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Research article

Cancer pain relief achieved by disrupting tumor-driven semaphorin 3A signaling in mice

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HIGHLIGHTS

- Femoral inoculation of LLC cells caused weight-bearing asymmetry of ipsilateral paw.
- Femoral inoculation of LLC cells upregulated *Sema3A* mRNA expression.
- *Sema3A*-knocked down LLC cell abrogated bone pain and femoral LLC cell proliferation.

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ABSTRACT

Cancer-induced bone pain (CIBP) is the most common pain arising from cancer and is inadequately managed with current standard therapeutics. While the etiology of CIBP remains to be fully elucidated, increasing evidence suggests that CIBP is uniquely complex. We tested whether semaphorin 3A (Sema3A) signals were involved in the development of CIBP in mice. The mouse model employed for CIBP – mice inoculated with Lewis lung carcinoma (LLC) cells injected into the femur intramedullary space – showed progressive decline in the weight bearing of the ipsilateral hind limb. The LLC cell inoculation resulted in a progressive increase in *Sema3A* mRNA expression over time and an increase in the number of Sema3A-immunoreactive cells in the ipsilateral femur. To define the role of Sema3A in development of CIBP, we employed a lentiviral expression system to establish a stable LLC cell line expressing scrambled shRNA for the control group (LLC/scramble) and shRNAs directed against *Sema3A* mRNA for the loss-of-function group (LLC/shSema3A). Inoculation of LLC/shSema3A did not cause upregulation of *Sema3A* mRNA expression and proliferation of the inoculated cells in the femur compared to that in mice inoculated with LLC/scramble. Mice inoculated with LLC/shSema3A, but not LLC/scramble, showed an attenuation of the significant decline in the weight bearing of the ipsilateral hind paw. Our findings indicate that Sema3A serves as a potential therapeutic target for CIBP.

1. Introduction

Most common cancer types, including breast cancer, prostate cancer and lung cancer, have a propensity to metastasize to the bone. Metastasis to the bone subsequently causes cancer-induced bone pain (CIBP) that is characterized as ongoing or breakthrough pain [1,2]. Pain can be present at any time during the course of the disease, but it generally increases with disease progression; thus, 75%–90% of patients with metastatic or advanced stage cancer will experience significant cancer-induced pain [3,4]. Although CIBP can severely compromise a patient's quality of life, strategies for curative treatment of metastatic bone tumors have not been established.

Of all semaphorin protein families, the class 3 semaphorin group is the only secreted type in vertebrates [5]. Semaphorin 3A (Sema3A) was originally discovered as a guidance factor for axons during the development of the central nervous system and several studies have reported that Sema3A inhibits tumor growth and metastasis [6–8]. In one study, decreased expression of *Sema3A* was shown to correlate with poor prognosis in non-small cell lung cancer patients [9]. In contrast, it was recently reported that Sema3A promotes tumor growth and angiogenesis via tumor-associated macrophage recruitment [10]. In addition, Sema3A promotes glioblastoma (GBM) dispersal and is highly expressed in

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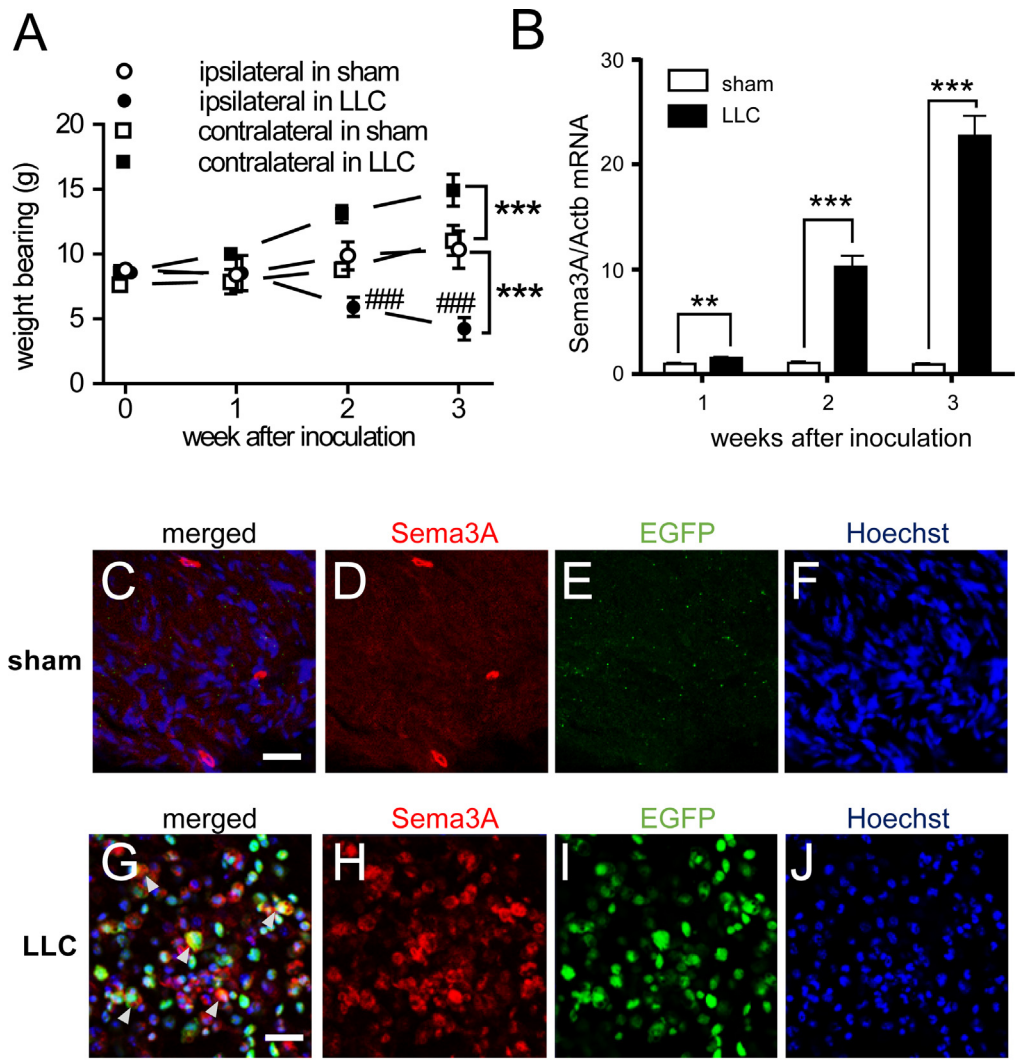

Fig. 1. Cancer-induced bone pain model showed upregulation of *Sema3a* mRNA in the tumor-bearing femur. LLC cells or vehicle were injected to the intramedullary space of the right femur of mice. (A) The weight bearing on the ipsilateral and contralateral hind limb to the inoculation was measured (n = 8). *** indicates $p < 0.001$ between ipsilateral in sham and ipsilateral in LLC, as determined by two-way repeated measure ANOVA. ### indicates $p < 0.001$ between ipsilateral in sham and ipsilateral in LLC, as determined by Bonferroni's multiple comparisons test. (B) *Sema3A* mRNA expression in the femora was evaluated by real-time quantitative PCR (n = 3). (C–J) The femora were harvested after three weeks of LLC cell inoculation, sectioned, subjected to immunohistochemistry staining, and imaged for Sema3A (red) (C); the nuclei were stained with Hoechst 33258 (blue). The fluorescence images were obtained with confocal microscopy. A representative image is shown, the arrowhead indicates the co-localization of Sema3A staining and LLC cells expressing GFP. Scale bar 50 μm. **, $p < 0.01$; ***, $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GBM tumors compared to normal brain tissue [11]. These findings indicate that Sema3A has complicated pathophysiological roles in cancer biology. More recently, our *in vitro* study demonstrated for the first time that autocrine Sema3A promoted proliferation of Lewis lung carcinoma (LLC) cells, a cell line derived from mouse lung cancer [12], through drive of the mTORC1 signaling pathway. However, the influence of Sema3A on tumor development in *in vivo* remains unclear.

There is a sole report on involvement of Sema3A in pain-related behavior of experimental animals; intrathecally administered Sema3A attenuated neuropathic pain behavior in rats with chronic constriction injury of the sciatic nerve [13]. However, the mechanism underlying the inhibitory effects of Sema3A on neuropathic pain in general and its specific involvement in CIBP is yet to be elucidated. In the present study, we used a CIBP mouse model to investigate temporal expression profile of *Sema3A* mRNA in the LLC-bearing femur and the functional role of autocrine Sema3A in ongoing pain.

2. Materials and methods

2.1. Lewis lung cancer cell lines

A stable, high GFP-expressing Lewis lung carcinoma (LLC; Anti-Cancer Japan, Osaka, Japan) cell line was used for inoculating mice, as previously reported [14]. After four days in culture, the cells were trypsinized and by re-seeded in culture plates or harvested for inoculation (Fig. 1).

2.2. Establishment of LLC cells with stable Sema3a silencing

Stable *Sema3A* silencing in LLC cells was achieved by lentiviral delivery of shRNA constructs, as previously reported [12]. Briefly, pLKO.1/TRC1-based MISSION short hairpin RNA (shRNA) Lentiviral Transduction Particles were purchased from Sigma-Aldrich. The following clones were used: TRCN0000067328 (#1) and TRCN0000067329 (#2). Lentivirus containing scramble shRNA