Cancer Research

HB-EGF Is a Potent Inducer of Tumor Growth and Angiogenesis

Pat P. Ongusaha, Jennifer C. Kwak, Andrew J. Zwible, et al.

Cancer Res 2004;64:5283-5290.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/64/15/5283

Cited Articles	This article cites by 49 articles, 19 of which you can access for free at: http://cancerres.aacrjournals.org/content/64/15/5283.full.html#ref-list-1
Citing articles	This article has been cited by 28 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/64/15/5283.full.html#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

[CANCER RESEARCH 64, 5283–5290, August 1, 2004]

HB-EGF Is a Potent Inducer of Tumor Growth and Angiogenesis

Pat P. Ongusaha,[1] Jennifer C. Kwak,[1] Andrew J. Zwible,[1] Salvador Macip,[2] Shigeki Higashiyama,[3] Naoyuki Taniguchi,[4] Li Fang,[5] and Sam W. Lee[1]

[1]*Cancer Biology Program, Hematology and Oncology Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts;* [2]*Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York;* [3]*Department of Medical Biochemistry, Ehime University School of Medicine, Ehime, Japan;* [4]*Department of Biochemistry, Osaka University Medical School, Osaka, Japan; and* [5]*Telik, Inc., Palo Alto, California*

ABSTRACT

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) has been shown to stimulate the growth of a variety of cells in an autocrine or paracrine manner. Although HB-EGF is widely expressed in tumors compared with normal tissue, its contribution to tumorigenicity is unknown. HB-EGF can be produced as a membrane-anchored form (pro-HB-EGF) and later processed to a soluble form (s-HB-EGF), although a significant amount of pro-HB-EGF remains uncleaved on the cell surface. To understand the roles of two forms of HB-EGF in promoting tumor growth, we have studied the effects of HB-EGF expression in the process of tumorigenesis using *in vitro* and *in vivo* systems. We demonstrate here that in EJ human bladder cancer cells containing a tetracycline-regulatable s-HB-EGF or pro-HB-EGF expression system, s-HB-EGF expression increased their transformed phenotypes, including growth rate, colony-forming ability, and activation of cyclin D1 promoter, as well as induction of vascular endothelial growth factor *in vitro*. Moreover, s-HB-EGF or wild-type HB-EGF induced the expression and activities of the metalloproteases, MMP-9 and MMP-3, leading to enhanced cell migration. *In vivo* studies also demonstrated that tumor cells expressing s-HB-EGF or wild-type HB-EGF significantly enhanced tumorigenic potential in athymic nude mice and exerted an angiogenic effect, increasing the density and size of tumor blood vessels. However, cells expressing solely pro-HB-EGF did not exhibit any significant tumorigenic potential. These findings establish s-HB-EGF as a potent inducer of tumor growth and angiogenesis and suggest that therapeutic intervention aimed at the inhibition of s-HB-EGF functions may be useful in cancer treatment.

INTRODUCTION

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a heparin-binding member of the EGF family (1), which was initially identified in the conditional medium of human macrophages (2). It is a potent mitogen and chemotactic factor for fibroblasts and smooth muscle cells (3–5). As with other EGF family members, HB-EGF binds and activates EGF receptors 1 and 4 (1, 6, 7). Moreover, HB-EGF has been shown to stimulate the growth of a variety of cells in an autocrine or paracrine manner and to be involved in stromal proliferation (8). HB-EGF is initially synthesized as a transmembrane protein of 208 amino acids (1). Although the membrane-anchored form of HB-EGF (pro-HB-EGF) is cleaved on the cell surface to yield a soluble growth factor of 75–86 amino acids, a considerable amount of pro-HB-EGF remains uncleaved on the cell surface (1). Pro-HB-EGF is not merely a precursor of the soluble form; it is biologically active such that it forms complexes with both CD9 (9) and integrin $\alpha_3\beta_1$ (10) and transduces biological signals to neighboring cells in a nondiffusible manner (7). The transmembrane form of HB-EGF is a juxtacrine growth factor, which is immobilized on the surface of the cell and interacts with neighboring cells (1). It has also been shown that the transmembrane HB-EGF synthesized by one type of cell can stimulate tyrosine phosphorylation of the EGF receptor in another type of cell in coculture (11). A recent study demonstrated that HB-EGF can bind to a novel 140-kDa receptor identified as *N*-arginine dibasic convertase, a metalloendopeptidase of the M16 family, and that binding to *N*-arginine dibasic convertase is highly specific for HB-EGF among EGF family members (12). Its specific binding modulates HB-EGF-induced cell migration via EGF receptor (12).

Several laboratories have described HB-EGF as being up-regulated in response to oncogenes and in oncogene-transformed cells (13, 14). In chicken embryo fibroblasts transfected with regulatable v-Jun, the expression of HB-EGF is greatly induced as v-Jun levels are increased (15), suggesting that HB-EGF plays an important part in mediating Jun-induced cell transformation. Furthermore, HB-EGF has been identified as an immediate-early response gene that can be activated by the Ras/Raf signaling pathway that mediates the autocrine activation of the c-Jun kinase in NIH3T3 cells (15). Phosphorylation of the transcription factor Ets-2 by activation of the Raf1/MAPK cascade regulates the induction of HB-EGF transcription in mouse fibroblasts (16). Additionally, in nontransformed human mammary epithelial cells, HB-EGF expression is induced by EGF and Ha-Ras overexpression (17), strongly implying that HB-EGF is a direct target of mitogen-activated protein kinase (MAPK). Our previous observations demonstrated that HB-EGF is induced in response to tumor suppressor p53, as well as DNA damage, and HB-EGF induction antagonizes apoptosis mediated by genotoxic stress through the activation of the Ras/Raf/MAPK cascade and the AKT pathway (18, 19), strongly suggesting a survival factor function for HB-EGF.

HB-EGF expression has been implicated in tumor progression because of its overexpression in many tumors, including hepatocarcinoma, colon, melanoma, myeloma, breast, prostate, and bladder tumors. It also has been implicated in increased proliferation and metastasis (20–27). Many tumor cells with HB-EGF overexpression are diphtheria toxin sensitive, suggesting that these cells accumulate the transmembrane form of HB-EGF. There is growing evidence of increased HB-EGF expression in tumors compared with normal tissue, *e.g.,* pancreatic (28), liver (23, 29), esophageal (30), melanoma (20), bladder (31), and gastric tumors (32). Although HB-EGF is widely expressed in tumors and may be enhanced compared with normal tissue, its contribution to tumorigenicity is unknown. Moreover, it is not known whether the membrane-bound or the soluble form of HB-EGF contributes to tumorigenic processes.

In this study, we examined the biological effects of the two forms of HB-EGF on tumor growth and angiogenesis, using a tetracycline (tet)-regulated expression system in human EJ bladder carcinoma cells, which have a low basal level of HB-EGF. EJ cells expressing soluble form HB-EGF (s-HB-EGF) or wild-type HB-EGF (wt-HB-EGF) resulted in an increased growth rate, activation of the cyclin D promoter, colony-forming ability, and tumor growth in athymic nude mice. We also found that s-HB-EGF induced vascular endothelial growth factor (VEGF) expression, implicating an autocrine loop that may play a role in regulating these growth factors. Additionally, in response to s-HB-EGF, cells induced the expression and activities of the metalloproteases MMP-9 and MMP-3, leading to enhanced cell

Received 3/17/04; revised 5/27/04; accepted 6/3/04.

Grant support: NIH Grants CA80058 and CA097216.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Sam W. Lee, Harvard Institutes of Medicine, 4 Blackfan Circle, Room 921, Boston, MA 02115. Phone: (617) 667-8563; Fax: (617) 667-0980; E-mail: slee2@bidmc.harvard.edu.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

migration. In this study, we provide evidence of the biological activity of HB-EGF in tumorigenesis.

MATERIALS AND METHODS

Cell Culture and Transfection of Different Forms of HB-EGFs. Human bladder carcinoma cell line, EJ, expressing a tet activator, was transfected with wt-, s-, or pro-HB-EGF cDNA/pTet-puro and selected by continuous growth in puromycin (2 μg/ml) to isolate stable tet-regulated clones. Mutant constructs and expression of s-HB-EGF and pro-HB-EGF were described previously (33). Tet-regulatable EJ-HB-EGF cells were maintained in the presence of tet (1–2 μg/ml) in DMEM plus 10% FBS, 75 μg/ml hygromycin, and 2 μg/ml puromycin. Induction of each form of HB-EGF expression was achieved by washing the cells three times with PBS followed by addition of culture media without tet. For zymograms for MMP activity, cell culture media was concentrated 50-fold and loaded on to a 10% zymogram gel (Invitrogen, Carlsbad, CA). The MMP gel was renatured by incubating in renaturing buffer (Invitrogen) for 30 min at room temperature, and the MMP activity was examined by incubating in developing buffer for 24 h. MMP bands were visualized by staining with simply blue dye reagent (Invitrogen). The concentrated culture media were also immunoblotted with the mouse anti-MMP-9 and anti-MMP-3 monoclonal antibodies (Neomarkers, Fremont, CA). For cell proliferation assay, three different EJ-HB-EGF cell lines expressing wt-HB-EGF, s-HB-EGF, and pro-HB-EGF, respectively, were seeded on 6-well plates with or without tet at a density of 2×10^4 cells/well. At days 2, 4, 6, and 8, cells were trypsinized, stained with trypan blue, and counted using a hemocytometer. Each data point was the average of three independent experiments.

Northern Blot and Western Blot Analyses. Total RNA was extracted, denatured, and electrophoresed through a 1% agarose-formaldehyde gel as described previously (19). Total protein extracts were immunoblotted with the respective antibodies. Tumors were dissected from the mice and snap frozen by liquid nitrogen. The tumors were then homogenized in lysis buffer containing 1% Triton X-100, 10 mM HEPES (pH 7.5), 130 mM NaCl, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, and 50 μg/ml aprotinin. Twenty μg of proteins were loaded into each well and separated by a 4–12% NuPAGE gel (Invitrogen).

Transwell Migration Assay. The cells were plated at a cell density of 2×10^5 cells in a 100-mm diameter dish. Each form of HB-EGF was induced by the removal of tet for 24 h, followed by serum starvation for another 24 h. Control cells were plated in similar conditions as HB-EGF-expressing cells, except that tet was added to suppress HB-EGF expression. Migration assays were performed using a chemotaxis chamber (Becton Dickinson and Co., Franklin Lakes, NJ) and transwell tissue culture plates (6.5 mm and 8-μm pore size). The bottom of the chamber was coated with either 10 μg/ml fibronectin, collagen I, or Matrigel (Sigma, St. Louis, MO). The uncoated sites were blocked with 10% BSA. One-hundred μl of 1×10^5 cells/ml were introduced into each well and were allowed to migrate for 6 h. Cells were then fixed with methanol and stained with crystal violet. The migrated cells were quantified by counting the number of cells in five random ×200 fields. Each experiment was done in triplicates, and the experiment was repeated twice.

Wound-Healing Assay. HB-EGF was expressed for 24 h in the absence of tet, and control cells were seeded in the same fashion, except in the presence of tet. Cells were then seeded in a 6-well plate at a density of 1×10^6 cells in the presence or absence of tet for 24 h. A wound was made using the tip of a

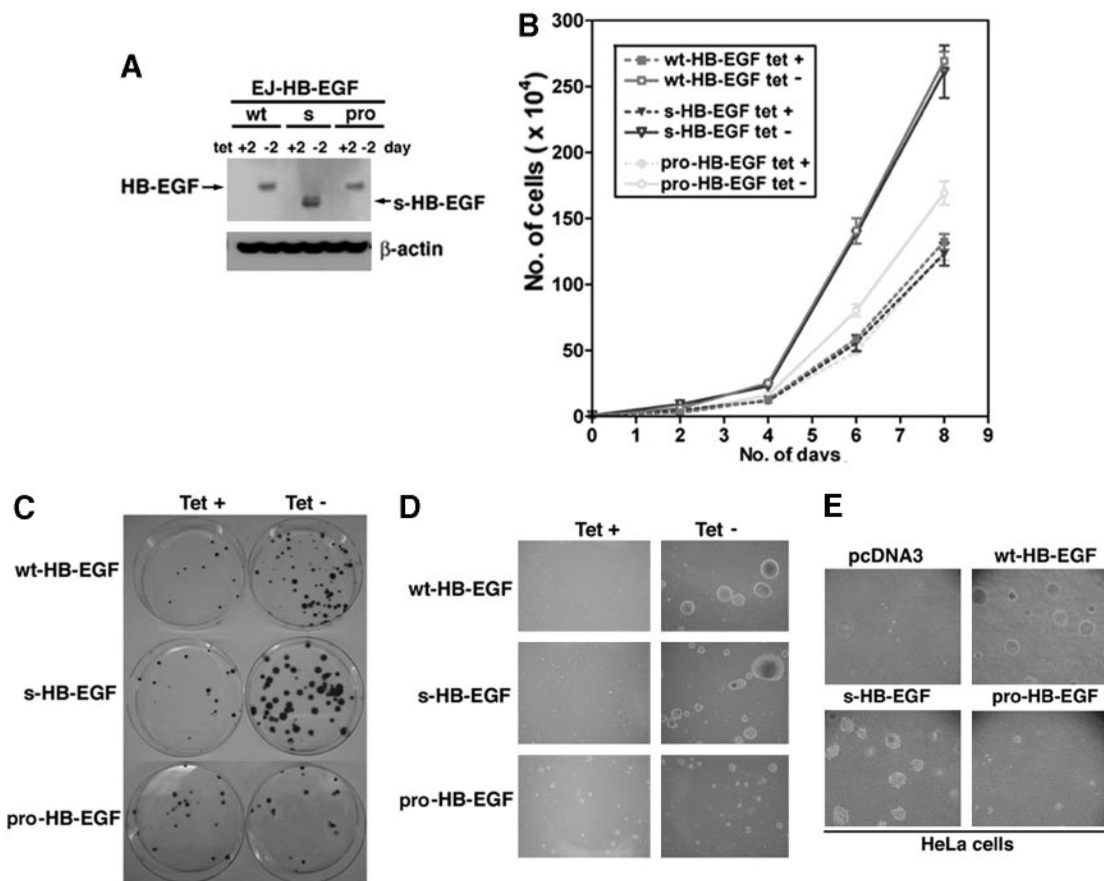

Fig. 1. Soluble heparin-binding epidermal growth factor-like growth factor (s-HB-EGF) enhances transformed phenotype in EJ human bladder carcinoma cells. *A*, inducible expression of three forms of HB-EGF in tetracycline (tet)-regulated expression system in EJ cells. Western blot analysis was performed in EJ-wt-HB-EGF, EJ-s-HB-EGF, or EJ-pro-HB-EGF cells grown in the absence or presence of tet (1 μg/ml). *B,* The growth rates of HB-EGF induced cells were measured in the absence or presence of tet. EJ cells expressing HB-EGF demonstrated significant increase in proliferation as compared with control cells. *C*, s-HB-EGF increases colony formation. *D*, tet-regulated s- or wild-type (wt)-HB-EGF expression enhances *in vitro*-transformed phenotype. HB-EGF expression enhanced anchorage-independent growth in soft agarose in tet-regulated EJ-s-HB-EGF and EJ-wt-HB-EGF cells but not in EJ-pro-HB-EGF cells. *E*, s-HB-EGF overexpression increases growth in soft agarose in HeLa cells. HeLa cells were transfected with constructs encoding wt-, s-, and membrane-anchored form (pro)-HB-EGF or the empty vector (pcDNA3). Cells were then harvested 36 h after transfection, equal cell numbers were seeded in 60-mm Petri dishes and grown under selection in G418 for 2 weeks.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

pipette. After 32 h, the cells were fixed with 3.7% formaldehyde in PBS for 20 min, and pictures of the wound were taken at the different field.

Soft Agar Colonogenic Assay. Soft agar assay was performed by seeding the cells at a density of 1×10^4 in a 60-mm diameter tissue culture dish containing 0.33% top low-melt agarose-0.6% bottom low-melt agarose. Cells were fed every 3 days. Colonies were counted and measured after 2 weeks.

Gene Reporter Assays. A total of 2×10^5 cells was seeded in each well of a 6-well dish and transfected with desired plasmids using Lipofectamine 2000 reagent (Invitrogen). The assays were performed with the dual luciferase reporter assay system from Promega. The internal controls used were pRL-TK and pRL-CMV to track transfection efficiency. The cells were harvested after 48 h. Each data point obtained is the mean of three independent experiments.

Xenograft Studies. EJ-HB-EGF and EJ-CAT cells were cultured in media with and without tet for 24 h. Cells were washed twice with DMEM without antibiotics and serum and finally resuspended at a density of 2×10^6 cells in 0.25 ml. The cell suspension was injected s.c. (bilaterally, 0.25 ml/site) into 6-week-old nude athymic mice [Taconic; Cr:(NCr)-nu f BR]. HB-EGF expression was suppressed by feeding mice with water containing 500 μg of doxycycline (dox)/ml in 1% sucrose. Mice were killed after 3 weeks. Tumors were excised and weighed, fixed for 1 h in 10% formalin, washing with PBS, followed by embedding in ornithine carbamyl transferase, and frozen in liquid nitrogen. Six mice were used for each sample.

Computer-Assisted Morphometric Analysis of Intratumoral Vasculature. Representative tumor sections were stained with anti-CD31 mouse monoclonal antibodies to visualize the blood vessels. Six tumors were evaluated/each sample, and five areas were evaluated at ×6 magnification. Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and blood vessels were quantified using IPLab software (Scanalytics, Billerica, MA). The two-sided unpaired *t* test was used to analyze differences in microvessel density and vascular size and area.

RESULTS

Effects of HB-EGF on the Transformed Phenotype. To elucidate the role of HB-EGF overexpression in the process of tumorigenesis in human cancer cells, a tet-regulated HB-EGF-inducible system was generated in EJ cells that contain a low basal level of HB-EGF. Three different forms (wt-, s-, and pro-HB-EGF) of HB-EGF expression induced by the removal of tet resulted in an increase of each form of HB-EGF (Fig. 1*A*). Using this system, we first examined the role of HB-EGF in cell proliferation. The growth rate of EJ-HB-EGF cells was measured in the presence or absence of tet at 1×10^4 cells in 6-well plates for up to 8 days. Induction of wt- or s-HB-EGF by removal of tet resulted in a substantial growth rate increase, as seen by the 75–100% increase, when compared with HB-EGF-repressed cells (tet+; Fig. 1*B*). However, pro-HB-EGF induction in EJ cells resulted in a slight increase of growth rate by ~25%. To examine the effect of HB-EGF on cell colony formation ability, EJ-HB-EGF cells expressing three different types of HB-EGF were seeded at 200 cells/100-mm diameter dish. Cells expressing s- or wt-HB-EGF produced approximately two to three times more colonies than that of pro-HB-EGF-induced cells (Fig. 1*C*). We next evaluated the effect of each HB-EGF on colony formation in soft agarose as an additional measure of tumorigenicity *in vitro* using tet-regulated EJ-HB-EGF cells. The results of agar colony tests are shown in Fig. 1*D*. Soluble- or wt-HB-EGF (tet-)-inducing cells significantly stimulated anchorage-independent growth, but anchorage-independent growth was not seen in pro-HB-EGF-induced cells. A colony formation assay in soft agarose was also used to examine whether HB-EGF could promote a transformed phenotype in a different cell line using HeLa cells. Transfection of HeLa cells with wt- or s-HB-EGF following a selection under G418 significantly increased sizes of colonies in soft agarose as compared with those in cells transfected with vector alone or a plasmid expressing pro-HB-EGF (Fig. 1*E*). Taken together, these results suggest that s-HB-EGF expression causes an enhanced transformed phenotype *in vitro* but pro-HB-EGF overexpression does not.

s-HB-EGF Elevates Cyclin D Expression via Cyclin D Promoter. It is well known that the signaling pathways of oncogenes, including ErbB2 and Ras, can up-regulate cyclin D expression. Recent

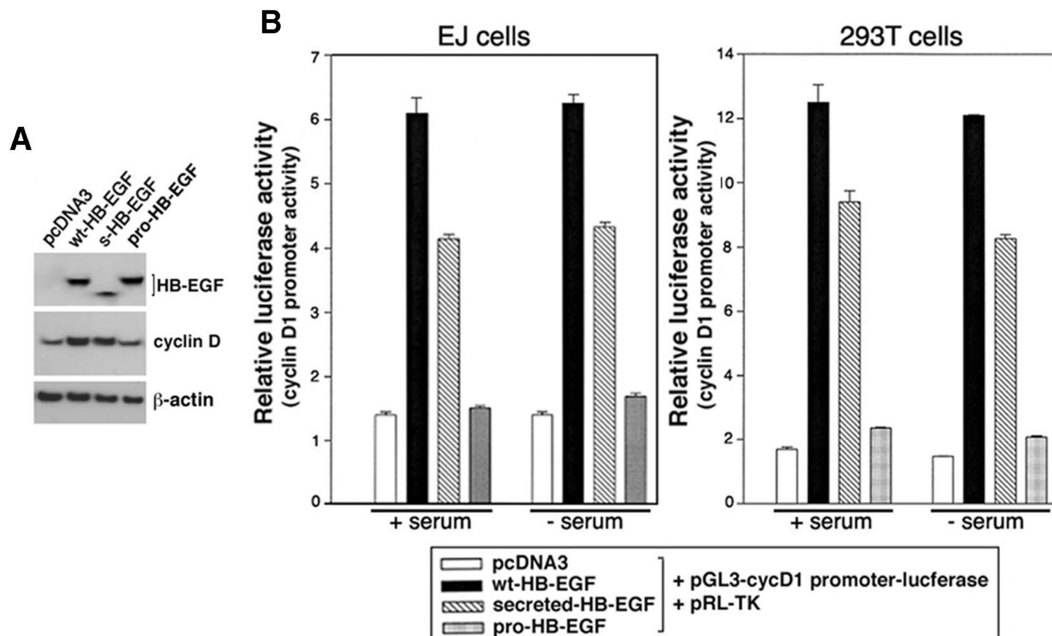

Fig. 2. Soluble heparin-binding epidermal growth factor-like growth factor (s-HB-EGF) elevates the cyclin D1 protein level and activates the cyclin D1 promoter. *A*, increase in cellular cyclin D1 protein by s- or wild-type (wt)-HB-EGF. Tetracycline (tet)-regulated EJ-HB-EGF cells were cultured in the absence or presence of tet followed by an immunoblot analysis of the cell lysates with antibodies against HB-EGF, cyclin D1, and β-actin (loading control). *B*, activation of cyclin D1 promoter by HB-EGF. EJ cells were transiently cotransfected with the cyclin D1 promoter reporter construct (−1745CD1) and three different forms of HB-EGF [wt-HB-EGF, s-HB-EGF, and membrane-anchored form (pro)-HB-EGF] in expression vector, respectively, or the empty vector then assayed for luciferase activity. pRL-TK Renilla luciferase reporter construct was cotransfected with each sample to normalize transfection efficiency. All results are expressed as x ± SD of three independent experiments with duplicates.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

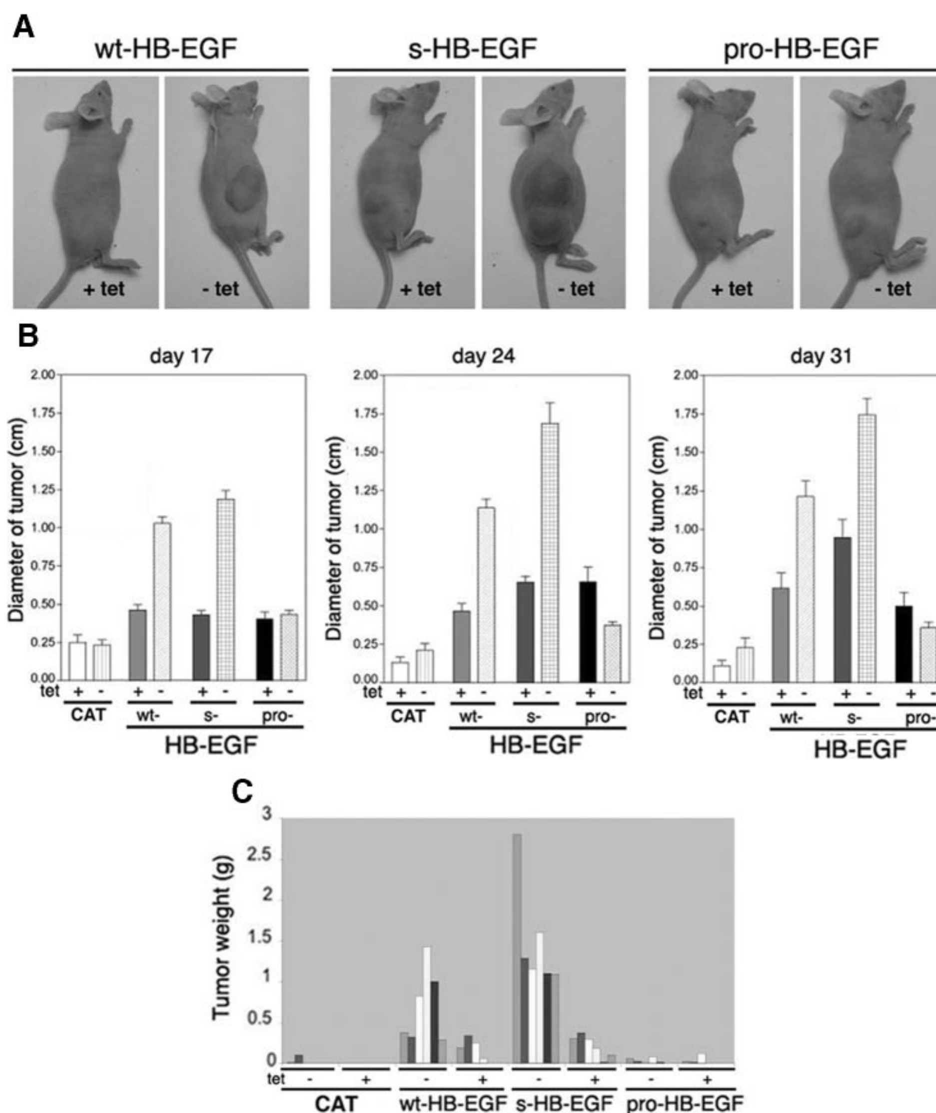

Fig. 3. Soluble heparin-binding epidermal growth factor-like growth factor (s-HB-EGF) overexpression increases tumorigenicity in nude mice. *A*, athymic nude mice received injections of 2×10^6 EJ-s-HB-EGF cells, EJ-wt-HB-EGF, EJ-pro-HB-EGF, or EJ-CAT cells mixed with an equal volume of Matrigel. The cell suspension was injected s.c. (bilaterally; 0.5 ml/site) into nude mice. Results are average tumor sizes for six animals into a site/experimental condition 3 weeks after injection. *B*, the mean tumor size of s- or wild-type (wt)-HB-EGF expressing EJ cells (−tet EJ-s-HB-EGF) was significantly larger than that of the EJ-CAT tumor (−tet EJ-CAT), EJ-pro-HB-EGF tumor (−tet EJ-pro-HB-EGF), or that of EJ-s-HB-EGF or EJ-wt-HB-EGF tumor from mice fed with doxycycline (500 μg/ml in water) to repress all forms of HB-EGF expression. *C*, representation of the mean tumor weight.

studies demonstrate that HB-EGF activates MAPK cascade through the activation of the Ras/Raf pathway (34). Therefore, we tested whether HB-EGF had any effect on cyclin D expression. To examine this possibility, we transiently transfected three different HB-EGF expression constructs, respectively, into EJ cells and then examined the effects on endogenous cyclin D1 levels. Forced expression of s- or wt-HB-EGF led to ~3-fold increase in cyclin D1 protein levels, whereas pro-HB-EGF did not result in an increase in cyclin D expression. (Fig. 2*A*). To determine whether HB-EGF regulates the transcription of cyclin D1, we also measured the effects of HB-EGFs on the cyclin D1 promoter using a cyclin D1-luciferase reporter construct (−1745CD1; Ref. 35). The reporter containing the cyclin D promoter was strongly activated in response to the expression of s- or wt-HB-EGF in EJ cells and 293T cells (Fig. 2*B*), whereas pro-HB-EGF did not show any significant effect on cyclin D promoter activity, regardless of serum presence. These results indicate that the activation of the cyclin D promoter and the −1745CD1 promoter fragment retains complete responsiveness to s-HB-EGF.

HB-EGF Overexpression Promotes Cancer Cell Growth *in Vivo*. To determine whether HB-EGF increases tumorigenicity *in vivo*, xenograft studies were conducted using EJ cells expressing s-HB-EGF, wt-HB-EGF, and pro-HB-EGF, respectively, and control cells expressing CAT (EJ-CAT) in the absence or presence of dox. Six mice per each HB-EGF form were injected with cells (2×10^6 cells mixed with Matrigel). As shown in Fig. 3, *A* and *B*, EJ cells expressing s- or wt-HB-EGF showed an increased overall tumor size when injected into nude athymic mice (EJ-HB-EGF tumors in −tet condition), compared with EJ cells expressing a control CAT protein (EJ-CAT tumors in −tet and +tet) and EJ-HB-EGF cells in tet+ conditions. The mean size of EJ-HB-EGF tumors maintained in the absence of dox was 1.5 g, significantly larger than tumors from control mice, ~0.1 g (Fig. 3*C*). However, tumors expressing pro-HB-EGF were similar to those of control mice. Data from the *in vitro* tumorigenicity assays and the xenograft assays support the conclusion that s-HB-EGF exerts a potent oncogenic potential but pro-HB-EGF does not.

HB-EGF Overexpression in Bladder Carcinomas Up-Regulates VEGF and Enhances Tumor Angiogenesis in Mice. The growth of tumors beyond a minimal size has been hypothesized to be dependent upon the induction of new blood vessel growth or angiogenesis, which in turn supplies needed nutrients to rapidly dividing tumor cells (36). Several studies indicated that HB-EGF can be up-regulated in some pathological states that may involve angiogenesis (37, 38). Some studies showed that VEGF, a critical factor in the development of new blood vessels, could induce HB-EGF in vascular endothelial cells and speculated that HB-EGF induction by VEGF may act in a paracrine fashion to promote angiogenesis (39). This prompted us to evaluate

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

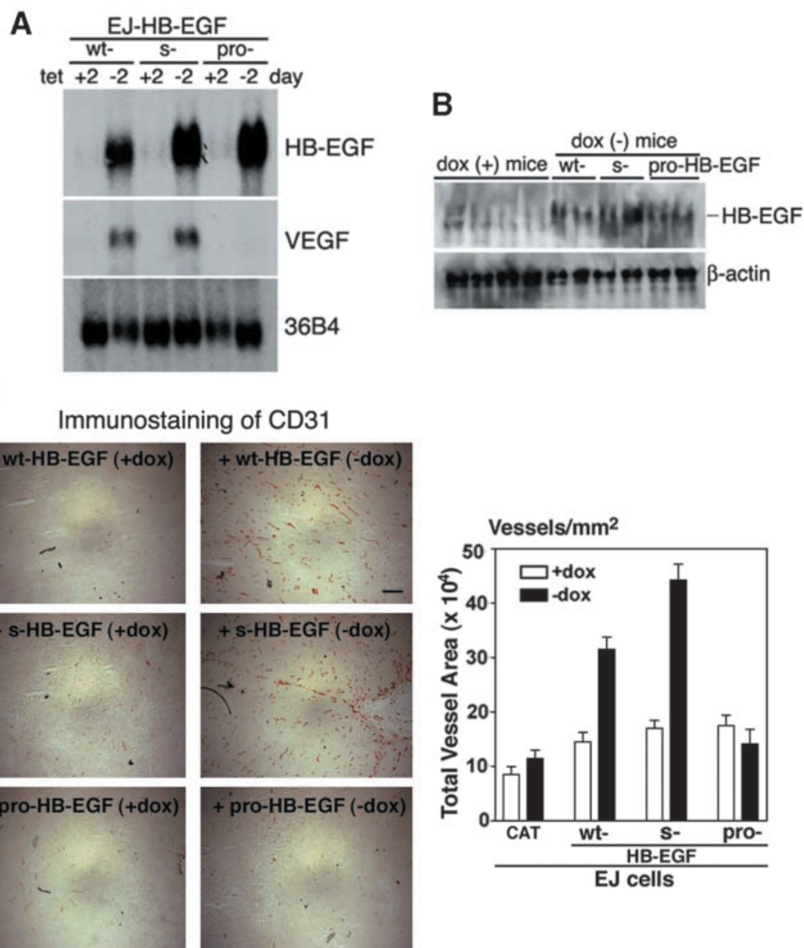

Fig. 4. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) increases tumor angiogenesis in nude mice. *A*, up-regulation of vascular endothelial growth factor (VEGF) by HB-EGF induction in EJ cells. Induction of VEGF mRNA after tetracycline removal in EJ-s- or wt-HB-EGF cells. Total RNA was prepared from EJ-HB-EGF cells grown in the presence or absence of tet for 2 days. Northern blot analyses were performed sequentially using a ^{32}P-labeled probe against HB-EGF, VEGF, and 36B4 (loading control). *B*, Western blot analysis of HB-EGF expressing tumors (−dox mice) or control tumors (+dox mice). Three weeks after injection, tumors were harvested, homogenized and examined for HB-EGF expression by Western blotting. *C*, immunostaining of CD31. Immunostaining of anti-CD31 (PECAM-1) monoclonal antibody demonstrated rarefication of tumor blood vessels in HB-EGF-overexpressing tumors as compared with control tumors (+/−CAT and +dox). *Bar* = 100 μm. CD31-stained blood vessels were evaluated in three different ×10 fields obtained from five different tumors for each condition. *Right panel* represents quantitative computer-assisted image analysis that revealed a significant increase of angiogenesis in soluble (s)- or wild-type (wt)-HB-EGF-overexpressing EJ tumors but not in membrane-anchored form (pro)-HB-EGF tumors, as measured by the number of blood vessels/mm^2 tumor area.

the effect of HB-EGF on the existence of a growth factor-mediated autocrine response, possibly involving VEGF up-regulation. Northern blot analysis was performed with total RNA from EJ cells with a tet-regulated HB-EGF-inducible system. Soluble-HB-EGF or wt-HB-EGF expression by removal of tet resulted in an increase of VEGF mRNA (Fig. 4*A*), but no increase of VEGF mRNA by pro-HB-EGF induction in EJ-pro-HB-EGF cells was seen. These results implicate the up-regulation of HB-EGF as an angiogenesis-related change, occurring through an autocrine response, which is closely associated with tumorigenesis of epithelial cells.

Next, we examined the effect of HB-EGFs on blood vessel growth/angiogenesis in mice. Three weeks after injection, tumors harvested from control and three forms of HB-EGF-expressing mice in the xenograft studies (Fig. 3) were first homogenized and examined for HB-EGF expression levels by Western blotting. Fig. 4*B* shows that mice maintained in the absence of dox-expressed HB-EGF at a higher level as compared with dox-fed mice. Cryostat sections of five different tumors expressing HB-EGF for each experimental condition include the following: +dox of EJ-HBEGF (no HB-EGF expression); −dox of EJ-HBEGF (HB-EGF expressed); +dox and −dox of EJ-CAT cells were processed and stained for CD31 (PECAM-1), an endothelial junction molecule (40). As shown in Fig. 4*C*, these sections demonstrated a dramatic increase of microvessels in s- or wt-HB-EGF expressing tumors (+wt-HB-EGF and +s-HB-EGF, -dox) when compared with the different control tumors (+dox, +CAT and −CAT, and +pro-HB-EGF). To achieve a more detailed quantification of the effects of HB-EGF on tumor angiogenesis, average vessel density, vessel size, and percentage of tissue area covered by vessels were determined by computer-assisted image analysis of representative digital images. The relative tumor area occupied by vessels increased ~2–3-folds in s- or wt-HB-EGF-expressing tumors (Fig. 4*C*, *right panel*).

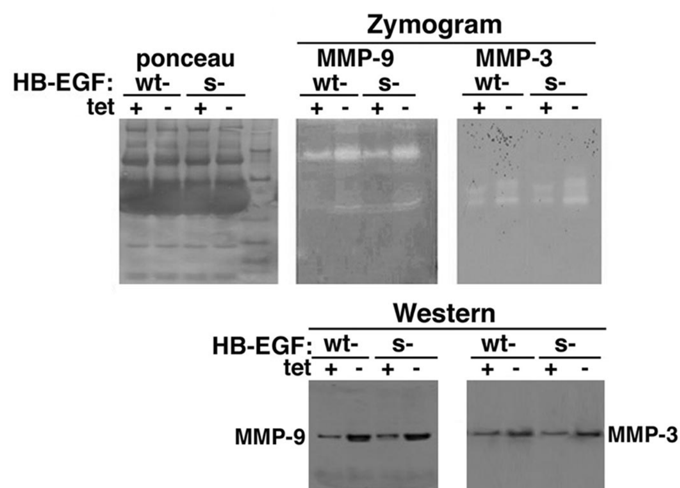

Fig. 5. Up-regulation of metalloprotease (MMP)-9 and MMP-3 in response to heparin-binding epidermal growth factor-like growth factor (HB-EGF) induction. EJ-HB-EGF cells expressing soluble (s)-, wild-type (wt)-, and membrane-anchored form (pro)-HB-EGF were grown in the absence or presence of tetracycline (tet) for 48 h, respectively, then analyzed by zymography for MMP-9 and MMP-3 activities. Expression levels of MMP-9 and MMP-3 were also determined by Western blotting. Ponceau staining shows the loading levels of total proteins.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

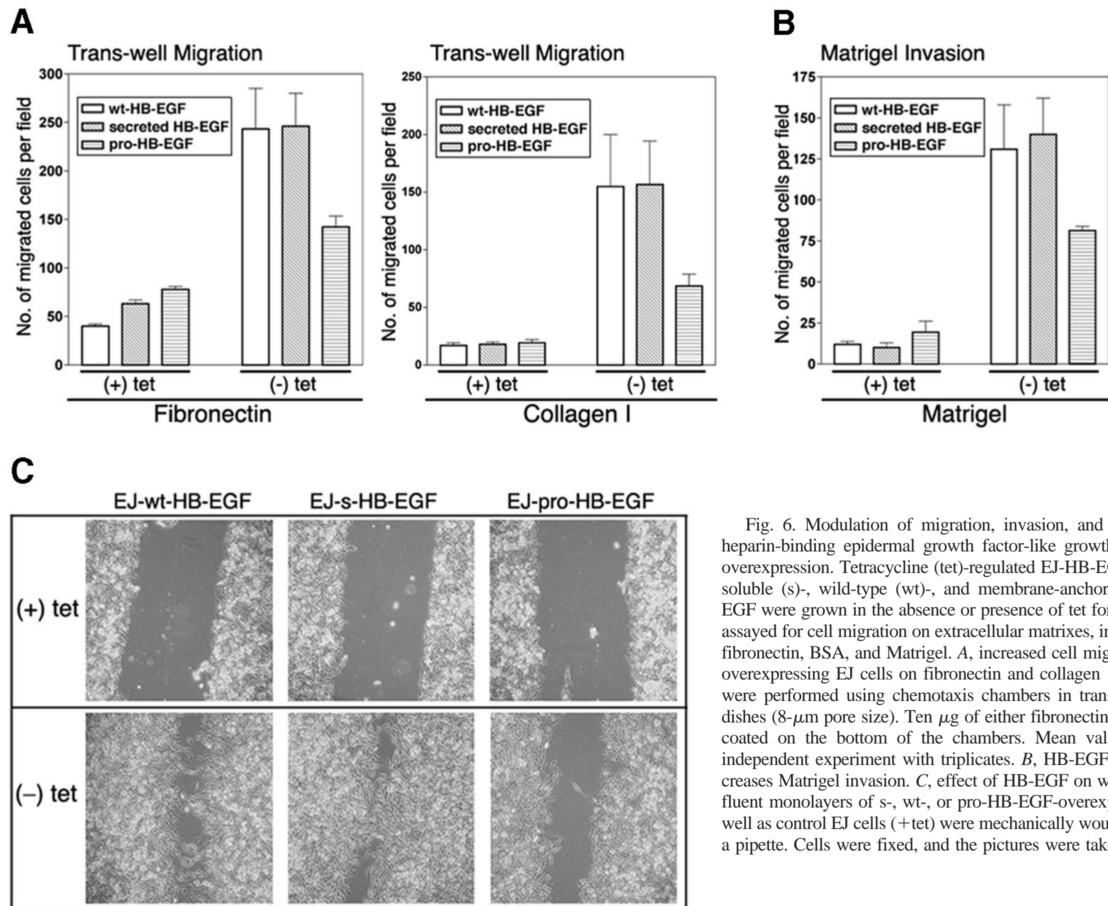

Fig. 6. Modulation of migration, invasion, and wound healing by heparin-binding epidermal growth factor-like growth factor (HB-EGF) overexpression. Tetracycline (tet)-regulated EJ-HB-EGF cells expressing soluble (s)-, wild-type (wt)-, and membrane-anchored form (pro)-HB-EGF were grown in the absence or presence of tet for 48 h, respectively, assayed for cell migration on extracellular matrixes, including collagen 1, fibronectin, BSA, and Matrigel. *A*, increased cell migration of HB-EGF-overexpressing EJ cells on fibronectin and collagen 1. Migration assays were performed using chemotaxis chambers in transwell tissue culture dishes (8-μm pore size). Ten μg of either fibronectin or collagen 1 were coated on the bottom of the chambers. Mean values ± SD of two independent experiment with triplicates. *B*, HB-EGF overexpression increases Matrigel invasion. *C*, effect of HB-EGF on wound healing. Confluent monolayers of s-, wt-, or pro-HB-EGF-overexpressing EJ cells as well as control EJ cells (+tet) were mechanically wounded with the tip of a pipette. Cells were fixed, and the pictures were taken after 32 h.

Up-Regulation of the MMP by HB-EGF. It is well documented that the up-regulation of MMP activities results in the proteolytic degradation of the extracellular matrix and the basement membrane, which promotes tumor growth and metastasis (41). To better evaluate the participation of HB-EGF in tumor migration and progression, we tested the effects of HB-EGF on MMP-9 activation. As shown in Fig. 5, HB-EGF induction after tet removal activated the 92-kDa pro-MMP-9 zymogen to the 67-kDa active form. The processing/activation was analyzed by zymography analysis. MMP-9 expression level was also determined by Northern and Western blotting, which showed that MMP-9 levels correlated with an increase of HB-EGF levels (Fig. 5). MMP-3, also known as a stromelysin-1, is the most efficient activator of MMP-9 *in vitro* and *in vivo*. Therefore, we evaluated the effect of HB-EGF on MMP-3 activity. Fig. 5 also shows the slightly increased activation and expression of MMP-3 in response to HB-EGF induction. However, HB-EGF-mediated activation of MMP-9 was not observed in response to pro-HB-EGF overexpression (data not shown). These results demonstrate that the expression level and activity of MMP-9 and MMP-3 can be enhanced in response to s-HB-EGF in EJ cells.

HB-EGF Effect on Migration and Wound Healing. We next investigated whether HB-EGF might be involved in extracellular matrix interactions in EJ cells and cause an increase in the migration of cells expressing HB-EGF because HB-EGF has been shown to be chemotactic (1, 12, 42) and to mediate migration in some cell types (12). Tet-regulated EJ-HB-EGF cells were used for migration assays in a transwell migration assay (haptotaxis). We tested for their ability to migrate through extracellular matrixes, including fibronectin, collagen 1, and Matrigel. As shown in Fig. 6*A*, HB-EGF expression in EJ cells after the removal of tet showed a strong increase in migration on all three substrates but not in cells grown in the presence of tet. Although s-HB-EGF and wt-HB-EGF were more effective, pro-HB-EGF expression also increased cell migration on all three matrixes.

It is well established that successful wound healing involves a number of processes, including cell proliferation, cell migration, vascular permeability, and angiogenesis (43). HB-EGF is known to be up-regulated in the wound-healing process of certain cell types, including keratinocytes (44, 45). Because HB-EGF expression triggers tumor angiogenesis and cell migration in EJ cells, we studied its effect on wound healing using the same cell system. One $\times 10^6$ EJ-HB-EGF cells of each form (EJ-wt-HB-EGF, EJ-s-HB-EGF, and pro-HB-EGF) were seeded in a 6-well plate. Then, the cells were grown with or without tet for 24 h. A wound was introduced by the use of a pipette tip. After 32 h, the cells were fixed, and pictures were taken at different regions of the wound (Fig. 6*C*). Soluble- or wt-HB-EGF expression (−tet condition) increased the effectiveness of wound healing, but no significant effect was seen in pro-HB-EGF-expressing cells.

DISCUSSION

In this article, we provide evidence that HB-EGF exerts oncogenic potentials *in vitro* and *in vivo*. We found that overexpression of HB-EGF enhanced the transformed phenotype *in vitro* as determined by cell proliferation, anchorage-independent growth, and foci formation assays. We also showed that HB-EGF can activate the cyclin D promoter. We established a tet-regulated HB-EGF expression system using three different forms of HB-EGF in EJ human bladder cancer cells to test the tumorigenicity *in vitro* and *in vivo* using a xenograft assay. Inducible overexpression of s- or wt-HB-EGF caused increased

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

tumor formation in mice, supporting the conclusion that s-HB-EGF but not the membrane-bound form of HB-EGF exerts a potent oncogenic ability. Our finding that the membrane-bound form of HB-EGF does not exert oncogenic potential may support previous speculation that pro-HB-EGF may act as a negative regulator of cell proliferation (46).

HB-EGF is known as a potent mitogen for keratinocytes, hepatocytes, smooth muscle cells, and fibroblasts (2, 29, 44). HB-EGF expression is elevated in human cancers, including hepatocellular and gastric carcinoma, breast carcinoma, melanoma, colon cancer, pancreatic cancer, glioma and glioblastoma (23, 24). Furthermore, HB-EGF is induced in NIH3T3 cells transformed by v-Ras or v-Raf (34). HB-EGF was identified as a target of v-Jun, a potent oncogene, and HB-EGF plays a role in v-Jun-mediated oncogenic transformation (15). The significance of HB-EGF overexpression in tumorigenesis is also supported by our findings that HB-EGF activates the cyclin D promoter, implying that v-Jun could stimulate cyclin D transcription through HB-EGF induction. We have previously reported the identification of *HB-EGF* as a p53 downstream target gene, and p53 induction of HB-EGF could activate cell survival signaling, including AKT and MAPK cascades (19). A recent article has demonstrated that inducible expression of oncogenic Raf in normal epithelial cells strongly induced autocrine expression of HB-EGF, transforming growth factor α, and amphiregulin, which were directly implicated in the ability of sustained Raf/MAPK pathway stimulation to protect cells from apoptosis (47).

It is now well established that VEGF is an endothelial cell-specific, multifunctional growth factor that plays a major role in the initiation of angiogenesis by acting directly as a mitogenic and chemotactic factor (48). Although HB-EGF is known to have no effect on the proliferation of endothelial cells, some studies demonstrated that VEGF induces expression of angiogenic growth factors, including HB-EGF, in vascular endothelial cells and suggested that HB-EGF induction in response to VEGF provides a critical endothelial cell-derived signal, perhaps the activation of the MAPK and AKT cascade, for the process of new blood vessel formation and maturation (39). In addition, the reviews have proposed that HB-EGF acts as one of the recruiting signals for mesenchymal cells during the late phase of angiogenesis (49), implying that HB-EGF plays a critical role in angiogenesis as well as tumor development. In this study, we also demonstrate that s- or wt-HB-EGF induces VEGF expression, whereas it inhibits thrombospondin-1 expression (data not shown), implying the presence of an autocrine growth factor stimulation. Moreover, HB-EGF promotes tumor angiogenesis *in vivo* and exerts the migration-stimulating and wound-healing potential for cancer cells.

It is now clear that the up-regulation of MMP activities results in the proteolytic degradation of the extracellular matrix and the basement membrane, which promotes tumor growth, angiogenesis, and metastasis (41). It is possible that the effect of HB-EGF on the functional interaction between VEGF and MMP-9 is a key mechanism for initiation and maintenance of angiogenesis. In our cell system, we found a significant difference in the levels of active MMP-9 and MMP-3 in s-HB-EGF-induced cells. Moreover, recent studies clearly demonstrated that MMP-9 mediates the release and accumulation of VEGF from the cell matrix and that MMP-9 triggers the angiogenic switch (50). The generation of HB-EGF overexpressing mice or a targeted deletion of HB-EGF should provide additional understanding of mechanisms underlying HB-EGF-associated functions in angiogenesis.

In summary, our findings provide evidence that HB-EGF enhances transformed phenotypes and is associated with the stimulation of MMP-9, MMP-3, and cyclin D activation, which promotes tumorigenesis and angiogenesis. Given its elevated expression in human cancers along with our findings of HB-EGF contribution to enhanced transformed phenotypes, we hypothesize that HB-EGF may behave as an oncogene and, as such, could have importance as a therapeutic target.

ACKNOWLEDGMENTS

We thank Michael Klasbrun for providing wt-HB-EGF cDNA, Rachel Hazan, Don Senger, Jeremy Brown, and Shanthy Nuti for helpful discussion, and Paula Velasco for technical help. We also thank Wendy Wong for technical help.

REFERENCES

1. Raab G, Klagsbrun M. Heparin-binding EGF-like growth factor. Biochim Biophys Acta 1997;1333:F179–99.
2. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun MA. Heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science (Wash. DC) 1991;251:936–9.
3. Blotnick S, Peoples GE, Freeman MR, Eberlein TJ, Klagsbrun MT. Lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: differential production and release by CD4+ and CD8+ T cells. Proc Natl Acad Sci USA 1994;91:2890–4.
4. Fukuda K, Inui Y, Kawata S, et al. Increased mitogenic response to heparin-binding epidermal growth factor-like growth factor in vascular smooth muscle cells of diabetic rats. Arterioscler Thromb Vasc Biol 1995;15:1680–7.
5. Dluz SM, Higashiyama S, Damm D, Abraham JA, Klagsbrun M. Heparin-binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. J Biol Chem 1993;268:18330–4.
6. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 1997;16:1647–55.
7. Iwamoto R, Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. Cytokine Growth Factor Rev 2000;11:335–44.
8. Hisaka T, Yano H, Haramaki M, Utsunomiya I, Kojiro M. Expressions of epidermal growth factor family and its receptor in hepatocellular carcinoma cell lines: relationship to cell proliferation. Int J Oncol 1999;14:453–60.
9. Murayama Y, Miyagawa J, Shinomura Y, et al. Significance of the association between heparin-binding epidermal growth factor-like growth factor and CD9 in human gastric cancer. Int J Cancer 2002;98:505–13.
10. Nakamura Y, Handa K, Iwamoto R, Tsukamoto T, Takahasi M, Mekada E. Immunohistochemical distribution of CD9, heparin binding epidermal growth factor-like growth factor, and integrin alpha$_3$beta$_1$ in normal human tissues. J Histochem Cytochem 2001;49:439–44.
11. Hashimoto K, Higashiyama S, Asada H, et al. Heparin-binding epidermal growth factor-like growth factor is an autocrine growth factor for human keratinocytes. J Biol Chem 1994;269:20060–6.
12. Nishi E, Prat A, Hospital V, Elenius K, Klagsbrun M. *N*-Arginine dibasic convertase is a specific receptor for heparin-binding EGF-like growth factor that mediates cell migration. EMBO J 2001;20:3342–50.
13. Suo Z, Risberg B, Karlsson MG, Villman K, Skovlund E, Nesland JM. The expression of EGFR family ligands in breast carcinomas. Int J Surg Pathol 2002;10:91–9.
14. Tarbe N, Losch S, Burtscher H, Jarsch M, Weidle UH. Identification of rat pancreatic carcinoma genes associated with lymphogenous metastasis. Anticancer Res 2002;22: 2015–27.
15. Fu S, Bottoli I, Goller M, Vogt PK. Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation. Proc Natl Acad Sci USA 1999;96:5716–21.
16. McCarthy SA, Chen D, Yang BS, et al. Rapid phosphorylation of Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. Mol Cell Biol 1997; 17:2401–12.
17. Martinez-Lacaci I, De Santis M, Kannan S, et al. Regulation of heparin-binding EGF-like growth factor expression in Ha-ras transformed human mammary epithelial cells. J Cell Physiol 2001;186:233–42.
18. Fang L, Li G, Liu G, Lee SW, Aaronson SA. p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. EMBO J 2001;20:1931–9.
19. Miyoshi E, Higashiyama S, Nakagawa T, Hayashi N, Taniguchi N. Membrane-anchored heparin-binding epidermal growth factor-like growth factor acts as a tumor survival factor in a hepatoma cell line. J Biol Chem 1997;272:14349–55.
20. Downing MT, Brigstock DR, Luquette MH, Crissman-Combs M, Besner GE. Immunohistochemical localization of heparin-binding epidermal growth factor-like growth factor in normal skin and skin cancers. Histochem J 1997;29:735–44.
21. Fishman S, Brill S, Papa M, Halpern Z, Zvibel I. Heparin-derived disaccharides modulate proliferation and Erb-B2-mediated signal transduction in colon cancer cell lines. Int J Cancer 2002;99:179–84.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.

22. Ito Y, Takeda T, Higashiyama S, Noguchi S, Matsuura N. Expression of heparin-binding epidermal growth factor-like growth factor in breast carcinoma. Breast Cancer Res Treat 2001;67:81–5.
23. Ito Y, Takeda T, Higashiyama S, et al. Expression of heparin binding epidermal growth factor-like growth factor in hepatocellular carcinoma: an immunohistochemical study. Oncol Rep 2001;8:903–7.
24. Jayne DG, Perry SL, Morrison E, Farmery SM, Guillou PJ. Activated mesothelial cells produce heparin-binding growth factors: implications for tumour metastases. Br J Cancer 2000;82:1233–8.
25. Kim J, Adam RM, Freeman MR. Activation of the Erk mitogen-activated protein kinase pathway stimulates neuroendocrine differentiation in LNCaP cells independently of cell cycle withdrawal and STAT3 phosphorylation. Cancer Res 2002;62:1549–54.
26. Thogersen VB, Sorensen BS, Poulsen SS, Orntoft TF, Wolf H, Nexo E. A subclass of HER1 ligands are prognostic markers for survival in bladder cancer patients. Cancer Res 2001;61:6227–33.
27. Wang YD, De Vos J, Jourdan M, et al. Cooperation between heparin-binding EGF-like growth factor and interleukin-6 in promoting the growth of human myeloma cells. Oncogene 2002;21:2584–92.
28. Kobrin MS, Funatomi H, Friess H, Buchler MW, Stathis P, Korc M. Induction and expression of heparin-binding EGF-like growth factor in human pancreatic cancer. Biochem Biophys Res Commun 1994;202:1705–9.
29. Ito N, Kawata S, Tamura S, et al. Heparin-binding EGF-like growth factor is a potent mitogen for rat hepatocytes. Biochem Biophys Res Commun 1994;198:25–31.
30. Sato M, Narita T, Kawakami-Kimura N, et al. Increased expression of integrins by heparin-binding EGF like growth factor in human esophageal cancer cells. Cancer Lett 1996;102:183–91.
31. Ruck A, Paulie S. EGF, TGF alpha, AR and HB-EGF are autocrine growth factors for human bladder carcinoma cell lines. Anticancer Res 1998;18:1447–52.
32. Murayama Y, Miyagawa J, Higashiyama S, et al. Localization of heparin-binding epidermal growth factor-like growth factor in human gastric mucosa. Gastroenterology 1995;109:1051–9.
33. Higashiyama S, Lau K, Besner GE, Abraham JA, Klagsbrun M. Structure of heparin-binding EGF-like growth factor. Multiple forms, primary structure, and glycosylation of the mature protein. J Biol Chem 1992;267:6205–12.
34. McCarthy SA, Samuels ML, Pritchard CA, Abraham JA, McMahon M. Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. Genes Dev 1995;9:1953–64.
35. Wulf GM, Ryo A, Wulf GG, et al. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO J 2001;20:3459–72.
36. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
37. Abramovitch R, Neeman M, Reich R, et al. Intercellular communication between vascular smooth muscle and endothelial cells mediated by heparin-binding epidermal growth factor-like growth factor and vascular endothelial growth factor. FEBS Lett 1998;425:441–7.
38. Abramovitch R, Marikovsky M, Meir G, Neeman M. Stimulation of tumour growth by wound-derived growth factors. Br J Cancer 1999;79:1392–8.
39. Arkonac BM, Foster LC, Sibinga NE, et al. Vascular endothelial growth factor induces heparin-binding epidermal growth factor-like growth factor in vascular endothelial cells. J Biol Chem 1998;273:4400–5.
40. Skobe M, Hawighorst T, Jackso DG, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 2001;7:192–8.
41. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161–74.
42. Elenius K, Paul S, Allison G, Sun J, Klagsbrun M. Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. EMBO J 1997;16:1268–78.
43. Clark RA. Biology of dermal wound repair. Dermatol Clin 1993;11:647–66.
44. Marikovsky M, Breuing K, Liu PY, et al. Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury. Proc Natl Acad Sci USA 1993;90:3889–93.
45. Tokumaru S, Higashiyama S, Endo T, et al. Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. J Cell Biol 2000;151:209–20.
46. Iwamoto R, Handa K, Mekada E. Contact-dependent growth inhibition and apoptosis of epidermal growth factor (EGF) receptor-expressing cells by the membrane-anchored form of heparin-binding EGF-like growth factor. J Biol Chem 1999;274:25906–12.
47. Gangarosa LM, Sizemore N, Graves-Deal R, Oldham SM, Der CJ, Coffey RJ. A raf-independent epidermal growth factor receptor autocrine loop is necessary for Ras transformation of rat intestinal epithelial cells. J Biol Chem 1997;272:18926–31.
48. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. Nature (Lond.) 1992;359:845–8.
49. Wells A. EGF receptor. Int J Biochem. Cell Biol 1999;31:637–43.
50. Hiratsuka S, Nakamura K, Iwai S, et al. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2002;2:289–300.

Downloaded from cancerres.aacrjournals.org on May 29, 2013. © 2004 American Association for Cancer Research.