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Role of Sema4D in Bone Metastasis of Breast Cancer

Linglin Zou, M.D., **Qinglian Wen,** M.D., **Zhu Wang,** M.M., **Yanping Wang,** M.M., and **Yi Shao,** M.D.

OBJECTIVE: To explore the role of Sema4D in the bone metastasis of breast cancer.

STUDY DESIGN: The expression of Sema4D in breast cancer MCF-7 cells was downregulated by siRNA transfection and detected by RT-PCR. Proliferation and invasion were detected by MTT and Transwell assays, respectively. A co-culture system of osteoblasts and breast cancer cells was constructed. Bone mineralization was detected by alizarin red staining. The expressions of AKT and p-AKT proteins were detected by western blot.

RESULTS: After transfection, the expression of the Sema4D siRNA group was significantly lower than those of the Control and Negative Control (NC) groups ($p<0.05$). The proliferation rate and number of invading cells in the Sema4D siRNA group from 36 to 72 hours were significantly lower than those of the NC and Control groups ($p<0.05$). The bone nodule area of the Sema4D siRNA+osteogenic medium (OM) group was significantly larger than that of the MCF-7+OM group ($p<0.05$). The expression level of p-AKT protein in the Sema4D siRNA+OM group significantly exceeded that of the MCF-7+OM group ($p<0.05$).

From the Laboratory of Molecular Diagnosis of Cancer, Clinical Research Center for Breast, West China Hospital, Sichuan University, Chengdu, Sichuan Province; the Department of Oncology, the Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan Province; and the Department of Medical Oncology, Tianjin Medical University General Hospital, Tianjin, China.

Dr. Zou is Attending Physician, Laboratory of Molecular Diagnosis of Cancer, Clinical Research Center for Breast, West China Hospital, Sichuan University, and Department of Oncology, Affiliated Hospital of Southwest Medical University.

Dr. Wen is Professor, Department of Oncology, Affiliated Hospital of Southwest Medical University.

Dr. Z. Wang is Senior Researcher, Laboratory of Molecular Diagnosis of Cancer, Clinical Research Center for Breast, West China Hospital, Sichuan University.

Dr. Y. Wang is Research Fellow, Laboratory of Molecular Diagnosis of Cancer, Clinical Research Center for Breast, West China Hospital, Sichuan University.

Dr. Shao is Attending Physician, Department of Medical Oncology, Tianjin Medical University General Hospital.

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Address correspondence to: Qinglian Wen, M.D., Department of Oncology, Affiliated Hospital of Southwest Medical University, Luzhou 646000, Sichuan Province, China (wenqlahsmu@foxmail.com).

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CONCLUSION: The Sema4D expression in MCF-7 cells was significantly upregulated. Downregulating this expression attenuated the inhibition of osteoblast differentiation, probably being associated with enhanced AKT phosphorylation. (Anal Quant Cytopathol Histpathol 2019;41:1–8)

Keywords: AKT phosphorylation; bone metastasis; breast cancer; phosphorylation; Sema4D; semaphorins.

Breast cancer is one of the most common malignant tumors in women, accounting for about 25% of all female tumors.[1] Breast cancer in the early and middle stages can be satisfactorily treated. However, the prognosis of the patient is seriously affected once metastasis occurs. Bone is the primary metastatic site for breast cancer.[2] After bone metastasis, symptoms such as bone pain, pathological fracture, and nerve compression appear, thereby significantly aggravating the quality of life and shortening the survival time. The pathogenesis of breast cancer bone metastasis includes loss of bone remodeling balance, osteogenesis, and osteolytic imbalance.[3] As a nerve axon growth-directing factor, semaphorin 4D (Sema4D) is highly expressed in many malignant tumors and closely related to tumor onset and progression.[4] Negishi-Koga et al reported that osteoclasts secreted Sema4D and participated in regulating the differentiation and metabolism of osteoblasts.[5] In addition, Zhang et al[6] found that knocking out Sema4D gene from mouse osteoblasts enhanced their differentiation ability, suggesting that this gene may play an important role in regulating bone metabolism balance. Nevertheless, it remains unclear whether Sema4D is involved in breast cancer bone metastasis. Thereby motivated, we herein aimed to explore the role of mediated AKT phosphorylation of osteogenic precursor cells in breast cancer bone metastasis by inhibiting Sema4D expression, and to provide a new strategy for metastasis prevention and treatment.

Materials and Methods

Cell Lines

Human breast cancer cell line MCF-7 was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (China). Human normal mammary cell line Hs578Bst was purchased from Shanghai Baili Biotechnology Co., Ltd. (China). Mouse embryonic osteogenic precursor cell line MC3T3-E1 was obtained from Shanghai Y-Y Biotechnology Co., Ltd. (China).

Antibodies and Reagents

MTT, crystal violet, ethidium bromide, fetal bovine serum, and DMEM were purchased from Gibco (USA). Dimethyl sulfoxide, diethyl pyrocarbonate, and alizarin red were purchased from Sigma (USA). RPMI-1640 medium, osteogenic medium (OM), complete α-MEM, and trypsin were obtained from HyClone (USA). RIPA lysis buffer, BCA protein quantification kit, and SDS-PAGE–related reagents were provided by Beyotime Institute of Biotechnology Co., Ltd. (China). Sema4D siRNA and lentiviral vector were purchased from Shanghai Genechem Co., Ltd. (China). Mouse-derived Sema4D and GAPDH polyclonal antibodies and HRP-labeled goat anti-mouse secondary antibody were purchased from Tiangen Biotech (Beijing) Co., Ltd. (China). RNAiso lysis buffer, Prime Script reverse transcription kit, Prime Script RT enzyme mix I, RT PrimerMix, and SYBR Premix Ex Taq II were obtained from Takara Biotechnology (Dalian) Co., Ltd. (China). Lipofectamine 2000 as well as mouse-derived AKT and p-AKT polyclonal antibodies were provided by Invitrogen (USA). The Transwell chamber was purchased from Costar (USA).

Cell Culture

MCF-7 and Hs578Bst cells were inoculated in DMEM and cultured in a 37°C incubator with 5% CO_2. When the confluence reached ≥90%, the cells were digested with trypsin, resuspended with serum-free medium, and passaged according to a proportion of 1:3.

Detection of Sema4D mRNA Expression by Real-Time Quantitative PCR

Cells in the logarithmic growth phase were washed 3 times with PBS, digested with 1 mL of trypsin, and centrifuged at 1,500 rpm for 5 minutes to discard the supernatant. Then 1 mL of cell lysis buffer was added to the precipitate and left still on ice for 10 minutes. Afterwards, the cells were transferred to an EP tube, added 0.2 mL of chloroform, completely shaken, left still on ice for 5 minutes, and centrifuged at 12,000 rpm for 10 minutes to collect the supernatant. After an equal volume of isopropanol was added, the

mixture was shaken for 10 seconds and centrifuged at 12,000 rpm for 10 minutes to discard the supernatant. Finally, the precipitate was washed with 75% ethanol solution. Extracted total RNA (5 μg) was reverse-transcribed according to the instructions of the Prime Script reverse transcription kit. Sema4D mRNA expression was detected by real-time quantitative PCR according to the instructions of the SYBR Premix Ex Taq II kit. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China). Sema4D: upstream, 5′-GAGAAGCAGCATGAGGTGTATTGGA-3′; downstream, 5′-GGATGTAGTTGAGGCACTCT GTCTG-3′. GAPDH: upstream, 5′-CCAGCAAGAG CACAAGAGGAAGAG-3′; downstream, 5′-GGTC TACATGGCAACTGTGAGGA-3′. PCR system (20 μL): 10 μL of SYBR Premix Ex Taq II, 1 μL of upstream and downstream primers each, 2 μL of template and distilled water. PCR conditions: predenaturation at 95°C for 30 seconds, denaturation at 95°C for 10 seconds, and extension at 60°C for 40 seconds, 35 cycles in total; extension at 74°C for 5 minutes. Using GAPDH as internal reference, relative Sema4D mRNA expression was measured.

Construction of MCF-7 Cell Line with Low Sema4D Expression by siRNA Transfection

MCF-7 cells were divided into a blank control (Control) group, a negative control (NC) group, and a Sema4D siRNA group. NC group: siRNA sense strand, 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense strand, 5′-ACGUGACAGCUUCGGAGA ATT-3′. Sema4D group: siRNA sense strand, 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense strand, 5′-ACGUGACACGUUCGGAGAATT-3′. Cells in the logarithmic growth phase were seeded in a 24-well plate at 1×10^5/well. When the confluence reached ≥50%, the culture medium was refreshed. The Control group was cultured without treatment. The NC group was added Lipofectamin 2000 and siRNA-NC, and the Sema4D siRNA group was added Lipofectamine 2000 and siRNA-Sema4D. Transfection was performed according to MOI determined in pre-experiment. Total RNA was extracted 48 hours after transfection, and the expression level of Sema4D mRNA was detected by real-time quantitative PCR.

Detection of Cell Proliferation by MTT Assay

Cells in the logarithmic growth phase were inoculated into a 96-well plate at 1.0×10^7/well, and 10 μL of 5 g/L MTT solution was added into each well at 12, 24, 36, 48, 60, and 72 hours, respectively. After 4 hours of culture the medium was discarded, and 150 μL of dimethyl sulfoxide was added into each well and completely shaken. The optical density was measured at 450 nm by a microplate reader.

Detection of Cell Invasion by Transwell Assay

Cells in the logarithmic growth phase (150 μL) were seeded into an upper Transwell chamber at 1.0×10^9/well, and 600 μL of RPMI-1640 medium containing 20% fetal bovine serum was added into a lower chamber. Six replicate wells were set for each group. After 48 hours of culture, the filter membrane of the Transwell chamber was fixed by 4% paraformaldehyde, and the cells on the surface were gently wiped off. The membrane-penetrating cells were observed under a microscope after 10 minutes of staining with crystal violet and washing with PBS 3 times.

Cell Co-Culture and Osteogenic Differentiation

According to a previous publication in the literature,[7] a Transwell chamber was used to establish a co-culture system of osteoblasts and breast cancer cells to simulate the bone metastasis microenvironment. The cells were divided into 3 groups: (1) Control group (Control+OM): to the upper Transwell chamber was added only OM, and to the lower chamber was added MC3T3-E1 cells; (2) breast cancer cell group (MCF-7+OM): to the upper chamber was added MCF-7 cells, and to the lower chamber was added MC3T3-E1 cells; (3) breast cancer cell with low Sema4D expression group (Sema4D siRNA+OM): to the upper chamber was added MCF-7 cells with low Sema-4D expression, and to the lower chamber was added MC3T3-E1 cells. In detail, MC3T3-E1 cells, normal MCF-7 cells, and MCF-7 cells with low Sema4D expression in the logarithmic growth phase were used. By using serum-free medium, the density of MC3T3-E1 cells was adjusted to 1×10^5/well, and those of normal MCF-7 cells and MCF-7 cells with low Sema4D expression were adjusted to 3×10^4/well. They were thereafter seeded into 12-well plates, added 0.5 mL of complete α-MEM and cultured at 37°C with 5% CO_2. After the cells reached complete confluence, the medium was replaced with 0.5 mL of OM (recorded as 0 hour) and further cultured. The culture medium was refreshed every 12 hours.

Detection of Bone Mineralization Capacity by Alizarin Red Staining

After 2 weeks of co-culture, the medium of the Transwell chamber was pipetted, and cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 10 minutes. After the liquid was pipetted, the cells were washed 3 times again with PBS. Alizarin red solution was then added into the culture plate at 200 μL/well. The cells were incubated for 5–10 minutes at room temperature, and staining was terminated when red particles appeared on the cell surface. After the staining solution was pipetted, the cells were washed with purified water 3 times and air-dried at room temperature. The stained cells were observed under an inverted microscope. The number and area of bone nodules of each group were analyzed by Quantity One software to reflect the bone mineralization capacity.

Detection of AKT and p-AKT Protein Expressions by Western Blot

After 24 hours of co-culture, cells in the logarithmic growth phase were lysed with 100 μL of lysis buffer containing 1% PMSF and centrifuged at 4°C and 15,000 rpm for 20 minutes. The supernatant was added into an EP tube and boiled with an equal amount of loading buffer for 5 minutes for protein denaturation. Then SDS-PAGE was performed, and the products were electronically transferred onto a PVDF membrane. Subsequently, the membrane was blocked with 5% nonfat milk for 2 hours, incubated overnight with AKT and p-AKT polyclonal antibodies (1:1000 diluted) and GAPDH polyclonal antibody (1:5000 diluted) at 4°C, washed 3 times with PBS, incubated with secondary antibodies (1:1000) at room temperature for 2 hours, washed 3 times again with PBS, and developed by using ECL reagent. The gray values of target genes were measured by gel imaging system, and the relative expression of target protein was the ratio of gray value of sample band to that of GAPDH band.

Statistical Analysis

All data were analyzed by SPSS 19.0 software. The categorical data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The means of multiple groups were compared by one-way analysis of variance, and subsequent pairwise test was conducted with the q test. $P < 0.05$ was considered statistically significant.

Results

Sema4D mRNA Expressions in MCF-7 and Hs578Bst Cells

Real-time quantitative PCR showed that the relative expression of Sema4D mRNA in MCF-7 cells was (7.331 ± 0.974), and that of Hs578Bst cells was (1.483 ± 0.341). The expression of MCF-7 cells was significantly higher than that of Hs578Bst cells ($p < 0.05$).

Sema4D mRNA Expression in MCF-7 Cells with siRNA Transfection

After transfection with Sema4D siRNA, the relative expression of Sema4D mRNA in the Sema4D siRNA group (0.492 ± 0.061) was significantly low-

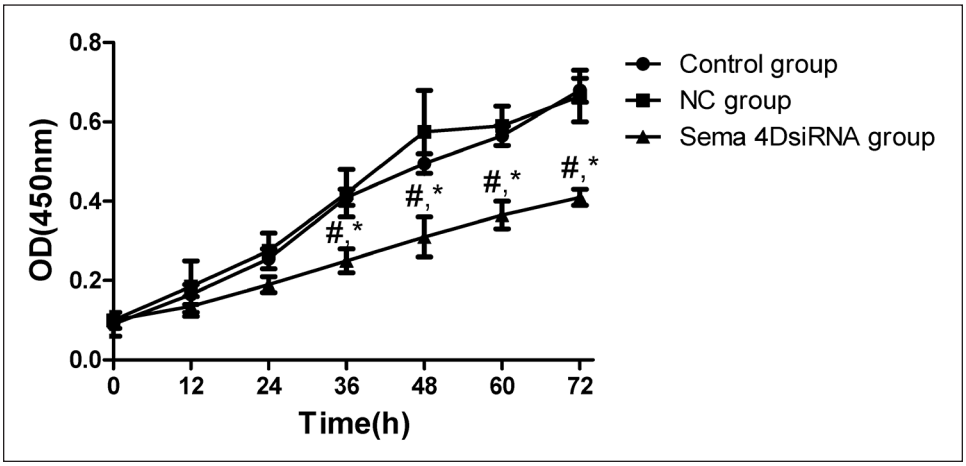

Figure 1
Changes of cell proliferation capacities. Compared with the Control group, #p<0.05; compared with NC group, *p<0.05.

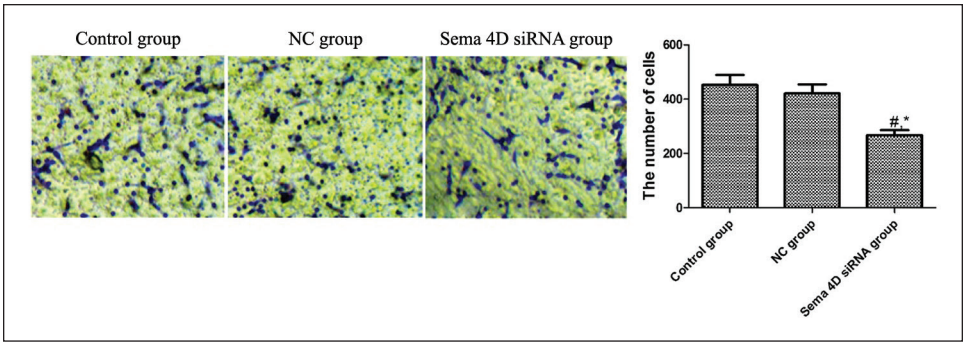

Figure 2
Changes of cell invasion capacities. Compared with the Control group, #p<0.05; compared with NC group, *p<0.05.

er than those of the Control (1.483±0.341) and NC groups (1.398±0.283) (p<0.05), but there was no significant difference between the latter 2 groups (p>0.05).

Effects of siRNA Transfection on MCF-7 Cell Proliferation

The proliferation rate of the Sema4D siRNA group from 36 to 72 hours was significantly lower than those of the NC and Control groups (p<0.05), whereas there was no significant difference between the latter 2 groups (p>0.05) (Figure 1).

Effects of siRNA Transfection on MCF-7 Cell Invasion

The number of invading cells in the Sema4D siRNA group (268±20) was significantly lower than those of the NC (422±31) and Control groups (451±36) (p<0.05), without significant difference between the latter 2 groups, either (p>0.05) (Figure 2).

Effects of siRNA Transfection on Bone Mineralization

The areas of bone nodules in the MCF-7+OM and Sema4D siRNA+OM groups were (1.27±0.19) μm² and (4.23±0.20) μm², respectively, which were significantly smaller than that of the Control+OM group (9.61±0.59) μm² (p<0.05). Additionally, the bone nodule area of the Sema4D siRNA+OM group was significantly larger than that of the MCF-7+OM group (p<0.05) (Figure 3).

Effects of siRNA Transfection on AKT and p-AKT Protein Expressions

The Control+OM, MCF-7+OM, and Sema4D siRNA+OM groups had similar AKT protein expressions (p>0.05). The expression levels of p-AKT protein in MCF-7+OM and Sema4D siRNA+OM groups were significantly lower than that of the Control+OM group (p<0.05). In addition, the expression level of p-AKT protein in the Sema4D siRNA+OM group was significantly higher than that of the MCF-7+OM group (p<0.05) (Figure 4).

Discussion

Sema4D is a member of the type IV semaphorins family. Semaphorins are capable of axonal targeting and maintaining normal neurophysiological functions. Sema4D is highly expressed in various malignant tumors such as breast cancer, prostate cancer, and lung cancer and can mediate tumor

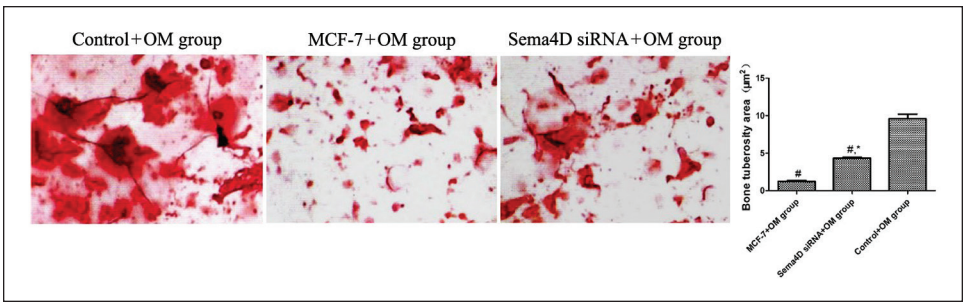

Figure 3
Alizarin red staining results. Compared with the Control+OM group, #p<0.05; compared with the MCF-7+OM group, *p<0.05.

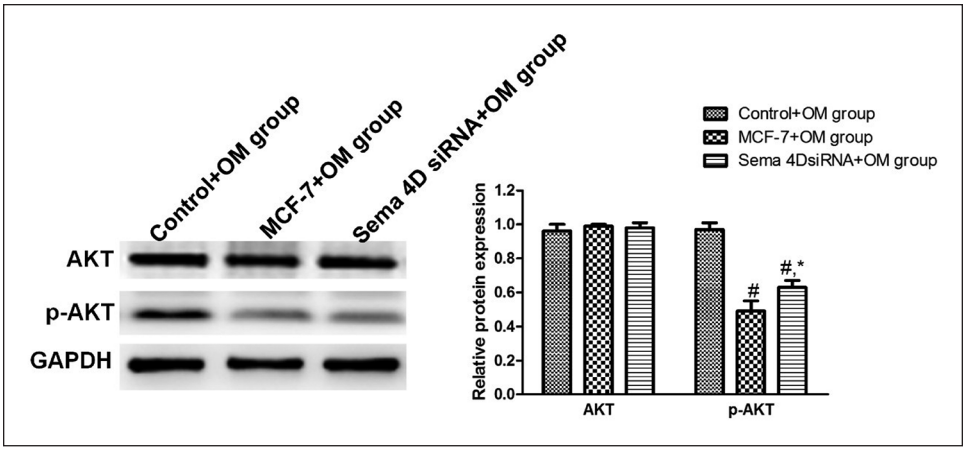

Figure 4
AKT and p-AKT protein expressions. Compared with the Control+OM group, #p<0.05; compared with the MCF-7+OM group, *p<0.05.

angiogenesis, progression, and cellular immunity.[8-10] Chen et al reported that Sema4D was highly expressed in breast cancer, and patients with higher expression levels had poorer prognosis.[11] Additionally, Shanks et al found that Sema4D mainly originated from tumor-associated macrophages and participated in tumor angiogenesis.[12] In this study the Sema4D mRNA expression in MCF-7 cells was significantly higher than that in Hs578Bst cells, which was consistent with the results of Binmadi et al,[13] suggesting that Sema4D may be involved in some biological behaviors of breast cancer.

Tasaka et al found that hypoxia-inducible factors promoted the expression of Sema4D in breast cancer and induced the migration of endothelial cells, leading to tumor proliferation, invasion, and angiogenesis.[14] In this study the Sema4D expression in breast cancer MCF-7 cells was subjected to interference by siRNA transfection. The Sema4D mRNA expression of the Sema4D siRNA group was significantly lower than those of the Control and NC groups after transfection, confirming that the MCF-7 cell model of low Sema4D expression was successfully constructed. Moreover, the proliferation and invasion rates of the Sema4D siRNA group were significantly lower than those of the NC and Control groups, suggesting that interference with Sema4D expression inhibited cell proliferation and invasion. Binmadi et al reported that Sema4D specifically bound filamentous protein B1 and tyrosine kinase receptors and activated the Rho A signaling pathway, which was crucial for tumor cell invasion and metastasis.[15] Also, Giordano et al verified that downregulating Sema4D expression inhibited the proliferation of breast cancer cells, arrested the cell cycle in the G phase,[16] and significantly suppressed their migration. Hence, Sema4D may function as an oncogene participating in the biological processes of breast cancer, such as proliferation, invasion, and metastasis.

Bone metastasis, as one of the serious complications of many malignant tumors, can lead to bone remodeling imbalance and further cause a variety of bone-related events.[17,18] Piccardo et al reported that breast cancer did not directly destroy bone after bone metastasis. Instead, it mainly inhibited the differentiation and maturation of osteogenic precursor cells through a series of regulatory mechanisms.[19] Osteoclasts can secrete Sema4D during differentiation, and the degree of osteoblast differentiation is attenuated with increasing Sema4D level.[20] Therefore, Sema-4D may be involved in osteolytic destruction and inhibition of osteogenic differentiation. Additionally, Wannemacher et al found that mice with Sema4D gene knockout underwent obvious osteosclerosis,[21] so Sema4D played an important role in osteogenic and osteoclastic balance. In this study a Transwell chamber was used to establish the microenvironment of breast cancer bone metastasis, and then osteogenic differentiation was observed by alizarin red staining. The stained areas of bone nodules in MCF-7+OM and Sema-4D siRNA+OM groups were significantly smaller than that of the Control+OM group, and the area of bone nodules in the Sema4D siRNA+OM group was significantly larger than that of the MCF-7+OM group. Thus, MCF-7 cells significantly inhibited the mineralization of osteogenic precursor

cells, whereas the inhibitory effects were weakened after interference with Sema4D expression.

As a key protein mediates osteoblast differentiation, AKT can induce osteogenic differentiation by activating the Wnt/β-catenin pathway.[22] Serafino et al[23] verified that osteogenic inducer promoted the differentiation of osteogenic precursor cells and significantly increased the degree of AKT phosphorylation. Herein, the expression levels of AKT protein in Control+OM, MCF-7+OM, and Sema4D siRNA+OM groups were similar. However, the expression level of p-AKT protein in the Sema4D siRNA+OM group was significantly higher than that of the MCF-7+OM group. Thus, MCF-7 cells may inhibit osteogenic differentiation by attenuating AKT phosphorylation, and interference of Sema4D expression enhanced AKT phosphorylation, thereby weakening the inhibition. Although we have demonstrated the possible role of Sema4D in the bone metastasis of breast cancer, the mechanism by which Sema4D regulates AKT phosphorylation and promotes osteoclast differentiation still needs further in-depth study.

In summary, the expression of Sema4D in human breast cancer cell line MCF-7 was significantly upregulated. Downregulating this expression attenuated the inhibition of osteoblast differentiation, which may be linked to augmented AKT phosphorylation.

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