ($P < 0.05$), which corresponds with the vitreous concentration of HGF measured in patients with PDR (5.7 to 6.0 ng/ml). $^{19,20}$ Vitreous concentrations of HGF exceeded 22 ng/ml in some patients with PDR, $^{19,20}$ a concentration that, in our studies, induced a 2.8-fold increase in REC growth after 4 days ($P < 0.001$) and a 70% increase in migration after 5 hours ($P < 0.001$). Local concentrations of HGF in the retina may be considerably greater than those measured in the vitreous, suggesting that the growth and migration responses could be even more marked, since we observed maximal HGF response at 50 to 100 ng/ml.

The bioactivity of HGF receptors on REC is supported by the rapid increase of MAPK phosphorylation, P3 kinase activity, and PKC activity induced by physiologically relevant concentrations of HGF. MAPK phosphorylation was rapid, marked, and prolonged, suggesting that it could play a significant role in the intracellular HGF signaling pathway. Indeed, inhibition of MAPK suppressed both HGF-induced migration and growth. Furthermore, multiple inhibitors of P3 kinase and PKC each partially suppressed HGF-induced MAPK phosphorylation, migration, and growth. When combined, the effects of P3 kinase and PKC inhibition were additive. These findings suggest that activation of P3 kinase and PKC occur upstream of MAPK activation and that the pathways may function at least partially in parallel. The $\beta$ isoform of PKC did not appear to be predominantly responsible for this effect, as assessed using the PKC-$\beta$ isoform-selective inhibitor LY335531. Activation of PKC by HGF in rat hepatocytes has been previously suggested using additional PKC inhibitors. $^{31}$

Of course, the caveats involved with the use of inhibitors, including inhibitor effects on other molecules or incomplete action, cannot be ruled out by these studies. Indeed, such issues may account for the finding in these studies that although 10 $\mu$m GFX and LY294002 each effectively suppressed migration, GFX did not substantially inhibit HGF phosphorylation. This could result from a diminished GFP effect on MAPK under MAPK phosphorylation conditions compared with migration conditions, or it could be that GFP only partially inhibits MAPK phosphorylation but also elicits another independent effect that suppresses migration, even though effective MAPK inhibition itself would significantly inhibit migration. However, the likelihood that both P3 kinase and PKC pathways are actually involved is increased by our observations of similar effects using multiple inhibitors of the same pathway and by directly demonstrating that HGF activates both P3 kinase and PKC. In addition, although these studies do not address the role, if any, of Ras, because simultaneous inhibition of PKC and P3 kinase did not completely suppress MAPK activity, it is possible that the Ras pathway may have a critical role in HGF signaling. $^{29,45,46}$

Several known monoclonal attributes of HGF further support its potential role in mediating intraocular angiogenesis. HGF-induced endothelial cell growth is augmented by bFGF, a factor that appears to potentiate retinal neovascularization, but that does not initiate it. $^{48}$ Although that report did not find an additive effect with VEGF, another study demonstrated that HGF and VEGF activity on endothelial cell proliferation and

![Image](https://iovs.arvojournals.org/pdfsaccess.ashx?url=/data/journals/iovs/932906/)
malignant tumors,49 and HGF expression is increased by myo-

valve cells,40 and human smooth muscle cells.41 VEGF is also known to activate PKC, MAPK, and PI3 kinase.23 These data suggest that HGF action may be mediated through increases in VEGF. However, VEGF-neutralizing antibody, which was capable of inhibiting VEGF-induced MAPK phosphorylation and mitogenesis, did not significantly effect these HGF-induced effects. Thus, at least the initial HGF-induced MAPK phosphorylation and early HGF-induced mitogenesis do not appear to be mediated through VEGF. These data suggest that HGF may have an independent role in mediating intraocular complications.

In summary, microvascular (RECs) express HGF and its receptor, and HGF induces REC growth and migration. These effects are observed at HGF concentrations known to occur in patients with PDR, in which vitreous concentrations of HGF are elevated. Furthermore, these responses appear to be at least partially mediated by HGF-induced activation of PI3 kinase, PKC and MAPK pathways, but appear not to be initially mediated by VEGF. These findings strongly suggest that HGF is capable of serving an important, perhaps independent role in the mediation of retinal neovascularization. Determination of the actual contribution of HGF to intraocular angiogenesis relative to other growth factors awaits evaluation of HGF effects in animal models of appropriate disease states and is the focus of ongoing studies.

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