Cancer Chemother Pharmacol (2011) 68:207–215
DOI 10.1007/s00280-010-1468-9

ORIGINAL ARTICLE

Lentivirus-mediated inhibition of Med19 suppresses growth of breast cancer cells in vitro

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Received: 8 May 2010 / Accepted: 10 September 2010 / Published online: 2 October 2010

Abstract
Purpose The mediator is a large multiprotein complex vital for transcription regulation. Human Med19 is a critical subunit of the mediator complex and plays an important role in stabilizing the whole mediator. To understand the role and mechanism of Med19 in breast cancer, we carried out studies on the impacts of lentivirus-mediated inhibition of Med19 on breast cancer cells in vitro.
Method The expression of Med19 in breast cancer tissue was detected using immunohistochemical analysis. The impacts of lentivirus-mediated inhibition of Med19 on breast cancer cells were detected using flow cytometric, cell proliferation, BrdU incorporation, and colony formation assays.
Results The upregulated expression of Med19 was found in breast cancer tissues. Med19 expression was significantly associated with tumor grade ($p = 0.026$). The expression of Med19 was strongly suppressed in human breast cancer MDA-MB-231 and MCF-7 cells infected with lentiviruses delivering small hairpin RNA (shRNA) against Med19. The inhibition of Med19 elicited augmentation of G0/G1 phase proportion and significantly attenuated the growth of MDA-MB-231 and MCF-7 cells in vitro.
Conclusion Med19 plays an important role in the proliferation of human breast cancer cells, which suggested that the lentiviruses delivering shRNA against Med19 could be a promising tool for breast cancer therapy.

Keywords Med19 · RNA interference · Lentivirus · Breast cancer

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Introduction

The mediator complex, a multiprotein complex conserved from yeast to man [1], is largely responsible for the elaborate regulation of transcription that underpins cell proliferation, development, differentiation, and maintenance. *Med19/ROX3*, an essential gene originally identified in a search for mutants increasing aerobic expression of the CYC7 gene [2], encodes a subunit of mediator and RNA polymerase II holoenzyme [3]. Biochemical and electron microscopic analysis of yeast core mediator have indicated the presence of three discrete areas that were referred to as the head, middle, and tail modules of the complex [4]. Med19/Rox3 is firstly referred to as a component of the yeast/mammalian mediator head module [1, 5, 6]. The deletion of Med19/Rox3 in *Saccharomyces cerevisiae* leads to dissociation of the middle module of mediator, leaving an intact mediator subcomplex consisting of head and tail modules. The absence of Med19/Rox3 destabilizes the association of middle module with intact mediator complex. Thus, Med19/Rox3 was proposed to function as a component of the middle module. Besides, the ΔMed19/Rox3 mediators have a decreased affinity for RNA Pol II

and are unable to stimulate phosphorylation of the carboxyl-terminal domain (CTD) of RNA Pol II by transcription factor II H (TFIIH) [7]. Recently, Med19, together with Med26, were identified as critical components of the regulatory apparatus employed by the repressor element 1 (RE1) silencing transcription factor. RE1 silencing transcription factor recruits mediator through a Med19/Med26 interface to facilitate epigenetic silencing of neuronal genes in non-neuronal cells [8].

RNA interference (RNAi) is a phenomenon where gene expression is specifically regulated by small double-stranded RNAs. The mechanism of RNAi has been extensively reviewed elsewhere [9, 10]. The RNAi machinery makes it possible to use exogenous small RNAs, like small hairpin RNAs (shRNAs) and synthetic small interfering RNAs (siRNAs) for silencing of almost any target gene in a specific way. There have been tremendous interests in applying these small RNAs as potential novel therapeutic drugs in the treatment of diseases like acquired immunodeficiency syndrome (AIDS), hepatitis B, respiratory syncytial virus infection, and solid tumors [9].

Worldwide, breast cancer is the most frequently diagnosed life-threatening cancer in women and the leading cause of cancer mortality among women. Its prevalence is about 13% in women, and it is extremely rare but highly lethal in men [11]. Breast cancer management has become increasingly complex, requiring the integration of data of the patient's history and specific tumor biomarkers. Targeted and biologic therapies in breast cancer continue to evolve rapidly. The field of molecular-targeted therapy for breast cancer has emerged. During the recent years, there has been an increasing and rapid development of molecular markers as targets for molecular-targeted therapy. A large number of molecules have become therapeutic targets already, like molecules of signal transduction, factors regulating cell survival, cell cycle and cell death, and molecules associated with invasion, metastasis, and angiogenesis [12]. Transcriptional dysfunction has been shown to elicit broad effects on cell proliferation, development, and differentiation. To elucidate the role of Med19 in breast cancer and to illustrate the potential of Med19 as a marker for molecular-targeted therapy of breast cancer, the expression of Med19 in breast cancer tissue was detected. The inhibition of Med19 mediated by lentivirus-delivered shRNA, which is capable of highly specific and stable silencing of gene expression in a variety of human cells [13, 14], was performed on human breast cancer MDA-MB-231 and MCF-7 cells. The effect of Med19 suppression on cell cycle and proliferation of these cells was investigated in vitro.

Materials and methods

Immunohistochemistry

Human breast tissue samples were taken from 50 patients with their written informed consent. This study was approved by the institutional review board. Tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned into 4-μm-thick slices. Immunohistochemical staining was carried out using NovoLink Polymer Detection Systems (Novocastra Laboratories Ltd.). All procedures followed the manufacturer's protocol. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

Cells lines and cell culture conditions

The human breast cancer MDA-MB-231 and MCF-7 cell lines, and human renal epithelial 293T cell line were purchased from American Type Culture Collection and maintained at 37°C and 5% CO_2. The MDA-MB-231 cell line was cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco) supplemented with 4 mM L-Glutamine (Sigma) and 10% fetal bovine serum (FBS, Gibco). The MCF-7 cell line was maintained in DMEM containing 4 mM L-Glutamine, 0.1 mM MEM Non-Essential Amino Acids (Sigma), and 10% FBS. The 293T cell line was cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma).

Lentiviral plasmids construction, lentiviruses production, and cell infection

The human *Med19*-specific targeting sequence is 5′-GG TGAAGGAGAAGCTAAGT-3′, designed using online siRNA tools provided by Invitrogen with *Med19* sequence (GenBank code: NM_153450.1) as a reference. The negative control (NC) sequence chosen in this study has been widely used [15, 16]. The shRNA cassette against human *Med19* is 5′-CCGGAAGGTGAAGGAGAAGCTAAGT TTCAAGAGAACTTAGCTTCTCCTTCACCTTTTTTT G-3′, comprised of 19-nucleotide targeting sequence separated by a loop (TTCAAGAGA) from the reverse complement of the targeting sequence, a U6 terminator, and two cohesive ends for ligation into pGCSIL-GFP vector (Genechem) linearized by *Age*I and *Eco*RI. Pairs of complementary oligonucleotides of these sequences were synthesized (Genechem), annealed, and ligated into linearized pGCSIL-GFP vector. The constructed lentiviral plasmids were hereafter denoted as pGCSIL-GFP-shMed19 for specific interfering of *Med19* and pGCSIL-GFP-NC for negative control.

Lentiviruses were generated in 293T cells by co-transfection of pGCSIL-GFP-shMed19 or pGCSIL-GFP-NC, with pHelper1.0 and pHelper2.0 plasmids. Briefly, these plasmids were amplified in *E. coli* DH5α, extracted with QIAGEN Plasmid Maxi Kit (Qiagen), and transfected into 70% confluent 293T cells using Lipofectamine 2000 (Invitrogen). Then lentiviral particles were harvested from the media 48 h after transfection, purified with ultracentrifugation [17, 18], and hereafter referred to as Lv-shMed19 for specific interfering of *Med19*, and Lv-NC for negative control. As the produced lentiviruses carry green fluorescence protein (GFP), the viral titer was measured using the method of serial dilutions via counting green cells under CKX41 fluorescence microscopy (Olympus) 120 h after infection [19].

For each cell line, there were three experimental groups: non-infected (CON) group, Lv-NC-infected (NC) group, and Lv-shMed19-infected (KD) group. For cell infection, 30% confluent MDA-MB-231 and MCF-7 cells were incubated with Lv-shMed19 or Lv-NC for 120 h, with a replacement of media 24 h after adding the lentiviruses.

RNA isolation, reverse transcription PCR, and real-time PCR

Total RNAs were prepared from cells using Trizol reagent (Invitrogen). The reverse transcription reactions were carried out following the protocol of the M-MLV Reverse Transcriptase (Promega). Each reverse transcription reaction mixture contained 2 μg total RNA. Twenty-five microliters of quantitative real-time PCR mixtures containing 0.1 μM primers, 10 μl 2 × SYBR Premix Ex Taq (Takara), and 20-100 ng cDNA sample was assayed on TP800 (Takara). The primers used were as follows: for *Med19*, 5′-GTAACTTCCTGCCTGACCTG-3′, and 5′-TG TGCTTGTGCTTATTCTTCTTC-3′; for *β*-actin, 5′-GGC GGCACCACCATGTACCCT-3′ and 5′-AGGGGCCGGA CTCGTCATACT-3′. The amplification program was comprised of polymerase activation at 95°C for 15 s and 45 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s. The relative expression of Med19 mRNA was calculated with the $2^{-\Delta\Delta Ct}$ method, using *β*-actin mRNA expression level for normalization [20]. All experiments were repeated at least three times.

Western blotting analysis

Cell lysates were separated on 12% SDS–PAGE gels and transferred onto PVDF membranes (Millipore). Proteins were probed overnight at 4°C with Med19 rabbit polyclonal antibody (1:200, Abcam, Cat. #Ab49271) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (1:5000, Santa Cruz Biotechnology, Cat. #sc-32233). The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology, Cat. #sc-2004) and goat anti-mouse IgG (1:5000, Santa Cruz Biotechnology, Cat. #sc-2005) at room temperature for 1 h. Signals were detected with enhanced chemiluminescence, using GAPDH as the internal standard.

Flow cytometric analysis

Cells in all three groups were harvested by centrifugation at 1200 rpm for 5 min after 8 days of infection. The pellets were washed twice with cold PBS, fixed with cold 70% ethanol, centrifuged at 1,500 rpm for 5 min to discard ethanol, and resuspended with PBS, sequentially. The suspensions were filtrated through 400-mesh membrane and centrifuged at 1,200 rpm for 5 min. The cells were stained with propidium iodide (PI) at 4°C for 30 min in dark for flow cytometric analysis. Each experiment was conducted in triplicate.

Cell proliferation assay

After 5 days of infection, cells were trypsinized, resuspended, seeded into 96-well plate in 100 μl (5×10^3 cells) per well, and incubated at 37°C. The number of viable cells in all three groups was measured at daily intervals (days 1, 2, 3, 4, and 5). At each time point, 10 μl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Dingguo Biotechnology) was added into each well, and cells were incubated for another 4 h. Then the media were removed carefully, and 100 μl dimethyl sulfoxide was added into each well at the end of incubation to solubilize the formazan crystals. The absorbance was measured at 570 nm on the spectrophotometer. Each experiment was performed in triplicate and repeated three times.

BrdU incorporation assay

After 5 days of infection, cells were trypsinized, resuspended, plated into 96-well tissue culture plate at a concentration of 2×10^4 cells per well in 100 μl cell culture media, and maintained at 37°C. After 24 and 48 h, the cell growth rate of all three groups was determined using BrdU Cell Proliferation Assay Kit (CHEMICON International), based on the enzyme-linked immunosorbent assay for a thymidine analog, BrdU. All procedures followed the manufacturer's protocol. Each experiment was performed in triplicate and repeated three times.

Cell colony formation assay

After 5 days of infection, cells in all three groups were trypsinized, resuspended, seeded into 6-well plate at a

concentration of 200 cells per well and cultured at 37°C for 14 days. The media were changed every 3–4 days. At the end of incubation, the cells were washed with PBS twice and fixed with paraformaldehyde. Then, the cells were washed with PBS twice, stained with Giemsa (Sigma) for 10 min, and washed with ddH_2O three times, sequentially. The plates were photographed with a digital camera.

Statistical analysis

Statistical analyses were carried out with the Student's *t* test and chi-square test with SPSS 12.0 (SPSS Inc.) software. Statistical significance was set at $p < 0.05$.

Results

Upregulated expression of Med19 in breast cancer

Since there was no reported study on determination of Med19 expression level in human breast cancers, we carried out immunohistochemical analysis to elucidate the expression of Med19 in breast cancer. Rabbit anti-Med19 antibody was used to probe endogenous Med19 protein and to characterize the in situ staining pattern of Med19 in breast cancer tissues. Med19 protein with positive staining was observed in cytoplasm. In histological normal tissues adjacent to tumors, Med19 was undetectable in most of epithelial cells. However, tumor cells exhibited intense staining by anti-Med19 antibody. Of the 50 patients with breast cancer, 25 (50.0%) were classified as positive expression of Med19 (Fig. 1). Med19 expression was significantly correlated with higher tumor grade ($p = 0.026$, Table 1), which have more potential to proliferation and metastasis.

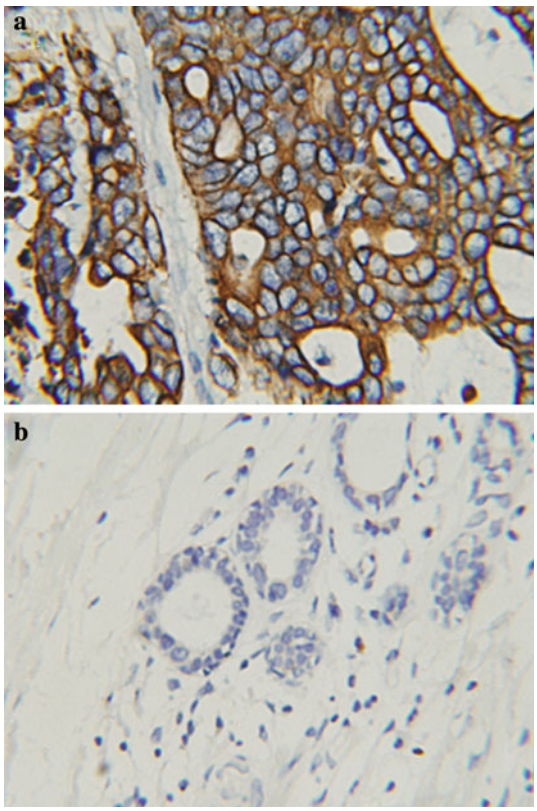

Fig. 1 The expression of Med19 in breast cancer. Representative immunohistochemical staining of the breast cancer samples showed elevated levels of Med19 in tumor tissues. Med19 protein with positive staining was shown in the cytoplasm of tumor cells (**a**), while no visible Med19 staining was detected in adjacent normal tissue from the same analyzed patients (**b**). Magnification ×400

Generation of stable cell lines expressing pGCSIL-GFP-shMed19 shRNA

To determine the lentiviral infection efficiency, expression of GFP was detected with fluorescence microscopy. The highest infection efficiency was obtained at a multiplicity of infection (MOI) of 10 for MDA-MB-231 cells and MOI of 20 for MCF-7 cells, resulting fluorescent expression identified in more than 90% of MDA-MB-231 cells (Fig. 2a) and MCF-7 cells (Fig. 2b) 120 h after infection. This demonstrates the high efficiency of pGCSIL-GFP-shMed19.

Lentivirus-mediated RNAi silencing specifically inhibits the expression of Med19 mRNA and protein

The mRNA and protein levels of Med19 in all three groups of MDA-MB-231 and MCF-7 cells were detected by real-time PCR and Western blotting (Fig. 3). The expression of Med19 mRNA in the KD group of MDA-MB-231 cells was dramatically decreased by 61% ($p < 0.01$) compared to the NC group (Fig. 3a). The Med19 mRNA level in the KD group of MCF-7 cells was significantly lower than that of the NC groups, with a reduction of 79% ($p < 0.01$) (Fig. 3b). No statistical significance was detected between the NC groups and the CON groups ($p > 0.05$). The Med19 protein expression in KD groups of MDA-MB-231 and MCF-7 cells was also significantly reduced (Fig. 3c, d), compared to the NC and CON groups. Thus, the constructed pGCSIL-GFP-shMed19 was demonstrated to be active and specific in inhibiting the expression of Med19.

Inhibition of Med19 augments the proportion of G0/G1 phase in human breast cancer cells in vitro

To elucidate whether Lv-shMed19-mediated knockdown of Med19 has any impact on the cell cycle of breast cancer cells, all three groups of MDA-MB-231 and MCF-7 cells were subjected to flow cytometry assay 8 days after infection. The KD groups of MDA-MB-231 (Fig. 4a, c)

Table 1 Associations between Med19 expression and clinicopathological characteristics of the 50 patients with breast invasive ductal carcinoma

Clinicopathological characteristics	*N*	Med19		χ^2 value	*p* value
		Positive	Negative		
Age at diagnosis (years)				0.397	0.529
<50	14	8	6		
≥50	36	17	19		
Pathological tumor size (mm)				0.347	0.556
<20	18	10	8		
≥20	32	15	17		
Grading				7.284	0.026
I	5	0	5		
II	29	14	15		
III	16	11	5		
TNM stage				1.268	0.737
I	10	6	4		
II	29	15	14		
III	8	3	5		
IV	3	1	2		
Lymph node status				0.325	0.569
Positive	22	10	12		
Negative	28	15	13		
Estrogen receptor status				0.089	0.765
Positive	33	17	16		
Negative	17	8	9		
Progesterone receptor status				0.397	0.529
Positive	36	19	17		
Negative	14	6	8		

and MCF-7 cells (Fig. 4b, d) displayed more proportion in G0/G1 phase compared to the NC groups ($p < 0.01$).

Inhibition of Med19 significantly suppresses growth of human breast cancer cells in vitro

To examine the effect of Lv-shMed19-mediated down-regulation of Med19 on the growth of breast cancer cells, MDA-MB-231 and MCF-7 cells were assayed to monitor cell proliferation. Five days after infection, all three groups of MDA-MB-231 and MCF-7 cells were plated for MTT, BrdU incorporation, and colony formation assays to monitor cell proliferation. The cell growth of KD groups was significantly inhibited compared to the NC groups (Fig. 5).

As determined in the MTT assay, the MDA-MB-231 KD group displayed significant cell proliferation defect with no obvious growth observed during the whole assay period (5 days) (Fig. 5a). Similarly, no obvious BrdU incorporation was detected for the MDA-MB-231 KD group during the whole assay period (48 h). Compared to the NC group, the BrdU incorporation rate of the MDA-MB-231 KD group was significantly decreased by 30% at 24 h and 50% at 48 h (Fig. 5b). The colony formation rate of the MDA-MB-231 KD group was dramatically suppressed compared to the NC group (Fig. 5c).

The MCF-7 cells represented similar results. The growth of the MCF-7 KD group was also dramatically inhibited in MTT assay, reduced by ~50% at each time point during the whole assay period (5 days), compared to the NC group (Fig. 5d). As determined in the BrdU incorporation assay, the MCF-7 KD group displayed significant DNA synthesis defect with a reduction of 50% at 24 h and 70% at 48 h, compared to the NC group (Fig. 5e). The colony formation rate of the MCF-7 KD group was dramatically inhibited compared to the NC group (Fig. 5f). No obvious difference between the NC group and the CON group was determined in all of these assays ($p > 0.05$). 2C). To rule out a possible off-target effect caused by the siRNA, relevant data were also confirmed with an additional RNAi sequences that deplete Med19 with a similar efficiency (data not shown).

Discussion

As a critical subunit of the mediator complex, Med19 plays an important role in structurally stabilizing the whole

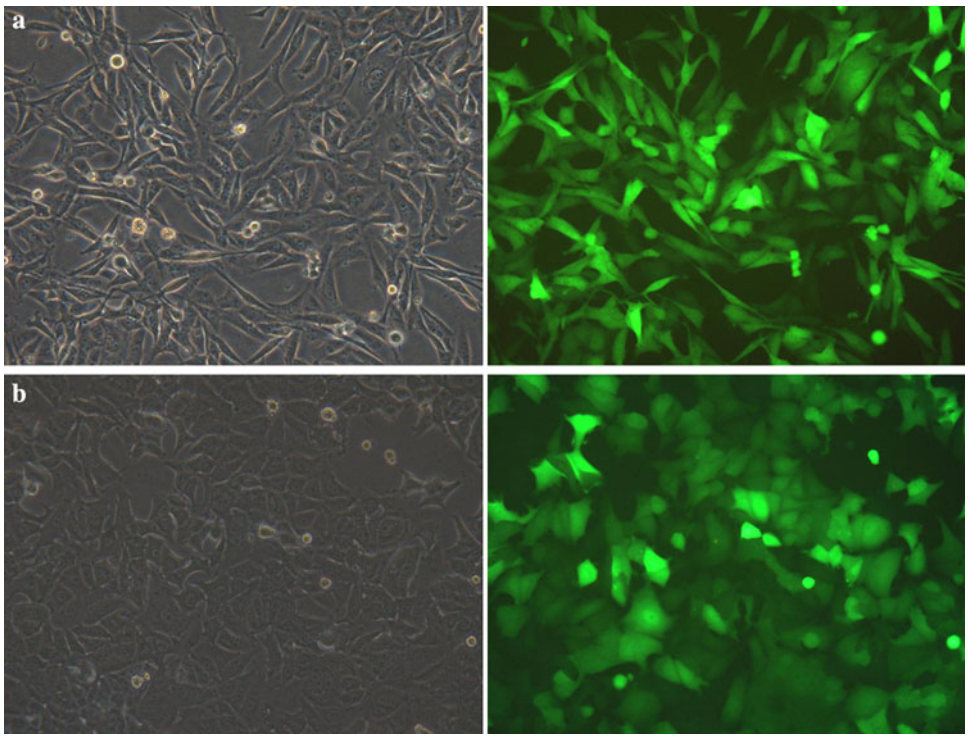

Fig. 2 Detection of lentiviral infection efficiency. MDA-MB-231 (**a**) and MCF-7 cells (**b**) were infected with Lv-shMed19, and the phase contrast (*left*) or GFP expression (*right*) was examined 120 h after infection. Magnification ×200

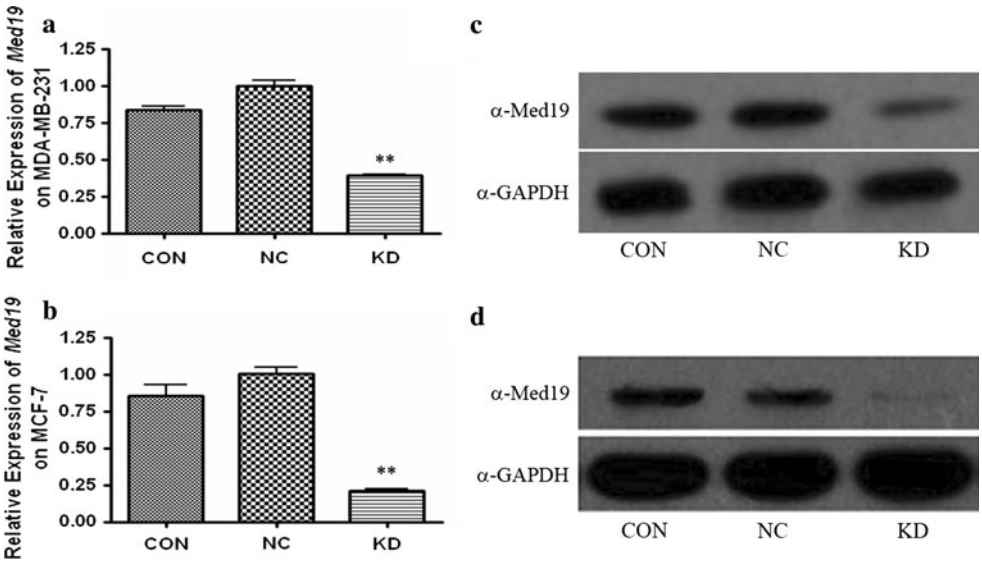

Fig. 3 Lv-shMed19-mediated RNAi silencing significantly suppressed the mRNA and protein expression of Med19. Med19 mRNA expression decreased dramatically in the Lv-shMed19-infected (KD) groups of MDA-MB-231 (**a**) and MCF-7 cells (**b**), when compared to the Lv-NC-infected (NC) groups. Med19 protein expression decreased significantly in the KD groups of MDA-MB-231 (**c**) and MCF-7 cells (**d**), when compared to the NC groups and non-infected (CON) groups. ** Significant difference from the NC groups ($p < 0.01$)

mediator complex [7], making it critical in elaborate regulation of transcription. To understand the role of Med19, in situ immunohistochemical staining analysis was carried out to detect the expression of Med19 in breast cancer. It was showed that the expression of Med19 was significantly upregulated in breast cancer tissues compared to adjacent normal tissues (Fig. 1) and was correlated with higher tumor grade (Table 1), indicating potential relationship between Med19 and breast cancer. This is the first report presenting the upregulated expression of Med19 in breast cancer tissues, and there is scope for the design of target-specific therapies in breast cancer.

Vector systems for expressing shRNA have been established to induce RNAi in mammalian cells [21]. Compared to the chemically synthesized siRNA, shRNA encoded within an expression vector offers advantages in silencing longevity, delivery options, and cost. But there are still some limitations, including transient shRNA

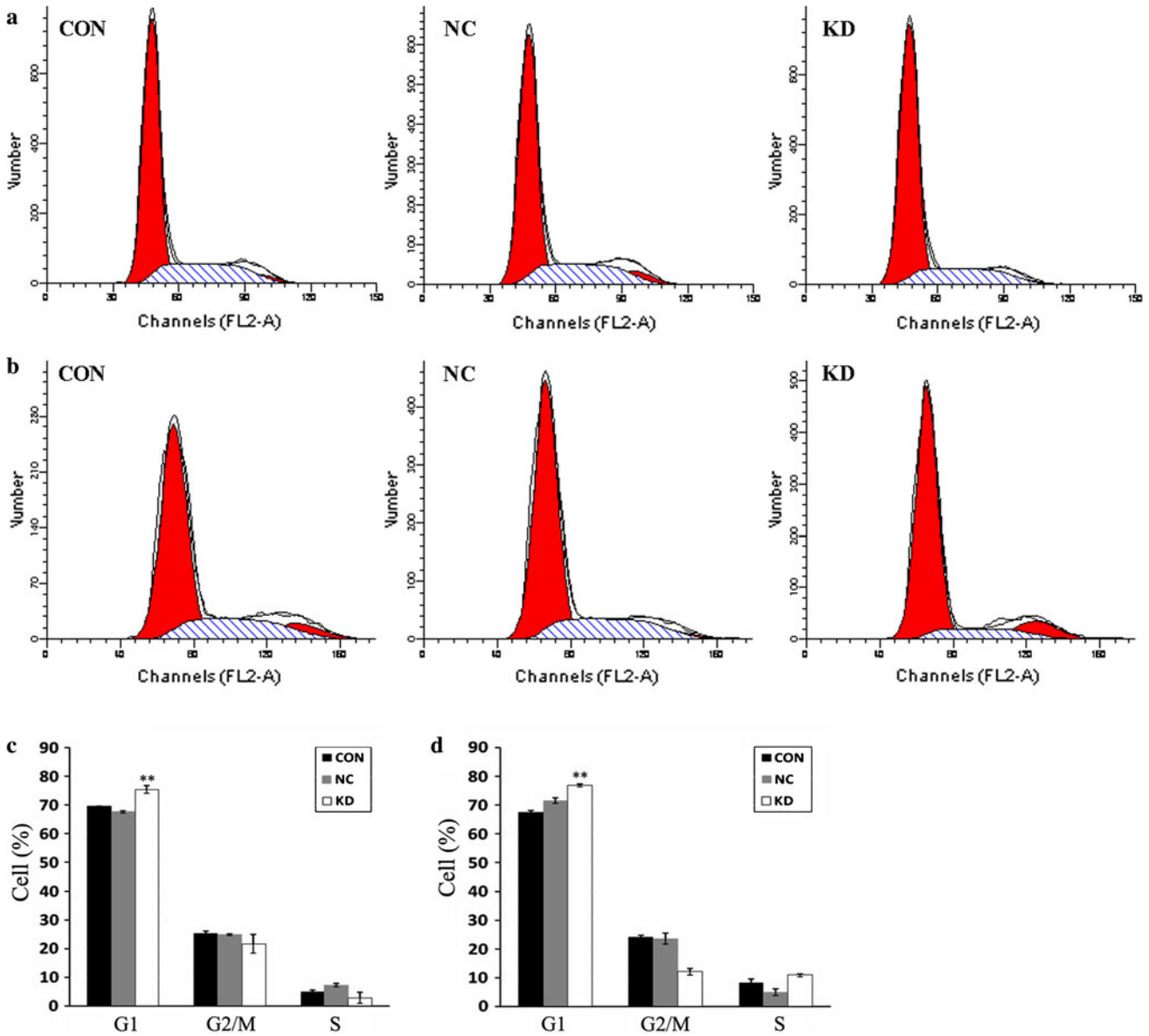

Fig. 4 Knockdown of Med19 augments the proportion of G0/G1 phase in MDA-MB-231 (**a, c**) and MCF-7 cells (**b, d**). The proportion of different cell cycle phases was quantitated by PI staining followed by flow cytometry analyses. KD, Lv-shMed19-infected; NC, Lv-NC-infected; CON, non-infected. ** Significant difference from the NC groups ($p < 0.01$)

expression and low transfection efficiency. To overcome these disadvantages, viral vectors have been developed, including adenoviral vectors [22], retroviral vectors [23], and lentiviral vectors [24]. Lentiviruses are particularly suited for gene therapy because they integrate into the host genome to provide life-long expression of the shRNA and also they can deliver large genetic payloads, a property that can be exploited for expressing multiple shRNAs simultaneously [25]. Furthermore, lentiviruses have been rendered progressively safer by the development of split plasmid systems for vector production to prevent generation of replication-competent virus [26]. Lentivirus-delivered shRNAs have been used in clinical trials with no significant side effects [27, 28].

In our study, lentiviral vector pGCSIL-GFP was chosen as the shRNA delivery vehicle to downregulate the endogenous expression of *Med19* gene in MDA-MB-231 and MCF-7 cells. Since the potency of the silencing effect of shRNA is related to the specificity to its target sequence, real-time PCR and Western blotting were used to determine the efficiency of Lv-shMed19 in MDA-MB-231 and MCF-7 cells. The significant inhibitory effect was observed and the expression of Med19 mRNA and protein were dramatically inhibited. Given this result, we concluded that

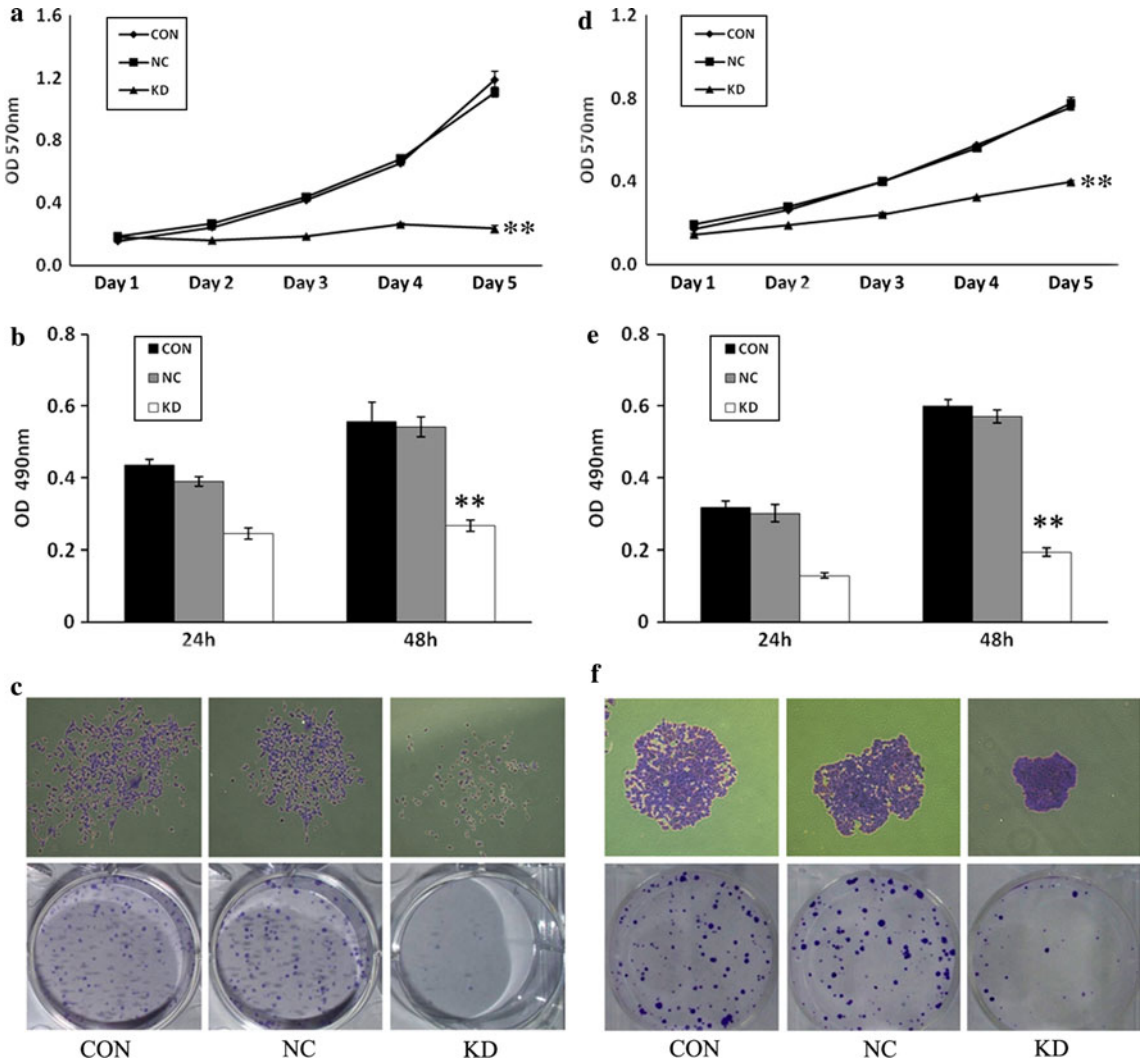

Fig. 5 Knockdown of Med19 inhibits the cell growth of MDA-MB-231 and MCF-7 cells. Effect of Med19 downregulation on proliferation of MDA-MB-231 (**a**) and MCF-7 cells (**d**) was assessed by the MTT assay. Effect of Med19 knockdown on DNA synthesis of MDA-MB-231 (**b**) and MCF-7 cells (**e**) was assessed by the BrdU incorporation assay. Effect of Med19 silencing on growth of MDA-MB-231 (**c**) and MCF-7 cells (**f**) was assessed by the colony formation assay. KD, Lv-shMed19-infected; NC, Lv-NC-infected; CON, non-infected. ** Significant difference from the NC groups ($p < 0.01$)

Lv-shMed19 had a high specificity against Med19 in MDA-MB-231 and MCF-7 cells thus was qualified to be used in subsequent studies.

Our study was the first report presenting a shRNA-mediated Med19 silencing strategy to inhibit cell growth and proliferation in breast cancer. Specific silencing of Med19 induced dramatic inhibition of cell growth and proliferation of MDA-MB-231 and MCF-7 cells, in the MTT, BrdU incorporation, and colony formation assays (Fig. 5). Besides, the downregulation of Med19 augments the proportion of G0/G1 phase in MDA-MB-231 and MCF-7 cells (Fig. 4).

Recent advances in biochemistry, cell, genetics, and molecular biology have greatly improved our understanding of the molecular mechanisms of breast cancer progression. There are now significant research opportunities to identify and exploit molecular targets for the discovery of anticancer agents. Some of these molecular-targeted agents are not only efficient as tumor therapeutics, but also improve the patient's quality of life. The list of targets for molecular therapy is growing daily. Our results in this report suggested the important role of Med19 in breast cancer and the potential of Med19 as a marker for molecular-targeted therapy of breast cancer.

In conclusion, our work provided the evidence that shRNA-mediated knockdown of *Med19* gene expression inhibited the growth of breast cancer cells. Lentivirus-mediated silencing of Med19 could be a potential molecular-targeted therapeutic strategy for breast cancer, although the molecular mechanism of Med19 in breast cancer still needs further investigation.

Acknowledgments The authors were thankful for the financial support from the Scientific and Technologic Bureau of Wuxi (CSE00708 and CAE00801-08-"333 project of Jiangsu Province").

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