DLX4 Homeoprotein Promotes PI3K/Akt Anti-Apoptosis Pathway in ER Negative Breast Cancer Cells through Upregulation of VEGFA

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B.S., June 2006, China Pharmaceutical University

A Thesis submitted to

The Faculty of Columbian College of Arts and Sciences
of The George Washington University in partial fulfillment of the requirements for
the degree of Master of Science

August 31th, 2010

Thesis directed by

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Abstract of Thesis

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The progression of breast cancer has been linked to a splice variant of DLX4 homeobox transcription factor, DLX4. Our ChIP-on-chip and bioinformatic analysis studies showed that VEGFA is a potential target of BP1, and it is upregulated in cells overexpressing DLX4. VEGFA has been implicated in tumor generation because it stimulates angiogenesis, cell proliferation and migration. This study aims to decipher the functional roles of DLX4 in VEGFA-PI3K/AKT pathways involving in inhibition of apoptosis in Estrogen Receptor negative breast cancer cell lines. Through Quantitative PCR and Western blot assays, we verified positive correlation of DLX4 and VEGFA expression, and their association with the activity status PI3K/Akt pathway in ER negative Breast Cancer cell lines.

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1.Introductions

In our previous study, we identified that one of the binding sites for BP1, a splice variant of DLX4 homeoprotein, is within the downstream region of VEGFA gene, using microarray and ChIP-on-chip (Chromotin Immunoprecipitation on chip) technology on Hs578T breast cancer cell line transfected by DLX4. VEGFA is a well-known molecule that promotes angiogenesis in tumor growth and progression. In this study, we verified the transcriptional regulatory action of DLX4 on VEGFA expression. We have demonstrated that DLX4, through the regulation of VEGFA, activates the PI3K/Akt survival pathway in ER negative breast cancer cell lines.

Breast cancer accounts for approximately 12.7% women born in the United States. Despite the progress in early diagnosis and treatment in breast cancer, it remains one of the main health threats. The 10-year survival of breast cancer varies greatly from 10% to 98% based on stage, grade, receptor status and age of the patients. Estrogen receptor (ER) serves as an indicator for estrogen blockage treatment (1, 2), such as tamoxifen, as well as a valuable favorable prognosis biomarker. On the other hand, although ER negative breast cancer accounts around 30% of all the breast cancer cases, limited therapies can be applied to patients with ER negative breast cancer (1).

ER negative breast cancer associates with poor prognosis and responses to anti-estrogen drugs, but also it dominates 90% of the inflammatory breast cancer (IBC) (1, 2). The IBCs are more invasive and aggressive compared with the non-inflammatory breast cancer. Also,

some of ER negative breast cancer patients fall into triple-negative breast cancer (TNBC), which accounts for 12-24% of breast cancer with worse relapse and survival rate, remains a huge challenge to clinicians for the lack of specific target for treatment (1,10). What's more, during the anti-estrogen therapy for ER positive tumors, some patients acquire anti-estrogen resistance via reduced ER expression on the surface of the cancer cells (1, 3). Therefore, this study focuses on the functional role of DLX4 in to unfold the survival pathway for potential drug target for ER negative breast cancer.

BP1, also referred to as DLX4 variant 1, belongs to distal-less (DLX) subfamily, encodes a homeodomain-containing transcription factor that involves in embryonic development, growth, differentiation as a transcription factor by via regulation of downstream gene (5, 6). Homeobox genes compose a highly conserved 180-base-pair segment encoding 60-amino-acid helix-turn-helix homeodomain with DNA binding capacity. Due to the lack of homeodomain's DNA binding specificity, they bind to target genes' promoter region more efficiently in presence of additional transcription factors complex to active or repress their transcription (4).

To elucidate the role of DLX4 in ER negative breast cancer as a transcription factor, ChIP-on-chip was applied in DLX4-transfected ER negative breast cancer cell line Hs578T, which expresses barely detectable DLX4. It is reported that 153 potential DLX4 binding sites were identified by genome-wide promoter analysis. Additionally, to ensure the DLX4's transcriptional regulation on these target genes, DLX4-containing and empty vector transfected Hs578T expression profile was compared by expression microarray assays, and a

list of 253 genes differentially expressed was generated via Partek Genomics Suite (Partek Incorporated, St. Louis, Missouri, USA). Eighteen genes appeared in common from both assays, including VEGFA, were verified by Chromatin Immunoprecipitation and QPCR (7). The 9-base-pair binding site of DLX4 was at Chr 6 43849244, downstream of VEGFA gene (7).

Intense studies have proved the essential role of Vascular Endothelial Growth Factor (VEGF) family in angiogenesis in many human tumor types; the establishment of tumor blood supply is considered the rate-limiting step in tumorigenesis. VEGFA, one of the best-characterized and major angiogenesis mediators of the VEGF family, is commonly overexpressed among various human solid tumors. Microvessel density in breast tumor was positively correlated with VEGF expression (8), and upregulated VEGF mRNA were detected within the malignant tissue compared with surrounding normal breast tissue (9).

Besides its role in angiogenesis (2), VEGF plays significant role in tumor cell survival, progression and metastasis, therefore it associates more malignant and invasive breast tumor type with poor survival. The epithelial cells in IBC, which exhibit high level of metastatic risk verse non-inflammatory breast cancer, express significantly higher level of mRNA of angiogenesis genes, including VEGFA (1). Within the triple-negative breast cancer tissue, the subtype that shows more aggressive and low responding rate to standard treatment, expresses significantly higher level of VEGF (10). What's more, compared to primary and regional breast tumor, VEGF overexpression was verified in distant metastatic tumor cells.

Right now, more than 20 anti-VEGF reagents were investigated in clinical trials for breast cancer in combination with other anti-angiogenesis drugs or lower dose chemotherapy (11). Anti-VEGF therapy not only works on vascular endothelial cells within solid tumor, but also diminishes VEGF's stimulation of survival on mammary epithelial cell in adenocarcinoma. The mechanism of anti-VEGF therapy is the blockage of VEGF or its receptor via humanized antibodies, or the impeded the activation of VEGFR2 by kinase inhibitors. The more we understand downstream signaling of VEGFR2, more ideas we could have to inhibit VEGF's action on tumor survival and progression. For instance, Bevacizumab, antibody to VEGF, showed significantly higher responding rate in all patients and increased tumor cell apoptosis in inflammatory breast cancer (11-13).

VEGFR2, as one of the three members of transmembrane tyrosine kinase receptors for VEGF in mammals, plays an essential role in inducing physical function, tumor angiogenesis and carcinogenesis. Although the well-studied actions of VEGFR2 generally involve in the proliferation, differentiation and migration of the endothelial cells, upregulation of VEGFR2 was confirmed in invasive primary and metastatic breast cancer (9,14, 15). VEGFR2 was expressed both in ER negative (MDA-MB-231, MDA-MB-468) and ER positive (MCF7, T47D) breast cancer cell lines (16, 17). It was also reported that the VEGFR2 on the surface of these breast cancer cell lines could be stimulated by external VEGFA (18). Furthermore, they demonstrated that VEGFR2 expression was positively correlated to VEGF activation in the primary cancer cell line, and hence endothelial-independent autocrine/paracrine VEGF/VEGFR-2 feedback loop in cancer cell survival was proposed (1, 19).

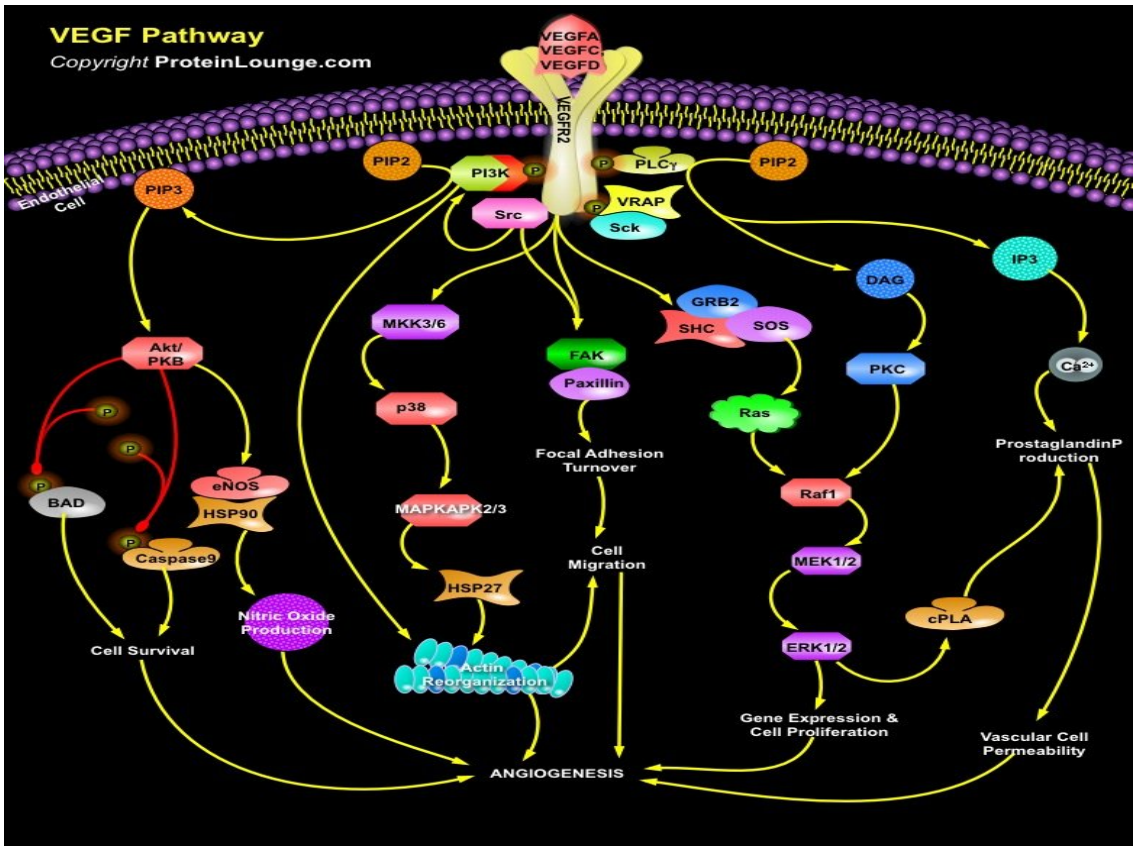


Figure 1 VEGF Pathway from the Proteinlounge.

VEGF family is composed of several isotypes, including VEGF-A, -B, -C and –D, which exist as numerous splice variants. VEGF-A and VEGF-B promote vascular angiogenesis primarily through activation of VEGFR-1 (Flt1) and VEGFR-2 (Flk1/KDR). These receptors are tyrosine kinases that are linked to cell survival, proliferation, and motility processes via phospholipase C (PLC)/calcium and PKC; PI3-K/AKT; and the RAS/RAF/MEK/ERK pathways.

PI3K (phosphatidylinositol 3-kinase)/Akt (Protein Kinase-B) pathway triggered by VEGFR2 (Figure 1) plays crucial role in survival of breast cancer epithelial cells. Hyperactivation of PI3K/Akt pathway has been reported in endocrine therapy acquired resistance in ER positive breast cancer cell lines (3), and it was demonstrated that other growth factor receptor tyrosine

kinase inhibitors, targeting PI3K/Akt, induced apoptosis of breast cancer cells (20). Therefore, this study is focusing on the cell survival pathway modulated via PI3K/Akt and its downstream significant player S196 phosphorylated pro-caspase-9 (21) and phosphorylated BAD (Bcl-2-associated death promoter). Through phosphorylation, both pro-caspase-9 and BAD acquire resistance to cleavage by proteases; and thus prevent cells from going through apoptosis upon stimulus.

2. Materials and Methods

2.1. Cell Culture

All of the breast cancer cell lines were all cultured in a 37ºC incubator with 5% of CO2. The DLX4 over-expressing stable Hs578T cell line was previously established and maintained in DMED medium containing 400μg/ml of G418 antibiotics (Invitrogen Corporation, Carlsbad, CA, USA) (Invitrogen Corporation, Carlsbad, CA, USA) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 50unit/ml of penicillin and 50μg/ml of streptomycin (Invitrogen Corporation, Carlsbad, CA, USA). The other ER- breast cancer cell lines, such as MDA-MB-231 and MDA-MB-468, were cultured in DMEM/F-12 50/50 (Mediatech, VA, USA) with 10% FBS and 50unit/ml of penicillin and 50μg/ml of streptomycin.

2.2. Small interfering RNA synthesis and Knockdown

The siRNA used for DXL4 knockdown in MDA-MB-231 and MDA-MB-468 was designed and synthesized by Silencer® siRNA Construction (Ambion, Austin, TX, USA). The siRNA concentration was determined by Nano Drop Spectrophotometer (The Thermo Scientific, Wilmington, DE, USA), and diluted to 200ng/μl using RNase-free water (Acros Organics, New Jersey, USA). Three pairs of template 29nt oligonucleotides were designed targeting three 21nt mRNA sequence within DXL4 following the instruction of the kit (Table 1). The knockdown efficiency was determined by real-time PCR in three cell lines.

Table 1. Template sequences for DLX4 siRNA synthesis

Template name	Template sequence
T1	Sense 5'-GAC CTA TGG GTA ATT TAT GCT CCT GTC TC-3' Antisense 5'-AAA GCA TAA ATT ACC CAT AGG CCT GTC TC-3'
T2	Sense 5'-AAG GAA CTG TGC AGA TTT AGA CCT GTC TC -3' Antisense 5'-GTT CTA AAT CTG CAC AGT TCC CCT GTC TC -3'
T3	Sense 5'- ATC TCC CCT TTT TGA ATA GAT CCT GTC TC -3' Antisense 5'-TTA TCT ATT CAA AAA GGG GAG CCT GTC TC-3'

The day before the siRNA transfection, cells that reached 65%-90% confluence in 10cm dishes (Sarstedt, Newton, NC, USA) were trypsinized by 0.05% trypsin-EDTA for 5 minutes in 37ºC, and harvested via Centrific Model 228 (Fisher Scientific, Fair Lawn, NJ, USA) at 3500 rpm for 5 minutes. An aliquot of 100µl of the resuspended cells was added to 10ml of 1x PBS and applied to Coulter Particle Count and Size Analyzer (Beckman Coulter, Brea, CA, USA) for cell counting. The size of counted particle was set up at 6.000-18.00µm, and the reading of cell density should fall into the range of $1x10^5$-$1.2x10^6$ cells/ml. Total of 350,000 cells/well were plated in a 6-well-plate antibiotic-free, serum-containing medium (Corning Incorporated, Corning, NY, USA) or $2X10^6$ cells when10cm dishes were used, and then incubated overnight. A non-targeting pooled siRNA (2µM) (Thermo Scientific, Wilmington, DE, USA) was used as a negative control. A total of 100µl transfection complex, includes 50nmol of DXL4 or non-targeting siRNAs, diluted in 95µl of serum-free medium (without antibiotics) and 5µl of FuGENE® HD Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany). After 25 minutes of incubation at RT, the transfection complex (100ul) was applied to a 6-well-plate, and 500ul was used for a10cm dish. The final concentration of DXL4 and non-targeting siRNAs in the medium would be 36nM.

2.3. RNA isolation and QPCR

After 24 hours of DLX4 siRNA or non-specific target siRNA treatment to MDA-MB-231 and MDA-MB-468, or the stable DLX4-transfected Hs578T cells and the empty vector controls, 1ml of Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) was applied to each well and incubated for 5 minutes at RT. Total RNAs were extracted from the cells following the manufacturer's protocol. 200µl of chloroform (Fisher Scientific, Fair Lawn, NJ, USA) was added to cell lysis in 1ml Trizol. The aqueous phase containing RNA was separated by centrifuge at 12,000 rcf and carefully transferred to a fresh tube. 1ml of isopropyl alcohol (Fisher Scientific, Fair Lawn, NJ, USA) was added to the aqueous for RNA precipitation. After the centrifuge at 12,000 rcf, the gel-like RNA pellet was colleted at the bottom of the tube. The RNA pellet was washed with 75% ethanol, and air-dried briefly to eliminate potential alcohol residue. 40µl of RNase-free water was utilized to dissolve the RNA at 55°C for 10 minutes before the concentration of the samples was measure by NanoDrop Spectrophotometer (Thermo Scientific, Rockford, IL, USA).

One microgram of each RNA sample was digested with DNase I (Invitrogen Corporation, Carlsbad, CA, USA) to eliminate potential DNA contamination before the reverse transcription was carried out using iScript kit (Bio-Rad Laboratories, Hercules, CA, USA). For each RNA sample, 1µl of reverse transcriptase and 4µl of 5X iScript Reaction Mix were mixed with 1µl sample. Then the reverse transcription reaction was carried out in the Thermal Cycler System (Applied Biosystem, Carlsbad, California, USA) following the

setting as 25ºC 5mins, 42ºC for 30mins and 85ºC for 5mins. A total of 20µl of cDNA was obtained for each sample.

Real-time PCR was performed with 2µl of cDNA produced in reverse transcription reaction as template. 1ul of both the forward and reverse primers, whose concentration is 20pmol/µl, was mixed with 12.5µl of 2xSYBR Green reagent (SABio, Fredrick, MD, USA) and brought into 25µl by RNase-free water. The amplification signals were detected and data was collected via ABI 7300 Real-time PCR system System (Applied Biosystem, Carlsbad, California, USA). 18s rRNA gene was used as an internal control. Due to its abundance, we diluted the cDNA samples by 100 times for 18s amplification. The primers used for DLX4, VEGFA, VEGFR2 and 18s QPCR are listed in Table 2.

Table 2. Primer Sequences Used for real-time PCR

Target Gene	Sequence
DLX4	Forward 5'-CAAAGCTGTCTTCCCAGACC-3' Reverse 5'-GTTGTAGGGGACAAGCCAAG-3'
VEGFA	Forward 5'-CTACCTCCACCATGCCAAGT-3' Reverse 5'-GCAGTAGCTGCGCTGATAGA-3'
VEGFR2	Forward 5'-GACTTGGCCTCGGTCATTTA-3' Reverse 5'-ACACGACTCCATGTTGGTCA-3'
18s	Forward 5'-CCGCAGCTAGGAATAATGGA-3' Reverse 5'-CCCTCTTAATCATGGCCTCA-3'

2.4. Protein Extraction and Quantitative Assays

siRNA treated cells were harvested using 4ml of 0.05% trypsin-EDTA (Atlanta Biologicals, Lawrenceville, GA, USA) for each plate. The cell pellets were resuspended in 1ml 1x PBS and centrifuged at 9000 rpm for 5 minutes. Then the cell pellets were resuspended in 80µl of tissue-protein extraction reagent (T-PER) (Thermo Scientific, Rockford, IL, USA), containing 1x protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail I and II (Sigma-Aldrich, St. Louis, MD, USA). After incubating at RT for 30 minutes, the reaction was centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and transferred to a fresh tube for quantization and long-term storage at -80C if not used immediately.

The concentration of the protein samples was measured via Pierce BCA Protein Assay Kit (Thermo Scientific, Rockland, IL, USA). The series of 9 standards were diluted from 2mg/ml of BSA stock solution in the kit by T-PER, and they ranged from 2.00mg/ml to 0mg/ml. BCA reagent working solution was prepared in 10:1 ratio of reagent A to B, and 200µl of the mixture was added to each well in a 96-well-plate (Corning Incorporated, Corning, NY, USA), before adding 10µl of each standard or diluted protein samples in triplicates. After 30-minute-incubation at 37ºC, the absorbance at 562nm was measured by Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), and the concentration of diluted unknown protein samples was determined by comparison with the standard curve.

2.5. Western Blot Assays

A total of 20µg of protein was used for each sample for Western blot assays. The samples were mixed with 6.67µl of 3x SDS dye and appropriate amount of T-PER for a total volume of 20ul before denaturing at 98°C for 5 minutes. The Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Hercules, CA, USA) was used as molecular standard. The samples were run on a 4-12% PAGEr® Gold Precast Gels (Lonza, Rockland, ME, USA) at 30mAmp for 1.5 hours using1x running buffer, which was diluted from 10x Running buffer composed of 250mM Tris (Fisher Scientific, Fair Lawn, NJ, USA), 1.92M glycine (Fisher Scientific, Fair Lawn, NJ, USA) and 1% SDS (Fisher Scientific, Fair Lawn, NJ, USA).

Sponges and Gel Block Paper (Whatman, Sanford, ME, USA) were soaked in 1x transfer buffer, which was freshly prepared from 10x transfer buffer, containing 25mM Tris and 192mM glycine, and 20% methanol (Fisher Scientific, Fair Lawn, NJ, USA). After the run, the gel was carefully removed and rinsed briefly with 1x transfer buffer. The polyscreen PVDF membrane (PerkinELmer Life Sciences, Waltham, MA, USA) was soaked in methanol for 15minutes before transfer sandwich was set up. After transfering with 20mAmp constant current at 4°C overnight, the membrane was removed from the gel. The membrane was then blocked at RT in 5% of non-fat dry milk (LabScientific, Livingston, NJ, USA) in PBST (1x PBS with 0.1% Tween 20) (Fisher Scientific, Fair Lawn, NJ, USA) buffer for 1 hour with slow shaking on Slow Speed Rotomix® Variable Speed Rotator (Barnstead Thermolyne Corporation, Hampton, NH, USA).

Both primary and secondary antibodies were distributed in blocking buffer with different ratio (Table 3) due to recommended protocols of antibodies and target protein abundance. The blot was incubated overnight at 4°C for primary antibody and 2 hours at RT for secondary antibody. After each incubation, the membrane was washed twice with PBST and once with PBS for 7 minutes. For visualization, 700µl each of A and B solution in SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) was mixed and applied evenly to the membrane for 5 minutes incubation in dark. The image was taken at 1 minute, 5 minutes and 20 minutes exposure depending on the strength of the fluorescence signal by Kodak 2000MM Image System (Kodak, Rochester, NY, USA), and analyzed using the Carestream MI (Carestream Health, Rochester, NY, USA). To measure other protein levels on the same blot, membranes were stripping by a solution of 2% of SDS (wt:vol), 100 mM of mercaptoethanol and 50mM of Tris for 5 minutes at 60°C.

Table 3. Antibodies Used for Western Blot Assays.

Primary Antibody	Dilution	Company/ Product#	Secondary Antibody	Dilution
Rabbit anit-DLX4	1:5000	Bethryl/ Customerized	Goat anti-Rabbit HRP Conjugated	1:10000
Rabbit-anti-VEGF	1:500	Biolegend/ 627501	Goat anti-Rabbit HRP Conjugated	1:1000
Rabbit-anti-PhosPho-PI3K p85 (Tyr458)/p55 (Tyr199)	1:500	Biolegend/ 627501	Goat anti-Rabbit HRP Conjugated	1:1000
Rabbit-anti-Phospho-AKT (Ser473)	1:500	Cell Signaling/ 9271	Goat anti-Rabbit HRP Conjugated	1:1000
Anti-Phospho-BAD (Ser136)	1:500	Cell Signaling/ 9295S	Goat anti-Rabbit HRP Conjugated	1:1000
Rabbit anit-Phospho-Caspase 9 (Ser196)	1:500	Abgent/ AP3044a	Goat anti-Rabbit HRP Conjugated	1:10000
Rabbit anti-Cleaved Caspase-3	1:500	Cell signaling/ 9661	Goat anti-Rabbit HRP Conjugated	1:1000
Mouse anti-β-Actin	1:10000	Sigma/A5441	Goat Anti-Mouse HRP Conjugated	1:1000

2.6. MTT Assays

For Hs578T DLX4 overexpressing cells, before the day of experiment, 30000 cells of 3 overexpressing cell lines along with 2 empty vector controls were plated in a 24-well-plate, and incubated overnight with 360µl selective medium with 1% FBS to induce apoptosis. Triplicates were prepared to minimize the deviation among samples. After 24 hours, the 24-well-plate was put on a shaker for 5 minutes after adding 40µl of 5mg/ml Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, St. Louis, MD, USA) to each well, and then incubated for 3 hours allowing cells to metabolize Thiazolyl Blue Tetrazolium Bromide (MTT). After the thorough removal of the medium, 200µl of Dimethyl Sulfoxide (Fisher Scientific, Fair Lawn, NJ, USA) was utilized to dissolve formazan, MTT metabolic product, in each well. The absorbance at 570nm was measured via Microplate Reader. For the MTT measurement after 48 or 72 hours, the same procedure was used except that the cells were fed with fresh medium on Day 2.

For MDA-MB-231 and MDA-MB-468 MTT assay, 20000 cells were plated and incubated overnight. Then the transfection reaction was composed of 25µl transfection complex, 1.25µl FuGENE HD reagent and the DLX4 or non-target siRNA. The cells were then incubated in the complete medium with 10% FBS overnight before MTT assays were performed.

3. Results

To determine the expression of DLX4 and VEGFA in DLX4-overexpressing Hs578T cell clones, quantitative PCR was performed on 3 overexpressors and 2 empty vector controls. Using 18s rRNA as an internal control, DLX4 expression was increased by an average of 15 folds, while VEGFA was up for 5.74 folds (Figure 2.). All of the experiments were done three times to eliminate experimental deviations and false positive result. This suggests that DLX4 may upregulate VEGFA expression transcriptionally. Since VEGFA exerts its function mainly through its binding to VEGFR2, we decided to test the proposed autocrine/paracrine loop between the expression of VEGF and VEGFR2 in DLX4-transfected Hs578T cells. We found that VEGFR2 mRNA expression was upregulated by 4.18 folds when DLX4 is overexpressed in Hs578T cells (Figure 2). This demonstrates that both VEGFA and VEGFR2 were upregulated in DLX4-overexpressing Hs578T cells. Although we cannot verify if DLX4 directly regulates VEGFR2 expression, it is most likely due to the upregulation of VEGFA, as has been demonstrated (1)(19).

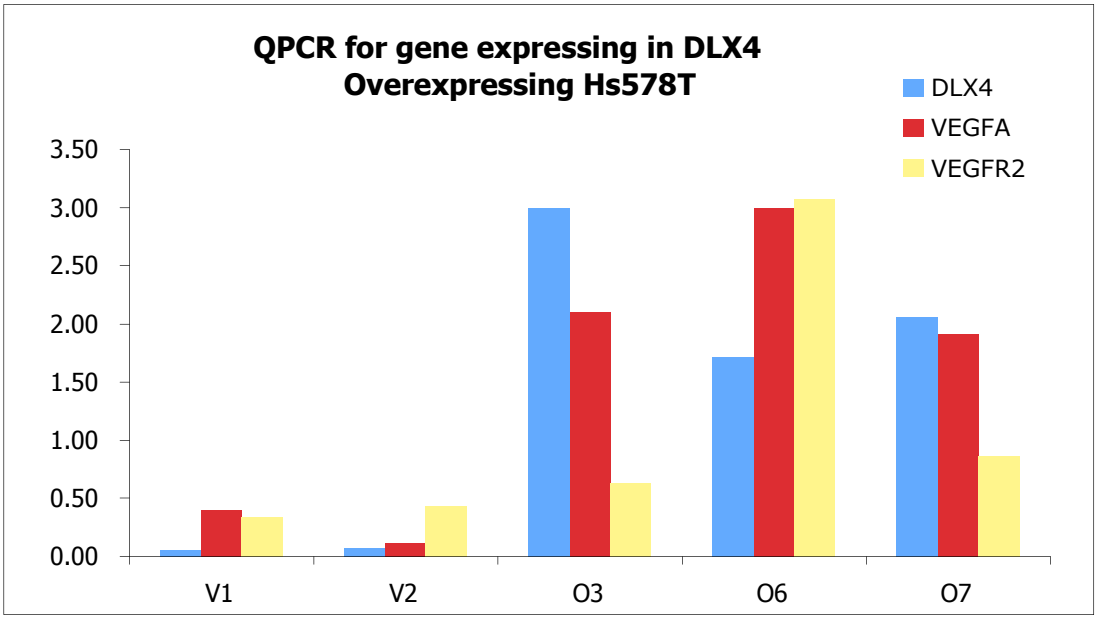


Figure 2. QPCR Analysis for DLX4, VEGFA and VEGFR2 in DLX4-transfected Hs578T cell lines. DLX4 expression in O3, O6 and O7 Hs578T cells was increased by 15 folds compared to V1 and V2 controls, while VEGFA and VEGFR2 mRNA expression was upregulated by 5.74 and 4.14 folds respectively. 18s was used to normalize the expression level of all three genes.

To further verify the transcriptional regulatory function of DLX4 on VEGFA, two ER negative adenocarcinoma epithelial cells lines, MDA-MB-231 and MDA-MB-468, which express different level of endogenous DLX4, were transiently transfected with siRNA targeting DLX4 mRNA along with non-specific target siRNA pool as a control. We successfully knocked down DLX4 mRNA expression by 44.8% in 231 cells and 38.0% in 468 cells relative to the non target controls. VEGFA mRNA expression was reduced by 14.2% and 29.9%, respectively. (Figure 3).

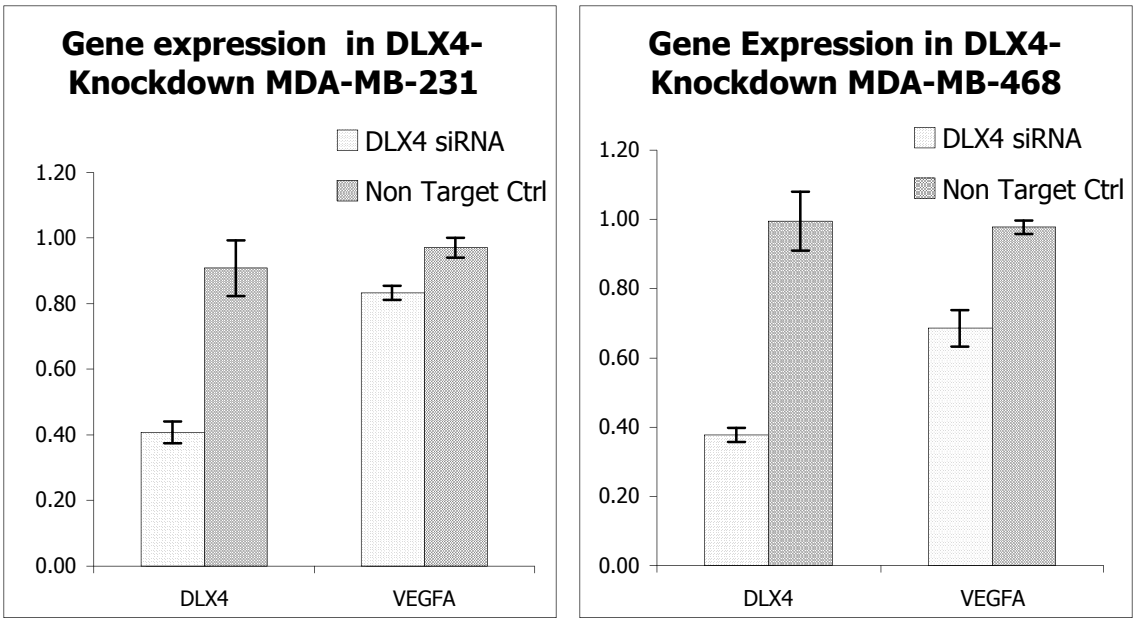
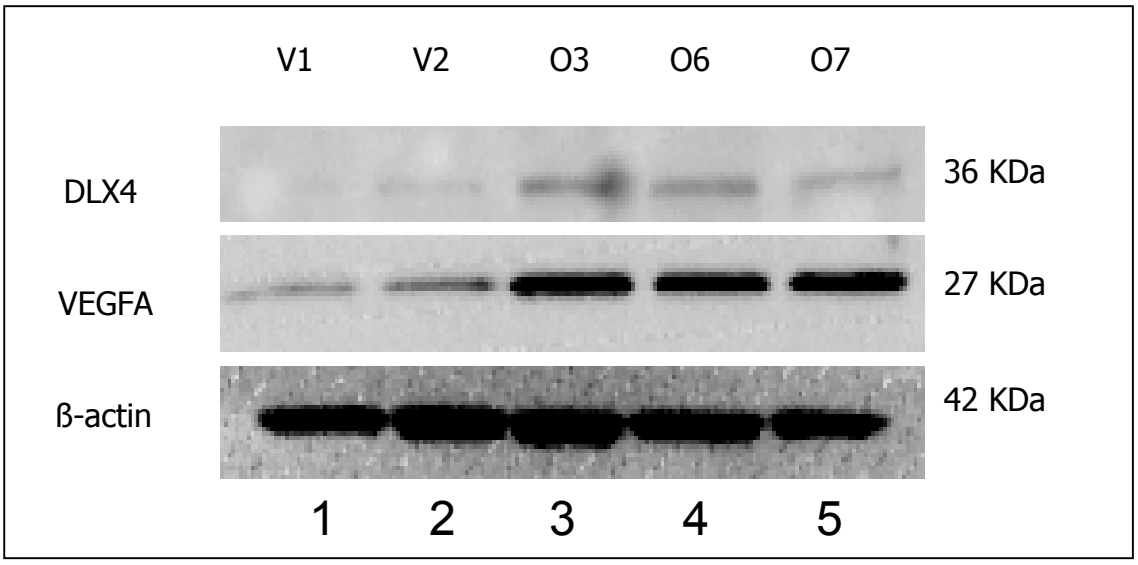


Figure 3. QPCR Analysis for DLX4 andVEGFA expression in MDA-MB-231 and MDA-MB-468 after DLX4-siRNA knockdown.. In DLX4 siRNA transfected MDA-MB-231 cells, 44.8 % DLX4 knockdown induced 14.2% decrease in VEGFA expression. In MDA-MB-468, 38% DLX4 knockdown reduces VEGFA expression by 29.9% decrease.

We have demonstrated that there is a positive correlation between DLX4 and VEGFA mRNA expression (Figure 2 & 3), and the fact DLX4 binds to the VEGFA 3' UTR, DLX4 may upregulate VEGFA. To determine the protein expression in those cell lines either overexpressing, or knocking-down DLX4, Western blots were performed targeting DLX4, VEGFA, as well as other targets involving the VEGFA/VEGFR2 mediated PI3K/Akt pathway.

In the Hs578T cells transfected with pcDNA3.2 containing no DLX4 gene sequence, there were barely any DLX4 detected in Western blot, while in DLX4-transfected Hs578T clones -- O3, O6, O7, the expressing DLX4 was significantly increased by 4.78 folds, while VEGFA raised by 6.17 folds (Figure 4).



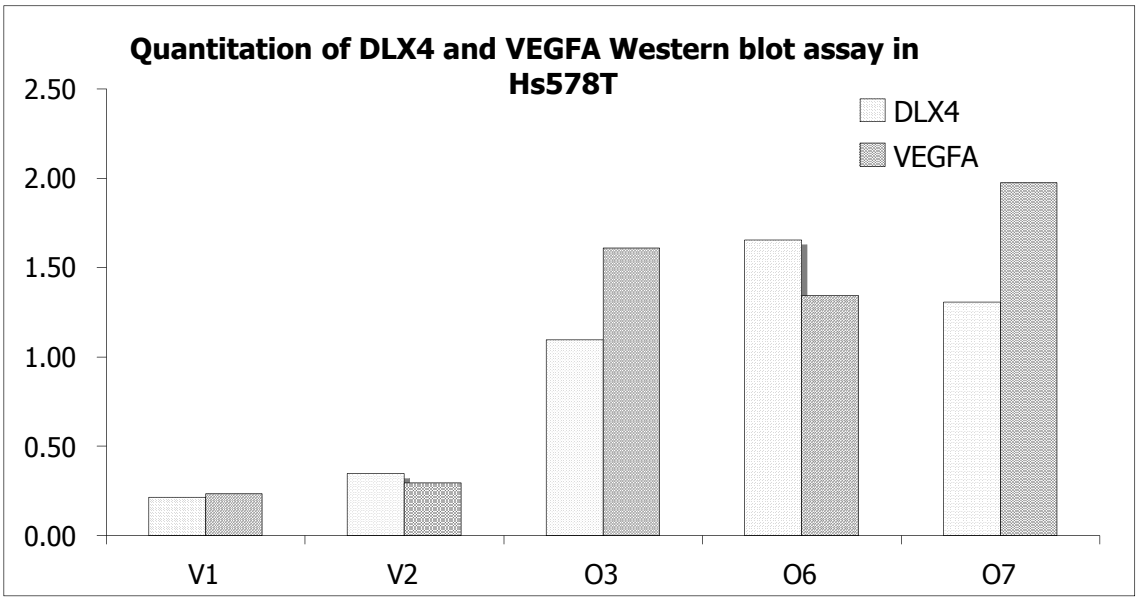


Figure 4. DLX4 upregulates VEGFA protein expression Hs578T breast cancer cells by Western blot assays. Three clones of DLX4transfected Hs578T cells along with two empty vector controls were selected in serum-containing medium with 800μg/ml G418 and maintained with 400μg/ml. Proteins were extracted by T-PER with added protease and phosphatase inhibitors. On average, 4.78 folds of DLX4 expression was detected in DLX4 transfected Hs578T cells, and VEGFA expression increased by 6.17 folds. The stripped blots were re-probed with ß-actin antibody as an internal control.

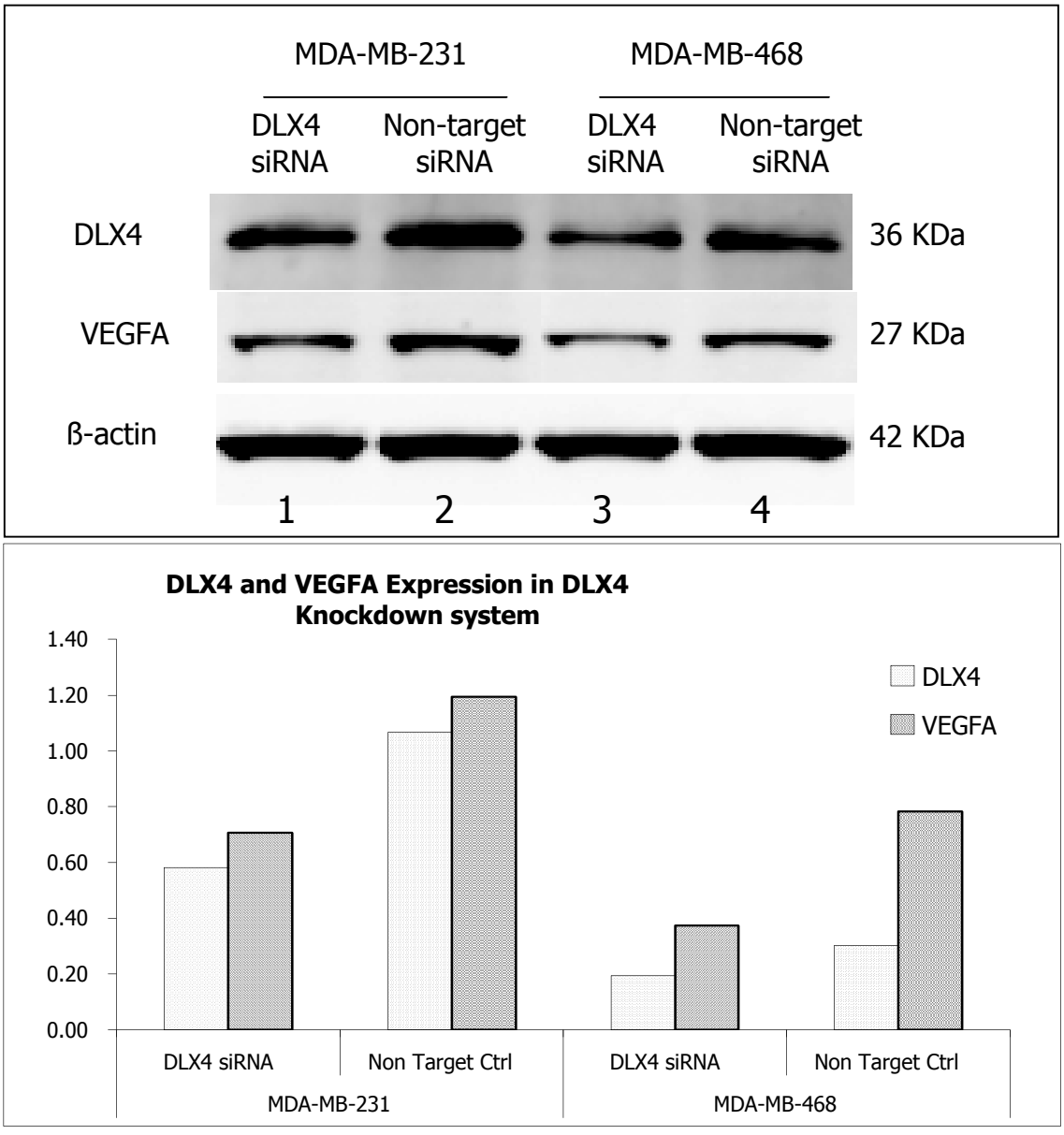


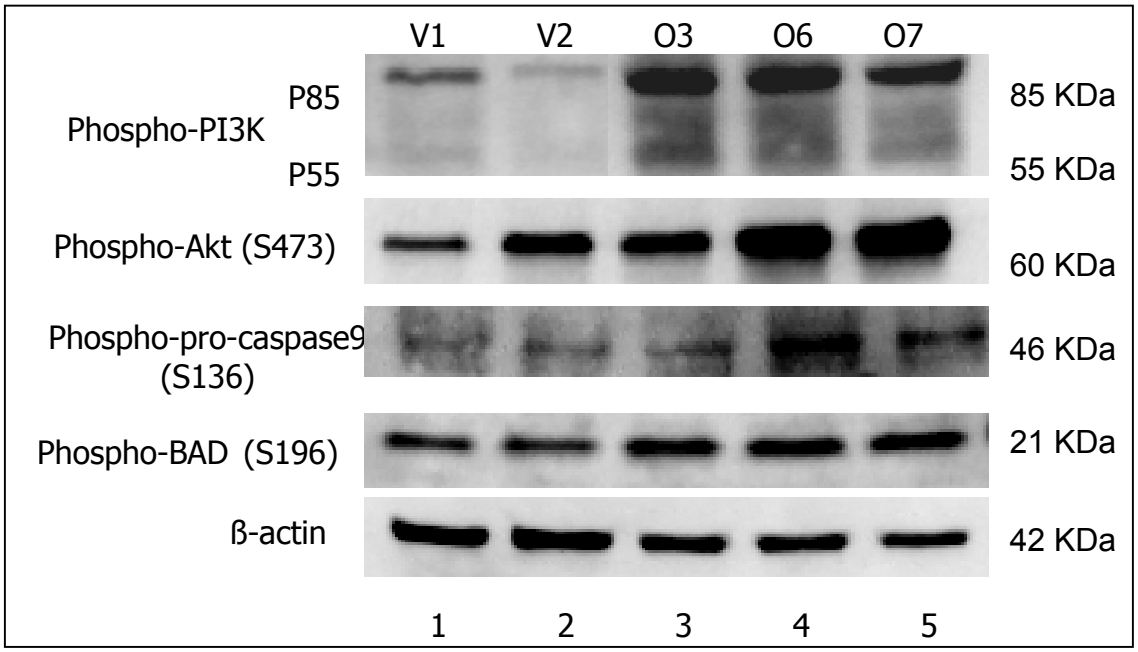
Figure 5. The siRNA knockdown of DLX4 suppresses the VEGFA expression in ER negative breast cancer cell lines MDA-MB-231 and MDA-MB-468. Cells were double transfected by DLX4 siRNA at the final concentration of 36nM within 48 hours' window. Proteins were extracted by T-PER reagent 24 hours after the second knock-down transfection. DLX4 and VEGFA expression were both assayed by Western blotting and quatified. 40.9% and 45.6% decline of DLX4 and VEGFA was demonstrated in MDA-MB-231, while 36.1% and 52.3% reduction of them was observed in MDA-MB-468 DLX4 knockdown cell compared with control.

In knockdown system, we demonstrated the significant reduction of VEGFA level by 40.9% with the 45.6% decreased DLX4 protein level in transient DLX4 siRNA knockdown in MDA-MB-231 via Western blot assays (Figure 5, left panels). 52.3% less VEGFA was detected with 36.1% DLX4 knockdown in MDA-MB-468 cell line (Fiugre 5, right panels). The results were consistant to the QPCR data in both DLX4 overexpressing and knockdown system of ER negative breast cancer cell lines (Figure 2, 3).

In addition, the evidence for positive correlation between DLX4 and VEGFA expression did not just come from the comparison of different level of DLX4 in the same cell line (Figure 4,5), but also between the two ER negative mammary adenocarcinoma cell lines transfected with non Target siRNAs, which express different level of endogenous DLX4 (Figure 5). Relatively higher VEGFA expression was observed in MDA-MB-231 cell line, which exhibits high basal DLX4 level relative to MDA-MB-468 (Figure.4).

Human breast cancer cells as well as other types of malignancies, employs diverse molecular strategies to evade apoptosis triggered by lack of nutrition and oxygen, immune system and anti-cancer drugs, and PI3K/Akt pathway obsess a dominant role in breast cancer cell survival (22) (23) (24)(18) (25). The binding of extra-cellular VEGFA upon trans-membrane VEGFR2 stimulated the recruitment and phosphorylation and activation of PI3K. Once activated, within the plasma membrane PI3K catalyzes the formation of lipid second messengers, such as PIP3 (phosphatidylinositol 3,4,5-trisphosphate) and PIP2 (phosphatidylinositol 3,4-bisphosphate), which facilitate the

translocation, phosphorylation and activation of Akt (26). After being activated, Akt phosphorylates and deactivates pro-caspase-9 and BAD (Figure 1), and obstacles this apoptosis machinery in epithelial cells within the breast tumor and cell proliferation among vascular endothelial cells.



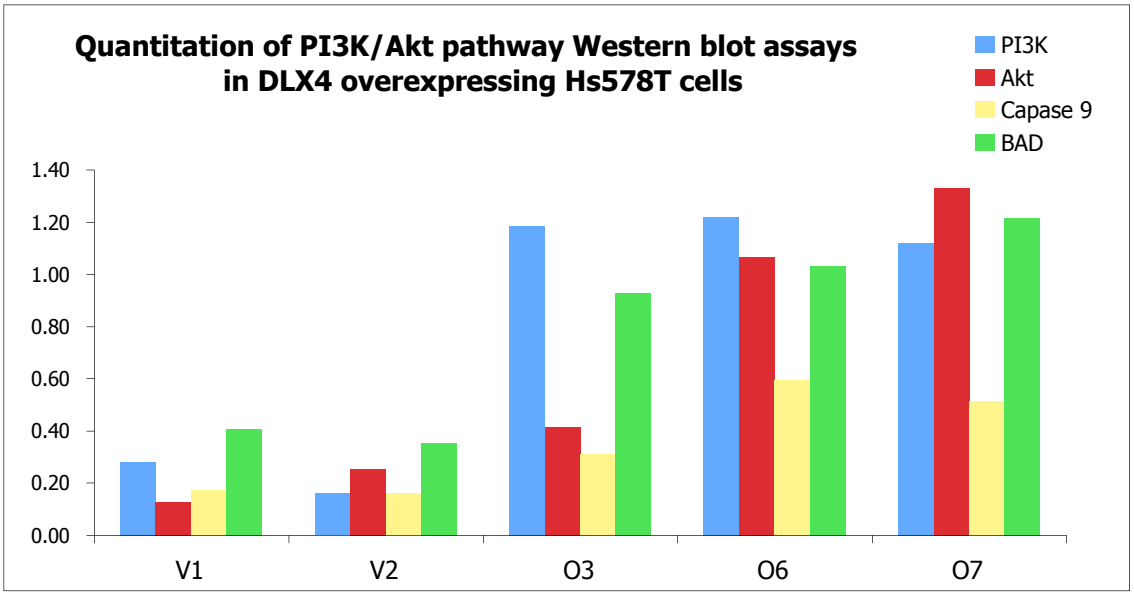
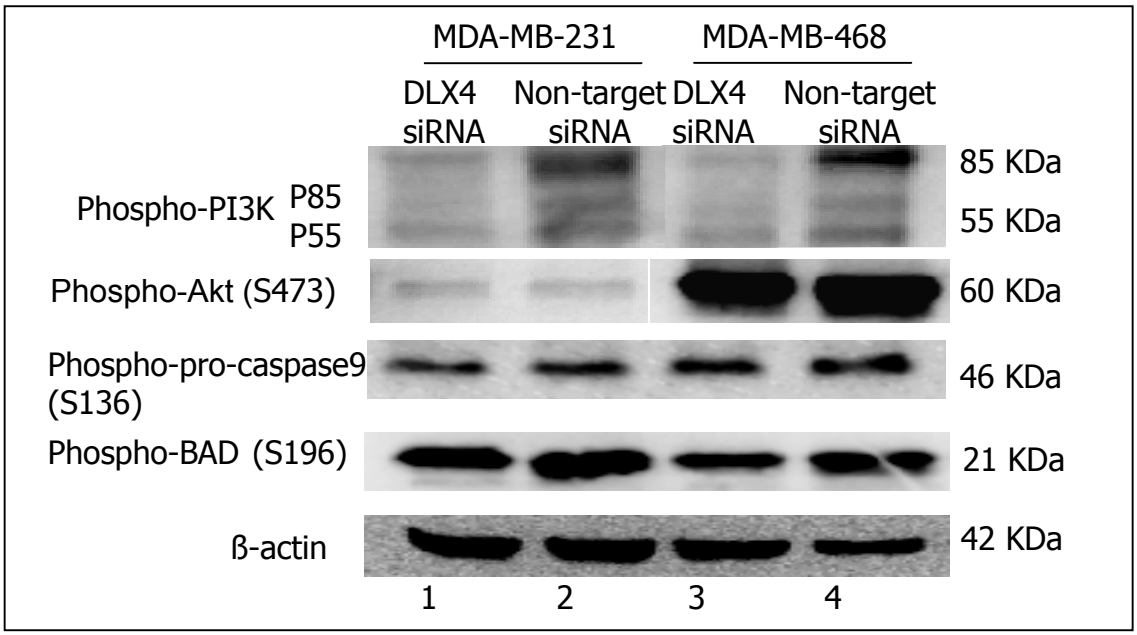


Figure 6. PI3K/Akt pathway activity was increased in DLX4-transfected Hs567T cell clones. Both the DLX4 transfected Hs578T cell clones and controls were cultured in 1% FBS medium, which induces apoptosis, before protein extraction. Specific antibodies targeting phosphorylated PI3K, Akt, pro-caspase-9 and BAD were utilized and the quantitative analysis was normalized by β-actin. Activated PI3K and Akt increased by 5.33 and 4.89 folds in DLX4 transfected clones, while deactivated pro-caspase-9 and BAD increased by 2.85 and 2.79 folds, respectively.



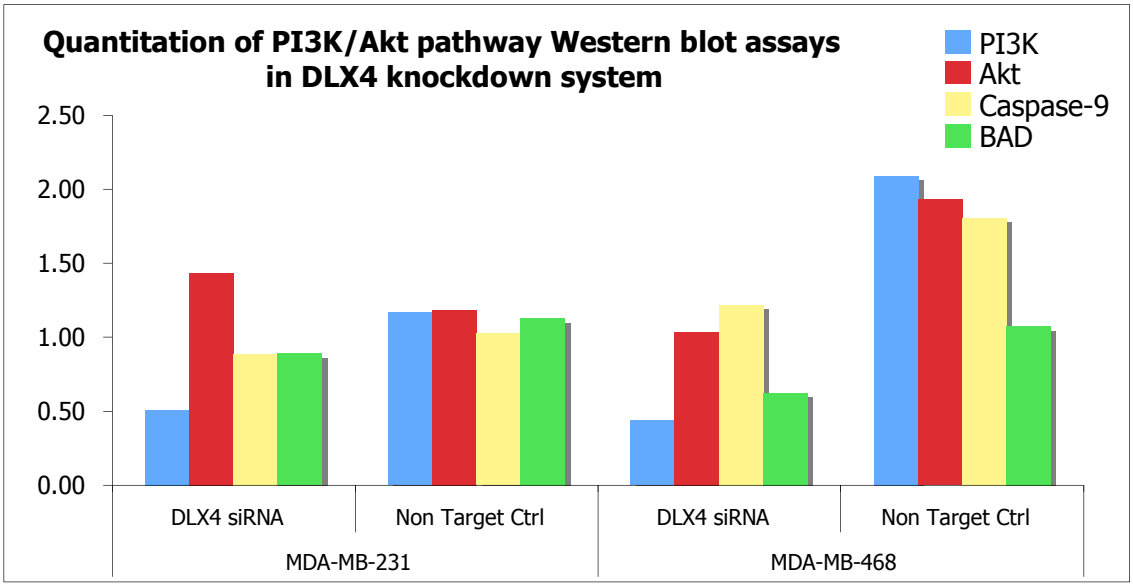


Figure 7. PI3K/Akt survival pathway was suppressed in DLX4 siRNA knockdown MDA-MB-231 and MDA-MB-468 cells. After and the confirmation of DLX4 knockdown at protein level in MDA-MB-231 and MDA-MB-468, PI3K/Akt pathway activity status was examined by Western blot. The overall PI3K/Akt activity was compromised in siRNA transfected cells. 20.9%, 53.6%, 67.4% and 58% of phosphorylated PI3k, Akt, pro-caspase-9 and BAD was detected in DLX4 siRNA transfected MDA-MB-468. Those components in PI3K/Akt pathway decreased slightly in MDA-MB-231 DLX4 knockdown system.

In our study, besides β-actin as loading control, the PI3K/Akt survival pathway activity status was investigated by western blot assays probing phosphorylated PI3K p85 (Tyr458)/p55 (Tyr199), phosphorylated Akt (Ser473), phosphorylated BAD (Ser 112/Ser136) (27) and phosphorylated pro-caspase-9 (Ser196) (21) within DLX4 transfected Hs578T as well as DLX4 siRNA knockdown MDA-MB-231 and MDA-MB-468 cells verse controls, respectively.

Shown in Figure 6 and 7, the amount of both the phosphorylated regulatory p85 subunit and p55 subunit with catalytic ability were increased by 5.33 folds in the DLX4 transfected Hs578T cells. Also, activated PI3K was suppressed by 43.4% and 20.9% in the DLX4 siRNA knockdown MDA-MB-231 and MDA-MB-468 cell lines relative to their controls, respectively. In general, PI3K activity associates with the DLX4 and VEGFA expression in ER negative breast cancer cell lines.

Specific antibody for fully activated S473 phosphorylated Akt (28) was utilized in western blot, and 4.89 folds of phosphorylated Akt was measured in DLX4 transfected Hs578T cells while 46.4% knockdown was observed in DLX4 siRNA transfected and MDA-MB-468 versus controls (Figure 6. & 7.). However, barely decrease of activated Akt was detected in DLX4 knockdown MDA-MB-231 (Figure 7, right panel).

PI3K/Akt survival signaling regulates cell survival mainly by phosphorylational deactivation of Pro-caspase and BAD, and therefore eliminate their ability to induce apoptosis (29).

Kinase Akt phosphorylates pro-caspase-9 at Ser196, inhibits its proteolytic activation by numerous apoptotic proteases induced by diverse pro-apoptosis signals. Hence, the inactive phospho-pro-Caspase-9 fails to cleave the downstream pro-Caspase-3 and that leads to the inhibition of this apoptosis machinery (30). In our study, phosphorylated pro-caspase-9 was suppressed to 86.2% and 67.4% in DLX4 siRNA transfected MDA-MB-231 and MDA-MB-468 relative to controls, respectively, indicating the lower activity status of this apoptosis machinery in DLX4 knockdown ER negative breast cancer cells (Figure 7). On the other hand, 2.85 times of phosphorylated pro-caspase-9 was detected in DLX4 overexpressing Hs578T cells relative to controls (Figure 6).

Bcl-2-associated death promoter (BAD), as a pro-apoptotic protein in Bcl-2 gene family, instead of directly involving in apoptosis by targeting or maintaining mitochondrial and nuclear envelope as other Bcl-2 family members, forms heterodimer with other anti-apoptotic proteins to prevent them from blocking apoptosis. However, phosphorylated BAD at Serine 112 and 136 by Akt tends to form protein homodimers and free the anti-apoptotic proteins to pretect mitochondria membrane, and the sub sequential release of cytochrome c and DNA fragmentation leading to cell Death (31). In short, Ser112/Ser 136 phosphorylated BAD serves as anti-apoptotic component prevents cell undergoing apoptosis cascade.

With the overexpression of DLX4 and VEGFA (Figure4), the phosphorylated pro-caspase-9 increased by 2.79 folds (Figure 6) in Hs578T cells. Furthermore, as Figure 7 shown, normalized by internal control β-actin, phosphorylated BAD decreased to 78.9%

and 58% in the DLX4 siRNA knockdown MDA-MB-231 and MDA-MB-468 cell lines compared with controls, respectively.

Therefore, our study indicated that the DLX4 expression status correlates with PI3K/Akt anti-apoptosis pathway triggered by VEGFA. Moreover, the downstream players of PI3K/Akt pathway, phosphorylated pro-caspase-9 and phosphorylated BAD associates with the activity of PI3K/Akt (Figure 6 & 7). By phosphorylation, both pro-caspase-9 and BAD obtain the resistance to proteolytic cleavage, and thus blocks the cellular apoptosis cascade.

The inhibition of PI3K/Akt via specific via specific kinase inhibitor induces dephosphorylation of BAD and pro-caspase-9, and promotes apoptosis in myeloid leukemias (32) kidney cancer (33) and virus HTLV-1 transformed malignant cells (34) A plethora of researches emphasizing on PI3K/Akt kinase inhibitors and specific phosphatases manifested positive outcomes concerning the growth inhibition, apoptosis induction and drug resistance prevention in cancers (32) (33) (34) (12) (20).

To estimate the overall impact of DLX4 on ER negative breast cancer cells' viability under survival stress, MTT assay was performed in both the DLX4 expressing Hs578T cells and DLX4 siRNA knockdown MDA-MB-231 and MDA-MB-468 cells versus respective controls.

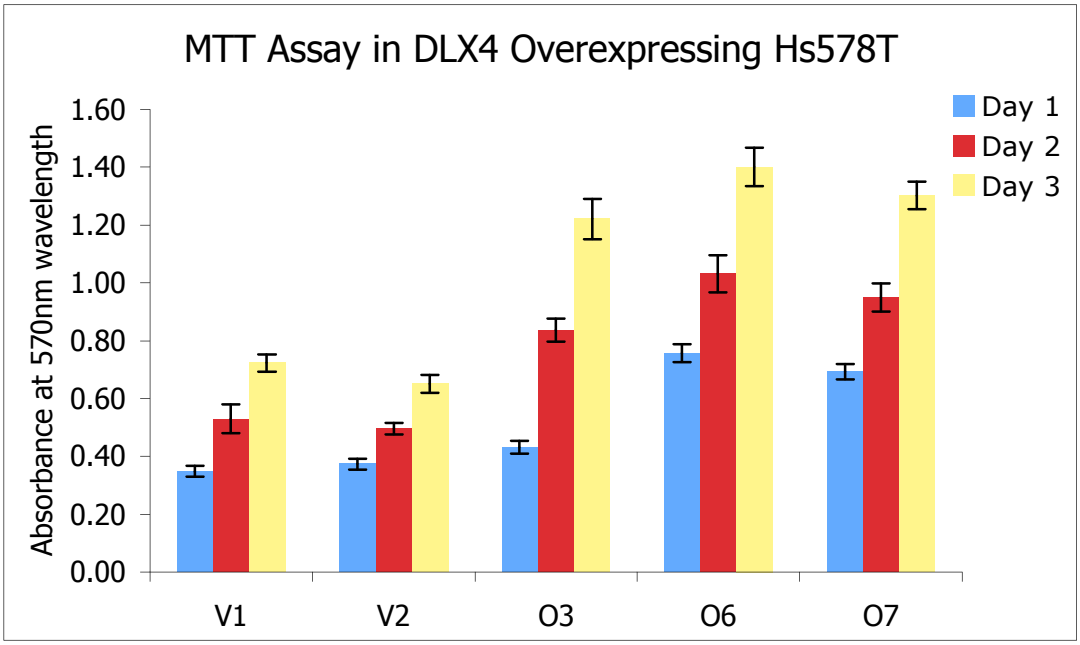


Figure 8. The viability of Hs578T breast cancer cells under survival stress correlated with DLX4 expression level confirming by cells' capacity to metabolize MTT. The data are the mean ± STDEV (n=3) Absorbance at 570nm of MTT metabolized blue/purple formazan. Over 3 days, DLX4 along with pcDNA3.2 transfected Hs578T cells were cultured in apoptosis-inducing, 1% FBS containing medium. On average, DLX4 transfected Hs578T cell clones exhibited 1.84 folds of viability relative to controls.

In DLX4 and control pcDNA transfected Hs578T cells, 1% instead of 10% FBS containing DMEM medium was utilized to address survival stress and induce apoptosis. On average 84% significant viability increase was observed in the DLX4 transfected cells over 3 days culturing in 1% FBS containing medium. The stress-enduring ability positively correlated with DLX4 expression in Hs578T cells. At the same time, DLX4 overexpressing Hs578T cells cultured in complete medium barely demonstrate higher cellular viability compared with controls (data not shown). It suggested that the viability advantage in Hs578T cells overexpressing DLX4 relies more on anti-apoptosis or other form of cell death, rather than proliferation.

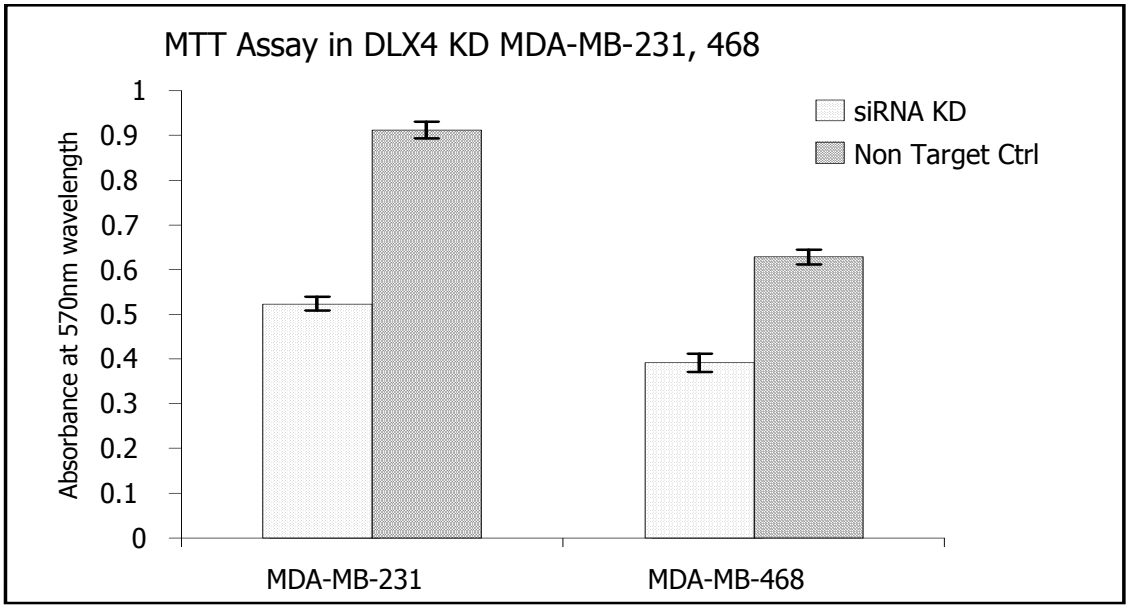


Figure 9. The viability of ER negative breast cancer cells correlated with DLX4 expression level confirming by cells' capacity to metabolize MTT. The data are the mean ± STDEV (n=3) Absorbance at 570nm of MTT metabolized blue/purple formazan. On average, DLX4 siRNA transfected MDA-MB-231 presented 57.4% viability versus non target siRNA control, while DLX4 knockdown MDA-MB-468 cells showed 62.3% viability compared with non target control, respectively.

In the case of DLX4 siRNA transfected MDA-MB-231 and MDA-MB-468, significant decreased cell viability was verified cells relative to controls, respectively (Figure 9.). MDA-MB-231 DLX4 siRNA transfected cells represents 57.4% viability in complete medium, and 62.3% cell viability was examined in DLX4 siRNA transfected MDA-MB-468 cells. In short, shown in Figure 8 &9, DLX4 assists the three ER negative cell lines to short-cut the survival stress in the environment.

However, due to the cellular toxicity of transfection reagent and the transient knockdown effect of the transfected siRNA, we did not establish the stable and consequential cell proliferation over 3 days. Also, when the transfection took place in 1% FBS containing medium, massive cell death was observed and the attachment of cells became too fragile to get gain accurate measurement for further MTT assay reading.

4. Discussion

DLX4, also referred as BP1, serving as a transcription factor, was overexpressed in breast cancer has been associated with tumor progression, invasion and metastasis (2, 35, 36). Our previous founding suggested it binding site at the downstream of VEGFA, and it upregulates the transcription of VEGFA. Even though pro-angiogenesis dominates the focus of study in VEGFA's function in tumorigenesis, we demonstrated its role in anti-apoptosis in ER negative epithelial adenocarcinoma cells.

In ER negative breast cancer cell line Hs578T, which expresses very few DLX4 endogenously, the expression of DLX4 resulted in higher VEGFA production, and the hyperactivation of VEGFR2 modulated downstream cell survival pathway was demonstrated. What's more, knockdown of DLX4 in ER negative breast cancer cell lines, MDA-MB-231 and MDA-MB-468, leads to suppressed PI3K/Akt pathway, and hence increased apoptosis. Hence, the epithelial cells in breast cancer gain survival advantage via anti-apoptosis activity in this pathway, while VEGFR2-modulated PI3 kinase/Akt plays significant role in tumor angiogenesis in vascular endothelial other blood vessel related cells (13, 37).

Besides that, in this study, the survival disadvantage in DLX4 knockdown ER negative breast cancer cell lines, MDA-MB-231 and MDA-MB-468, was confirmed via MTT assay (Figure 9). However, we were not able to establish the MTT assay over three days culture, in neither 1% nor 10% FBS containing medium. This might be due to both the transient effect of DLX4 siRNA knockdown and the toxicity of transfection reagent. Also, in 1% FBS medium

apoptosis inducing condition, higher viability in DLX Hs578T cells compared with controls was observed in MTT assay. At the same time, the survival advantage of Hs578T DLX4 transfected cells was not observed under 10% FBS containing complete medium. Therefore, proliferation may not render the cellular viability advantage in DLX4 overexpressing Hs578T cells.

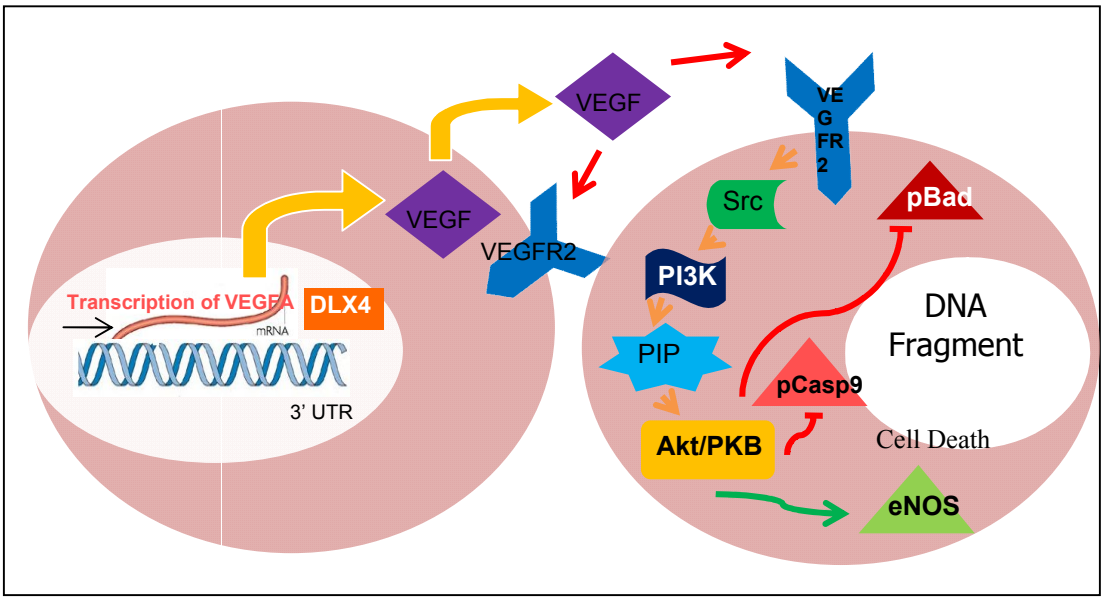


Figure 10. Diagram of DLX4 regulating PI3K/Akt pathway through VEGFA/VEGFR2 in Breast cancer. DLX4 binds to the 3'-UTR of VEGFA and up-regulates its transcription. VEGFA in turn activates the PI3K/Akt pathway via binding to VEGFR2 on both vascular endothelial and other cells within the tumor.

Third, besides DLX4, other transcription factors and post-transcriptional modification may stimulate the expression of VEGFA in ER negative epithelial adenocarcinoma cells. Without DLX4 expressing in empty pcDNA3.2 transfected Hs578T cells, we can still detect VEGFA Western blot (Figure 4).

The secretion of VEGFA by both endothelial and epithelial cells can trigger VEGFR2-mediated signaling cascade in autocrine and/or paracrine fashion. In our study, the focus is on the effect of VEGFR2-mediated PI3K/Akt survival pathway in ER negative epithelial adenocarcinoma cells. Other tyrosine kinase growth factors' receptors, such as HER2 and Estrogen Receptor, also effect PI3K/Akt pathway, which contributes to progression, required assistance to endocrine therapy in breast cancer and metastasis (14, 15, 24).

PI3K/Akt plays multiple roles in diverse cellular activity and gets activated upon plethora of stimulus besides VEGFA, and thus a lack of effective knockdown of DLX4 and VEGFA may be submerged. Together with complicated regulatory network of VEGFA, these might partially account for the 21% increase of phosphorylated Akt detected in DLX4 siRNA transfected MDA-MB-231 (Figure 7).

In further study, the different apoptosis rate among cells expressing different level of DLX4 may be better investigated under stress, for instance, pro-apoptosis cytokines or chemotherapy. Also, to annotate the significant function of DLX4 concerning anti-apoptosis in ER negative breast cancer cell lines, the interrogation ought to enroll the stable DLX4 knockdown ER negative cell lines. Apoptosis assays that illustrate degree of apoptosis via DNA fragmentation or mitochondria cytochrome c release and etc, such as TUNEL, would provide direct evidence for DLX4 modulated VEGFA's anti-apoptosis effect in ER negative epithelial breast cancer cells. The investigation of specific kinase inhibitors, such as LY294002, which particularly inhibits the activity of Akt, could further unravel VEGFA's effect on cellular survival in ER negative breast cancer cells.

On the other hand, other pathways triggered by VEGFR2 may contribute to DLX4's role in ER negative breast cancer, such as cell proliferation, cytokine resistance, cell survival and metastasis. Furthermore, like other transcription factors, DLX4 regulates the transcription of a series of genes that involve in breast cancer progression and invasion. For instance, IL2R, which we identified in previous DLX4 promoter study, may effect the immune status in breast cancer micro-environment.

Targeting VEGF, VEGFR2 and downstream player in PI3K/Akt pathway, may not just diminish angiogenesis in endothelial cells within solid breast tumor, but also may obstruct VEGF induced anti-apoptosis in epithelial cells. Targeting the DLX4 or the components in PI3K/Akt pathway would provide multi-point inhibition of anti-apoptosis survival advantage, required drug resistance as well as angiogenesis modulated by VEGFA/VEGFR2.

Last, The positive correlation between DLX4 and VEGFA expression in ER negative breast caner cells we established in this study shield light on the potential regulatory role of DLX4 in VEGFA expression and its PI3K/Akt pathway activity. .To further verify the binding of DLX4 on VEGFA 3' UTR, we can utilize gel-shifting and super gel-shifting of the proposed binding site.

5. Conclusions

Through the investigation into the PI3K/Akt pathway activity in DLX4 overexpressing and knockdown ER negative cell lines, we can conclude that the DLX4 upregulates the VEGFA gene transcription in ER negative Breast Cancer, and ensure the hyperactivity of VEGFR2 promoted PI3K/Akt pathway which contributes to survival advantage. Also, it proffered some evidence to support the autocrine/paracrine VEGFA/VEGFR2 feed back loop theory, which proposed the positive correlation between the expression of VEGFR2 and the amount of VEGFA.

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