

Heparin-Binding EGF-Like Growth Factor Is a Promising Target for Ovarian Cancer Therapy

Shingo Miyamoto,¹ Michinari Hirata,² Ayano Yamazaki,² Takuya Kageyama,² Hidetoshi Hasuwa,³ Hiroto Mizushima,² Yoshihiro Tanaka,¹ Hiroshi Yagi,¹ Kenzo Sonoda,¹ Masahiro Kai,⁴ Hideo Kanoh,⁴ Hitoo Nakano,¹ and Eisuke Mekada^{2,3}

¹Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; ²Department of Cell Biology, Research Institute for Microbial Diseases and ³Department of Experimental Genome Research, Genome Information Research Center, Osaka University, Osaka, Japan; and ⁴Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo, Japan

ABSTRACT

Ovarian cancer is the most frequent cause of cancer death among all gynecologic cancers. We demonstrate here that lysophosphatidic acid (LPA)-induced ectodomain shedding of heparin-binding EGF-like growth factor (HB-EGF) is a critical to tumor formation in ovarian cancer. We found that among the epidermal growth factor receptor (EGFR) family of growth factors, HB-EGF gene expression in cancerous tissues and HB-EGF protein levels in patients' ascites fluid were significantly elevated. The human ovarian cancer cell lines SKOV3 and RMG-1 form tumors in nude mice. Tumor formation of these cells was enhanced by exogenous expression of pro-HB-EGF and completely blocked by pro-HB-EGF gene RNA interference or by CRM197, a specific HB-EGF inhibitor. Transfection with mutant forms of HB-EGF indicated that the release of soluble HB-EGF is essential for tumor formation. LPA, which is constitutively produced by ovarian cancer cells, induced HB-EGF ectodomain shedding in SKOV3 and RMG-1 cells, resulting in the transactivation of EGFR and the downstream kinase extracellular signal-regulated kinase/mitogen-activated protein kinase. LPA-induced transactivation was abrogated by HB-EGF gene RNA interference or by CRM197. Introduction of lipid phosphate phosphohydrolase, which hydrolyzes LPA, decreased the constitutive shedding of HB-EGF, EGFR transactivation, and the tumorigenic potential of SKOV3 and RMG-1 cells. These results indicate that HB-EGF is the primary member of the EGFR family of growth factors expressed in ovarian cancer and that LPA-induced ectodomain shedding of this growth factor is a critical step in tumor formation, making HB-EGF a novel therapeutic target for ovarian cancer.

INTRODUCTION

Ovarian cancer is the most frequent cause of cancer death among all gynecologic cancers, and therapies over the last 30 years have not improved cure rates (1). To develop a novel effective therapy for ovarian cancer, further understanding of the processes and molecules leading to the initiation and progression of ovarian cancer is required.

Activation of the ErbB family of receptors is involved in the progression of various cancers; therefore, epidermal growth factor receptor (EGFR) and ErbB2 are recognized as effective targets for the therapy of various cancers (2, 3). In the case of ovarian carcinoma, impairments of the epidermal growth factor (EGF) system, including EGFR ligands and ErbB family receptors, have also been reported to be involved in autonomous proliferation of ovarian carcinoma cells. As described in the literature (2, 3), EGFR overexpression occurs in 35–70% of all primary ovarian cancers. Statistical analyses have

confirmed that EGFR overexpression is significantly associated with a high risk of progression in ovarian cancer patients (4). Although the frequency of ErbB2 overexpression is low, it has been correlated with clinical outcome (2, 3). Seven ligands have been described for EGFR: EGF; transforming growth factor (TGF) α ; heparin-binding EGF-like growth factor (HB-EGF); amphiregulin; betacellulin; epiregulin; and epigen. Although a significant correlation between EGFR activation and ovarian cancer progression has been documented, which EGFR ligands contribute to cancer progression is largely unknown. The expression of TGF- α and amphiregulin was described in ovarian carcinomas, although expression levels varied in tumors (5, 6). Pro-HB-EGF is also expressed in ovarian cancer cells, because ovarian cancer cells are sensitive to diphtheria toxin (DT) (7), and DT utilizes pro-HB-EGF as the DT receptor (8). However, no comprehensive studies examining the significance of EGFR ligands in ovarian cancer progression have been reported.

HB-EGF is initially synthesized as a membrane-bound precursor (pro-HB-EGF; Ref. 9). The soluble form of HB-EGF (sHB-EGF) is released from the cell membrane by ectodomain shedding of pro-HB-EGF (10), in a manner similar to that for other EGFR ligands (11). Ectodomain shedding of pro-HB-EGF is critical for growth factor activity, and unregulated release of sHB-EGF results in lethal severe hyperplasia in mice (12). A number of physiological and pharmacological stimuli, which include G protein-coupled receptor (GPCR) ligands such as lysophosphatidic acid (LPA), induce ectodomain shedding of pro-HB-EGF (13–15), indicating that overabundance of such stimuli may cause excess release of sHB-EGF.

Ascites from ovarian cancer patients are considered to be a rich source of growth factor activity for ovarian cancer cells. The molecules involved in this process have been termed ovarian cancer activating factors (16). Recent biochemical analysis has revealed that LPA is one possible ovarian cancer activating factor candidate (17, 18). LPA is a simple phospholipid with numerous cellular effects including growth promotion, cell cycle progression, and cytoskeletal organization (19–21). LPA levels are markedly elevated in the plasma and ascites of patients with ovarian cancer (22). LPA signals are generally mediated by LPA receptors, members of the GPCR family. Recent studies indicated that LPA and other GPCR ligands induce proteolytic cleavage of the extracellular domain (ectodomain shedding) of EGFR ligands at the cell surface (14, 15). The secreted EGF family ligands then activate EGFR on ligation (14, 23). Thus, LPA signals and the EGFR system are thought to be closely connected.

To search for novel effective therapeutic targets, in this work we studied the expression of EGFR ligands in ovarian cancer patients and found HB-EGF to be the primary EGFR ligand altered in ovarian cancer. Based on this evidence, we examined the role of HB-EGF in tumor formation of ovarian cancer cells in nude mice. Results showed that HB-EGF, especially in its soluble form, is essential for tumor growth of ovarian cancer cells in nude mice; therefore, inhibition of gene expression or inhibition of growth factor activity can block tumor growth. We also show evidence that LPA, which is produced

Received 3/5/04; revised 6/14/04; accepted 6/21/04.

Grant support: Grant-in-Aid 14032202 from the Ministry of Education, Culture, Sports, Science and Technology (to E. Mekada).

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.

Note: Supplementary data for this article can be found at Cancer Research Online (<http://cancerres.aacrjournals.org>).

Requests for reprints: Eisuke Mekada, Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8286; Fax: 81-6-6879-8289; E-mail: emekada@biken.osaka-u.ac.jp.

by ovarian cancer cells, causes enhanced ectodomain shedding of pro-HB-EGF and consequent tumor formation.

MATERIALS AND METHODS

Reagents and Antibodies. DT and CRM197 were prepared as described previously (24). To administer CRM197 to mice, LPS-like materials contaminating the CRM197 preparations were removed using Detoxi Gel (Pierce Biotechnology, Rockford, IL). LPA 18:1 (1-oleoyl-sn-glycerol-3-phosphate, sodium salt) and LPA 18:0 (1-stearoyl-sn-glycerol-3-phosphate, sodium salt) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Suramin, GM6001, PD98059, and PD153035 were obtained from Calbiochem (San Diego, CA). Polyclonal rabbit anti-EGFR and anti-mitogen-activated protein kinase (MAPK) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against phospho-tyrosine and phospho-MAPK were acquired from Upstate Biotechnology Inc. (Lake Placid, NY). Sheep antimouse IgG was obtained from Amersham Corp. (Arlington Heights, IL). Peroxidase-conjugated goat antirabbit IgG was purchased from Zymed (San Francisco, CA).

Cell Culture. SKOV3, RMG-1, OVMG1, and the transfected cells were maintained in RPMI 1640 supplemented with 100 units/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin, and 10% fetal bovine serum (ICN Biomedical, Irvine, CA).

Plasmid Constructions and Transfection. Constructions of plasmids encoding human pro-HB-EGF cDNA, the *uc* mutant, and the ΔTM mutant (12) inserted into the eukaryotic expression vector pRC/CMV (Invitrogen, Carlsbad, CA) have been described previously. Plasmids encoding FLAG-tagged lipid phosphate phosphohydrolase (LPP)-1 and FLAG-tagged LPP-3 were generated by polymerase chain reaction (PCR) using human LPP-1 and LPP-3 complete cDNA (25) as templates. These fragments were then inserted into the *Bam*HI-*Not*I sites of pCX4pur vector. To construct the HB-EGF small interfering RNA (siRNA) vector (pH1-PUR/siRNAHB-EGF), we synthesized the following DNA oligonucleotides: 5'-CCCGTCCGTGACTTGCAAGAGGT-TCAAGAGACCTCTTGCAAGTACGGACTTTTGGAAA and 5'-CTA-GTTTCCAAAAAGTCCGTGACTTGCAAGAGGTCTCTTGAACCTC-TTGCAAGTACGGACGGGTGCA. After amplification of the cDNA fragments by PCR, the fragments were cloned into the *Pst*I and *Xba*I site of the pH1 RNA interference vector (26). The product was digested with *Bam*HI and *Xho*I and then cloned into a pPUR selection vector (Clontech, Palo Alto, CA) to confer puromycin resistance. Transfections of all constructs into SKOV3 and RMG-1 cells were performed using LipofectAMINE 2000 reagent (Invitrogen), according to the manufacturer's instructions. Transfected cells were selected with 400 $\mu\text{g/ml}$ G418 or 5 $\mu\text{g/ml}$ puromycin.

Assays of Diphtheria Toxin Binding and Protein Tyrosine Phosphorylation. To remove extracellular matrix components, cells were detached with trypsin-EDTA and then allowed to recover for 30 min in RPMI 1640 with 10% fetal bovine serum (27). To assess the effect of each pharmacological agent on DT binding, after rinsing with serum-free medium, cells were incubated with serum-free medium at 37°C for 30 min. Cells (1×10^6) were seeded on polylysine-coated 6-cm dishes. Samples were incubated with serum-free RPMI 1640 at 37°C for 1 h to assure the complete adherence of cells to the polylysine-coated dishes. Binding of ^{125}I -labeled DT to cells was measured, and values of the specific binding were determined as described previously (8). To examine the activation of EGFR and MAPK, cells were treated as described above. Cells were rinsed in phosphate-buffered saline containing 1 mM sodium orthovanadate and then lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris (pH 8.0), 0.2 unit/ml aprotinin, 2 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin A, 2 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate]. Extracts and immunoprecipitants were then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting analysis (28).

Tumor Growth in Nude Mice. Subconfluent cell cultures were detached from plates with trypsin-EDTA. A total volume of 250 μl containing 5×10^6 cells suspended in serum-free RPMI 1640 was injected into female BALB/c nu/nu mice at 5 weeks of age (Charles River Laboratories). Injection-treated mice were examined every week for tumor apparition. Tumor volume was calculated as described previously (29). To assess the effect of CRM197 inhibition, CRM197 dissolved in 1 ml of 20 mM HEPES and 0.15 M NaCl (pH

7.2) [1 mg/week] was injected intraperitoneally into tumor-bearing mice each week. All experimental use of animals complied with the guidelines of Animal Care of Kyushu University.

Reverse Transcription-PCR. For the cell lines used in this study, total cellular RNA was purified. First-strand cDNA synthesis was performed as described previously (30). PCR analysis for the expression of EGFR ligands and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control was performed using the primers described (31). For real-time PCR analysis, total cellular RNA was purified from frozen tumor tissues; first-strand cDNA synthesis was performed according to the manufacturer's protocol. Primers specific for HB-EGF (5'-CTCCCTCTGCATCTGCCACCC-3'), TGF- α (5'-CCAGCATGTGTCTGCCATTCTGGG-3'), amphiregulin (5'-TCA-GAGTTGAACAGGTAGTTAAGCCCC-3'), and GAPDH (5'-CCGACTCT-TGCCCTTCGAAC-3') were labeled with 6-carboxyfluorescein fluorescent spectrum as a reporter. The amplification primer pairs were 5'-TGGAGAAT-GCAAATATGTGAAGGA-3' and 5'-AGGATGGTTGTGTGGTCATAGG-TAT-3' for HB-EGF, 5'-GATTCCCACACTCAGTTCTGCTT-3' and 5'-CAC-AGCGTGACCAACGT-3' for TGF- α , 5'-CCTGGCTATATTGTCGATGAT-TCA-3' and 5'-GTATTTTCACTTCCGCTTGTGTTT-3' for amphiregulin, and 5'-GAAGTGAAGTCCGGAGTC-3' and 5'-CTTTAGGGTAGTGGTA-GAAG-3' for GAPDH. PCR reactions were carried out using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). The expression index for each messenger RNA was defined as the EGFR ligand messenger RNA copy number/GAPDH messenger RNA copy number $\times 10^4$.

Assay of Cell Surface Activities Hydrolyzing LPA of LPP-Transfected SKOV3 Cells. Radiolabeled LPA was prepared by *Escherichia coli* diacylglycerol kinase-catalyzed phosphorylation of monoolein (Sigma-Aldrich, St. Louis, MO) as described previously (25, 32). Cells (3×10^5 cells/well in a 12-well culture plate) were starved for 24 h in RPMI 1640 containing 0.1% bovine serum albumin. The cells were then incubated for 10 min at 37°C in medium containing 3% bovine serum albumin and radioactive LPA (20 μM ; specific radioactivity, ~ 7000 cpm/nmol; Ref. 33). The culture medium was then rapidly aspirated, and the cells were washed once with 0.5 ml of Tris-buffered saline. The inorganic phosphate liberated was extracted from the aliquots (250 μl) of the combined medium as described previously (25, 32).

The Amount of EGFR Ligands in Ascitic Fluid. The quantity of HB-EGF in patient ascites fluids was determined by ^{125}I -DT binding. One milliliter of ascites fluid was incubated with 20 μl of heparin-Sepharose CL-6B (Amersham Biosciences, Uppsala, Sweden) for 5 h at 4°C. Gels were washed in phosphate-buffered saline, incubated with ^{125}I -DT (100 ng/ml) in the presence or absence of excess unlabeled DT for 12 h at 4°C, and then washed with phosphate-buffered saline containing 1 mg/ml bovine serum albumin. The radioactivity bound to the gels was then counted in a gamma counter; the specific binding of ^{125}I -DT was determined. The amount of HB-EGF was determined by normalization to a standard curve obtained by assessment of recombinant HB-EGF. The amounts of TGF- α and amphiregulin were determined by enzyme-linked immunosorbent assay systems provided by R&D Systems, Inc. (Minneapolis, MN), and TECHNE Corp. (Minneapolis, MN), respectively. The sensitivity of these assays was <2.2 pg/ml for TGF- α and <5 pg/ml for amphiregulin.

Patients and Statistical Analysis. All of the patients examined had undergone surgery between 1997 and September 2002 at the Department of Obstetrics and Gynecology, Kyushu University Hospital. All tissue samples were obtained at surgery from 34 patients with stage III/IV ovarian cancer derived from coelomic epithelium and 10 patients with normal ovaries who had surgery for benign disease or other gynecologic cancer. Ascitic fluid was also obtained from a total of 37 patients with normal ovaries ($n = 10$), benign ovarian cysts ($n = 10$), and stage III/IV ovarian cancers ($n = 17$). In this study, informed consent was obtained from all patients. The values for each EGFR ligand were analyzed using the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

RESULTS

Enhanced Expression of HB-EGF in Human Ovarian Cancer. In the beginning of this study, we determined the expression levels of EGFR ligand genes in ovarian cancer. Among EGFR ligands, the expression of HB-EGF, TGF- α , amphiregulin, and epiregulin was

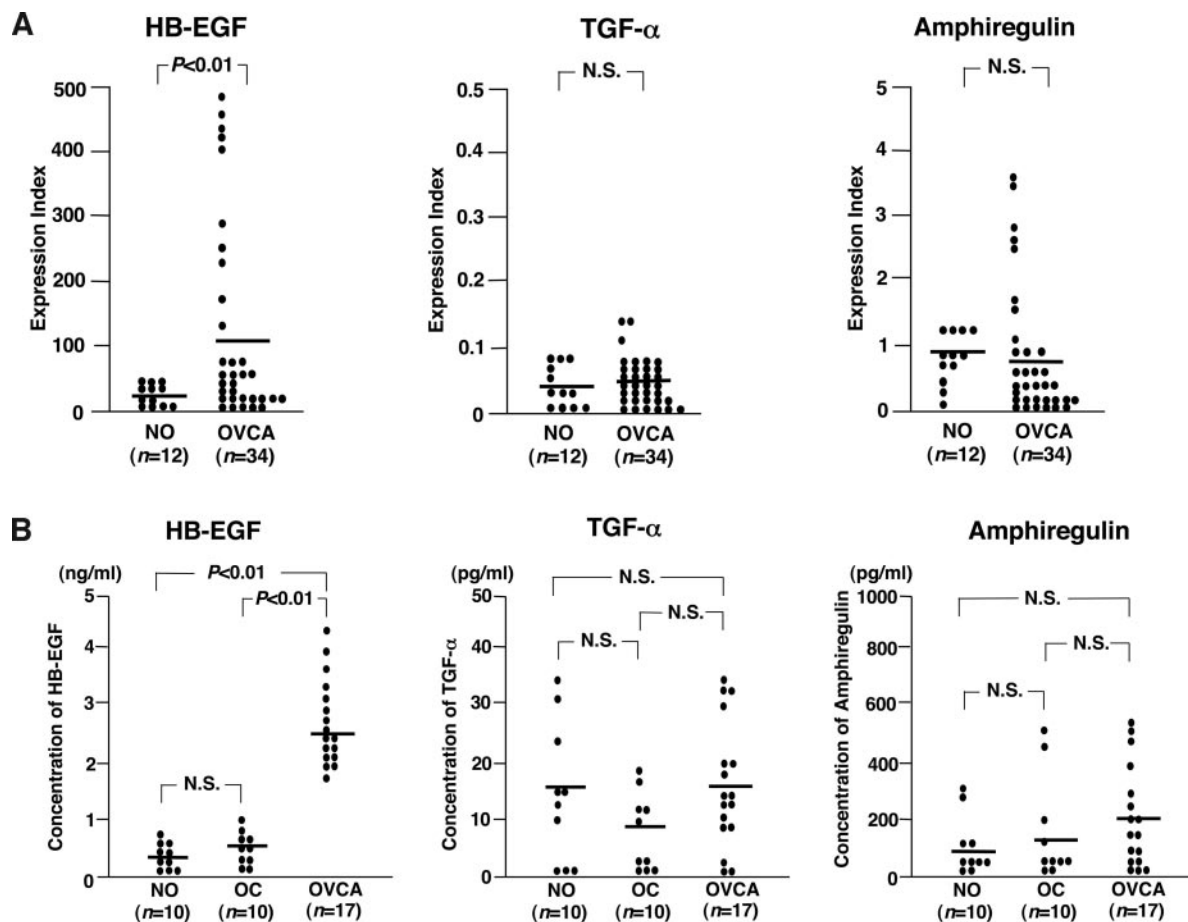


Fig. 1. Association of EGFR ligand expression levels with clinical outcome in human ovarian cancer. A, expression index of HB-EGF, TGF- α , and amphiregulin in patients with either normal ovaries (NO) or ovarian cancer (OVCA). B, the ascites fluid levels of HB-EGF, TGF- α , and amphiregulin in patients with normal ovaries (NO), ovarian cysts (OC), and ovarian cancer (OVCA). Bars indicate mean. NS, not significant.

measured by nonquantitative reverse transcription-PCR (RT-PCR) in specimens from patients with ovarian cancer. EGF and betacellulin expression were not detected (data not shown). Then, we determined the expression levels of these four EGFR ligand genes in a quantitative manner by real-time PCR using specimens from patients with normal ovaries or ovarian cancer. Expression indices of HB-EGF, TGF- α , and amphiregulin in patients with normal ovaries or ovarian cancer are shown in Fig. 1A. The expression level of epiregulin was similar to that of TGF- α .⁵ Large differences between the expression of HB-EGF and the other three EGFR ligands were observed in the tissue specimens examined. In addition to gene expression, we also determined the protein levels of HB-EGF, TGF- α , and amphiregulin in ascites fluids (Fig. 1B). The amount of epiregulin was not determined because appropriate antibodies for enzyme-linked immunosorbent assay were not available. Consistent with the PCR data, the amount of HB-EGF in the peritoneal fluid of patients with normal ovaries or ovarian cancer was much higher than the amount of TGF- α and amphiregulin. Furthermore, and more importantly, HB-EGF gene expression levels in tissue specimens and protein levels in ascitic fluid from patients with ovarian cancer increased significantly from the levels observed in normal and cystic ovary tissues, although such enhanced expression was not observed for other EGFR ligands. These

results indicated that HB-EGF may play a role in human ovarian cancer.

HB-EGF Is Essential for Tumor Formation of Ovarian Cancer Cells. As shown above, HB-EGF was the only EGFR ligand with particularly enhanced RNA and protein levels in ovarian cancer. To examine whether HB-EGF contributes to tumor formation in human ovarian cancer, we studied the relationship between HB-EGF expression levels and tumor-forming ability of ovarian cancer cell lines in nude mice. SKOV3 and RMG-1 are cell lines derived from human epithelial ovarian carcinomas. RT-PCR revealed that HB-EGF is the primary EGFR ligand expressed in these cells, although expression of TGF- α , amphiregulin, and epiregulin was also detected (Fig. 2, A and B). A DT binding assay confirmed the presence of pro-HB-EGF on the surface of SKOV3 and RMG-1 cells and indicated that the amount of pro-HB-EGF on the surface of RMG-1 cells was about 2.5-fold higher than that on the surface of SKOV3 cells (Fig. 2, C and D). When SKOV3 and RMG-1 cells were injected subcutaneously (5×10^6 cells/mouse) into nude mice, both cells formed tumors (Fig. 3, A and B). Consistent with HB-EGF expression levels, RMG-1 cells formed tumors more rapidly than SKOV3 cells.

To gain evidence of the involvement of HB-EGF in tumor formation, ovarian cancer cells ectopically expressing pro-HB-EGF were isolated. SK-HB cells were obtained by transfecting SKOV3 cells with wild-type pro-HB-EGF. SK-HB-1-3, stable clones of SK-HB cells, expressed pro-HB-EGF on the cell surface at levels approximately 7-fold higher than that of the parental SKOV3 cells (Fig. 2C).

⁵ Y. Tanaka, S. Miyamoto, S. O. Suzuki, E. Oki, H. Yagi, K. Sonoda, A. Yamazaki, H. Mizushima, E. Mekada, and H. Nakano. Clinical significance of heparin-binding EGF-like growth factor and ADAM 17 expression in human ovarian cancer, manuscript in preparation.

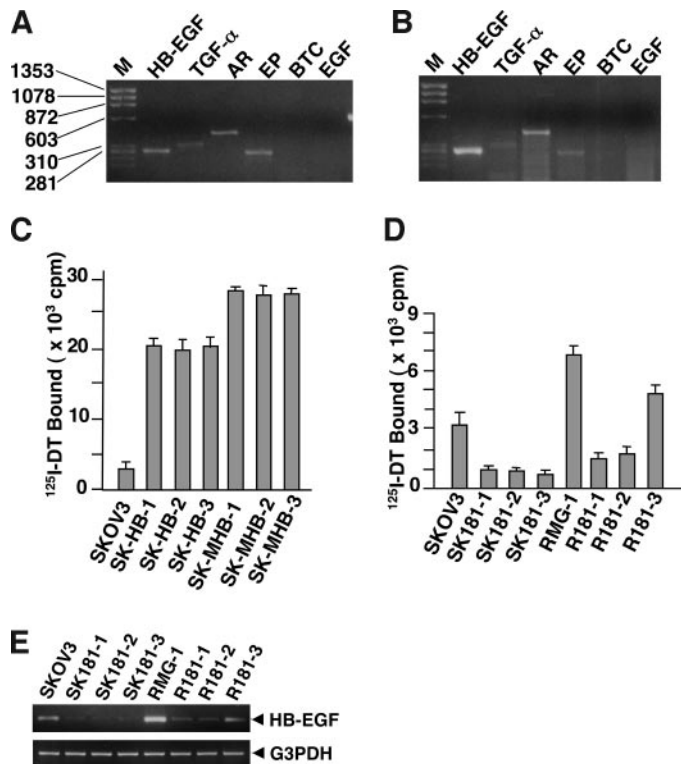


Fig. 2. Expression of HB-EGF in SKOV3, RMG-1, and transfectant cells and inhibition by siRNA. *A* and *B*, expression of EGF family ligands in SKOV3 (*A*) and RMG-1 (*B*) cells was assessed by RT-PCR. The positions of size markers (bp) are shown in *Lanes M*. *C*, quantitation of cell surface pro-HB-EGF expressed in SKOV3, SK-HB, and SK-MHB cells as measured by the binding of ^{125}I -DT. The values of specific binding represent the average \pm SD of three independent experiments. *D*, quantitation of cell surface pro-HB-EGF expressed in SKOV3, RMG-1, and HB-EGF siRNA vector-expressing cells as measured by the binding of ^{125}I -DT. SK181-1, SK181-2, and SK181-3 cells, transfectants derived from SKOV3 cells, or R181-1, R181-2, and R181-3 cells, transfectants derived from RMG-1 cells, express HB-EGF siRNA. The values of specific binding represent the average \pm SD of three independent experiments. *E*, semiquantitative RT-PCR analysis for pro-HB-EGF and GAPDH expression from transcripts derived from SKOV3 cells, RMG-1 cells, and cells expressing HB-EGF siRNA vector.

All SK-HB cell clones formed larger tumors in nude mice than the parental SKOV3 cells. Mice that received injection with SK-HB cells died within 6 weeks of subcutaneous injection (Fig. 3C). Similar results, *i.e.*, enhanced tumorigenicity, were also observed in R-HB cells obtained by transfecting RMG-1 cells with wild-type pro-HB-EGF (data not shown).

To confirm the requirement for pro-HB-EGF in tumor formation of ovarian cancer cells, pro-HB-EGF expression was suppressed by vector-encoded siRNA. A plasmid coexpressing the pro-HB-EGF siRNA and a puromycin resistance gene was transfected into either SKOV3 or RMG-1 cells. After selection in puromycin-containing media, we isolated the surviving colonies. The specific knockdown of pro-HB-EGF expression in the isolated clones (referred to as SK181-1, SK181-2, and SK181-3 for SKOV3 transfectants and R181-1, R181-2, and R181-3 for RMG-1 transfectants) was confirmed by RT-PCR (Fig. 2E). DT binding also revealed reduced levels of pro-HB-EGF on the cell surface in these siRNA-expressing cells, although the reductions observed were minimal in clone R181-3 (Fig. 2D). SKOV3, RMG-1, and the respective transfectants were injected subcutaneously (5×10^6 cells/mouse) into nude mice. Whereas SKOV3 cells rapidly formed tumors in nude mice, the pro-HB-EGF knockdown cell clones, SK181-1, SK181-2, and SK181-3, did not form tumors until 10 weeks after subcutaneous injection (Fig. 3A). Similarly, whereas RMG-1 cells developed into tumors more rapidly than SKOV3 cells, R181-1 and R181-2 did not form tumors until 6

weeks after injection, and tumors were slow to develop after this point (Fig. 3B). R181-3, a clone expressing higher levels of HB-EGF than the other R181 clones, formed smaller tumors than the parental RMG-1 cells over a similar time scale. The small tumor formation of R181-3 cells may be due to the partial reduction of pro-HB-EGF expression in R181-3 cells, but we cannot exclude the possibility that the introduced siRNA construct was lost from the R181-3 cells under nonselective conditions, and therefore the cells re-formed tumors. Expression of empty vector containing only the H1 promoter (26) or vector containing an irrelevant DNA fragment did not affect the tumorigenicity of either SKOV3 or RMG-1 cells (data not shown). These results indicate that expression levels of pro-HB-EGF are critical for tumor formation of ovarian cancer cells.

Suppression of Tumor Growth by Exogenously Administered CRM197. To further examine the tumorigenic activity of sHB-EGF, we studied the effect of CRM197 on tumor growth. CRM197, a nontoxic mutant protein of DT, is a specific inhibitor of sHB-EGF (34). One week after subcutaneous injection of either SKOV3 or RMG-1 cells, mice were given CRM197 (1 mg/week) every week by intraperitoneal injection. Tumor formation was completely suppressed by CRM197 treatment (Fig. 4, *A* and *B*). Similar results were also observed with OVMG1, another ovarian cancer cell line (Ref. 35; Fig. 4C). Furthermore, CRM197 also completely suppressed tumor formation from SK-HB-1 (Fig. 4D) and R-HB-1 cells (data not shown), even though these cells expressed high levels of surface pro-HB-EGF. These results confirm that HB-EGF is required for tumor formation of ovarian cancer cell lines *in vivo*. In comparison, Taxol, a widely used chemotherapeutic agent for ovarian cancer, was used for nude mice with SKOV3 or SK-HB-1 cell tumors. Taxol inhibited tumor formation of SKOV3 cells to a degree similar to that of CRM197 but only weakly inhibited tumor formation by SK-HB-1 cells (Fig. 4, *E* and *F*).

Ectodomain Shedding of Pro-HB-EGF Is Essential for Tumor Formation. A previous study indicated that ectodomain shedding of pro-HB-EGF is critical for growth factor activity (12). To examine the

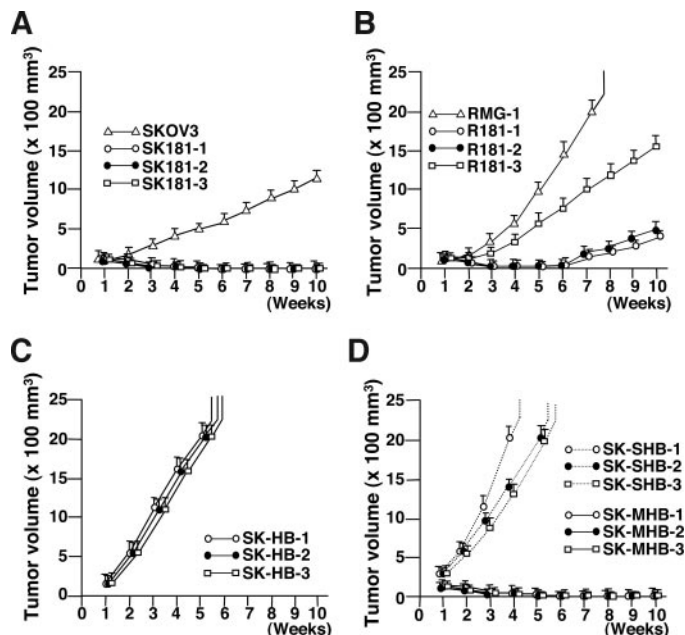


Fig. 3. Tumor formation of ovarian cancer cells in nude mice. SKOV3 cells and HB-EGF-targeting siRNA-expressing SK-181 cells (*A*), RMG-1 and HB-EGF-targeting siRNA-expressing R181 cells (*B*), SK-HB cells (*C*), and SK-MHB and SK-SHB cells (*D*) were injected subcutaneously into nude mice. Every week, beginning 1 week after injection of cells, tumor size at the injection sites was measured. Tumor volume was then calculated as described in "Materials and Methods." Each tumor volume represents the mean \pm SD ($n = 10$).

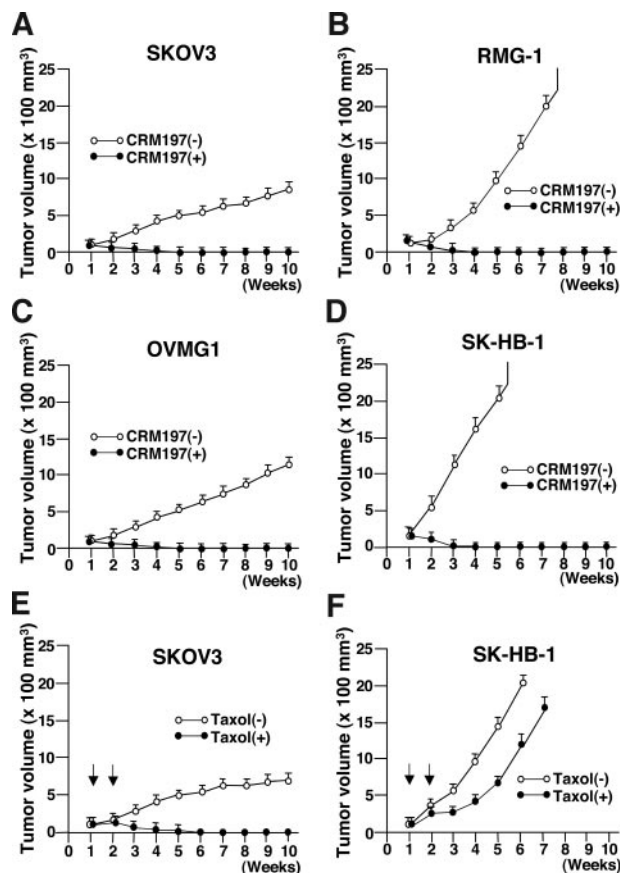


Fig. 4. Suppression of tumor growth after administration of CRM197. One week after the injection of SKOV3 (A), RMG-1 (B), OVMG1 (C), and SK-HB-1 cells (D), 1 mg of CRM197 or control saline was injected intraperitoneally each week for 10 weeks. Taxol (40 mg/kg) was also administered to mice with SKOV3 (E) or SK-HB-1 cells (F) twice. Each week, beginning 1 week after the subcutaneous injection of cells, tumor size at the injection sites was measured. Tumor volume was calculated as described in "Materials and Methods." Each tumor volume represents the mean \pm SD ($n = 10$).

requirement for pro-HB-EGF ectodomain shedding for tumor formation, we prepared SKOV3 cells ectopically expressing mutant forms of pro-HB-EGF. SK-MHB and SK-SHB cells were obtained by transfecting SKOV3 cells with an uncleavable pro-HB-EGF mutant (*uc*) and an HB-EGF form with its transmembrane domain deleted (ΔTM). The *uc* form is resistant to ectodomain shedding resulting from induction by various shedding-inducing stimuli, whereas the ΔTM secretes sHB-EGF in the absence of shedding stimuli (12). SK-MHB cell clones expressed the *uc* form of pro-HB-EGF mutants on the cell surface at levels approximately 9-fold higher than that of the parental SKOV3 cells (Fig. 2C). However, SK-MHB cell clones did not form tumors until 10 weeks after subcutaneous injection (Fig. 3D), despite endogenous expression of wild-type pro-HB-EGF. SK-SHB cell clones developed the largest tumors of all of the cells tested, and the majority of mice that received injection with SK-SHB cells died between 4 and 6 weeks after injection (Fig. 3D). These results indicate that ectodomain shedding of pro-HB-EGF, leading to the liberation of sHB-EGF, is essential for tumor formation of ovarian cancer cells *in vivo*.

LPA Induces Pro-HB-EGF Ectodomain Shedding and Transactivation of EGFR in Ovarian Cancer Cells. As shown in the above sections, pro-HB-EGF and ectodomain shedding are essential for tumor formation of ovarian cancer cells in nude mice. Considering that ectodomain shedding of pro-HB-EGF is a regulated process that does not occur without shedding-inducing stimuli (15, 36), some factor(s) seems to enhance ectodomain shedding of pro-HB-EGF in

ovarian cancer cells. In ovarian cancer patients, LPA levels are markedly elevated in plasma and ascites (22). In addition, using different cell lines, we and others have shown that LPA induces ectodomain shedding of pro-HB-EGF (14, 15, 36). Thus, we next focused on the contribution of LPA to the ectodomain shedding of pro-HB-EGF in ovarian cancer cells.

We first confirmed whether exogenously added LPA actually induces pro-HB-EGF ectodomain shedding in ovarian cancer cells. SKOV3, RMG-1, and OVMG1 cells were treated with saturated or unsaturated LPA, and pro-HB-EGF ectodomain shedding was determined. Ectodomain shedding was quantitated by measuring the amount of pro-HB-EGF remaining on the cell surface using the DT binding assay (8). Addition of LPA with unsaturated fatty acids (18:1), but not LPA with saturated fatty acids (18:0), to these ovarian cancer cells induced ectodomain shedding of pro-HB-EGF (Supplementary Fig. 1). The metalloprotease inhibitor GM6001, which inhibits ectodomain shedding of EGFR ligands, blocked LPA-induced decreases in pro-HB-EGF surface levels, confirming that the reduction resulted from ectodomain shedding.

LPA-induced pro-HB-EGF ectodomain shedding resulted in transactivation of EGFR and the downstream kinase extracellular signal-regulated kinase (ERK)/MAPK. As shown in Fig. 5, A and B, addition of LPA 18:1 (unless otherwise specified, LPA 18:1 was used in all of the following experiments) enhanced EGFR and ERK/MAPK activation, and GM6001 blocked LPA-induced EGFR and ERK/MAPK activation. In contrast, the activation of EGFR and ERK/MAPK by exogenously added sHB-EGF was not inhibited by GM6001 treatment. By preventing the binding of EGFR ligands to the EGFR extracellular domain, an anti-EGFR antibody, ICR-3R, also blocked LPA-induced activation of EGFR and ERK/MAPK. These results confirm that LPA-induced activation of EGFR and ERK/MAPK in SKOV3 and RMG-1 cells occurred via a ligand-dependent mechanism. SKOV3 and RMG-1 cells express other EGFR ligands including TGF- α , amphiregulin, and epiregulin at levels lower than that of HB-EGF (Fig. 2, A and B). However, inhibition of HB-EGF protein by CRM197 or HB-EGF gene expression by siRNA prevented LPA-induced transactivation of EGFR and ERK/MAPK (Fig. 5, C and D). These results indicate that HB-EGF is the primary EGFR ligand contributing to LPA-induced transactivation of EGFR and ERK/MAPK in ovarian cancer cells.

Self-Production of LPA in Ovarian Cancer Cells Results in Constitutive HB-EGF Shedding and EGFR Activation. LPA is constitutively produced in ovarian cancer cells, including SKOV3 and RMG-1 (22, 37). This constitutive production of LPA potentially contributes to autonomous induction of pro-HB-EGF shedding. The DT binding assay revealed that incubation of SKOV3 cells with GM6001 or suramin, a broad specificity inhibitor of LPA-LPA receptor interaction (15, 38), increased the amount of pro-HB-EGF on the cell surface, even in the absence of exogenously added LPA (Supplementary Fig. 2A). SKOV3 and RMG-1 cells demonstrated constitutive weak phosphorylation of EGFR and ERK/MAPK (Fig. 5, A, B, and E). The elevated background levels of EGFR and ERK/MAPK activation were due to constitutive shedding of pro-HB-EGF because treatment with suramin or GM6001 abrogated the constitutive phosphorylation of EGFR and ERK/MAPK. Transactivation of EGFR and ERK/MAPK by constitutively produced LPA, through autocrine stimulation of pro-HB-EGF shedding, was more clearly demonstrated with SK-HB-1 cells. These cells demonstrate strong phosphorylation of EGFR and ERK/MAPK, and treatment with suramin or GM6001 abrogated the constitutive phosphorylation of EGFR and ERK/MAPK (Fig. 5F). These results indicate that EGFR and ERK/MAPK are constitutively activated by the constitutive cleavage of pro-HB-EGF after endogenous LPA production.

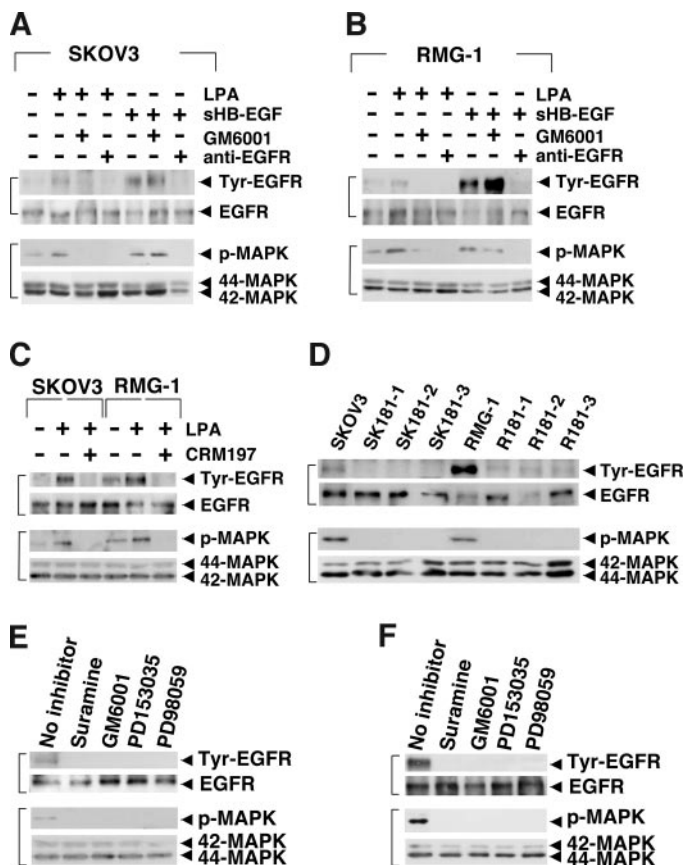


Fig. 5. LPA-induced EGFR transactivation requires pro-HB-EGF ectodomain shedding. *A* and *B*, tyrosine phosphorylation of EGFR and ERK/MAPK induced by LPA. SKOV3 (*A*) or RMG-1 (*B*) cells were treated with or without LPA in the presence or absence of GM6001 (5 μ M) or anti-EGFR monoclonal antibody. Soluble HB-EGF (*sHB-EGF*; 10 ng) was also added where indicated. *Top panel*, lysates were immunoprecipitated with antibodies specific for EGFR; immunoprecipitates were probed with antibodies to phospho-tyrosine or EGFR. *Bottom panel*, lysates were immunoblotted with antibodies to phospho-MAPK and ERK. *C*, inhibition of LPA-induced transactivation by CRM197 treatment. SKOV3 and RMG-1 cells were incubated with or without CRM197 (10 μ g/ml) and stimulated with LPA. Tyrosine phosphorylation of EGFR and ERK/MAPK was detected by immunoblotting. *D*, loss of LPA-induced tyrosine phosphorylation of EGFR and ERK/MAPK in HB-EGF siRNA-expressing cells. Cells were incubated with LPA (30 μ M) for 60 min, and then cell lysates were analyzed for EGFR and ERK activation. *E* and *F*, effects of various pharmacological inhibitors on EGFR and ERK/MAPK phosphorylation. SKOV3 cells (*E*) and SK-HB-1 cells (*F*), a SKOV3-derived clone overexpressing wild-type pro-HB-EGF, were incubated with suramin (10 μ M), GM6001 (5 μ M), PD153035 (5 μ M), or PD98059 (50 μ M) for 1 h. The phosphorylation status of EGFR and ERK/MAPK was determined by immunoblotting. LPA was not added exogenously in these experiments.

As we have shown previously (36), activation of the MAP/ERK kinase (MEK)-ERK pathway results in pro-HB-EGF shedding. Released HB-EGF causes EGFR activation, subsequently stimulating the Ras-MEK-ERK pathway. Thus, the induction of pro-HB-EGF shedding by constitutive LPA signaling could be amplified by an autocrine loop, as discussed previously (36). Both an inhibitor of EGFR tyrosine kinase, PD153035, and a MEK inhibitor, PD98059, inhibited pro-HB-EGF ectodomain shedding in SKOV3 and SK-HB-1 cells, resulting in increases in surface pro-HB-EGF (Supplementary Fig. 2). These inhibitors prevented EGFR and ERK/MAPK activation in these cells at levels comparable with the inhibition of shedding (Fig. 5, *E* and *F*). These results indicate that constitutive production of LPA induces autonomous cleavage of pro-HB-EGF and subsequent activation of EGFR in ovarian cancer cells.

Degradation of Extracellular LPA Reduces Pro-HB-EGF Shedding and Tumor Formation. To confirm the relationship between LPA production and pro-HB-EGF shedding in ovarian cancer cells,

we isolated stable clones of SKOV3 cells ectopically expressing a phosphatidic acid phosphatase family phosphatase. LPP-1/PAP2A and LPP-3/PAP2B, both membrane-associated phosphatases, degrade extracellular LPA (25, 32, 39). SK-LPP1 cells (SKOV3 transfectants expressing LPP-1) and SK-LPP3 cells (those expressing LPP-3; Fig. 6*A*) degraded added LPA in the medium more effectively than the parental SKOV3 cells (Fig. 6*B*). Both SK-LPP1 and SK-LPP3 cells demonstrated increased DT binding (Fig. 6*C*) and reduced EGFR and ERK/MAPK activation at steady state (Fig. 6*D*), indicating that ectodomain shedding of pro-HB-EGF was suppressed by LPP expression. Furthermore, expression of LPP-1 or LPP-3 in SKOV3 cells partially suppressed tumor formation from these cells in nude mice (Fig. 6, *E* and *F*), in accordance with previous reports (33). These results further confirm that LPA-induced ectodomain shedding of pro-HB-EGF functions in tumor formation in ovarian cancer.

DISCUSSION

We have demonstrated here that among the EGF family of growth factors, HB-EGF is a key ligand in ovarian cancer. A number of

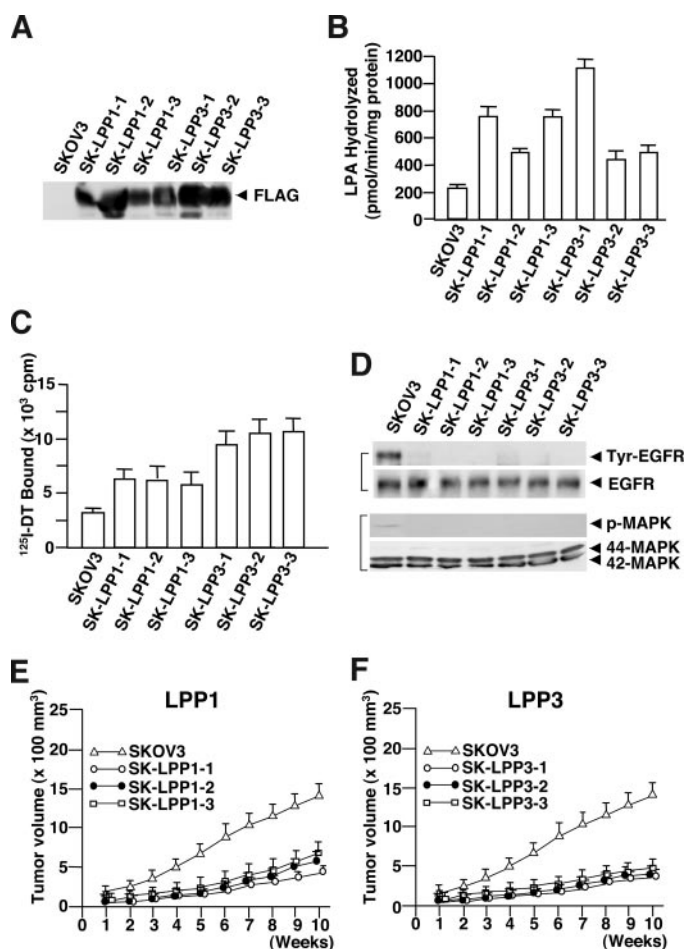


Fig. 6. The effect of LPA degradation mediated by overexpression of LPP-1 or LPP-3 in SKOV3 cells on pro-HB-EGF ectodomain shedding and tumor formation. *A*, expression of LPP-1 or LPP-3 in SKOV3 cells. Cell lysates of either SKOV3 or stable clones of SKOV3 expressing either FLAG-tagged LPP-1 (SK-LPP1-1, SK-LPP1-2, and SK-LPP1-3) or FLAG-tagged LPP-3 (SK-LPP3-1, SK-LPP3-2, and SK-LPP3-3) were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with an anti-FLAG antibody. *B*, LPA hydrolysis by SKOV3 cells and SKOV3 cells expressing either LPP-1 or LPP-3. The results are the averages of eight determinations \pm SD. *C*, quantitation of cell surface pro-HB-EGF assessed by binding of ¹²⁵I-DT. *D*, suppression of constitutive phosphorylation of EGFR and ERK/MAPK in cells cultured under serum-free conditions. *E* and *F*, tumor formation by SKOV3 cells and SKOV3 cells expressing LPP-1 or LPP-3 (*n* = 6).

tumors isolated from ovarian cancer patients expressed high levels of HB-EGF (Fig. 1A), and the ascites fluid obtained from ovarian cancer patients contained higher concentrations of HB-EGF protein than those from normal control subjects and patients with benign tumors (Fig. 1B). Although TGF- α and amphiregulin have been reported to contribute to the formation and progression of other tumors (2, 3, 40–42), the expression levels of TGF- α and amphiregulin in ovarian cancer samples and the protein levels in ascites fluids from ovarian cancer patients were 1 or 2 orders of magnitude lower than those of HB-EGF. The present study also demonstrated that HB-EGF is an essential molecule for tumor formation of ovarian cancer cell lines in nude mice. Suppression of endogenous HB-EGF through siRNA knockdown or inhibition by CRM197 completely inhibited tumor formation. Such strong inhibition of tumor formation supports the notion that HB-EGF is a key molecule in the tumor-forming activity of ovarian cancer cells.

CRM197, a nontoxic DT mutant (24), binds to human HB-EGF to block its mitogenic activity (34). Because CRM197 does not inhibit the mitogenic activity of other EGFR ligands, CRM197 has been used as a specific inhibitor of HB-EGF (14). This study also indicates that CRM197 or similar molecules exerting HB-EGF inhibition could be effective therapeutics for ovarian cancer. Because CRM197 effectively suppressed the growth of Taxol-resistant SK-HB cells, CRM197 or similar drugs may be an effective treatment for patients with recurrent, Taxol-resistant cancer. The present study also suggests that overexpression of the *uc* form of pro-HB-EGF inhibits tumor formation of SKOV3 cells. The inhibitory effect of the *uc* form, possibly due to the dominant-negative effect of the membrane-bound pro-HB-EGF (28), could also be useful in tumor suppression. Additional studies will be necessary to clarify this mechanism.

We have also demonstrated that LPA-induced ectodomain shedding of pro-HB-EGF contributes to tumor formation in ovarian cancer. This is the first study directly demonstrating that LPA-induced HB-EGF ectodomain shedding is critical for tumor formation. The concentrations of LPA required to induce pro-HB-EGF shedding in these ovarian cancer cells were comparable with the concentrations of LPA observed in the ascites of patients with epithelial ovarian cancer (16, 37). Previous studies indicate that unsaturated fatty acids are increased in plasma LPA in patients with late-stage or recurrent ovarian cancer (43) and that LPA-containing unsaturated fatty acids are more biologically active than LPA with saturated fatty acid chains (19–21). Consistent with the above-mentioned reports, LPA with unsaturated fatty acids (18:1) induced ectodomain shedding of pro-HB-EGF, but saturated fatty acids (18:0) showed a minimal effect (Supplementary Fig. 1).

Our results indicate that autonomous LPA production by ovarian cancer cells induces constitutive pro-HB-EGF shedding and EGFR activation. Shedding would be amplified by two positive feedback loops, one for LPA production and the other for pro-HB-EGF ectodomain shedding. LPA is produced from ovarian cancer cells by sequential degradation of membrane phospholipids through the combined action of phospholipase D, phospholipase A2, phospholipase A1, and lysophospholipase A2 (37, 44–46). Phospholipase D and lysophospholipase A2 are activated by EGFR activation (47, 48), suggesting that LPA-induced ectodomain shedding of HB-EGF and subsequent EGFR stimulation may enhance LPA production. At steady-state conditions, LPA is rapidly degraded, primarily by dephosphorylation to monoacylglycerol. The homeostasis of LPA production and LPA degradation may be perturbed in ovarian cancer. Overexpression of LPP-3 in ovarian cancer cells decreased LPA levels, thereby reducing *in vivo* tumorigenicity (33). We demonstrated that LPP-1 and LPP-3 decrease HB-EGF ectodomain shedding and EGFR activation under serum-free conditions and confirmed the inhibitory effect of these

molecules on tumor formation. Thus, targeted suppression of the autocrine loop of LPA production may be an effective approach to ovarian cancer therapy. The other positive feedback loop acts to enhance ectodomain shedding. LPA-induced ectodomain shedding of pro-HB-EGF results in EGFR activation and subsequent ERK/MAPK activation. Activation of the EGFR-MEK-ERK/MAPK pathway in turn promotes pro-HB-EGF shedding as reported previously (36). EGFR signals also induce transcription of pro-HB-EGF and other EGFR ligands (49), resulting in repeated activation of pro-HB-EGF shedding and EGFR activation. LPA signaling may trigger multiple positive feedback loops ensuring substantial EGFR activation.

The standard care for ovarian cancer is a combination of surgery and chemotherapy. Cytotoxic agents including carboplatin and paclitaxel form the cornerstone of chemotherapy in ovarian cancer. However, most patients with advanced ovarian cancer relapse within 2 years of initial treatment and ultimately die due to the development of drug resistance. In ovarian cancer, overexpression of EGFR has been associated with chemoresistance and poor prognosis (4). Targeting HB-EGF, as the abundant EGFR ligand, may be favorable for treatment of chemoresistant ovarian cancer. It is also noted that HB-EGF activates ErbB4 and ErbB2 as well as EGFR (50). Therefore, HB-EGF might be a better target for the therapy of ovarian cancer than EGFR.

In conclusion, HB-EGF is a key molecule connecting the signals from LPA to EGFR in ovarian cancer cells. We argue that, among all of the members of the EGFR family, HB-EGF and the signaling pathway to induce pro-HB-EGF ectodomain shedding are a novel target for the therapy of ovarian cancer. EGF family receptors are widely accepted as effective targets for cancer therapy. This study indicates that EGFR ligands are also good candidates for targeted cancer therapies.

ACKNOWLEDGMENTS

We thank Dr. Yasuhiro Ohashi for critical advice.

REFERENCES

1. Penson RT, Shannon KE, Sharpless NE, Seiden MV. Ovarian cancer: an update on genetics and therapy. *Compr Ther* 1998;24:477–87.
2. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995; 19:183–232.
3. Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS. Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. *Endocr Relat Cancer* 2003;10:1–21.
4. Scambia G, Benedetti Panici P, Battaglia F, et al. Significance of epidermal growth factor receptor in advanced ovarian cancer. *J Clin Oncol* 1992;10:529–35.
5. D'Antonio A, Losito S, Pignata S, et al. Transforming growth factor alpha, amphiregulin and cripto-1 are frequently expressed in advanced human ovarian carcinomas. *Int J Oncol* 2002;21:941–8.
6. Morishige K, Kurachi H, Amemiya K, et al. Evidence for the involvement of transforming growth factor alpha and epidermal growth factor receptor autocrine growth mechanism in primary human ovarian cancers *in vitro*. *Cancer Res* 1991;51: 5322–8.
7. Morimoto H, Safrit JT, Bonavida B. Synergistic effect of tumor necrosis factor-alpha and diphtheria toxin-mediated cytotoxicity in sensitive and resistant human ovarian tumor cell lines. *J Immunol* 1991;147:2609–16.
8. Iwamoto R, Higashiyama S, Mitamura T, et al. Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO J* 1994;13:2322–30.
9. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science (Wash DC)* 1991;251:936–9.
10. Goishi K, Higashiyama S, Klagsbrun M, et al. 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* 1995;6:967–80.
11. Massague J, Pandiella A. Membrane-anchored growth factors. *Annu Rev Biochem* 1993;62:515–41.
12. Yamazaki S, Iwamoto R, Saeki K, et al. Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. *J Cell Biol* 2003;163:469–75.

13. Takenobu H, Yamazaki A, Hirata M, Umata T, Mekada E. The stress- and inflammatory cytokine-induced ectodomain shedding of heparin-binding epidermal growth factor-like growth factor is mediated by p38 MAPK, distinct from the 12-O-tetradecanoylphorbol-13-acetate- and lysophosphatidic acid-induced signaling cascades. *J Biol Chem* 2003;278:17255–62.
14. Prenzel N, Zwick E, Daub H, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of pro-HB-EGF. *Nature (Lond)* 1999;402:884–8.
15. Hirata M, Umata T, Takahashi T, et al. Identification of serum factor inducing ectodomain shedding of pro-HB-EGF and studies of noncleavable mutants of pro-HB-EGF. *Biochem Biophys Res Commun* 2001;283:915–22.
16. Mills GB, May C, McGill M, Roifman CM, Mellors A. A putative new growth factor in ascitic fluid from ovarian cancer patients: identification, characterization, and mechanism of action. *Cancer Res* 1988;48:1066–71.
17. Westermann AM, Havik E, Postma FR, et al. Malignant effusions contain lysophosphatidic acid (LPA)-like activity. *Ann Oncol* 1998;9:437–42.
18. Xu Y, Gaudette DC, Boynton JD, et al. Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. *Clin Cancer Res* 1995;1:1223–32.
19. Moolenaar WH, Kranenburg O, Postma FR, Zondag GC. Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr Opin Cell Biol* 1997;9:168–73.
20. Fang X, Schummer M, Mao M, et al. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. *Biochim Biophys Acta* 2002;1582:257–64.
21. Goetzl EJ, Lee H, Dolezalova H, et al. Mechanisms of lysolipid phosphate effects on cellular survival and proliferation. *Ann N Y Acad Sci* 2000;905:177–87.
22. Xu Y, Shen Z, Wiper DW, et al. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *JAMA* 1998;280:719–23.
23. Gschwind A, Hart S, Fischer OM, Ullrich A. TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J* 2003;22:2411–21.
24. Uchida T, Pappenheimer AM Jr, Greany R. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J Biol Chem* 1973;248:3838–44.
25. Kai M, Wada I, Imai S, Sakane F, Kanoh H. Cloning and characterization of two human isozymes of Mg²⁺-independent phosphatidic acid phosphatase. *J Biol Chem* 1997;272:24572–8.
26. Hasuwa H, Kaseda K, Einarsdottir T, Okabe M. Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett* 2002;532:227–30.
27. Miyamoto S, Akiyama SK, Yamada KM. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash DC)* 1995;267:883–5.
28. 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.
29. Bissery MC, Guenard D, Gueritte-Voegelein F, Lavelle F. Experimental antitumor activity of taxotere (RP 56976, NSC 628503), a Taxol analogue. *Cancer Res* 1991;51:4845–52.
30. Fujita T, Miyamoto S, Onoyama I, et al. Expression of lysophosphatidic acid receptors and vascular endothelial growth factor mediating lysophosphatidic acid in the development of human ovarian cancer. *Cancer Lett* 2003;192:161–9.
31. Adam RM, Borer JG, Williams J, Eastham JA, Loughlin KR, Freeman MR. Amphiregulin is coordinately expressed with heparin-binding epidermal growth factor-like growth factor in the interstitial smooth muscle of the human prostate. *Endocrinology* 1999;140:5866–75.
32. Ishikawa T, Kai M, Wada I, Kanoh H. Cell surface activities of the human type 2b phosphatidic acid phosphatase. *J Biochem (Tokyo)* 2000;127:645–51.
33. Tanyi JL, Morris AJ, Wolf JK, et al. The human lipid phosphate phosphatase-3 decreases the growth, survival, and tumorigenesis of ovarian cancer cells: validation of the lysophosphatidic acid signaling cascade as a target for therapy in ovarian cancer. *Cancer Res* 2003;63:1073–82.
34. Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M, Mekada E. Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J Biol Chem* 1995;270:1015–9.
35. Okugawa K, Kobayashi H, Hirakawa T, et al. In vivo establishment and characterization of a paclitaxel-resistant human ovarian cancer cell line showing enhanced growth properties and drug-resistance only in vivo. *J Cancer Res Clin Oncol* 2004;130:178–86.
36. Umata T, Hirata M, Takahashi T, et al. A dual signaling cascade that regulates the ectodomain shedding of heparin-binding epidermal growth factor-like growth factor. *J Biol Chem* 2001;276:30475–82.
37. Eder AM, Sasagawa T, Mao M, Aoki J, Mills GB. Constitutive and lysophosphatidic acid (LPA)-induced LPA production: role of phospholipase D and phospholipase A2. *Clin Cancer Res* 2000;6:2482–91.
38. van der Bend RL, Brunner J, Jalink K, et al. Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. *EMBO J* 1992;11:2495–501.
39. Hooks SB, Ragan SP, Lynch KR. Identification of a novel human phosphatidic acid phosphatase type 2 isoform. *FEBS Lett* 1998;427:188–92.
40. Jhappan C, Stahle K, Harkins RN, et al. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell* 1990;61:1137–46.
41. Sandgren EP, Luetteke NC, Palminter RD, Brinster RL, Lee DC. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell* 1990;61:1121–35.
42. Ma L, Gauville C, Berthois Y, et al. Antisense expression for amphiregulin suppresses tumorigenicity of a transformed human breast epithelial cell line. *Oncogene* 1999;18:6513–20.
43. Shen Z, Wu M, Elson P, et al. Fatty acid composition of lysophosphatidic acid and lysophosphatidylinositol in plasma from patients with ovarian cancer and other gynecological diseases. *Gynecol Oncol* 2001;83:25–30.
44. Pages C, Simon MF, Valet P, Saulnier-Blache JS. Lysophosphatidic acid synthesis and release. *Prostaglandins Other Lipid Mediat* 2001;64:1–10.
45. Shen Z, Belinson J, Morton RE, Xu Y. Phorbol 12-myristate 13-acetate stimulates lysophosphatidic acid secretion from ovarian and cervical cancer cells but not from breast or leukemia cells. *Gynecol Oncol* 1998;71:364–8.
46. Tokumura A, Tominaga K, Yasuda K, et al. Lack of significant differences in the corrected activity of lysophospholipase D, producer of phospholipid mediator lysophosphatidic acid, in incubated serum from women with and without ovarian tumors. *Cancer (Phila)* 2002;94:141–51.
47. Yeo EJ, Exton JH. Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. *J Biol Chem* 1995;270:3980–8.
48. Leslie CC. Properties and regulation of cytosolic phospholipase A2. *J Biol Chem* 1997;272:16709–12.
49. 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.
50. Iwamoto R, Yamazaki S, Asakura M, et al. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc Natl Acad Sci USA* 2003;100:3221–6.