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REVIEW

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Hepatocyte growth factor in lung morphogenesis and tumor invasion: role as a mediator in epithelium-mesenchyme and tumor-stroma interactions

Abstract Hepatocyte growth factor (HGF), a ligand for Met tyrosine kinase, is a mesenchyme- or stroma-derived multipotent factor that regulates the growth, motility, and morphogenesis of various types of cells. During lung development, Met/HGF receptor mRNA was localized in lung epithelial cells, whereas HGF mRNA was localized in lung mesenchymal cells in rat embryos. Antisense HGF oligonucleotides specifically inhibited epithelial branching morphogenesis in cultured lung rudiment isolated from day-13 rat embryos, whereas recombinant HGF stimulated branching morphogenesis. Thus, HGF seems to be at least one of the mesenchyme-derived factors that support branching morphogenesis during lung development. Together with the finding that HGF plays important roles in organogenesis and morphogenesis of organs such as the liver and kidney, HGF seems to be a mediator in epithelium-mesenchyme interactions during organogenesis. Extending the conceptual framework of epithelium-mesenchyme (or epithelium-stroma) interactions, we next examined the possible involvement of HGF in tumor-stroma interactions, because the growth and motility of carcinoma cells are regulated through their interactions with host stromal cells. HGF induced in vitro migration and invasion of GB-d1 gallbladder carcinoma cells into basement membrane components and collagen-gel matrix; however, several other growth factors did not induce marked migration and invasion of the carcinoma cells. GB-d1 cells do not produce HGF, but they produce an inducing factor for HGF production in fibroblasts; the inducing molecule was identified as interleukin 1β. Cocultivation of GB-d1 cells with stromal fibroblasts embedded in a collagen-gel matrix induced invasion of GB-d1 cells into the collagen gels, but invasion was inhibited by a specific antibody against HGF. This indicates that in vitro invasion of GB-d1 cells depends on stromal fibroblasts and that the fibroblast-derived invasion factor is HGF. Since HGF stimulated in vitro migration and invasion of various carcinoma cells and several carcinoma cells produced inducing factors for HGF production in stromal fibroblasts, the looped interaction of carcinoma cells and stromal fibroblasts mediated by HGF and HGF inducers may be a mechanism responsible for acquisition of the malignant phenotype through tumor-stroma interactions.

Key words Epithelium-mesenchyme interactions · Hepatocyte growth factor · Met · Tumor invasion · Tumor-stroma interactions

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Introduction

Hepatocyte growth factor (HGF) was initially identified as a potent mitogen for mature hepatocytes in primary culture [17]. By 1987, HGF had been purified [18], and it was molecularly cloned in 1989 [19]. HGF is a heterodimeric molecule consisting of a 4-kringle-containing α-chain and a serine protease-like β-chain [18, 19]. HGF binds to the c-met protooncogene product, which is a heterodimeric tyrosine kinase composed of a 50-kDa α-chain and a 145-kDa β-chain [3, 20].

In addition to its initial role as a potent hepatotrophic factor responsible for vigorous regeneration of the liver, HGF has multipotent characteristics [9, 12, 36]. Characterization and molecular cloning of cDNAs for scatter factor [5, 21, 33] and tumor cytotoxic factor [28] revealed that these molecules are identical to HGF. Scatter factor was originally identified as a fibroblast-derived factor with the ability to scatter tightly growing epithelial cell colonies [6]. Subsequently, a fibroblast-derived epithelial morphogen that induces branching tubulogenesis was identified as HGF [16]. HGF has trophic roles for the regeneration and maintenance of various tissues and organs [9, 12]. Recent extensive studies on HGF expression and functional anal-

ysis of HGF during embryogenesis have revealed a distinct role of HGF as a mediator in morphogenic epithelium-mesenchyme interactions [1, 10, 16, 30, 31].

Interactions between epithelium and mesenchyme mediate crucial aspects of normal development, affecting tissue induction, organogenesis, and morphogenesis of specific multicellular structures [7, 25]. The development and morphogenesis of various organs and tissues, including the kidney, lung, liver, pancreas, limb, tooth, and hair follicle, among others, depend on epithelium-mesenchyme interactions, and such interactions (or epithelium-stroma interactions in adults) are also believed to be important in regeneration, morphogenesis, and functional differentiation of epithelial tissues. Importantly, an extended interpretation of the conceptual framework of epithelium-mesenchyme (or epithelium-stroma) interactions is likely to be applied to the specific interactions between carcinoma cells (tumor cells originating from epithelial cells) and host stromal cells. In vivo growth of carcinoma cells is markedly accelerated by a broad spectrum of fibroblasts [4, 22], and in vitro invasion of oral squamous-cell carcinoma cells is induced by cocultivation with stromal fibroblasts [13]. These findings have led to the notion that the growth, invasion, and metastatic potential of tumor cells are influenced by their interaction with normal stromal fibroblasts; thus, molecular mechanisms underlying these tumor-stroma interactions are of current interest in tumor biology.

In this paper we show that HGF is a mesenchyme-derived factor that supports epithelial branching morphogenesis during lung development and that the looped interaction of carcinoma cells and stromal fibroblasts mediated by HGF and HGF inducers is a mechanism in tumor-stroma interactions that confers an invasive phenotype on carcinoma cells.

HGF as a mediator in morphogenic epithelium-mesenchyme interactions

During lung development, lung buds, the origin of the lung, sprout from the foregut endodermal tissue on embryonic day 10 in rats, and the primitive bronchial epithelia undergo branching tubulogenesis during embryonic days 13–18. Subsequently, epithelial cells at the bronchial tips differentiate to form alveolar structures. Classic experiments using in vitro organ-culture systems have shown that the isolated lung bud epithelium does not undergo branching morphogenesis, but when lung bud epithelium is cultured with lung-bud mesenchymal cells, it does. This indicates that a mesenchyme-derived factor is responsible for lung epithelium morphogenesis, but such a mesenchyme-derived factor has not yet been identified.

To determine the involvement of HGF in lung organogenesis, we first analyzed the in situ localization of HGF and Met/HGF receptor mRNA in the developing lungs of day-13 rat embryos. Met/HGF receptor mRNA is specifically expressed in lung epithelia, whereas HGF mRNA is localized to mesenchymal cells. Thus, HGF is produced in lung mesenchyme, but it targets developing lung epithelium.

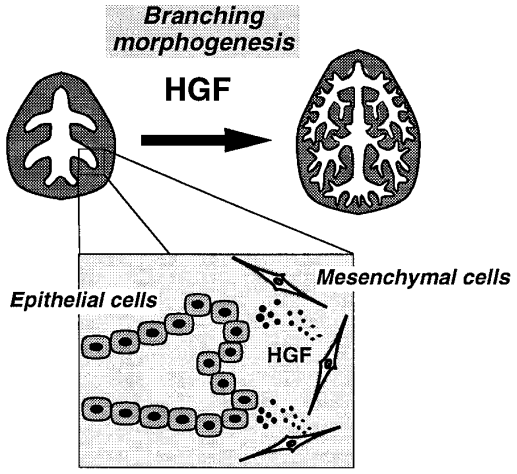

Fig. 1 Possible function of HGF as a mesenchyme-derived factor that induces branching morphogenesis during lung organogenesis

For analysis of the biological function of HGF, lung rudiments were isolated from day-13 rat embryos and cultured in the presence of sense or antisense HGF oligonucleotide to inhibit the expression of HGF in lung mesenchyme. In the presence of sense HGF oligonucleotide, lung epithelia underwent extensive branching morphogenesis within 48 h. However, branching morphogenesis in cultured lung rudiments was inhibited by antisense HGF oligonucleotide. Consistent with this finding, when lung rudiments were cultured in the presence of recombinant HGF, exogenous HGF stimulated extensive branching morphogenesis of lung epithelium. On the basis of these results, we conclude that HGF is at least one of the mesenchyme-derived factors that induce branching tubulogenesis during lung organogenesis (Fig. 1).

The involvement of HGF in organogenesis and morphogenesis of the kidney and mammary gland has been noted [1, 31], and targeted disruption of the HGF gene in mice results in impaired development of the placenta and liver [26, 32]. The essential role of HGF in liver development has recently been demonstrated using in vivo loss-of-function mutation in *Xenopus* embryos. Overexpression of mutant tyrosine kinase-minus (TK$^-$Met) c-Met/HGF receptor in *Xenopus* embryos resulted in liver defects, in impaired development of the pronephros and gut, and in impaired skeletal morphogenesis in the tail regions (Matsumoto et al., unpublished results). These results indicate that HGF and the c-Met/HGF receptor are highly conserved molecules that are active in the development of internal organs such as the liver in species ranging from amphibians to mammals, functioning as mediators in morphogenic epithelium-mesenchyme interactions.

HGF in tumor invasion and tumor-stroma interactions

On the basis of the notion that HGF is a mesenchyme- or stroma-derived multipotent factor that regulates organogenesis and organ regeneration, we hypothesized that it might

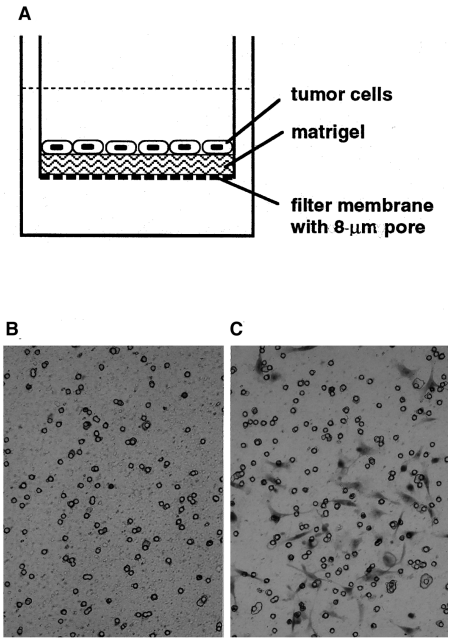

Fig. 2 A–C Induction of in vitro invasion of GB-d1 gallbladder carcinoma cells through Matrigel basement-membrane components. **A** Schematic representation of the in vitro invasion assay method. **B**, **C** Appearance of cells that have invaded through the gel to the lower side of a membrane with 8-μm pores in **B** the absence or **C** presence of HGF at 30 ng/ml. GB-d1 cells were plated on Matrigel basement-membrane components at a density of 10^5 cells/cm^2 and then cultured for 24 h. The medium was replaced with William's E medium supplemented with 5% fetal calf serum, HGF was added, and the cells were cultured for a further 24 h

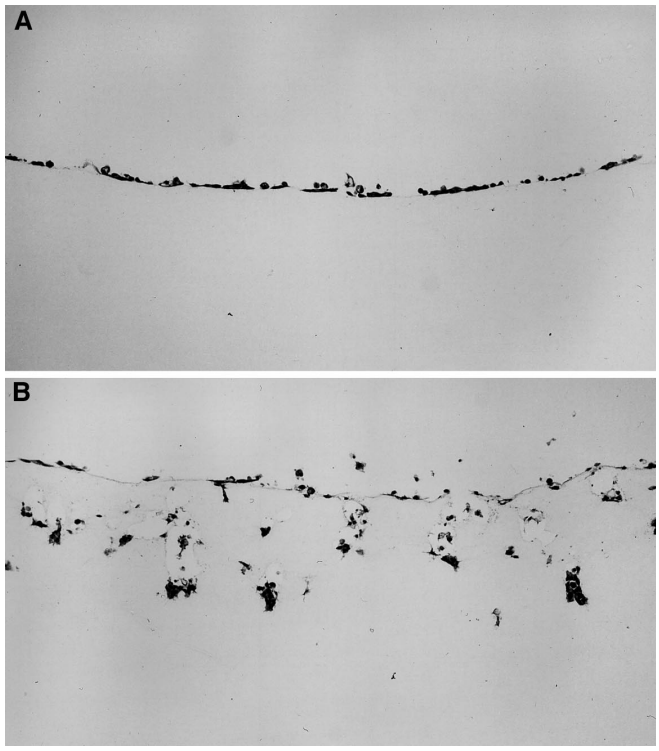

Fig. 3 A, B Induction of in vitro invasion of GB-d1 gallbladder carcinoma cells into a collagen-gel matrix. GB-d1 cells were plated on the collagen-gel matrix (2.1 mg/ml type I collagen) at a density of 10^5 cells/cm^2 and then cultured for 24 h. The medium was replaced with William's E medium supplemented with 5% fetal calf serum and the cells were cultured in the **A** absence or **B** presence of HGF at 10 ng/ml for 12 days. Cells were fixed, sectioned, and stained with hematoxylin

have an important function in tumor-stroma interactions. In an initial study, we utilized the GB-d1 gallbladder-carcinoma cell line, established from a patient with gallbladder cancer, because gallbladder cancer is a digestive tract cancer showing rapid invasive and metastatic progression of cancer cells [29] and, thus, has a high mortality rate.

The motogenic activity of HGF implied that HGF might affect the invasiveness of GB-d1 cells, and we tested this possibility using an in vitro invasion model (Fig. 2A). GB-d1 gallbladder carcinoma cells were cultured on Matrigel basement-membrane components, and growth factors were added into the outer cup such that they reached the cells via the lower basement-membrane components (Fig. 2A). In this system, when the invasive potential of cells is up-regulated, the cells migrate through the basement-membrane components and the 8-μm pores of the filter membrane to the lower side of the membrane.

In the absence of growth factors, GB-d1 cells did not migrate through the membrane (Fig. 2B). However, the numbers of migrating cells were increased by the addition of HGF (Fig. 2B). HGF dose-dependently stimulated migration of cells; maximal stimulation was seen with HGF at 10–30 ng/ml. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) stimulated low-level migration of GB-d1 cells, but platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) had no effect.

We next designed an in vitro invasion model to analyze invasion of cancer cells into stromal tissue. GB-d1 cells were cultured on the top of a 1-mm-thick collagen-gel matrix, with HGF being allowed to penetrate the gel and reach the cells from below. Although the GB-d1 cell line was originally established from a lymph node metastasis of gallbladder carcinoma and invades surrounding tissues when intradermally implanted into athymic mice, the cells did not invade the collagen-gel matrix in the absence of HGF (Fig. 3). In contrast, GB-d1 cells invaded collagen gels when cultured in the presence of HGF at 10 ng/ml. Collagen gels were locally degraded by invading cells, indicating that HGF may induce (and/or activate) proteases responsible for enzymatic degradation of collagen gels.

Since several growth factors are known to influence cell motility, we next investigated whether the invasiveness of GB-d1 cells was affected by other typical growth factors in the same culture system. Although HGF induced significant invasion by carcinoma cells, such a marked effect was not seen with other growth factors. EGF had only a minimal inducing effect on invasiveness, and its potency was less than that of HGF; TGF-β1, PDGF, and bFGF did not induce invasion. These results indicate that HGF is a potent invasion-inducing factor for GB-d1 cells and that the

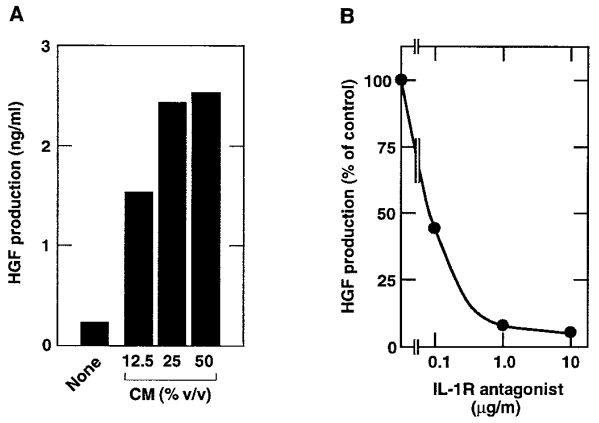

Fig. 4 A, B HGF-inducing activity of conditioned medium from GB-d1 gallbladder carcinoma cells and its inhibition by an IL-1 receptor antagonist. **A** Dose-dependent stimulation of HGF production in normal human skin fibroblasts by the addition of GB-d1 cell-conditioned medium (*CM*). HGF production by fibroblasts over a 24-h culture period was measured by enzyme-linked immunosorbent assay. **B** Inhibition of HGF-inducing activity in GB-d1 cell CM by an IL-1 receptor (*IL-1R*) antagonist. Human skin fibroblasts in CM were cultured for 24 h in the absence or presence of human recombinant IL-1R antagonist

Table 1 HGF inducers produced by various human carcinoma cell lines[a] (*ND* Not determined)

Cell line	Origin	HGF-inducing activity	HGF inducer
Lu99A	Small-cell lung carcinoma	+++	IL-1
SBC-3	Small-cell lung carcinoma	+++	bFGF, PDGF
EBC-1	Small-cell lung carcinoma	+	ND
A549	Lung adenocarcinoma	+++	IL-1, bFGF
A431	Epidermoid carcinoma	+++	IL-1
ME180	Uterus cervical carcinoma	++	IL-1, bFGF
KB	Oral squamous-cell carcinoma	++	ND
SAS	Tongue squamous-cell carcinoma	+++	IL-1
MKN-28	Stomach adenocarcinoma	+++	IL-1α
HuCCT1	Cholangiocarcinoma	+++	bFGF
T98G	Glioblastoma	+	ND
Bows	Melanoma	+++	IL-1
NEC-8	Testicular germ-cell tumor	++	bFGF, PDGF
GB-d1	Gallbladder carcinoma	+++	IL-1β

[a] Conditioned media from various carcinoma cell lines were added to normal human skin-fibroblast cultures, and HGF produced by the fibroblasts during a 24-h culture period was measured using an enzyme-linked immunosorbent assay as described elsewhere 14

induction of invasiveness is fairly specific to HGF, at least among the growth factors tested.

Although GB-d1 gallbladder carcinoma cells do not produce HGF, we found that HGF production in normal human dermal fibroblasts was markedly stimulated by a soluble factor derived from GB-d1 gallbladder carcinoma cells. The conditioned medium of GB-d1 cells was added to cultures of normal skin fibroblasts, and the HGF concentration in the culture medium was determined after 24 h of culture (Fig. 4A). Addition of the GB-d1 cell-derived conditioned medium resulted in dose-dependent stimulation of HGF production by normal fibroblasts, indicating that GB-d1 cells secrete a soluble factor(s) that stimulates HGF production.

Of the various growth factors and cytokines identified, interleukin 1α (IL-1α), IL-1β [14], EGF, transforming growth factor-α, acidic fibroblast growth factor (aFGF), bFGF, HST-1/FGF-4, and PDGF stimulated HGF production in the human skin fibroblasts used in the present study (data not shown). Therefore, we tested whether GB-d1 cell-derived soluble inducer(s) for HGF is a known growth factor or cytokine using specific antibodies for the ligand or receptor. Among the antibodies tested, the stimulatory activity of the conditioned medium for HGF production was almost completely abrogated by an IL-1 receptor antagonist (Fig. 4B), and further analysis using specific antibodies indicated that the soluble inducer derived from GB-d1 cells was IL-1β.

On the basis of the above-mentioned results, we designed an in vitro model for tumor-stroma interactions. Fibroblasts obtained from gallbladder stromal tissue were cultured in collagen gels and GB-d1 cells were cultured on the gel. When the cells were cultured for 10 days under these conditions, GB-d1 cells invaded the collagen gel (Fig. 5). Notably, GB-d1 cell invasion was almost completely inhibited by anti-HGF antibody. These results indicate that GB-d1 cell invasion depends on cocultured stromal fibroblasts and that the fibroblast-derived invasion factor for GB-d1 cells is HGF.

To extend our hypothesis that tumor-stroma interactions may be mediated by stroma-derived HGF and carcinoma cell-derived inducers for HGF expression, we analyzed the HGF-inducing activity in conditioned media from various carcinoma cells. Of 40–50 carcinoma cell types derived from various tissues, 14 produced inducing factors for HGF production in skin fibroblasts (Table 1). Using specific antibodies and antagonistic molecules, the HGF inducers derived from these carcinoma cells were identified as IL-1 (IL-1α or IL-1β), bFGF, and PDGF. Since HGF stimulates cell migration and these cells do not produce HGF (not shown), HGF inducers derived from these carcinoma cell types may up-regulate HGF production in stromal cells. Thus, HGF seems to function as a stroma-derived invasion factor for these cells.

Discussion

The growth, invasion, and metastatic potential of tumor cells are influenced by interactions between tumor cells and host stromal tissues. Molecular and cellular mechanisms for tumor-stroma interactions involve highly complicated processes, but stimulation of the growth and invasion of carcinoma cells through interactions with stromal cells has been noted in vivo and in vitro. Cocultivation of carcinoma cells on fibroblast-embedded collagen gels induces invasion of tumor cells [13], and coinoculation of carcinoma cells and fibroblasts into athymic mice results in augmented proliferation and increased invasive potential of

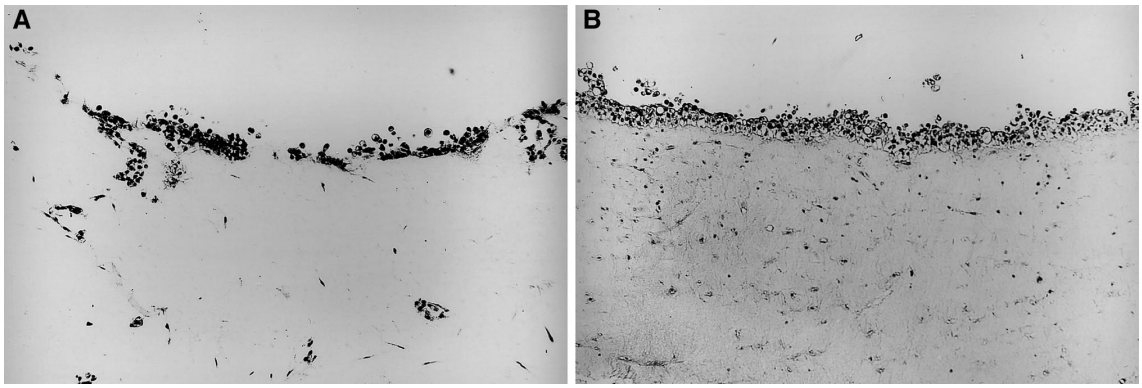

Fig. 5 A, B Inhibition of in vitro invasion of GB-d1 gallbladder carcinoma cells cocultured with human fibroblasts embedded in a collagen-gel matrix. Fibroblasts obtained from gallbladder stromal tissue were cultured in a collagen-gel matrix (10^4 cells/cm^3) and GB-d1 cells were plated on the gel. The cells were cultured for 10 days in the presence of **A** preimmune IgG or **B** anti-HGF IgG

the tumor cells [13, 22]. These results indicate the particular importance of interactions between carcinoma cells and stromal fibroblasts in regulating the growth and invasion of tumor cells. We recently identified a fibroblast-derived invasion factor for oral squamous-cell carcinoma cells as HGF [15]. The present study provides evidence that HGF is the predominant fibroblast-derived factor inducing the migration and invasion of GB-d1 gallbladder carcinoma cells. Moreover, it establishes that the carcinoma cells secrete an inducer of HGF production in fibroblasts and identifies the carcinoma-derived HGF-inducer as IL-1β. In a previous study we have shown that HGF acts as a mitogen for GB-d1 gallbladder carcinoma cells [29].

The establishment of the autocrine loop of growth factors and their receptors is known frequently to result in tumorigenic transformation of cells, and gene-transfer experiments indicate that autonomous activation of the Met/HGF receptor results in tumorigenic transformation [2, 11, 23]. Such autocrine activation of Met/HGF receptors may be involved in malignant transformation in certain tumor cells [8]; however, most carcinoma cells do not secrete HGF (Matsumoto et al., unpublished results). Nevertheless, apart from the autocrine activation of Met/HGF receptors, our present investigation and previous studies indicate that a paracrine interaction of HGF and Met/HGF receptor is important for the malignant behavior of carcinoma cells. It is noteworthy that GB-d1 gallbladder cancer cells secrete an inducing factor for HGF production in stromal fibroblasts. This finding has led to the hypothesis that a looped interaction between tumor cells and stromal fibroblasts may exist, in which a tumor-derived factor regulates stromal HGF production while stroma-derived HGF regulates the growth and invasion of tumor cells (Fig. 6). Importantly, we also found that various types of carcinoma cells secrete HGF inducers, including IL-1β, IL-1α, bFGF, and PDGF. Other groups have also noted the presence of tumor-derived inducing molecules for HGF [24, 27]. In this context, it is noteworthy that HGF levels in breast-tumor tissue extracts are a strong predictor of recurrence and survival in human breast cancer [35]. Since breast carcinoma cells do not express HGF [34], this finding suggests that stroma-derived HGF may affect malignant phenotypes of breast carcinoma cells in a paracrine manner.

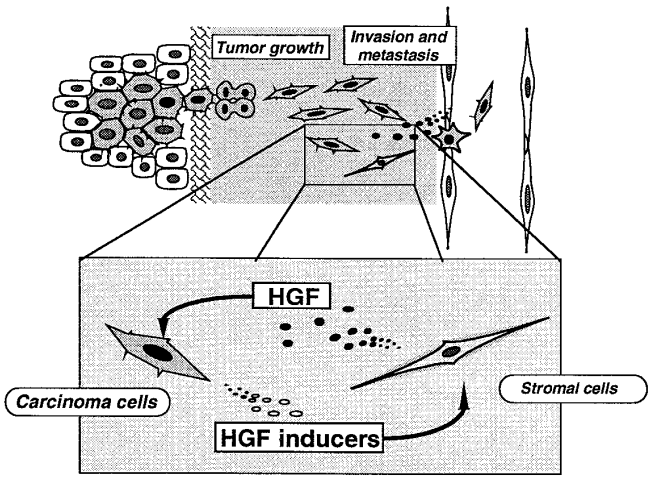

Fig. 6 Possible mechanisms of tumor-stroma interactions mediated by host stroma-derived HGF and carcinoma cell-derived HGF inducer

HGF is currently considered to be crucial as a mediator in epithelium-mesenchyme (or epithelium-stroma) interactions affecting cell growth and movement as well as the morphogenesis of specific tissue architecture during embryogenesis and organ regeneration. Our present study implies that epithelium-stroma interactions may function in tumor-stroma interactions that affect the growth and invasion of tumor cells via signals from surrounding stromal cells. Looped interaction between tumor cells and stromal cells mediated by HGF and its inducers may be involved in the acquisition of malignant phenotypes in various carcinoma cells. Identification and characterization of known and unknown HGF inducers may lead to the elucidation of further molecular mechanisms for tumor-stroma interactions; as based on our current results, the generation of antagonistic molecules may be a unique therapeutic tool to prevent tumor invasion and metastasis.

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