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*Original Article*

# Down-regulation of CXCL12 by hypermethylation induces cell proliferation, growth and invasion in triple-negative breast cancer

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**Abstract:** We previously reported that CXCL12 was reduced in breast cancer, and its expression was associated with clinicopathological factors and prognosis. However, the epigenetic regulation and molecular functions of CXCL12 in Triple-negative breast cancer (TNBC) remain unknown. In this study, CXCL12 expression levels and methylation status of its promoter region in TNBC cell lines and TNBC tissues (n=105) as well as normal breast tissues (n=105) were assessed by RTq-PCR and methylation analysis, respectively. The cellular functions of CXCL12 on TNBC cell proliferation, growth and invasion were investigated *in vivo* and *in vitro*. Our data revealed that CXCL12 was frequently silenced by promoter hypermethylation in both tested TNBC cell lines and primary TNBC, and correlation analysis between methylation status and clinicopathological parameters found that TNBC methylation was significantly correlated with lymph node status and TNM stage, while no significant correlation was found in other parameters. In addition, demethylation treatment resulted in re-expression of CXCL12 in TNBC cell lines, and cellular function experiments revealed that restoration of CXCL12 inhibited MDA-MB-231 cell proliferation and suppressed cell invasion. Furthermore, animal studies revealed that nude mice injected with the Lentivirus-CXCL12 cell lines featured a lighter weight than the control cells. These data suggest that CXCL12 may function as a tumor suppressor gene, which is down-regulated through promoter hypermethylation in TNBC development.

**Keywords:** Triple-negative breast cancer, CXCL12, DNA mehtylation, cell proliferation, cell invasion

## Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive disease characterized by a high number of relapses and poor overall survival [1]. The heterogeneity of the disease and the limited treatment options compared to other breast cancer subtypes mainly explain these clinical outcomes [2]. New biomarkers are urgently needed to improve the management of TNBC. In the development of TNBC growing numbers of tumor suppressor genes have been reported to be inactivated by genetic and epigenetic alteration. Promoter hypermethylation, one of the well-studied epigenetic modifications, has been recognized as an important mechanism for gene silencing during the early stage of TNBC development, which highlights the importance of promoter hypermethylation in the tumorigenesis of TNBC and provide important biomarkers for TNBC [3].

Chemokines have multiple roles in many kinds of physiologic processes, such as hematopoiesis, lymphocyte development, and wound healing [4, 5]. Recent findings demonstrated that there is a close relationship between tumor cells and chemokines [6-8]. Cancer cell invasion and metastasis shares many similarities with the process by which leukocytes enter inflamed tissues [9]. Numerous studies indicate that chemokine receptors are expressed by tumor cells, while chemokines are expressed at organs that turn into metastatic targets [10, 11]. To date, the CXCL12-CXCR4 pair was found to be involved in almost all malignancies that

were studied, including many solid cancers and tumors of a hematopoietic origin. In most cases, the CXCL12-CXCR4 pair was found to be associated and/or involved with increased malignancy and metastasis, acting at many different levels [12, 13], and another CXCL12 receptor, CXCR7, was shown to promote the survival of tumor cells by preventing apoptosis, increased adhesion properties and dissemination, but did not mediate chemotaxis towards CXCL12 [14, 15].

Recent studies have also reported that the epigenetic down-regulation of CXCL12 modulates the metastatic potential of gastric, colon carcinoma, and non-small cell lung cancer [16-18]. And we previously reported that CXCL12 was reduced in breast cancer, and its low-expression was associated with tumor size, lymph node metastasis, TNM stage and Her-2 expression [19]. However, the mechanism of CXCL12 gene silencing in primary tumors, especially in TNBC, is largely unknown. In this study, we first analyzed the mRNA expression level and promoter methylation status of CXCL12 in TNBC cell lines and tumor specimens. We also explored whether the decreased CXCL12 expression occurred as a result of aberrant methylation of the gene. Then, we investigated the cellular functions of CXCL12 on cell proliferation, invasion and xenograft growth in TNBC *in vivo* and *in vitro*.

## Materials and methods

### *Cell line and culture*

Three breast cancer cell lines with a basal-like transcriptional profile (MDA-MB-435, MDA-MB-231, and HCC-1937) and a normal mammary epithelial cell line (MCF-10A) were obtained from the Cancer Research Institute of Beijing, China. These cells were cultivated in T75 tissue culture flasks in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM hydroxyethyl piperazine ethanesulfonic acid, and incubated in humidified incubator containing 5% $CO_2$ at 37°C.

### *Clinical tissue samples*

TNBC primary breast carcinoma samples and normal breast tissues were collected in the Affiliated Hospital of Qingdao University from 2014 to 2015. None of the patients had undergone preoperative chemotherapy or radiation. Written informed consent was obtained from all participants, and research protocols for the use of human tissue were approved by and conducted in accordance with the policies of the Institutional Review Boards at Qingdao University. The histological subtype was determined according to the World Health Organization classification. The TNM stage was determined postoperatively according to the American Joint Committee on Cancer (7th edition), and the histological grade was determined according to the Scarff-Bloom-Richardson grading system.

### *Lentivirus production and transduction*

Virus particles were harvested 48 h after cotransfecting pWPXL-CXCL12 with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G into HEK-293T cells using Lipofectamine 2000 reagent (Invitrogen, USA). MDA-MB-231 cells were infected with recombinant Lentivirus-transducing units plus 6 μg/mL polybrene (Sigma, USA).

### *RNA extraction and real-time RT-PCR*

The cells were grown to subconfluence and then starved for 15 h in serum-free medium to attain quiescence. Total RNA was isolated from these cells using the TRIzol reagent according to the manufacturer's instructions. One microgram of the total cellular RNA was then reverse-transcribed into cDNA for PCR amplification using a kit from Sigma. Amplification consisted of initial 5 min incubation at 95°C and then 30 cycles of amplification using 30 s of denaturation at 95°C, 30 s at 56°C, and 60 s at 72°C. The final extension was set for 10 min at 72°C. All data were expressed as the relative differences between control and treated cells after normalization to GAPDH expression.

### *Protein extraction and Western blotting*

Total cellular protein was extracted using a lysis buffer and quantified using protein quantification reagents from Bio-Rad. Next, 50 μg of the protein were suspended in 5 × reducing sample buffer, boiled for 5 min, electrophoresed on 10% SDS-PAGE gels, and then transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was blocked in 1%

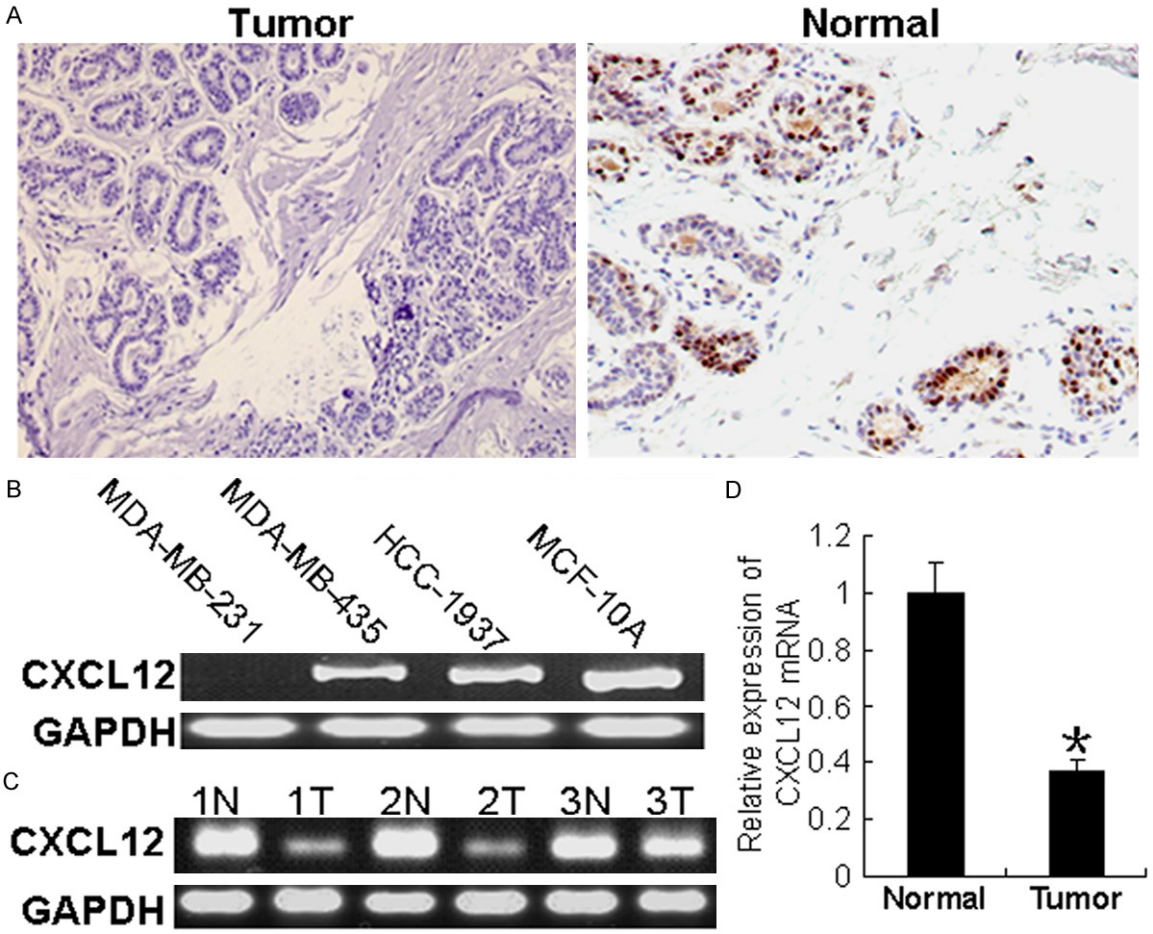


**Figure 1.** CXCL12 expression in TNBC cells and tissues, taking GAPDH as control. A. Immunohistochemical analysis of CXCL12 protein in primary breast carcinomas and corresponding nonmalignant tissues. Original magnification × 100. B. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) analyses on the expression of CXCL12 in MCF-10A and three TNBC cell lines (HCC-1937, MDA-MB-435, and MDA-MB-231). C. CXCL12 mRNA expression in three cases of breast cancer tissues and corresponding non-tumors tissues. D. The expression of CXCL12 was analyzed by RT-PCR in 105 paired breast cancer specimens and corresponding nonmalignant breast tissues. N nonmalignant breast tissues, T primary breast tissues.

BSA/0.05% Tween/PBS solution overnight at 4°C, followed by incubation with the primary antibody for 24 h. A horseradish peroxidase-labelled goat anti-mouse IgG was used as the secondary antibody. The blots were then developed by incubation in a chemiluminescence substrate and exposed to X-ray films.

*Immunohistochemistry*

For immunohistochemical detection of CXCL12, 4 μm histological sections were deparaffinized with xylene and rehydrated through a graded series of alcohol. The sections were then boiled for 10 min in 0.01 M citrate buffer and endogenous peroxidase was blocked by incubation in 0.3% $H_2O_2$ in methanol for 30 min. Nonspecific binding was blocked by incubating slides with normal goat serum for 30 min at room temperature. The sections were incubated overnight at 4°C with a 1:150 dilution of rabbit-CXCL12 antibody. The sections were exposed to biotinlabeled secondary antibody for 1 h, to a streptavidin-peroxidase reaction system, and then developed with DAB-$H_2O_2$.

*Methylation-specific PCR*

The genomic DNA was prepared from cell lines and tissues by the phenol/chloroform protocol and was modified by bisulfite treatment as described previously [20]. Then, DNA (2 μg) was

**Table 1.** Correlation of CXCL12 expression and clinicopathological parameters of TNBC samples

| Clinicopathological variables | Cases (n=105) | Expression of CXCL12 | | *P* value |
|---|---|---|---|---|
| | | Low | High | |
| Age (years) | | | | 0.297 |
| ≤45 | 42 | 21 | 21 | |
| >45 | 63 | 25 | 38 | |
| Tumor Size (cm) | | | | 0.496 |
| ≤2 | 60 | 28 | 32 | |
| >2 | 45 | 18 | 27 | |
| Histological grade | | | | 0.035* |
| Well/moderate | 44 | 14 | 30 | |
| Poor | 61 | 32 | 29 | |
| Lymph node status | | | | 0.031* |
| No | 49 | 16 | 33 | |
| Yes | 56 | 30 | 26 | |
| TNM stage | | | | 0.062 |
| I | 50 | 16 | 34 | |
| II | 27 | 14 | 13 | |
| III | 28 | 16 | 12 | |

*$P<0.05$.

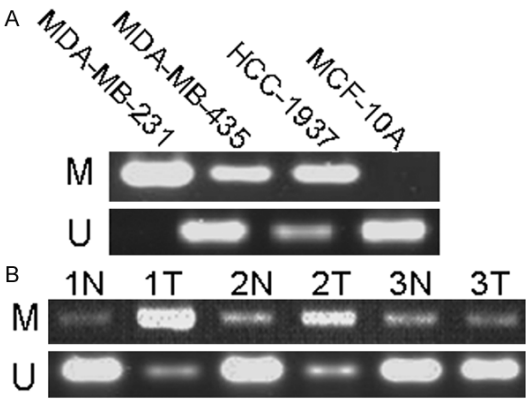


**Figure 2.** CXCL12 methylation analyses in TNBC cell lines and tumors and their corresponding nonmalignant breast tissues by methylationspecific PCR. A. DNA methylation of CXCL12 in MCF-10A and breast cancer cell lines. B. PCR showing DNA methylation of CXCL12 in TNBC specimens and corresponding nonmalignant breast tissues. M methylation, U unmethylation, N nonmalignant breast tissue, T tumor specimens.

purified using a Wizard DNA Clean-Up System, precipitated with ethanol, and resuspended in 30 μl of Tris-EDTA buffer. PCR amplification was performed using 2.0 μl bisulfite-modified DNA in a volume of 50 μl containing 10 × DreamTaq buffer, 2 mM dNTP Mix, 0.4 μM of each primer, and 1.25 U of DreamTaq. The PCR conditions were as follows: 95°C for 10 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing for 30 s at 60°C, extension for 30 s at 72°C, and then a final extension for 10 min at 72°C. CpG genome Universal Methylated and Unmethylated DNA was used as a positive control for the methylated and unmethylated genes, respectively. The amplification products were separated on 2.5% agarose gels.

*Invasion assay*

Invasion assays were performed using the Chemicon Cell Invasion Assay Kit according to the manufacturer's protocol. Briefly, cells ($1 \times 10^4$) were plated onto a Matrigel-coated transwell invasion chamber and incubated at 37°C for 24 h. Non-invading cells were removed by wiping the upper side of the transwell. Invading cells were fixed with methanol and stained with hematoxylin. Three independent invasion assays were performed in triplicate. Five random fields on average were counted using a light microscope.

*In vivo tumor model*

Six-week-old female athymic nude mice were subcutaneously injected with $5 \times 10^6$ cells in 0.2 ml PBS into right scapular region. Three groups (7 each) of mice were tested. Group 1 was injected with MDA-MB-231 cells alone; group 2 was injected with MDA-MB-231 cells stably transfected with Lv-CXCL12; and group 3 was injected with cells stably transfected with Lv-Control. Tumor size was measured every 2 days with calipers. After the mice were killed at 3 weeks, the weight of the tumors was measured.

*Statistics*

For statistical analysis, we used the $X^2$ test and Fisher's exact test for categorical variables, and the Student's *t* test or one-way ANOVA test for continuous variables. Relative mRNA expression levels (CXCL12/GAPDH) were calculated from quantified data. Data are expressed as mean ± SD. For statistical analysis SPSS version 18.0 was used throughout, and *P* values <0.05 were considered significant.

**Table 2.** Clinicopathological parameters of TNBC samples and CXCL12 methylation

| Clinicopathological variables | Cases (n=105) | CXCL12 methylation | | *P* value |
|---|---|---|---|---|
| | | Positive | Negative | |
| Age (years) | | | | 0.265 |
| ≤45 | 42 | 24 | 18 | |
| >45 | 63 | 29 | 34 | |
| Tumor Size (cm) | | | | 0.499 |
| ≤2 | 60 | 32 | 28 | |
| >2 | 45 | 21 | 24 | |
| Histological grade | | | | 0.527 |
| Well/moderateII | 44 | 23 | 21 | |
| Poor | 61 | 30 | 31 | |
| Lymph node status | | | | 0.008* |
| No | 49 | 16 | 33 | |
| Yes | 56 | 37 | 19 | |
| TNM stage | | | | 0.001* |
| I | 50 | 14 | 36 | |
| II | 27 | 18 | 9 | |
| III | 28 | 21 | 7 | |

*$P<0.05$.

## Results

*CXCL12 expression is down-regulated in TNBC cell lines and tissues*

The mRNA expression of CXCL12 was down-regulated in HCC-1937, MDA-MB-435, and MDA-MB-231 cells compared to the normal mammary epithelial cell line MCF-10A. The lowest CXCL12 expression was found in MDA-MB-231. We then examined 105 paired breast cancer specimens and corresponding nonmalignant breast tissues by RT-PCR. We examined the relationship between CXCL12 mRNA exprression and clinicopathological factor. CXCL12 mRNA expression was related to histological grade and lymph node metastasis. CXCL12 mRNA expression level in patients with lymph node metastasis were significantly lower than those in no lymph node metastasis tumors ($P$=0.031) (**Figure 1**; **Table 1**).

*Low expression of CXCL12 is related to DNA methylation*

To identify whether low CXCL12 expression was due to DNA methylation, we first examined the DNA methylation of CXCL12 in breast cancer cell lines. Data from our MSP analysis showed hypermethylation of CXCL12 in MDA-MB-231 cells, and partial methylation in HCC-1937 and MDA-MB-435 cells. No methylation was observed in MCF-10A cells (**Figure 2**). Meanwhile, the results of MSP showed that both unmethylated and methylated bands existed in two breast cancer lines, HCC-1937 and MDA-MB-435 cells, but unmethylated bands did not appear in the MSP was also used to examine CXCL12 methylation in breast specimens, including 105 tumor and corresponding nonmalignant breast tissues. DNA methylation occurred in 50.5% (53/105) of primary breast cancer tissues and 19.5% (20/105) of nonmalignant breast tissues. The difference in methylation between primary breast cancer and nonmalignant breast tissue specimens was significant ($P$<0.01). Furthermore, DNA methylation of CXCL12 was related to the lymph node status and TNM stage. Compared with negative lymph node metastasis, the tumor with lymph node metastasis were more frequently methylated (66.1 vs. 32.7%, $P$=0.008). For TNM stage, only 28.0% (14/50) of tumor showed hypermethylation of CXCL12 in stages I, compared with stages II (66.7%) and III (75.0%) (**Table 2**).

*Reactivation of CXCL12 expression after treatment with 5-Aza-dC*

To confirm that aberrant methylation was responsible for silencing CXCL12 expression, we treated the MDA-MB-231 cell lines with the demethylating agent 5-Aza-dC. The methylation status of CXCL12 of breast cancer cells was modified from methylated to unmethylated by the 5-Aza-dC treatment. As shown in **Figure 3A**, CXCL12 expression significantly increased in MDA-MB-231 cells after treatment with 5-Aza-dC, with the highest expression occurring at a concentration of 10 μM (**Figure 3**).

*Restoration of CXCL12 expression with 5-Aza-dC inhibits the invasion ability of highly metastatic MDA-MB-231 cells*

To further examine whether the reactivation of CXCL12 expression can regulate breast cancer invasion, we analyzed the invasion capability of the highly metastatic MDA-MB-231 cells using the methods described above. The number of MDA-MB-231 cells in the untreated group that migrated through the membrane was 124.1 ±

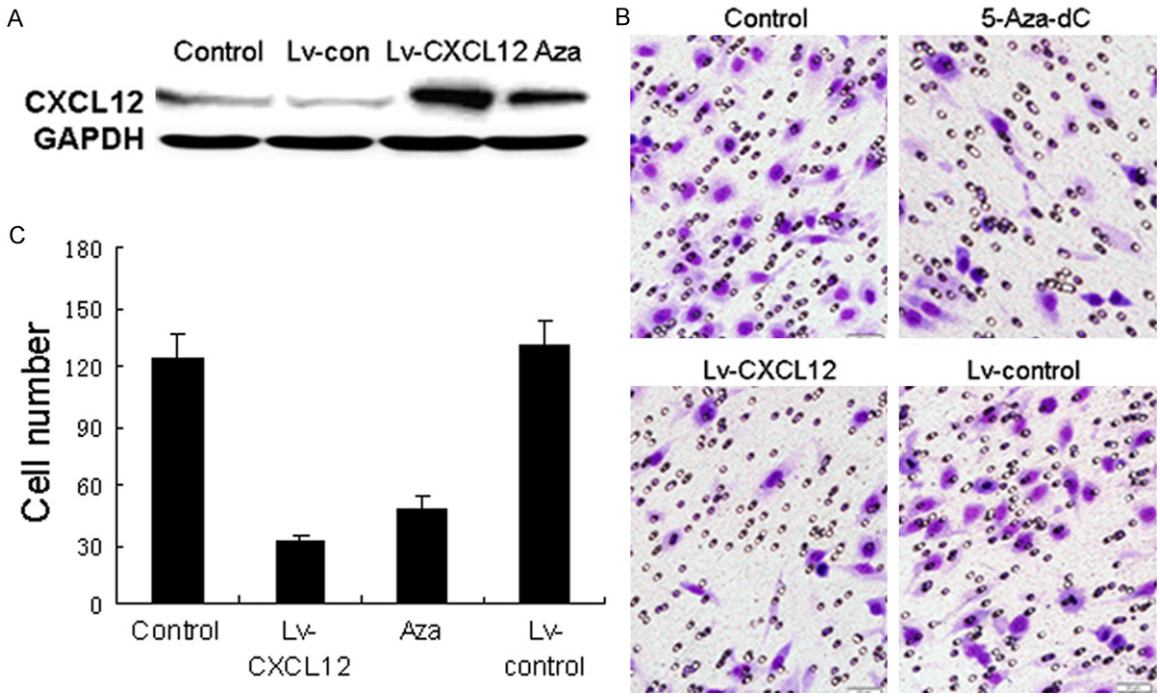


**Figure 3.** Restoration of CXCL12 suppresses cell invasion of MDA-MB-231 cells. A. Comparison of CXCL12 protein expression in human breast cancer cell line MDA-MB-231 after treatment with 5-Aza-dC (10 μM) and Lv-CXCL12. B. Demethylation effect of CXCL12 on the invasion ability of the highly metastatic cell line MDA-MB-231 was observed by the invasion assay after treatment with 5-Aza-dC or Lv-CXCL12. Original magnification × 100. C. The columns indicate the number of cells invaded at the 24 h time point. The values represent the mean values ± SD.

8.4/HP. The number of invading cells was significantly decreased when MDA-MB-231 cells were treated with 5-Aza-dC (49.1 ± 5.2/HP). A significant reduction in the number of invasive cells was observed for 24 h when the cells were treated with 72 h of 5-Aza-dC exposure compared to the control ($P$<0.01). We further used an Lv-CXCL12 vector to restore CXCL12 expression in the MDA-MB-231 cell line. Western blot conformed CXCL12 was effectively up-regulated after 48 h transfection. Invasion assay showed the number of invading cells was significantly decreased after transfection Lv-CXCL12 vector (32.4 ± 9.3/HP) compared with the control group (**Figure 3**).

*Restoration of CXCL12 inhibits the proliferation and growth of MDA-MB-231 cells in vivo and in vitro*

We then investigated the functional role of CXCL12 in MDA-MB-231 cells. To do so, we constructed CXCL12 expressing vector and transfected to MDA-MB-231 cells. Restoration of CXCL12 in the cells was confirmed by RTq-PCR. Then, the colony formation assay was used to evaluate the growth of the cells in which infected with a lentivirus containing a CXCL12 vector. As shown in **Figure 4A**, Lv-CXCL12 cells formed significantly fewer colonies on soft agar compared to MDA-MB-231 and Lv-control cells. To further test the effect of CXCL12 on breast cancer cell growth, MTT assay was performed and growth curves were generated (**Figure 4B**). As shown by the curves, Lv-CXCL12 cells proliferated lower than MDA-MB-231 and Lv-control cells during the first 96 h after the cells were plated. The dramatic reduction of colony formation and growth of Lv-CXCL12 cells suggested CXCL12 expression might negatively regulate breast cancer cell growth. Since CXCL12 confers an inhibitory effect on growth of MDA-MB-231 cells *in vitro*, its effect in vivo was also investigated. **Figure 4C** showed that Lv-CXCL12 cells formed substantially smaller tumors in nude mice than the control groups. At the time of death, the mean tumor weight at the end of the experiment was remarkably lower in the Lv-CXCL12 cells group (0.21 ± 0.05 g) than in the MDA-MB-231 group (0.65 ± 0.07 g, $P$<0.05) or Lv-control group (0.60 ± 0.08 g, $P$<0.05) (**Figure 4D**).

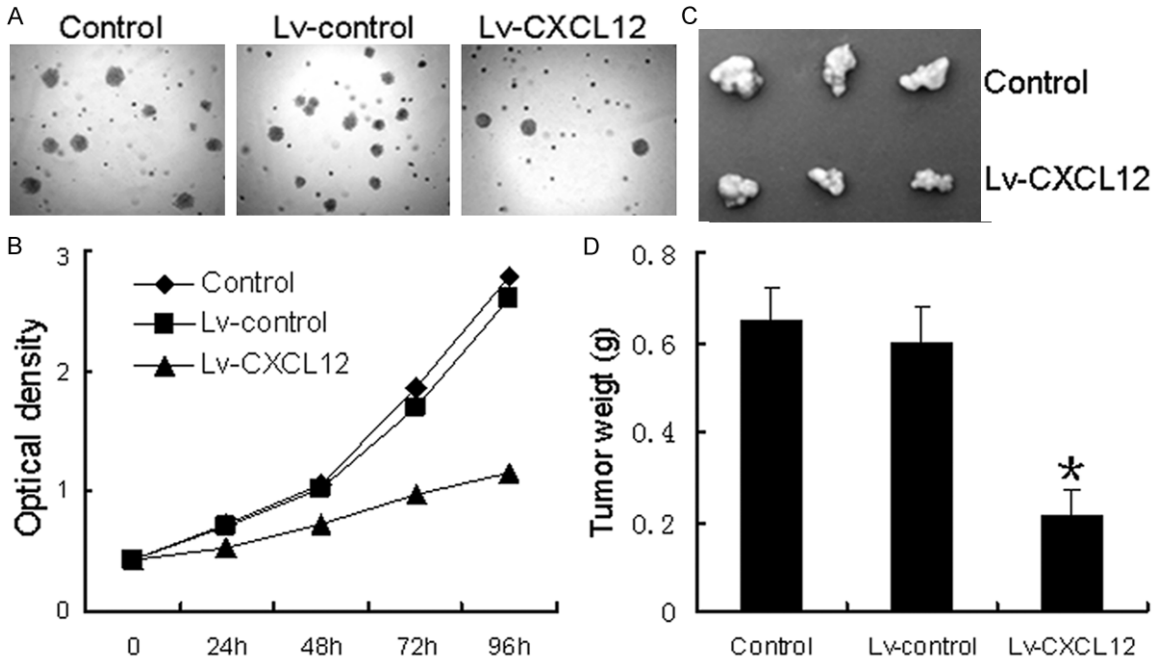


**Figure 4.** Restoration of CXCL12 inhibits the proliferation of MDA-MB-231 cells *in vivo* and *in vitro*. MDA-MB-231 cells were transfected with Lv-CXCL12 or Lv-Control as negative control, then the effects on cell proliferation were determined using colony formation assay (A) and MTT assay (B). MDA-MB-231 or Lv-CXCL12 cell lines were injected into right scapular region of nude mice (each group =7). 3 weeks later, the mice were sacrificed, photographed, dissected and the weight of tumor were counted. (C) The photograph of excised tumors from each group terminated in 3 weeks. (D)The mean weight of each group. **P*<0.05 versus the control.

## Discussion

It is well known that carcinoma results from the combined forces of both genetic and epigenetic events. DNA methylation is the most widely studied epigenetic event. DNA methylation, especially in CpG islands, leads to transcriptional gene silencing [21, 22]. There is increasing evidence that the inactivation of most tumor suppressor genes (TSGs) is related to DNA methylation, and the silencing of these genes possibly contributes to the development and progression of tumors [23]. It has also been shown that aberrant methylation of TSGs is a common event in the development and progression of cancer. Thus, DNA methylation has been a promising biomarker in detecting the disease-associated changes in cells.

In this study, we demonstrated that lower expression levels of CXCL12 mRNA occur in TNBC cell lines. Moreover, we also found that the expression of CXCL12 was frequently reduced in breast primary carcinomas when compared to corresponding normal breast tissues. Other researchers found the same phenomenon in colon cancer and non-small lung cancer, and the CpG islands of the CXCL12 gene was hypermethylated [16, 17]. One of our objectives was to verify whether the aberrant methylation findings were in breast carcinomas. We evaluated the CpG islands of the CXCL12 gene that had already been analyzed by another group that found DNA methylation in non-small cell lung cancer and gastric carcinoma [24, 25]. In our research, the CpG islands of the CXCL12 gene was hypermethylated in TNBC cells, but not in normal mammary epithelial cell line MCF-10A cells, and as high as 50.5% in the 105 primary TNBC tumors, whereas only 19.5% hypermethylation of CXCL12 was observed in corresponding normal breast tissues. To further explore the DNA methylation, we treated cancer cells with 5-Aza-dC, a DNA methyltransferase inhibitor, which was sufficient to cause demethylation of the promoter region and reactivate the expression of the hypermethylated silenced gene. After 5-Aza-dC treatment, we observed a complete reversal of CXCL12 protein expression in MDA-MB-231 cells. As shown in this study, the action of 5-Aza-dC on the breast can-

cer cell lines MDA-MB-231 resulted in the demethylation of the CXCL12 gene, accompanied by the up-regulation of CXCL12 expression. This confirmed that 5-Aza-dC regulated the transcription of the CXCL12 gene. In addition, the effect of 5-Aza-dC on the invasion and growth ability of the highly metastatic breast cancer cells MDA-MB-231 was demonstrated. Our data suggest that restoration of CXCL12 in MDA-MB-231 cells inhibited cell proliferation and suppressed cell invasion. The down-regulation of CXCL12 was correlated with its promoter methylation in both breast cancer cell lines and breast tumor tissues. These results suggest that the aberrant methylation of CXCL12 might be involved in the progression of TNBC and CXCL12 may function as a tumor suppressor gene in TNBC cells.

In summary, we analyzed the mRNA expression level and promoter methylation status of CXCL12 in TNBC cell lines and tumor specimens. We also explored whether the decreased CXCL12 expression occurred as a result of aberrant methylation of the gene. Then, we investigated the cellular functions of CXCL12 on cell proliferation and invasion in TNBC. Our data revealed that CXCL12 was frequently silenced by promoter hypermethylation in both tested TNBC cell lines and primary TNBC. In addition, functional experiments reveled that CXCL12 inhibited TNBC cell proliferation and suppressed cell invasion and growth *in vivo* and *in vitro*. Taken together, CXCL12 may function as a tumor suppressor gene, which is down-regulated through promoter hypermethylation in TNBC development.

## Acknowledgements

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## Disclosure of conflict of interest

None.

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