BPB Reports

Report

Plexin A1 Is Essential for Tumorigenic Capacity of Mouse Lewis Lung Carcinoma Cell-Derived Cancer Stem Cells

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Received August 27, 2025; Accepted December 8, 2025

Plexin (Plxn) family molecules are the receptor for semaphorins and their involvement in tumor malignancy is also demonstrated. However, the regulatory roles of Plxns in cancer-stemness have not been completely understood. Previously we found that Sema3a and its receptor PlxnA1 confer the high proliferative capacity and the resistance against epidermal growth factor receptor (EGFR) inhibitors to mouse-derived lewis lung cancer (LLC) cells constitutively expressing green fluorescent protein (GFP) (LLC-GFP). Here we show that PlxnA1 is essential for maintaining cancer stem-like property of LLC-GFP-derived cancer stem cells (LLC-GFPstem). *Plxna1* knockdown downregulated stem cell markers, *Sox2* and *Cd44,* in LLC-GFPstem cells. Importantly, the sphere-forming and tumorigenic capacity of LLC-GFPstem cells was lost by *Plxna1* knockdown. These results demonstrate that PlxnA1 is essential for LLC-GFPstem cells to maintain their cancer stem-like properties.

Key words lung cancer, bone metastatic tumor, plexin, cancer stem-like cell

INTRODUCTION

Lung cancer is the secondary frequent occurring cancer and the leading cause of cancer-related death worldwide.[1)] During tumor progression or advanced stages, patients with bone metastasis increase and they show poor prognosis compared with those without bone metastasis.[2,3)] Although several therapeutic strategies, including bone-targeted agents management, are tested,[4)] the complete eradication of bone metastatic lung cancer has not been achieved.

Cancer stem-like cells (CSCs) are characterized by their stem cell-like properties including self-renewal, tumorigenic capacity, and drug-resistance.[5-8)] Tumor microenvironment and paracrine or autogenous signaling have supportive roles in maintaining CSCs[9-11)] and the therapeutic targeting of those mechanisms have been tested to improve the quality of life of cancer patients.[8)] Although several signaling pathways related to lung cancer stem cell markers are also demonstrated,[12)] the regulatory mechanism for maintaining the stemness of lung cancer has not been completely understood.

Plexin (Plxn) molecules are single-pass transmembrane receptors for semaphorin family that regulate axon guidance.[13)] Several semaphorins are aberrantly expressed in cancers and regulate malignant phenotypes by controlling the downstream signaling pathways, including mammalian target of rapamycin complex 1 (mTORC1), ERK or AKT signaling.[14,15)] Plxns are subgrouped into four types, including type-A, type-B, type-C and type-D, and their involvement for tumor malignancy have also been reported.[16)] However, Plxn receptors that are essential for maintaining cancer stem-like property have not been completely defined.

One of the secreted type of semaphorin molecules, Semaphorin 3A (Sema3A), binds to plexin A1-A4 (PlxnA1-PlxnA4) and co-receptor neuropilin1 (NRP1) to show its regualtory role in axon guidance, bone metabolism and tumor malignancy.[17-20)] Previously we reported that Sema3a and its receptor PlxnA1 positively regulates the proliferation and resistance against epidermal growth factor receptor (EGFR) inhibitors of mouse Lewis lung carcinoma (LLC) cells constitutively expressing green fluorescent protein (GFP) (LLC-GFP).[21,22)] In addition, we revealed that Sema3A is essential for maintaining cancer-stem like property of LLC-GFP derived cancer stem cells (LLC-GFPstem),[10)] but the regulatory effect of PlxnA1 on LLCstem-GFP has not been investigated. Here we show that PlxnA1 has critical roles for maintaining the self-renewal and tumorigenic capacity of LLC-GFPstem.

MATERIALS AND METHODS

Cell Culture LLC-GFP cells (AntiCancer Japan, Chiba, Japan) were maintained in DMEM high glucose (Sigma-Aldrich, St. Louis, Missouri, USA) / 10% FBS (JRH bio-

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sciences, St. Lenexa, Kansas, USA) / 50 U/mL penicillin/0.5% streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C in 5% CO_2 atmosphere. Cells were stripped with 0.05% Trypsin/EDTA (Thermo Fisher Scientific, Waltham, Massachusetts, USA) every 4 days and passaged onto collagen-coated culture plates (Iwaki, Shizuoka, Japan).

How to Establish LLC-GFPstem Cells LLC-GFP cells were striped with 0.05% Trypsin/EDTA and suspended in DMEM/F12 (Sigma-Aldrich, St. Louis, Missouri, USA) / B27/RA(-) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) / 10 μg/mL EGF (Sigma-Aldrich, St. Louis, Missouri, USA) / 10 μg/mL FGF2 (Peprotech, Rocky Hill, New Jersey, USA) / 50 U/mL penicillin and 0.5% streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cell suspension was seeded onto 6 well ultra-low attachment plate (Corning, Corning, New York, USA) at 37°C in a 5% CO_2 atmosphere. To maintain LLC-GFPstem, spheroids were dissociated into single cells with Accutase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and passaged every 4 days.

How to Infect Lentivirus pLKO.1/TRC1-based MISSION shRNA lentivirus particles (Sigma-Aldrich, St. Louis, Missouri, USA) were used and the following clones previously used[22] were chosen; TRCN0000079188 and TRCN0000079189. Scramble shRNA was used as control. After infecting LLC-GFPstem cells with shRNA-encoding lentivirus for 24 hr, LLC-GFPstem cells were treated with 10 μg/mL puromycin (FUJIFILM Wako Pure Chemical, Osaka, Japan) to select puromycin-resistant clones. Cells stably expressing shRNA were maintained with 10 μg/mL puromycin.

RT-qPCR Total RNA samples were extracted using TRIZOL (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total RNA (1 μg) was used to prepare cDNA samples with PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan). GoTaq®qPCR Master Mix (Promega, Madison, Wisconsin, USA) and MiniOpticon real-time PCR system (BioRad, Barkeley, California, USA) were used for performing RT-qPCR reaction. PCR cycle used in this study was as follows: denaturation 95°C for 30 sec, annealing at 58˚C for 30 sec, and elongation 72°C for 30 sec. 2ΔΔCt method was used to compare the expression level of each gene. *Actb* Ct values were used for normalizing the data. The following primers were used; *PlexA1* sense;5`-GGGTGTGTGGATAGCCATCA-3`, *PlexA1* antisense;5`- GCCAACATATACCTCTCCTGTCT-3`, *Sox2* sense: 5′- GCGGAGTGGAAACTTTTGTCC-3′, *Sox2* antisense: 5′-CGGGAAGCGTGTACTTATCCTT-3′, *Cd44* sense: 5′-TCGATTTGAATGTAACCTGCCG-3′, *Cd44* antisense: 5′-CAGTCCGGGAGATACTGTAGC-3′, *Actb* sense: 5′-GGCTGTATTCCCCTCCATCG-3′, *Actb* antisense: 5′-CCAGTTGGTAACAATGCCATGT-3′

Sphere Forming Assay LLC-GFPstem cells dissociated with Accutase were suspended in DMEM/F12 (Sigma-Aldrich, St. Louis, Missouri, USA) containing B27/RA (-) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 10 μg/mL EGF (Sigma-Aldrich, St. Louis, Missouri, USA), 10 μg/mL FGF2 (Peprotech, Rocky Hill, New Jersey, USA), 50 U/mL penicillin / 5% streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1% methylcellulose (FUJIFILM Wako Pure Chemical, Japan) (10^3 cells / 200 μL). 200 μL of cell suspension was seeded onto 96 well Ultra-low attachment plates (Corning, Corning, New York, USA) and cultured for 1 week. Spheroids with diameter of greater than 100 μm were counted.

Intrafemoral Inoculation LLC-GFPstem cells were dissociated with Accutase and suspended in 5% FBS/ 1x PBS at a concentration of 2 x 10^3 cells/μL. The femoral cartilage of mice under inhalation anesthesia with 3% isoflurane was penetrated using 26-gauge needles (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to make small holes. 5 μL cell suspension was intrafemorally inoculated and mice were sacrificed at 3 weeks after transplantation.

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 (Shizuoka, Japan) and used as recipients for LLC/scramble or LLC/shPlxnA1.

GFP Detection Three weeks after transplantation, mice were deeply anesthetized with 3% isoflurane and perfused with 4% paraformaldehyde (PFA, Nacalai Tesque, Kyoto, Japan). Femurs were soaked in 4% PFA overnight and OSTEOSOFT solution (Sigma-Aldrich, St. Louis, Missouri, USA) was used to decalcify samples. After dehydration with 30% sucrose (FUJIFILM Wako Pure Chemical, Osaka, Japan), samples were embedded in OCT compound (Sakura Finetek, Tokyo, Japan). Samples were sectioned into 20 μm slices and nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, Missouri, USA). All photos were taken with LSM700 (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis Data were analyzed using Prism 10 (GraphPad software, Boston, Massachusetts, USA). Two or three biologically independent experiments were performed to obtain all data in this study. Statistical significance was evaluated with unpaired one-way analysis of variance (ANOVA) using the Dunnett`s method. 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 can be provided by the corresponding author when required.

RESULTS

LLC-GFPstem Cells Lose Self-Renewal Capacity After *Plxna1* Knockdown Our previous work found that autocrine Sema3a signaling is essential for LLC-GFPstem cells[10] but the receptor responsible for this effect was not identified. As *Plxna1* knockdown in LLC-GFP cells showed the same effect as those of *Sema3a* knockdown,[22] we focused on the effect of PlxnA1 on LLC-GFPstem cells. To investigate the regulatory role of PlxnA1 on the self-renewal capacity of LLC-GFPstem cells, cells were infected with lentivirus encoding scramble or shPlxna1 to knockdown *Plxna1*. Each cell line was designated as LLC-GFPstem/scramble, LLC-GFPstem/shPlxna1#1 and LLC-GFPstem/shPlxna1#2. As shown in Fig. 1A, *Plxna1* mRNA level was decreased by less than half. Importantly, shPlxna1 significantly downregulated stem cell marker genes, such as *Sox2* and *Cd44* (Fig. 1B and 1C). When the sphere-forming assay, a gold standard to evaluate the self-renewal of stem cells[23], was performed, LLC-GFPstem/scramble formed spheroids but LLC-GFPstem/shPlxna1#1 and LLC-GFPstem/shPlxna1#2 completely lost the sphere-forming capacity

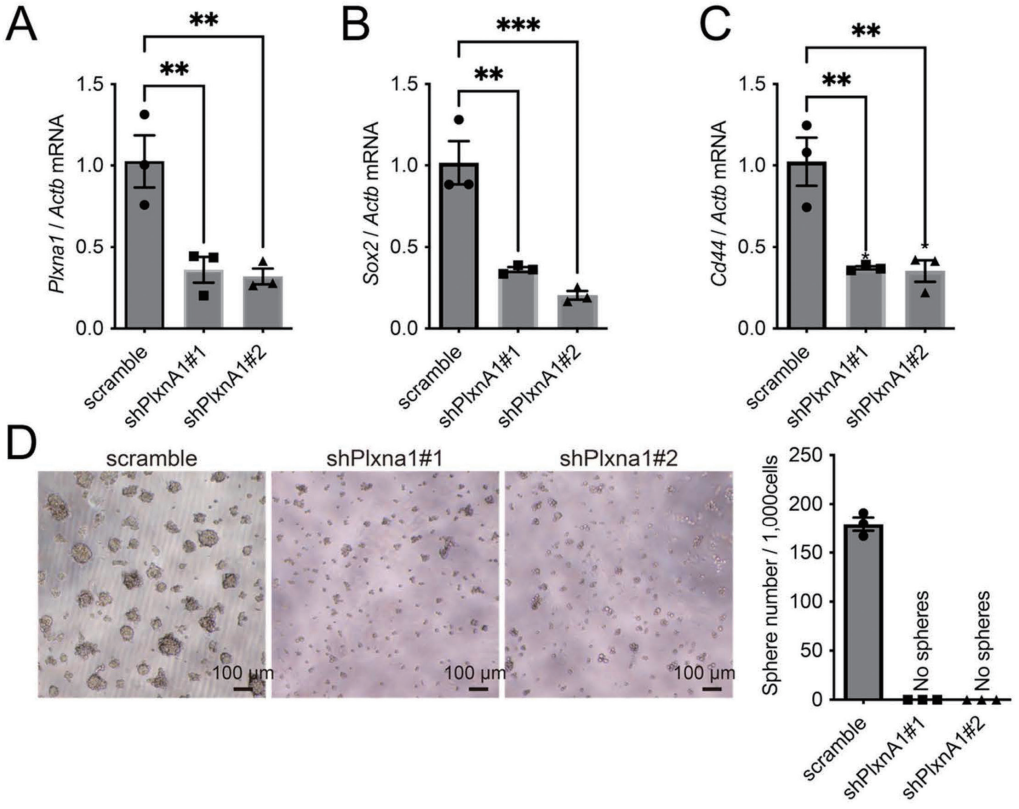

Fig. 1. Plxna1 Is Essential for Cancer Stem-Like Properties of LLC-GFPstem Cells

(A-C) Expression level of *Plxna1, Sox2* and *Cd44* mRNA after *Plxna1* knockdown. LLC-GFPstem cells infected with lentivirus were selected with 10 μg/mL puromycin and total RNAs were extracted. The expression level of *Plxna1, Sox2* and *Cd44* was compared by qRT-PCR. Each value was normalized by *Actb* level. (n = 3, three independent experiments) (D) Sphere forming assay. LLC-GFPstem/scramble, LLC-GFPstem/shPlxna1#1 and LLC-GFPstem/shPlxna1#2 were dissociated and 1,000 cells were seeded onto ultra-low attachment 96-well culture plate. After culturing for 1 week, the number of spheroids that have more than 100 μm in diameter was counted. (n = 3, three independent experiments) Data shown represent the mean ± SEM. **P*<0.05 and ***P*<0.01.

(Fig. 1D). These results indicate that Plxna1 is essential for the self-renewal of LLC-GFPstem cells.

LLC-GFPstem Cells Lose Tumorigenic Capacity After *Plxna1* Knockdown Tumorigenic capacity is one of the characteristic features of CSCs.[8] To assess the effect of *Plxna1* knockdown on tumorigenesis of LLC-GFPstem cells after bone metastasis, each cell line was transplanted into femoral bone marrow cavity and GFP positive cells were counted at 3 weeks after experiment. As shown in Fig. 2, the engraftment and proliferation of LLC-GFPstem/scramble cells was observed, but LLC-GFPstem/shPlxna1#1 and LLC-GFPstem/shPlxna1#2 engrafted but could not proliferate in bone marrow cavity. This result demonstrates that PlxnA1 positively regulates the tumorigenicity of LLC-GFPstem cells in bone marrow cavity.

DISCUSSION

In this study, we found the critical roles of Plxna1 in maintaining cancer stem-like properties of lung cancer stem cells. Several autocrine signaling pathways positively support the self-renewal or tumorigenic capacity of CSCs[9,10,24-26] and the therapeutic strategies against lung cancer stem cells is also demonstrated.[12] Our previous work revealed that autocrine Sema3a signaling maintains the stem-like properties of LLC-GFPstem cells.[10] Sema3a binds to Plxn type A (1-4) but the Plxn receptor which is responsible for maintaining LLC-GFPstem cells has not been defined. The same as the results of *Sema3a* knockdown,[10] *Plxna1* knockdown abolished the sphere-forming or tumorigenic capacity of LLC-GFPstem cells (Fig. 1D and Fig. 2). Sema3a activates mammalian target of rapamycin complex 1 (mTORC1) to promote the proliferation and tumorigenesis of LLC-GFP cells,[10,15] and the regulatory role of mTORC1 in cancer stemness has been demonstrated.[11,27] Although further studies are needed to investigate the mTORC1 activity after *Plxna1* knockdown, our data indicate that Plxna1 is one of the essential receptors for Sema3a to exert its regulatory effects on LLC-GFPstem cells. Importantly, our previous works showed that Sema3a or PlxnA1 knockdown do not affect apoptosis.[15,22] Although LLC-GFP cells were used in these studies, the inhibition of Sema3a/PlxnA1 signaling may induce the dormancy of LLC-GFPstem cells because the engraftment of LLC-GFPstem/shPlxna1#1 or LLC-GFPstem/shPlxna1#2 was detected (Fig. 2). As the engraftment of LLC-GFPstem cells in bone marrow cavity was completely lost by *Sema3a* knockdown,[10] other receptors that are responsible for inhibiting cell death after intrafemoral transplantation should be identified. Further studies should be performed to investigate the role of Sema3a/PlxnA1 pathway in regulating the dormancy of LLC-GFPstem. In summary our data demonstrate that Sema3a/PlxnA1 signaling would be the potential therapeutic target of lung cancer-derived bone meta-

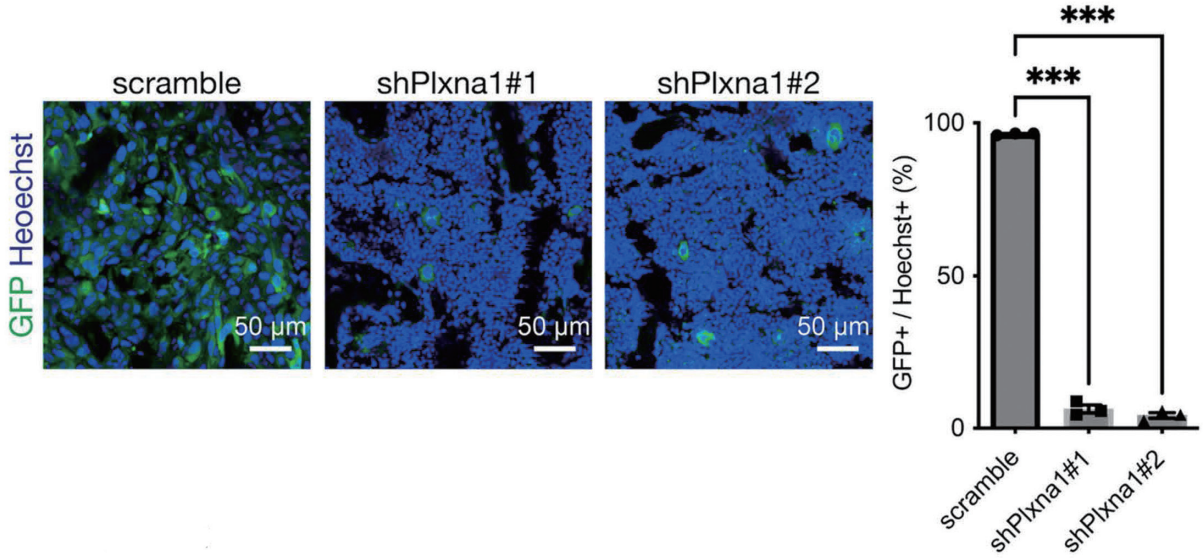

Fig. 2. Plxna1 Knockdown Suppressed the Tumorigenicity of LLC-GFPstem Cells

Tumorigenic capacity of LLC-GFPstem cells after *Plxna1* knockdown. Three weeks after intra-femoral inoculation of each cell line, the number of GFP-positive cells in bone marrow cavity was compared. Nuclei were counterstained with Hoechst33342. (n = 3, three independent experiments) Data shown represent the mean ± SEM. ***P<0.001.

static tumors.

Acknowledgments This study was supported by JSPS KAKENHI Grant Number 15K08682 (T.M.), 15K18449 (D.Y.) and 19K16964 (T.H.).

Conflict of interest The authors declare no conflict of interest.

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