Lung Cancer-derived Galectin-1 Enhances Tumorigenic Potentiation of Tumor-associated Dendritic Cells by Expressing Heparin-binding EGF-like Growth Factor

Background: Communication between cancer cells and immune cells in their microenvironment leads to cancer progression.

Results: Lung cancer-derived galectin-1 stimulates HB-EGF expression and triggers a release mechanism in tumor-associated dendritic cells that stimulates tumor cell growth and invasion.

Conclusion: The galectin-1/HB-EGF cycle between cancer and dendritic cells enhances lung cancer progression.

Significance: Galectin-1 is a potential therapeutic target for inhibiting tumor-promoting immune cells in the tumor microenvironment.

The interaction between cancer cells and their microenvironment is a vicious cycle that enhances the survival and progression of cancer, resulting in metastasis. This study is the first to indicate that lung cancer-derived galectin-1 secretion is responsible for stimulating tumor-associated dendritic cells (TADCs) production of mature heparin-binding EGF-like growth factor (HB-EGF), which, in turn, increases cancer progression. Treatment of galectin-1, present in large amounts in lung cancer conditioned medium and lung cancer patient sera, mimicked the inductive effect of lung cancer conditioned medium on the expression and ectodomain shedding of HB-EGF by TNFα-converting enzyme/a disintegrin and metalloproteinase 9 (ADAM9) and ADAM17. Significant up-regulation of HB-EGF has been seen in tumor-infiltrating CD11c+ dendritic cells in human lung cancer samples. Active cleavage of HB-EGF in TADCs by ADAM9 and ADAM17 is associated with increased protein kinase C δ and Lyn signaling. Enhancement of HB-EGF production in TADCs increased the proliferation, migration, and epithelial-to-mesenchymal transition abilities of lung cancer. In contrast, inhibiting HB-EGF by siRNA suppressed TADC-mediated cancer progression. Moreover, mice injected with galectin-1 knockdown Lewis lung carcinoma showed decreased expression and ectodomain shedding of HB-EGF and reduced incidence of cancer development, resulting in increased survival rates. We demonstrate here for the first time that human and mouse DCs are a source of HB-EGF, an EGF ligand with tumorigenic properties. Antagonists of the effect of lung cancer-derived galectin-1 on DCs and anti-HB-EGF blocking antibodies could, therefore, have therapeutic potential as antitumor agents.

Lung cancer has been one of the leading causes of death worldwide and is associated with very poor prognoses, even after tumor resection (1). Interaction between cancer cells and their microenvironment causes a vicious cycle of tumor progression (2, 3). Tumor-associated dendritic cells (TADCs)2 are speculated to be one of the cells that interact with cancer. Dendritic cells (DCs) located in the microenvironment of the tumor not only inhibit the T cell-based anticancer immune response but also promote the progression of tumor growth and invasive and proangiogenic abilities (4–6). However, despite these findings, the mechanisms by which cancer cells modify TADCs and by which TADCs mediate cancer progression have remained elusive. Failure to understand the interaction between TADCs and cancer has hindered progress in developing effective immunotherapy strategies.

Heparin-binding EGF-like growth factor (HB-EGF) is frequently produced as membrane-anchored precursor proteins that require cleavage by cell surface proteases, such as TNFα-converting enzyme/a disintegrin and metalloproteinases 9 (TNFα-converting enzyme/ADAM9) and 17 (TNFα-converting enzyme/ADAM17), into soluble ligands (7–10). Aberrant expression or ectodomain shedding of HB-EGF results in the development of various cancers: ovarian, pancreatic, liver, esophageal, melanoma, colon, gastric, and bladder, together with glioblastoma (9, 11). HB-EGF has been demonstrated to promote cancer cell proliferation and migration by binding

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2 The abbreviations used are: TADC, tumor-associated dendritic cell; DC, dendritic cell; HB-EGF, heparin-binding EGF-like growth factor; ADAM, a disintegrin and metalloprotease; EGFR, EGF receptor; MdDC, monocyte-derived dendritic cells; EMT, epithelial-mesenchymal transition; Q-PCR, quantitative PCR; ANOVA, analysis of variance.
Recent studies have shown that tumor-infiltrated immune cells can produce certain tumorigenic factors, such as growth and inflammatory cytokines, as well as chemokines, to enhance cancer progression (13, 14). Tumor-associated macrophages express high angiogenic factors, VEGF, TGF-β, and IL-6, which increase cancer growth and metastasis (13–15). However, the tumorigenic properties of TADCs have thus far been poorly described.

FIGURE 1. Lung cancer increased the expression of HB-EGF, ADAM9, and ADAM17 in mDcCs. A, the up-regulation of HB-EGF, ADAM9, and ADAM17 in A549-TADCs. Elevated expressions of HB-EGF, ADAM9, and ADAM17 in A549-TADCs and H460-TADCs at mRNA (B) and protein levels (C). D, the ectodomain shedding of HB-EGF. The mDCs, A549-TADCs, and H460-TADCs were generated by culturing CD14+ monocytes with RPMI, A549-CM (20%), and H460-CM (20%) presenting in GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 5 days. The expression of mRNA and protein was assessed by Q-PCR and ELISA. The ectodomain shedding of HB-EGF determined the level of HB-EGF in supernatants of mDCs, A549-TADCs, and H460-TADCs. Results are representative of at least three independent experiments. *, p < 0.05; mean ± S.D. between the two test groups as analyzed by ANOVA with Student’s t test.
In this study, we discovered that lung cancer cells secrete high amounts of galectin-1, a critical mediator in the promotion of cancer by means of its interaction with TADCs. Our data show that galectin-1 increases the expression and ectodomain shedding of HB-EGF in monocyte-derived dendritic cells (MdDCs) by an ADAM9 and ADAM17-mediated cleavage.

**FIGURE 2. Galectin-1 is involved in lung cancer-mediated HB-EGF up-regulation.** A, galectin-1 increases the mRNA expression of HB-EGF, ADAM9, and ADAM17. Galectin-1 increases the protein expression of HB-EGF (B), ADAM9 (C), and ADAM17 (D). E, galectin-1 enhances the ectodomain shedding of HB-EGF. Galectin-1 knockdown decreases the enhancement of A549-CM on HB-EGF expression (F) and ectodomain shedding (G). Galectin-1 knockdown of A549 cells was performed by the pLKO-AS2-LGALS1 shRNA lentiviral expression system. The condition medium was collected after A549 stable clone establishment. The galectin-1-mdDCs, control shRNA-A549-TADCs, and galectin-1-shRNA-A549-TADCs were generated by culturing CD14+/H11001 monocytes with RPMI, galectin-1 (2 μg/ml), control shRNA-A549-CM (20%), and galectin-1-shRNA-A549-CM (20%) presenting in GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 5 days. The expression of mRNA and protein was assessed by Q-PCR and ELISA. The ectodomain shedding of HB-EGF was determined by ascertaining the levels of HB-EGF in supernatants of various mdDCs and TADCs. Results are representative of at least three independent experiments. *, p < 0.05; data are mean ± S.D. between the two test groups as analyzed by ANOVA with Student’s t test.
which is regulated in a Lyn- and PKCδ-dependent manner. Our findings have also revealed that DCs infiltrating tumor tissues of mice and human patients express high levels of HB-EGF and ADAM9 and 17, suggesting both a novel role of galectin-1 in the tumor DC vicious cycle and its candidacy as a potential target for developing therapeutic strategies to strengthen the immune surveillance of the host when fighting lung cancer.

**EXPERIMENTAL PROCEDURES**

**Lung Cancer Cells and Conditioned Media**—Human lung cancer cells A549 and NCI-H460 and mouse Lewis lung carcinoma (LLC) cells were obtained from the Bioresource Collection and Research Center (Hsinchu City, Taiwan). To obtain A549 and H460 conditioned medium (CM), cells were seeded at 2 × 10^6 cells/100-mm dish and cultivated for 24 h. The medium was replaced, and the supernatants were harvested after 48 h of incubation.

**Serum Samples from Lung Cancer Patients**—Preoperative blood samples were obtained from 58 lung cancer patients and 20 healthy donors admitted to the Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan. The serum was separated by centrifugation and frozen at −80 °C. Approval for these studies was obtained from the Institutional Review Board of Kaohsiung Medical University Hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

**Isolation of CD14^+ Monocytes and Differentiation of MdDCs**—Monocytes were purified from peripheral blood mononuclear cells obtained from healthy consenting donors. Mononuclear cells were isolated from blood by Ficoll-Hypaque gradient (GE Healthcare). CD14^+ monocytes were purified using CD14^+ monoclonal antibody-conjugated magnetic beads (MACS MicroBeads, Miltenyi Biotec), according to the manufacturer’s protocol. MdDCs were generated by culturing CD14^+ monocytes in RPMI 1640 medium containing 10% FBS (Invitrogen) and 20 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 5 days. The medium was replaced with fresh medium containing GM-CSF and IL-4 on day 3. For maturation of MdDCs, immature MdDCs were stimulated with LPS (100 ng/ml) after priming with IFN-γ for 3 h. A549 tumor-associated mdDCs (A549-TADCs) and H460-TADCs were generated by culturing CD14^+ monocytes in RPMI 1640 medium containing FBS, GM-CSF, and IL-4 presenting in A549-CM and H460-CM and then stimulated as described above (supplemental Fig. 1 A).

**Real-time RT-PCR (Q-PCR)**—RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed using SYBR Green on the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Each PCR reaction mixture contained 200 nM of each primer, 10 μl of SYBR Green PCR Master Mix (Applied Biosystems), and 5 μl cDNA and RNase-free water with a total volume of 20 μl. The experiments, *p < 0.05; data are mean ± S.D. between the two test groups as analyzed by ANOVA with Student’s t test.
PCR reaction was carried out with a denaturation step at 95 °C for 10 min and then for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All PCRs were performed in triplicate and normalized to internal control GAPDH mRNA. Relative expression was presented using the \(2^{-\Delta\Delta CT}\) method.

Measurement of Secreted Factors—Supernatants from DCs were collected. Shedding HB-EGF, ADAM9, and ADAM17 levels were quantified using the DuoSet ELISA development systems (R&D Systems) according to the manufacturer’s instructions.

3D Culture, Migration, and Invasion Assay—The effect of TADCs on A549 and H460 cell proliferation was assessed by AlgiMatrix 24-well plates (Invitrogen). TADCs were cocultured with A549 or H460 for 14 days, and cell spheroids were

FIGURE 4. TADCs increase the progression of lung cancer. TADCs increase growth (A) in A549 and H460 cells. HB-EGF enhances cell growth (B), migration (C), invasion (D), and EMT (E) in A549 and H460 cells. For cell growth, A549 and H460 cells were cocultured with mDC for TADCs (A549-TADCs for A549 cells and H460-TADCs for H460 cells) in a three-dimensional culture system for 14 days. Alternatively, A549 and H460 cells were treated with or without HB-EGF (250 ng/ml) in an AlgiMatrix three-dimensional culture system. The sphere of cancer cells was visualized by microscope. The invasiveness and migration ability of A549 and H460 cells were quantified by QCM™ 24-well cell invasion and migration assay. A549 or H460 cells were seeded in the upper insert and HB-EGF (250 ng/ml) were placed in the bottom. Cancer cell migration or invasion was allowed for 24 h. E, A549 and H460 cells were treated with HB-EGF for 24 h, and the expressions of various proteins were assessed by immunoblot analysis. Results are representative of at least three independent experiments. *, \(p < 0.05\); data are mean ± S.D. between the two test groups as analyzed by ANOVA with Student’s t test.
HB-EGF Involved in Cancer-Immune System Interaction

A

Relative mRNA expression (fold of control)

Control siRNA  HB-EGF siRNA

B

Colony formation

Control siRNA  HB-EGF siRNA

A549

H460

C

Colony formation

mdDCs  Control shRNA  A549-TADCs  Galectin-1 shRNA  A549-TADCs

D

Cell migration (OD at 540 nm)

mdDCs  Control shRNA  A549-TADCs  Galectin-1 shRNA  A549-TADCs

E

Cell invasion (OD at 540 nm)

mdDCs  Control shRNA  A549-TADCs  Galectin-1 shRNA  A549-TADCs
visualized by microscope. Cell migration and invasion assay was conducted using QCM® 24-well cell migration and invasion assay kits (Millipore, Bedford, MA). Briefly, 2 × 10⁵ cells were seeded into the top insert, and various types of TADCs (2 × 10⁵) were placed in the bottom wells for 24 h. At the end of the treatment, the cells were post-stained with CyQuant GR® dye in cell lysis buffer for 15 min at room temperature. Fluorescence of the migrated and invaded cells was read using a fluorescence plate reader at excitation/emission wavelengths of 485/540 nm.

**Immunoblot Analysis**—Cells were lysed on ice for 15 min by M-PER® lysis reagent (Pierce). Cell lysate was centrifuged at 14,000 × g for 15 min, and the supernatant fraction was collected for immunoblot analysis. Equivalent amounts of protein were resolved by SDS-PAGE (8–12%) and transferred to PVDF membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1–16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Millipore) according to the manufacturer's instructions.

**Gene Knockdown by siRNA**—Monocytes were transfected with 1 µmol/L non-target, ADAM9, ADAM17, or HB-EGF Accell siRNAs pool (Dharmacon) in Accell delivery media (B-005000) according to the manufacturer's instructions. Positive controls Accell GAPDH siRNA and scrambled Accell siRNA pool were used in the experiments. After 72-h transfection, the medium was changed to whole medium. The changes of ADAM9, ADAM17, HB-EGF, and c-Met were measured by real-time PCR as described previously. Knockdown of galectin-1 in A549 and LLC was performed by using the lentiviral expression system provided by the National RNAi Core Facility (Taipei, Taiwan). Lentiviruses were produced by cotransfecting HEK293T with pLKO-AS2 or pLKO-AS2-LGALS1 shRNA and two packaging plasmids (pCMVVD8R9.81 and pMD.G).

**Animal Models and Isolation of CD11c⁺ Cells from Lungs**—LLC cells were injected into C57BL/6 mice via the tail vein. Lung tissue was collected 14 days after injection and then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Millipore) according to the manufacturer's instructions.

**Shedding of HB-EGF**—To better understand the involvement of ADAMs on the ectodomain shedding of HB-EGF, knockdown of galectin-1 and expression of ADAM9, and ADAM17 in mdDCs at both mRNA and protein levels. The ectodomain shedding of HB-EGF was also enhanced in galec-1-treated mdDCs (Fig. 2E). Significantly, galectin-1 knockdown A549-CM lost its activity in up-regulation and ectodomain shedding of HB-EGF, suggesting that galectin-1 is involved in lung cancer-mediated HB-EGF up-regulation and ectodomain shedding in mdDCs (Fig. 2D). Statistical Analysis—Data were expressed as mean ± S.D. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (p < 0.05) between the means of the two test groups were analyzed by Student’s t test.

**RESULTS**

**Lung Cancer-CM and Galectin-1 Increases MdDCs to Produce HB-EGF**—To investigate which factor is responsible for TADC-mediated lung cancer development, we assessed the gene profile of A549-TADCs by microarray. The data showed that several soluble factors were up-regulated in A549-TADCs when compared with mdDCs. Among these up-regulated genes, levels of HB-EGF, a lung cancer-related growth factor, increased 3.89-fold in A549-TADCs (Fig. 1A). In addition, levels of ADAM9 and ADAM17, enzymes responsible for HB-EGF release, also increased in A549-TADCs (Fig. 1A). Q-PCR analysis also showed that A549-CM or H460-CM increased the expression of HB-EGF, ADAM9, and ADAM17 in A549-TADCs and H460-TADCs (Fig. 1B). In addition, the protein levels of HB-EGF, ADAM9, and ADAM17 were enhanced in A549-TADCs and H460-TADCs, as determined by ELISA analysis (Fig. 1C). Furthermore, the ectodomain shedding of HB-EGF was also enhanced in both A549-TADCs and H460-TADCs (Fig. 1D).

We have reported that lung cancer-derived galectin-1 causes Dcs energy in cancer microenvironments (16). We further investigated whether galectin-1 is involved in lung cancer-mediated HB-EGF up-regulation. As shown in Fig. 2, A–D, galectin-1 increases the expression of HB-EGF, ADAM9, and ADAM17 in mdDCs at both mRNA and protein levels. The ectodomain shedding of HB-EGF was also enhanced in galec-1-treated mdDCs (Fig. 2E). Significantly, galectin-1 knockdown A549-CM lost its activity in up-regulation and ectodomain shedding of HB-EGF, suggesting that galectin-1 is involved in lung cancer-mediated HB-EGF up-regulation and ectodomain shedding in mdDCs (Fig. 2D). Specific knockdown of ADAM9 or ADAM17 expression decreased the shedding of HB-EGF in A549-TADCs, H460-TADCs, and galectin-1-mdDCs, suggesting that ADAM9 and ADAM17 are involved in lung cancer-mediated HB-EGF up-regulation and ectodomain shedding in mdDCs (Fig. 2D). Previous studies have reported that TADCs increase lung cancer progression. A, the effect of HB-EGF siRNA. B, HB-EGF knockdown decreased the promotion of TADCs on lung cancer growth. Galectin-1 knockdown decreased the enhancement of TADC on A549 growth (C), migration (D), and invasion (E). HB-EGF knockdown of monocytes was performed by Accell siRNA at 1 µmol concentration of scramble or HB-EGF siRNA. After 72-h transfection, HB-EGF mRNA was assessed by Q-PCR. The control shRNA-A549-TADCs, galectin-1-shRNA-A549-TADCs, and cell growth, migration, and invasion were performed as described above. Results are representative of at least three independent experiments. *, p < 0.05; data are mean ± S.D. between the two test groups as analyzed by ANOVA with Student's t test.

**TADCs Increase the Progression of Cancer by HB-EGF**—Previous studies have reported that TADCs increase lung cancer progression. A, the effect of HB-EGF siRNA. B, HB-EGF knockdown decreased the promotion of TADCs on lung cancer growth. Galectin-1 knockdown decreased the enhancement of TADC on A549 growth (C), migration (D), and invasion (E). HB-EGF knockdown of monocytes was performed by Accell siRNA at 1 µmol concentration of scramble or HB-EGF siRNA. After 72-h transfection, HB-EGF mRNA was assessed by Q-PCR. The control shRNA-A549-TADCs, galectin-1-shRNA-A549-TADCs, and cell growth, migration, and invasion were performed as described above. Results are representative of at least three independent experiments. *, p < 0.05; data are mean ± S.D. between the two test groups as analyzed by ANOVA with Student's t test.
FIGURE 6. PKCζ and Lyn mediate the ectodomain shedding of HB-EGF in TADCs. A, the expression of Lyn in DCs was determined by Q-PCR analysis. B, the phosphorylation of PKCζ and Lyn. C, Lyn is the upstream factor of PKCζ. D, the PKCζ and Lyn inhibitor decrease ectodomain shedding of HB-EGF. E, knockdown of Lyn and PKCζ by siRNA confirmed that Lyn is the upstream factor of PKCζ. F, inhibition of Lyn and PKCζ by siRNA reduced ectodomain shedding of HB-EGF.

CD14⁺ monocytes were treated with various inhibitors for 1 h, and then cells were treated with RPMI 1640, A549-CM (20%), and H460-CM (20%) or galectin-1 (2 μg/ml) presenting in IL-4 and GM-CSF for 5 days. Lyn and PKCζ knockdown of monocytes was performed by Acell siRNA at 1 μM concentration of scramble or Lyn or PKCζ siRNA. After 72-h transfection, siRNA-transfected CD14⁺ monocytes were treated with RPMI, galectin-1, A549-CM, or H460-CM for the indicated times: 2 days for the activation of Lyn and PKCζ and 5 days for HB-EGF ectodomain shedding. The supernatant was collected, and the ectodomain shedding of HB-EGF was assessed by ELISA kit. Monocytes were treated with RPMI, galectin-1, A549-CM, or H460-CM for the indicated times, and the various proteins were assessed by immunoblot assay. Data are representative of three independent experiments. *, p < 0.05; data are mean ± S.D. between the two test groups as analyzed by ANOVA with Student’s t test.
HB-EGF Involved in Cancer-Immune System Interaction

A

Relative mRNA expression

| CD11c⁺ cells of non-tumor region | CD11c⁺ cells of tumor region |
|----------------------------------|-----------------------------|
| HB-EGF                           | ADAM17                       |

B

Relative mRNA expression (fold of control)

| CD11c⁺ cells of control mice | CD11c⁺ cells of LLC-bearing mice |
|------------------------------|---------------------------------|
| HB-EGF                       | ADAM17                          |

C

Ectodomain shedding HB-EGF level (pg/ml)

| CD11c⁺ cells of control mice | CD11c⁺ cells of LLC-bearing mice |
|------------------------------|---------------------------------|
| HB-EGF                       | ADAM17                          |

D

Ectodomain shedding HB-EGF level (pg/ml)

| CD11c⁺ cells of LLC-bearing mice | CD11c⁺ cells of Galectin-ko-LLC-bearing mice |
|----------------------------------|---------------------------------------------|
| HB-EGF                           | ADAM17                                      |
progression by producing soluble factors (2, 6). We therefore assessed whether HB-EGF is involved in TADC-mediated cell progression. We first assessed the effects of TADCs on proliferation, migration, invasion, and EMT in A549 and H460 cells. Coculturing of A549-TADCs with A549 cells or coculturing of H460-TADCs with H460 cells increases colony formation ability in a three-dimensional culture system (Fig. 4A). In addition, coculturing of TADCs with lung cancer also enhances the migration, invasion, and EMT of cancer cells (supplemental Fig. 1, B–D). We next assessed the effect of HB-EGF protein on the proliferation, migration, invasion and EMT of A549 and H460 cells. HB-EGF increases the proliferation of A549 and H460 cells in a concentration-dependent manner (Fig. 4B). HB-EGF not only increases the migratory ability of A549 and H460 (Fig. 4C) but also enhances their invasive ability (D). Moreover, HB-EGF also causes A549 and H460 cells to undergo EMT, including the down-regulation of epithelial marker (ZO-1, E-cadherin, and claudin3), and up-regulation of fibroblast (migratory) markers (vimentin, N-cadherin, and fibronectin) (Fig. 4E).

To evaluate the contribution of HB-EGF to the enhancement of TADCs on cancer progression, we interfered with the effects of HB-EGF by means of siRNA transfection. Compared with non-target siRNA-transfected TADCs, HB-EGF siRNA effectively decreased HB-EGF expression in A549-TADCs and H460-TADCs by 85% (Fig. 5A). Knockdown of HB-EGF by siRNA was sufficient to reverse the effect of TADCs on the enhancement of cancer growth (Fig. 5B), migration, and invasion (supplemental Fig. 2, A and B).

To better understand the involvement of tumor-derived galectin-1 on TADC-mediated cancer progression, we assessed the effect of galectin-1 knockdown A549-TADCs on cancer cell proliferation, migration, and invasion. Galectin-1 knockdown A549-TADC lost its ability to enhance cell proliferation, migration, and invasion in a coculture system (Fig. 5, C–E), suggesting that HB-EGF, induced by galectin-1, is involved in TADCs-mediated cancer progression.

PKCδ and Lyn Signaling Is Involved in Lung Cancer-derived Galectin-1-mediated HB-EGF in TADCs—Previous studies have reported that PKCδ and Src family kinase is involved in the ectodomain shedding of HB-EGF (17, 18). We therefore assessed the roles of PKCδ and Lyn, a member of Src kinase, on the activation of HB-EGF. Earlier microarray data show that A549-CM increases the transcript of Lyn, as has also been confirmed by Q-PCR (Fig. 6A). Immunoblot data also shows that A549-CM, H460-CM, and galectin-1 increase the phosphorylation of PKCδ and Lyn, as well as the amount of Lyn protein (Fig. 6B). The specific PKCδ inhibitor decreases the ectodomain shedding of HB-EGF but not the phosphorylation of Lyn (Fig. 6, C and D). In contrast, Lyn inhibitors not only decrease the ectodomain shedding of HB-EGF but also affect the phosphorylation of PKCδ (Fig. 6, C and D).

To avoid the nonspecific effect of chemical inhibitors, we also assessed the role of PKCδ and Lyn by using siRNA. Transfection of CD14+ monocyte with PKCδ and Lyn siRNA reduces basal levels of PKCδ and Lyn (Fig. 6E). Knockdown of PKCδ reduces the ectodomain shedding of HB-EGF but not the phosphorylation of Lyn (Fig. 6, E and F). Indeed, blockade of Lyn by siRNA not only decreases the ectodomain shedding of HB-EGF but also the phosphorylation of PKCδ (Fig. 6, E and F).

**High Amounts of HB-EGF in CD11c+ DCs Are Found in the Tumor Sections of Lung Cancer-bearing Mice and Human Lung Cancer Patients**—To confirm the role of galectin-1 on lung cancer-mediated immune suppression in humans, we characterized the amount of HB-EGF in tumor-infiltrating CD11c+ DCs. Compared with CD11c+ DCs from non-tumor regions, CD11c+ DCs isolated from fresh lung cancers show a stronger ability to express HB-EGF and ADAM17 mRNA (Fig. 7A). However, no statistical differences could be found between lung cancer patient sera and healthy donor sera (supplemental Fig. 3), suggesting that the changes of HB-EGF are found only in the affected lung.

Finally, we used animal experiments to determine whether lung cancer increased HB-EGF and ADAM17 expression in DCs in vivo and whether galectin-1 acts as a major regulator on up-regulation of HB-EGF in TADCs. First, we assessed the LLC-CM and murine galectin-1 on the expression of HB-EGF and ADAM17. As shown in supplemental Fig. 4, A–C, LLC-CM and murine galectin-1 increased the expression of HB-EGF and ADAM17 in mouse bone marrow cells. In addition, LLC-CM and murine galectin-1 also enhance the ectodomain shedding of HB-EGF in mouse bone marrow cells (supplemental Fig. 4C). These data correlate with human lung cancer data.

Next, we injected the mouse lung cancer cell line LLC into mice and allowed the tumor to develop for 14 days. Q-PCR and ELISA data show that lung tumor-infiltrating CD11c+ DCs are able to produce elevated levels of HB-EGF and ADAM17 on gene expression and protein secretion (Fig. 7, B and C). We further investigated the association of tumor-derived galectin-1 and DCs by using galectin-1 shRNA. Transfection of LLC cells with galectin-1 shRNA reduced basal galectin-1 levels by 80% (supplemental Fig. 4D). As expected, the levels of HB-EGF and ADAM17 mRNA on CD11c+ DCs isolated from the lung tumors of galectin-1-knockdown LLC-bearing mice was significantly lower than that of CD11c+ DCs obtained from lung tissues of control shRNA-transfected LLC-bearing mice (supplemental Fig. 4E). Moreover, ELISA analysis of HB-EGF and ADAM17 protein levels also confirmed the reduction of HB-EGF and ADAM17 in LLC-bearing mice treated with galectin-1 shRNA.
ADAM17 also exhibited similar results (Fig. 7D), suggesting that galectin-1 is involved in the up-regulation of HB-EGF and ADAM17.

**DISCUSSION**

The microenvironment of the tumor is widely known to be an important regulator in cancer development (2, 3, 19). Tumor cells consistently release immunosuppressive and proinflammatory factors that facilitate tumor immune-escape and tumor progression (20, 21). In contrast, tumor-surrounding cells, such as dendritic cells, also secrete certain factors to modulate the development of cancer (22). In this study, we found that galectin-1, present in conditioned media obtained from lung cancer cell lines or sera from lung cancer patients, induces TADCs to produce HB-EGF and promote cell progression. Galectin-1 increases the expression of ADAM9 and ADAM17, which, in turn, releases HB-EGF. Furthermore, TADC-derived HB-EGF enhances the proliferation, migration, and invasion abilities of lung cancer cells. These findings suggest that lung cancer-derived galectin-1 may be a novel candidate in preventing the vicious cycle between lung cancer and TADCs.

We demonstrated previously that lung cancer is capable of modifying the DC phenotype and impair DC function by expressing elevated levels of galectin-1 (16). Galectin-1 has also been implicated in regulating immune responses by controlling T cell survival, cytokine secretion, and transendothelial migration (23–25). However, whether galectin-1 is involved in the interaction of DCs and cancer in the tumor microenvironment has not yet been determined. Our data demonstrate that galectin-1 is involved in the TADC-mediated potential effect of DCs on lung cancer development by inducing HB-EGF expression and ectodomain cleavage. Several lines of evidence support this view. First, galectin-1-containing lung cancer cell medium elevates the expression of cell growth factor HB-EGF in mDCs. A similar high expression of HB-EGF has also been found in sections of the regions of the tumor. Second, knockdown of galectin-1 by specific siRNA in A549 cells reverses the effect of A549-CM on the up-regulation and release of HB-EGF protein in TADCs. Furthermore, HB-EGF-producing CD11c+ DCs have been found to infiltrate cancerous lung tissue of LLC-bearing mice, whereas this phenomenon is significantly less in the lung sections of galectin-1 knockdown LLC-bearing mice. These results, from experimental cell studies, mouse models, and clinical patient sections, strongly suggest that galectin-1 is an important effector factor that strengthens the feedback of lung cancer cells with the immune system.

HB-EGF, a member of the EGF family, has been described as a mitogenic factor of various types of cells, including cancer cells (9, 26). Overexpression of HB-EGF is correlated with tumor progression and poor patient survival in ovarian, colon, head and neck squamous cell carcinoma, stomach, and breast cancers (27, 28). In addition, up-regulation of HB-EGF has also been associated with chemoresistance to various chemotherapeutic drugs (29). HB-EGF is a membrane-bound EGF ligand that is activated by ADAM9 or ADAM17 to form soluble HB-EGF (8, 10). The soluble form of HB-EGF contains the EGF-like domain and can bind EGFR and ErbB4 (12). Increased expression of EGFR has been reported in over 70% of non-small-cell lung carcinoma and is correlated with poor outcome (30). In addition, abnormal expression of cognate EGFR ligand expression is involved in the development of the EGFR resistance of the antagonist, including receptor mutations, constitutive activation of downstream signaling, and activation of alternative pathways (31). Our data are the first to demonstrate that TADCs are one of the sources of HB-EGF, which, in turn, increases cell proliferation and migration and causes lung cancer cells to undergo EMT. That knockdown of HB-EGF decreases the potential effect of TADCs on cancer progression is further supported by the evidence that TADC-derived HB-EGF plays an important role in the vicious cycle of TADCs and cancer development.

All EGF ligand family members, including HB-EGF, are formed as inactive transmembrane precursors that undergo ectodomain proteolytic cleavage to release active soluble forms (32). ADAM17 has been implicated in playing an important role in ectodomain shedding of HB-EGF (33). The trigger of EGF signaling is also regulated by EGFR transactivation, which mediates several critical downstream signals, such as PKC and cytosolic non-receptor Src family kinase (33, 34). We found that treatment of galectin-1 and lung cancer CMs increased PKCδ phosphorylation and Lyn expression, as well as Lyn activation in mDCs. Inhibition of Lyn with a specific inhibitor and siRNA decreases PKCδ phosphorylation and ectodomain shedding of HB-EGF, whereas PKCδ inhibitor does not affect the phosphorylation of Lyn. Indeed, genetic blockade of ADAM9 and ADAM17 also attenuates the ectodomain shedding of HB-EGF, suggesting that sequential activation of Lyn, PKCδ, and ADAM9/17 is associated with the ectodomain shedding of mature HB-EGF.

Taken together, our findings suggest a new mechanism for TADCs-lung cancer interaction in which cancer-derived galectin-1 modifies DCs to product mitogenic and proinvasive factors, even to the point of causing cancer progression. Galectin-1 induces TADCs to produce ADAM9/17, which cleaves HB-EGF and in turn promotes cancer progression by paracrine effects. We also detected HB-EGF-expressing CD11c+ DCs infiltrating mouse tumors and human lung cancers. Knockdown of galectin-1 decreases the level of infiltrating HB-EGF-expressing DCs in lung cancer and significantly decreases the incidence of cancer development in mice. In light of these findings, inhibition of galectin-1 or HB-EGF is an attractive therapeutic target for the paracrine signaling networks between TADCs and lung cancer.

**REFERENCES**

1. Andrews, J., Yeh, P., Pao, W., and Horn, L. (2011) Molecular predictors of response to chemotherapy in non-small cell lung cancer. *Cancer J. 17*, 104–113
2. Sautès-Fridman, C., Cherfils-Vicini, J., Damotte, D., Fisson, S., Fridman, W. H., Cremer, I., and Dieu-Nosjean, M. C. (2011) Tumor microenvironment is multifaceted. *Cancer Metastasis Rev. 30*, 13–25
3. Bremnes, R. M., Al-Shibli, K., Donnem, T., Sirera, R., Al-Saad, S., Andersen, S., Stenvold, H., Camps, C., and Buslund, L. T. (2011) The role of tumor-infiltrating immune cells and chronic inflammation at the tumor site on cancer development, progression, and prognosis. Emphasis on non-small cell lung cancer. *J. Thorac. Oncol. 6*, 824–833
4. Ikawa, S., Ohue, Y., Kitakoa, K., Aji, T., Uenaka, A., Oka, M., and Nakayama, E. (2010) Enrichment of Foxp3+ CD4 regulatory T cells in mi-
HB-EGF Involved in Cancer-Immune System Interaction

grated T cells to IL-6- and IL-8-expressing tumors through predominant induction of CXCR1 by IL-6. J. Immunol. 185, 6734–6740

5. Domínguez-Soto, A., Sierra-Filardi, E., Puig-Kröger, A., Pérez-Maceda, B., Gómez-Aguado, F., Corcuera, M. T., Sánchez-Mateos, P., and Corbi, A. L. (2011) Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. J. Immunol. 186, 2192–2200

6. Mantovani, A., Savino, B., Locati, M., Zammataro, L., Allavena, P., and Bonecchi, R. (2010) The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev. 21, 27–39

7. Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Raska, C. S., Peschon, J. J., Castner, B. I., Gerhart, M. J., Paxton, R. J., Black, R. A., and Lee, D. C. (2002) Tumor necrosis factor α converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. J. Biol. Chem. 277, 12838–12845

8. Miyamoto, S., Yagi, H., Yotsutomo, F., Kawarabayashi, T., and Mekada, E. (2008) Heparin-binding epidermal growth factor-like growth factor as a new target molecule for cancer therapy. Adv. Exp. Med. Biol. 622, 281–295

9. Sagmeister, S., Drucker, C., Losert, A., Grusch, M., Daryabeigi, A., Parvez, W., Rohr-Udilova, N., Bichler, C., Smedsrod, B., Kandioler, D., Grünberger, T., Wrbka, F., Schulte-Herman, R., and Grasl-Krappe, B. (2008) HB-EGF is a paracrine growth stimulator for early tumor prestages in inflammation-associated hepatocarcinogenesis. J. Hepatol. 49, 955–964

10. Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S., and Mekada, E. (1998) A metalloproteinase-disintegrin, MDC9/melrin-α/ADAM9 and PKCζ are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. EMBO J. 17, 7260–7272

11. Yotsutomo, F., Yagi, H., Suzuki, O. O., Oki, E., Tsujioka, H., Hachisuga, T., Sonoda, K., Kawarabayashi, T., Mekada, E., and Miyamoto, S. (2008) Validation of HB-EGF and amphiregulin as targets for human cancer therapy. Biochem. Res. Commun. 365, 555–561

12. Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G., Nanba, D., Higashiyama, S., Horii, M., Klagsbrun, M., and Mekada, E. (2003) Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart development. Proc. Natl. Acad. Sci. U.S.A. 100, 3221–3226

13. Nakao, S., Noda, K., Zandi, S., Sun, D., Taher, M., Schering, A., Xie, F., and Natarajan, V. (2006) Regulation of lysophosphatidic acid-induced epidermal growth factor receptor transactivation and interleukin-8 secretion in human bronchial epithelial cells by protein kinase Cφ, Lyn kinase, and matrix metalloproteinases. J. Biol. Chem. 281, 19501–19511

14. Kveiborg, M., Instrell, R., Rowlands, C., Howell, M., and Parker, P. J. (2011) PKCo and PKCφ regulate ADAM17-mediated ectodomain shedding of heparin binding EGF through separate pathways. PLoS ONE 6, e17168

15. Box, C., Rogers, S. J., Mendiola, M., and Eccles, S. A. (2010) Tumour-microenvironmental interactions. Path to progression and targets for treatment. Semin. Cancer Biol. 20, 128–138

16. Kwon, C., Gilman-Sachs, A., and Beaman, K. (2011) Tumor-associated a2 vacuolar ATPase acts as a key mediator of cancer-related inflammation by inducing pro-tumorigenic properties in monocytes. J. Immunol. 186, 1781–1789

17. Cao, Y., Zhang, L., Kamiyama, Y., Ritzprajak, P., Hashiguchi, M., Hirose, S., and Azuma, M. (2011) B7-H1 overexpression regulates epithelial-mesenchymal transition and accelerates carcinogenesis in skin. Cancer Res. 71, 1235–1243

18. Hu, C. E., Gan, J., Zhang, R. D., Cheng, Y. R., and Huang, G. J. (2011) J. Gastroenterol. 46, 156–164

19. Koh, H. S., Lee, C., Lee, K. S., Park, E. J., Seong, R. H., Hong, S., and Jeon, S. H. (2009) Twist2 regulates CD7 expression and galectin-1-induced apoptosis in mature T-cells. Mol. Cells 28, 553–558

20. Gandhi, M. K., Moll, G., Smith, C., Dua, U., Lambley, E., Ramuz, O., Gill, D., Marltoron, P., Seymour, J. F., and Khanna, R. (2007) Galectin-1 mediated suppression of Epstein-Barr virus-specific T-cell immunity in classic Hodgkin lymphoma. Blood 110, 1326–1329

21. Kiss, J., Kunstár, A., Fajka-Boja, R., Dudics, V., Tóvári, I., Légrádi, A., Monostori, E., and Uher, F. (2007) A novel anti-inflammatory function of human galectin-1. Inhibition of hematopoietic progenitor cell mobilization. Exp. Hematol. 35, 305–313

22. Yasumoto, K., Yamada, T., Kawashima, A., Wang, W., Li, Q., Donev, I. S., Tacheuchi, S., Mouri, H., Yamashita, K., Ohtsubo, K., and Yano, S. (2011) The EGFR ligands amphiregulin and heparin-binding EGF-like growth factor promote peritoneal carcinomatosis in CXCX4-expressing gastric cancer. Clin. Cancer Res. 17, 3619–3630

23. Tanaka, Y., Miyamoto, S., Suzuki, S. O., Oki, E., Yagi, H., Sonoda, K., Yamazaki, A., Mizushima, E., Maehara, Y., Mekada, E., and Nakano, H. (2005) Clinical significance of heparin-binding epidermal growth factor-like growth factor and a disintegrin and metalloprotease 17 expression in human ovarian carcinoma. Clin. Cancer Res. 11, 4783–4792

24. Yin, Y., Grabowska, A. M., Clarke, P. A., Whelband, E., Robinson, K., Argent, R. H., Tobias, A., Kumari, R., Atherton, J. C., and Watson, S. A. (2010) Helicobacter pylori potentiates epithelial-mesenchymal transition in gastric cancer. Links to soluble HB-EGF, gastrin and matrix metalloprotease-7. Gut 59, 1037–1045

25. Yotsutomo, F., Oki, E., Tokunaga, E., Maehara, Y., Kuroki, M., and Miyamoto, S. (2010) HB-EGF orchestrates the complex signals involved in triple-negative and trastuzumab-resistant breast cancer. Int. J. Cancer 127, 2707–2717

26. Zhang, Z., Stiegler, A. L., Boggon, T. J., Kobayashi, S., and Halmos, B. (2010) EGFR-mutated lung cancer. A paradigm of molecular oncology. Oncotarget 1, 497–514

27. Pines, G., Köstler, W. J., Yarden, Y. (2010) Oncogenic mutant forms of EGFR. Lessons in signal transduction and targets for cancer therapy. FEBS Lett. 584, 2699–2706

28. Nakagawa, M., Nabeshima, K., Zhang, P., Chen, W., Flanders, K. C., Gutkind, J. S., Wakefield, L. M., and Kulkarni, A. B. (2009) Progressive tumor formation in mice with conditional deletion of TGF-β signaling in head and neck epithelia is associated with activation of the PISK/Akt pathway. Cancer Res. 69, 5918–5926

29. Kuo, P. J., Sato, Y. J., Huang, S. K., Chou, S. H., Cheng, D. E., Jong, Y. J., Hung, C. H., Yang, C. I., Tsai, Y. M., Hsu, Y. L., and Huang, M. S. (2011) Lung cancer-derived galectin-1 mediates dendritic cell energy through inhibitor of DNA binding 3/IL-10 signaling pathway. J. Immunol. 186, 1521–1530

30. Zhao Y., He, D., Saatian, B., Watkins, T., Spannhake, E. W., Pyne, N. I., and Natarajan, V. (2006) Regulation of lysophosphatidic acid-induced epidermal growth factor receptor transactivation and interleukin-8 secretion in human bronchial epithelial cells by protein kinase Cφ, Lyn kinase, and matrix metalloproteinases. J. Biol. Chem. 281, 19501–19511