Genetics and Molecular Research

Effects of siRNA-mediated silencing of Sal-like 4 expression on proliferation and apoptosis of prostate cancer C4-2 cells

K.F. Liu and Y.X. Shan

Department of Urology, The Second Hospital Affiliated to Suzhou University, Suzhou, Jiangsu Province, China

Corresponding author: Y.X. Shan
E-mail: shanyuxidu@yeah.net

Genet. Mol. Res. 15 (2): gmr.15027885
Received October 22, 2015
Accepted December 28, 2015
Published May 13, 2016
DOI http://dx.doi.org/10.4238/gmr.15027885

ABSTRACT. The aim of this study was to evaluate the effects of small interfering RNA (siRNA)-inhibited expression of the Sal-like 4 (*SALL4*) gene on the proliferation, colony formation, and apoptosis of prostate cancer C4-2 cells. C4-2 cells were cultured and divided into a si-SALL4 group, a negative control siRNA group, and a blank control group. *SALL4* mRNA levels and protein expression were detected by real-time polymerase chain reaction and western blot, respectively. Changes in the cell proliferation and colony formation capacities were observed by using the MTS colorimetric method and colony formation assay, respectively. The influence of SALL4 on apoptosis was assessed with flow cytometry, and the expression of apoptosis-related proteins B-cell lymphoma 2 (Bcl-2) and bcl-like-protein 4 (Bax) were detected by western blot. The si-SALL4 group had significantly lower mRNA and protein levels of SALL4 as well as decreased proliferation and colony formation capacities than the negative control group ($P < 0.05$). There were significantly more apoptotic cells in the si-SALL4 group compared to the negative control ($P < 0.05$), and the expression of Bcl-2 and Bax decreased and increased, respectively, after treatment with

si-SALL4. Silencing *SALL4* expression by using siRNA technology inhibited the proliferation and colony formation of C4-2 cells, and promoted apoptosis likely mediated by Bcl-2 and Bax expression. These results provide experimental basis for further elucidating the role of SALL4 in prostate cancer cells.

Key words: Small interfering RNA; Sal-like 4; Prostate cancer; Gene silencing

INTRODUCTION

The mortality rate of prostate cancer ranks second among those of all male malignant tumors worldwide (Jemal et al., 2011). Without obvious early symptoms, many patients with prostate cancer have already experienced metastasis upon diagnosis, resulting in poor treatment outcomes. Therefore, it is necessary to find specific markers for early prediction of the onset and progression of prostate cancer and to develop targeted drugs.

The Sal-like 4 (*SALL4*) gene was first cloned from the fruit fly in 2006 because it is a widely expressed transcription factor. It encodes proteins with several C2H2-type zinc finger double domains. In the normal physiological state, *SALL4* plays an important role in embryo development and organ formation as an embryonic stem cell factor (Rao et al., 2010). Recently, it has been closely associated with tumor onset and progression. In general, SALL4 is highly expressed in germ cell tumors, gastric cancer, lung cancer, breast cancer, and liver cancer (Cao et al., 2009; Kobayashi et al., 2011; Yong et al., 2013). We have previously reported that SALL4 was highly expressed in prostate cancer tissues and its levels closely correlated with Gleason score, clinical stage, prognosis, and expression of prostate specific antigens. Therefore, this protein may be a crucial marker for the diagnosis and prognostic evaluation of prostate cancer (Lai et al., 2013), and its biological role in the development of this cancer should be further examined. In this study, *SALL4* gene expression in the prostate cancer cell line C4-2 was silenced by RNA interference. Cell proliferation and colony formation were detected using the MTS colorimetric assay and colony formation assay, respectively, and apoptosis was detected by flow cytometry. The aim of this study was to assess the effects of the SALL4 protein on the proliferation and apoptosis of prostate cancer cells.

MATERIAL AND METHODS

Reagents and culture conditions

RNAiso Plus total RNA extraction kit, reverse transcription kit, polymerase chain reaction (PCR) reagent and Lipofectamine™ 2000 transfection reagent were purchased from Life Technologies (USA). SALL4 rabbit anti-human polyclonal antibody was from Santa-Cruz (USA). Bcl-2 monoclonal antibody was obtained from Abcam (USA). MTS reagent, apoptosis-related reagent, and serum were provided by Sigma (USA). Incomplete RPMI 1640 medium was purchased from Gibco (USA). All other reagents were from Beyotime Institute of Biotechnology Co., Ltd. (China). Human prostate cancer C4-2 cells were provided by China Center for Type Culture Collection (China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and incubated at 37°C in 5% CO_2 atmosphere with saturated humidity.

C4-2 cell transfection

C4-2 cells were inoculated onto 6-well plates at a density of 2 x $10^9/m^2$ 24 h before transfection, which was initiated at 70-80% confluence. Then, the cells were divided into an untreated blank control group, a negative control (NC) group treated with NC-small interfering (siRNA), and a si-SALL4 group treated with SALL4 siRNA (RiBo-Bio, Guangzhou, China). Lipofectamine™ 2000 (5 mL/L) and different groups of siRNA (100 pM) were dissolved in serum- and antibiotic-free culture medium, mixed, and left at room temperature for 20 min. After the original culture medium was discarded, the cells were washed twice with fresh serum-free culture medium and then the mixture of Lipofectamine™ 2000 and siRNA was added. Culture medium containing serum was used 6 h later to further culture the cells for 24-72 h.

Detection of *SALL4* mRNA levels by real-time PCR

Total RNA was extracted with TRIzol reagent 48 h after transfection, and *SALL4* and internal reference *GAPDH* were amplified by using real-time PCR. Primers for *SALL4* were: forward 5'-CCGGCAGTAAGGACTGTC-3', and reverse 5'-TCTCTGTCTTTAGGTACACCACA-3' (PCR product: 97 bp). *GAPDH* primers: forward 5'-GGAGTCAACGGATTTGGTCGTAT-3', and reverse 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (PCR product: 500 bp). PCR was performed at 98°C for 20 s, 55°C for 30 s, and 72°C for 30 s for a total of 32 cycles.

Detection of SALL4, B-cell lymphoma 2 (Bcl-2), and bcl-2-like protein 4 (Bax) protein levels by western blot

Cells were collected and total protein was quantified 72 h after transfection, resolved on 8% SDS-PAGE, and transferred to a PVDF membrane. The membrane was blocked in skim milk at room temperature for 1 h and incubated overnight with GAPDH (1:1000), SALL4 (1:1000), Bcl-2 (1:1000) and Bax (1:1000) primary antibodies (SantaCruz, Dallas, TX, USA) at 4°C. The next day, the membrane was washed with TBS-T, incubated with goat anti-rabbit IgG/horseradish peroxidase (1:2000 dilution) at room temperature for 1 h, washed again with TBS-T, and treated with ultrasensitive enhanced chemiluminescence reagent.

Detection of cell proliferation by MTS assay

C4-2 cells were cultured on 96-well plates, and 20 μL MTS was added to each well 24, 48, 72, or 96 h after transfection. The cells were then cultured for another 4 h at 37°C, and the absorbance was measured at 490 nm. Three replicate were performed for each group and the mean was used as the final result.

Detection of colony formation by colony formation assay

C4-2 cells were cultured on 6-well plates and those cells adhering to the wall were digested 24 h after transfection and counted. Two thousand cells from each group were inoculated on 6-well plates and incubated, and the culture medium was discarded 14 days later. The cells were thereafter fixed with 1 mL paraformaldehyde for 20 min and stained by adding 1 mL crystal violet staining solution for 30 min after removal of paraformaldehyde.

Subsequently, the cells were washed with pure water, air-dried at 37°C and counted under a microscope. A colony was defined where there were more than 50 cells.

Detection of apoptosis by flow cytometry

Cells (10^6) were collected 72 h after transfection, washed with PBS, and centrifuged at 1000 *g* for 5 min. This procedure was then repeated. After the supernatant was discarded, the cells were collected, mixed, resuspended in 500 μL binding buffer, mixed with 10 μL propidium iodide (Beyotime, Beijing, China), left at room temperature in the dark for 5-15 min for the reaction to occur, mixed with 5 μL AnnexinV-FITC (Beyotime, Beijing, China), and finally detected with flow cytometry. The binding buffer was prepared by Beyotime (Beijing, China).

Statistical analysis

All data were analyzed by the SPSS19.0 software (Chicago, IL, USA) and are reported as means ± standard deviation. Inter-group comparisons were performed by using the Student *t*-test or one-way analysis of variance.

RESULTS

SALL4 siRNA reduced *SALL4* mRNA levels in transfected cells

Total RNA was extracted 48 h after transfection and *SALL4* gene expression level was detected by real-time PCR (Figure 1A). The relative expression level of *SALL4* mRNA of the NC group was set at 100% and the si-SALL4 group was then 47.92% ($P < 0.05$). As expected, si-SALL4 inhibited *SALL4* expression at the mRNA level.

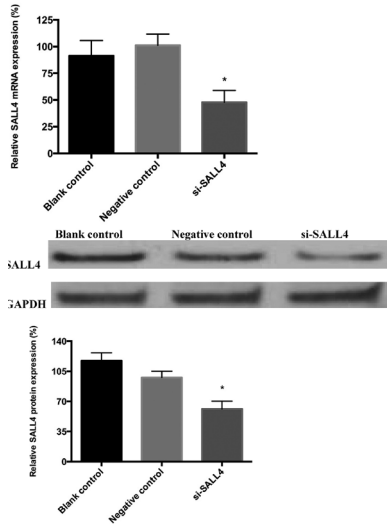

Figure 1. Effects of SALL4 siRNA transfection on SALL4 mRNA levels (top) and protein expression (bottom). A representative western blot for protein expression of SALL4 and GAPDH is shown. All graph values are reported as means ± SD, N = 3, *P < 0.05 *vs* negative control.

SALL4 siRNA inhibited SALL4 protein expression in transfected cells

Total protein was extracted 72 h after transfection and detected by western blot (Figure 1B). The expression of SALL4 protein was significantly lower in the si-SALL4 group compared to the control group (49.71%) ($P < 0.05$). Therefore, si-SALL4 also suppressed SALL4 expression at the protein level.

Transfection with SALL4 siRNA inhibited cell proliferation

Compared with the blank control and NC groups, the proliferative activity of the si-SALL4 group was significantly inhibited 72 h after transfection ($P < 0.05$) (Figure 2).

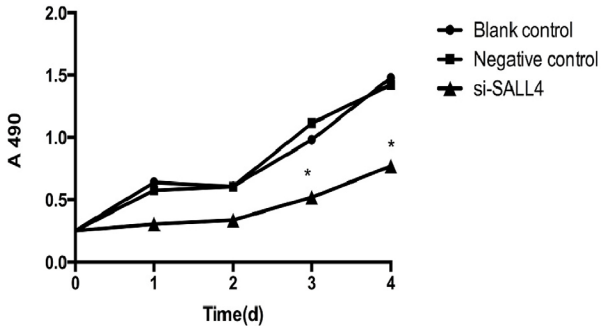

Figure 2. Changes in cell proliferation after SALL4 siRNA transfection. Means±SD, N=3, *P<0.05 *vs* negative control.

Transfection with SALL4 siRNA inhibited colony formation

The colony formation assay was performed 24 h after transfection and the colony formation rate was detected after 14 days of culture. The si-SALL4 group had significantly fewer colonies than the NC group (47.72%) ($P < 0.05$) (Figure 3).

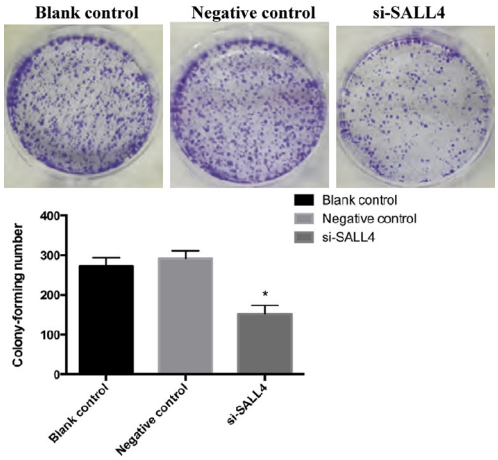

Figure 3. Changes in colony formation after SALL4 siRNA transfection. Representative colonies on plates (top) and graphed values (bottom) are shown. Graph values are reported as means ± SD, N = 3, *P < 0.05 *vs* negative control.

Detection of cell apoptosis by flow cytometry

The apoptotic rate of the si-SALL4 group was 16.57%, which was significantly higher than the NC group ($P < 0.05$) (Figure 4).

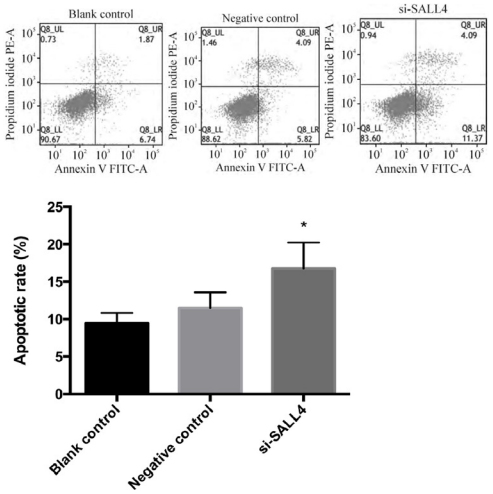

Figure 4. Effects of SALL4 siRNA on cell apoptosis. Results from flow cytometry (top) and graphed values (bottom) are shown. Graph values are reported as means ± SD, N = 3, *P < 0.05 *vs* negative control.

Si-SALL4 downregulated Bcl-2 and upregulated Bax protein expression

Compared with the NC group, the expression of the anti-apoptotic protein Bcl-2 in the si-SALL4 group was significantly downregulated ($P < 0.05$), while the expression of the pro-apoptotic protein Bax was significantly upregulated ($P < 0.05$) (Figure 5).

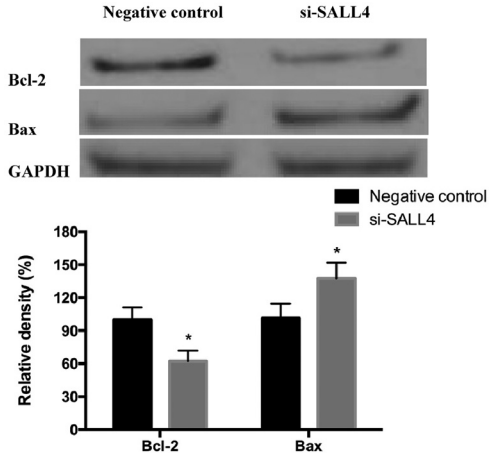

Figure 5. Effects of SALL4 siRNA on Bcl-2 and Bax protein expression in C4-2 cells. Representative western blot (top) and its quantitation (bottom) are shown. Graph values are reported as means ± SD, N = 3, *P < 0.05 *vs* negative control.

DISCUSSION

Prostate cancer is one of the most devastating malignant tumors in the urogenital system threatening men worldwide and has a rapidly increasing morbidity rate (Liu et al., 2013). However, the pathogenesis of the disease is currently unknown. Malignant tumors, which are typified by uncontrollable proliferation and resistance to apoptosis (Chisholm et al., 2012), are not regulated by defined mechanisms.

The *SALL4* gene, an important transcription factor and stem cell factor, plays key roles in the differentiation and development of embryonic organs. Depletion or mutation of SALL4 is associated with diseases such as acro-renal-ocular Okihiro and IVIC syndromes that are characterized by ear deformities, hearing loss, and kidney, heart and limb defects. Highly expressed in many malignant tumors, SALL4 protein may regulate the proliferation and apoptosis of tumor cells, particularly tumor stem cells. SALL4 is commonly overexpressed in malignant tumors. For example, SALL4 is overexpressed in about 90% of samples of colorectal cancer and its levels closely correlate with lymphatic metastasis and degree of differentiation (Forghanifard et al., 2013). SALL4 expression is increased by 86.1% in breast cancer samples, even in those at an early stage of disease (Kobayashi et al., 2011). In addition, SALL4 is highly expressed in liver cancer stem cells but not in liver cancer cells; therefore, disrupted expression of SALL4 inhibits proliferation and promotes apoptosis (Oikawa et al., 2013). Yang et al. (2008) reported that SALL4 was crucial to survival and apoptosis in cancer stem cells that were isolated from the promyelocytic leukemia cell line NB4. As shown by gene expression microarray data, inhibiting the *SALL4* gene significantly downregulated anti-apoptotic gene Bcl-2 and significantly upregulated pro-apoptotic genes tumor protein 53 and tumor necrosis factor, suggesting that SALL4 maintains the resistance of leukemia cells to apoptosis.

Prostate-specific membrane antigen may positively regulate the growth and migration of prostate cancer cells by upregulating the activity of extracellular signal-related kinase (ERK) protein, and the expression of ERK remarkably resembles that of SALL4 under different conditions. Since phosphorylated ERK may initiate rapid proliferation of tumor cells by activating SALL4 after entering cells, high SALL4 expression may be regulated by prostate-specific membrane antigen in prostate cancer (Brumbaugh et al., 2012). Currently, the regulatory effects of SALL4 on the biological functions of prostate cancer cells have never been reported. Herein, when SALL4 protein expression was inhibited, the proliferation and colony formation capacities of C4-2 cells significantly decreased and the number of apoptotic cells increased. Accordingly, inhibiting SALL4 may suppress cell proliferation by facilitating apoptosis. Nevertheless, the mechanism by which this occurs is still unclear.

Cell apoptosis, as a multi-step procedure, is synergistically regulated by molecules and genes of various functions (Eum and Lee, 2011). Bcl-2 is a well-known anti-apoptotic gene and its overexpression decreases its downstream product Bax, which inhibits cell apoptosis and leads to disequilibrium between enhanced proliferation and weakened programmed cell death (Akl et al., 2014; Czabotar et al., 2014). Highly expressed in progressive and metastatic prostate cancers, Bcl-2 enhances the anti-apoptotic ability and eventually increases the tumor drug resistance of cancer cells (Furuya et al., 1996; Chan and Yu, 2004; Karnak and Xu, 2010). In this study, transient transfection of SALL4 siRNA promoted cell apoptosis, decreased Bcl-2 protein expression level and elevated that of Bax, suggesting that SALL4 was conducive to the apoptosis of prostate cancer cells. This is most likely mediated by the regulation of the expression of apoptosis-related proteins Bcl-2 and Bax.

In summary, SALL4 is able to regulate several biological functions of prostate cancer cells. Downregulating SALL4 suppressed cell proliferation and colony formation as well as enhanced apoptosis most likely through Bcl-2 and Bax proteins. These findings pave the way for clarifying the mechanism by which SALL4 regulates cancer cells and its role in prostate cancer. However, this is only an *in vitro* study, so further experiments using animal models are still necessary.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank all the authors for their great contributions to this study.

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