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Report

Autocrine Semaphorin 6B Signaling Is Essential for Tumorigenesis in Lung Cancer

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Semaphorin family belongs to secreted or membrane anchored proteins. Recent studies have demonstrated the involvement of semaphorins and their receptors in cancer biology, but it remains unclear how each semaphorin molecule regulates tumorigenesis. Previously we reported that Semaphorin 3A (Sema3A) and its receptor Plexin A1 (PlxnA1) regulate the malignant phenotypes of mouse-derived lewis lung cancer (LLC) cells constitutively expressing GFP (LLC-GFP). Here we show that Semaphorin 6B (Sema6b) serves as one of the oncogenic semaphorin molecules in lung cancer using LLC-GFP. *Sema3a* or *Plxna1* knockdown downregulated *Sema6b*, and their suppressive effect on proliferation was significantly recovered by recombinant SEMA6B (rSEMA6B) treatment. *Sema6b* knockdown suppressed the proliferation and tumorigenicity of LLC-GFP. Interestingly, the self-renewal capacity of LLC-GFP derived cancer stem-like cells (LLC-GFPstem) was completely lost by *Sema6b* knockdown. These results demonstrate that Sema6B would be the novel therapeutic target of lung cancer.

Key words Lung cancer, Bone metastatic tumor, Semaphorin, Cancer induced bone pain, Cancer stem-like cell

INTRODUCTION

Lung cancer is one of the leading causes of cancer death and characterized by the high frequency for bone or brain metastasis.[1)] Genetic mutations including EGFR, PTEN and K-RAS and excess production of several cytokines such as IL-6 and HGF has been demonstrated to be involved in lung cancer malignancy.[2)] However, the molecule responsible for lung cancer development has not been completely defined yet.

Semaphorin family members (vertebrate - class 3 to 7)[3)] are secreted or trans-membrane proteins and discovered as axonal guidance molecules during the developmental process of central nervous system. Recent studies have demonstrated the involvement of semaphorins and its receptor Plexins in various pathological disorders.[4)] In basic cancer research, semaphorins have been shown to regulate tumorigenicity and metastatic capacity of tumor cells and reconstitute tumor microenvironment via angiogenesis.[5)] Semaphorin 3A (Sema3A) is a secreted form of semaphorins and acts in a opposite way because of its promoting or suppressive effect on tumorigenicity.[6-10)] Sema3A binds to its receptor Plexin A1-A4 (PlxnA1-A4). Several groups have shown that Sema3A, 3B or 3F signaling plays the critical regulatory roles in tumor malignancy.[11-15)] Semaphorin 6B (Sema6b) is a membrane-anchored or secreted form of semaphorin family molecule[16)] and its functional inhibition was shown to suppress the tumorigenicity of U87MG glioblastoma cells.[17)] However, the oncogenic role of Sema6B in lung cancer has not been investigated.

Our previous studies revealed that Sema3A and PlxnA1 positively regulate the malignant phenotypes, including tumorigenesis and cancer stem cell-like properties, of mouse LLC cells.[12,18,19)] Here we identified the functional crosstalk between Sema3A and Sema6B signaling and show Sema6B as a novel therapeutic target against lung cancer.

MATERIALS AND METHODS

Cell Culture LLC-GFP cells were purchased from Anti-Cancer Japan. DMEM high glucose (Sigma-Aldrich) / 10% FBS (JRH biosciences) / 50 U/mL penicillin/0.5% streptomycin (Life technologies) was used to maintain cells at 37°C in 5% CO_2 atmosphere. Every 4days, cells were passaged with 0.05% Trypsin/EDTA (Life technologies) onto collagen-coated culture plates (Iwaki). In the case of LLC-GFP-derived cancer stem-like cells (LLC-GFPstem), spheroids were dissociated into single cells with Accutase (Life technologies) and re-seeded onto ultra-low attachment plate every 4 days. Including lentivirus infection and procedures of analysis, detailed infor-

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mation can be found in Doc S1.

Animal Experiments Animal experiments performed in this study were approved by the Committee on Animal Experimentation of Niigata University of Pharmacy and Applied Life Sciences. All procedures were followed by the University's Guidelines for the Care and Use of Laboratory animals. C57BL/6J mice (male, 5 weeks old) were purchased from SLC (Japan) and used as recipients for LLC/scramble or LLC/shSema6b. Detailed information can be found in Doc S1.

Other Experiments Detailed information can be found in Doc S1.

Statistical Analysis Prism 10 was used to analyze data. All data were obtained from two or three biologically independent experiments. Unpaired one-way or two-way analysis of variance (ANOVA) using the Bonferroni method was used to test statistical significance. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

Data Availability The raw data supporting the results of this study will be provided according to the demands.

RESULTS

The Functional Crosstalk between Sema3A/PlxnA1 and Sema6B Signaling Sema6B positively regulates tumorigenicity via PlxnA4,[17] but its functional cross-talk with other semaphorin signaling have not been defined. In this study, we used LLC cells constitutively expressing GFP (LLC-GFP) and the same shRNA clones against *Sema3a* or *Plxna1* that have been validated in our previous works.[12,18,20] Interestingly, *Sema3a* or *Plxna1* knockdown significantly downregulated the expression of *Sema6b* (Fig. 1A and 1B). Importantly, recombinant SEMA6B (rSEMA6B) significantly suppressed the inhibitory effect of shSema3a or shPlxna1 on proliferation (Fig. 1C). These results suggest that Sema6B partially mediate the Sema3A/Plxna1 signaling to promote cell proliferation.

Assessment of Oncogenic Role of Sema6B in Lung Cancer To test the role of Sema6B in lung cancer, shRNAs against *Sema6b* was transduced into LLC-GFP and their effects were assessed. Here we designed two shRNAs and established cell lines, designated as LLC-GFP/shSema6b#1 and LLC-GFP/shSema6b#2. LLC-GFP expressing scrambled shRNA was used as control (LLC-GFP/scramble). As shown in Fig. 2A, each shRNA clone decreased *Sema6b* mRNA level by less than 20%. When cell numbers were compared at each indicated time point, LLC-GFP/shSema6b#1 and LLC-GFP/shSema6b#2 showed lower proliferative capacity than that of LLC-GFP/scramble (Fig. 2B). Consistent with this result, the expression level of cell-cycle regulatory genes, such as *Ccnd1* and *Pcna*, was also decreased by *Sema6b* knockdown (Fig. 2C and 2D). Importantly, the proliferative capacity of LLC-GFP/shSema6b#1 and LLC-GFP/shSema6b#2 became comparable to that of LLC-GFP/scramble after rSEMA6B treatment (Fig. 2E), indicating that the result with shSema6b is not off-target effects. To assess the effect of shSema6b on tumorigenesis, we transplanted each cell line into bone marrow cavity and the number of GFP positive cells was compared after 3 weeks (Fig. 2F). The engraftment of LLC-GFP/scramble was confirmed but GFP positive cells could not be detected in mice inoculated with LLC-GFP/shSema6b#1 and LLC-GFP/shSema6b#2 (Fig. 2G). As shown in Fig. 2H, when Cancer-induced bone pain (CIBP) was evaluated by weight bearing test, the ipsilateral hindpaw of mice inoculated with LLC/scramble showed a significant decline in weight bearing at 2 weeks and 3 weeks after inoculation compared to the contralateral hindpaw. However, the wight bearing of mice with LLC/shSema6b#1 or LLC/shSema6b#2 was comparable between ipsilateral and contralateral hindpaws. These results demonstrate that Sema6B secreted by LLC-GFP itself serves as a malignant factor in lung cancer.

Loss of Cancer Stem-Like Properties after *Sema6b* Knockdown Finally, we investigated the effect of Sema6b knockdown on self-renewal capacity of cancer stem-like cells (CSCs). As described previously,[18] we established LLC-GFP-derived stem-like cells (LLC-GFPstem) by culturing LLC-GFP under floating condition. The knockdown efficiency of *Sema6b* in LLC-GFPstem was comparable to that of LLC-GFP (Fig. 2A and 3A). Here we designated each cell line as LLC-GFPstem/scramble, LLC-GFPstem/shSema6b#1 and LLC-GFPstem/shSema6b#2. As shown in Fig. 3B and 3C, shSema6b significantly downregulated stem cell marker gene, such as *Sox2* and *Cd44* (Fig. 3B and 3C). Sphere forming assay is a gold standard to evaluate the self-renewal of stem cells.[21]

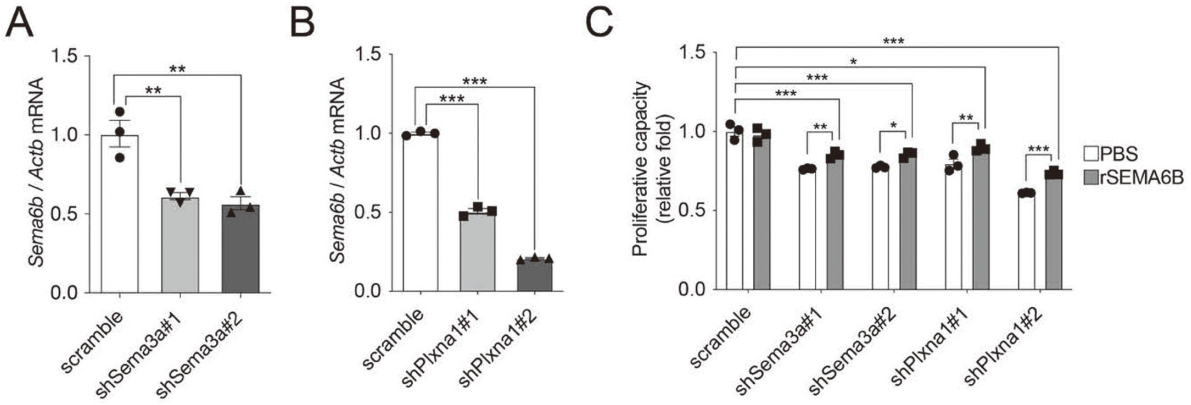

Fig. 1. *Sema3a* or *Plxna1* Knockdown Downregulated *Sema6b*

(A, B) Quantification of *Sema6b* mRNA level after *Sema3a* or *Plxna1* knockdown. Lentivirus-infected cells were selected with 10 μg/mL puromycin and total RNAs were extracted from LLC-GFP/scramble, LLC-GFP/shSema3a#1, LLC-GFP/shSema3a#2, LLC-GFP/shPlxna1#1 and LLC-GFP/shPlxna1#2, and the expression level of *Sema6b* was compared by qRT-PCR. Values were normalized to *Actb* level. (n = 3, three independent experiments) (C) The recovery effect of rSEMA6B on proliferation after *Sema3a* or *Plxna1* knockdown. Cells were cultured in the absence or presence of 100 ng/mL rSEMA6B for 4 days and their proliferative capacities were compared by WST-8 assay. (n = 3, three independent experiments) Data shown represent the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001.

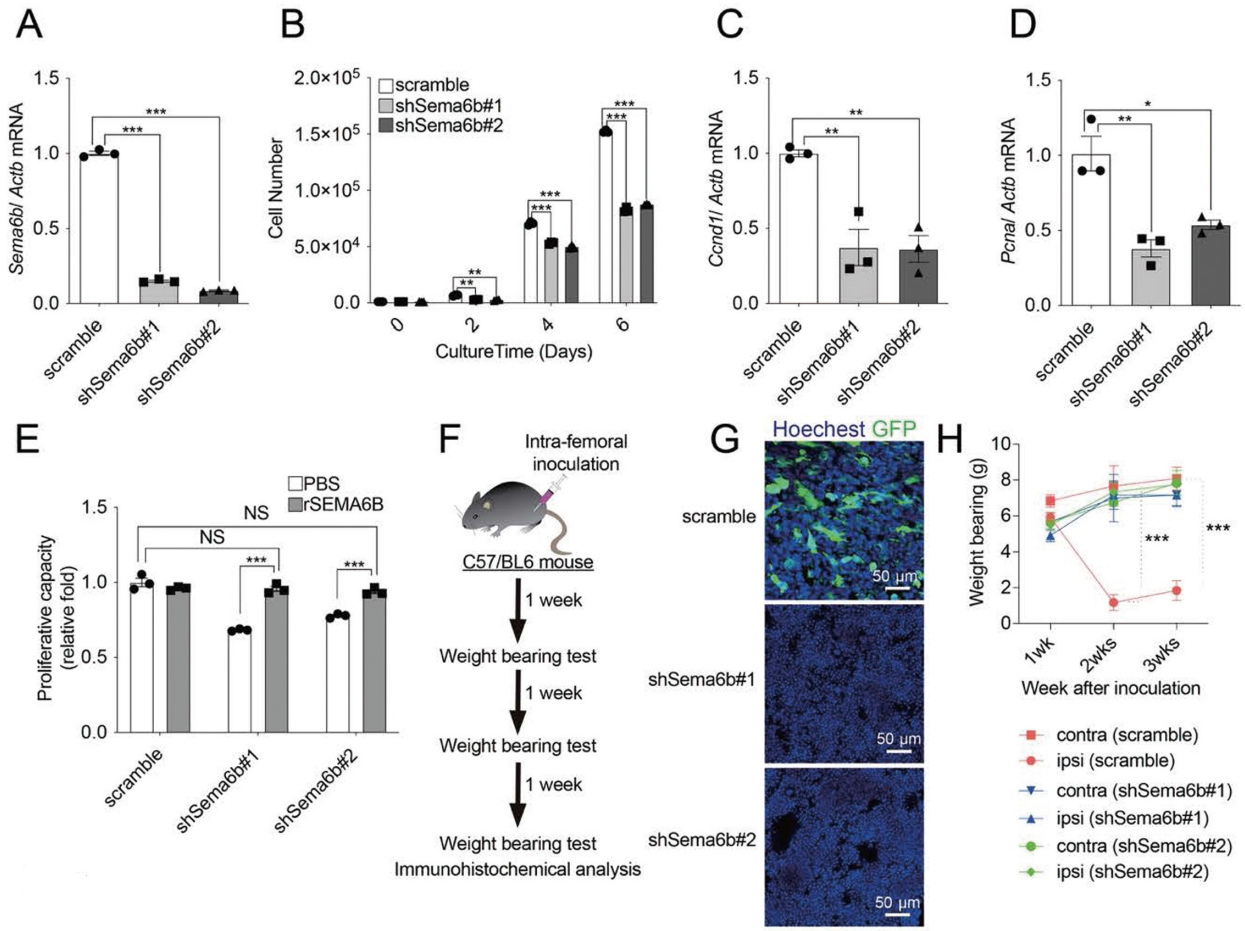

Fig. 2. Sema6b Knockdown Suppressed the Tumorigenicity of LLC-GFP

(A) Quantification of *Sema6b* mRNA level after shSema6b transduction. Lentivirus-infected cells were selected with 10 μg/mL puromycin and total RNAs were extracted from LLC-GFP/scramble, LLC-GFP/shSema6b#1 and LLC-GFP/shSema6b#2 and the expression level of *Sema6b* was compared by qRT-PCR. Values were normalized to *Actb* level. (n = 3, three independent experiments) (B) The proliferative capacity of LLC-GFP after *Sema6b* knockdown. 1x10^3 cells were seeded onto 96-well culture plate and the cell number was counted at each indicated time point. (n = 3, three independent experiments) (C, D) Quantification of *Ccnd1 or Pcna* mRNA level after *Sema6b* knockdown. Total RNAs were extracted from LLC-GFP/scramble, LLC-GFP/shSema6b#1 and LLC-GFP/shSema6b#2 and the expression level of *Ccnd1* and *Pcna* was compared by qRT-PCR. Values were normalized to *Actb* level. (n = 3, three independent experiments) (E) The recovery effect of rSEMA6B on proliferation after *Sema6b* knockdown. Cells were cultured in the absence or presence of 100 ng/mL rSEMA6B for 4 days and their proliferative capacities were compared by WST-8 assay. (n = 3, three independent experiments) (F) Schematic of intra-femoral inoculation of LLC-GFP. (G) Assessment of tumorigenic capacity after *Sema6b* knockdown. Three weeks after intra-femoral inoculation, the number of GFP-positive cells was compared. Nuclei were counterstained with Hoechst33342. (H) Weight bearing capacity of the ipsilateral limb of mice inoculated with LLC-GFP/scramble, LLC-GFP/shSema6b#1 or LLC-GFP/shSema6b#2 (n = 4, two independent experiments). Data shown represent the mean ± SEM. **P*<0.05, ***P*<0.01 and ****P*<0.001.

LLC-GFPstem/scramble formed spheroids, the sphere-forming capacity was completely lost by Sema6b knockdown (Fig. 3D). Although the concentration may not be sufficient, 100 ng/mL recombinant SEMA6B could not recover the sphere forming capacity (data not shown), suggesting that continuous Sema6B signaling is essential for maintaining LLC-GFPstem. These results demonstrate that Sema6B is essential for the self-renewal of LLC-GFPstem cells.

DISCUSSION

CSCs secrete cytokines that support their tumorigenic or self-renewal capacity[18,22-24] and the therapeutic strategies against CSCs, including lung cancer stem cells, have been demonstrated.[25] We previously revealed that autocrine Sema3A signaling contributes to the acquisition of stem-like properties in LLC.[18] In our knowledge, this is the first study demonstrates that autocrine Sema6B signaling is also essential for CSCs to maintain their stem-like properties. Although the detail mechanism how Sema6B regulates the cancer stem-like properties is unclear, the dual inhibition of Sema3A and Sema6B signaling would be more effective strategy to eradicate CSCs in lung cancer.

In conclusion, the inhibition of Sema6B signaling decreases malignant phenotypes of lung cancer cells. We expect that our results will contribute to the detail understanding of semaphorin signaling in basic and clinical cancer research field.

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Author contribution D.Y. and T.M. conceived and designed the experiments. D.Y. and T.H. performed the experiments. D.Y. and T.H. analyzed data. D.Y. and T.M. contributed reagents / materials / analysis tools. D.Y., T.H., K.K. and T.M. wrote the paper.

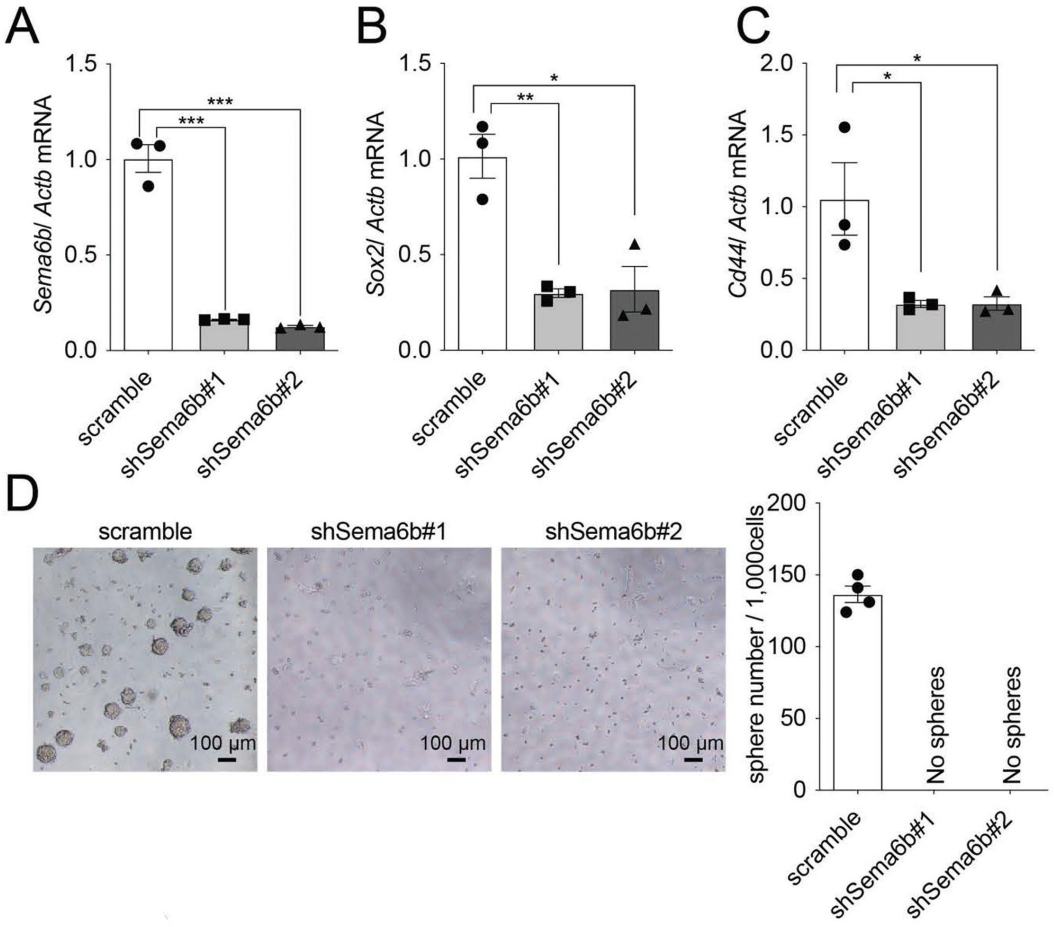

Fig. 3. Sema6B Is Essential for Cancer Stem-Like Properties of LLC-GFPstem.

(A-C) Quantification of *Sema6b, Sox2* and *Cd44* mRNA level after shSema6b transduction. Lentivirus-infected cells were selected with 10 μg/mL puromycin and total RNAs were extracted from LLC-GFPstem/scramble, LLC-GFPstem/shSema6b#1 and LLC-GFPstem/shSema6b#2 and the expression level of *Sema6b, Sox2* and *Cd44* was compared by qRT-PCR. Values were normalized to *Actb* level. (n = 3, three independent experiments) (D) Assessment of self-renewal capacity by sphere forming assay. LLC-GFPstem/scramble, LLC-GFPstem/shSema6b#1 and LLC-GFPstem/shSema6b#2 were dissociated into single cells and seeded at low density onto ultra-low attachment 96-well culture plate. After culturing for 1 week, the spheres with more than 100 μm in diameter were counted. (n = 4, two independent experiments) Data shown represent the mean ± SEM. **P*<0.05, ***P*<0.01 and ****P*<0.001.

Conflict of interest The authors declare no conflict of interest.

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