Effect of specific silencing of EMMPRIN on the growth and cell cycle distribution of MCF-7 breast cancer cells

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Genet. Mol. Res. 14 (4): 15730-15738 (2015)
Received August 8, 2015
Accepted October 3, 2015
Published December 1, 2015
DOI http://dx.doi.org/10.4238/2015.December.1.24

ABSTRACT. The extracellular matrix metalloproteinase inducer (EMMPRIN, CD147) is a member of the immunoglobulin family and shows increased expression in tumor cells. We examined the effect of RNAi-mediated *EMMPRIN* gene silencing induced by lentiviral on the growth and cycle distribution of MCF-7 breast cancer cells. Lentiviral expressing EMMPRIN-short hairpin RNA were packaged to infect MCF-7 cells. The inhibition efficiency of EMMPRIN was validated by real-time fluorescent quantitation polymerase chain reaction and western blotting. The effect of EMMPRIN on cell proliferation ability was detected using the MTT assay and clone formation experiments. Changes in cell cycle were detected by flow cytometry. EMMPRIN-short hairpin RNA-packaged lentiviral significantly down-regulated EMMPRIN mRNA and protein expression, significantly inhibited cell proliferation and *in vitro* tumorigenicity, and induced cell cycle

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abnormalities. Cells in the G0/G1 and G2/M phases were increased, while cells in the S phase were decreased after infection of MCF-7 cells for 3 days. The *EMMPRIN* gene facilitates breast cancer cell malignant proliferation by regulating cell cycle distribution and may be a molecular target for breast cancer gene therapy.

Key words: Breast cancer; Extracellular matrix metalloproteinase inducer; Targeting silence

INTRODUCTION

Breast cancer is a frequent malignant tumor that acutely influences women's physical and mental health and is a serious threat to life. Breast cancer incidence rate accounts for 7-10% of all malignant tumors (Howlader et al., 2014; Jansen et al., 2014). Similar to other malignant tumors, breast cancer generation and development are associated with functional changes in various genes (Lin et al., 2014; Cao et al., 2014a). The main function of extracellular matrix metalloproteinase inducer (EMMPRIN) is to participate in cell recognition and to regulate the matrix metalloproteinase (MMP) expression by regulating 5-lipoxygenase and phospholipase A1 (Cannistra and Niloff, 1996; Guo et al., 1998). EMMPRIN is abundant on tumor cell surfaces and is present in the normal tissues; levels in tumor tissues are much higher than those in normal tissues. In addition to promoting tumor infiltration, EMMPRIN can also facilitate malignant tumor cell proliferation and metastasis and is associated with the survival of some tumor patients (Yang et al., 2003; Rosenthal et al., 2005). The *EMMPRIN* gene is located on chromosome 19q13.3. The EMMPRIN protein is a transmembrane glycoprotein composed of 248 amino acids (Guo et al., 1998; Hao et al., 2010). This protein induces MMP production through the mitogen-activated protein kinase (MAPK) pathway, promotes tumor invasion and metastasis (Fan et al., 2011; Cao et al., 2014b), facilitates blood vessel neogenesis through the vascular endothelial growth factor pathway (Bougatef et al., 2009), facilitates tumor cell resistance by inducing hyaluronan production (Marieb et al., 2004; Toole and Slomiany, 2008), and inhibits tumor cell nested apoptosis by inhibiting proapoptosis-associated protein. EMMPRIN can also interact with cyclophilin A to promote pancreatic cancer proliferation by activating extracellular signal-regulated kinase and p38 MAPKs (Takahashi et al., 2012).

It remains unclear how *EMMPRIN* gene silencing affects the pathogenesis and invasion of breast cancer. In this study, we examined the role of the *EMMPRIN* gene in breast cancer malignant proliferation and cell cycle progression using a loss-of-function strategy to further understand the mechanism of breast cancer.

MATERIAL AND METHODS

Materials

The human breast cancer cell line MCF-7 and the cell line HEK293T were purchased from the cell bank of the Biochemistry and Cell Biology Institutes, Shanghai Institutes for Biological Sciences (Shanghai, China). The lentiviral vector system pLVTHM, psPAX2, and pMD2G were purchased from Tronolab (www.tronolab.com/lentivectors.php). *Bam*HI and *Eco*RI restrictive endonuclease and T4 ligase were purchased from New England Biolabs

(Ipswich, MA, USA). Cell culture media RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). The plasmid extract kit was purchased from Qiagen (Hilden, Germany). TRIzol reagent and Lipofectamine™ 2000 Oligofectamine reagent were purchased from Invitrogen (Carlsbad, CA, USA). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA). SYBR Master Mixture were purchased from TaKaRa (Shiga, Japan). The BCA protein analysis kit was purchased from HyClone-Pierce (Logan, UT, USA). Rabbit anti-EMMPRIN antibody was purchased from Abcam (Cambridge, UK). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody, peroxidase-labeling goat anti-rabbit IgG, and goat anti-mouse IgG were purchased from Santa Cruz Biotechnology. The ECL-Plus™ Western Blotting System was purchased from Amersham-Pharmacia Biotech (Amersham, UK).

Construction of short hairpin RNA expressing EMMPRIN (*EMMPRIN*-shRNA) and virus package

EMMPRIN-shRNA sequence and control shRNA were all synthesized by Sigma-Aldrich (St. Louis, MO, USA). Lentiviral backbone plasmid pLVTHM were double-digested and renatured to generate double-stranded DNA and then the plasmids were transferred to competent *Escherichia coli*. Positive recombinant clones were selected and detected by polymerase chain reaction (PCR). The successfully connected vectors were sequenced by Shanghai YingJun and confirmed clones were cultured and amplified to extract plasmid DNA.

HEK293T cells in the logarithmic phase were collected and inoculated on a 15-cm Petri dish at a density of 6 x 10^5/mL. When the cell coverage rate reached 80%, culture media containing 10% FBS was replaced with media without FBS and co-incubated with cells for 2 h before transfection. Vector DNA solution contained 20 μg recombined vector (or control vector), 15 μg psPAX2 vector, 10 μg pMD2G vector, and Opti-MEM solution to a final volume of 2.5 mL. This solution was incubated at room temperature for 5 min. Lipofectamine™ 2000 reagent and Opti-MEM were mixed in a volume ratio of 1:24 and then incubated at room temperature for 5 min. The vector solution was mixed with diluted Lipofectamine™ 2000 solution and then incubated at room temperature for 20 min. Transfection compound was added to the cell culture supernatant and cultured for 8 h before removing the culture solution with Oligofectamine Reagent. The 293T cells were washed with phosphate-buffered saline (PBS), DMEM culture solution containing 10% FBS was added, and the cells were cultured at 37°C with 5% CO_2 for 48 h. The supernatant was collected by centrifugation at 4°C, 4000 *g* for 10 min and filtered through a 0.45-μm filter. The filtered virus solution was transferred to a 50-mL ultracentrifugation tube and centrifuged at 4000 *g* for 15 min to collect the concentrated virus solution. Virus titer was detected using well-by-well dilution method to determine the best multiplicity of infection of breast cancer MCF-7 cells.

EMMPRIN knockdown efficiency verification

MCF-7 cells in good condition were inoculated on 6-well plates at a concentration of 2000 cells per well. After culture for 24 h, *EMMPRIN*-shRNA lentivirus or control lentivirus were added with a multiplicity of infection of 50. Cells expressing green fluorescent protein were evaluated to determine the efficiency of infection of lentivirus into MCF-7 cells after 72 h cell culture. After continued culturing for 3 days, cells were collected to extract total RNA and

total protein. Based on the method described by Fan et al. (2011), we detected the expression changes of *EMMPRIN* mRNA using real-time PCR (Takara). After the reaction, the Ct value of each sample was calculated by subtracting the blank and baseline fluorescence signals from Ct values and then the relative expression of the *EMMPRIN* gene was analyzed. Primers for *EMMPRIN* were as follows: upstream primer: 5'-CGAGATCTATGGCGGCTGCGCTGTTC-3', downstream primer: 5'-GCGAATTCTCAGGAAGAGTTCCTCTG-3'; primers for reference gene β-actin: upstream primer: 5'-GTGGACATCCGCAAAGAC-3', downstream primer: 5'-AAAGGGTGTAACGCAACTA-3'. Western blotting was utilized to determine the inhibition efficiency of *EMMPRIN*-shRNA lentivirus of the expression of the EMMPRIN protein. An ECL-plus^{TM} kit was utilized for coloration.

MTT detection

Cells infected by lentivirus for 3 days were collected and inoculated on a 96-well plate with 2000 cells in 100 μL culture medium per well (5 duplicates for each group) and cultured at 37°C and 5% CO_2 for 1, 2, and 3 days. After incubation, 20 μL 5 mg/mL MTT was added to each well and the cells were cultured for 4 h. The culture medium was removed and 200 μL dimethyl sulfoxide was added to each well. Absorbance (A) at 490 nm was detected using a microplate reader instrument (Mailang Company, Shenzhen, China).

Clone formation experiment

Cells infected by lentivirus for 5 days were collected and inoculated on 6-well plates at a concentration of 500 cells in 2 mL culture medium per well (3 duplicates per group) and cultured at 37°C and 5% CO_2 for 10 days. The culture medium was removed and 1 mL 4% paraformaldehyde was used to fix the cells at room temperature for 60 min. After removing the fixative solution, the cells were treated with Giemsa staining solution for 20 min, washed with ddH_2O, and clones were counted from photographs acquired using a fluorescence microscope.

Flow cytometry detection of cell cycle

Cells infected by lentivirus for 3 days were inoculated on 6-well plates at a concentration of approximately 50%. Supernatants were transferred to 5-mL centrifuge tubes.The plate wells were washed with D-Hanks solution and this solution was mixed with the supernatant. Cells were digested with pancreatin collected into the same centrifuge tube. Cells were centrifuged at 1500 *g* for 5 min after washing with precooling PBS and then re-suspended into 100 μL PBS. Next, 900 μL precooled 70% alcohol was used to fix the cells at 4°C for 1 h. Samples were centrifuged at 1500 rpm for 5 min, the supernatant was removed, and the cells were washed with PBS. Cells were stained with 100 μg/mL propidium iodide and 10 μg/mL RNase A solution, and the cell suspension was filtered through a 300-mesh screen cloth.Cell cycle was evaluated by flow cytometry (B&D Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Data were analyzed using the SPSS 16.0 statistics software (SPSS, Inc., Chicago,

IL, USA). Experimental data are reported as means ± SD. The paired *t*-test was utilized to evaluate interclass differences. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Construction and package of shRNA lentiviral expression vectors targeting *EMMPRIN* gene and infection into MCF-7 cells

The *EMMPRIN* gene shRNA was inserted into the lentiviral plasmid pLVTHM, which had been double-digested by *Bam*HI and *Eco*RI. The products were transferred to *E. coli* and the positive clones were detected by PCR. Successfully linked clones were sequenced. The pLVTHM shRNA vector, lentivirus plasmid psPAX2, and envelope plasmid pMD2G were transformed into 293T cells to construct the virus, which was used to infect breast cancer MCF-7 cells. Infection rate was determined by identifying green fluorescent protein-positive cells using a fluorescence microscope after incubation for 96 h. The results showed that more than 90% MCF-7 cells expressed green fluorescent protein (Figure 1).

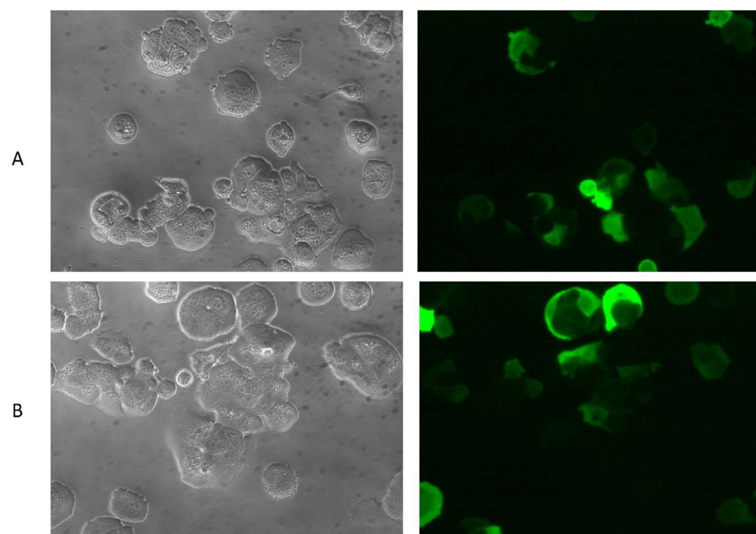

Figure 1. MCF-7 cells infected with *EMMPRIN*-shRNA-expressing lentivirus. **A.** 200X; **B.** 400X.

shRNA lentivirus targeting *EMMPRIN* gene inhibited *NOB1* gene mRNA and protein expression

RNA and protein from MCF-7 cells infected with shRNA lentivirus targeting the *EMMPRIN* gene were collected. Real-time quantitative PCR and western blotting were used to detect changes in *EMMPRIN* gene and protein expression. *EMMPRIN* mRNA expression decreased by 75.8% compared with that in the negative control lentivirus group (Figure 2A), and the difference was statistically significant ($P < 0.01$). Western blotting confirmed that EMMPRIN protein expression was decreased significantly (Figure 2B).

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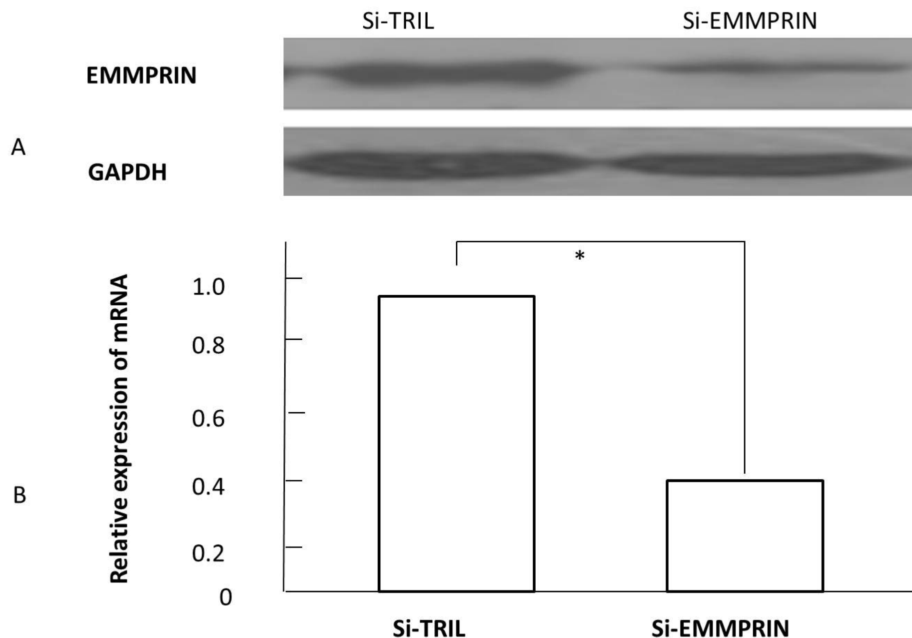

Figure 2. Expression of *EMMPRIN* mRNA and protein in MCF-7 cells infected with *EMMPRIN*-shRNA-expressing lentivirus. **A.** Analysis of relative gene expression data using real-time quantitative PCR and $2^{-\Delta\Delta Ct}$ method. **B.** Analysis of protein expression using western blot assay. Si-CTRL: control cells infected with control lentivirus; Si-EMMPRIN: MCF-7 cells infected with *EMMPRIN*-shRNA-expressing lentivirus. *P < 0.001.

shRNA lentivirus targeting *EMMPRIN* gene inhibited *EMMPRIN* gene expression and the proliferation of breast cancer MCF-7 cells

The MTT assay showed that the proliferation of MCF-7 cells in the *EMMPRIN* gene silencing group was significantly inhibited (Figure 3). The difference in the absorbance at A490 nm after cell culture for 2-3 days and that in the negative control group was statistically significant ($P < 0.01$), suggesting that shRNA lentivirus targeting the *EMMPRIN* gene can decrease MCF-7 cell proliferation.

shRNA lentivirus targeting the *EMMPRIN* gene inhibited *EMMPRIN* gene expression and the cell cycle distribution of breast cancer MCF-7 cells

Cells stained with propidium iodide were used to observe cell cycle distribution changes by flow cytometry after *EMMPRIN* silencing. Table 1 shows that the inhibition of *EMMPRIN* gene expression was increased in G1/G0 and G2/M phase cells, but decreased in S phase cells. All differences were statistically significant ($P < 0.01$), suggesting that down-regulation of *EMMPRIN* gene expression affected the cell cycle distribution and thus induced a decrease in cell replication ability, indicating that the EMMPRIN protein can promote breast cancer cell growth by regulating the cell cycle. The cell cycle distribution is shown in Figure 4.

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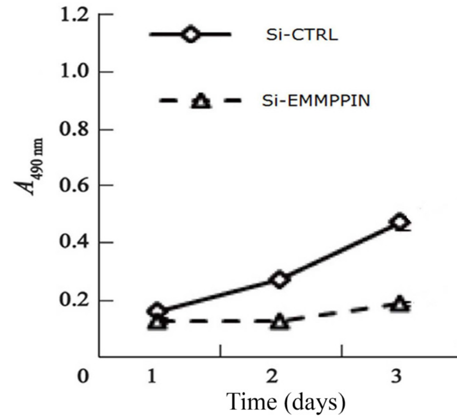

Figure 3. Effect of specific silencing of *EMMPRIN* on cell proliferation of MCF-7 breast cancer cells. Si-CTRL: control cells infected with control lentivirus; Si-EMMPRIN: MCF-7 cells infected with *EMMPRIN* shRNA-expressing lentivirus. *P < 0.01.

Table 1. Flow cytometry cell cycle analysis using propidium iodide-stained DNA staining was performed on MCF-7 cells.

Group	G1/G0	S	G2/M
Si-CTRL	62.3 ± 0.44	32.5 ± 1.21	5.3 ± 0.66
Si-EMMPRIN	72.9 ± 0.98*	16.2 ± 0.94*	12.1 ± 0.41*

Compared to Si-CTRL, *P < 0.001.

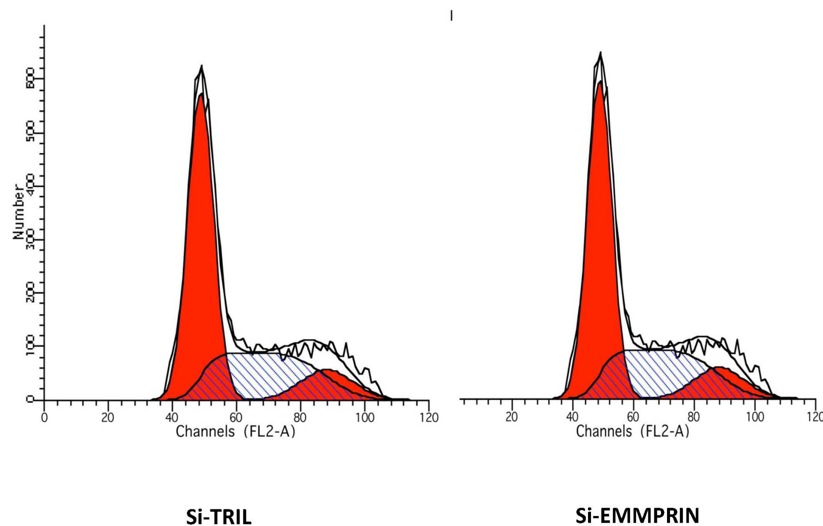

Figure 4. Effect of specific silencing of *EMMPRIN* on cell cycle distribution of MCF-7 breast cancer cells. Si-CTRL: control cells infected with control lentivirus; Si-EMMPRIN: MCF-7 cells infected with *EMMPRIN* shRNA-expressing lentivirus.

DISCUSSION

EMMPRIN was first purified from human lung cancer LX-1 cells by Ellis et al. (1989) and named the tumor cell-derived collagenase-stimulatory factor. Subsequent studies revealed that this protein exists in human normal tissues as well. Biswas et al. (1995) renamed tumor cell-derived collagenase-stimulatory factor to EMMPRIN in 1995 because this protein can significantly induce MMP.

EMMPRIN is widely present in human normal tissues such as keratinocytes, embryo epithelial cells, and vascular endothelial cells under normal physiological conditions and participates in many processes, such as the construction of the human blood-brain barrier, wound healing, tissue repair, pregnancy, and embryo development (Lee et al., 2013). EMMPRIN mainly participates in the adhesion between cells or ground substances in a pathological state, such as in a malignant tumor. *In vitro* studies showed that EMMPRIN from tumor cells can stimulate human fibroblasts and endotheliocyte to secrete MMP and thus participate in the destruction of the natural tissue mechanical barrier to help tumor cell invasion and diffusion (Xiong et al., 2014).

In this study, we silenced the *EMMPRIN* gene in breast cancer MCF-7 cells using the lentivirus-induced RNAi technique to study changes in breast cancer proliferation ability under conditions of *EMMPRIN* gene deletion. The results show that the expression level of EMMPRIN mRNA and protein in MCF-7 cells was significantly inhibited after infection with the *EMMPRIN*-shRNA lentivirus. The MTT assay and clone formation results showed that down-regulation of *EMMPRIN* expression significantly inhibited reproductive activity and tumor formation ability in MCF-7 cells. To further study the inhibition mechanism of EMMPRIN on cell proliferation, we detected the cycle distribution of MCF-7 cells under EMMPRIN deletion conditions. The results revealed G0/G1 phase blocking in cells in which *EMMPRIN* gene had been silenced, suggesting that cell division was inhibited. This indicates that EMMPRIN plays an important role in breast cancer malignant proliferation by regulating the cell cycle.

In summary, malignant proliferation of human breast cancer MCF-7 cells was significantly inhibited after silencing of the *EMMPRIN* gene using RNAi, and cells in the G1/G0 and G2/M phases were increased. However, the molecule mechanism regarding how EMMPRIN inhibition affected malignant breast cancer cell proliferation and regulated the cell cycle distribution should be further examined.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (grant #81001176).

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