RESEARCH ARTICLE

Tumor-suppressive miRNA-135a inhibits breast cancer cell proliferation by targeting ELK1 and ELK3 oncogenes

Akhlaq Ahmad[1] · Weijie Zhang[1] · Mingming Wu[1] · Sheng Tan[1] · Tao Zhu[1]

Abstract Breast cancer is the most common malignant disease amongst women. miRNAs are small, non-coding RNAs that regulate gene expression, thus have the potential to play an important role during cancer development. Emerging evidence shows that miR-135a is down-regulated in breast cancer cells, but the functional roles of miR-135a in breast cancer cells remains unexplored. For this purpose, we investigated the expression of miR-135a in breast cancer cells and explored its functional role during breast cancer progression. In vitro study showed that miR-135a may be a novel tumor suppressor. Further studies showed that transcription factors ELK1 and ELK3 are direct target genes of miR-135a that modulates the suppressive function of miR-135a in breast cancer cells. Induced expression of miR-135a significantly downregulated the expression of ELK1 and ELK3 both at mRNA and protein levels. Furthermore, the effect of miR-135a in MCF-7 and T47D cells was investigated by the overexpression of miR-135a mimics. In vitro, induced expression of miR-135a in breast cancer cells inhibited cell Proliferation and clongenicity. Moreover, a luciferase activity assay revealed that miR-135a could directly target the 3′-untranslated region (3′ UTRS) of ELK1 and ELK3 oncogenes. In addition, rescue experiment demonstrated that the promoted cell growth by transcription factors ELK1 and ELK3 was attenuated by the over-expression of miR-135a. Our study demonstrates that miR-135a regulates cell proliferation in breast cancer by targeting ELK1 and ELK3 oncogenes, and suggests that miR-135a potentially can act as a tumor suppressor.

Keywords Breast cancer · MiR-135a · ELK1 · ELK3 · Proliferation

Introduction

Generally, breast cancer is the most common type of cancer in women. After lung cancer, breast cancer is ranked as the second leading cause of death in women. Breast cancer is a complicated process and is a highly heterogeneous disease. It is associated with different genetic alterations involving either up-regulation of oncogenes or down-regulation of tumor suppressor genes (Colozza et al. 2005; Pantel et al. 2009). RNA exists in two different forms i.e. protein coding RNA and non-coding RNA (ncRNA). To elucidate the involvement of ncRNA in human diseases including cancer, it is important to examine the expression of ncRNA in both normal and diseased tissues. miRNAs are small non-coding cellular RNAs (20–25 nts) that can negatively regulate gene expression by mRNA silencing either by inhibiting the translation of mRNAs or promoting their degradation. Previous research has established that a large number of miRNAs have abnormal expression patterns in different cancers including breast cancer (Iorio et al. 2007; Zhang et al. 2006). In addition to recent data revealed that miRNAs act as tumor promoters or suppressors and thus modulate cancer relevant processes such as angiogenesis, proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism (Calin and Croce 2006; Dumont and Tlsty 2009). Furthermore, miRNA-135a acts as a tumor suppressor in many types of cancer including epithelial ovarian cancer, malignant glioma, renal carcinoma and lung cancer

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(Mao et al. 2015; Shi et al. 2015; Tang et al. 2014; Wu et al. 2012b). Moreover, miR-135a has oncogenic role in many types of cancer such as, human Hodgkin's lymphoma, gastric cancers, hepatocellular carcinoma, bladder and colorectal cancer (Liu et al. 2012; Navarro et al. 2009). ELK1 and ELK3 are ternary complex factors (TCFs) that belong to the sub-family of erythroblast transformation specific (Ets) domain proteins which bind to a DNA specific sequence through a purine-rich GGA core sequence. The three proteins Elk-1, Net and Sap-1, form a subfamily of e-twenty-six ETS domain transcription factors. ELK1 and ELK3 regulate the expression of a variety of genes including proto oncogenes (Hipskind et al. 1991). When ELK1 is phosphorylated, it translocates to the nucleus and activates downstream targets, while ELK3 normally acts as a repressor of gene expression but it can become an activator after being targeted by the MAPK cascade following activation of the Ras signaling pathway (Boros et al. 2009; Giovane et al. 1994). In this study, we found that the level of miR-135a is reduced in breast cancer cells. Both ELK1 and ELK3 were found to be the direct targets of miR-135a. Moreover, ELK1 or ELK3 over-expressions in breast cancer cells were rescued by miR-135a overexpression resulting in a significant reduction of cell proliferation and colony formation abilities of breast cancer cells, and also caused the down regulation of ELK1 and ELK3 ectopic expression at protein level. Further study about the ELK1 and ELK3 transcription factors as the potential target genes of miR-135a is of great importance.

Materials and methods

Cell culture

MCF-7 and T47D cells were purchased from the cell bank (Shanghai institute of cell biology). These cells were cultured in RPMI 1640 medium plus 10% heat-inactivated fetal bovine serum. All cells were maintained at 37 °C under 5% CO_2 atmosphere in a humidified incubator.

RNA oligonucleotides and plasmid transfection

The miRNAs mimics were obtained from Gene Pharma, Shanghai, China. miRNAs mimics and plasmid co-transfection were performed using lipofectamine 2000 (QIAGEN). Twenty nano molar shRNA or miRNA mimics were used for transfection. Total RNA and protein were prepared 48–72 h after transfection and were further used for quantitative PCR and Western blot analysis, respectively.

The sequences of miRNA mimics used were as follows: miR-135a mimics:

5′-UAUGGCUUUUUAUUCCUAUGUGAACAUAGG AAUAAAAAGCCAUAUU-3′

miR-135a mimics negative control: Sense strand: 5′-UUG UACUACACAAAAGUACUG-3′.

Colony formation assay

MCF-7 and T47D cells were transfected with miR-135a mimics or shRNA and their respective control using Lipofectamine 2000 (QIAGEN), for 48 h in 6-well plates. Cells were seeded in six well plate (in triplicate) at 1000 cells per well for colony formation assay. During this assay, fresh culture medium was replaced every 3 days. Experiments were performed in triplicates. The number of viable cell colonies was determined after 8–10 days depending upon the cell types. Colonies were fixed with 4% formaldehyde, stained with 0.1% crystal violet, photographed and megascopic colonies were counted.

Cell proliferation assay

Cell viability was determined by MTT (3-(4, 5-dic-Methylthiazol-2-yl)-2, 5 dipenyltetrazolium bromide) assay. MCF-7 and T47D cells were suspended in RPMI 1640 medium containing 10% fetal bovine serum. The cells were seeded in 96-well plates at 2000 or 1000 cells/well. At indicated time point, 10 µl of MTT solution (5 mg/ml) was added into each well and cells were incubated at 37 °C for 2 h. The colorimetric assay was read at 570 nm in a microplate reader (Model 3550 Bio-Rad). All experiments were performed in triplicates. The data was plotted as means ± SD of three separate experiments.

Luciferase reporter assay

The full-length 3′UTRs of ELK1 and ELK3 was amplified and cloned downstream of Renilla luciferase in a psiCHECK2 vector (Promega). Cells were plated in 24-well plates and transfected with 100 ng of psi-CHECK2-ELK1-3′-UTR or psi-CHECK2-ELK3-3′-UTR and their mutant's psi-CHECK2-ELK1-mut or psi-CHECK2-ELK3-mut or psiCHECK2 and miR-135amimics or negative control. After 48 h, cells were lysed and assayed with a dual luciferase assay (Promega), according to manufacturer's instruction. Three independent experiments were performed in triplicate.

Quantitative analysis of miRNAs and mRNAs

Total RNA from the culture cells was isolated using TRIzol reagent, and 1 µg of total RNA was reverse-transcribed using reverse transcriptase (Trans, AQ131 catalog). Then the

generated cDNAs were synthesized using TransStart TOP Green qPCR SuperMix (Trans) following the manufacturers protocol. The reaction mixture was consist of 100 ng CDNA template, 10 µM PCR primers, and $2\times$TransStart Top Green qPCR SuperMix (Trans). For the quantitative analysis of ELK1 and ELK3 transcription factor expressions, the relative amount of gene transcripts was normalized by GAPDH. Three independent experiments were performed in triplicate. All primers for quantitative PCR of ELK1, ELK3 transcription factors and GAPDH were designed by Primer Premier 5.0 and were synthesized by Sangon (Shanghai, China):

ELK1 (forward):   5′-GGC TAC GCA AGA ACA AGA CCAAC-3′

ELK1 (reverse):   5′-AGACGAACTTCTGGCCGCTCA-3′

ELK3 (forward):   5′-ATT TGA TCT GCT GGA CCT CGAAC-3′

ELK3 (reverse):   5′-CAAACTTCTGCCCGATCACCT-3′

GAPDH (forward):   5′-TGCACCACCACCTGCTTAGC-3′

GAPDH (reverse):   5′-GGC ATG GAC TGT GGT CAT GAG-3′.

Protein extraction and western blot

Cells were lysed using lysis buffer after 48 h of transfection. Protein concentration was determined with the BSA protein assay kit (Pierce) and equal amounts of total proteins were separated in 10% SDS–polyacrylamide gels, transferred to poly vinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 1 h with 5% BSA in Tris-buffer saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed and incubated with secondary antibody, and visualized by chemiluminescence Tanon (*GIS-2009*). The antibodies used were anti-ELK1 (diluted at 1:1000; Proteintech catalog), anti-ELK3 (diluted at 1:1000; Proteintech catalog), anti DDX3X (diluted at 1:1000; Proteintech catalog), and anti-GAPDH (diluted at 1:5000; Proteintech catalog).

Statistical analysis

All experiments were replicated for at least three times. Data was presented as the mean ± standard deviation. Raw data were analyzed with GraphPad PRISM software (version 5.0; San Diego, CA, USA). Regardless of statistical test used, the differences were considered statically significant when $P < 0.05$.

Results

Overexpression of miR-135a inhibits colony formation and cell proliferation, in breast cancer cells

To better understand the functionality of miR-135a in breast cancer cells, it was investigated whether miR-135a exerts an effect on colony formation and proliferation in breast cancer cells progression. MCF-7 and T47D cells were transfected with miR-135a mimics, or a negative control.

The expression of miR-135a was examined via real time qPCR in breast cancer cells, miR-135a was found to be markedly upregulated in MCF-7 and in T47D cell lines as shown in (Fig. 1a, b) compared with negative control. In addition, clonogenic assays indicated that the ability of breast cancer cells to form colonies was significantly reduced in miR-135a overexpression cells (Fig. 1c, d), while induced expression of miR-135a significantly decreased the proliferation of breast cancer cells in MTT assay (Fig. 1e, f). These results suggest that overexpression of miR-135a decreased both the proliferation and colony formation ability of breast cancer cells in vitro.

ELK1 and ELK3 are direct targets of miR-135a

ELK1 and ELK3 transcription factors were identified as potential targets of miR-135a using two algorithms Target scan (Lewis et al. 2005). And miRanda (Enright et al. 2003). Twelve putative target genes of miR-135a were generally predicted by the two algorithms (Fig. 2a).The potential binding sites were further described for miR-135 at 3′UTRs region of ELK1 and ELK3 transcription factors mRNA. Both of these two sites were conserved among different species as in (Fig. 2b, d). We constructed both miR-135a binding site wild-type and mutated 3′UTR of ELK1 and ELK3 in to dual-luciferase reporter plasmid, psi-Check2 (Fig. 2e, f). To confirm the true binding of miR-135a with ELK1 and ELK3, we co-transfected psi-CHECK2, psi-CHECK2-ELK1-3′UTR, psi-CHECK2-ELK1-3′UTR-mutant, psi-CHECK2-ELK3-3′UTR, psi-CHECK2-ELK3-3′UTR-mutant with miR-135a mimics or negative control oligonucleotides in MCF-7 cells. Induced expression of miR-135a in MCF-7 cells dramatically reduced the activity of luciferase reporter gene fused with wild type ELK1 and ELK3 3′UTRs. On the other hand, mutant constructs of ELK1 and ELK3 abolished the translation inhibition (Fig. 3a). Hence, the luciferase assay demonstrated that miR-135a binds directly and specifically to ELK1 and ELK3 3′UTRs and repressed the translation of ELK1 and ELK3 in MCF- 7 cells.

We further examined the effect of miR-135a on the endogenous ELK1 and ELK3 expression level by qPCR and western blot in MCF-7 and T47D cells, while DDX3X was used as negative control. Transfection with miR-135a

Fig. 1 miR-135a inhibited proliferation of breast cancer cells. MCF-7 and T47D cells were transfected with NC and miR-135a. **a**, **b** The relative miR-135a expression level in MCF and T47D cell lines via qRT-PCR. **c**, **d** Induced expression of miR-135a abrogates ELK1 and ELK3 stimulated colony formation ability. **e**, **f** MTT assay results showed that the cell proliferation of MCF-7 and T47D cells were inhibited after transfection with miR-135a. Cell proliferation assays were performed in triplicates with mean ± SD and representative images are displayed in (**c**) and (**d**).*P, 0.05, **P, 0.01, ***P, 0.001
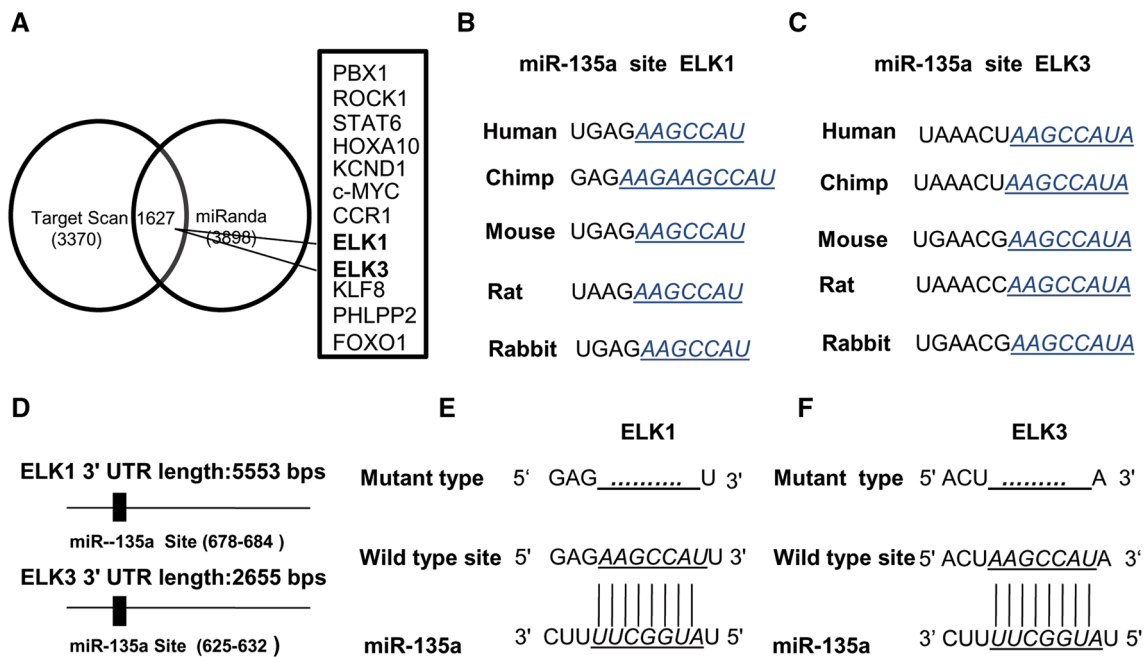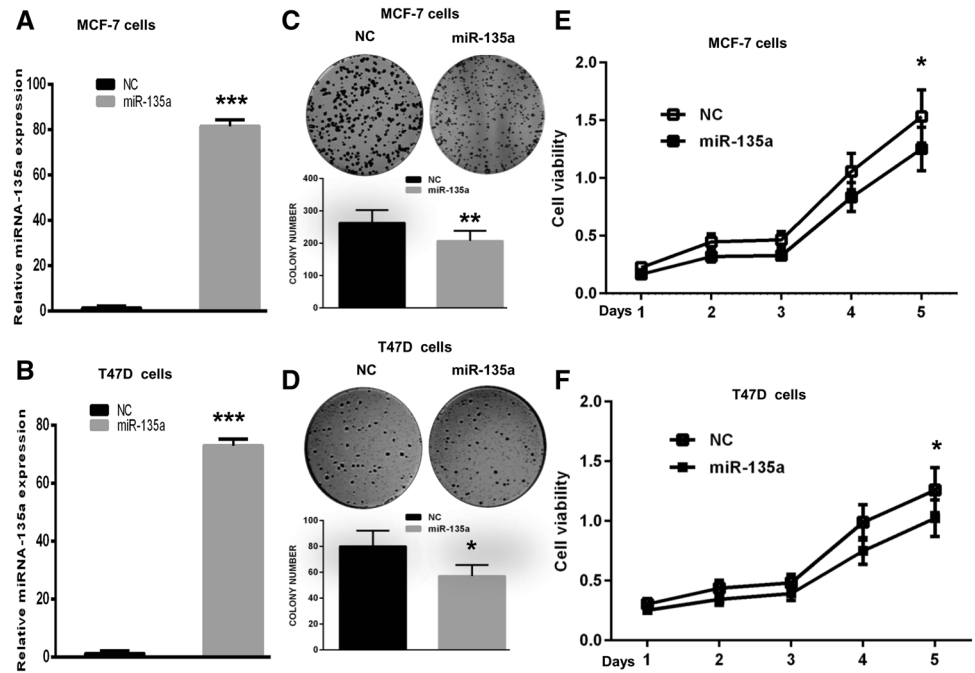

Fig. 2 Bioinformatics analysis of miR-135a. **a** Schema of the candidate genes predicted by different prediction methods. Each circle represents the number of genes identified by one algorithm. One thousand six hundred and twenty-seven genes listed in the overlap of these two circles are simultaneously predicted by different algorithms. Alignment of potential miR-135a binding site in the 3′UTRs of ELK1 and ELK3 mRNA from different mammalian species. Seed sequences are in italics and underlined as shown in **b**, **c** Predicted miR-135a binding sites in the 3′UTRs of ELK1 and ELK3. **e**, **f** The sequences of two predicted miR-135a binding sites within the 3′UTRs of ELK1 and ELK3 transcription factors and their cognate seed region. **d** Mutations on the seed sequence are exhibited above the seed region

mimics led to an obvious down regulation of ELK1 and ELK3 protein levels in both type of cells, as compared to negative control (Fig. 3d). Moreover ELK1 and ELK3 mRNA expression levels in the miR-135a mimic's

transfected cells were potently downregulated in both type of cells (Fig. 3b, c). Taken together these results indicated that miR-135a suppresses ELK1 and ELK3 expression by directly targeting its 3′UTRs.
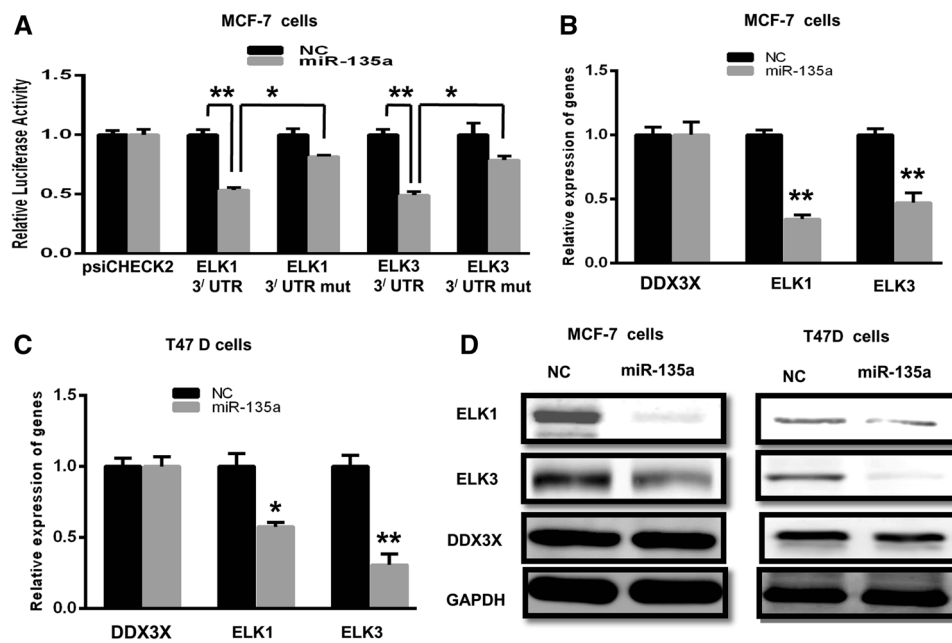
Fig. 3 Experimental identification of miR-135a target ELK1 and ELK3 transcription factors. **a** MCF-7 cells were co-transfected with miR-135a mimics, plasmid reporter containing wild type or mutant 3′UTRs of either ELK1 or ELK3. After 48 h luciferase assay was performed and normalized to the international firefly luciferase activity. MCF-7 and T47D cells were transfected with either NC or miR-135a mimics. **b**, **c** Relative expression of ELK1 and EKL3 mRNA in MCF-7 and T47D cells treated as described above were measured by q-PCR. **d** The expression level of ELK1 and ELK3 in MCF-7 and T47D cells were examined by western blotting. (62 kDa), ELK3 (44 kDa) and GAPDH (37 kDa) used as an inner control. DDX3X was used as negative control. *P, 0.05, **P, 0.01, ***P, 0.001

Silencing of ELK1 and ELK3 inhibits cell proliferation in breast cancer cells

To further confirm the function of endogenous ELK1 and ELK3, we designed two independent short hairpin RNAs (shRNAs) targeting ELK1 and ELK3. Forty eight hours after transfection of cells with ELK1, ELK3 and empty vector shPLKO.1, it was evident that ELK1 and ELK3 mRNA levels were reduced in the transfected cells, as compared to shPLKO.1 (Fig. 4a, b). Western blot analysis (Fig. 4c–f). Further confirmed the silencing of ELK1 and ELK3 proteins in MCF-7 and T47D cell lines. In addition, clonogenic assays showed that shRNAs transfected cells formed fewer colonies as compared to shPLKO.1 as in (Fig. 5a, c). Moreover, the function of ELK1 and ELK3 silencing was also measured with the cell viability assay in transfected MCF-7 and T47D cells (Fig. 5b, d). These studies demonstrated that knockdown of ELK1 and ELK3 genes resulted in decreased proliferation of breast cancer cells compared with cells transfected with shPLKO.1 plasmid. Collectively these results show that ELK1 and ELK3 transcription factors promote proliferation and colony formation abilities of breast cancer cells.

Effects of ELK1 and ELK3 restore miR-135a repressed cell proliferation in breast cancer cells

In order to determine the importance of ELK1 and ELK3 transcription factors as a functional target of miR-135a, MCF-7 cells were transfected with miR-135a mimics or negative control with or without ELK1 and ELK3 over-expression plasmid. Induced expression of miR-135a in MCF-7 cells resulted in a significant reduction in cell proliferation. On the other hand, overexpression of ELK1 and ELK3 led to increased cellular viability and colony formation ability. Overall, this suggests that miR-135a inhibits colony formation ability and cell viability through its repression of ELK1 and ELK3 transcription factors as illustrated in (Fig. 6a, b). Finally, we assessed the effect of miR-135a on the protein level of ELK1 and ELK3 by performing western blot analysis in MCF-7 cells. The reduction of ELK1 and ELK3 protein levels by overexpression of miR-135a can be restored by ectopic expression of ELK1 and ELK3 plasmids which possess no miR-135a binding site as indicated in (Fig. 6c). Thus, ELK1 and ELK3 are the functionally relevant targets downstream of miR-135a which mediate the cell proliferation.
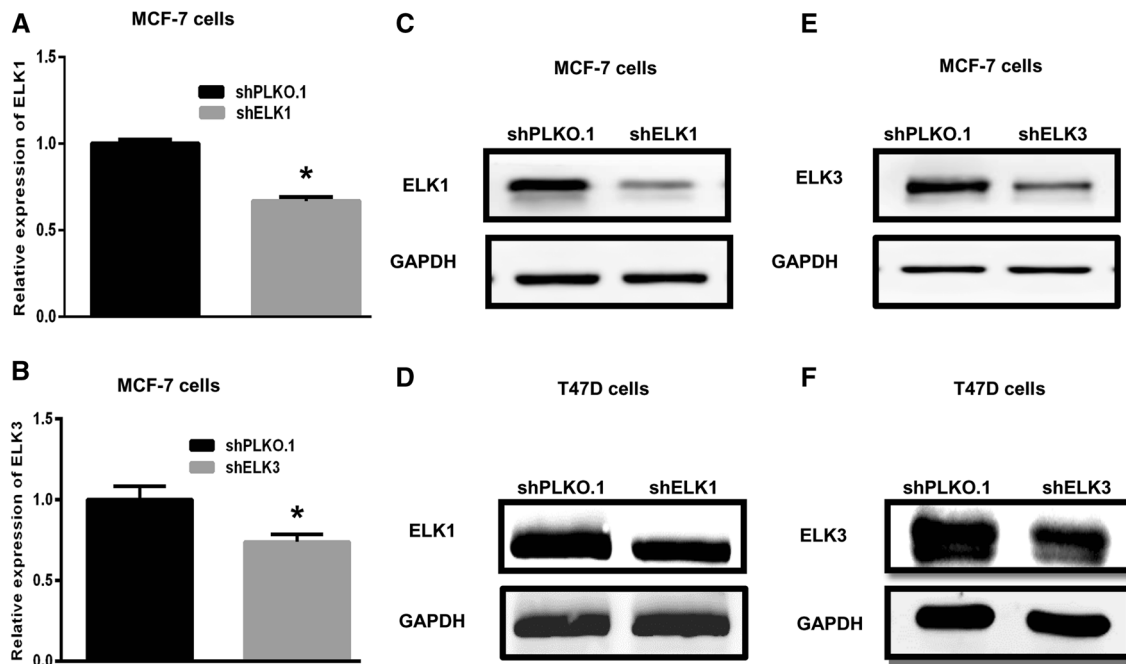
Fig. 4 Silencing ELK1 and ELK3 gene in breast cancer cells. ShRNA mediated down-regulation of ELK1 and ELK3 mRNA and protein expression in MCF-7 and T47D cells. **a**, **b** MCF-7 cells were harvested 48 h after transfection with shRNA for total RNA extraction. qPCR was employed to determine the levels of ELK1 or ELK3 mRNA in shRNA transfected cells. Expression of GAPDH was used as internal control gene. **c–f** MCF-7 and T47D cells were harvested 48 h transfection with shRNA. ELK1 and ELK3 protein levels was analyzed by western blotting. GAPDH was used as loading control. Results show that shRNA constructs, shELK1 and shELK3 targeting ELK1 and ELK3 drastically reduce both mRNA transcription and translation of ELK1 and ELK3 compared to shPLKO.1

Discussion

Discrepant results shows that aberrant expression of miR-NAs contributes to the initiation, development, and metastasis of cancer (Nelson and Weiss 2008). However, in human cancer cells the aberrant expression of miRNAs causes the destruction of miRNA-mediated molecular pathways. miR-135a has been reported to promote or inhibit traits of cancer aggressiveness (Nagel et al. 2008; Ren et al. 2015). Recent data shows that the deregulation of miRNA plays an important role in breast carcinogenesis (Bartel 2009). miR-135a regulates the JAK2 expression and inhibits gastric cancer cell proliferation (Wu et al. 2012a). Emerging evidences suggested that many miRNAs exhibit dysregulated expression in primary breast cancer cells as compared with their normal tissues, such as miR-21, miR-125b, miR-10B, miR-26a, miR-155, miR-301. Although the tumor suppressor role of miR-135a in breast cancer is largely unknown. Our research displayed that miR-135 is a potential suppressor for MCF-7 and T47D cells, as miR-135a significantly diminishes ELK1 or ELK3 stimulated colony formation ability and cell proliferation. Furthermore miRNA potentially relate with numerous mRNA targets through perfect or imperfect base pairing in the 3′UTR portion. However, these predictions usually yield a large number of false-positive candidates and experimental justification is thus strictly required (Migliore et al. 2008). In the present study, we have identified ELK1 and ELK3 as the key targets. We have subsequently mapped the potential miR-135a binding site in the 3′UTRS of ELK1 and ELK3 mRNA and further determined the critical sites which are responsible for the functional interaction with miR-135a. In addition, induced expression of miR-135a could inhibit cell proliferation and increase apoptosis through targeting c-MYC, STAT6, SMAD5, BMPR2 and HOXA10 in different types of cancers, the target genes of miRNA-135a were revealed to be involved in different type of cellular process, such as cell proliferation, cell cycle or apoptosis in many type of human cancers (Tang et al. 2013; Yamada et al. 2013). These results showed that miR-135a could act as a predictor of treatment in some cancers.

In this study, we describe the potential use of plasmid based shRNA targeting ELK1 and ELK3 oncogenes against breast cancer cells in vitro, using MCF-7 and T47D cells as a model system, and thus the efficacy of the down-regulation of both ELK1 and ELK3 genes was detected by qPCR and western blot analysis. The mRNA and protein levels of ELK1 and ELK3 were decreased in the ELK1 and ELK3 shRNA transcription group but not in the empty vector shRNA group.
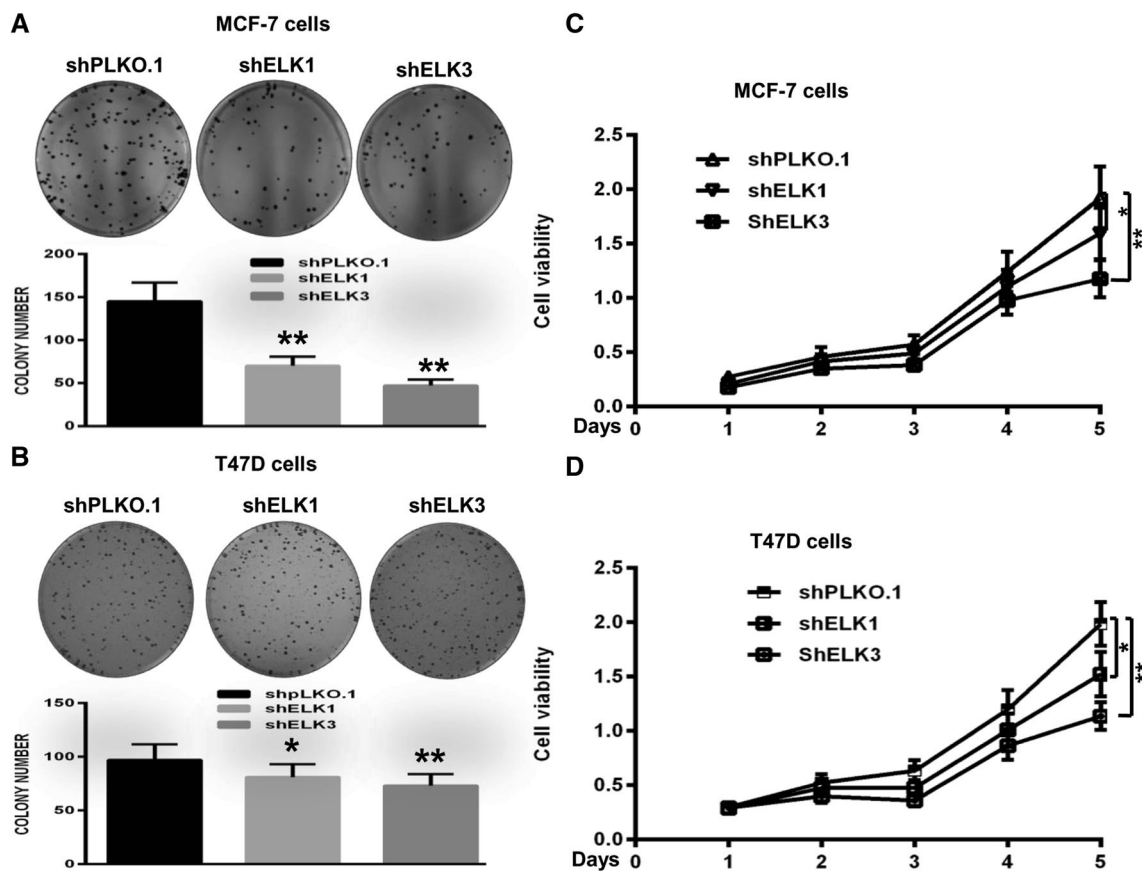
Fig. 5 Function of ELK1 and ELK3 gene in breast cancer cells. **a, c** ELK1 and ELK3 specific shRNAs reduces the colony formation ability of MCF-7 and T47D cells. MCF-7 and T47D cells were transfected with ELK1 and ELK3 shRNA and shPLKO.1 plasmid. After transfection cells were allowed to grow for 8–10 days. **b, d** Cell proliferation was determined by MTT assay. At days 15, the OD value in each group of cells was measured by absorbance at 450 nm. Each sample was tested in triplicate. Cell transfected with shELK1 and shELK3 plasmids showed a remarkable reduction of cell viability compared to shPLKO.1 plasmid. Each value represents the mean ($\pm$ SD). *P, 0.05, **P, 0.01, ***P, 0.001

Further to evaluate whether silencing the ELK1 and ELK3 genes in both type of cells affects cell growth and proliferation, the MTT assay and colony formation assay were performed as a result a significant increase in growth was observed between the empty vector shPLKO.1 and shRNA group cells, respectively. Cell proliferation was also examined using MTT analysis. The cells were significantly lower in the ELK1 and ELK3 shRNA transfected cells than that of empty vector shPLKO.1. These results showed that the knock down of ELK1 and ELK3 genes resulted in decreased proliferation and colony formation abilities of MCF-7 and T47D cells.

Transcription factors play an important role in the activation of specific network of genes. ELK1 has been identified as an important regulator of immediate—early genes such as FOS, while ELK3 normally acts as a repressor of gene expression but it can become an activator after being targeted by the MAPK cascade following activation of the Ras signaling pathway (Giovane et al. 1994; Sharrocks 2002).

In the present study, we characterized ELK1 and ELK3 as functional targets of miR-135a by the luciferase reporter gene assay, qPCR and western blot analysis. We found that miR-135a negatively regulated ELK1 and ELK3 expression at mRNA and protein level in MCF-7 and T47D cells, suggesting a role for miR-135a dysregulation in the pathogenesis of breast cancer. To address whether overexpression of ELK1 and ELK3 plasmid effects cellular proliferation and colony formation abilities of breast cancer cells, a rescue experiment was performed. miR-135a inhibited MCF-7 cells proliferation and colony formation abilities which could be rescued by overexpression of ELK1 and ELK3 oncogenes. Furthermore, we confirmed that ELK1 and ELK3 overexpression level was attenuated by miR-135a in MCF-7 cells. However, the inability to describe a particular mechanism of miR-135a function in MCF-7 and T47D cell apoptosis and the related signaling pathway is still a major challenge.

Fig. 6 ELK1 and ELK3 restore miR-135a repressed cell proliferation. MCF-7 cells were transfected with miR-135a or negative control with or without ELK1 or ELK3 overexpression. **a** ELK1 and ELK1 overexpression was rescued by miR-135a reduces the colony formation ability of breast cancer cells in vitro. **b** Overexpression of miR-135a inhibits the proliferation of breast cancer cells. **c** miR-135a targets ELK1 and ELK3, western blot analysis was performed to detect the expression of ELK1 and ELK3 oncogenes. GAPDH was used as a loading control
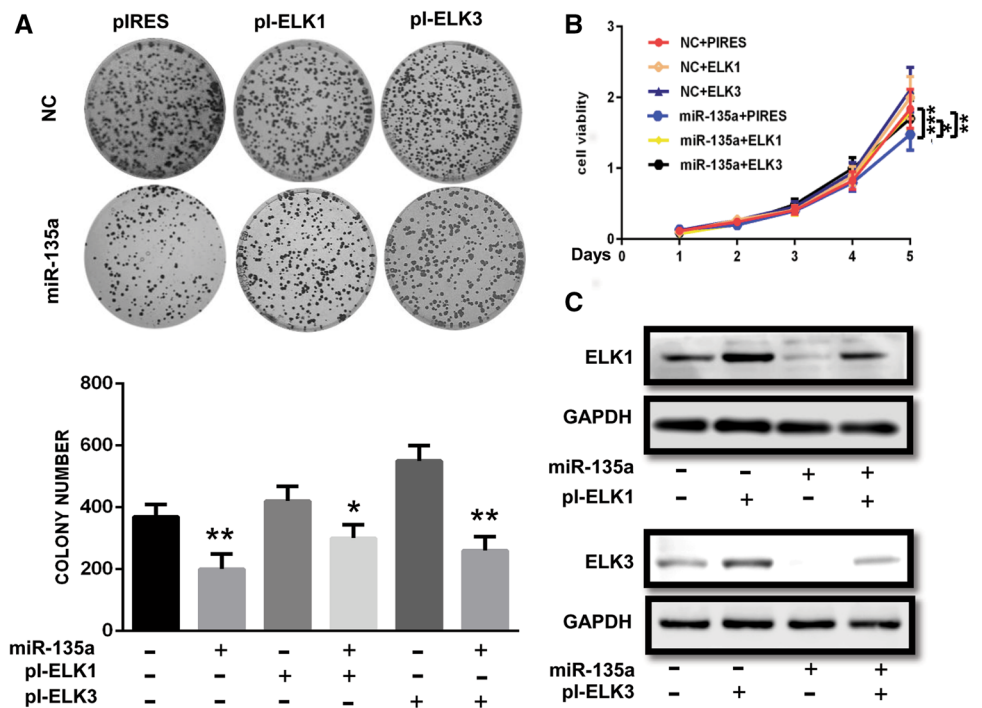
Further research will be required to investigate the biological function of miR-135a.

In conclusion our study provides mechanistic insight of the consequence of reduced expression of miR-135a in breast cancer cells. Overexpression of miR-135a inhibits the proliferation of MCF-7 and T47D cells through the regulation of proto-oncogenes i.e. ELK1 and ELK3 transcription factors. Moreover, the identification of miR-135a as a regulator of ELK1 and ELK3 transcription factors gives the insight for the establishment of a new approach and valuable miRNA-based therapies for the treatment of breast cancers.

Compliance with ethical standards

Conflict of interest   Akhlaq Ahmad, Weijie Zhang, Mingming Wu, Sheng Tan and Tao Zhu declare that they have not any conflict of interest.

Ethical approval   This article does not contain any studies with human subjects or animals performed by any of the authors. All the experimental procedures were approved by Scientific Ethics Committee and Review Board of the School of Life Sciences, University of Science and Technology of China.

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