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ORIGINAL ARTICLE

VEGFA Involves in the Use of Fluvastatin and Zoledronate Against Breast Cancer

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Abstract Our study aimed to identify key genes involved in the use of fluvastatin and zoledronate against breast cancer, as well as to investigate the roles of vascular endothelial growth factor A (VEGFA) in the malignant behaviors of breast cancer cells. The expression data GSE33552 was downloaded from Gene Expression Omnibus database, including mocked-, fluvastatin- and zoledronate-treated MDA-MB-231 cells. Differentially expressed genes (DEGs) were identified in fluvastatin- and zoledronate-treated cells using limma package, respectively. Pathway enrichment analysis and protein-protein interaction (PPI) network analysis were then performed. Then we used shRNA specifically targeting VEGFA (shVEGFA) to knock down the expression of VEGFA in MDA-MB-231 cells. Cell viability assay, scratch wound healing assay, Transwell invasion assay and flow cytometry were performed to explore the effects of VEGFA knockdown on the malignant behaviors of breast cancer cells. VEGFA was up-regulated in both fluvastatin- and zoledronate-treated breast cancer cells. Moreover, VEGFA was a hub node in PPI network. In addition, VEGFA was successfully knocked down in MDA-MB-231 cells by shVEGFA. Suppression of VEGFA promoted the migration and invasion of breast cancer MDA-MB-231 cells. Suppression of VEGFA inhibited the apoptosis of MDA-MB-231 cells. Our results indicate that up-regulation of VEGFA may prevent the progression of breast cancer after fluvastatin and zoledronate treatment via inducing cell apoptosis and inhibiting migration and invasion. VEGFA may serve as a potential prognostic indicator for clinical outcome in the management of breast cancer.

Keywords Fluvastatin · Zoledronate · Breast cancer · Genes

Introduction

Breast cancer is a leading cause of cancer death in women, accounting for 29% of all new cancer cases annually among women. It is always diagnosed as a invasive malignant tumor without curable therapy [1]. Regretfully, the molecular mechanisms underlying breast cancer are still ambiguous to data. Therefore, better understanding of the genetic underpinnings of breast cancer has great significance.

Statins and bisphosphonates (BPs) are two distinct classes of isoprenoid pathway inhibitors and have been well established in the management of breast cancer [2]. Fluvastatin is a member of lipophilic statins, which can inhibit cell proliferation and induce cell apoptosis in women with breast cancer [3]. Zoledronate, one of BPs, can inhibit bone resorption and induce cell apoptosis in breast cancer. In addition, several molecules have been identified in the use of statins and BPs against breast cancer. For instance, stimulation of inducible nitric oxide synthase by statins can enhance the proapoptotic effects of statins in breast cancer cells [4]. Fluvastatin can inhibit breast cancer progression via down-regulation of TfR1, matrix metalloproteinase-2 and matrix metalloproteinase-9 [5]. BPs can induce apoptosis in human breast cancer cells via down-regulation of bcl-2 protein and

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proteolytic cleavage of Poly (ADP)-ribose polymerase (PARP) [6]. Zoledronate is also shown to modulate functional properties of breast cancer cells by regulating the expression of matrix macromolecules [7]. Besides, accumulating studies have highlighted the roles of other key genes and pathways in the progression of breast cancer. Vascular endothelial growth factor (VEGF) family members are reported to play key roles of in the progression of breast cancer [8]. The transcription factor Twist Family BHLH Transcription Factor 1 (TWIST1) promotes bone metastasis formation in breast cancer via a mechanism dependent of miR-10b [9]. However, the key mechanisms involved in the use of statins and BPs against breast cancer are largely unknown. Therefore, identification of key genes involved in the use of statins and BPs against breast cancer will help to the development of effective drug targets for breast cancer.

In the present study, we downloaded microarray data GSE33552 to identify the differentially expressed genes (DEGs) after MDA-MB-231 cells were treated with fluvastatin and zoledronate respectively. Comprehensive bioinformatics was used to investigate key genes associated with anticancer potential of fluvastatin and zoledronate in the management of breast cancer. We then performed experimental validations to verify the roles of key molecules in breast cancer. Our study aimed to identify the key molecule involved in the use of fluvastatin and zoledronate against breast cancer, as well as to investigate the key roles of vascular endothelial growth factor A (VEGFA) in the malignant behaviors of breast cancer cells, thus to elucidate the potential regulatory mechanism.

Materials and Methods

Microarray Data

The expression data GSE33552 deposited by Vintonenko et al. [10] was downloaded from NCBI Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/). MDA-MB-231 cells were treated with 2 μM fluvastatin for 12 h and 24 h. Moreover, MDA-MB-231 cells were treated with 30 μM zoledronate for 12, 24 and 48 h or with higher concentration (100 μM zoledronate) for 24 h. Mock-treated cells were considered as control. Each treatment had 4 independent replicates. Therefore, a total of 40 samples were used for the development of this microarray data. The raw CEL data were obtained based on the platform of Affymetrix Hμman Gene 1.0 ST Array (Affymetrix Inc., Santa Clara, California, USA).

Data Preprocessing and DEGs Screening

The raw CEL data was preprocessed using affy [11] analysis in R package. Compared with corresponding mock-treated control, the DEGs were identified in fluvastatin-treated cells and zoledronate treated cells using limma [12] package in R, respectively. The significant *p*-value was adjusted as false discovery rate (FDR) by Benjamini and Hochberg (BH) method. The cutoff value for significant differences was FDR < 0.05 and fold change ≥1.5.

Pathway Enrichment Analysis

SubpathwayMinner [13] (freely available at http://cran.r-project.org/web/packages/SubpathwayMiner/) is a software for annotating gene sets and flexible identifying pathways (entire pathways and K-clique sub-pathways) automatically. The characteristics of K-clique sub-pathway are that the distance between the nodes in this sub-pathway is not more than K. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) [14] database is widely utilized for classification of large-scale genes into their respective pathways. In this study, KEGG pathway enrichment analysis for DEGs was performed using SubpathwayMinner software. K = 4 was used for the identification of the involved sub-pathways. The *p*-value <0.01 was set as the threshold value.

Protein-Protein Interaction (PPI) Network Construction

Search Tool for the Retrieval of Interacting Genes (STRING) [15] database collects comprehensive predicted and experimental information of protein interactions. The reliability of PPIs is displayed with a combined score. In this study, the PPIs with combined score ≥ 0.7 were identified based on the information of STRING database. The PPI network was then built using Cytoscape [16] software.

Cell Culture

Human breast cancer cell line MDA-MB-231 was obtained from the State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai Jiao Tong University (Shanghai, China). 293 T cells were purchased from Invitrogen (Carlsbad, CA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) 37 °C in a humidified incubator with 5% CO_2.

Plasmids, Lentiviral Package and Transfection

The control shRNA (no silencing) and shRNA specifically targeting VEGFA (shVEGFA) were synthesized by GenePharma Co (Shanghai, China). The primers used were: shVEGFA-forward: 5′-ccggGCAGATTATGCGGATCAAACCTTCAAGAGAGGTTTGATCCGCATAATCTGCTTTTT TGGTACC-3′, shVEGFA-reverse: 3′-CGTCTAATACGCCTAGTTTGGAAGTTCTCTCCAAACTA

GGCGTATTAGACGAAAAAACCATGGttaa-5′; NC shRNA-forward: 5′-ccggGTTCTCCGAACGTGTCACGT CAAGAGATTACGTGACACGTTCGGAGAATTTTTTGG TACC-3′, NC shRNA-reverse: 3′-CAAGAGGCTTGCAC AGTGCAGTTCTCTAATGCACTGTGCAAGCCTCTTAA AAAACCATGGttaa-5′, which were designed using BLOCK-iT™ RNAi Designer (Thermo Fisher Scientific, USA). The sequence of shVEGFA was inserted into Plko.1-puro to construct a shVEGFA expression vector, which was confirmed by sequencing.

We then used lentiviral expression system to package these plasmids. Briefly, the lentivirus packaging vectors pCDH, psPAX2 and pMD2.G were obtained from Addgene (http://www.addgene.org/). Then the lentivirus packaging vectors pCDH, psPAX2 and pMD2.G and recombinant expression plasmid plko.1-puro-shVEGFA were cotransfected into 293 T cells using the Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) method following the manufacturer's protocol. Twenty four hours after transfection, the culture supernatants were collected and filtered through 0.45 μm pore size filters. Viral particles were concentrated by ultracentrifugation and the titer of virus was tested using large-scale real time titration (LaSRT).

Plasmids plko.1-puro-shVEGFA were transfected into MDA-MB-231 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommended protocol. Forty eight hours after transfection, the green fluorescent protein (GFP)-positive cells were detected by fluorescence microscope. MDA-MB-231 cells that can successfully express plko.1-puro-shVEGFA were defined as MDA-SH group. The MDA-MB-231 cells transfected with plko.1-puro-shRNA were considered as a negative control (MDA-PLKO group), and MDA-MB-231 cells without any treatment were defined as blank control (MDA-MB-231 group).

qRT-PCR Analysis

Total mRNA was extracted from MDA-MB-231 cells using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the instructions of manufacturer. Reverse transcription of mRNA was then performed with PrimeScript RT Master Mix Kit (#RR036A, TaKaRa Bio, Inc., Otsu, Shiga, Japan). qRT-PCR was performed on an ABI ViiA7 PCR System to detect the expression levels of VEGFA mRNA with SYBR Green Master mix (#4367659, Invitrogen, Carlsbad, CA, USA). The expression value of β-actin was used as internal control and the relative expression values of VEGFA were calculated using the comparative threshold (Ct) cycle ($2^{-\Delta\Delta Ct}$) method.

Cell Viability Assay

Cells (5×10^4/ml) were harvested after transfection and then seeded in a 96-well plate. After incubation for 0, 24, 48 and 72 h, cell proliferation was determined using the Cell Counting Kit 8 (CCK-8, biosharp, China) following the manufacturer's instructions. Briefly, 10 μl of CCK-8 solution was added to each well, and the plates continued to incubate for 1–4 h at 37 °C. Absorbance at 450 nm was measured on a microplate reader (Epoch Etock, BioTek, USA). All experiments were performed in triplicate.

Scratch Wound Healing Assay

The scratch wound healing assay was performed to determine cell migration capacity [17]. Briefly, cells were seeded in a 12-well plate and continued to incubate until forming a confluent monolayer. A "scratch" of the cell monolayer was scraped in a straight line with a sterile pipette tip. The cell fragments caused by scratch were washed by PBS. The plate continued to incubate for 24 h. After 0, 12, 24 and 36 h, the scratch wounds were observed by phase-contrast microscope (Nikon, TS100, Japan) respectively. Each experiment was conducted in triplicate.

Transwell Invasion Assay

Transwell assay was carried out to assess cell invasive ability. Briefly, 200 μl of cells (5×10^5cells/well) were seeded in the upper compartment of Transwell chamber (BD Biosciences, Mountain View, CA). The Transwell chamber (8-μm pore size) was pre-coated with Matrigel (BD Biosciences, Mountain View, CA). Chambers were placed into 24-well plates, and their lower compartments were loaded with 500 ul of RPMI-1640 medium containing 20% FBS as the nutritional attractant. After incubating for 24 h, the chambers were fixed with methanol and stained with 0.1% crystal violet, and non-invaded cells were removed with a cotton swab. Finally, the cells penetrating across membrane were counted under a microscope (CKX 31, Olympus Corp, Japan).

Flow Cytometry for Cell Apoptosis Analysis

Cell apoptosis analysis was assayed by flow cytometry using FITC Annexin V Apoptosis Detection Kit (BD PharMingen, San Diego, CA, USA). Briefly, cells with different treatment were suspended in 1 × Binding Buffer. Cells were then stained with 5 μl annexin V-FITC and 5 μl PI (50 μg/ml) for 15 min in the dark at 25 °C. After mixing with 400 μL 1 × Binding Buffer, cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). The percentage of apoptotic cells was defined as the sum of the apoptotic cells in the early stage (annexin V positive/PI negative) and late stage (annexin V positive/PI positive).

Statistical Analysis

Data presented as mean ± SEM were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). The differences between groups were performed with t-test or one-way ANOVA. A value of $P < 0.05$ was defined as statistically significant.

Results

DEGs Screening

After 12 h of 2 μM fluvastatin treatment, only 1 up-regulated gene was identified in MDA-MB-231 cells compared with corresponding mock-treated control. However, we screened 435 DEGs (339 up- and 96 down-regulated) in MDA-MB-231 cells after 24 h of 2 μM fluvastatin treatment compared with corresponding mock-treated control. In addition, compared with corresponding mock-treated control, 1635 DEGs (806 up- and 829 down-regulated) were identified in MDA-MB-231 cells after 24 h of 100 μM zoledronate treatment, and 32 DEGs (10 up- and 22 down-regulated) were screened out in MDA-MB-231 cells after 48 h of 30 μM zoledronate treatment respectively. There were no DEGs identified after 12 and 24 h of 30 μM zoledronate treatment.

Pathway Enrichment Analysis

In our study, we first performed KEGG pathway analysis for DEGs in MDA-MB-231 cells after 24 h of 2 μM fluvastatin treatment (Table 1). The results showed that these DEGs were significantly enriched in starch and sucrose metabolism, metabolism of xenobiotics by cytochrome P450 and reductive carboxylate cycle (CO2 fixation). In addition, we performed KEGG pathway analysis for DEGs in MDA-MB-231 cells after 24 h of 100 μM zoledronate treatment (Table 2). We found that these DEGs were significantly enriched in ECM-receptor interaction, pyrimidine metabolism, and pathways in cancer.

PPI Network Analysis

Based on the information of STRING database, PPI network containing 128 nodes and 171 edges were constructed by DEGs identified from fluvastatin-treated cells (Fig.1a). Thereinto, 100 nodes (red) were up-regulated genes and 28 nodes (green) were down-regulated gene. Based on node degrees, the top 5 nodes with higher node degree (from high to low) were Vascular Endothelial Growth Factor A (VEGFA, degree = 17), V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC, degree = 16), Prostaglandin-Endoperoxide Synthase 2 (PTGS2, degree = 13), Endothelin 1 (EDN1, degree = 11) and Coagulation Factor II (Thrombin) Receptor-Like 1 (F2RL1, degree = 8).

Table 1 The significant pathways enriched by differentially expressed genes identified from cells after 24 h of 2 μM fluvastatine treatment

PathwayID	PathwayName	AnnGeneRatio	*P* value
path:00500_3	Starch and sucrose metabolism	8/435	2.54E-07
path:00500	Starch and sucrose metabolism	8/435	5.25E-06
path:00500_1	Starch and sucrose metabolism	8/435	1.03E-05
path:00500_7	Starch and sucrose metabolism	10/435	3.87E-05
path:00980	Metabolism of xenobiotics by cytochrome P450	7/435	2.65E-04
path:00720	Reductive carboxylate cycle (CO2 fixation)	3/435	6.34E-04
path:04512	ECM-receptor interaction	7/435	8.07E-04
path:00100	Biosynthesis of steroids	4/435	8.29E-04
path:00980_1	Metabolism of xenobiotics by cytochrome P450	7/435	9.29E-04
path:05200	Pathways in cancer	15/435	9.77E-04
path:00720_1	Reductive carboxylate cycle (CO2 fixation)	3/435	2.25E-03
path:04510	Focal adhesion	10/435	3.92E-03
path:05222	Small cell lung cancer	6/435	4.62E-03
path:04012	ErbB signaling pathway	6/435	4.89E-03
path:04350	TGF-beta signaling pathway	6/435	4.89E-03
path:00620	Pyruvate metabolism	4/435	6.22E-03
path:00620_1	Pyruvate metabolism	4/435	7.37E-03
path:00590_5	Arachidonic acid metabolism	5/435	8.61E-03
path:05212	Pancreatic cancer	5/435	9.67E-03

'_number' represents the subway of signal pathways

Table 2 The significant pathways enriched by differentially expressed genes identified from cells after 24 h of 100 μM zoledronate treatment

PathwayID	PathwayName	AnnGeneRatio	*P* value
path:04512	ECM-receptor interaction	21/1635	1.29E-07
path:00240_2	Pyrimidine metabolism	21/1635	1.17E-05
path:00240_6	Pyrimidine metabolism	20/1635	3.92E-05
path:00240_5	Pyrimidine metabolism	20/1635	4.49E-05
path:00240	Pyrimidine metabolism	18/1635	4.52E-05
path:05200	Pathways in cancer	42/1635	5.37E-05
path:05322	Systemic lupus erythematosus	22/1635	2.77E-04
path:03030	DNA replication	9/1635	5.18E-04
path:04510	Focal adhesion	27/1635	6.01E-04
path:05222	Small cell lung cancer	15/1635	6.14E-04
path:04110	Cell cycle	18/1635	1.08E-03
path:00030	Pentose phosphate pathway	7/1635	1.35E-03
path:00030_1	Pentose phosphate pathway	7/1635	1.72E-03
path:00030_3	Pentose phosphate pathway	7/1635	1.72E-03
path:00230	Purine metabolism	20/1635	2.59E-03
path:00030_2	Pentose phosphate pathway	6/1635	2.77E-03
path:04350	TGF-beta signaling pathway	13/1635	5.60E-03
path:04010	MAPK signaling pathway	30/1635	6.82E-03
path:00380_4	Tryptophan metabolism	8/1635	9.89E-03
path:03440	Homologous recombination	6/1635	9.90E-03

'_number' represents the subway of signal pathways

Furthermore, PPI network containing 691 nodes and 2202 edges were constructed by DEGs identified from zoledronate-treated cells (Fig.1b). Thereinto, 319 nodes (red) were up-regulated genes and 372 nodes (green) were down-regulated gene. Based on node degrees, the top 5 nodes with higher node degree (from high to low) were

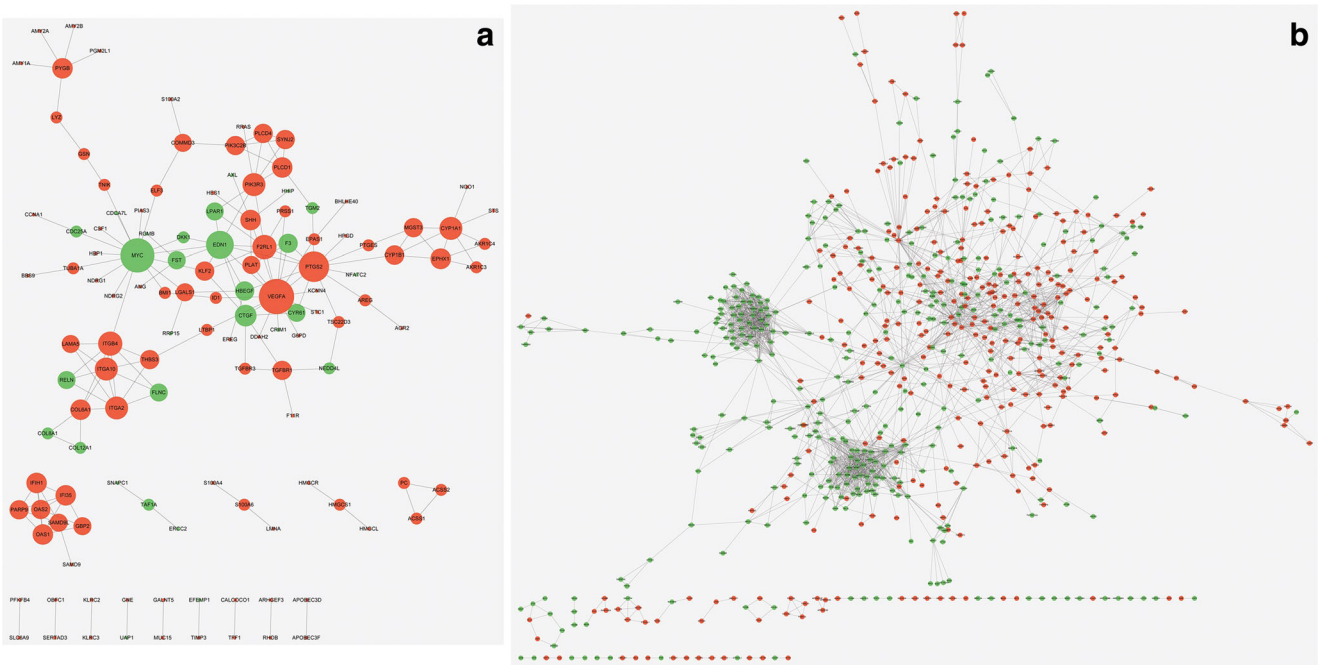

Fig. 1 The PPI networks constructed by DEGs identified from fluvastatin-treated cells (**a**) and from zoledronate-treated cells (**b**). *Red node* represents up-regulated genes and *green node* represents down-regulated gene

MYC (degree = 45), INS-IGF2 Readthrough (INS-IGF2, degree = 44), Cell Division Cycle 6 (CDC6, degree = 42), NOP58 Ribonucleoprotein (NOP58, degree = 41), and VEGFA (degree = 40).

Analysis of the Expression of VEGFA mRNA

From the above bioinformatics analysis, we found VEGFA were up-regulated in both fluvastatin-treated and zoledronate-treated MDA-MB-231 cells. Therefore, we used shRNA specifically targeting VEGFA (shVEGFA) to knock down the expression of VEGFA in MDA-MB-231 cells. As shown in Fig.2, the expression of VEGFA mRNA in MDA-SH group were significantly lower than MDA-PLKO group or MDA-MB-231 group ($P < 0.05$), indicating that VEGFA expression were successfully suppressed in MDA-MB-231 cells.

The Effects of VEGFA Suppression on Cell Proliferation

We performed CCK8 assay to analyze cell viability of different group in an experimental period of 72 h of transfection. As shown in Fig.3, there were no significant differences in cell viability between different groups ($P > 0.05$).

The Effects of VEGFA Suppression on Cell Migration

The scratch wound healing assay was performed to determine cell migration capacity in an experimental period of 36 h of transfection (Fig.4). The results showed that the migration rate of MDA-SH group were significantly higher than MDA-PLKO group or MDA-MB-231 group ($P > 0.05$, Fig.4); indicating that suppression of VEGFA could promote cell migration.

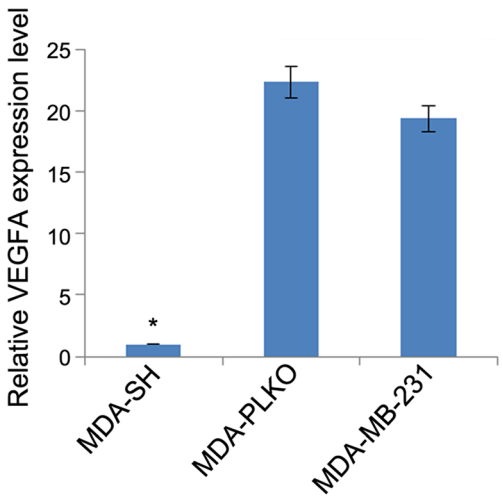

Fig. 2 The expression of VEGFA mRNA in different transfected groups. The data were presented as the mean ± SD and * indicated a significant difference compared with MDA-MB-231 cells without any treatment ($P < 0.05$)

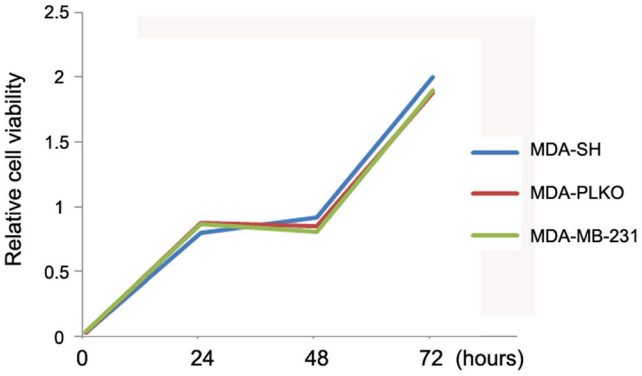

Fig. 3 CCK8 assay showed cell viability of different transfected groups in an experimental period of 72 h of transfection

The Effects of VEGFA Suppression on Cell Invasion

Transwell assay displayed cell invasive ability of different group. As shown in Fig.5, the invasive cell number in MDA-SH group

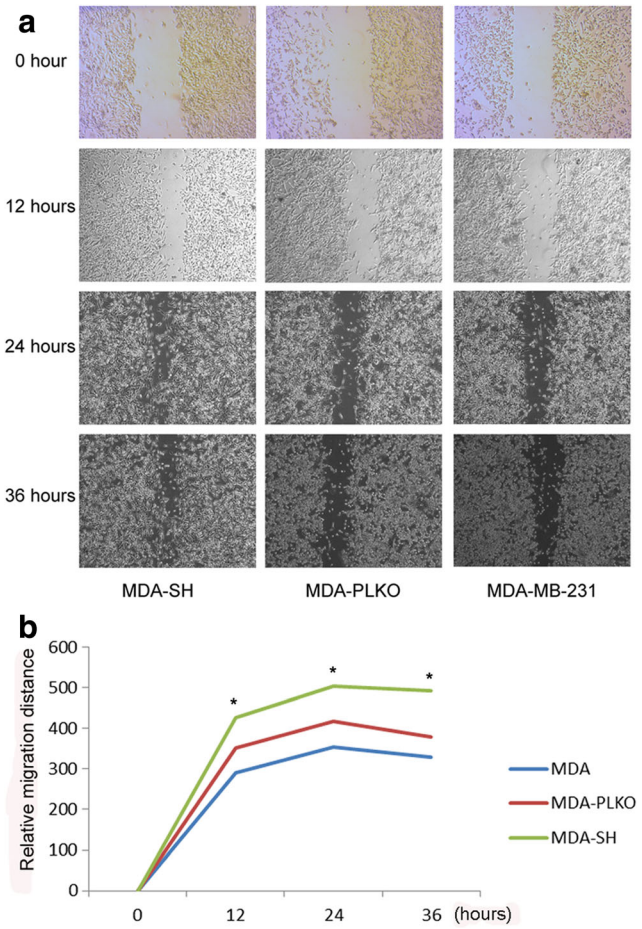

Fig. 4 The scratch wound healing assay dispalyed cell migration capacity of different transfected groups in an experimental period of 36 h of transfection. * indicated a significant difference compared with MDA-MB-231 cells without any treatment ($P < 0.05$)

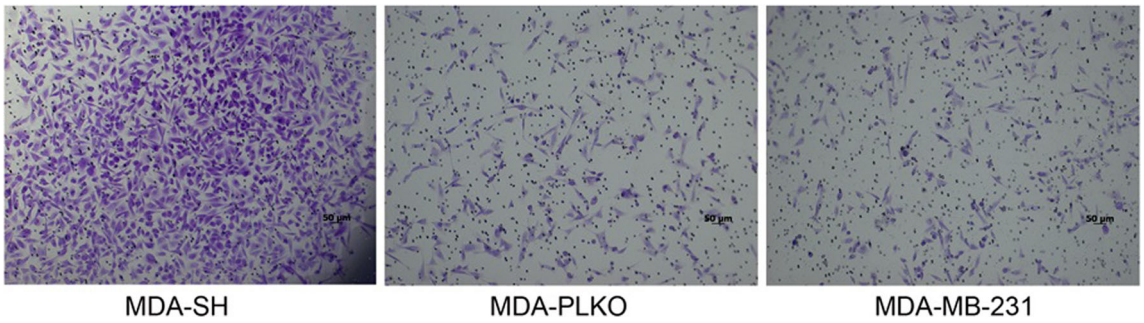

Fig. 5 Transwell assay displayed cell invasive ability of different group

was significantly higher than MDA-PLKO group or MDA-MB-231 group ($P > 0.05$), indicating that suppression of VEGFA could promote cell invasion.

The Effects of VEGFA Suppression on Cell Apoptosis

Flow cytometry showed the apoptotic cells of different groups in the early stage and late stage. The results showed that, compared with MDA-PLKO group or MDA-MB-231 group, the apoptotic cells in the early stage were significantly reduced in MDA-SH group ($P < 0.05$, Fig.6), indicating that suppression of VEGFA inhibited cell apoptosis.

Discussion

In the present study, we first applied comprehensive bioinformatics methods to identify key genes associated with anticancer potential of fluvastatin and zoledronate in the management of breast cancer. The results showed that VEGFA was the hub node that was up-regulated in breast cancer cells after fluvastatin and zoledronate treatment. To further verify the key roles of VEGFA, we used shVEGFA to knock down the expression of VEGFA in MDA-MB-231 cells and performed experimental validations to verify the VEGFA suppression on the malignant behaviors of breast cancer cells. Expected results were obtained that suppression of VEGFA promoted cell migration and invasion, and inhibited cell apoptosis. The roles of VEGFA in the fluvastatin- and zoledronate- treated breast cancer cells merit further discussion.

VEGFA, as a key angiogenic factor, is considered as an important tumor specific factor in breast cancer, which plays a crucial role in tumor angiogenesis and cancer progression [18]. VEGFA can induce adhesion and migration of cancer cells via binding to integrin α9β1 [19]. Upregulation of VEGFA is shown to modulate cell migration and invasion in lung cancer through PI3K/AKT pathway [20]. Furthermore, a polymorphism of VEGFA is shown to contribute to the extrathyroidal invasion of papillary thyroid cancer [21]. Gong et al. demonstrated that VEGFA was essential for regulating invasion of glioblastoma [22]. In addition, miR-29a can inhibit the invasion of gastric cancer cells via targeting VEGFA [23]. VEGFA is also shown to mediate the unappreciated role of miR-497 in the invasion of non-small cell lung cancer [24]. Besides, anti-VEGFA therapy can suppress tumor lymphangiogenesis in an orthotopic breast tumor model, thus to control the metastasis of breast cancer [25]. VEGFA may function as a prognostic indicator for clinical outcome in

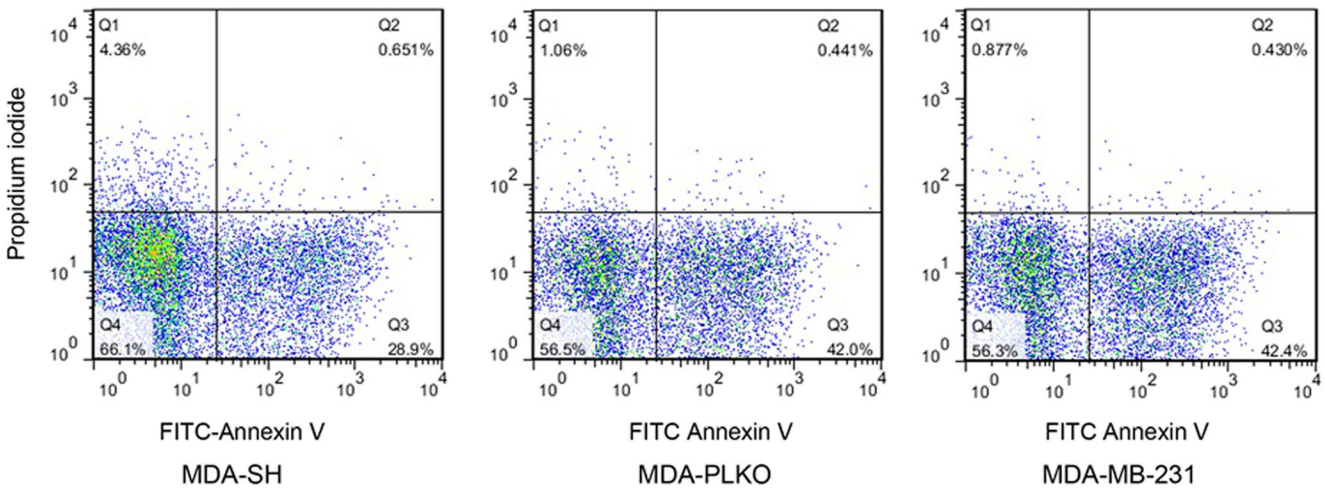

Fig. 6 Flow cytometry showed the apoptotic cells of different groups in the early stage and late stage

breast cancer [26]. In our study, VEGFA was up-regulated in breast cancer cells after fluvastatin and zoledronate treatment, and down-regulation of VEGFA promoted migration and invasion in breast cancer cells. Therefore, we speculate that VEGFA may control cell migration and invasion in the treatment of breast cancer with fluvastatin and zoledronate.

Furthermore, the effects of VEGFA on apoptosis were also explored by means of flow cytometry in our study. The results showed that the apoptotic cells in the early stage were significantly reduced after knockdown of VEGFA, implying suppression of VEGFA could inhibit breast cancer cell apoptosis, which were in line with previous study that inhibition of VEGFA could ameliorate podocyte apoptosis in diabetes through suppression of activating protein 1 [27]. In addition, VEGFA is shown to be the key component for supporting chondrocyte survival during bone development [28]. VEGFA gene variation is likely to be associated with the susceptibility and severity of breast cancer [29]. Besides, VEGFA can play a crucial role in miR-185 mediated cell apoptosis in clear cell renal cell carcinoma [30]. Although the association of VEGFA with cell apoptosis in breast cancer has not been fully investigated, we speculate that up-regulated VEGFA may induce cell apoptosis in the treatment of breast cancer with fluvastatin and zoledronate.

In conclusion, our results indicate that knockdown of VEGFA may promote migration and invasion and inhibit the apoptosis of breast cancer MDA-MB-231 cells. Up-regulation of VEGFA may be a key event involved in the management of breast cancer with fluvastatin and zoledronate treatment. VEGFA may serve as a potential prognostic indicator for clinical outcome in the management of breast cancer. However, due to up-regulation of VEGFA after fluvastatin and zoledronate treatment, it is different to detect the effects of VEGFA overexpression. In addition, we lacked of direct evidence to verify the anti-cancer effects of VEGFA induction by fluvastatine and zoledronate. Whether a knock-down experiment of VEGFA with fluvastatine and zoledronate treatment can suppress anti-cancer effects will merit further investigated. Besides, anti-VEGFA therapy can be used to treat patients with cancer and control the metastasis of breast cancer, which is contradictory to our results that knockdown of VEGFA promote migration and invasion in breast cancer cells. Whether VEGFA has different regulatory mechanism in the use of fluvastatin and zoledronate against breast cancer is still needed to be elucidated.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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