

## RESEARCH ARTICLE

# ALEX1 Regulates Proliferation and Apoptosis in Breast Cancer Cells

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### Abstract

**Background:** Arm protein lost in epithelial cancers, on chromosome X (ALEX) is a novel subgroup within the armadillo (ARM) family, which has one or two ARM repeat domains as opposed to more than six-thirteen repeats in the classical Armadillo family members. **Materials and Methods:** In the study, we explore the biological functions of ALEX1 in breast cancer cells. Overexpression of ALEX1 and silencing of ALEX1 were performed with SK-BR3 and MCF-7 cell lines. Cell proliferation and colony formation assays, along with flow cytometry, were carried out to evaluate the roles of ALEX1. **Results:** ALEX1 overexpression in SK-BR3 breast cancer cells inhibited proliferation and induced apoptosis. Furthermore, depletion of ALEX1 in MCF-7 breast cancer cells increased proliferation and inhibited apoptosis. Additional analyses demonstrated that the overexpression of ALEX1 activated the intrinsic apoptosis cascades through up-regulating the expression of Bax, cytosol cytochrome c, active caspase-9 and active caspase-3 and down-regulating the levels of Bcl-2 and mitochondria cytochrome c. Simultaneously, silencing of ALEX1 inhibited intrinsic apoptosis cascades through down-regulating the expression of Bax, cytosol cytochrome c, active caspase-9, and active caspase-3 and up-regulating the level of Bcl-2 and mitochondria cytochrome c. **Conclusions:** Our data suggest that ALEX1 as a crucial tumor suppressor gene has been involved in cell proliferation and apoptosis in breast cancer, which may serve as a novel candidate therapeutic target.

**Keywords:** Breast cancer - ALEX1- cell proliferation - apoptosis - active caspase-3

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### Introduction

Cancer is a global problem that accounts for almost 13% of deaths worldwide, there will be between 15 and 17 million new cases of cancer every year, 60% of which will be in developing countries (Lopez-Gomez et al., 2013). Breast cancer is one of the most prevalent cancers in women worldwide, accounting for 23% of the total cancer cases. It is a major cause of morbidity and mortality among women accounting for 14% of all cancer deaths. Each year, more than one million newly diagnosed cases worldwide appeared and brought serious harm to women health (Jemal et al., 2011). Surgery together with chemotherapy is the main method for breast cancer treatment but brings serious side effects to the patients. Therefore, new diagnostic and treatment strategies with low toxicity and high efficiency for breast cancer are shortly needed.

Arm protein lost in epithelial cancers, on chromosome X (ALEX; also known as armadillo repeat containing, X-linked; ARM CX), characterized by the presence of a repeating 42 amino acid motif (arm repeat) is a novel subgroup within the ARM family. The ALEX gene family

consists of at least three variants (ALEX1, ALEX2, and ALEX3), which have one or two ARM repeat domains. Though the classical ARM family was involved in a variety of processes such as cell adhesion, embryogenesis and tumorigenesis (Peifer et al., 1994; Hatzfeld, 1999), little is known about the ALEX genes. Recent report demonstrates that the ALEX3 directly interacts with the sex determining region Y (Sry)-box 10 (SOX10) transcription factor via the ARM repeat domains and alters its subcellular localization and transcriptional activity (Mou et al., 2009). In addition, the study have shown that the ALEX3 gene encodes a mitochondrial-targeted protein and the non-canonical Wnt/PKC pathway regulates mitochondrial distribution through ALEX3 protein degradation (Serrat et al., 2013). gene expression analysis revealed that both ALEX1 and ALEX2 mRNA is expressed in a variety of adult human tissues, but dramatically reduced or even undetectable in several human carcinoma cell lines and tissues, including breast cancer (Kurochkin et al., 2001). Recent studies have shown that ALEX1 gene is transcriptionally regulated by CREB and Wnt $\beta$ -catenin signaling and ALEX1 suppresses colony formation ability of human colorectal carcinoma cell lines (Iseki et al., 2010; 2012). Despite that,

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the function of ALEX1 in cancer is still poorly understood, especially, in breast cancer.

With this goal, we examined the expression of ALEX1, cell proliferation and apoptosis of SK-BR3 cells and MCF-7 cells after overexpression and silencing of ALEX1. The results showed that ALEX1 overexpression in breast cancer cells inhibited proliferation and induced apoptosis. However, depletion of ALEX1 increased proliferation and inhibited apoptosis.

## Materials and Methods

### Materials

SK-BR3 and MCF-7 cells were obtained from American Type of Culture Collection. Dulbecco's modified Eagle's medium (DMEM) was from Gibco. Lipofectamine 2000 and fetal bovine serum (FBS) were from Invitrogen. Primary and secondary antibodies for ALEX1, Bcl-2, Bax and  $\beta$ -actin for Western blot analysis were purchased from Santa Cruz Biotechnology and antibodies for active caspase-3, active caspase-9 and cytochrome c were obtained from Abcam Biotechnology. All the other chemicals and reagents were obtained from local commercial sources.

### Cell culture

SK-BR3, MDA-MB-231, T47D and MCF-7 cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin in a cell culture incubator at 37°C with 5% CO<sub>2</sub>. MCF-10A were grown in DMEM/F12 supplemented with 5% Horse serum, 10  $\mu$ g/ml Insulin, 20ng/ml EGF, 100ng/ml Cholera Toxin, 0.5  $\mu$ g/ml Hydrocortisone, 100 units/ml Penicillin and 100  $\mu$ g/ml Streptomycin in a cell culture incubator at 37°C with 5% CO<sub>2</sub>.

### siRNA sequences

The Promega software system was used to select siRNA against ALEX1 mRNA (NM\_016608). the ALEX1-siRNAs and control siRNA were designed as follows: RNAi-1: 5'-CCUGGAGCGAACAAAUGAUTT-3' (sense) and 5'-AUCAUUUGUUCGCUCCAGGTT-3' (anti-sense); RNAi-2: 5'-GCCUGCUACUGUGUAUACATT-3' (sense) and 5'-UGUAUACACAGUAGCAGGCTT-3' (anti-sense); RNAi-3: 5'-GCUGGGCUAAGACUGUUAATT-3' (sense) and 5'-UUAACAGUCUUAGCCCAGCTT-3' (anti-sense) for three of ALEX1 siRNAs; and 5'-UUCUCCGAACGU GUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense) for control siRNA. All of the siRNA sequences were chemically synthesized by Shanghai Genepharma Co. Ltd.

### Construction of recombinant lentiviral vector

The ALEX1 coding region fragment DNA was cloned into shuttle plasmid LV5 (Genepharma, China). The recombinant construct (LV5-ALEX1) was confirmed by sequencing. The recombinant construct and the negative control (LV5-NC) were transfected into 293T cells with three packaging components (pGag/Pol, pRev and pVSV-G) by Lipofectamine 2000 (Invitrogen, USA), respectively. The recombinant lentivirus expressing

ALEX1 and the negative control lentivirus were obtained from the cells supernatant and named as LV5-ALEX1, LV5-NC, respectively.

### Transient transfection of siRNA oligonucleotides

One day before transfection, cells were seeded at a density of 100,000 cells in 1.0 ml of growth medium without antibiotics in 6-well plates so that cells would grow to 50-60% confluence at the time of transfection. MCF-7 cells were transfected with ALEX1-siRNA (SiALEX1) or control siRNA (SiCon) for 48 h via Lipofectamine 2000. Two groups were set up: (i) The 90 nmol/l SiALEX1 group, (ii) The 90 nmol/l SiCon group.

### Stable transfection of lentivirus LV5

SK-BR3 cells were infected with lentivirus LV5-ALEX1 or LV5-NC at an MOI (multiplicity of infection) of 5 for 72h. Cell line was divided into two experimental groups: (i) LV5-ALEX1 group, (ii) LV5-NC group.

### RNA isolation and real-time PCR

Total RNA was isolated using E.Z.N.A. Total RNA Kit I (Omega, USA). cDNA was synthesized using PrimeScript<sup>®</sup> RT reagent Kit with gDNA Eraser (Takara, Japan). Real-time PCR was carried out by SYBR<sup>®</sup> Premix ExTaq<sup>™</sup>II (Takara, Japan) and analyzed on MiniOpticon TM Real-Time PCR Detection System (Bio-Rad, USA). The sequences used for primers were as follows: 5'-TGATATTCTGAGTGCTCCCGACC-3' (sense) and 5'-TGTTACCCAGAGTGACCAAGGCT-3' (antisense) for ALEX1; and 5'-CTTTGGTATCGTGGGAAGGACTC-3' (sense) and 5'-GTAGAGGCAGGGATGAGTTCT-3' (antisense) for GAPDH.

### Cell proliferation assay

Cell proliferation was measured by a CCK8 kit (Beyotime, China) according to the manufacturer's protocol.

### Colony formation assay

SK-BR3 and MCF-7 cells were treated as described above. For each group of cells, 0.5 $\times$ 10<sup>3</sup> cells were plated in 12-well plates and incubated for 10 days. Medium was changed every 3 days. And then, colonies were fixed with 4% polyformaldehyde and stained with 0.2% crystal violet solution and counted.

### Apoptosis analysis

SK-BR3 and MCF-7 cells were treated as described above. Two distinct methods were applied to get the convincing results of cell apoptosis. First, cell apoptosis was measured by fluorescence microscopy to identify apoptotic nuclear changes (cell shrinkage, chromatin condensation and fragmentation, nuclear membrane blebbing) after staining SK-BR3 and MCF-7 cells with Hoechst 33258. The percentage of apoptotic cells was determined with at least 100 cells per treatment group. Next, Cell apoptosis was evaluated by staining with AnnexinV-FITC/PI according to the protocol provided in the AnnexinV-FITC/PI apoptosis detection kit (ABGAB, USA) for 5 min at 25°C, immediately analyzed by flow

cytometry, where apoptotic cells were defined as annexin V+ and PI-.

#### Western blot

SK-BR3 and MCF-7 cells were treated as described above. Total proteins from cells were extracted in RIPA Lysis Buffer (Beyotime, China) plus Phenylmethanesulfonyl fluoride. Mitochondrial and cytosolic proteins were isolated using Mitochondria/cytosol Fractionation Kit (Beyotime, China) following the manufacturer's protocol. Proteins were probed with anti-ALEX1, anti- $\beta$ -actin, anti-Bax, anti-Bcl-2 (Santa Cruz, USA) and anti-active caspase 3, anti-active caspase 9, anti-cytochrome c (Abcam, USA), 40  $\mu$ g of protein were separated in 12% SDS-PAGE gels, transferred to nitrocellulose membrane, and blocked with 5% nonfat milk in TBST on a shaker at room temperature for 1.5 h. The membranes were incubated with primary antibody on a shaker overnight at 4°C. Goat anti-mouse and anti-rabbit, secondary antibodies conjugated to HRP (Santa Cruz, USA) were used to visualize the stained bands with an ECL kit (Santa Cruz, USA).

#### Immunofluorescence

Immunofluorescence staining with anti-ALEX1 antibody was performed in SK-BR3 cells. SK-BR3 cells were infected as described above and treated on coverslips. After fixation with 4% polyformaldehyde, cells were blocked in a buffer containing 10% goat serum and 1% BSA for 1h at room temperature and washed with PBS for three times; the slides were incubated with anti-ALEX1 antibody (1:50 dilution) overnight at 4°C, then with Alexa Fluor 549-conjugated anti-mouse IgG (Invitrogen, USA) for 2h at 37°C. After the final washes, DAPI (Sigma, USA) was added and used as counterstain for nuclei. Fluorescence images were acquired by an Olympus-BX51 microscope.

#### Statistical analysis

All statistical analyses were performed using the SPSS 17.0. data were represented as mean $\pm$ SD. Student's t test was used to compare data between control group and test group.  $p < 0.05$  was considered as statistically significant. Treatments of cells were analyzed in triplicate wells and the experiments were repeated three times.

## Results

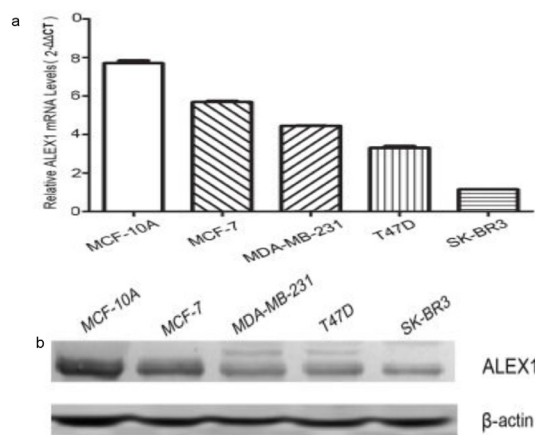
#### Expression of ALEX1 in breast cancer cells

To evaluate the expression of ALEX1 in breast cancer cells, we have examined the expression level of ALEX1 in five breast cell lines (MCF-10A, MCF-7, MDA-MB-231, T47D and SK-BR3) (Figure 1), Endogenous ALEX1 was underexpressed with different levels in all of these four breast cancer cell lines compared with MCF-10A, but relatively high expression of ALEX1 was observed in MCF-7 and low expression of ALEX1 in SK-BR3 cell lines by real-time PCR and Western blot.

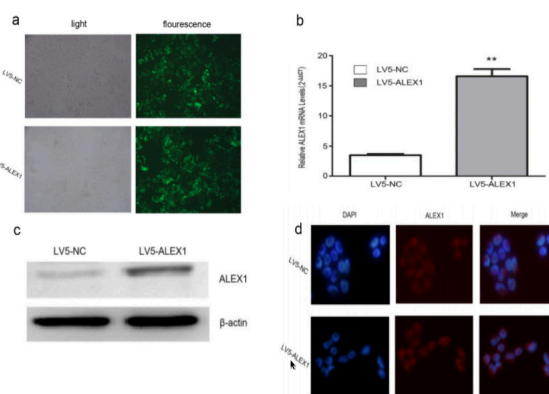
#### LV5-ALEX1 increases ALEX1 expression in SK-BR3 cells

To explore the role of ALEX1 in breast cancer, we have overexpressed ALEX1 in SK-BR3 cells by

recombinant lentiviral infection. SK-BR3 cells were treated as described above. The efficiency of infection was at least 90 % as indicated by the fluorescence intensity of GFP (Figure 2a). To further confirm the efficiency of ALEX1 overexpression, real-time PCR, Western blot and immunofluorescence were performed. The result revealed that the level of ALEX1 mRNA in the LV5-ALEX1 group was significantly higher than that in the LV5-NC group ( $p < 0.01$ , Figure 2b). Western blot showed the same result in the protein levels (Figure 2c). In LV5-ALEX1 cells, immunofluorescence staining with anti-ALEX1 antibody showed that ALEX1 protein was expressed in a higher



**Figure 1. The Expression of ALEX1 in Normal Breast Cell MCF-10A and Breast Cancer Cells MCF-7, MDA-MB-231, T47D and SK-BR3.** a) The expression of ALEX1 in breast cells by Real-time PCR. b) The expression of ALEX1 in breast cells by Western blot,  $\beta$ -actin was used as an internal control for equal loading



**Figure 2. The overexpression of ALEX1 in MCF-7 cell line infected with lentiviruses.** a) The infection efficiency of LV5-ALEX1 in SK-BR3. The morphology of SK-BR3 cells was measured by a light microscope (Magnification,  $\times 200$ ) and fluorescence microscope (Magnification,  $\times 200$ ) at 72 h after infection with LV5-ALEX1. b) Real-time PCR revealing relative ALEX1 mRNA levels normalized to GAPDH. Data shown are fold changes over the LV5-NC group and presented as mean values of three replicates with standard errors (\*\* $p < 0.01$  vs LV5-NC). c) Western blot showing protein levels of ALEX1.  $\beta$ -actin was used as an internal control for equal loading. d) Immunofluorescence with an anti-ALEX1 antibody showing the overexpression of ALEX1 in SK-BR3 infected with LV5-ALEX1, compared to the LV5-NC. DAPI staining was used to show the nucleus (Magnification,  $\times 400$ )

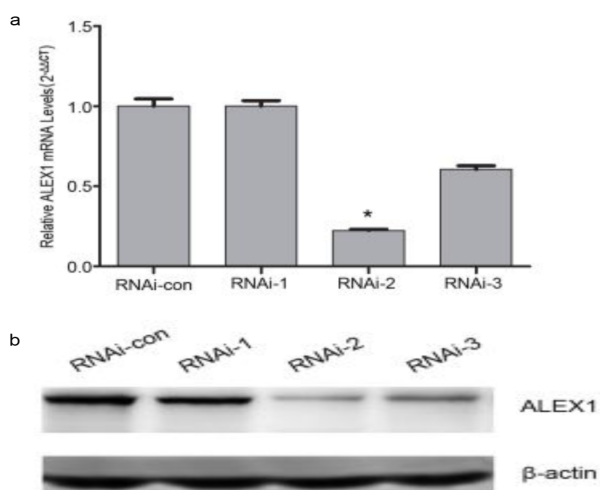
level in cytoplasmic fraction, compared with LV5-NC group (Figure 2d).

*SiALEX1 inhibits ALEX1 expression in MCF-7 cells*

MCF-7 Cells were transfected with ALEX1 RNAi-1, ALEX1 RNAi-2, ALEX1 RNAi-3 and RNAi-con . After 48 h, ALEX1 mRNA and protein was examined by real-time PCR and Western blot. The real-time PCR data showed that the lowest expression of ALEX1 in MCF-7 cells treated with ALEX1 RNAi-2 among three siRNAs compared to RNAi-con group (Figure 3a). Western blot showed the same result in the protein levels (Figure 3b). Therefore, ALEX1 RNAi-2 (SiALEX1) was used to perform further tests.

*Overexpression of ALEX1 inhibited proliferation and clonogenic ability of SK-BR3 cells and silencing of ALEX1 increased proliferation and clonogenic ability of MCF-7 cells*

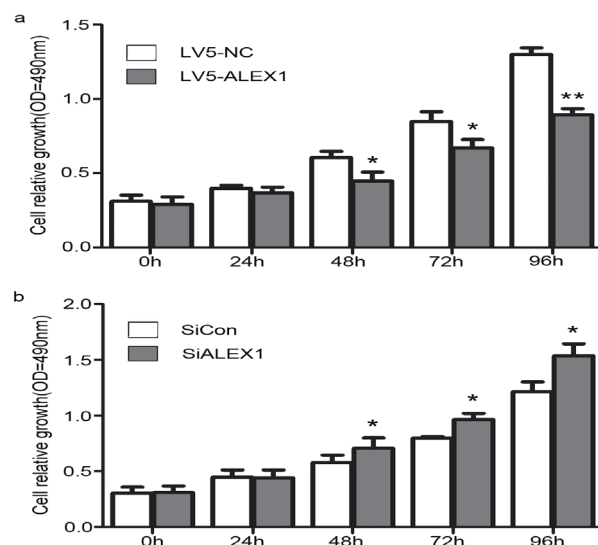
Cell proliferation assays were performed for SK-BR3 cells that were infected with either LV5-ALEX1 or LV5-NC. According to results from the CCK-8 assay, the absorbance of SK-BR-3 cells infected with LV5-ALEX1 was significantly decreased when compared with LV5-NC group at 48, 72 and 96h ( $p<0.05$ ; Figure 4a). However, the absorbance of MCF-7 cells transfected with SiALEX1 was markedly increased , compared with SiCon group at 48, 72 and 96h ( $p<0.05$ ; Figure 4b). In the colony formation assay, the results revealed that the overexpression of ALEX1 in SK-BR3 cell line significantly inhibited the number of foci, relative to LV5-NC group ( $p<0.01$ ; Figure 5a, b). But, the number of foci in SiALEX1 group increasingly increased, compared to SiCon group in MCF-7 cells ( $P<0.05$ ; Figure 5c, d).



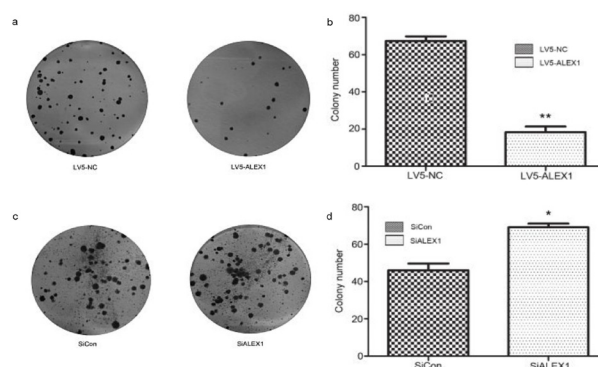
**Figure 3. The Silencing of ALEX1 in MCF-7 Cell Line Transfected with ALEX1-siRNAs.** a) ALEX1 expression in MCF-7 cells transduced with ALEX1 RNAi-1, 2, 3 for 48h. Data shown are fold changes over the RNAi-con group analyzed by Real-time PCR. RNAi-2 treatment remarkably decreased the ALEX1 mRNA levels in MCF-7 cells and results are representative of three independent experiments ( $*p<0.05$  vs RNAi-con). b) RNAi-2 treatment decreased the protein expression levels of ALEX1 in MCF-7 cells analyzed by Western blot

*Overexpression of ALEX1 induced SK-BR3 cells apoptosis and silencing of ALEX1 resisted MCF-7 cells apoptosis*

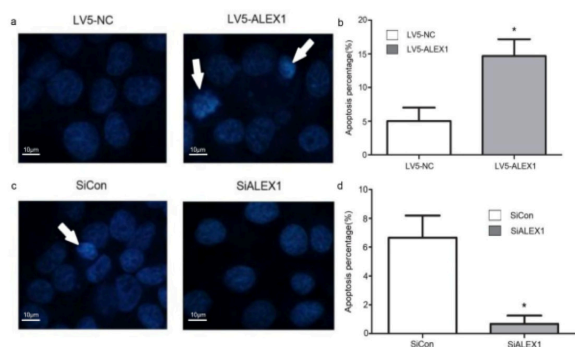
We speculated that ALEX1 could induce breast cancer cells apoptosis. First, Hoechst 33258 staining was performed. The result exhibited typical apoptosis changes with chromatin condensation and fragmentation in LV5-ALEX1 group. However, the typical apoptosis changes were not found in SiALEX1 group of MCF-7 cells (Figure 6). To quantify the apoptotic cells with overexpression and silencing of ALEX1, SK-BR3 and MCF-7 cells were stained with Annexin-V/PI and analyzed by flow cytometry, which showed markedly increased apoptotic cells in ALEX1 overexpression cells, whereas the apoptotic rate was significantly less in ALEX1 silencing cells ( $p<0.05$ ; Figure 7).



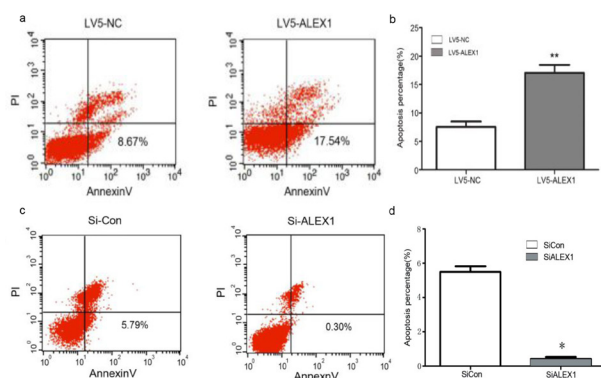
**Figure 4. Effects of ALEX1 Overexpression or Depletion on Cell Growth in MCF-7 Cells.** a) Inhibition of SK-BR3 cells proliferation with LV5-ALEX1 were performed by CCK8 assay. ( $*p<0.05$ ,  $**p<0.01$  vs LV5-NC). b) Promotion of MCF-7 cells proliferation with SiALEX1 ( $*p<0.05$  vs SiCon). Each bar represents the mean±SD (n=3)



**Figure 5. Colony Formation Assay was Performed in MCF-7 Cells with LV5-ALEX1 or SiALEX1.** a) Colony number was decreased in the group with LV5-ALEX1 treatment. b) The Colony number in each group, was shown ( $**p<0.01$  vs LV5-NC). c) Colony number was increased in the group with SiALEX1 treatment. d) The Colony number in each group, was shown ( $*p<0.05$  vs SiCon). Each bar represents the mean±SD (n=3)



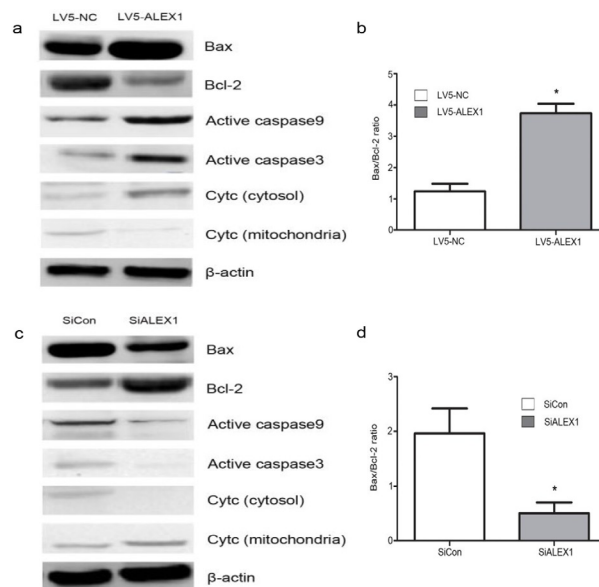
**Figure 6. Hoechst 33258 Staining was Performed in SK-BR3 or MCF-7 Cells with LV5-ALEX1 or SiALEX1.** a) SK-BR3 cells were stained with Hoechst, showed chromatin condensation (arrows, Magnification,  $\times 1,000$ ). b) increase of apoptotic cells was observed in the group with LV5-ALEX1 treatment in SK-BR3 cells ( $**p < 0.05$  vs LV5-NC). c) MCF-7 cells were stained with Hoechst, showed chromatin condensation (arrows, Magnification,  $\times 1,000$ ). d) decrease of apoptotic cells was observed in the group with SiALEX1 treatment in MCF-7 cells ( $*p < 0.05$  vs SiCon). Each bar represents the mean  $\pm$  SD (n=3)



**Figure 7. ALEX1 Regulates Apoptosis in SK-BR3 and MCF-7 Cells.** Apoptotic cell numbers were determined by flow cytometric analysis with Annexin-V and PI staining. a) SK-BR3 cells with LV5-ALEX1 were subjected to apoptosis assay. b) The percentage of apoptosis in each group, was shown ( $**p < 0.01$  vs LV5-NC). c) MCF-7 cells with SiALEX1 were subjected to apoptosis assay. d) The percentage of apoptosis in each group, was shown ( $*p < 0.05$  vs SiCon), each bar represents the mean  $\pm$  SD (n=3)

*Effects of ALEX1 on protein expression levels of intrinsic apoptosis pathway in SK-BR3 and MCF-7 cells*

To determine whether breast cancer cells apoptosis is modulated by intrinsic apoptosis pathway, we analyzed the expression of Bax, Bcl-2, active caspase-9, active caspase-3 proteins with Western blot after overexpression or silencing of ALEX1. The results showed that overexpression of ALEX1 in SK-BR3 cells promoted the expression of Bax, active caspase-9, active caspase-3 and reduced Bcl-2 expressions (Figure 8a, b). However, silencing of ALEX1 in MCF-7 cells decreased the expression of Bax, active caspase-9, active caspase-3 and increased Bcl-2 expressions,  $\beta$ -actin was the internal control, suggesting that the protein loading amount was the same (Figure 8c, d). Collectively, these results suggest that ALEX1 induced apoptosis by causing to increase the



**Figure 8. ALEX1 regulated the expression of apoptosis proteins in SK-BR3 and MCF-7 cells.** a) Western blot analysis of the effect of ALEX1 overexpression in SK-BR3 cells on the expression of Bax, Bcl-2, active caspase-9, active caspase-3, cyt c (mitochondrial and cytosolic),  $\beta$ -actin was used as an internal control for equal loading. b) Quantification of the results from panel a. Shown here are the ratios of Bax/Bcl-2 as determined by the relative intensities of protein bands ( $*P < 0.05$  vs LV5-NC). c) Western blot analysis of the effect of ALEX1 silencing in MCF-7 cells on the expression of Bax, Bcl-2, active caspase-9, active caspase-3, cyt c (mitochondrial and cytosolic),  $\beta$ -actin was used as an internal control for equal loading. d) Quantification of the results from panel c. Shown here are the ratios of Bax/Bcl-2 as determined by the relative intensities of protein bands. Each bar represents the mean  $\pm$  SD of three experiments ( $*p < 0.05$  vs SiCon)

ratio of Bax/Bcl-2 and then release cytochrome c from mitochondria to cytoplasm and activate caspase-9 and caspase-3, ultimately, triggered SK-BR3 cells apoptosis.

**Discussion**

The arm repeats were first found in the segment polarity gene armadillo in *Drosophila* in 1989 (Riggelman et al., 1989). Since then a number of proteins containing arm repeats have been noted and studied. Studies have revealed that arm repeats regulate protein-protein interaction with multiple binding partners involved in nuclear transport, transcription activation and cell junction (Ozawa et al., 1995; Rubinfeld et al., 1995; Troyanovsky et al., 1996). Recently, the biological function of some classical arm repeat proteins have been well clarified. Such as  $\beta$ -catenin, a classical member of ARM family plays important roles both at cells adherence junctions and in Wnt signaling through interaction with E-cadherin and T cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors (Xu et al., 2007; Bass-Zubek et al., 2009). Tumor suppressor adenomatous polyposis coli (APC) is also an ARM family member and acts with casein kinase I, glycogen synthase kinase-3 $\beta$  and AXIN to regulate Wnt signaling through  $\beta$ -catenin degradation (Munemitsu et al., 1995; Rubinfeld et al.,

1996; Goss et al., 2000), APC promoter methylation was found to be significantly associated with a higher risk of developing CRC and the findings indicate that APC promoter methylation may be a potential biomarker for the carcinogenesis of CRC (Ding et al., 2015). But that of the ALEX family members is largely unknown.

To explore the biological function of ALEX1 on breast cancer, a series of studies were performed in SK-BR3 and MCF-7 cells. Infecting SK-BR3 with lentivirus LV5-ALEX1, we were able to increase the expression of ALEX1 at the mRNA and protein levels (Figure 2). In contrast, using the siRNA-targeting ALEX1 gene, we were able to silence the expression of ALEX1 in MCF-7 at the protein and mRNA levels (Figure 3). Next, the effects of ALEX1 overexpression or silencing on cell proliferation, apoptosis were investigated in SK-BR3 or MCF-7 cells. Our results showed that the overexpression of ALEX1 in SK-BR3 cells suppressed cell growth and colony formation (Figure 4a, 5a, b). In addition, the silencing of ALEX1 in MCF-7 cells promoted cell growth and colony formation (Figure 4b, 5c, d). Iseki H et al. (2012) also demonstrated that ALEX1 inhibits colony formation in human colorectal carcinoma cell lines (Kurochkin et al., 2001). Our studies support the conclusion. The study revealed that ALEX1 transcript was found to be lost or reduced in carcinomas derived from several tissues types including primary ovarian carcinoma, lung, prostate, colon, pancreas and also in cell lines originating from human carcinomas through Northern blot hybridization and RT-PCR. ALEX1 mRNA were, however, detectable in cells lines and human samples originating from neuroblastomas, gliomas or sarcomas (Iseki et al., 2012). The loss of expression specifically in carcinomas from epithelial tissues suggests that the ALEX genes is linked to this type of malignancy. Therefore, ALEX1 as tumor suppressor might be involved in regulation of cell growth. However, previous studies have not yet investigated that the molecular mechanisms of ALEX1 underlying the anti-cancer effects.

Apoptosis, the planned cell death, plays key roles in several physiologic processes in cell development (Chen et al., 2013). Failure in apoptosis leads to tumor growth and development as well as resistance to most oncologic therapies (Lowe et al., 2000). Therefore, it is of interest to understand whether ALEX1 is involved in the physiological progression of breast cancer cells apoptosis. In this study, we observed that ALEX1 overexpression induced cell apoptosis in SK-BR3 breast cancer cells by Hoechst staining and flow cytometric analysis, conversely, ALEX1 depletion inhibited MCF-7 breast cancer cells apoptosis (Figure 6, 7). One of the important apoptotic pathways is mitochondrial-mediated signal pathway (Martin et al., 2014). Mitochondria participate in the so-called intrinsic pathway of apoptosis. For intrinsic pathway, apoptosis is regulated by the Bcl-2 family proteins. The ratio of Bax/Bcl-2 is a determinative factor and the high ratio results in mitochondrial outer membrane permeabilization and cytochrome c release and finally activities caspase cascades (Wang et al., 2009; Zhang et al., 2013). Further studies by Western blot showed that ALEX1 overexpression increased the expression of Bax

and decreased the expression of Bcl-2 in SK-BR3 cells. conversely, ALEX1 silencing decreased the expression of Bax and increased the expression of Bcl-2 in MCF-7 cells (Figure 8). In addition, we observed that ALEX1 overexpression reduced the protein level of cytochrome c in mitochondria and increased cytochrome c in cytosol. However, we came to the opposited conclusion in ALEX1 depletion (Figure 8). These results have suggested that ALEX1 promote the ratio of Bax/Bcl-2 and then result in cytochrome c release from mitochondria to cytosol. It is well known that caspase-8 and caspase-9 are essential proteases of extrinsic and intrinsic apoptotic pathways (Chen et al., 2014). Reports showed that the intrinsic apoptosis pathway is characterized as mitochondrial dysfunction and stimulation of caspase-9 and caspase-3 and caspase-3 is the cuultimate executioner for the nucleic changes with apoptosis (Chen et al., 2002). Therefore, mitochondrial damage is associated with the high level of caspase-9 and caspase-3. Our results displayed the higher level of active caspase-9 and active caspase-3 in LV5-ALEX1 group compared with in LV5-NC group (Figure 8). it is reasonable to infer that the elevated level of active caspase-3 is responsible for the increased apoptosis in SK-BR3 cells infected LV5-ALEX1. Taken together, ALEX1 overexpression induces cell apoptosis or ALEX1 silencing inhibits cell apoptosis in SK-BR3 or MCF-7 cells through intrinsic apoptosis depended on caspase activation.

In summary, we demonstrated that the overexpression of ALEX1 inhibited SK-BR3 breast cancer cells growth through upregulating the expression of apoptosis precursor protein Bax, downregulating anti-apoptotic protein Bcl-2 expression, increasing mitochondrial membrane permeability, triggering the caspase-3 cascade and inducing cell apoptosis. On the other hand, the silencing of ALEX1 promoted MCF-7 breast cancer cells growth by the same signal. Results in the study suggest that ALEX1 as a novel tumor suppressor gene could be involved in tumorigenesis and progression of human breast cancer and be utilized as a potential biomarker and therapeutic target in breast cancer. We will further study the molecular mechanisms of ALEX1 on apoptosis, migration and invasion of human breast cancer.

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