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Original Article

Sema4D/Plexin-B1 promotes the progression of osteosarcoma cells by activating Pyk2-PI3K-AKT pathway

Changhui Li[1,2], Lei Wan[1], Peng Wang[2], Xiliang Guan[1], Congda Li[1], Xishan Wang[1]

[1]Department of Orthopedics, People's Hospital of Rizhao, Rizhao, China;
[2]Department of Rehabilitation Medicine, People's Hospital of Rizhao, Rizhao, China

Abstract

Objectives: Osteosarcoma (OS) is one of the two most common malignant bone tumors among children and teens but it is still a rare disorder. Semaphorin 4D (Sema4D) has been reported to play a specific role in human cancers. The aim of this study was to explore the function of Sema4D in the tumorigenesis and development of OS. **Methods**: 10 pairs of OS tissues and paracancerous normal tissues from human OS samples and OS cell lines were used. Western blot assay was performed to detect the protein expression of Sema4D, Plexin-B1, and associated proteins of Pyk2-PI3K/AKT pathway. To explore the effect of Sema4D in the progression of OS, we reduced the expression of Sema4D. The effect of Sema4D knockdown on cell proliferation was explored by CCK-8 assay and clone formation assay. The effect of Sema4D knockdown on cell migration and invasion was assessed by Transwell assay. **Results**: Sema4D was overexpressed in OS tissues and cell lines. Sema4D knockdown notably suppressed cell proliferation in OS cells. Cell migration and invasion were reduced by Sema4D knockdown. Sema4D/Plexin-B1 facilitated OS, progression by promoting Pyk2-PI3K/AKT pathway. **Conclusion**: Sema4D/Plexin-B1 promoted the development of OS so Sema4D might be a potential target of treatment for patients with OS.

Keywords: Osteosarcoma, Plexin-B1, Pyk2-PI3K-AKT Pathway, Sema4D

Introduction

Osteosarcoma (OS) is a malignant tumor originating in mesenchymal tissue. OS is more common in children and adolescents, and is more likely to occur in the long tubular epiphyseal region with rich blood circulation[1]. Clinically, OS presents only as local pain and swelling, with occasional joint dysfunction[2]. The etiology of OS is complex and is still not fully elucidated. The pathogenesis of OS is closely related to genetics and is highly heterogeneous. The risk of OS is also related to a variety of diseases. Therefore, it is imperative to explore new treatments. In addition to surgery, chemotherapy and radiotherapy, the comprehensive treatment of OS has also included molecular targeted therapy, immunotherapy, gene therapy, embolization therapy, radiofrequency ablation therapy and stem cell therapy[3]. With the improvement of surgical methods and the use of chemotherapy, the 5-year survival rate of OS patients has improved significantly.

Semaphorins, also known as axon-directed molecules, are a large family of secreted or membrane-bound signaling proteins. Up to now, more than 20 types of semaphorins have been found, which are expressed in various tissues such as nervous system, immune system and cardiovascular system[4]. Semaphorins are related to immune response, cell migration, neuronal development and synaptic transmission, tumor angiogenesis and thrombosis[5,6]. Semaphorin 4D (Sema4D) is an important member of the Semaphorin IV subfamily, also known as CD100, and is a cross-model homodimer glycoprotein. Sema4D was first discovered to bind to T lymphocytes of the immune system[7]. Moreover, Sema4D was found to be another important proangiogenic factor after VEGF. Sema4D has been reported to induce vascular endothelial cell migration and neovascularization. Sema4D plays an important role in inhibiting osteocyte function and blocking the signal transduction pathway of bone formation. Sema4D mediates the occurrence of biological responses by binding

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Corresponding author: Xishan Wang, Department of Orthopedics, People's Hospital of Rizhao, No. 126 Taian Road, Rizhao, China
E-mail: wupu34132@163.com

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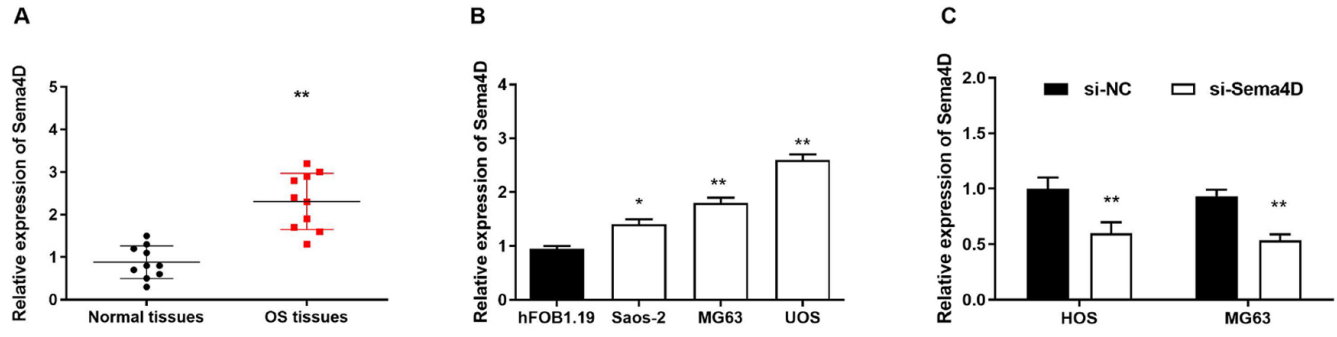

Figure 1. Sema4D was in a high expression in OS cells. (A) Sema4D was upregulated in 10 OS tissues; (B) Sema4D was overexpressed in HOS, MG63, U2OS, SaOS2 cells compared with hFOB1.19 cells; (C) The expression of Sema4D was reduced in HOS cells and MG63 cells. **p<0.01

to their receptors, neuroplexin B1 (Plexin-B1) and leukocyte differentiation antigen cluster 72 (CD72).

In recent years, the role of Sema4D in human cancers has been identified. Sema4D was found to promote cell migration and proliferation, but inhibit cell apoptosis in esophageal squamous cell carcinoma[8]. Sema4D promoted angiogenesis by collaborating with VEGF in epithelial ovarian cancer[9]. Furthermore, Sema4D was discovered to suppress osteogenesis in lung cancer patients with bone metastasis[10]. In this study, we investigated the expression and function of Sema4D in the occurrence and development of OS. The effect of Sema4D on the Pyk2-PI3K/AKT signaling pathway in the progression of OS was also studied. We found that Sema4D affected the development of OS, suggesting that Sema4D might be a diagnostic target of OS.

Materials and Methods

Clinical samples

A total of 10 pairs of OS tissue and paracancerous normal tissue specimens from patients with OS were collected from People's Hospital of Rizhao. The surgically resected tissues were stored in liquid nitrogen tanks, and then transferred to -80°C refrigerators after surgery. Written informed consent from each patient was obtained prior to the enrolment in the current study, which was approved by the Ethics Committee of the People's Hospital of Rizhao. The study design complies with the Declaration of Helsinki ethical standards.

Cell culture

OS cell lines (MG63, HOS, Saos2) and human normal osteoblasts cells hFOB1.19 were purchased from Shanghai Slack Laboratory Animal Co., Ltd (Shanghai, China). The procedure of cell resuscitation was carried out in the ultra-clean workbench. After thawing, the frozen cells were added to the cell medium and centrifuged in a low-speed centrifuge. The cells collected after centrifugation were cultured in an incubator containing 5% CO_2 at 37°C. MG63, HOS and hFOB1.19 cells were cultured in DMEM medium containing with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin Saos2 cells were cultured in McCoy's 5A medium with 15% FBS at 37°C and 5% CO_2. Cell culture and passage also followed the aseptic procedures strictly. The resuscitated cells were thoroughly washed with phosphate buffer and digested with trypsin solution. The scattered cells were collected into new culture flasks for further culture.

Cell transfection

For Sema4D knockdown, Sema4D si-RNA and control siRNA (si-NC) were transfected into human osteosarcoma (HOS) cells and MG63 cells. For Sema4D overexpression, Sema4D pcDNA3.1 vector and pcDNA3.1 vector were transfected into Saos-2 cells. The transfections were conducted by Lipofectamine 2000 according to the instructions.

RT-qPCR assay

RNA was extracted by TRIZOL method. TRIZOL and 0.2 mL chloroform were added to the cell suspension. The supernatant was extracted after high-speed centrifugation, then added with isopropyl alcohol. After centrifugation at high-speed again, the precipitate was collected. RNA was dissolved in DEPC treated pure water. Using 1 μg total RNA as a reverse transcription template, the first strand of cDNA was synthesized. Agilent Stratagene MX3000P was used for RT-qPCR experiment. Primer sequences were as followed: β–actin: Forward 5'-GTGCGGCGATTTCATCTTCC-3', Reverse 5'-CTGCGGCTGAGTTAACAGGA-3'; Sema4D Forward 5'-AGCTCTGCACAAAGCCATCAGC-3', Reverse 5' CCAGCATAGACAAACCTGTTGCC-3'.

Western blot assay

The target cells were lysed by RIPA and the protein concentration was determined by BCA method. After boiling

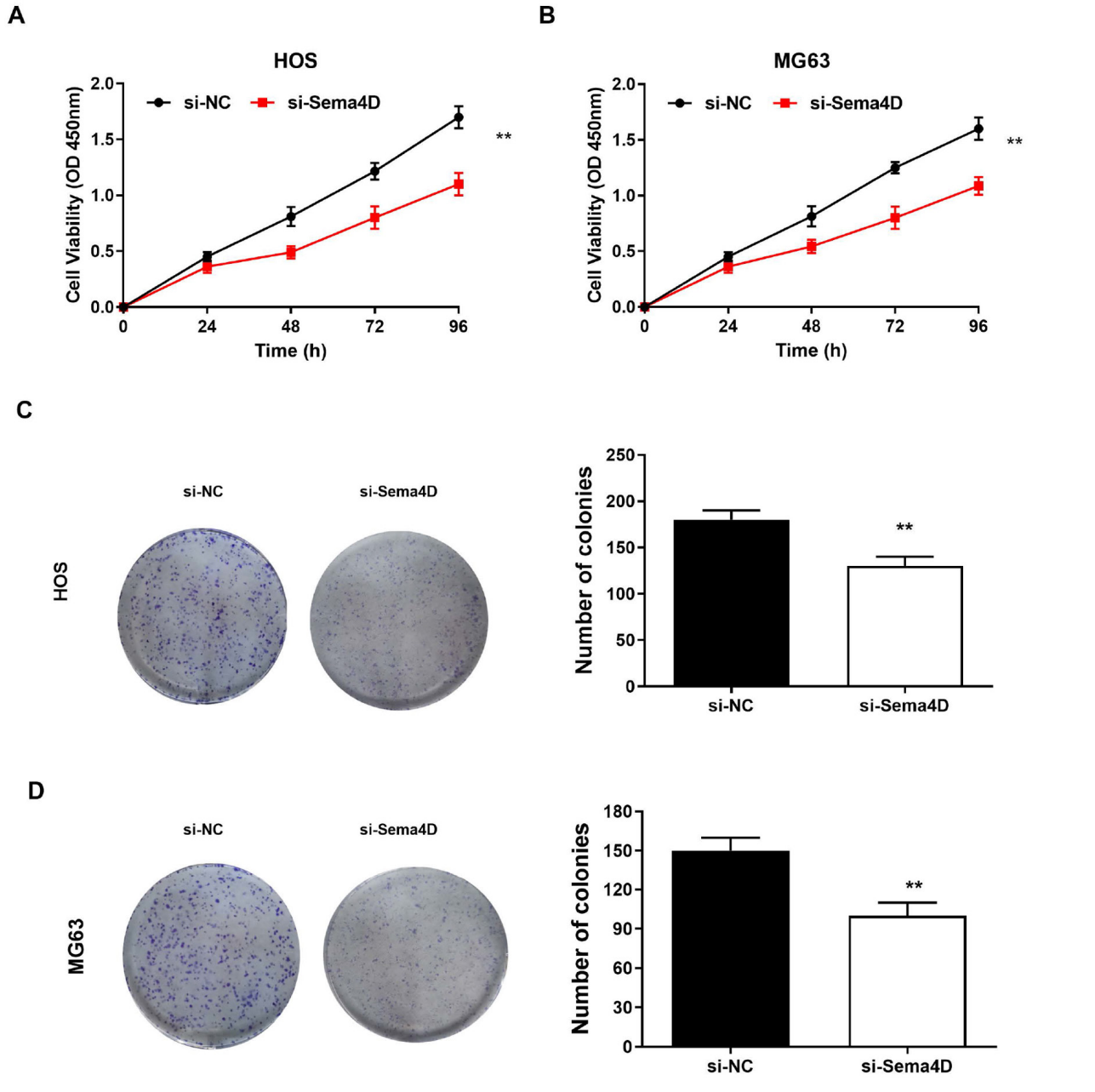

Figure 2. Depletion of Sema4D suppresses cell proliferation in OS cells. CCK-8 assay was performed to detect the effect of Sema4D silencing on cell proliferation in HOS and MG63 cells: (A) Sema4D knockdown suppressed cell proliferation in HOS cells; (B) Similarly, Sema4D knockdown suppressed cell proliferation in MG63 cells. Clone formation assay was used to detect the effect of Sema4D knockdown on cell clones in HOS and MG63 cells: (C) Sema4D knockdown reduced the number of HOS cell clones; (D) Moreover, Sema4D knockdown reduced the number of MG63 cell clones. **p<O.O1.

and denaturation of proteins, 40 μg proteins were added to the sample well of SDS·PAGE glue. After polyacrylamide gel electrophoresis, the gel was peeled off the glass plate and transferred to methanol activated PVDF membrane. PVDF membrane was sealed with 5% BSA at room temperature for 2 h, and incubated with the primary antibodies at 4°C overnight. After washing with TBST for 3 times, the membrane was incubated with the secondary antibodies for 2 h at room temperature. Finally, after washing with TBST for three times, ECL (BioRad) was used for chemical development.

CCK-8 assay

Cell suspension of Sema4D si-RNA and si-NC transfected cells was added into 96-well plates. Added 10μl CCK-

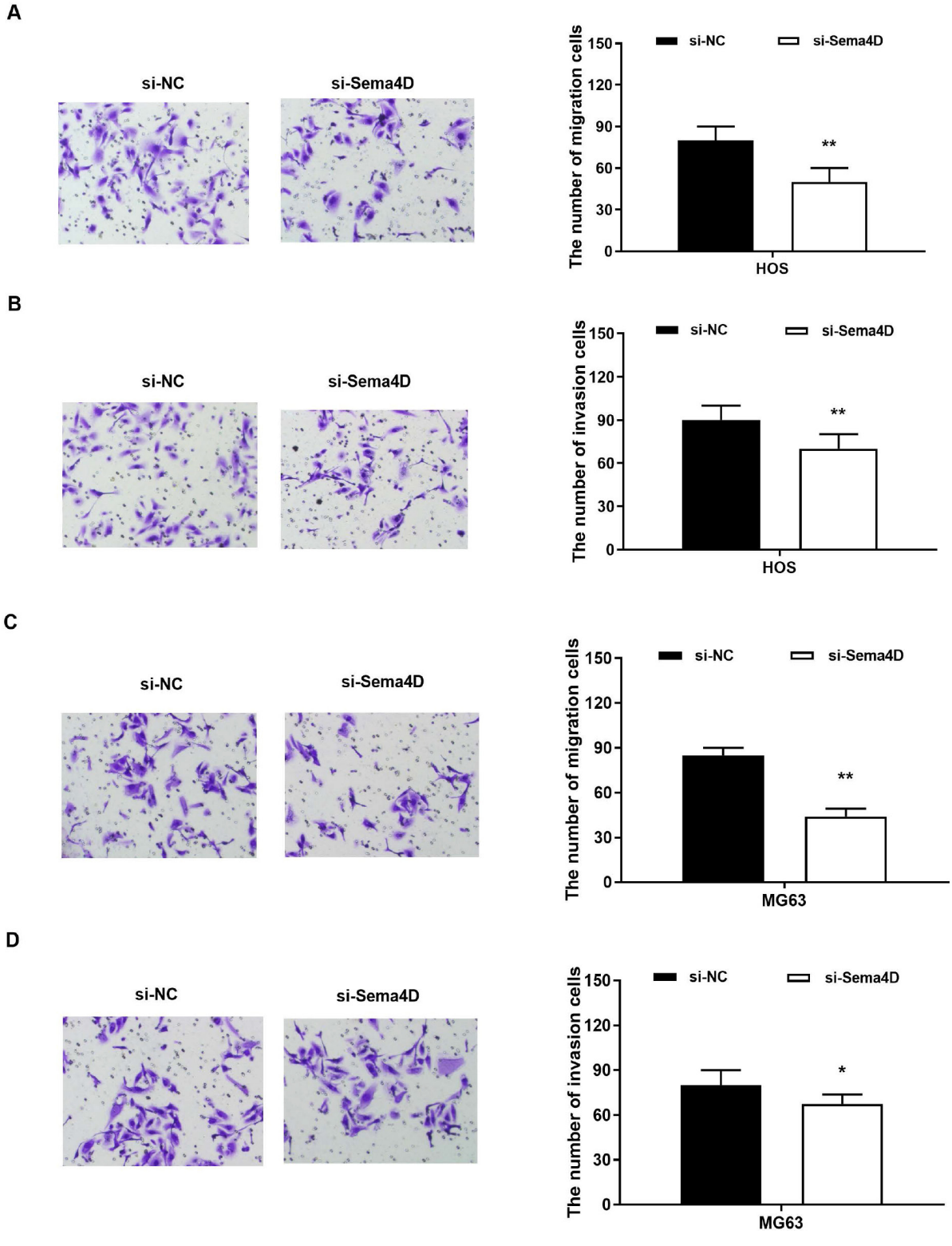

Figure 3. Depletion of Sema4D suppresses cell migration and invasion in OS cells. Transwell assay was used to detect the effect of Sema4D knockdown on cell migration and invasion in HOS and MG63 cells: (A, B) Sema4D knockdown notably suppressed cell migration and invasion in HOS cells; (C, D) Sema4D knockdown reduced the number of migrated and invasive cells in MG63 cells. *p<0.05; **p<0.01.

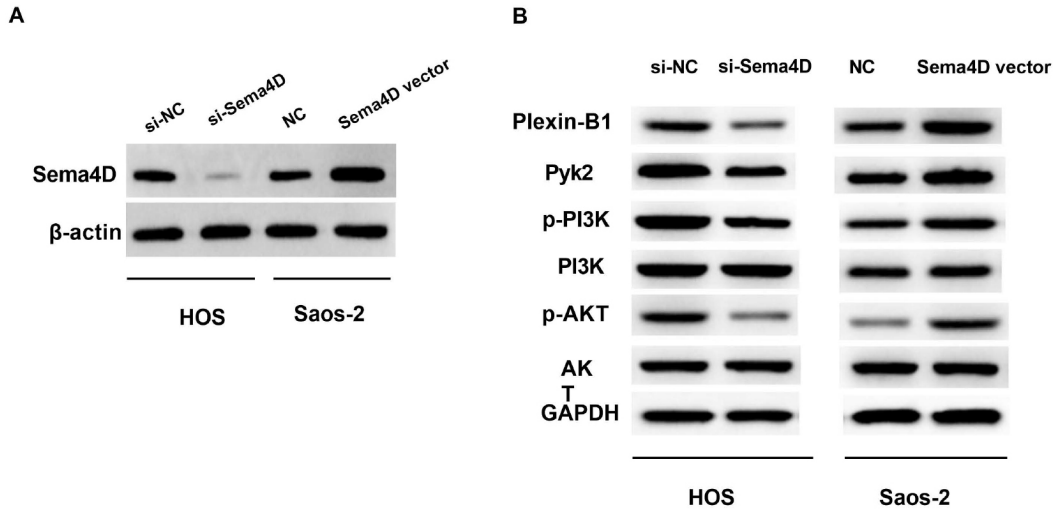

Figure 4. Sema4D-Plexin B1 promotes the OS progression by activating Pyk2-PI3K/AKT pathway. (A) Western blot detected the protein of Sema4D in HOS cells and Saos-2 cells. (B) The expressions of Plexin-B1, Pyk2, and proteins associated with the PI3K/AKT pathway were detected after transfected with Sema4D knockdown or Sema4D overexpression. **p<0.01.

8 reagent to each well, and incubated in carbon dioxide incubator at 37°C for 1h. OD value was detected at 450nm by using a microplate analyzer.

Clone formation assay

A single cell can form clones or colonies after successive proliferation for more than 6 generations *in vitro*. The proliferation ability of cells can be analyzed by counting the number of clone formation. OS cells were routinely digested, centrifuged and resuspended, and were seeded into 6-well plates and incubated at 37°C and 5% CO_2 for 10-14 days. After washing with PBS, cells were fixed with pure methanol for 20 minutes. Then, cells were stained with 0.1% crystal violet solution for 10-15 min at room temperature. The clones with more than 50 cells were counted.

Transwell assay

Cell migration and invasion were assessed by Transwell assay. For invasion, 100 μL Matrigel was added into the upper chamber of Transwell. Unlike invasion, the addition of Matrigel was not required for migration. Besides that, the steps of the migration and invasion experiments were the same. 100 μL cell suspension (2×10^5 cells/mL) was added to the upper chamber. Then, 700 μL complete medium containing 10% FBS was added to the lower chamber of the Transwell. The Transwell chamber was placed in an incubator under the following conditions: 5% CO_2 and 37°C. After 48 h for culture, the cells in the upper chamber were gently wiped off with cotton swabs. The Transwell was fixed with 4% paraformaldehyde for 20 min, and strained with crystal violet. Olymous CX41 microscope was used to photograph. Five visual fields were selected for measurement, and IPP software was used to count the migratory and invasive cells.

Statistical analysis

SPSS19.0 software was used to conduct statistical analysis on the data. The experiments were performed 3 times, and the data were expressed as mean ± standard deviation. The t-test was used for comparison between two groups. P<0.05 was considered statistically significant.

Results

Sema4D was in a high expression in OS cells

The expression level of Sema4D was examined by RT-qPCR. Sema4D was showed to be in a high expression in OS tissues (Figure 1A). Also, Sema4D was up-regulated in HOS, MG63, Saos-2 cells compared with hFOB1.19 cells (Figure 1B). To further investigate the specific role of Sema4D in OS, we reduced the expression of Sema4D in HOS cells and MG63 cells (Figure 1C). According to the high expression of Sema4D, we speculated that Sema4D might be involved in the cancer process of OS.

Depletion of Sema4D suppresses cell proliferation in OS cells

In order to study the role of Sema4D on OS progression, the effect of Sema4D knockdown on HOS and MG63 cells proliferation and motility was investigated. Cell proliferation was detected by CCK-8 assay and clone formation assay.

Sema4D knockdown obviously reduced the cell proliferation in HOS cells (Figure 2A). Besides, MG63 cell proliferation was blocked by Sema4D silencing (Figure 2B). Next, Sema4D knockdown notably reduced the colony number of HOS cells (Figure 2C). Similarly, the colony number of MG63 cells was reduced by Sema4D knockdown (Figure 2D). These results indicated that Sema4D knockdown suppressed the ability of OS cells to proliferate.

Depletion of Sema4D suppresses cell migration and invasion in OS cells

Transwell assay was performed to measure the effect of Sema4D on cell migration and invasion abilities in OS cells. We found that Sema4D knockdown played an inhibitory role in cell migration of HOS cells (Figure 3A). Likewise, cell invasion of MG63 cells was blocked by Sema4D knockdown (Figure 3B). In addition, Sema4D knockdown blocked cell migration ability in HOS cells (Figure 3C). Simultaneously, Sema4D knockdown suppressed cell invasion ability in MG63 cells (Figure 3D). All data manifested that Sema4D knockdown blocked cellular motility in OS cells.

Sema4D-Plexin B1 promotes the OS progression by activating Pyk2-PI3K-Akt pathway

Sema4D was usually associated with Plexin B1to exert its oncogenic effect. As shown in Figure 4A, Sema4D knockdown decreased the expression of Plexin B1. Furthermore, Pyk2, p-PI3K and p-AKT was reduced by Sema4D depletion in HOS cells. Interestingly, Pyk2, p-PI3K and p-AKT was accelerated by Sema4D overexpression in Saos-2 cells. However, the total levels of PI3K and AKT did not change by Sema4D depletion or overexpression (Figure 4B). Therefore, our results showed that Sema4D-Plexin B1 promoted the progression of OS cells by activating Pyk2-PI3K-Akt pathway.

Discussion

Sema4D can be cleaved into an extracellular soluble fragment of sema4D (s Sema4D) by membrane matrix metalloproteinase, to act as a soluble form in body fluid or blood[11]. Sema4D was involved in the functional system of bone cells to regulate bone metabolism, and considered to be an important inhibitor of bone formation. Sema4D was confirmed to play an important role in inhibiting osteocyte function and blocking the signal transduction pathway of bone formation by binding to Plexin-B1. Furthermore, Sema4D/Plexin-B1 can also induce vascular endothelial cell migration and neovascularization. And simultaneously, Sema4D was abnormally expressed in a variety of cancers and are associated with poor prognosis. Sema4D was highly expressed in prostate, glioma, lung, ovary, skin squamous cell carcinoma and other solid tumors[12]. Sema4D was highly expressed in early breast cancer, while downregulated in advanced breast cancer. In breast cancer, Sema4D knockdown reduced bone metastasis[13]. Current studies have demonstrated that Sema4D promoted angiogenesis and tumor growth. In this study, we showed that Sema4D was overexpressed in OS tissues and cells. Moreover, knockdown of Sema4D remarkably blocked the proliferation and motility of OS cells. Our results are in line with the findings by Jiang et al.[14], which reported that Sema4D was upregulated in breast cancer, and Sema4D knockdown suppressed cell proliferation, migration, invasion, angiogenesis and tumor growth. Furthermore, Sema4D overexpression enhanced cell migration and invasion in gastric carcinoma cells[15].

Plexin-B1 is a high-affinity receptor for Sema4D, which is mainly expressed in endothelial cells and epithelial cells[16]. Plexin-B1 can mediate the effects of Sema4D on a variety of biological functions, such as nerve development[17], cell invasion[18], blood vessel formation[19], cell migration, bone resorption[20] and other aspects. Sema4D was confirmed to cooperate with Plexin-B1 to facilitate cell growth and metastasis in cutaneous squamous cell carcinoma[21]. As a member of the FAK family, Pyk2 is mainly distributed in the central nervous system and hematopoietic system. Extensive studies have shown that the main biological functions of Pyk2 are involved in the transduction of multiple intracellular signaling pathways[22], the reorganization of cytoskeleton, and the regulation of proliferation, migration and apoptosis of tumor cells[23]. As an upstream signals, Pyk2 was involved in PI3K/AKT signaling pathway. In this paper, we investigated the effect of Sema4D on the Pyk2-PI3K/AKT signaling pathway. We found that the differential expression of Sema4D affected the phosphorylation levels of PI3K and AKT, and the expressions of Plexin-B1 and Pyk2. In line with our results, in bladder cancer, Sema4D was confirmed to stimulate cell proliferation and metastasis *in vivo* and *in vitro* by inducing the PI3K/AKT pathway[24].

Conclusion

Our results showed that Sema4D was overexpressed in OS, and was associated with oncogenesis and progression in OS. Our findings also suggested that Sema4D might be a potential target for the diagnosis and treatment of patients with OS.

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Authors' contributions

CL and XW conceived and designed this study. XW offered administrative support.

CL and LW dealt with the experimental materials for study. LW, PW and XG helped with data collection and summary. CL, XG and CoL were responsible for data analysis and interpretation. All authors wrote the manuscript. All authors read and approved the final manuscript.

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