The heparin-binding domain of HB-EGF mediates localization to sites of cell-cell contact and prevents HB-EGF proteolytic release

Robin N. Prince[1], Eric R. Schreiter[2], Peng Zou[3], H. Steven Wiley[4], Alice Y. Ting[3], Richard T. Lee[5] and Douglas A. Lauffenburger[1,*]

[1]Department of Biological Engineering, and [3]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[2]Department of Chemistry, University of Puerto Rico, Rio Piedras, San Juan, PR 00931, USA
[4]Systems Biology Program, Pacific Northwest National Laboratory, Richland, WA 99354, USA
[5]Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
*Author for correspondence (lauffen@mit.edu)

Accepted 6 April 2010
Journal of Cell Science 123, 2308-2318

doi:10.1242/jcs.058321

Summary

Heparin-binding EGF-like growth factor (HB-EGF) is a ligand for EGF receptor (EGFR) and possesses the ability to signal in juxtacrine, autocrine and/or paracrine mode, with these alternatives being governed by the degree of proteolytic release of the ligand. Although the spatial range of diffusion of released HB-EGF is restricted by binding heparan-sulfate proteoglycans (HSPGs) in the extracellular matrix and/or cellular glycocalyx, ascertaining mechanisms governing non-released HB-EGF localization is also important for understanding its effects. We have employed a new method for independently tracking the localization of the extracellular EGF-like domain of HB-EGF and the cytoplasmic C-terminus. A striking observation was the absence of the HB-EGF transmembrane pro-form from the leading edge of COS-7 cells in a wound-closure assay; instead, this protein localized in regions of cell-cell contact. A battery of detailed experiments found that this localization derives from a *trans* interaction between extracellular HSPGs and the HB-EGF heparin-binding domain, and that disruption of this interaction leads to increased release of soluble ligand and a switch in cell phenotype from juxtacrine-induced growth inhibition to autocrine-induced proliferation. Our results indicate that extracellular HSPGs serve to sequester the transmembrane pro-form of HB-EGF at the point of cell-cell contact, and that this plays a role in governing the balance between juxtacrine versus autocrine and paracrine signaling.

Key words: Heparin-binding epidermal-growth-factor-like growth factor (HB-EGF), Heparan-sulfate proteoglycan (HSPG), Heparin binding, Cell-cell contact, Juxtacrine

Introduction

Heparin-binding epidermal-growth-factor (EGF)-like growth factor (HB-EGF) is a ligand in the EGF receptor (EGFR) family that activates the EGFR (Higashiyama et al., 1991) and ErbB4 (also known as HER4) (Elenius et al., 1997), and binds heparan-sulfate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix (Higashiyama et al., 1991) by means of a heparin-binding domain (HBD) (Thompson et al., 1994). As with all EGFR-family ligands, HB-EGF is synthesized in pro-form as a transmembrane protein that is proteolytically cleaved from the cell surface to yield a soluble form that can activate receptors in autocrine and/or paracrine mode (Harris et al., 2003; Singh and Harris, 2005; Higashiyama et al., 2008) or bind to HSPGs. However, HB-EGF also has the ability to signal in juxtacrine mode by activating receptors on a neighboring cell while still being anchored to the plasma membrane. HB-EGF associates with the tetraspanin protein CD9, which enhances its juxtacrine activity (Higashiyama et al., 1995; Iwamoto et al., 1991) and links HB-EGF to $\alpha 3\beta 1$ integrins (Nakamura et al., 1995). CD9 also interacts with HB-EGF via its HBD (Sakuma et al., 1997).

HB-EGF juxtacrine signaling and autocrine and/or paracrine signaling have been shown to elicit different phenotypes, with autocrine and/or paracrine activity leading to cell proliferation and migration (Higashiyama et al., 1991; Yahata et al., 2006), and juxtacrine activity causing growth inhibition and in some cases apoptosis (Iwamoto et al., 1999; Pan et al., 2002). HB-EGF proteolytic cleavage and subsequent autocrine signaling drives physiologically beneficial migration of a variety of tissue cells, such as keratinocytes (Shirakata et al., 2005; Tokumaru et al., 2000), corneal epithelial cells (Block et al., 2004; Xu et al., 2004), smooth-muscle cells (Bakken et al., 2009), peritoneal mesothelial cells (Faull et al., 2001) and mesenchymal stem cells (Ozaki et al., 2007) during wound healing. Soluble HB-EGF stimulation is also crucially involved in invasive motility of aggressive solid tumors, particularly ovarian cancer (Yagi et al., 2008; Matsuzaki et al., 2005; Tanaka et al., 2005; Miyamoto et al., 2004) among others (Normanno et al., 2001).

We have previously shown that soluble HB-EGF can operate in a highly localized spatial domain following its proteolytic release (Yoshioka et al., 2005), owing to a combination of restricted diffusion and fast binding to cell surface receptors. Both of these localization processes might arise from interactions with HSPGs – residing in extracellular matrix for the former, or on the cellular glycocalyx for the latter. Moreover, human mammary epithelial cells, driven by proteolytic release of the soluble autocrine EGF ligand, migrate in a more directionally persistent manner compared with cells treated with EGF ligand exogenously (Maheshwari et al., 2001). We have shown by computational modeling that the

EGFR system might be capable of producing spatially localized autocrine signaling loops (Maly et al., 2004), and have found in ensuing experimental studies that autocrine EGF drives migration effectively (Joslin et al., 2007).

Because of the disparity in cell phenotypic responses to juxtacrine HB-EGF signaling versus autocrine and/or paracrine HB-EGF signaling, and the prospective importance of spatial localization of both the transmembrane pro-form as well as the soluble mature form, ascertaining mechanisms responsible for governing localization and proteolytic cleavage of this ligand is important for understanding its physiological and pathological effects. Previous work has provided crucial insights into the roles played by the membrane-anchoring domain of the ligand (Dong et al., 2005), regulatory signaling pathways (Herrlich et al., 2008) and extracellular-matrix HSPGs (Forsten-Williams et al., 2008). Thus, we endeavor here to offer a contribution elucidating how HSPGs in the cellular glycocalyx might influence localization and/or release of HB-EGF.

Our goal is complicated by the growing evidence that the C-terminal fragment of HB-EGF, consisting of the transmembrane and cytoplasmic domains, might provide signaling information separately from the extracellular domain (Higashiyama et al., 2008). This fragment can translocate to the nucleus and reverse gene repression of the transcriptional repressors promyelocytic leukemia zinc finger protein (PLZF) and Bcl6 (Kinugasa et al., 2007; Nanba et al., 2003). Therefore, in exploring processes governing HB-EGF localization, it is imperative to distinguish the extracellular EGF-like domain from the intracellular C-terminal fragment, because they are both signaling molecules that can have different localization after ligand cleavage. To address this challenge, we incorporated different visualization tags at the extracellular and intracellular domains of pro-HB-EGF. For the former, we employed an acceptor peptide enabling biotinylation for subsequent visualization with a streptavidin-conjugated fluorophore and attached green fluorescent protein (GFP) to the C-terminus for the latter. This construct facilitated novel examination of interactions between HSPGs and membrane-anchored HB-EGF (pro-HB-EGF), and consequent of the effects on ligand behavior. A key finding is that pro-HB-EGF binding to HSPGs on adjacent cells (in *trans*) is responsible for localization of pro-HB-EGF to sites of cell-cell contact, prevents proteolytic cleavage and promotes growth inhibition. This suggests a novel negative control mechanism for HB-EGF autocrine and/or paracrine signaling through sequestration of the pro-form by HSPGs.

Results

Pro-HB-EGF is localized at sites of cell-cell contact and is absent from the wound edge

In order to independently track the C-terminal tail and the extracellular domain of HB-EGF, a construct [acceptor-peptide (AP)–HB-EGF–GFP] was designed to allow two-color tracking of the localization of each fragment. A 14-amino-acid acceptor-peptide tag was inserted before the shortest of the five N-terminal cleavage sites in the extracellular domain of murine HB-EGF (Fig. 1A). The acceptor-peptide tag allows for biotinylation of one specific lysine residue in the sequence with the enzyme biotin ligase (BirA) (Beckett et al., 1999) and subsequent fluorescent labeling with a streptavidin-conjugated fluorophore (Fig. 1B). GFP was conjugated to the cytoplasmic C-terminus of HB-EGF. Acceptor-peptide labeling has the advantage that only the cell-surface pool of the protein is labeled, because streptavidin is not permeable to the cell membrane, whereas the GFP signal represents the total intracellular and plasma-membrane fraction of AP–HB-EGF–GFP. Insertion of the acceptor peptide near the N-terminus of HB-EGF and biotinylation of the tag did not affect the autocrine- and/or paracrine-growth-factor activity of the protein, as assessed by phosphorylation of the EGFR (Fig. 1C). Biotinylated AP–HB-EGF was purified from the conditioned media of COS-7 cells with heparin beads and used to stimulate naïve COS-7 cells. The purified growth factor phosphorylated the EGFR at tyrosine 1148 in the same manner as recombinant human HB-EGF (rhHB-EGF). Additionally, a heparin-bead eluant from cells lacking AP–HB-EGF expression to control for other heparin-binding growth factors in the media showed little activation of the EGFR. The activity of streptavidin-bound AP–HB-EGF was not assessed, because streptavidin labeling was only performed immediately before image acquisition. The localization of AP–HB-EGF (no GFP) and HB-EGF–GFP (no AP) were identical, suggesting that neither insertion of the AP nor GFP conjugation disrupted the localization of the protein (see supplementary material Fig. S1).

AP–HB-EGF–GFP was successfully expressed in COS-7 cells and labeled with a streptavidin-conjugated fluorophore. Cells were co-transfected with the AP–HB-EGF–GFP construct and a plasmid encoding BirA with an endoplasmic-reticulum localization sequence (BirA-ER). BirA-ER expression leads to biotinylation of acceptor-peptide-tagged proteins in the endoplasmic reticulum, utilizing endogenous ATP and biotin supplemented in the media. After 24 hours of transfection, biotinylated proteins on the cell surface were visualized with an engineered monovalent streptavidin-fluorophore conjugate that does not promote protein crosslinking (Howarth et al., 2006; Howarth and Ting, 2008) (mSA-AF568) in all experiments with the exception of those in Fig. 2, in which streptavidin-Cy5 was utilized after paraformaldehyde fixation to

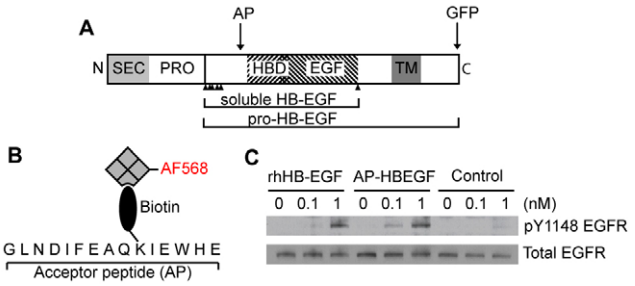

Fig. 1. Acceptor-peptide labeling of HB-EGF. (**A**) Schematic representation of mouse HB-EGF and the AP–HB-EGF–GFP construct. The arrows show sites of modification, where EGFP was inserted at the C-terminus, and a 15-amino-acid acceptor-peptide sequence (AP) was inserted before the HBD, allowing biotinylation of all size isoforms of HB-EGF. Triangles represent sites of cleavage at the N- and C-terminus of the human protein. The protein is broken up into the secretory region (SEC), the pro-region (PRO), the heparin-binding domain (HBD), the EGF-like domain (EGF),and the transmembrane domain (TM). (**B**) The 15-amino-acid acceptor peptide (AP) sequence is specifically biotinylated at one lysine residue by the enzyme BirA. After biotinylation, the monovalent streptavidin tetramer with Alexa-Flour-568 attached, whose biotin-binding ability is dead in three of the four streptavidin subunits, is used to visualize the acceptor peptide. (**C**) Biotinylated AP–HB-EGF, purified from conditioned media with heparin beads, phosphorylated the EGFR at tyrosine 1148 in the same manner as recombinant human HB-EGF (rhHB-EGF) after 1 minute of stimulation. Treatment with an equal volume of the control heparin-bead eluant, to control for other heparin-binding growth factors in the media, shows little EGFR phosphorylation.

allow for co-staining with N-cadherin to visualize sites of cell-cell contact. In some of the cells, only the GFP fluorescence (green, Fig. 2) but no acceptor peptide labeling (red, Fig. 2) was observed, which is probably due to inefficient co-transfection with BirA-ER. In most cells, however, the colocalization of the HB-EGF extracellular acceptor peptide (red, Fig. 2A), intracellular HB-EGF C-terminal GFP tail (green, Fig. 2A), and the cell-cell adhesion protein N-cadherin (white, Fig. 2A), showed that AP–HB-EGF–GFP was present on the cell surface in the uncleaved pro-form at sites of cell-cell contact, which is consistent with previous reports (Goishi et al., 1995; Singh et al., 2004). The localization of the two HB-EGF tags occasionally differed in the perinuclear region, where only the GFP signal was present in intracellular vesicles, which are probably part of the secretory pathway.

To determine the localization of the extracellular and intracellular domains of HB-EGF during cell migration, a wound-closure assay was employed to directionally control cell migration. Confluent monolayers of COS-7 cells expressing AP–HB-EGF–GFP were scratch-wounded and allowed to close for 4 hours, then the extracellular biotinylated acceptor peptide was labeled with streptavidin-Cy5 and sites of cell-cell contact were visualized with N-cadherin immunostaining. We observed that both the extracellular EGF-like domain (red, Fig. 2B) and cytoplasmic tail (green, Fig. 2B) of HB-EGF were completely absent from the wound edge, and remained only at sites of cell-cell contact. Because of the well-established role of HB-EGF in wound healing and chemotaxis, we hypothesized that HB-EGF was preferentially cleaved from the wound edge, inducing a polarized autocrine loop of EGFR activation and cleavage at the leading edge of the cell. To test this hypothesis, treatments aimed at blocking the autocrine signaling loop were used on COS-7 cells before induction of the wound. These included inhibition of proteolytic cleavage of pro-HB-EGF with batimastat and GM6001, blocking of EGFR-ligand binding with mAb225, inhibition of EGFR kinase activity with AG1478, and inhibition of intracellular pathways downstream of the EGFR using inhibitors to ERK (PD98059), Src (PP2) and PI3K (LY294002). However, none of these treatments prevented the loss of HB-EGF from the leading edge of transfected cells after wounding (see supplementary material Fig. S2). Pro-HB-EGF was also localized only at sites of cell-cell contact and was absent from any free edges in sparsely plated COS-7 cells (Fig. 2C). This suggested that, rather than loss by cleavage, it was loss of cell-cell contact that led to the absence of HB-EGF from the leading edge of cells at the wound edge. The fraction of HB-EGF at sites of cell-cell contact was quantified by measuring the amount of extracellular AP–HB-EGF–GFP colocalized with N-cadherin immunostaining normalized to the total HB-EGF area in cells that were sparse, at the wound edge or in confluent monolayers. Additionally, the fraction of the cell perimeter in cell-cell contact was calculated by measuring the length of the cell periphery positive for N-cadherin immunostaining, and normalizing this to the total cell periphery. These data indicate that the fraction of HB-EGF at sites of cell-cell contact remains constant at approximately 80%, independent of the degree of cell-cell contact (Fig. 2D).

Role of the HBD in pro-HB-EGF localization to sites of cell-cell contact

Because pro-HB-EGF was observed primarily at sites of cell-cell contact, the question arose of what molecular interactions between cells led to pro-HB-EGF concentration in this area. Because the extracellular domain of pro-HB-EGF has the ability to interact with cell-surface HSPGs, we hypothesized that this interaction might control localization of HB-EGF to sites of cell-cell contact. To test this hypothesis, we sought to compete for HSPG binding to pro-HB-EGF with exogenous heparin and heparan sulfate. We found that heparin and heparan sulfate (100 μg/ml) dramatically changed the localization of AP–HB-EGF–GFP (Fig. 3A,B). The extracellular (red, Fig. 3) and intracellular (green, Fig. 3) domain of AP–HB-EGF–GFP changed from localization primarily at sites of cell-cell contact to a homogenous distribution over the entire cell surface. Although the results shown in Fig. 3 are after 4 hours of treatment, changes in pro-HB-EGF localization were observed

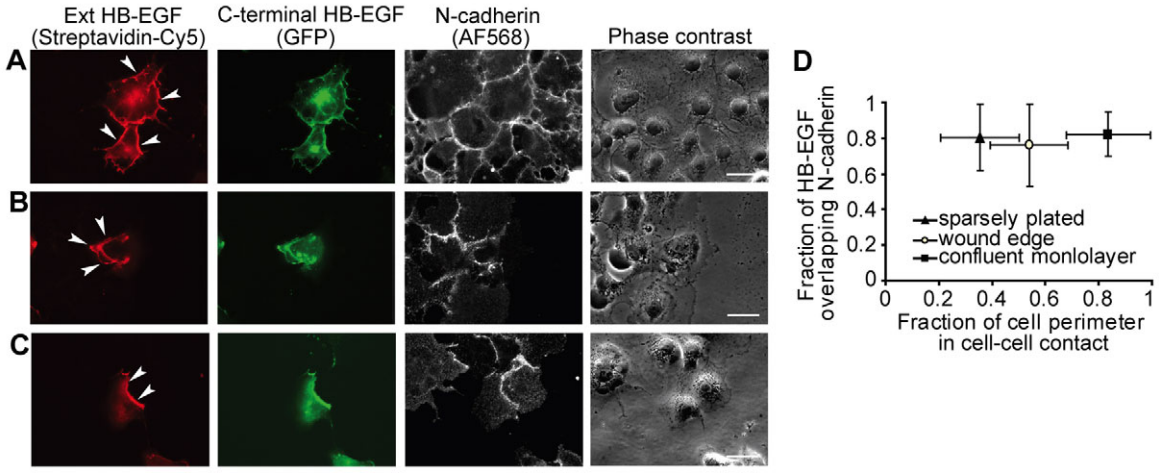

Fig. 2. Pro-HB-EGF is localized to sites of cell-cell contact. After 24 hours of transfection of COS-7 cells with AP–HB-EGF–GFP, the biotinylated extracellular acceptor peptide in AP–HB-EGF–GFP was labeled with streptavidin-Cy5 (red) and imaged alongside the cytoplasmic tail conjugated to EGFP (green), N-cadherin immunostaining (white) and phase contrast (**A**) in a confluent monolayer, (**B**) after 4 hours of wound healing and (**C**) in sparsely plated cells. Both the extracellular and intracellular domains of HB-EGF were localized to sites of cell-cell contact (arrowheads) and absent from free edges. In B, cells are migrating from left to right to close the wound. Each row represents the same field. Scale bars: 40 μm. (**D**) The fraction of HB-EGF colocalized with N-cadherin is relatively constant between cells that were sparsely plated (triangles), at the wound edge (circles) or in a confluent monolayer (squares), despite different values for the fraction of the cell perimeter in cell-cell contact. Data shown are average and standard deviation for $n \geq 8$.

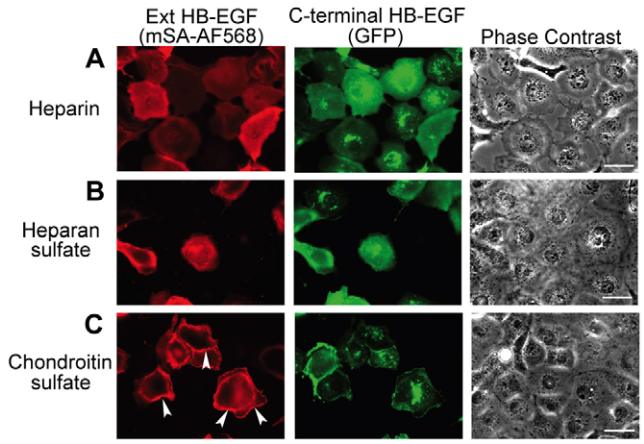

Fig. 3. Heparin and heparan sulfate changed the localization of pro-HB-EGF from sites of cell-cell contact to a homogenous distribution over the cell surface. After 24 hours of transfection of COS-7 cells with AP–HB-EGF–GFP, the biotinylated, extracellular acceptor peptide in AP–HB-EGF–GFP was labeled with monovalent streptavidin–Alexa-Fluor-568 (red, left) and imaged alongside the cytoplasmic tail conjugated to EGFP (green, middle), and phase contrast (right). Addition of (**A**) heparin (100 μg/ml) or (**B**) heparan sulfate (100 μg/ml) for 4 hours changed the localization of both the extracellular and intracellular domains of AP–HB-EGF–GFP to a diffuse distribution over the cell surface, rather than at sites of cell-cell contact. (**C**) Addition of chondroitin sulfate (100 μg/ml) for 4 hours had no effect on localization of AP–HB-EGF–GFP. Each row represents the same field. Arrowheads indicate sites of cell-cell contact. Scale bars: 40 μm.

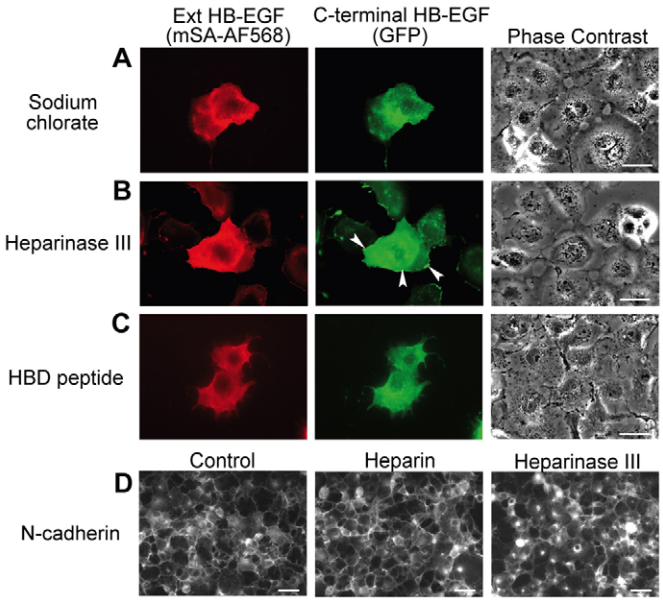

Fig. 4. HSPGs target pro-HB-EGF to sites of cell-cell contact. After 24 hours of transfection of COS-7 cells with AP–HB-EGF–GFP cells were (**A**) cultured for 24 hours with 50 mM sodium chlorate in media without penicillin-streptomycin, (**B**) treated with heparinase III (1.6 mU/ml) for 4 hours, or (**C**) incubated with a HBD peptide (100 μM) for 4 hours. The biotinylated, extracellular acceptor peptide in AP–HB-EGF–GFP was labeled with monovalent streptavidin–Alexa-Fluor-568 (red, left) and imaged alongside the cytoplasmic tail conjugated to EGFP (green, middle), and phase-contrast images were acquired (right), showing a reduction in the amount of pro-HB-EGF at sites of cell-cell contact and a more homogenous distribution over the cell surface. (A-C) Each row represents the same field. Arrowheads indicate sites of cell-cell contact. (**D**) Confluent monolayers of COS-7 cells were treated with heparin (100 μg/ml) or heparinase III (10 mU/ml) for 4 hours, then immunostained for endogenous N-cadherin. There was no change in the appearance of cell-cell junctions compared with an untreated controls. Scale bars: 40 μm.

as soon as 5 minutes following heparin addition (see supplementary material Fig. S3). The minimum concentration required to change the localization of HB-EGF after 4 hours was determined to be approximately 0.1 μg/ml of heparin and 10 μg/ml of heparan sulfate. The addition of the glycosaminoglycan chondroitin sulfate (100 μg/ml) did not affect the localization of AP–HB-EGF–GFP (Fig. 3C). At 24 hours after the removal of heparin, AP–HB-EGF–GFP localized back to sites of cell-cell contact as observed before the addition of heparin (R.N.P., unpublished). These data indicate that an interaction with HSPGs was responsible for localizing HB-EGF to sites of cell-cell contact.

To further test our hypothesis, we aimed to diminish HSPG–pro-HB-EGF interactions by manipulating HSPGs. Culture of COS-7 cells expressing AP–HB-EGF–GFP for 24 hours with 50 mM sodium chlorate, an inhibitor of protein sulfation that blocks the enzyme ATP sulfurylase (Baeuerle and Huttner, 1986), led to a homogeneous distribution of AP–HB-EGF–GFP over the cell surface (Fig. 4A), similar to the effect seen with the addition of heparin. Additionally, digestion of cell-surface heparan sulfate with heparinase III (1.6 mU/ml) for 4 hours similarly changed the localization of AP–HB-EGF–GFP, although some AP–HB-EGF–GFP remained at sites of cell-cell contact (Fig. 4B). It might be that some heparan-sulfate chains bound to HB-EGF are protected from degradation by heparinase, as is the case with bFGF and mammalian heparanase (Tumova and Bame, 1997). Competition for 4 hours with an HBD peptide (100 μM) consisting of the 21-amino-acid sequence of the HBD of murine HB-EGF also changed the localization of HB-EGF (Fig. 4C). Neither treatment with heparin nor heparinase III affected cell-cell junctions, as assessed by N-cadherin immunostaining after 4 hours of treatment (Fig. 4D). Thus, the reduction of all sulfated proteins with sodium

Table 1. Mutations in the HBD of HB-EGF

Name	HBD sequence	Number of basic residues
AP–HB-EGF–GFP	KKKKKGKGLGKKRDPCLRKYK	12
AP–delHBD-HB-EGF–GFP	----------DPCLRKYK	3
AP–97A-HB-EGF–GFP	AAAAAGKGLGKKRDPCLRKYK	7
AP–105A-HB-EGF–GFP	AAAAAGAGLGAAADPCLRKYK	3
AP–113A-HB-EGF–GFP	AAAAAGAGLGAAADPCLAAYA	0

Mutations of basic lysine and arginine residues to alanine in the 21-amino-acid HBD of murine HB-EGF, with the unaltered HBD listed in the AP–HB-EGF–GFP row. The number of basic amino acids left after mutation is shown for each mutant. –, represents amino acid deletion, rather than replacement.

chlorate, the removal of cell-surface heparan sulfate with heparinase III and competition with an HBD peptide reduced the amount of AP–HB-EGF–GFP at sites of cell-cell contact, leading to a more homogenous distribution over the cell surface.

As a complementary approach to reduce the interaction of pro-HB-EGF with cell-surface HSPGs, we made four different mutants of AP–HB-EGF–GFP to inhibit the ability of HB-EGF to interact with heparan sulfate (Table 1). The HBD of HB-EGF consists of amino acids 93-113 of the mouse protein, which contains 12 basic lysine and arginine residues that are responsible for the HB-EGF interaction with heparin (Thompson et al., 1994). Residues 108-113 lie within the EGF-like domain and contain three of the basic amino acids. Deletion of the portion of the HBD that lies outside the EGF-like domain (93-105) (AP–delHBD-HB-EGF–GFP) led to a homogeneous distribution of HB-EGF over the cell surface (Fig. 5A), similar to that seen with the addition of heparin. By contrast, mutation of the first five positive lysine residues (93-97) in the HBD to non-polar alanine (AP–97A-HB-EGF–GFP) had no effect on the localization of HB-EGF (Fig. 5B). Mutation of an additional four lysine and arginine residues to alanine (93-105) (AP–105A-HB-EGF–GFP) was nonetheless sufficient to change the localization of AP–HB-EGF–GFP to a homogenous distribution over the cell surface (Fig. 5C). The same result was observed with the mutation of all 12 basic amino acids in the HBD to alanine, including those within the EGF-like domain (AP–113A-HB-EGF–GFP) (Fig. 5D). Correct localization of HB-EGF to the plasma-membrane fraction was observed in all HBD mutants, as well as after treatment with heparin and heparinase III (see supplementary material Fig. S4A). Additionally, paracrine stimulation with the AP–113A-HB-EGF mutant activated the EGFR in the same manner as AP–HB-EGF, suggesting that mutation of the HBD caused no gross changes in molecular conformation (see supplementary material Fig. S4B). The fraction of cell-surface HB-EGF at sites of cell-cell contact was quantified with fluorescence microscopy by measuring the area of acceptor-peptide-labeled HB-EGF that overlapped with N-cadherin immunostaining divided by the total HB-EGF area per cell. Treatment with heparin, heparinase III, or mutation of the HBD (AP–113A-HB-EGF–GFP) led to a reduction in the fraction of HB-EGF at sites of cell-cell contact from 0.8 to between 0.25 and 0.35 (Fig. 5E). These data suggest that binding of HB-EGF via the HBD to HSPGs on the cell surface is required for HB-EGF localization to sites of cell-cell contact.

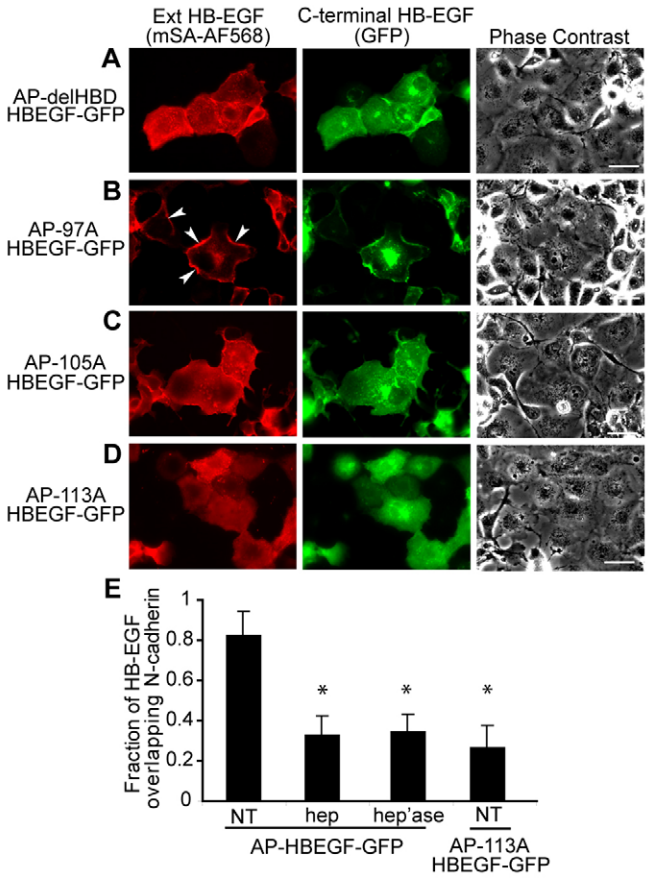

Fig. 5. The HBD targets pro-HB-EGF to sites of cell-cell contact. After 24 hours of plasmid transfection, the biotinylated, extracellular acceptor peptide in the HB-EGF mutants was labeled with monovalent streptavidin–Alexa-Fluor-568 (red, left) and imaged alongside the cytoplasmic tail of HB-EGF conjugated to EGFP (green, middle) and phase contrast (right). (**A**) Deletion of the portion of the HBD of AP–HB-EGF–GFP that lies outside of the EGF-like domain (AP–delHBD-HB-EGF–GFP) led to a homogenous distribution of HB-EGF over the cell surface. (**B**) Mutation of the first five positively charged lysine residues in the HBD of HB-EGF to alanine (AP–97A-HB-EGF–GFP) had no effect on the localization of the protein. (**C**) Mutation of all nine positive lysine and arginine residues of the HBD, which lie outside the EGF-like domain (AP–105A-HB-EGF–GFP), led to a more diffuse distribution of HB-EGF over the cell surface; however, some remained localized at sites of cell-cell contact. (**D**) Mutation of all 12 positive lysine and arginine residues in the HBD of HB-EGF to alanine (AP–113A-HB-EGF–GFP), both those outside and inside the EGF-like domain, led to a homogenous distribution of HB-EGF over the cell surface. Each row represents the same field. Scale bars: 40 μm. Arrowheads indicate sites of cell-cell contact. (**E**) The fraction of extracellular streptavidin-labeled AP–HB-EGF–GFP that colocalized with N-cadherin immunostaining was quantified from fluorescence micrographs. Mutation of the HBD (AP–113A-HB-EGF–GFP) and treatment with heparin (100 μg/ml; hep) or heparinase (10 mU/ml; hep'ase) significantly decreased the fraction of HB-EGF that colocalized with N-cadherin at sites of cell-cell contact ($n \geq 10$, $^*P < 0.0005$). NT, no treatment.

HSPGs interact with pro-HB-EGF in *trans*

Demonstrating that HSPGs were required for HB-EGF localization to sites of cell-cell contact raised the question of the source of HSPGs for this interaction. HB-EGF could interact in *cis* with HSPGs on the same cell that expresses HB-EGF, holding it at sites of cell-cell contact. Alternatively, HB-EGF might interact with HSPGs in *trans*, and therefore HSPGs would be required on the neighboring cell for localization to sites of cell-cell contact. To distinguish between these two possibilities, CHO-K1 cells (wild type) and CHOpgsD-677 cells (Lidholt et al., 1992), which are heparan-sulfate deficient, were transfected with AP–HB-EGF–GFP (green, Fig. 6) or mCherry (red, Fig. 6) and co-cultured together in different combinations. The positive control (Fig. 6A) showed AP–HB-EGF–GFP at sites of cell-cell contact between mCherry-transfected and AP–HB-EGF–GFP-transfected wild-type CHO-K1 cells. Additionally, the negative control (Fig. 6D) showed no cell-cell-contact localization of AP–HB-EGF–GFP between mCherry-transfected and AP–HB-EGF–GFP-transfected CHOpgsD-677 cells. However, CHOpgsD-677 cells transfected with AP–HB-EGF–GFP showed localization of HB-EGF to the sites of cell-cell contact with a wild-type CHO-K1 mCherry-transfected neighbor (Fig. 6B). Wild-type CHO-K1 cells transfected with AP–HB-EGF–GFP showed no HB-EGF localization to sites of cell-cell contact with an mCherry-transfected CHOpgsD-677 neighbor (Fig. 6C). Additionally, the

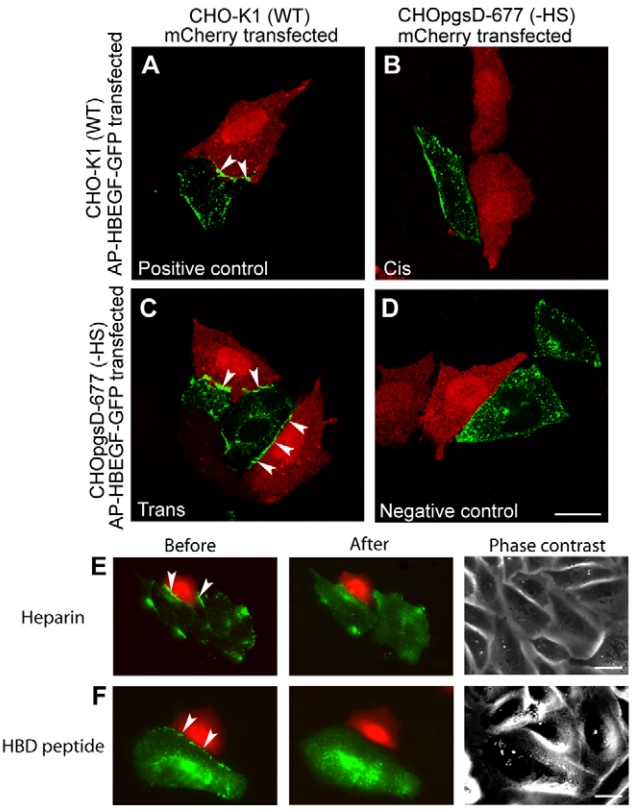

Fig. 6. HSPGs interact in *trans* with pro-HB-EGF. Confocal imaging of the localization of HB-EGF (green) at the junction of an mCherry (red)-transfected cell and an AP–HB-EGF–GFP-transfected cell. (**A**) Positive control sample with AP–HB-EGF–GFP and mCherry both in CHO-K1 (wild-type) cells had HB-EGF at sites of cell-cell contact (arrowheads). (**B**) *Cis* binding with AP–HB-EGF–GFP in CHO-K1 (wild-type) cells and mCherry in CHO-pgsD677 cells [– heparan sulfate (HS)] showed little HB-EGF at sites of cell-cell contact. (**C**) *Trans* binding with AP–HB-EGF–GFP in CHOpgsD-677 (–HS) cells and mCherry in CHO-K1 (wild-type) cells showed HB-EGF present at the cell-cell junction (arrowheads). (**D**) Negative control sample with AP–HB-EGF–GFP and mCherry in CHOpgsD-677 (–HS) cells showed no concentration of HB-EGF at the cell-cell junction. *Trans* binding epifluorescence sample images were taken before and after treatment with (**E**) heparin (100 μg/ml) for 15 minutes or (**F**) an HBD peptide (100 μM) for 2 hours, which dissociated the ligand from sites of cell-cell contact between an AP–HB-EGF–GFP transfected CHOpgsD-677 (–HS) cell and an mCherry-transfected CHO-K1 (wild-type) cell. Each experiment was repeated three or more times. Scale bars: 20 μm.

trans-bound AP–HB-EGF–GFP was dissociated from sites of cell-cell contact with the addition of heparin (100 μg/ml) for 15 minutes or the HBD peptide (100 μM) for 2 hours (Fig. 6E,F), resulting in a diffuse localization. Because CHO cells do not express cadherins (Niessen and Gumbiner, 2002), we were unable to study endogenous cell-cell junctions. These data demonstrate that the localization of pro-HB-EGF to sites of cell-cell contact is dependent on interaction with HSPGs on a neighboring cell only, and thus pro-HB-EGF interacts with HSPGs in *trans*.

Pro-HB-EGF interaction with HSPGs prevents ligand cleavage

Because heparin dramatically changed the localization of pro-HB-EGF from sites of cell-cell contact to a homogeneous distribution over the cell surface, we hypothesized that this localization change might increase access to proteases and affect ligand cleavage. To assess release of HB-EGF into the media, human placental alkaline phosphatase was inserted into the extracellular domain of AP–HB-EGF–GFP and AP–113A-HB-EGF–GFP near the N-terminus. The addition of heparin (100 μg/ml) increased alkaline-phosphatase activity in the media (Fig. 7A) of confluent monolayers of COS-7 cells transfected with wild-type HB-EGF (AlkPhos–AP–HB-EGF–GFP), suggesting that the heparin-induced localization change of pro-HB-EGF away from sites of cell-cell contact upregulates ligand cleavage. Treatment with the protease inhibitor batimastat (10 μM) inhibited heparin-induced cleavage of both wild-type and mutant HB-EGF. Interestingly, the HBD-mutant alkaline-phosphatase fusion (AlkPhos–AP–113A-HB-EGF–GFP) had higher levels of cleavage compared with wild-type HB-EGF and was unaffected further by the addition of heparin. Heparin increased the release of HB-EGF into the media in a dose-dependent manner, with concentrations above 1 μg/ml leading to maximum release after 2 hours of treatment (Fig. 7B). These data suggest that the *trans* interaction of pro-HB-EGF with HSPGs at sites of cell-cell contact prevents proteolytic release of the ligand. The interaction of HB-EGF with CD9, which also involves the HBD, might also serve to inhibit proteolytic release of the ligand, because we found a similar increase in alkaline-phosphatase activity in the medium for AlkPhos–AP–113A-HB-EGF–GFP when using the HSPG-lacking CHOpgsD-677 cells (see supplementary material Fig. S5). Because the CD9 interaction seems to operate in *cis* (Sakuma et al., 1997), and therefore ought not to depend on HB-EGF localization to sites of cell-cell contact, the two HBD interactions might work in series to provide a multi-layer control on ligand release. Despite the upregulation in AP–113A-HBEGF release, no detectable difference in EGFR phosphorylation at tyrosine 1148 was detected in cells transfected with AP–113A-HB-EGF–GFP, AP–HB-EGF–GFP or GFP (see supplementary material Fig. S4C). However, transfection with AP–HB-EGF–GFP led to growth inhibition, because [^{3}H]thymidine incorporation was decreased 48% compared with a GFP-transfected control (Fig. 7C). Interestingly, mutation of the HBD of HB-EGF not only reversed the growth inhibition of HB-EGF transfection, but also led to cell proliferation, with a 42% increase in [^{3}H]thymidine incorporation. Because HB-EGF juxtacrine signaling has been reported to be growth inhibitory (Iwamoto et al., 1999; Pan et al., 2002), we hypothesize that mutation of the HBD decreases the juxtacrine interaction by decreasing the concentration of pro-HB-EGF at sites of cell-cell contact. Additionally, because mutation of the HBD increased the rate of ligand cleavage from the cell surface, we hypothesize that an increase in autocrine signaling stimulates cell proliferation. These data would suggest that HSPGs might act as an important regulator in the balance between juxtacrine and autocrine HB-EGF, which can induce opposite cell fates of growth inhibition versus proliferation.

Heparin binding controls amphiregulin localization, but engineered heparin binding is insufficient for cell-cell-contact localization

HB-EGF is not the only ligand in the EGFR family capable of heparin binding, because amphiregulin, betacellulin and some isoforms of neuregulin also interact with HSPGs. HB-EGF and amphiregulin have similar HBDs located at the N-terminus of the protein before the EGF-like domain. These domains are both 21 amino acids in length, with over half of the residues represented by basic lysine or arginine, which allow the domain to interact

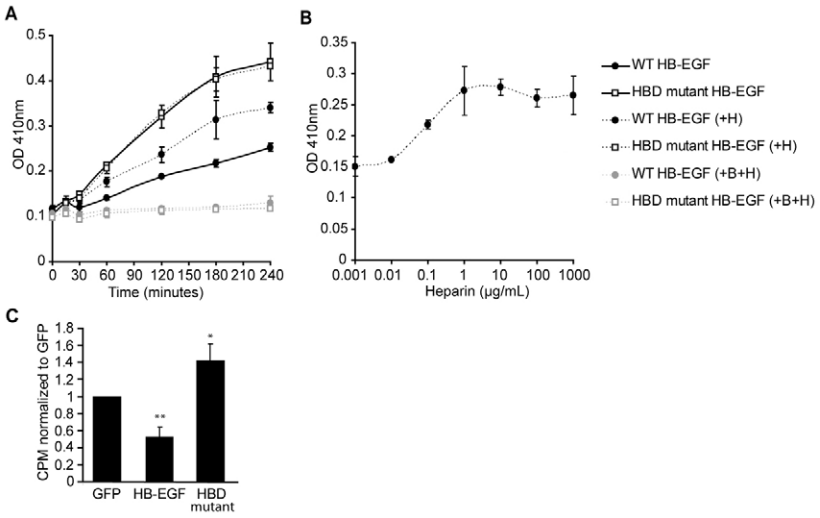

Fig. 7. Interaction with HSPGs reduces pro-HB-EGF cleavage and cell proliferation. (**A**) COS-7 cells transfected with wild-type HB-EGF (AlkPhos–AP–HB-EGF–GFP) (circles) or the HBD mutant (AlkPhos–AP–113A-HB-EGF–GFP) (squares) were either pre-treated with 10 μM batimastat (+B) or PBS alone for 1 hour. Cells were stimulated with either 100 μg/ml of heparin (+H) alone (black dashed line) or heparin and batimastat (gray dashed line), or unstimulated (black solid line) and media was collected at various time points, then cells were incubated with a 1.5 M salt solution for 60 seconds to release any soluble HB-EGF bound to HSPGs. AlkPhos–AP–HB-EGF–GFP release is increased by treatment with heparin, and cleavage is further increased by mutating the HBD (AlkPhos–AP–113A-HB-EGF–GFP). Pre-treatment with batimastat before the addition of heparin blocks both AlkPhos–AP–HB-EGF–GFP and AlkPhos–AP–113A-HB-EGF–GFP cleavage. (**B**) Treatment for 2 hours with heparin (100 μg/ml) increases AlkPhos–AP–HB-EGF–GFP release in a dose-dependent manner. (A,B) Data shown are average and standard deviation of three biological replicates from one of three independent experiments in which the same trend was observed. (**C**) Incorporation of [^{3}H]thymidine into cells transfected with GFP, AP–HB-EGF–GFP (HB-EGF) or AP–113A-HB-EGF–GFP (HBD mutant) for 24 hours shows that HB-EGF transfection induces growth inhibition compared with GFP, whereas mutation of the HBD reverses the growth inhibition and promotes proliferation. Results shown are normalized to GFP control and represent the average and standard deviation from four independent experiments with three or more biological replicates per experiment (*P<0.05, **P<0.005).

with negatively charged heparin and heparan sulfate (Thompson et al., 1994). We sought to test whether heparin changed the localization of amphiregulin in the same manner as it does HB-EGF to determine whether HSPG-mediated localization was a common control mechanism for heparin-binding EGFR ligands. Acceptor-peptide labeling of the extracellular portion of amphiregulin, and a C-terminal GFP tag, showed that amphiregulin is similar to HB-EGF in that the extracellular and intracellular fluorophores were colocalized, suggesting that amphiregulin is primarily present in the pro-form (Fig. 8A). Additionally, amphiregulin was also concentrated around the perimeter of the cell at sites of cell-cell contact and the addition of heparin (100 μg/ml) for 4 hours led to a homogenous distribution of amphiregulin over the cell surface (Fig. 8B). Similarly, mutation of all lysine and arginine residues in the HBD of amphiregulin to alanine led to an identical distribution (R.N.P., unpublished), suggesting that the HBD of amphiregulin is also responsible for its localization to sites of cell-cell contact.

To test whether the HBD of HB-EGF was sufficient to localize any transmembrane protein to sites of cell-cell contact, we engineered an artificial protein consisting of an extracellular acceptor-peptide sequence followed by the portion of the HBD of HB-EGF that lies outside the EGF-like domain (residues 93-105), a 15-amino-acid flexible linker, cyan fluorescent protein (CFP), then the transmembrane domain (TM) of the PDGF receptor. This construct (AP-HBD-CFP-TM) (Fig. 8D) showed the same homogenous localization over the cell surface as a control construct (Fig. 8C) lacking an HBD (AP-CFP-TM). Therefore, the HBD alone was insufficient to localize any protein to sites of cell-cell contact. This suggests that an additional cofactor or specific structural feature of EGFR ligands is required for HB-EGF and amphiregulin localization to sites of cell-cell contact.

Discussion

HSPGs, which are present on the cell surface and in the extracellular matrix, are capable of binding many growth factors. Traditionally, this interaction has been proposed to restrain soluble, diffusible ligands, thus increasing their local concentration and ability to activate receptors (Schlessinger et al., 1995). Additionally, heparan-sulfate binding is known to modulate the activity of signaling molecules and protect them from proteolytic degradation (Conrad, 1998). Most ligands that have been reported to interact with HSPGs are soluble secreted factors, such as fibroblast growth factors (Gospodarowicz et al., 1984; Maciag et al., 1984; Shing et al., 1984), vascular endothelial growth factor (Ferrara and Henzel, 1989), hepatocyte growth factor (Nakamura et al., 1984; Zhou et al., 1999) and platelet-derived growth factor (Schilling et al., 1998). However, HSPGs also interact with some growth factors that remain anchored to the cell surface via a transmembrane domain, particularly those that belong to the EGFR-ligand family, including HB-EGF (Higashiyama et al., 1991), amphiregulin (Cook et al., 1991), betacellulin (Shing et al., 1993) and certain isoforms of neuregulin (Holmes et al., 1992; Loeb and Fischbach, 1995).

To extend our knowledge and understanding of the role of HSPGs on growth-factor regulation, we investigated their interactions with transmembrane pro-HB-EGF. One of our key findings was that a *trans* interaction between HSPG and the HB-EGF HBD is responsible for localizing pro-HB-EGF to sites of cell-cell contact (Figs 2-6). HSPGs also modulated the localization

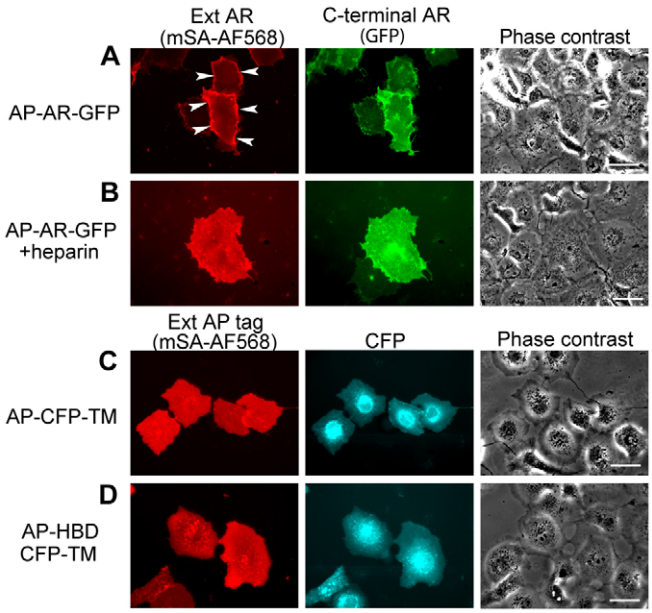

Fig. 8. Pro-amphiregulin localization is changed by heparin, but the addition of an HBD to other proteins was insufficient for localization to sites of cell-cell contact. After 24 hours of plasmid transfection, the biotinylated, extracellular acceptor peptide was labeled with monovalent streptavidin–Alexa-Fluor-568 (red, left) and imaged alongside the cytoplasmic tail of amphiregulin conjugated to EGFP (green, middle) or the CFP-tagged control construct (cyan, middle), and phase contrast (right). (**A**) AP-AR-GFP showed localization of the extracellular and intracellular domains to sites of cell-cell contact (arrowheads). (**B**) Addition of heparin (100 μg/ml) for 4 hours changed the localization of both the extracellular and intracellular domains of AP-AR-GFP to a diffuse distribution over the cell surface, rather than at sites of cell-cell contact. (**C**) The control construct AP-CFP-TM had a diffuse localization of the protein over the cell surface, and (**D**) the addition of an HBD to the control construct (AP-HBD-CFP-TM) did not alter this localization. Each row represents the same field. Scale bars: 40 μm.

of pro-amphiregulin in a similar manner (Fig. 8), suggesting that this might be a common mechanism for transmembrane heparin-binding ligands of the EGFR family. Because HB-EGF (Higashiyama et al., 1995), amphiregulin (Inui et al., 1997) and betacellulin (Tada et al., 1999) are heparin-binding and also capable of signaling in a juxtacrine mode, the role of this interaction could be to assist in sequestering prospective juxtacrine ligands at sites of cell-cell contact to facilitate receptor binding, probably in concert with CD9, to bind a receptor on a neighboring cell. This idea is supported by the observation that the HBD of HB-EGF and amphiregulin inhibits binding to the receptor except when in complex with heparin or heparan sulfate (Higashiyama et al., 1993; Johnson and Wong, 1994; Piepkorn et al., 1994; Takazaki et al., 2004), and that presence of an intact HB-EGF N-terminus prevents the ligand from activating its receptor during intracellular transport (Dong et al., 2005). Together, these data support the idea that HSPGs act as a permissive factor for juxtacrine signaling at sites of cell-cell contact.

Using wild-type and mutant CHO cells lacking cell-surface heparan sulfate, we demonstrated that the interaction of HSPGs with pro-HB-EGF occurs in *trans* (Fig. 6). The localization of HB-EGF to sites of cell-cell contact is unlikely to depend on interaction with EGFR, because CHO-K1 cells lack endogenous EGFR yet still show strong localization of HB-EGF to sites of cell-cell contact. This experiment also suggests that HSPGs are not required as an intracellular chaperone for pro-HB-EGF during transport, because cells lacking heparan sulfate were still able to localize HB-EGF to the cell surface. HSPGs have previously been shown to interact in *trans* with VEGFR-2 in VEGFR-mediated angiogenesis (Jakobsson et al., 2006) and with the *Xenopus* receptor caALK4 via the cofactor Vg1 during mesoderm migration in early left-right development (Kramer and Yost, 2002). Additionally, this interaction probably plays a role in blastocyst implantation during pregnancy, because the interaction between HB-EGF on the lumenal epithelium with EGFR and HSPGs on the adjacent blastocyst is required for successful attachment, which is reduced by exogenous heparin or blastocyst heparinase treatment (Farach et al., 1987; Farach et al., 1988; Raab et al., 1996).

A further key finding from our work here is that exogenous heparin and mutation of the HBD can upregulate HB-EGF proteolytic release in COS-7 cells, suggesting that association with HSPGs at sites of cell-cell contact protects pro-HB-EGF from proteolysis (Fig. 7A). However, this protection is not likely to be due to physical association of pro-HB-EGF with HSPGs, because soluble heparin increased proteolytic cleavage of the ligand. We speculate that the sequestration of pro-HB-EGF at sites of cell-cell contact reduces access to metalloproteinases, consistent with evidence that ectodomain cleavage is regulated at the substrate level (Herrlich et al., 2008). Mutation of the HBD not only reversed the growth inhibition observed with expression of pro-HB-EGF (Fig. 7C), but also stimulated cell proliferation. We hypothesize that the reversal of growth inhibition is due to a reduction in juxtacrine signaling, because less HB-EGF is present at sites of cell-cell contact when the HBD is not intact. Additionally, because the HBD mutant is cleaved at a higher rate, this probably increases autocrine HB-EGF signaling, stimulating cell growth. These data suggest that interaction with HSPGs plays an important role in the balance between juxtacrine and autocrine HB-EGF signaling and the resulting opposite cell fates of growth inhibition versus proliferation.

It is conceivable that heparin and heparan sulfate also operate in vivo to dissociate pro-HB-EGF from HSPGs, thus stimulating ligand cleavage. However, results reported here should be treated with some caution, because experiments were performed in transformed cells with overexpression of the ligand. In vivo, free heparin is secreted by mast cells upon degranulation, and both mast cells and HB-EGF signaling are implicated in the biological processes of wound healing (for a review, see Noli and Miolo, 2001) (Tokumaru et al., 2000), as well as angiogenesis (for a review, see Galinsky and Nechushtan, 2008) (Ongusaha et al., 2004) and the pathogenesis of atherosclerosis (for a review, see Kalesnikoff and Galli, 2008) (Nakata et al., 1996). Additionally, dermal mast cells themselves express *HB-EGF* mRNA (Artuc et al., 2002). Extracellular free heparan sulfate is generated by degradation of cell-surface HSPGs with heparanase, the expression of which is upregulated in all analyzed human cancers (Vlodavsky et al., 2007). It is possible that heparanase leads to a local concentration of free heparan sulfate that is high enough in the interstitial space to dissociate pro-HB-EGF from sites of cell-cell contact and stimulate cleavage of pro-HB-EGF. Fully understanding these complicated effects will probably require detailed mathematical modeling studies in the future along the lines analogously undertaken for the fibroblast-growth-factor system (Forsten-Williams et al., 2008).

Materials and Methods

Reagents

The following reagents were used: biotin (Sigma B4501), heparin (Sigma H3149), heparan sulfate (Sigma H7640), chondroitin sulfate (Sigma C4384), heparinase III (Sigma H8891), sodium chlorate (Sigma 244147), HBD peptide KKKKKGKGLGKKRDPCLRKYK (Neopeptide),

Cell culture

COS-7 cells were cultured in high-glucose Dulbecco's modified eagle medium (DMEM) with L-glutamine (Gibco 11965), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. CHO-K1 and CHOpgsD-677 cells (Lidholt et al., 1992) were cultured in Ham's F12 media with L-glutamine (BioWhittaker 12-615F), supplemented with 10% FBS and 1% penicillin-streptomycin. For microscopy experiments, cells were plated on 35-mm glass-bottom dishes (Mattek) coated with 0.1% gelatin (COS-7) or 1 μg/ml of fibronectin (CHO cells) for 1 hour at 37°C.

Plasmid constructs

The murine *HB-EGF* coding region was subcloned into pEGFP-N1 (Clontech) using the *Eco*RI and *Xho*I restriction sites from an adenoviral plasmid (Yoshioka et al., 2005). The acceptor peptide was inserted via site-directed mutagenesis (Geiser et al., 2001) with the primer 5′-GTGAAGTTGGGCGTGGCTAGCTCCCACC-GAATTCTGCAGTCGACGGTACCGCGGGCC-3′ and its reverse complement. From here the following primers and their reverse complements were used to mutate the HBD: AP-delHBD-HBEGF-GFP with 5′-AAATCGAATGGCACGAA-GGGGACCCATGCCTCAGGAAATA-3′, AP-97A-HBEGF-GFP with 5′-AAATC-GAATGGCACGAAGGGGCGGCAGCCGCTGCGGGAAAGGGGTTAGGGAA-GAAGA-3′, AP-105A-HBEGF-GFP with 5′-AAATCGAATGGCACGAA-GGGGCGGCAGCCGCTGCGGGAGCAGGGTTAGGGGCGGCAGCCGACC-CATGCCTCAGGAAATA-3′ and AP-105A-HBEGF-GFP was used as the base plasmids to make AP-113A-HBEGF-GFP with 5′-CAGCCGACCCATGC-CTCGCAGCGTACGCAGACTACTGCATCCACGGGGA-3′. Human amphiregulin cDNA was subcloned into pEGFP-N1 and the acceptor peptide inserted after V107 with the primer 5′-TCAGTCAGAGTTGAACAGGTAGTTGGCCTGAACGAC-ATCTTCGAAGCCCAGAAAATCGAATGGCACGAAAAGCCCCCCCAAAAC-AAG-3′ and its reverse complement. The HBD of HB-EGF (93-105) was inserted into AP-CFP-TM (Chen et al., 2005) between CFP and the AP sequence to make AP-HBD-CFP-TM with the primer 5′-AGTGGCACGAGGGCGCGCCGAAAAAGAAGA-AGAAAGGAAAGGGGTTAGGGAAGAAGAGCGGGCGGCATGGTGAG-CAAGGGCGAGGA-3′ and its reverse complement. Then, a flexible linker (SEGGGSEGGTSGATG) was added between CFP and AP to allow the HBD better access to HSPGs. This was done by PCR on AP-HBD-CFP-TM with the primer 5′-GAAGAAGAGAGCGGGCGGCTCTGAAGGCGGCGGCAGCGAAGGCGG-CACCAGCGGCGCGACCGGAATGGTGAGCAAGGGCGAGGA-3′ and its reverse complement. Human placental alkaline phosphatase was amplified from a human HB-EGF alkaline-phosphatase fusion protein (Raab et al., 1996) with forward primer 5′-CCTGGCCACCCCAAGCAAAGAAAGGAATATCATCCCAGTTGAG-GAGGAGAACCCGGACTTCTGGAACCGC-3′ and reverse primer 5′-TTTCT-GGGCTTCGAAGATGTCGTTCAGGCCGTCGGTGGTGCCGGCGGGG-3′. The PCR product was gel purified and then used as the primer for a QuikChange insertion modified for large inserts on the base plasmid AP–HB-EGF–GFP and AP–113A-HB-EGF–GFP.

Purification of AP–HB-EGF

Three confluent 150-cm^2 cell-culture dishes of COS-7 cells with 10 μM biotin in the media were transfected with AP–HB-EGF and BirA-ER or BirA-ER alone. After 24 hours, cell-culture media was replaced with serum-free media with 50 mM sodium chlorate and 10 μM biotin. After an additional 24 hours, the media was replaced with phosphate buffered saline (PBS) with sodium and magnesium, 50 mM sodium chlorate, 4 μM phorbol 12-myristate 13-acetate (Sigma P8139), 0.0003% H_2O_2 and 10 μM biotin. The following day, the PBS solution was collected after spinning out dead cells and incubated at 4°C overnight with 50 μl of heparin acrylic beads (Sigma H5263). After washing with PBS, the biotinylated AP–HB-EGF was eluted from the heparin beads with 50 μl of 2 M NaCl in PBS. The concentration of biotinylated AP–HB-EGF was estimated by western blotting with streptavidin-HRP in comparison with a standard of biotinylated acceptor-peptide-tagged maltose-binding protein (Avidity BIS-300).

Bioactivity of AP–HB-EGF

COS-7 cells were serum starved for 24 hours and stimulated for 1 minute with recombinant human HB-EGF (R&D Systems, 259-HE), heparin-bead-purified biotinylated AP–HBEGF or an equal volume of a control heparin-bead eluant. Cells were washed with PBS, then lysed with RIPA buffer [PBS with 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 0.57 mM PMSF, protease inhibitor cocktail (Sigma P8340), and phosphatase inhibitor cocktail II (Sigma P5725)]. Lysates were subjected to SDS-PAGE and immunoblotted for total EGFR (C74B9) (Cell Signaling Technology, 2464) and phospho-EGFR Tyr1148 (Cell Signaling Technology, 4404) according to standard protocols. This experiment was repeated twice.

Biotinylation and cell-surface labeling of the acceptor peptide

Acceptor-peptide fusion constructs and a variant of BirA that was engineered to localize to the ER (BirA-ER) (Howarth and Ting, 2008) were co-transfected into COS-7 cells with Fugene6 or Mirus LTI Transfection reagent at equal molar ratios and cell-culture media was supplemented with 10 μM biotin. After 24 hours, cells were washed with PBS with calcium and magnesium and the acceptor peptide was labeled with mSA-AF568 or streptavidin-Cy5 (Invitrogen 43-8316) at 1 μg/ml in 5% dialyzed bovine serum albumin (BSA) in PBS for 10 minutes on ice. Cells were washed twice with PBS before imaging and imaged in PBS. Monovalent streptavidin–Alexa-Fluor-568 was produced as previously published (Howarth et al., 2006; Howarth and Ting, 2008). Each fluorescent labeling experiment was repeated three or more times, with 20 or more cells evaluated per experiment.

Immunostaining and fluorescence quantification

Cells were fixed with 2% paraformaldehyde for 10 minutes, blocked with 5% horse serum, incubated with anti-N-cadherin (H-63) antibody (Santa Cruz 7939) at 1:50, then anti-rabbit AF568 (Invitrogen A11011) at 1:500. The area in pixels for N-cadherin immunostaining and streptavidin-fluorophore-labeled extracellular HB-EGF was measured from fluorescence micrographs using thresholding with the ImageJ software package to define positive fluorescence. An N-cadherin mask was then applied to the HB-EGF images to quantify the number of pixels per cell that overlapped N-cadherin compared with the total number of HB-EGF-positive pixels.

Alkaline-phosphatase cleavage assay

COS-7 cells were plated on 96-well dishes (6500 cells per well) for 1 day, then transfected with AlkPhos–AP–HB-EGF–GFP or AlkPhos–AP–113A-HB-EGF–GFP. The following day, designated samples were pre-treated with batimastat (10 μM) in PBS with calcium and magnesium for 1 hour, then stimulated with a 100 μl solution of heparin in PBS supplemented with 1% BSA. Supernatants were collected at various time points, then washed for 1 minute with 100 μl of 1.5 M NaCl in PBS supplemented with 1% BSA to remove any soluble HB-EGF bound to HSPGs. The salt wash was combined with the supernatant and 40 μl was combined with 100 μl of p-nitrophenyl phosphate (Millipore ES009), incubated for 2 hours at 37°C, then optical density was read at 410 nm.

[^{3}H]thymidine incorporation

COS-7 were plated at 25,000 cells per well in a 12-well dish (BD Falcon) for 24 hours, then transfected and serum starved. After 1 day, 1 μCi/ml of [^{3}H]thymidine (Perkin Elmer NET027E001MC) was added for 3 hours, then the cells were washed with PBS and 5% tricholoroacetic acid, and lysed with 1 ml of 0.1 N NaOH + 0.1% SDS. After the addition of 4 ml of scintillation cocktail (Ecolume 88247005), samples were read on a Beckman Coulter LS6500 scintillation counter.

Microscopy

Phase-contrast and fluorescent images were obtained with a digital CCD camera (CoolSNAP HQ, Roper Scientific) and an inverted microscope (Olympus IX-70) with a 20× phase-contrast objective (Olympus LCPlanF NA 0.40) or 40× water-immersion objective (Olympus UApo/340 NA 1.15) for fluorescence. Confocal images were acquired at 60× with a Nikon TE2000-U inverted microscope.

Statistical analysis

A two-tailed, unpaired Student's *t*-test assuming unequal variance was utilized.

We wish to thank our funding sources, including NIBIB Grant EB003805, NCI Grant CA96504, MIT Poitras Predoctoral Fellowship and the National Science Foundation (NSF) Graduate Research Fellowship. Additionally, we would like to thank Lee Opresko, Rosalyn Adam, Leslie Mebane, Rachel Miller and Alan Grodzinsky for reagents. For helpful scientific discussion, we are grateful to Matthew Nugent. Deposited in PMC for release after 12 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/13/2308/DC1

References

Artuc, M., Steckelings, U. M. and Henz, B. M. (2002). Mast cell-fibroblast interactions: human mast cells as source and inducers of fibroblast and epithelial growth factors. *J. Invest. Dermatol.* **118**, 391-395.

Baeuerle, P. A. and Huttner, W. B. (1986). Chlorate-a potent inhibitor of protein sulfation in intact cells. *Biochem. Biophys. Res. Commun.* **141**, 870-877.

Bakken, A. M., Protack, C. D., Roztocil, E., Nicholl, S. M. and Davies, M. G. (2009). Cell migration in response to the amino-terminal fragment of urokinase requires EGF receptor activation through an ADAM-mediated mechanism. *J. Vasc. Surg.* **49**, 1296-1303.

Beckett, D., Kovaleva, E. and Schatz, P. J. (1999). A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci.* **8**, 921-929.

Block, E. R., Matela, A. R., SundarRaj, N., Iszkula, E. R. and Klarlund, J. K. (2004). Wounding induces motility in sheets of corneal epithelial cells through loss of spatial

constraints: role of heparin-binding epidermal growth factor-like growth factor signaling. *J. Biol. Chem*. **279**, 24307-24312.

Chen, I., Howarth, M., Lin, W. and Ting, A. Y. (2005). Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. *Nat. Methods* **2**, 99-104.

Conrad, H. E. (1998). *Heparin-Binding Proteins*. San Diego, CA: Academic Press.

Cook, P. W., Mattox, P. A., Keeble, W. W., Pittelkow, M. R., Plowman, G. D., Shoyab, M., Adelman, J. P. and Shipley, G. D. (1991). A heparin sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. *Mol. Cell. Biol*. **11**, 2547-2557.

Dong, J., Opresko, L. K., Chrisler, W., Orr, G., Quesenberry, R. D., Lauffenburger, D. A. and Wiley, H. S. (2005). The membrane-anchoring domain of epidermal growth factor receptor ligands dictates their ability to operate in juxtacrine mode. *Mol. Biol. Cell* **16**, 2984-2998.

Elenius, K., Paul, S., Allison, G., Sun, J. and Klagsbrun, M. (1997). Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J*. **16**, 1268-1278.

Farach, M. C., Tang, J. P., Decker, G. L. and Carson, D. D. (1987). Heparin/heparan sulfate is involved in attachment and spreading of mouse embryos in vitro. *Dev. Biol*. **123**, 401-410.

Farach, M. C., Tang, J. P., Decker, G. L. and Carson, D. D. (1988). Differential effects of p-nitrophenyl-D-xylosides on mouse blastocysts and uterine epithelial cells. *Biol. Reprod*. **39**, 443-455.

Faull, R. J., Stanley, J. M., Fraser, S., Power, D. A. and Leavesley, D. I. (2001). HB-EGF is produced in the peritoneal cavity and enhances mesothelial cell adhesion and migration. *Kidney Intl.* **59**, 614-624.

Ferrara, N. and Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun*. **161**, 851-858.

Forsten-Williams, K., Chu, C. L., Fannon, M., Buczek-Thomas, J. A. and Nugent, M. A. (2008). Control of growth factor networks by heparan sulfate proteoglycans. *Ann. Biomed. Eng.* **36**, 2134-2148.

Galinsky, D. S. and Nechushtan, H. (2008). Mast cells and cancer-No longer just basic science. *Crit. Rev. Oncol. Hematol*. **68**, 115-130.

Geiser, M., Cebe, R., Drewello, D. and Schmitz, R. (2001). Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *Biotechniques* **31**, 88-90.

Goishi, K., Higashiyama, S., Klagsbrun, M., Nakano, N., Umata, T., Ishikawa, M., Mekada, E. and Taniguchi, N. (1995). Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. *Mol. Biol. Cell* **6**, 967-980.

Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A. and Bohlent, P. (1984). Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **81**, 6963-6967.

Harris, R. C., Chung, E. and Coffey, R. J. (2003). EGF receptor ligands. *Exp. Cell Res.* **284**, 2-13.

Herrlich, A., Klinman, E., Fu, J., Sadegh, C. and Lodish, H. F. (2008). Ectodomain cleavage of the EGF ligands HB-EGF, neuregulin1-beta, and TGF-alpha is specifically triggered by different stimuli and involves different PKC isoenzymes. *FASEB J*. **22**, 4281-4295.

Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C. and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936-939.

Higashiyama, S., Abraham, J. A. and Klagsbrun, M. (1993). Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J. Cell Biol*. **122**, 933-940.

Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). The membrane protein CD9/DRAP 27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J. Cell Biol*. **128**, 929-938.

Higashiyama, S., Iwabuki, H., Morimoto, C., Hieda, M., Inoue, H. and Matsushita, N. (2008). Membrane-anchored growth factors, the EGF family: beyond receptor ligands. *Cancer Sci.* **99**, 214-220.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D. et al. (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205-1210.

Howarth, M. and Ting, A. Y. (2008). Imaging proteins in live mammalian cells with biotin ligase and monovalent streptavidin. *Nat. Protocols* **3**, 534-545.

Howarth, M., Chinnapen, D. J., Gerrow, K., Dorrestein, P. C., Grandy, M. R., Kelleher, N. L, El-Husseini, A. and Ting, A. Y. (2006). A monovalent streptavidin with a single femtomolar biotin binding site. *Nat. Methods* **3**, 267-273.

Inui, S., Higashiyama, S., Hashimoto, K., Higashiyama, M., Yoshikawa, K. and Taniguchi, N. (1997). Possible role of coexpression of CD9 with membrane-anchored heparin-binding EGF-like growth factor and amphiregulin in cultured human keratinocyte growth. *J. Cell. Physiol*. **171**, 291-298.

Iwamoto, R., Senoh, H., Okada, Y., Uchida, T. and Mekada, E. (1991). An antibody that inhibits the binding of diphtheria toxin to cells revealed the association of a 27-kDa membrane protein with the diphtheria toxin receptor. *J. Biol. Chem*. **266**, 20463-20469.

Iwamoto, R., Handa, K. and Mekada, E. (1999). Contact-dependent growth inhibition and apoptosis of EGF receptor-expressing cells by the membrane-anchored form of heparin-binding EGF-like growth factor. *J. Biol. Chem*. **274**, 25906-25912.

Jakobsson, L., Kreuger, J., Holmborn, K., Lundin, L., Eriksson, I., Kjellen, L. and Claesson-Welsh, L. (2006). Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev. Cell* **10**, 625-634.

Johnson, G. R. and Wong, L. (1994). Heparan sulfate is essential to amphiregulin-induced mitogenic signaling by the epidermal growth factor receptor. *J. Biol. Chem*. **269**, 27149-27154.

Joslin, E. J., Opresko, L. K., Wells, A., Wiley, H. S. and Lauffenburger, D. A. (2007). EGF receptor-mediated mammary epithelial cell migration is driven by sustained ERK signaling from autocrine stimulation. *J. Cell Sci.* **120**, 3688-3699.

Kalesnikoff, J. and Galli, S. J. (2008). New developments in mast cell biology. *Nat. Immunol*. **9**, 1215-1223.

Kinugasa, Y., Hieda, M., Hori, M. and Higashiyama, S. (2007). The carboxyl-terminal fragment of pro-HB-EGF reverses Bcl6-mediated gene repression. *J. Biol. Chem*. **282**, 14797-14806.

Kramer, K. L. and Yost, H. J. (2002). Ectodermal syndecan-2 mediates left-right axis formation in migrating mesoderm as a cell-nonautonomous Vg1 cofactor. *Dev. Cell* **2**, 115-124.

Lidholt, K., Weinke, J. L., Kiser, C. S., Lugemwa, F. N., Bame, K. J., Cheifetz, S., Massague, J., Lindahl, U. and Esko, J. D. (1992). A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc. Natl. Acad. Sci. USA* **89**, 2267-2271.

Loeb, J. A. and Fischbach, G. D. (1995). ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. *J. Cell Biol*. **130**, 127-135.

Maciag, T., Mehlman, T., Friesel, R. and Schreiber, A. B. (1984). Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science* **225**, 932-935.

Maheshwari, G., Wiley, H. S. and Lauffenburger, D. A. (2001). Autocrine EGF signaling stimulates directionally persistent mammary epithelial cell migration. *J. Cell Biol*. **155**, 1123-1128.

Maly, I., Lee, R. T. and Lauffenburger, D. A. (2004). A model for mechanotransduction in cardiac muscle: effects of extracellular matrix deformation on autocrine signaling. *Ann. Biomed. Eng.* **32**, 1319-1335.

Matsuzaki, H., Kobayashi, H., Yagyu, T., Wakahara, K., Kondo, T., Kurita, N., Sekino, H., Inagaki, K., Suzuki, M., Kanayama, N. et al. (2005). Reduced syndecan-1 expression stimulates HB-EGF-mediated invasion in ovarian cancer cells in a urokinase-independent mechanism. *Oncol. Rep.* **14**, 449-457.

Miyamoto, S., Hirata, M., Yamazaki, A., Kageyama, T., Hasuwa, H., Mizushima, H., Tanaka, Y., Yagi, H., Sonoda, K., Kai, M. et al. (2004). Heparin-binding EGF-like growth factor is a promising target for ovarian cancer therapy. *Cancer Res.* **64**, 5720-5757.

Nakamura, T., Nawa, K. and Ichihara, A. (1984). Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun*. **122**, 1450-1459.

Nakamura, K., Iwamoto, R. and Mekada, E. (1995). Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha 3 beta 1 at cell-cell contact sites. *J. Cell Biol*. **129**, 1691-1705.

Nakata, A., Miyagawa, J., Yamashita, S., Nishida, M., Tamura, R., Yamamori, K., Nakamura, T., Nozaki, S., Kameda-Takemura, K., Kawata, S. et al. (1996). Localization of heparin-binding epidermal growth factor-like growth factor in human coronary arteries. Possible roles of HB-EGF in the formation of coronary atherosclerosis. *Circulation* **94**, 2778-2786.

Nanba, D., Mammoto, A., Hashimoto, K. and Higashiyama, S. (2003). Proteolytic release of the carboxy-terminal fragment of proHB-EGF causes nuclear export of PLZF. *J. Cell Biol*. **163**, 489-502.

Niessen, C. M. and Gumbiner, B. M. (2002). Cadherin-mediated cell sorting not determined by binding or adhesion specificity. *J. Cell Biol*. **156**, 389-399.

Noli, C. and Miolo, A. (2001). The mast cell in wound healing. *Vet. Dermatol*. **12**, 303-313.

Normanno, N., Bianco, C., DeLuca, A. and Salomon, D. S. (2001). The role of EGF-related peptides in tumor growth. *Front. Biosci.* **6**, D685-D707.

Ongusaha, P. P., Kwak, J. C., Zwible, A. J., Macip, S., Higashiyama, S., Taniguchi, N., Fang, L. and Lee, S. W. (2004). HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res*. **64**, 5283-5290.

Ozaki, Y., Nishimura, M., Sekiya, K., Suehiro, F., Kanawa, M., Nikawa, H., Hamada, T. and Kato, Y. (2007). Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. *Stem Cells Dev.* **16**, 119-129.

Pan, B., Sengoku, K., Goishi, K., Takuma, N., Yamashita, T., Wada, K. and Ishikawa, M. (2002). The soluble and membrane-anchored forms of heparin-binding epidermal growth factor-like growth factor appear to play opposing roles in the survival and apoptosis of human luteinized granulosa cells. *Mol. Hum. Reprod*. **8**, 734-741.

Piepkorn, M., Lo, C. and Plowman, G. (1994). Amphiregulin-dependent proliferation of cultured human keratinocytes: autocrine growth, the effects of exogenous recombinant cytokine, and apparent requirement for heparin-like glycosaminoglycans. *J. Cell. Physiol*. **159**, 114-120.

Raab, G., Kover, K., Paria, B. C., Dey, S. K., Ezzell, R. M. and Klagsbrun, M. (1996). Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. *Development* **122**, 637-645.

Sakuma, T., Higashiyama, S., Hosoe, S., Hayashi, S. and Taniguchi, N. (1997). CD9 antigen interacts with heparin-binding EGF-like growth factor through its heparin-binding domain. *J. Biochem.* **122**, 474-480.

Schilling, D., Reid, I. J., Hujer, A., Morgan, D., Demoll, E., Bummer, P., Fenstermaker, R. A. and Kaetzel, D. M. (1998). Loop III region of platelet-derived growth factor (PDGF) B-chain mediates binding to PDGF receptors and heparin. *Biochem. J*. **333**, 637-644.

Schlessinger, J., Lax, I. and Lemmon, M. (1995). Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* **83**, 357-360.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M. (1984). Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* **223**, 1296-1299.

Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K. and Folkman, J. (1993). Betacellulin: a mitogen from pancreatic beta cell tumors. *Science* **259**, 1604-1607.

Shirakata, Y., Kimura, R., Nanba, D., Iwamoto, R., Tokumaru, S., Morimoto, C., Yokota, K., Nakamura, M., Sayama, K., Mekada, E. et al. (2005). Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. *J. Cell Sci*. **118**, 2363-2370.

Singh, A. B. and Harris, R. C. (2005). Autocrine, pararine, and juxtacrine signaling by EGFR ligands. *Cell Signal.* **17**, 1183-1193.

Singh, A. B., Tsukada, T., Zent, R. and Harris, R. C. (2004). Membrane-associated HB-EGF modulates HGF-induced cellular responses in MDCK cells. *J. Cell Sci*. **117**, 1365-1379.

Tada, H., Sasada, R., Kawaguchi, Y., Kojima, I., Gullick, W. J., Salomon, D. S., Igarashi, K., Seno, M. and Yamada, H. (1999). Processing and juxtacrine activity of membrane-anchored betacellulin. *J. Cell. Biochem*. **72**, 423-434.

Takazaki, R., Shishido, Y., Iwamoto, R. and Mekada, E. (2004). Suppression of the biological activities of the epidermal growth factor (EGF)-like domain by the heparin-binding domain of heparin-binding EGF-like Growth Factor. *J. Biol. Chem*. **279**, 47335-47343.

Tanaka, Y., Miyamoto, S., Suzuki, S. O., Oki, E., Yagi, H., Sonoda, K., Yamazaki, A., Mizushima, H., Maehara, Y., Mekada, E. et al. (2005). Clinical significance of heparin-binding EGF-like growth factor and a disintegrin and metalloprotease 17 expresion in human ovarian cancer. *Clin. Cancer Res.* **11**, 4783-4792.

Thompson, S. A., Higashiyama, S., Wood, K., Pollitt, N. S., Damm, D., McEnroe, G., Garrick, B., Ashton, N., Lau, K., Hancock, N. et al. (1994). Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. *J. Biol. Chem*. **269**, 2541-2549.

Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J. I., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y. et al. (2000). Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. *J. Cell Biol*. **151**, 209-220.

Tumova, S. and Bame, K. J. (1997). The interaction between basic fibroblast growth factor and heparan sulfate can prevent the in vitro degradation of the glycosaminoglycan by Chinese hamster ovary cell heparanases. *J. Biol. Chem*. **272**, 9078-9085.

Vlodavsky, I., Ilan, N., Nadir, Y., Brenner, B., Katz, B. Z., Naggi, A., Torri, G., Casu, B. and Sasisekharan, R. (2007). Heparanase, heparin and the coagulation system in cancer progression. *Thromb. Res*. **120**, S112-S120.

Xu, K. P., Ding, Y., Ling, J., Dong, Z. and Yu, F. S. (2004). Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. *Invest. Ophthalmol. Vis. Sci*. **45**, 813-820.

Yagi, H., Yotsumoto, F. and Miyamoto, S. (2008). Heparin-binding EGF-like growth factor promotes transcoelomic metastasis in ovarian cancer through epithelial-mesenchymal transition. *Mol. Cancer Ther.* **7**, 3441-3451.

Yahata, Y., Shirakata, Y., Tokumaru, S., Yang, L., Dai, X., Tohyama, M., Tsuda, T., Sayama, K., Iwai, M., Horiuchi, M. et al. (2006). A novel function of angiotensin II in skin wound healing. Induction of fibroblast and keratinocyte migration by angiotensin II via heparin-binding epidermal growth factor (EGF)-like growth factor-mediated EGF receptor transactivation. *J. Biol. Chem*. **281**, 13209-13216.

Yoshioka, J., Prince, R. N., Huang, H., Perkins, S. B., Cruz, F. U., MacGillivray, C., Lauffenburger, D. A. and Lee, R. T. (2005). Cardiomyocyte hypertrophy and degradation of connexin43 through spatially restricted autocrine/paracrine heparin-binding EGF. *Proc. Natl. Acad. Sci. USA* **102**, 10622-10627.

Zhou, H., Casas-Finet, J. R., Heath Coats, R., Kaufman, J. D., Stahl, S. J., Wingfield, P. T., Rubin, J. S., Bottaro, D. P. and Byrd, R. A. (1999). Identification and dynamics of a heparin-binding site in hepatocyte growth factor. *Biochemistry* **38**, 14793-14802.