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The correlations between CXCL12, CXCR4, EAAT1 and GS in malignant pleural mesothelioma, and CXCL12 regulates cell invasion and migration via CXCR4/EAAT1/GS pathway

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Simple Summary:

CXCL12/CXCR4 axis is known closely related to tumors such as malignant pleural mesothelioma (MPM). Since we have recently found EAAT1/GS (Excitatory amino acid transporter 1/Glutamine synthetase) were highly-expressed in MPM tissues. This study aims to analyze the correlations between them in MPM and normal tissues, and to elucidate the mechanism of CXCR4/EAAT1/GS pathway in CXCL12 regulating invasion and migration. We confirmed significant correlations between CXCL12 and CXCR4, CXCL12 and EAAT1, CXCR4 and EAAT1, CXCR4 and GS, EAAT1 and GS in MPM samples. CXCL12 upregulated CXCR4, EAAT1 and GS expression in H2052 cells. The EAAT1 and GS expression upregulated or not by CXCL12 were decreased by CXCR4 and EAAT1 knockdown. CXCR4 antagonist AMD3100 and EAAT1 antagonist TFB-TBOA also resulted in the same effects. CXCL12 promoted cell invasion and migration. CXCR4 and EAAT1 knockdown suppressed all these functions, suggesting that CXCL12 regulated the invasion and migration through CXCR4/EAAT1/GS pathway in H2052 cells.

Abstract:

Purpose: To elucidate the mechanism of CXCR4/EAAT1/GS pathway in CXCL12 regulating invasion and migration in malignant pleural mesothelioma (MPM).

Methods: Immunohistochemistry for CXCL12, CXCR4, EAAT1 and GS stainings and correlation analysis between them were conducted in MPM and normal tissues. Western blot and real-time PCR were performed to examine the CXCR4, EAAT1 and GS expression in H2052 cells. Wound healing and transwell assay were applied to determine the cell migration and invasion. MTT was utilized to assess cell viability.

Results: CXCL12, CXCR4, EAAT1 and GS were highly expressed in MPM tissues and correlated with each other. CXCL12 upregulated both in protein and mRNA levels of CXCR4, EAAT1 and GS in H2052 cells. The EAAT1 and GS expression upregulated or not by CXCL12 were decreased by CXCR4 and EAAT1 knockdown. CXCR4 antagonist AMD3100 and EAAT1 antagonist TFB-TBOA also resulted in the same effects as CXCR4 and EAAT1 knockdown, respectively. CXCL12 promoted cell invasion and migration and increased the Matrix metalloproteinase 9 (MMP9) mRNA level. CXCR4 and EAAT1 knockdown suppressed all these functions. Furthermore, CXCL12 promoted H2052 cells growth in nude mice, both AMD3100 and TFB-TBOA inhibited this promotion.

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Conclusions: CXCL12 regulated the invasion and migration through CXCR4/EAAT1/GS pathway in H2052 cells.

Keywords: Malignant pleural mesothelioma; CXCL12/CXCR4; EAAT1; Glutamine synthetase; Invasion; Migraiton

1. Introduction

Malignant pleural mesothelioma (MPM) is a relatively rare and aggressive cancer, originating from mesothelial surfaces of the pleural lining. The prognosis of MPM is poor for decades due to its nonspecific clinical manifestations and the relatively late diagnosis [1]. Treatments for MPM in the past rarely extend patients survival beyond 12 months [2,3]. However, recent studies have elucidated genetic alterations in mesothelioma. Some genes have been found to be significantly espressed in MPM, such as BAP1, NF2, TP53, SETD2, SMARCC1, PBRM1, SF3B1 and TRAF7 [4-7]. Pastorino et al. [8] reported that a subset of MPM patients who were not aware of asbestos exposure and carried pathogenic germline mutations of BAP1 had significantly improved survival. Nevertheless, recent clinical trials targeting molecular pathways have failed to significantly improve the prognosis of MPM [9]. Therefore, a better understanding of the cytogenetic and molecular pathogenesis of MPM is still needed to develop effective therapeutic approaches for the MPM patients [10-12].

Chemokines are a class of chemotactic cytokines that are categorized based on the positioning of well-conserved N-terminal cysteine residues and classified as C, CC, CXC, CX3C chemokines. Chemokines play a critical role in many biological processes, including hematopoiesis, immune cell trafficking and stem and progenitor cell mobility by activating their corresponding chemokine receptors, which belong to the rhodopsin-like seven transmembrane G protein-coupled receptor (GPCR) family [13,14]. Among these receptors, CXCR4, a kind of very important receptor, is widely expressed in various types of tissues, where it plays a central role in development-related processes [15,16] and tumor metastasis. CXCR4 is the specific chemokine receptor which can interact with the chemokine

CXCL12, also known as Stromal-Derived Factor-1 (SDF-1) ligand [17]. CXCL12 was expressed in breast tumor, brain tumor and colorectal cancer tissues [18-20], etc. and also usually expressed in fibroblasts and endothelial cells [21,22] or produced by stromal cells of lymph nodes, lung, liver, and bone marrow, which were the most commom sites for metastasis [23]. CXCL12/CXCR4 axis is also a key factor in the tumor development, metastasis and cross - talking between tumor cells and their microenvironment of various malignancies [24,25]. This axis not only promotes the survival and proliferation of cancer cells but also attracts the CXCR4-expressed tumor tissue/organ-specific metastasis [26,27]. In addition, previous work suggests that CXCL12 and CXCR4 are involved in MPM tumorigenesis [28,29].

Glutamate (Glu) is an important excitatory neurotransmitter of mammalian central nervous system (CNS) [30]. The presynaptic concentration of Glu in human CNS is regulated by Glutamate transporters (excitatory amino acid transporters, EAATs) [31], which utilize the transmembrane gradients of Na^+, K^+, and H^+ as a driving force to uptake glutamate from extracellular space into cells [32], and subsequently converted into glutamine by glutamine synthetase (GS), which is a glia - specific enzyme [33,34], and transported back to the neurons for recycling into glutamate. To data, five EAATs (EAAT1 ~ 5) have been characterized [35,36]. Evidence has suggested that EAATs play a critical role in maintaining extracellular glutamate homeostasis and regulating glutamate neurotransmission [37-39]. Among these EAATs, EAAT1 is a ubiquitous subtype. GS is the only key enzyme that is capable of de novo synthesis of glutamine and also functions to detoxify glutamate and ammonia [40], which is involved in nitrogen metabolism [41] and in the metabolic pathway of glutamate [42]. Nevertheless, there is evidence showed that the glutamatergic system may also have an important role in non-neuronal tissues, such as in cancer biology [31].

CXCL12/CXCR4 axis is known closely related to tumors such as malignant pleural mesothelioma (MPM). Our previous research showed that CXCL12 was a functional activation factor of Akt downstream pathways and could induce cell proliferation in several mesothelioma cell lines and the CXCL12/CXCR4 antagonist AMD3100 blocked these

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effects [43]. Moreover, we also found that EAAT1 was highly-expressed in most of human MPM cell lines and some MPM tissues. The degree of EAAT1 staining by IHC was correlated with pathological type but inversely correlated with the survival time of patients [44]. However, no reports described the functions of EAAT1 in MPM and whether CXCL12 could regulate cell invasion and migration via CXCR4/EAAT1/GS pathway is still unknown.

Therefore, in the present study, we have included more clinical samples and completely examined the expression of CXCL12, CXCR4, EAAT1 and GS in normal pleura and MPM samples, and analyze the correlations between them. Additionally, *In vitro* experimental studies were designed to investigate the mechanism of CXCR4/EAAT1/GS pathway in CXCL12 regulating invasion and migration in malignant pleural mesothelioma (MPM).

2. Results

2.1. Immunohistochemistry (IHC) staining of CXCL12, CXCR4, EAAT1 and GS in normal pleura and MPM samples

The positive or negative results of CXCL12, CXCR4, EAAT1, GS and HE staining in MPM samples in the tissue microarray sections were shown in **Figure. 1** and **Table 1** and **2**. In MPM samples, the percentages of strong positive (+++) in CXCL12, CXCR4, EAAT1 and GS staining were 7.3%, 39.1%, 23.1% and 8.6%, respectively and the strong positive ratio in CXCR4 staining was the highest. While the percentages of negative (−) in CXCL12, CXCR4, EAAT1 and GS staining were 22%, 2.4%, 2.7% and 8.6%, respectively and the negative ratio in CXCR4 staining was the lowest. In normal pleura samples, the percentages of strong positive (+++) in CXCL12, CXCR4, EAAT1 and GS staining were negative (−). While the percentages of negative (−) in CXCL12 staining is higher (60%) than CXCR4 (30%), EAAT1 (50%) and GS (20%) staining, respectively.

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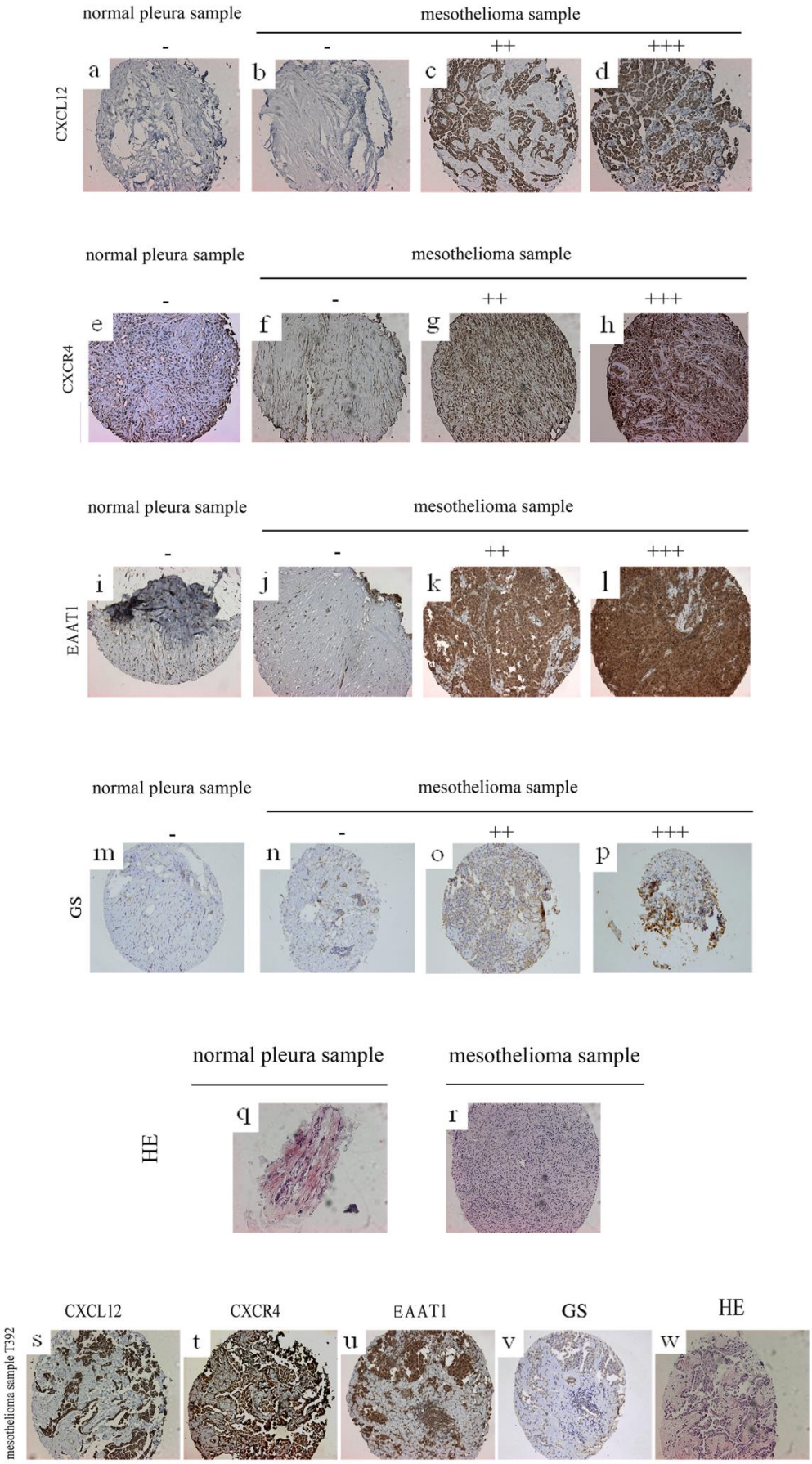

Figure 1

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Figure 1. IHC staining of CXCL12, CXCR4, EAAT1, GS and HE staining in mesothelioma and normal pleura samples (200 ×). a-d: IHC staining of CXCL12. a: Normal pleura sample. b-d: Mesothelioma samples. b: Negative. c: Moderate. d: Strong. e-h: IHC staining of CXCR4. e: Normal pleura sample. f-h: Mesothelioma samples. f: Negative. g: Moderate. h: Strong. i-l: IHC staining of EAAT1. i: Normal pleura sample. j-l: Mesothelioma samples. j: Negative. k: Moderate. l: Strong. m-p: IHC staining of GS. m: Normal pleura sample. n-p: Mesothelioma samples. n: Nnegative. o: Moderate. p: Strong. q, r: HE staining. q: Normal pleura sample. r: Mesothelioma samples. s-w: IHC staining in one mesothelioma sample. s: IHC staining of CXCL12. t: IHC staining of CXCR4. u: IHC staining of EAAT1. v: IHC staining of GS. w: HE staining.

Table 1. Characteristics of primary human MPM and normal pleura samples

Number of the sample	IHC of anti-CXCL12	IHC of anti-CXCR4	IHC of anti-EAAT1	IHC of anti-GS
N233	-	-	-	+
N237	+	++	+	+
T237	+	+	++	+
T241	+	+	++	++
N242	-	+	+	+
T242	++	+++	++	+
N244	+	+	++	+
T246	++	+++	+++	++
T264	+	+	+	++
T300	+	+	++	ND
T324	-	+	++	+

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N331	+	+	-	-
T331	-	+	+	-
T370	+++	+++	++	+
T374	++	+++	+++	ND
T383	-	+	ND	ND
T392	++	++	++	+
T394	++	+++	++	++
T396	+	+	+++	++
T401	-	+	-	ND
T410	++	+++	+	++
T416	+	++	+++	++
T439	-	+	+	+
T453	++	+++	+++	++
N472	+	+	-	+
T472	+	+	+	+
T473	++	+++	++	+
T484	++	+++	+++	+++
T501	++	+++	++	+
T514	++	+++	++	++
T660	++	+++	ND	ND
T696	+++	+++	ND	ND

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T713	+++	+++	+++	+++
T737	+	+	++	+
N745	-	+	++	++
T773	-	+	+	+
N775	-	-	-	+
T775	++	+++	++	++
N777	-	+	+	+
T777	+	++	+++	++
T795	+	+	+	+
T809	+	+++	+++	++
T813	-	+	+	++
T829	+	+	++	++
T839	-	-	+	-
T858	+	++	+	++
T862	+	++	+	+
N869	-	-	-	-
T869	+	++	++	+++
T905	-	+	+	-
T936	+	++	ND	++

N, normal tissue; T, tumor tissue; IHC, immunohistochemistry; -, no stain; +, weak stain; ++, moderate stain; +++, strong stain; ND, not done.

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Table 2. Positive and negative staining for CXCL12, CXCR4, EAAT1 and GS in MPM and normal pleura samples

	-	+	++	+++
	Number (%)	Number (%)	Number (%)	Number (%)
CXCL12				
MPM samples	9(22.0%)	16(39.0%)	13(31.7%)	3(7.3%)
Normal pleura samples	6(60.0%)	3(30.0%)	1(10.0%)	0(0%)
CXCR4				
MPM samples	1(2.4%)	18(43.9%)	6(14.6%)	16(39.1%)
Normal pleura samples	3(30.0%)	6(60.0%)	1(10.0%)	0(0%)
EAAT1				
MPM samples	1 (2.7%)	12 (30.8%)	15 (40.5%)	9 (23.1%)
Normal pleura samples	5 (50%)	3 (30%)	2 (20%)	0 (0%)

GS				
MPM samples	3 (8.6%)	13 (37.1%)	13 (37.1%)	3 (8.6%)
Normal pleura samples	2 (20%)	7 (70%)	1 (10%)	0 (0%)

-, no stain; +, weak stain; ++, moderate stain; +++, strong stain

The overall positive ratios of CXCL12, CXCR4, EAAT1 and GS staining were 78%, 97.6%, 97.3% and 91.4% in MPM samples, respectively and were 40%, 70%, 50.0% and 80.0% in normal pleura samples, respectively. Multiple correlation analysis were shown in **Table 3,** CXCL12 was strongly correlated with both CXCR4 (R^2 = 0.8590, p < 0.01) and EAAT1 (R^2 = 0.6409, p < 0.01) and CXCR4 was strongly correlated with EAAT1 (R^2 = 0.6311, p < 0.01). The correlations were slightly weaker between CXCR4 and GS (R^2 = 0.3648, p = 0.0287), EAAT1 and GS (R^2 = 0.4377, p = 0.0076), respectively in MPM samples, CXCL12 was not correlated with GS (R^2 = 0.2879, p = 0.0886) in MPM samples. As shown in **Table 4,** CXCL12, CXCR4, EAAT1 and GS have no strong correlations with each other in normal pleura samples, In addition, the positive staining areas of CXCL12, CXCR4, EAAT1 and GS were similar in some of other mesothelioma tissue samples.

Table 3. Results of multiple correlation analysis of MPM samples

	CXCL12 staining	CXCR4 staining	EAAT1 staining	GS staining
CXCL12 staining	---	R^2 = 0.8590, P < 0.01	R^2 = 0.6409, p < 0.01	R^2 = 0.2879, p = 0.0886

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CXCR4 staining	---	---	R^2 = 0.6311, p < 0.01	R^2 = 0.3648, p = 0.0287
EAAT1 staining	---	---	---	R^2 = 0.4377, p < 0.01
GS staining	---	---	---	---

---: no data or not done.

Table 4. Results of multiple correlation analysis of normal pleura samples

	CXCL12 staining	CXCR4 staining	EAAT1 staining	GS staining
CXCL12 staining	---	R^2 = 0.6099, P = 0.0611	R^2 = 0.0386, p = 0.9157	R^2 = -0.2196, p = 0.5421
CXCR4 staining	---	---	R^2 = 0.5873, p = 0.0742	R^2 = 0.2615, p = 0.4656
EAAT1 staining	---	---	---	R^2 = 0.6262, p = 0.0527
GS staining	---	---	---	---

---: no data or not done.

2.2. The effects of CXCL12 on the CXCR4, EAAT1 and GS expression

In order to determine an optimal time point for the CXCL12 stimulation. H2052 cells were treated with various times (0 - 24 h) of CXCL12. Exposure to CXCL12 obviously increased the protein expression levels of CXCR4, EAAT1 and GS in a time dependent

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manner and all three proteins reached their highest peak in 24 h (**Figure 2A**). Meanwhile, QPCR was also performed to detect their expressions at the transcriptional level. Similarly, as shown in **Figure 3A**, treatments of H2052 cells with CXCL12 increased the mRNA levels of *CXCR4*, *EAAT1* and *GS* in a time dependent manner and all three genes reached their highest peak in 24 h.

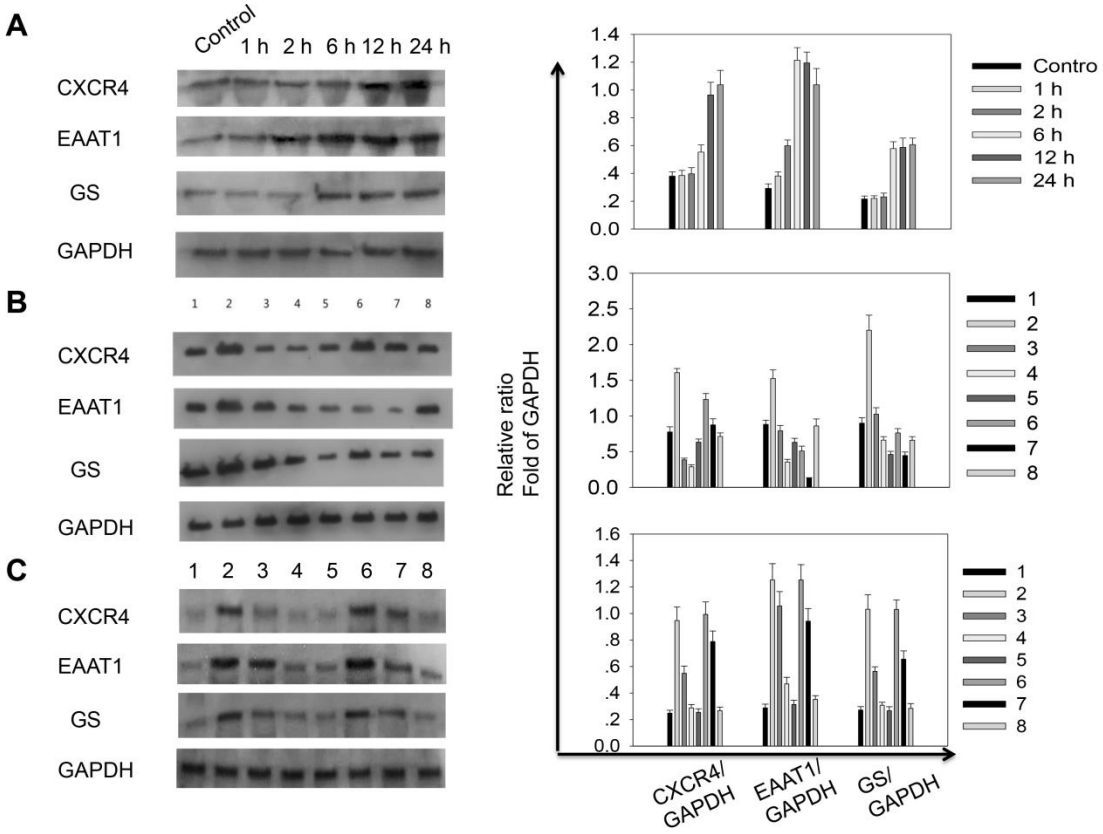

Figure 2. Effects of CXCL12 on the levels of CXCR4, EAAT1 and GS protein in MPM cell line. (A), H2052 cells were incubated in the presence of CXCL12 (100 ng/mL) for various times (0 – 24 h) and subsequently the cells were lysed and the CXCR4, EAAT1 and GS were detected by western blot. (B), H2052 cells were transfected with CXCR4-RNAi or EAAT1-RNAi or respective controls and then treated with CXCL12 (100 ng/mL) for 24 h. The cells were lysed and the CXCR4, EAAT1 and GS were detected by western blot. 1: Control. 2: CXCL12. 3: CXCL12+CXCR4-RNAi. 4: CXCR4-RNAi. 5: CXCR4-RNAi control. 6: CXCL12+EAAT1-RNAi. 7: EAAT1-RNAi. 8: EAAT1-RNAi control. (C), H2052 cells were pretreated with AMD3100 (1.11 μM) or TFB (25 nM) for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h. The CXCR4, EAAT1 and GS were then determined by western blot. 1: Control. 2: CXCL12. 3: CXCL12+AMD3100. 4:

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AMD3100. 5: Control. 6: CXCL12. 7: CXCL12+TFB. 8: TFB. The GAPDH was used as a control to ensure the same loading volume. The data shown were representative of three experiments, and the right panel of each example denoted the densitometry relative to GAPDH.

To explore the potential regulatory effects of CXCL12/CXCR4 axis on EAAT1/GS. First, we included four double-strands of candidate short interfering (siRNA) oligonucleotide sequences (siRNA#1, #2, #3 and #4) specific for CXCR4 and EAAT1 (**Table S2**) and then transiently transfected H2052 cells using CXCR4 or EAAT1 siRNA, respectively for 48 h. The results showed that CXCR4 or EAAT was knockdowned by CXCR4 siRNA#2 or EAAT siRNA#1 with the best inhibitory effect, respectively (**Figure S1**). Therefore, CXCR4 siRNA#2 and EAAT siRNA#1 were selected for the future experiments.

Next, H2052 cells were transfected with CXCR4 siRNA or EAAT siRNA alone, in the presence or absence of CXCL12 treatments or vehicle. As shown in **Figure 2B**, CXCR4 and EAAT1 silencing apparently suppressed CXCL12-upregulated the levels of EAAT1 and GS proteins. CXCR4 and EAAT1 silencing alone also obviously decreased the expression levels of both EAAT1 and GS proteins compared to control siRNAs, respectively. While, EAAT1 silencing slightly inhibited CXCL12-upregulated the expression of CXCR4, but failed to decrease the CXCR4 protein level compared to control siRNA. These findings suggest that EAAT1 locates downstream of CXCR4 and GS locates downstream of EAAT1. CXCL12 upregulates the expression of EAAT1 and GS proteins via CXCR4, and CXCL12 upregulates GS protein via EAAT1. In addition, to further confirm this finding, H2052 cells were treated with the CXCL12, AMD3100 and TFB alone, AMD3100 or TFB followed by CXCL12. The results were similar to the effects of CXCR4 and CXCL12 silencing. While, AMD3100 or TFB alone fail to decrease the protein levels of CXCR4, EAAT1 and GS compared with the control groups, respectively (**Figure 2C**). Similarly, as shown in **Figure 3B**, QPCR results showed that TFB obviously inhibited CXCL12-increased the expression of *CXCR4*, *EAAT1* and *GS* mRNA.

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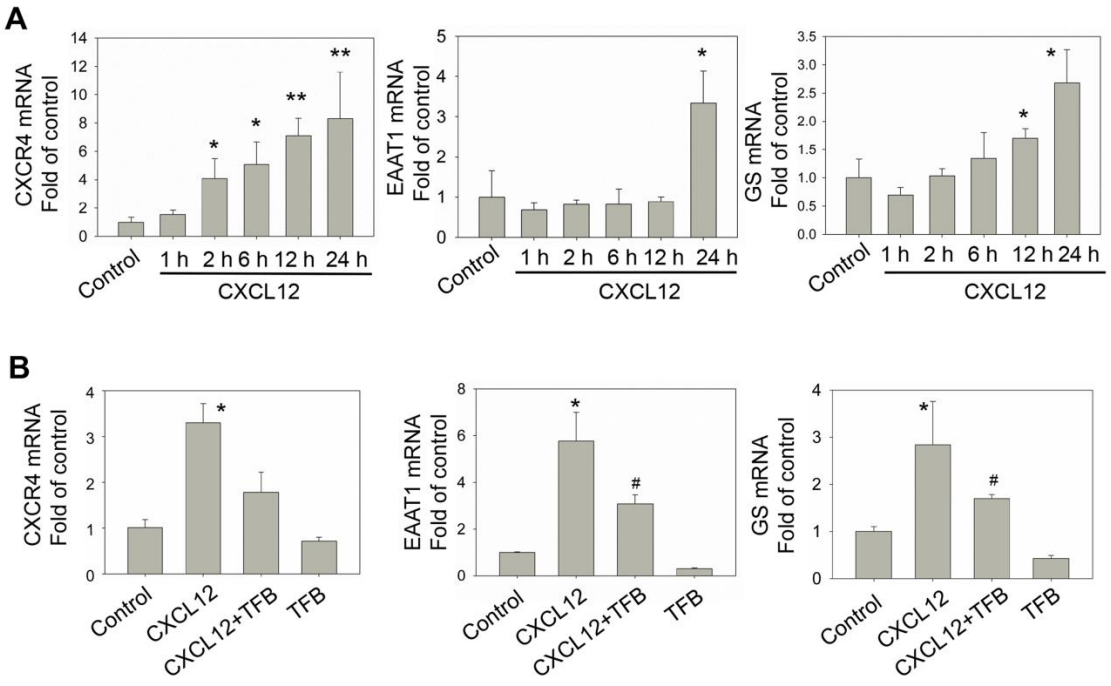

Figure 3. Effects of CXCL12 on the levels of CXCR4, EAAT1 and GS mRNA in MPM cell line. (A), H2052 cells were incubated in the presence of CXCL12 (100 ng/mL) for various times (0 – 24 h) and the levels of CXCR4, EAAT1 and GS mRNA were detected by QPCR. (B), H2052 cells were pretreated with TFB for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h. The levels of CXCR4, EAAT1 and GS mRNA were then determined by QPCR. Values were expressed as fold of untreated control levels. * $p < 0.05$, ** $p < 0.01$ versus control. # $p < 0.05$ versus CXCL12 treatment, as indicated. The experiment data were expressed as mean ± SD from triplicates/quadruplicates and repeated three times independently.

2.3. The effects of CXCL12 on cell invasion and migration

The transwell assay was carried out to determine the ffects of CXCL12 on the cell invasion and migration. Exposure to various concentrations of CXCL12 (0 - 100 ng/mL) promoted the invasion (**Figure 4A**) and migration (**Figure 5A**) in H2052 cells in a does dependent manner and both of them reached their highest peak in 100 ng/mL. In addition,

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MTT was also performed to evaluate the cell proliferation. As shown in **Figure S2A** and **S2B**, CXCL12 (0 - 100 ng/mL) gradually promoted the proliferation in H2052 cells. AMD3100 (0 - 10 μM) gradually inhibited the proliferation in H2052 cells (**Figure S2C**).

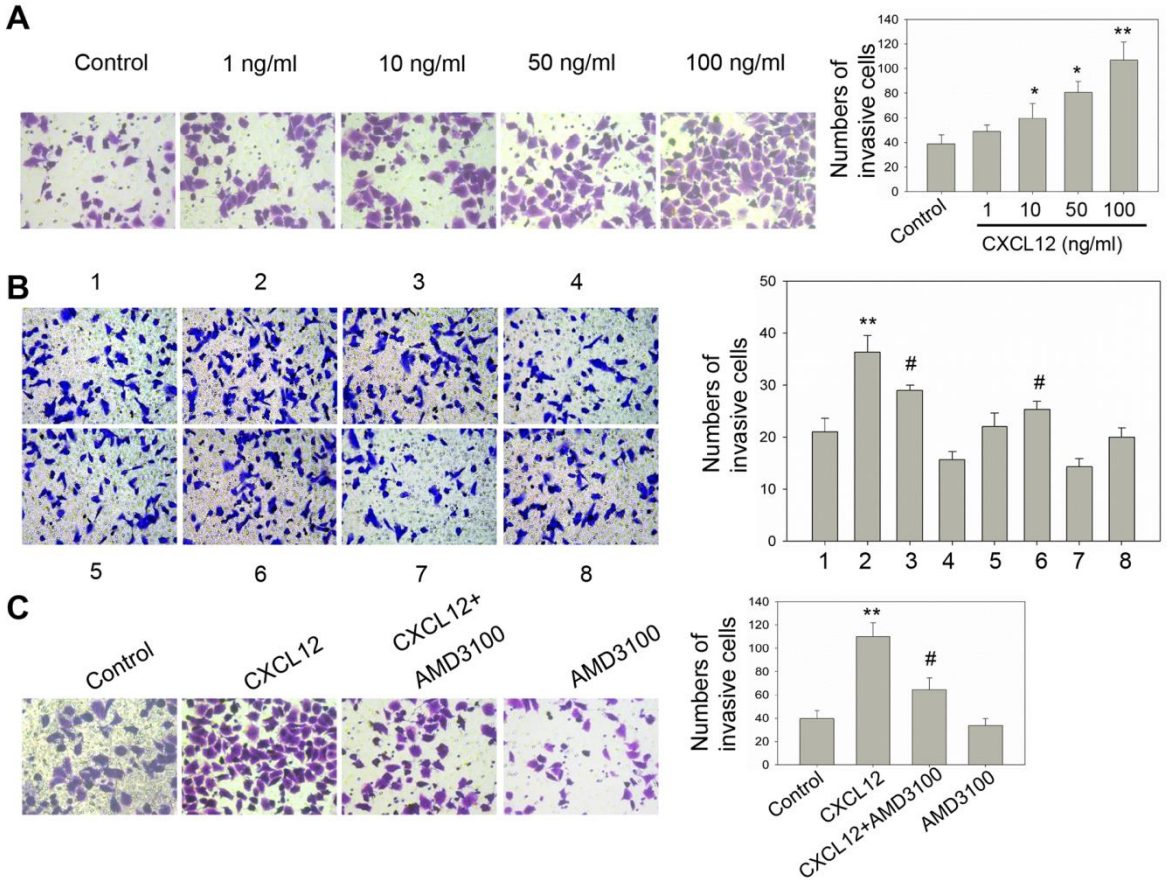

Figure 4. Effects of CXCL12 on the invasion in MPM cell line. (A), H2052 cells were treated with various concentrations of CXCL12 (0 - 100 ng/mL) for 24 h and subsequently the cells invasion were evaluated by transwell assay. Representative images from different groups were shown in left panal and the corresponding quantitative analysis was also presented in right panal. (B), H2052 cells were transfected with CXCR4-RNAi or EAAT1-RNAi or respective controls and then treated with CXCL12 (100 ng/mL) for 24 h. The cells invasion were evaluated by transwell assay. 1: Control. 2: CXCL12. 3: CXCL12+CXCR4-RNAi. 4: CXCR4-RNAi. 5: CXCR4-RNAi control. 6: CXCL12+EAAT1-RNAi. 7: EAAT1-RNAi. 8: EAAT1-RNAi control. Representative images from different groups were shown in left panal and the corresponding quantitative analysis was also presented in right panal. (C), H2052 cells were pretreated with AMD3100 for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h. The cells invasion were evaluated by transwell assay.

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Representative images from different groups were shown in left panal and the corresponding quantitative analysis was also presented in right panal. * $p < 0.05$, ** $p < 0.01$ versus control. # $p < 0.05$ versus CXCL12 treatment, as indicated. The data were expressed as mean ± SD from repeated three independent experiments.

To further define the effects of CXCR4 and EAAT1 on the cell invasion and migration, H2052 cells were transfected with CXCR4 siRNA or EAAT siRNA alone, in the presence or absence of CXCL12 treatments or vehicle. The results showed that CXCR4 siRNA or EAAT1 siRNA significantly suppressed CXCL12-promoted the H2052 cells invasion. CXCR4 siRNA or EAAT1 siRNA alone also slightly inhibited H2052 cells invasion (**Figure 4B**). Similar effects were also observed in H2052 cells migration (**Figure 5B**). Furthermore, H2052 cells were treated with the CXCL12, AMD3100 alone, AMD3100 followed by CXCL12. The results showed that CXCL12-promoted the cells invasion remarkably suppressed by AMD3100 (**Figure 4C)**. Similar effects were also observed in H2052 cells migration (**Figure 5C**). These results indicated that CXCL12 regulated the invasion and migration also through CXCR4/EAAT1 pathway in H2052 cells.

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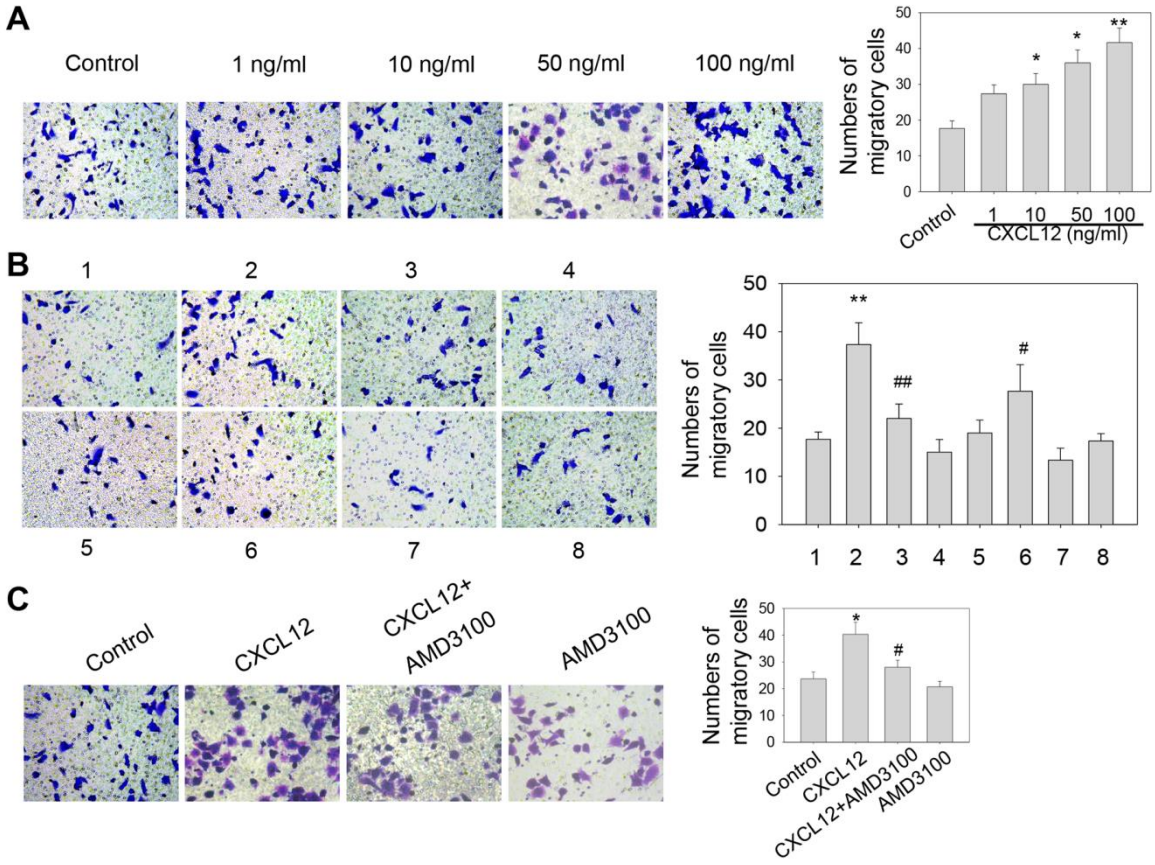

Figure 5. Effects of CXCL12 on the migration in MPM cell line. (A), H2052 cells were treated with various concentrations of CXCL12 (0 - 100 ng/mL) for 24 h and subsequently the cells migration were examined by transwell assay. Representative images from different groups were shown in left panal and the corresponding quantitative analysis was also presented in right panal. (B), H2052 cells were transfected with CXCR4-RNAi or EAAT1-RNAi or respective controls and then treated with CXCL12 (100 ng/mL) for 24 h. The cells invasion were examined by transwell assay. 1: Control. 2: CXCL12. 3: CXCL12+CXCR4-RNAi. 4: CXCR4-RNAi. 5: CXCR4-RNAi control. 6: CXCL12+EAAT1-RNAi. 7: EAAT1-RNAi. 8: EAAT1-RNAi control. Representative images from different groups were shown in left panal and the corresponding quantitative analysis was also presented in right panal. (C), H2052 cells were pretreated with AMD3100 for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h. The cells invasion were examined by transwell assay. Representative images from different groups were shown in left panal and the corresponding quantitative analysis was also presented in right panal. * $p < 0.05$, ** $p <$

0.01 versus control. # $p < 0.05$, ## $p < 0.01$ versus CXCL12 treatment, as indicated. The data were expressed as mean ± SD from repeated three independent experiments.

In addition, wound healing assay in vitro was also performed to assess the cell migration ability. H2052 cells were treated with the indicated agents for 24 h and 48 h. As shown in **Figure S3A** and **3B,** the results were consistent with H2052 cells invasion and migration by transwell assay.

2.4. QPCR for MMP9

As is known to all, MMPs are frequently expressed in nearly all tumors, where they accelerates tumor progression. Among these MMPs, MMP9 is particularly known to play an important role in tumor progression, including tumor angiogenesis and growth as well as metastasis to distant organs [45,46]. Therefore, we focused on MMP9 and detected its expression in H2052 cells invasion. The result showed that CXCL12 slightly promoted the MMP9 mRNA level in 5 min (**Figure S4**). Moreover, H2052 cells were transfected with CXCR4 siRNA or EAAT siRNA alone, in the presence or absence of CXCL12 treatments or vehicle. As shown in **Figure 6A**, the CXCL12 significantly increased the MMP9 mRNA level in 24 h, while this effect was obviously suppressed by CXCR4 siRNA or EAAT1 siRNA. CXCR4 siRNA or EAAT1 siRNA alone also decreased the MMP9 mRNA level, respectively. Similarly, AMD3100 also inhibited CXCL12 - increased the MMP9 mRNA level (**Figure 6B**). All these data indicates that MMP9 is involved in the cell invasion.

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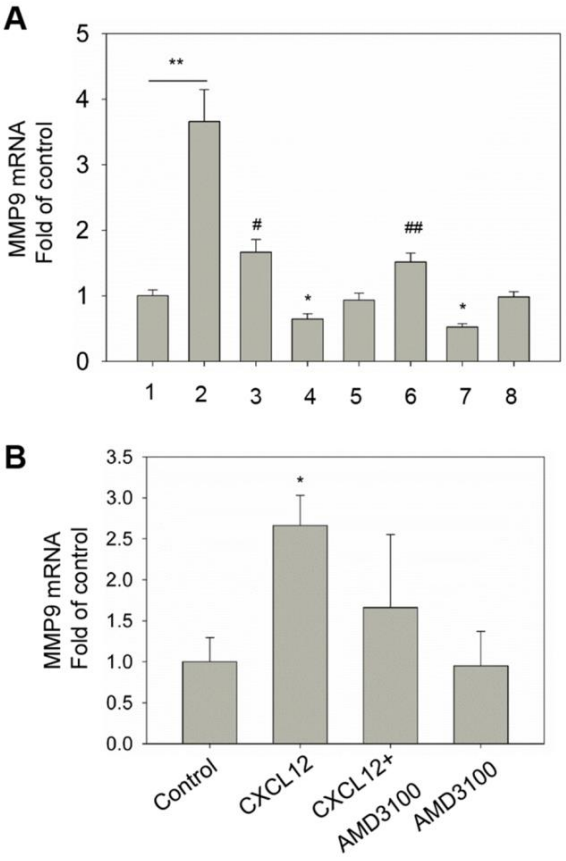

Figure 6. Effect of CXCL12 on the MMP9 mRNA level in MPM cell line. (A), H2052 cells were transfected with CXCR4-RNAi or EAAT1-RNAi or respective controls and then treated with CXCL12 (100 ng/mL) for 24 h and the MMP9 mRNA was determined by QPCR. 1: Control. 2: CXCL12. 3: CXCL12+CXCR4-RNAi. 4: CXCR4-RNAi. 5: CXCR4-RNAi control. 6: CXCL12+EAAT1-RNAi. 7: EAAT1-RNAi. 8: EAAT1-RNAi control. (B), H2052 cells were pretreated with AMD3100 for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h. The MMP9 mRNA was then determined by QPCR. Values were expressed as fold of untreated control levels. * $p < 0.05$, ** $p < 0.01$ versus control. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ versus CXCL12 treatment, as indicated. The data shown were representative of three independent experiments.

2.5. In vivo experiments

Two weeks after inoculation, the tumors were obviously visible on more than half of the mice's back (**Figure S5A**). The mice were excluded and not counted if no tumor could be visible on the mice back. The final number of each group was six (n = 6). The tumor

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sizes of all four groups increased. Fluorescence signal (**Figure S5B**) was enhanced from 2 weeks to 5 weeks in all groups. The changes of fluorescence signal values from four groups in 1 week, 2 weeks and 3 weeks after the treatments were shown in **Table S3**. The changes of fluorescence signal values had no significant difference between four groups in 1 week and 2 weeks. After 3 weeks of treatment, the changes of fluorescence signal values from each treatment group were remarkably different from the control group (**Table S4, $p < 0.05$**). Compared with control group, CXCL12 promoted the growth of H2052 cells in nude mice, both AMD3100 and TFB inhibited the growth of H2052 cells in nude mice.

3. Discussion

Chemokines are thought to play a critical role in tumor growth, invasion and progression [47]. Previous studies have showed that there is obvious correlation between multiple chemokine receptors in many kinds of human malignant tumor metastases, including gastric carcinoma [48], T-cell leukemia [49], osteosarcoma [50], colorectal cancer [51,52], hepatocellular carcinoma [53], ovarian cancer [54], malignant melanoma [55,56], and renal cell carcinoma [57]. Our previous studies [43,44] indicated that both chemokines (CXCL12/CXCR4) and Glu system (EAAT1/GS) were involved in MPM tumorigenesis. In the present study, we found that CXCL12, CXCR4, EAAT1 and GS staining were more positive in MPM samples than that of in normal pleura samples. These results indicated that CXCL12, CXCR4, EAAT1 and GS were highly expressed in MPM samples. According to the multiple correlation analysis, CXCL12, CXCR4, EAAT1 and GS had strong correlations with each other in MPM samples except between CXCL12 and GS. In normal pleura samples, there were no strong correlations between them according to p values, but there were weaker correlations between them according to R^2 values. This difference may be due to the limited sample sizes.

In order to explore the potential regulatory effects of CXCL12/CXCR4 axis on EAAT1/GS. We performed siRNA knockdown and specific antagonists blocking experiments. Our results for the first time demonstrated that CXCL12 upregulated the

 doi:10.20944/preprints202009.0380.v1

expression of EAAT1 and GS via CXCR4, and CXCL12 upregulated the expression of GS protein via EAAT1. CXCR4 silencing alone decreased the expression levels of EAAT1 and GS proteins compared to control siRNA, but conversely, EAAT1 silencing alone failed to decrease the CXCR4 protein level compared to control siRNA, which indicates that EAAT1 is a downstream factor of CXCR4 and GS is a downstream factor of EAAT1. It is noticeable that according to our results (**Figure 2B**), EAAT1 silencing conversely slightly inhibited CXCL12-upregulated the expression of CXCR4. This suggests that one possible mutual regulatory relationship exists between them. However, the impact of CXCR4 on EAAT1 is greater than that of EAAT1 on CXCR4.

Many recent studies reported that CXCL12/CXCR4 axis induces cell proliferation, invasion and migration, including cancer cells, such as human gastric cancer [58], endometrial cancer [59], epithelial ovarian cancer [60], cervical cancer [61], ovarian cancer [62], breast cancer [63], adamantinomatous craniopharyngiomas [64] and non-cancer cells, such as osteoblast [65], fibroblasts [66] and bone marrow stem cells [67]. In the present study, we also investigated the roles of CXCL12/CXCR4 axis in MPM cells proliferation, invasion and migration. Our results from **Figure 4, 5** and **6** and **Figure S2, S3** and **S4** showed that CXCL12/CXCR4 axis promoted proliferation, invasion and migration in MPM cells, which were consistent with those previous results. Additionally, we detected the invasion-associated protease, such as MMP9, which was involved in cells invasion. Moreover, to assess the role of EAAT1 in invasion and migration of MPM cells, we used specific EAAT1 siRNA to kncockdown the EAAT1 expression and found that CXCL12-promoted the H2052 cells invasion and migration were significantly suppressed by EAAT1 silencing, and to a certain extent, EAAT1 silencing alone also inhibited H2052 cells invasion and migration. Besides, CXCL12-increased the expression of MMP9 mRNA was remarkably inhibited by EAAT1 silencing. These results denoted that CXCL12 regulated the invasion and migration also through CXCR4/EAAT1/GS pathway in H2052 cells.

Our recent study showed that AMD3100 could inhibit the growth of MPM in vivo [68]. In this study, the results showed that CXCL12 promoted the growth of MPM in vivo. AMD3100 also inhibited the growth of MPM in vivo, which was consistent with our

 doi:10.20944/preprints202009.0380.v1

previous report. This indicates that the progression of MPM is associated with CXCL12/CXCR4. Besides, in vivo results showed that TFB treatment also inhibited the growth of MPM in vivo. All these results suggested that CXCL12/EAAT1 may be another axis that affects the cell proliferation, invasion and migration in MPM cells. The CXCL12 binding the CXCR4 receptor activated divergent signals on multiple pathways, such as ERK1/2, p38, SAPK/JNK, AKT, mTOR [17], which reminds us that CXCL12/EAAT1 axis proposed also may activate certain signaling pathway molecules in cancer cell, and thus represents a potential target for cancer treatment.

In sum up, Although we obtained the first evidance that EAAT1 is a downstream factor of CXCR4 and GS is a downstream factor of EAAT1. CXCL12 regulated the invasion and migration through CXCR4/EAAT1/GS pathway in H2052 cells. There are still some limitation in our research, for example, CXCL12/CXCR4- or CXCL12/EAAT1-mediated downstream signaling pathways involved in cell invasion and migration needs further study. Therefore, our next research will attempt to explore the molecular signal pathways and mechanisms involved. It will bring us a deeper insights of clinical significance and provide us with many potential candidate targets and theoretical basis for drug development.

4. Materials and Methods

4.1. Reagents, drugs and antibodies

RNA extraction reagent and real-time quantitative PCR (QPCR) kit were purchased from Invitrogen (Carlsbad, CA, USA). Reverse transcriptional system (First Strand cDNA Synthesis Kit) was purchased from Promega (Medison, WI, USA). Fetal bovine serum (FBS), RPMI 1640 medium and phosphate buffer saline (PBS) were from Gibco (Carlsbad, CA, USA). Trypsin was purchased from Life Technologies (Waltham, MA, USA). Human CXCL12 was purchased from Upstate (Lake Placid, NY, USA). AMD3100 and TFB-TBOA (TFB) were purchased from Sigma (St. Louis, MO, USA). Matrigel and Propidium

 doi:10.20944/preprints202009.0380.v1

Iodide (PI) were obtained from BD Pharmingen (Franklin Lakes, NJ, USA). Transwell chambers were purchased from Corning Costar (Corning, NY, USA).

4.2. Cell culture

Human malignant pleural mesothelioma (MPM) cell line H2052 was purchased from the Cell Culture Core Facility at Harvard University (Boston, MA, USA). The H2052 cells had been transfected with luciferase reporter gene and could stably express luciferase activity. The H2052 cells were maintained and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml) and incubated at 37 ºC with humidified air containing 5% CO_2.

4.3. Immunohistochemistry (IHC) staining

The tissue samples from 41 patients diagnosed with MPM were fixed in formalin and embedded in paraffin in 4 - μm tissue microarray sections, which contained normal pleura tissue samples from another three patients. After routine processing, the sections were incubated with corresponding primary anti-CXCR4 (R&D Systems, Minneapolis, MN, USA), anti-CXCL12 (R&D Systems, Minneapolis, MN, USA), anti-EAAT1 (Cambridge, UK) or anti-GS antibody (Cambridge, UK), respectively, and then followed by routine immunostaining progress with the commercial kit (Maixin Biotechnology, Fuzhou, China) according to the manufacturer's instructions. The IHC, HE staining procedures and the scoring system were carried out referring to our previous study [43].

4.4. Western blot

The H2052 cells in 6-well culture plates were washed three times with cold PBS and directly lysed with lysis buffer (Pierce, Rockford, IL, USA). The lysates were then centrifuged at 4° C, 15,000 rpm, for 15 min. Protein concentration was quantified using the Bradford reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Equal amounts of total protein were resolved by standard 4 ~ 15% sodium

dodecyl sulfate polyacrylamide gels electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose membranes (0.45 μm, Millipore Millex, USA). Membranes were blocked with 5% non-fat milk for 1 hour at room temperature, then washed with TBST and incubated overnight at 4˚ C with appropriate primary anti-CXCR4 (Abcam, Cambridge, UK), anti-glutamine synthetase (GS) (Abcam, Cambridge, UK), anti-EAAT1 (CST, Danvers, MA, USA) and anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibody (CST, Danvers, MA, USA) at the dilution recommended. The membranes were washed with TBST to remove the primary antibodies and then incubated for 1 hour with appropriate horseradish peroxidase (HRP)- conjugated secondary antibody IgG (Santa Cruz, CA, USA). GAPDH was used as a loading control. The protein bands were detected by SuperSignal west Femto (Thermo, Rockford, IL, USA) and Chemilmager 5500 (Alpha Innotech, Santa Clara, CA, USA).

4.5. RNA isolation and real-time PCR (QPCR)

Total RNA was extracted from the H2052 cells by TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was reverse transcribed from total RNA by First Strand cDNA Synthesis Kit (Promega, Medison, WI) according to the manufacturer's instructions. QPCR was carried out with the QPCR kit (Invitrogen, Carlsbad, CA) with the cDNA as a template. According to the protocol, cocktails (2X Real-Time SYBR PCR Master Mix, Template cDNA, Forward and Reverse primer pairs and Nuclease-Free water) were incubated at 50˚ C for 2 min and then denatured at 95˚C for 10 min followed by 40 cycles at 95˚ C for 15 s, and then 1 min of final extension at 60˚C. PCR for GAPDH was used as a control for RNA quantity. The ABI7500 real-time PCR system was performed to determine the mRNA expression. The forward and reverse primer pairs of CXCL12, CXCR4, GS, EAAT1, MMP9 and GAPDH were shown in **Table S1.**

4.6. Cell invasion and migration

Transwell chambers were dipped in 500 μL (1:100) Matrigel for 5 min, dried in the air, and inserted in 24-well flat-bottom plates overnight, then 200 μl invasion buffer was

added into the each well. The H2052 cells were serum-starved for 12 h, harvested, washed, resuspended in invasion buffer, and the density was adjusted to 5×10^5 cell/mL with RPMI 1640 media. The invasion buffer of the upper Transwell chamber was abandoned carefully. 600 μl medium containing 1% FBS was added into the lower chamber. Subsequently, 100 μL of cell suspension was added into the upper chamber carefully to bestrew the cells on the filter. The cells were subjected to various treatments as indicated in figure. And then, the Transwell chambers were incubated in 37°C and 5% CO_2 for 24 h. The transwell membrane of upper chamber was collected and the matrigel was removed by cotton tips. After fixation with methanol and staining with 0.1% crystal violet, the number of the invaded cells were counted randomly under a microcopy in 5 fields of visions (200 × magnification). For cell migration, no Matrigel is needed and the experimental procedures are the same with cell invasion assay.

4.7. Statistical analysis

Statistical analysis was carried out with SPSS 21.0 (SPSS Inc, Chicago, USA). Data were expressed as mean ± S.D. Student's t-test or one way ANOVA was used to detect the differences between different groups. For correlation analysis, 0, 1, 2 and 3 represented -, +, ++ and +++, respectively during the calculation. $P < 0.05$ was considered to be of significance.

5. Conclusions

CXCL12 regulated the invasion and migration through CXCR4/EAAT1/GS pathway in H2052 cells.

6. Patents

Fresh primary human MPM samples from 41 patients were obtained (the patients diagnosed with MPM underwent surgical removal of the primary tumor). The normal pleura tissue samples were obtained simultaneously to serve as controls from seven of these patients. We

 doi:10.20944/preprints202009.0380.v1

confirmed that we had the informed consents from all patients and all tissue samples were analyzed complying with procedures approved by the institutional review board of the University of California, San Francisco (IRB H8714-22942-01) and Capital Medical University, Beijing.

Supplementary Materials

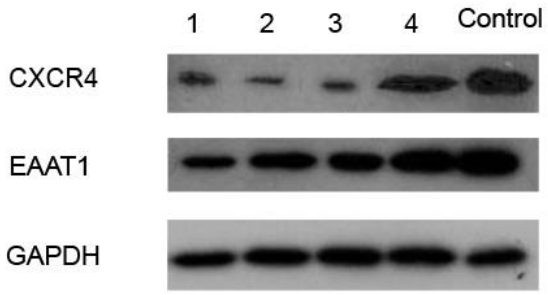

Figure S1. Western blot analysis was performed to validate the transfection effects of respective siRNA. H2052 cells were transfected with negtive control siRNA or siRNA targeted to CXCR4 and EAAT1, respectively. Both the targeted CXCR4 and EAAT1 were downregulated. 1, 2, 3, 4 represents siRNA1, siRNA2, siRNA3, siRNA4 targeted to CXCR4 and EAAT1, respectively.

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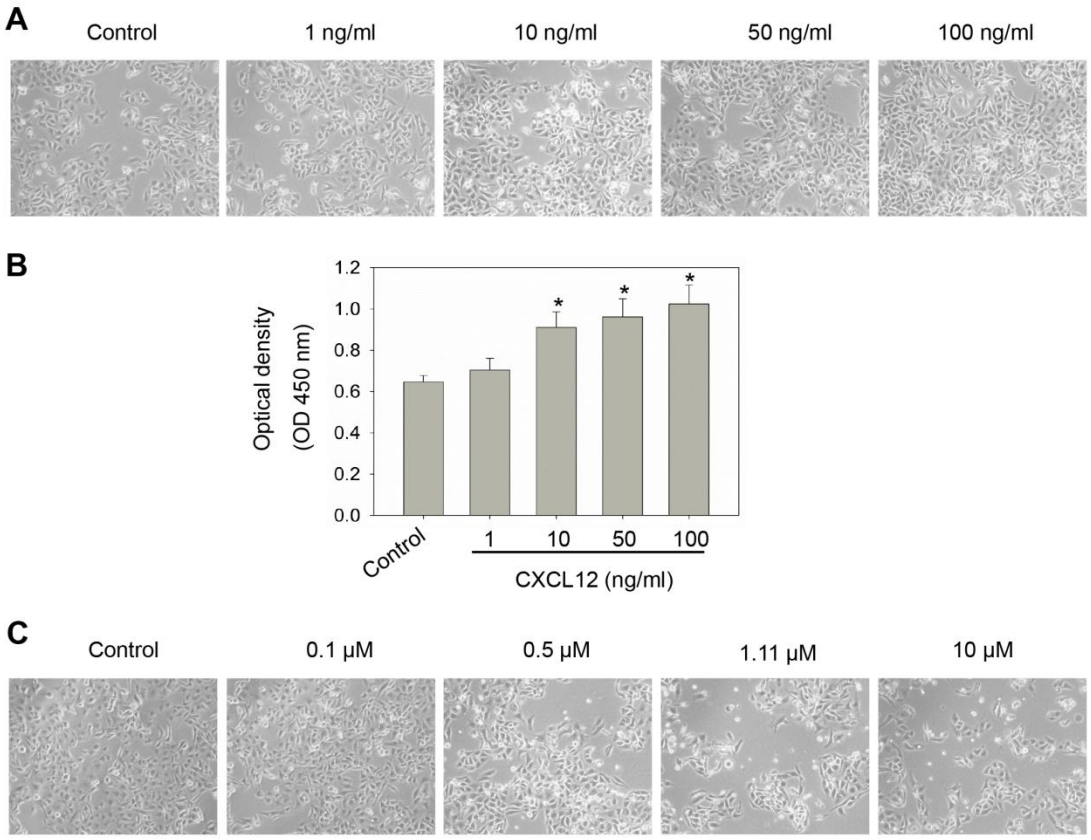

Figure S2. Effect of CXCL12 on the viability in MPM cell line. H2052 cells were treated with various concentrations of CXCL12 (0 - 100 ng/mL) (A) or AMD3100 (0 - 10 μM) (C) for 24 h and morphological images in vitro from different treatment groups were shown. (B), The cell viability was evaluated by MTT assay. * $p < 0.05$ versus control, as indicated. The data were expressed as mean ± SD from quadruplicates and repeated three times independently.

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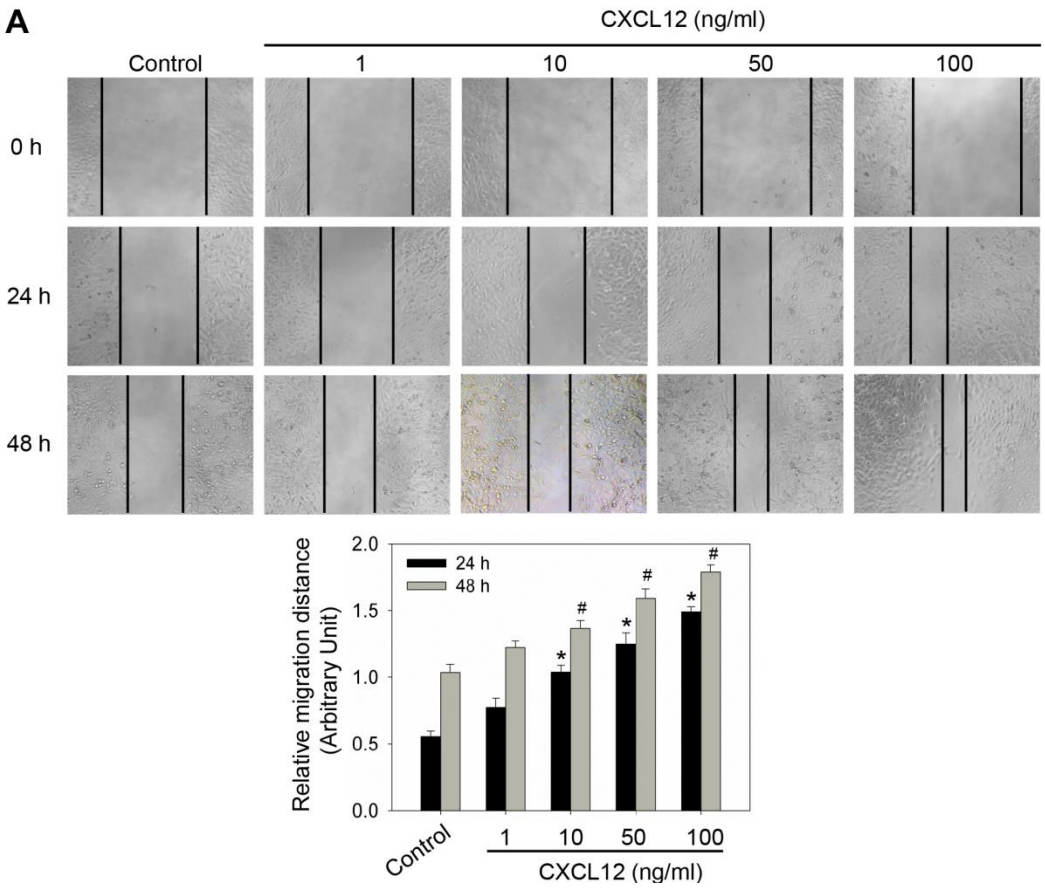

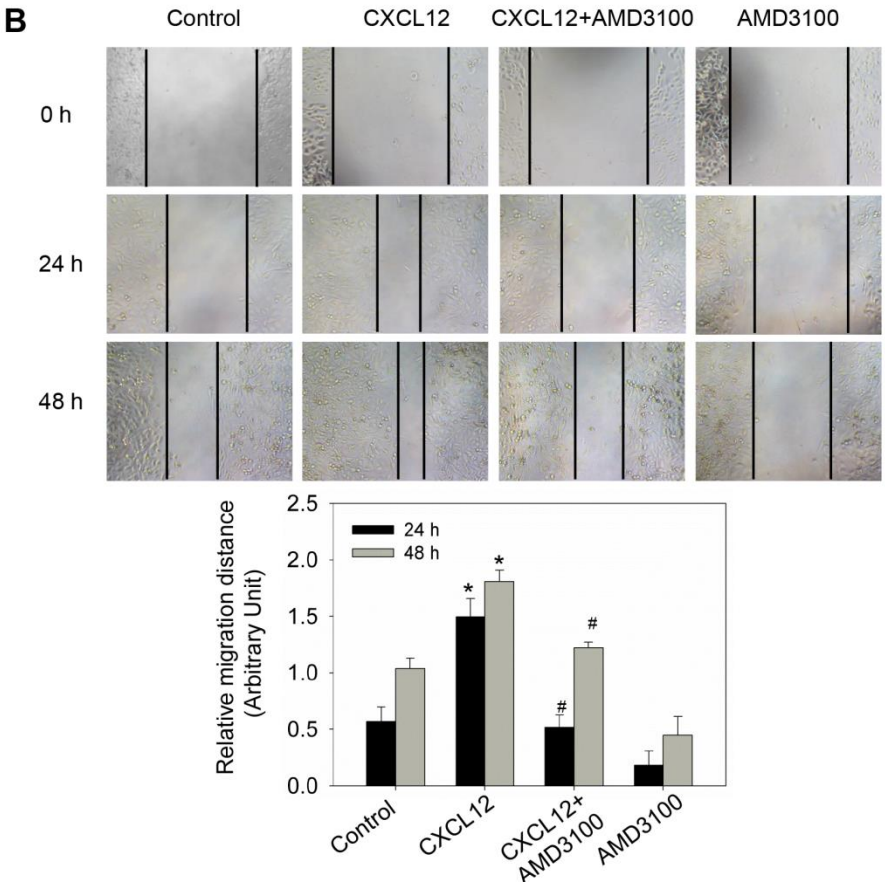

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Figure S3. Effects of CXCL12 on the migration in MPM cell line. (A), H2052 cells were treated with various concentrations of CXCL12 (0 - 100 ng/mL) for 24 h, 48 h and subsequently the cells migration were examined by wound healing assay. Representative images (upper panal) and the corresponding quantitative analysis (lower panal) from different groups were shown. (C), H2052 cells were pretreated with AMD3100 for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h and 48 h. The cells migration were examined by wound healing assay. Representative images (upper panal) and the corresponding quantitative analysis (lower panal) from different groups were pesented. Values were expressed as relative migration distance (relative ratio of different treatment groups to untreated control). * $p < 0.05$ versus control. # $p < 0.05$ versus CXCL12 treatment, as indicated. The data were expressed as mean ± SD from repeated three independent experiments.

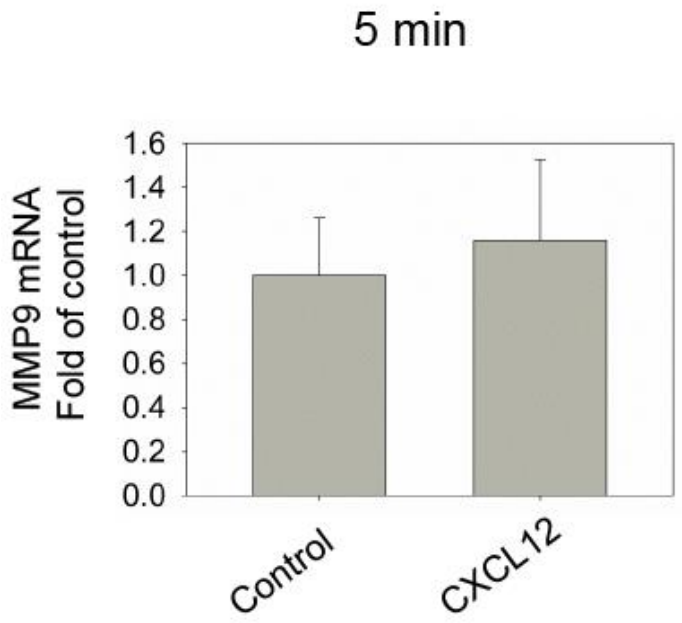

Figure S4. Effect of CXCL12 on the MMP9 mRNA level in MPM cell line. H2052 cells were treated with or without CXCL12 (100 ng/mL) for 5 min and the MMP9 mRNA was determined by QPCR. Values were expressed as fold of untreated control levels. The data shown were representative of three independent experiments.

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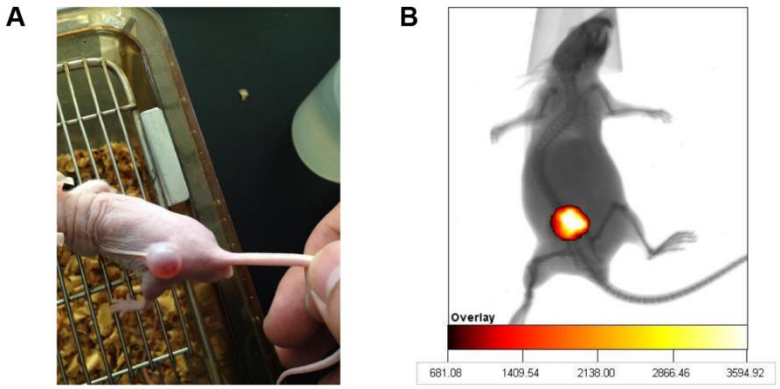

Figure S5. Effect of CXCL12 on tumor growth in nude mice. 2×10^6 H2052 cells were inoculated subcutaneously (S.C.) into the left dorsal of nude mice, and the mice were treated with AMD3100, TFB, CXCL12 or vehicle starting 2 weeks after implantation, as described in **Materials and methods**. Obvious tumor appearance and (A) and bioluminescence/fluorescence signal value (B) could be visible on the mouse back.

Table S1. Primer pairs of CXCR4, GS, EAAT1, MMP9 and GAPDH

Target gene	Primer pairs	
	Forward primer (5' - 3')	Reverse primer (5' - 3')
CXCR4	TCAGTCTGGACCGCTACCTG	GGGATCCAGACGCCAACATA
GS	CAATCGAAGGCCTGCAGAGA	ATACTCCTGCTCCATGCCAA
EAAT1	TTGCTGCAAGCACTCATCAC	GCTTGTCCACGCCATTGTTC
MMP9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
GAPDH	CTTCTTTTGCGTCGCCAGCC	GGCGCCCAATACGACCAAA

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Table S2. CXCR4 and EAAT1 siRNA oligonucleotide sequences

Gene	SiRNA oligos (5'-3')	
	Sense	Anti-sense
CXCR4(1)	GGCUGAAAAGGUGGUCUAUTT	AUAGACCACCUUUUCAGCCTT
CXCR4(2)	GCCUUACUACAUUGGGAUCTT	GAUCCCAAUGUAGUAAGGCTT
CXCR4(3)	CAAGCAAGGGUGUGAGUUUTT	AAACUCACACCCUUGCUUGTT
CXCR4(4)	GCACAAGUGGAUUUCCAUCTT	GAUGGAAAUCCACUUGUGCTT
CXCR Control	ACCAGGUUAUUGCAGUACGTT	CGUACUGCAAUAACCUGGUTT
EAAT1(1)	CGGGGAAUAUUAUCAGAUGTT	CAUCUGAUAAUAUUCCCCGTT
EAAT1(2)	CCAUAACCAGCUAUACCUUTT	AAGGUAUAGCUGGUUAUGGTT
EAAT1(3)	CCAAGAAGAAAGUGCAGAATT	UUCUGCACUUUCUUCUUGGTT
EAAT1(4)	CACUGAAGUGCAAAGAAGATT	UCUUCUUUGCACUUCAGUGTT
EAAT1 Control	ACCAGGUUAUUGCAGUACGTT	CGUACUGCAAUAACCUGGUTT

Table S3. The changes of fluorescence signal values of four groups

After starting of treatments	Control	CXCL12	AMD3100	TFB
1 week	0.98 ± 1.46	1.31 ± 1.31	-0.16 ± 0.97	0.75 ± 1.23

 doi:10.20944/preprints202009.0380.v1

2 weeks	2.80 ± 3.20	58.44 ± 63.82	2.13 ± 3.50	3.82 ± 3.24
3 weeks	6.89 ± 3.26	43.00 ± 36.58	0.97 ± 0.82	2.35 ± 2.49

Table S4. p values between groups at 3 weeks after starting of treatments

	AMD3100	Control	CXCL12	TFB
AMD3100		P = 0.0021	P = 0.0002	P > 0.05
Control			P = 0.0368	P = 0.0157
CXCL12				P = 0.0014
TFB				

Author Contributions

Shuo Li, Tong Li and Qiang Lin: Conceptualization, Methodology, Software; Shuo Li, Tong Li and Qiang Lin: formal analysis; Debing Shi, Shengcai Hou, Orli Kadoch, Yilin Yang and Zhidong Xu: resources; Shuo Li, Tong Li, Qiang Lin and Debing Shi: Data curation; Shuo Li, Tong Li and Qiang Lin: Writing- Original draft preparation; Tong Li: Visualization; Shuo Li, Tong Li, Qiang Lin, Debing Shi, Haishi Zheng, Jin Zhang and Hui Li: Investigation; Tong Li: Supervision, Tong Li: Validation, Tong Li: Writing- Reviewing and Editing, Tong Li: project administration, Tong Li: funding acquisition

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Conflicts of Interest

The authors declare no conflict of interest and the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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