Estrogen Induces Vav1 Expression in Human Breast Cancer Cells

Ming-juan Du¹, Xiang-dong Chen¹, Xiao-li Zhou¹, Ya-juan Wan¹, Bei Lan², Cui-zhu Zhang², Youjia Cao¹,²

¹ Key Laboratory of Microbial Functional Genomics of Ministry of Education, College of Life Sciences, Nankai University, Tianjin, P. R. China, ²State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin, P. R. China

Abstract

Vav1, a guanine nucleotide exchange factor (GEF) for Rho family GTPases, is a hematopoietic protein involved in a variety of cellular events. In recent years, aberrant expression of Vav1 has been reported in non-hematopoietic cancers including human breast cancer. It remains to be answered how Vav1 is expressed and what Vav1 does in its non-resident tissues. In this study, we aimed to explore the mechanism for Vav1 expression in breast cancer cells in correlation with estrogen-ER pathway. We not only verified the ectopic expression of Vav1 in human breast cancer cell lines, but also observed that Vav1 expression was induced by 17β-estradiol (E2), a typical estrogen receptor (ER) ligand, in ER-positive cell lines. On the other hand, Tamoxifen, a selective estrogen receptor modulator (SERM), and ICI 182,780, an ER antagonist, suppressed the expression of Vav1. The estrogen receptor modulating Vav1 expression was identified to be α form, not β. Furthermore, treatment of E2 increased the transcription of vav1 gene by enhancing the promoter activity, though there was no recognizable estrogen response element (ERE). Nevertheless, two regions at the vav1 gene promoter were defined to be responsible for E2-induced activation of vav1 promoter. Chromatin immunoprecipitation (ChIP) and co-immunoprecipitation (Co-IP) analyses suggested that ERα might access to the vav1 promoter via interacting with transcription factors, c-Myc and ELF-1. Consequently, the enhanced expression of Vav1 led to the elevation of Cyclin D1 and the progression of cell cycle. The present study implies that estrogen-ER modulates the transcription and expression of Vav1, which may contribute to the proliferation of cancerous cells.

Introduction

Breast cancer is the most common death-causing cancer in females [1]. The persistent exposure to estrogen has been observed to closely correlate with the development of breast cancer [2–5]. The expression and responsiveness of estrogen receptor (ER) has been applied as one of the most important markers for the breast cancer classification and prognosis [6]. Two forms of estrogen receptors, ERα and ERβ, have been identified [7]. ERα is the dominant form in the breast and uterus, whereas ERβ has a wider distribution profile that expands in tissues such as prostate, ovary, lung, and spleen [8]. As a ligand, estrogen binds to ER, and induces its conformational change to activate it. The activated ER associates with the estrogen response element (ERE) at the promoter regions of a variety of genes [9], or complexes with other transcription factors, such as AP1 [10], SP1 [11], or E2F1 [12], modulating the expression of target genes that are involved in cell cycle checkpoint [12,13], cell proliferation [14,15], and apoptosis [16].

Vav1 is first identified as a proto-oncogene in hematopoietic cells [17], with the renowned character as a guanine nucleotide exchange factor (GEF) for RhoGTPases. A plethora of studies revealed that Vav1 is a multidomain protein which not only activates RhoGTPases for cytoskeleton reorganization during lymphocytes activation [18], but also plays a GEF-independent role in diverse cellular processes including calcium mobilization in T cells [19]. The oncogenic form, lacking the N-terminal Calponin homology (CH) domain, is obtained by its transforming effect on NIH3T3 fibroblast cells [20]. Meanwhile, evidence unveils the non-hematopoietic expression profile of Vav1, which associates with several human tumor malignancies, such as neuroblastoma [21], lung cancer [22], and pancreatic ductal adenocarcinomas [23]. Knocking down of vav1 gene in lung cancer and pancreatic cells leads to the decreased cell proliferation and reduces tumor size in nude mice [22,23]. In addition, patients with Vav1-positive pancreatic tumors exhibit poorer prognosis and lower survival rate than those with Vav1-negative tumors [23], suggesting that the ectopic expression of Vav1 plays an inextricable role in tumor development and progression.

Recently, the aberrant expression of Vav1 has been reported and its correlation with estrogen receptor has been addressed in human breast cancer [24–27]. Herein we aim to investigate the modulation of Vav1 expression in breast cancer cells, and the effect of Vav1 on breast cancer cell proliferation. By confirming the increased vav1 mRNA and protein in several ER-positive cell lines, we found that the transcription and expression of Vav1 was significantly enhanced by E2 treatment in a time- and dose-dependent manner via ERα. We further addressed that E2-
induced vav1 transcription involved the complex of ERα with other transcription factors. Finally, we showed that the amount of Vav1 expression correlated with the expression of Cyclin D1 and influenced the cell cycle progression in breast cancer cells. Our data suggested that estrogen may promote breast cancer cell growth partially by triggering the aberrant expression of Vav1.

Methods and Materials

Antibodies and reagents

The anti-ERα (sc-73479), anti-ERβ (sc-373853), anti-Vav1 (sc-132), anti-c-Myc (sc-517 X), and anti-ELF-1 (sc-631X) antibodies and normal IgG (rabbit, sc-2027; mouse, sc-2025) were purchased from Santa Cruz Biotechnology (CA, USA). The anti-Cyclin D1 antibody (MA0770) was purchased from Boster (Wuhan, China). ICI 182,780 (Fulvestrant), Tamoxifen, 17β-estradiol (E2), Dimethyl sulfoxide (DMSO), 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), 2,3-Bis(4-hydroxyphenyl) propionitrile (DPN), and anti-α-tubulin antibody were purchased from Sigma (MO, USA).

Cell lines and culture

Human breast cancer cell lines (ER positive: MCF7 and T47D; ER negative: MDA-MB-231 and MDA-MB-157) were originally from American Type Culture Collection (ATCC) and maintained in phenol free RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, NY, USA). MCF7 and T47D cells, 0.1 mg/ml human recombinant insulin was added to the medium. The immortalized breast epithelial line 184A1 was maintained in MEGM (Cambrex, NJ, USA) that consisted of 7.5% fetal bovine serum (FBS), 5 μg/mL hydrocortisone, 5 μg/mL insulin, 50 μg/mL gentamycin sulfate, and 50 μg/mL amphotericin B. Jurkat T leukemia cells and vav-null Jurkat T cells (J.Vav1) were cultured in lab as described previously [28] and were grown in RPMI 1640 medium containing 10% FBS. HEK293T cell line was obtained from ATCC, and grown in DMEM supplemented with 10% FBS.

RNA isolation and reverse transcription

MCF7 and T47D cells were cultured in phenol free RPMI 1640 medium for 24 h to deprive estrogen, and then treated with 10^{-7} mol/L of E2 for 24 h. The mRNA was extracted from the cells using PolyATtract System 1000 (Promega, WI, USA) and reverse-transcribed with Reverse Transcription System (Promega, WI, USA).

Luciferase reporter assay

The vav1 promoter constructs were obtained as described [30]. MCF7 cells were transfected with 2 μg of total plasmid DNA containing Renilla luciferase vector pRL-TK (Promega, WI, USA) and vav1 reporter construct, pVav1-Luc, per 1×10⁶ cells by Lipofectamine 2000 (Invitrogen, CA, USA). MCF7 cells were transfected with pVav1-Luc and treated with Tamoxifen (10⁻² mol/L) for 48 h, then lysed for luciferase activity analyses with Dual Luciferase Assay kit (Promega, WI, USA) and TD20/20 luminometer (Turner Designs Inc, CA, USA). The promoter activity was presented as the ratio of the firefly luciferase activity to Renilla luciferase activity. To determine the effect of ERα on the promoter activity of vav1, MCF7 cells transfected with pVav1-Luc were pre-treated with Tamoxifen or ICI 182,780 for 30 min in prior to E2 treatment or treated with ER type-specific agonists, PPT or DPN. The cells were then harvested for luciferase assay as described above.

Chromatin immunoprecipitation (ChiP) assay

ChiP assay was carried out as previously described [31]. Briefly, T47D cells were pre-cultured in serum-free medium and then treated with DMSO or E2 (10⁻⁷ mol/L) for 4 h or pre-treated with Tamoxifen (10⁻⁶ mol/L) for 30 min followed by E2 (10⁻⁷ mol/L) for 4 h. Then, cells were crosslinked, lysed, and sonicated by Sonicator JY92-II (SCIENTZ, Ningbo, China). The lysate was pre-absorbed with protein A/G-agarose (Santa Cruz Biotechnology, CA, USA), then incubated with indicated antibodies. The immunocomplexes were precipitated by protein A/G-agarose and the crosslinked DNA samples were amplified by PCR. The primers sequences corresponding to position +59 to +340 of the vav1 relative to TSS were: sense, 5'-CTGCGAGGGTGCAGCAGG-3'; and antisense, 5'-GTCTCAACCTGTAGCAA-3'. The primers for position -232 to +4 were: sense, 5'-GAGGAGCTTCCATCTCA-3'; and antisense, 5'-TGACACCTTGAGGAATCTGCA-3'.

Knockdown of Vav1 expression by lentivirus-based transduction

The lentiviral plasmids were constructed as described [32]. The shRNA sequence targeting Vav1 or control RNA of scrambled sequence were cloned into pLKO.1-TRC vector (Addgene, http://www.addgene.org/), respectively. The lentivirus particles were produced in 293T cells using the Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions.
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A

Relative Level of vav1 mRNA

E2 - +

** MCF7  T47D

B

MCF7

E2 0 24 48 72 (h)

Vav1

Tubulin

T47D

E2 0 24 48 72 (h)

Vav1

Tubulin

C

MCF7

E2 conc. 0 10^6 10^7 10^8 (M)

Vav1

Tubulin

T47D

E2 conc. 0 10^6 10^7 10^8 (M)

Vav1

Tubulin

D

MCF7

E2 - + + +

ICI - - + +

Vav1

Tubulin

Lane 1 2 3 4 5 6

E

E2 - + + + +

Tam 0 0 10^-7 10^-6 10^-5 (M)

Vav1

Tubulin

Lane 6 7 8 9 10
Figure 2. ER-mediated Vav1 expression. (A) MCF7 and T47D cells were treated with E2 (10^{-7} mol/L) or DMSO for 24 h. The relative level of vav1 mRNA was determined by qRT-PCR and was presented by the ratio of vav1 mRNA of E2-treated samples to that of DMSO-treated control samples and presented as y-axis. The data represented the mean value ± S.D. of three independent experiments. (B and C) MCF7 and T47D cells were exposed to E2 (10^{-7} mol/L) for 0 to 72 h (B), or increasing concentration of E2 for 48 h (C). (D and E) MCF7 and T47D cells were pre-treated with ICI 182,780 (4 × 10^{-7} mol/L) (D) or increasing concentration of Tamoxifen for 30 min (E) before adding E2 (10^{-7} mol/L) for 48 h. The DMSO treatment was used as a solvent control. The Vav1 expression in above treated samples was analyzed by Western Blot with anti-Vav1 antibody, with tubulin as protein loading control. The bar chart below each example blot represents the normalized protein level of Vav1 to Tubulin of three independent experiments. The statistical significance was set at P < 0.01 versus E2 treatment by unpaired student T test.

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Results

The aberrant expression of Vav1 in human breast cancer tissue and cell lines

It was reported previously that Vav1 was detected in ER-positive breast cancer tissue by immunohistochemistry [27]. Here we examined the expression of Vav1 in human breast cancer cell lines (Fig. 1), using Vav1 abundant Jurkat cells as positive control and its derived vav1-null cells (J.Vav1) as negative control (Fig. 1, left two lanes). As shown in Figure 1, Vav1 expression appeared high in the two ER positive cell lines, MCF7 and T47D cells, whereas it was barely detectable in MDA-MB-231 and MDA-MB-157, and not detected in the immortalized breast epithelial line 184A1 cells. The estrogen receptors expression of the cell lines was also detected.

Estrogen enhances Vav1 expression through ERs in MCF7 and T47D cell lines

A correlation has been observed between the progression of breast cancer and the exposure to estrogen, which modulates the transcription of many genes by binding and activating ERs [2–5]. From the results of tissue immunohistochemistry [27] and cell lines Western blot (Fig. 1), higher Vav1 expression was visualized in ER positive breast tumors or cells than that in ER negative samples or cells. We speculated that estrogen-ER was involved in the control of vav1 gene expression. Two ER-positive cell lines, MCF7 and T47D, were tested for Vav1 expression in the presence or absence of 17β-estradiol (E2). After treatment with 10^{-7} mol/L of E2 or DMSO as control, the mRNA transcript of vav1 was measured by qRT-PCR. As shown in Figure 2A, E2 induced an increase in vav1 mRNA expression by 3.15-fold in MCF7 and 2.39-fold in T47D, in reference to the DMSO control (P < 0.01), suggesting that E2 enhanced the transcription of vav1 gene.

We further explored the effects of time and concentration of E2 on Vav1 expression. MCF7 and T47D cells were exposed to E2 (10^{-7} mol/L) for different time points, or to the indicated concentration of E2, respectively. As shown in Figure 2B, the E2-induced Vav1 expression increased to about 2.2 fold at 48 h (P < 0.01) and plateaued at 72 h in both cell lines. The expression of Vav1 reached to nearly maximum at 10^{-7} mol/L of E2, as only limited increases of Vav1 from 10^{-7} mol/L to 10^{-6} mol/L were observed, namely from 2.29 to 2.59 fold for MCF7 and 2.15 to 2.21 fold for T47D, respectively (Fig. 2C). The above data indicated that the induction of Vav1 expression is dependent on the time and dose of the ER ligand treatment.

Given that ICI 182,780 and Tamoxifen have been applied in endocrine therapy for ER-positive breast cancer due to their inhibitory effects on ER activation [35–37], we used these drugs to address the role of ER in the estrogen regulation of Vav1. As shown in Figure 2D, E2 alone induced Vav1 protein expression by 2.39-fold and 2.08-fold in MCF7 and T47D cells (Fig. 2D, Lane 2 and 5, P < 0.01), respectively. However, this expression was generated according to the standard protocol [33]. Briefly, HEK293T were co-transfected with the vectors containing shRNAs together with vectors pCMV-HSV-G, pMDLgp/pRRE, and pRSV-REV. At 48 h post-transfection, the supernatants were harvested, and the viral particles were collected to infect T47D cells at 37°C for 18 h. The transduced cells were selected by 0.5 μg/mL puromycin for 7 days.

Cell cycle analysis

Cells were synchronized to G0/G1 phase by cultured in serum-free medium in the presence of Tamoxifen [34]. After 36 h of treatment with E2 (10^{-7} mol/L) or DMSO as control, cells were collected and fixed with ice-cold 70% ethanol, then incubated with 100 μg/mL RNase A (Transgene Biotech, Beijing, China) for 30 min. Cells were then stained with 30 μg/mL propidium iodide (PI) (Sigma, MO, USA) in the dark for 30 min, and subjected to flow cytometer analysis (Calibur, NJ, USA). The DNA contents and cell numbers were plotted using Cell Quest software (Becton Dickinson, NJ, USA).

Western Blot analysis

Cells (1 × 10^6 per sample) were harvested and lysed in RIPA buffer (50 mmol/L Tris- HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L PMSF, 1 mmol/L NaF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mmol/L NaVO_4). Protein concentration was measured by Bradford assay. Equal protein amounts of cell lysates were separated by 7.5% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and blotted with indicated antibodies. The density of each band was quantitated by Quantity One software (Bio-Rad, version 4.4.0, CA, USA).

Co-immunoprecipitation (Co-IP)

T47D cells were cultured in RPMI 1640 medium for 24 h before adding E2 (10^{-7} mol/L) or DMSO for 4 h. Then cells were harvested and lysed in RIPA buffer. 1.5 mg of lysate was precleared with protein A/G-agarose beads and subsequently added in 4 μg of indicated antibodies overnight at 4°C. The immunocomplexes were precipitated by protein A/G-agarose, washed 3 times with RIPA buffer, subjected to SDS-PAGE, and analyzed by Western Blot.

Statistics analysis

Graphical data values are presented as mean values of triplicate experiments ± standard deviations. Each experiment was carried out independently for at least 3 times, and unpaired student T tests were performed. The statistical significance was set at P < 0.05 (marked with *) and P < 0.01 (marked with **).
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The involvement of ERα in estrogen-induced vav1 transcription led us to examine the vav1 proximal promoter for conserved ERE sequence in silico by rVista2.0 (http://rvista.dcode.org/) and TRANSFAC (http://www.cbr.nsc.ru/hbim/nph-tfsearch). However, the search result revealed no perfect ERE at the vav1 proximal promoter region, rather, there were two half-ERE sites (hERE) located at the positions +165 to +169 bp and +273 to +277 bp to TSS, respectively (Fig. 4A). As previously reported, ERE-like sequence, such as two half ERE sites, can bind with estrogen activated ER even though they were separated by hundreds of base pairs [9,38]. Thus we set to verify if ERα bound to the hERE site at vav1 promoter by ChIP analysis. The primers corresponding to the hERE region spanning the two hERE sites (+340) were designed accordingly. As shown in Figure 4B upper panel, the sample prior to immunoprecipitation (Input) exhibited a positive hERE region, whereas was detected negative in the post-immunoprecipitated sample (ERα), indicating that ERα did not interact with the hERE region.
Unexpectedly, the region -232 to +71 was found in association with ER\(\alpha\) (Fig. 4B, lower panel, third lane from the left), though there was no consensus binding site for ER. Furthermore the recruitment of ER\(\alpha\) was increased by \(\sim 1.7\) fold upon E2 treatment (Fig. 4B, lower panel, sixth lane from the left, \(P<0.01\)), and reduced by Tamoxifen treatment (Fig. 4C, \(P<0.01\)).
Figure 5. Analysis of transcription factors involved in ERα-activated vav1 promoter. (A) Depicted deletion mutations in vav1 promoter reporter gene. The deleted regions were indicated by break lines. (B) MCF7 cells were transfected with plasmids containing luciferase under WT or mutated vav1 promoters (D1, D2, and D3) and then treated with E2 (10^{-7} mol/L) or DMSO for 48 h. The relative fold induction of each group was calculated as the ratio of the luciferase activity induced by E2 to that induced by DMSO, respectively, and plotted as y-axis, and the deletion mutants were presented as in x-axis. All the data represented the mean±S.D. of three independent experiments. “N.S.” and “**” indicates P>0.05 and P<0.01, respectively, versus D1 group by unpaired student T test. (C) ChIP assay. T47D cells were treated with E2 (10^{-7} mol/L) for 4 h or pre-treated with Tamoxifen (10^{-6} mol/L) for 30 min before treating with E2 (10^{-7} mol/L) for 4 h. The immunoprecipitation was performed using antibodies against c-

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with c-Myb and ELF-1. T47D cells were treated with E2 (10^{-7} mol/L) or DMSO for 4 h or pretreated with Tamoxifen (10^{-6} mol/L) for 30 min in prior to E2 treatment and lysed. Antibodies against c-Myb (upper panels) and ELF-1 (lower panels) or control IgG (Lane 1) were used to immunoprecipitate

The above results demonstrated that ERα was involved in the transcriptional activation of vav1 gene by association with the promoter region other than the hEREs sites, implying an indirect binding of ERα to the promoter region, perhaps through other transcription factors.

ERα associates with −38 to −5 region at vav1 promoter via other transcription factors

The above results indicated that ERα was in complex with the 5’ region of vav1 gene promoter. Several transcription factors were predicted to bind at the 5’ minimal regulatory region of the human vav1 gene, including E2F, Sp1, E2F, NF-c, c-Myb, TCF/β, PU.1, and ELF-1 [30]. We therefore attempted to locate the regions that respond to estrogen. The wild type vav1 promoter (WT) and the truncated mutants (D1, D2, D3) that lack the predicted transcription factor binding sites were depicted in Figure 5A, and the reporter plasmids were constructed [30]. As shown in Figure 5B, the wild type promoter activity was elevated to 3 fold by E2. The deletion mutant D1 that lacks region −143 to −152 exhibited similar extent of induction (2.6 fold), implying that the region −143 to −152 was dispensable in E2-induced vav1 expression. In contrast, the E2 induction of truncated promoters D2 and D3 was severely suppressed to less than 1.5 fold (P<0.01), indicating that these two regions, −(−25 to −38) and −(−5 to −22), were required for E2-induced vav1 transcription. As these regions were reported to possess putative binding sites for transcription factors E2F/NF-c/c-Myb at −(−25 to −38) and TCF/β/PU.1/ELF-1 at −(−5 to −22), respectively, ERα may associate with certain transcription factors within these regions. As reported previously, c-Myb affects vav1 transcription in lung cancer cells [30] and is also involved in the E2-ER regulated gene expression in breast cancer cells [39]. Meanwhile, another breast cancer related transcription factor, ELF-1, is identified to interact with the promoter of vav1 (Genome browser, http://genome.ucsc.edu/) [40]. Firstly, to confirm the binding of these two transcription factors to vav1 promoter, the ChIP assay was performed. As shown in Figure 5C, both c-Myb and ELF-1 presented positively in complex with the vav1 promoter (Fig. 5C, upper two panels), and the presence of E2 or Tamoxifen had no effects on the complex (Fig. 5C, bottom panel).

Next we investigated whether ERα associated with c-Myb and/or ELF-1 to form the transcriptional complex, and if the complex formation was E2-dependent. By co-immunoprecipitation analyses shown in Figure 5D, detectable amount of ERα was pulled down with anti-c-Myb (upper panels) or anti-ELF-1 (lower panels) antibodies, respectively, in comparison with control IgG (Fig. 5D, lane 2 versus lane 1). And the amount of co-immunoprecipitated ERα increased with the presence of E2 (Lane 3 in both upper and lower panels), indicating the E2-activated ERα associated with the transcription factors c-Myb and ELF-1. In the presence of Tamoxifen, the co-immunoprecipitated ERα was barely detectable (Fig. 5D, lane 4), further suggesting that it was the activated form of ERα that bound to these transcription factors.

Given that the existing interaction of c-Myb/ELF-1 with vav1 promoter was constitutive (Fig. 5C), and ERα association with -292 to +71 region was E2 inducible (Fig. 4C), we proposed that the E2 dependent activation of vav1 promoter was achieved by the association of resident transcription factors such as c-Myb and ELF-1, with E2-activated ERα. As the model shown in Figure 5E, E2 induced activation of ERα, which, instead of binding directly to vav1 promoter, interacted with the existing transcription factors to control the vav1 transcription. Of course, our results did not exclude the involvement of other regulators in the complex. Nevertheless, the recruitment of ERα and the formation of the transcriptional complex enhanced the transcription of vav1 gene.

The expression of Vav1 promotes cell cycle progression in breast cancer cells

E2 is identified as a causative factor of breast cancers and well-characterized to induce cell growth in ER-positive breast tumors [34]. As Vav1 is also involved in cell proliferation in lung cancer and pancreatic cancer cells [22,22], we speculated that Vav1 participated in cell cycle progression of breast cancer cells under the control of E2. Stable cell lines, T47D-ShVav1 expressing short hairpin RNA for Vav1, and T47D-Ctl expressing a scrambled sequence, were established by lentivirus-based transduction. The expression of Vav1 in these cell lines was verified as shown in Figure 6A, and the level of Vav1 expressed in shVav1 cells was reduced (Fig. 6A, top panel, the left two lanes). The E2 treatment elevated the Vav1 expression proportionally (Fig. 6A, top panel, the right two lanes). The expression of Cyclin D1 was also determined as a commonly recognized factor for cell cycle progression (Fig. 6A, middle panel). In the absence of E2, shVav1 reduced Cyclin D1 by 50% (P<0.01) in comparison with the control (left lane). E2 induced a significant 2.31-fold increase of Cyclin D1 (Fig. 6A, third lane from the left, P<0.01) in coordination with Vav1, and that was reduced by the shRNA knockdown of Vav1 (P<0.01 versus E2 treatment of Control).

Furthermore, we examined the effects of Vav1 on the cell proliferation by flow cytometry analysis. The shRNA-transduced breast cancer cell lines were synchronized to G0/G1 phase and the G0/G1 arrest was released by E2 or DMSO treatment as described in Methods. The decreased percentage of cells in G0/G1 phase represented the cells progressing to cell cycle. As in Figure 6B, in the absence of E2, 5.20% of T47D-Ctl cells and 1.41% of T47D-ShVav1 cells were released from the G0/G1 checkpoint (Fig. 6B, left bars), respectively. With E2 treatment, 13.36% of the T47D-Ctl cells reentered cell cycle, whereas only 3.91% of T47D-ShVav1 cells were progressed (Fig. 6B, right bars). Thus, knockdown of Vav1 resulted in a 3.79% reduction in DMSO group and a 9.45% reduction in E2 group. The differences of the percentage of proliferating cells with shVav1 or not reckoned for the relative amount of Cyclin D1 of those cells (Fig. 6A), and revealed that knockdown of Vav1 decreased E2-induced cell proliferation. In addition, overexpression of Vav1 in T47D cells also exhibited an enhanced Cyclin D1 by 2.5 fold (Fig. S1A), and a higher cell growth rate was also shown by WST-1. 
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A plethora of genes are reported to be modulated by estrogen-ER pathway via estrogen response elements. We analyzed the vav1 promoter sequence in silico, and identified two half ERE sites. However, it was not supported by the ChIP analysis, as these sequences did not bind and recruit ERα (Fig. 4B). Rather, promoter sequences stretching from −232 to +71 bp to TSS associated with ERα indirectly. Further, two regions in the vav1 promoter were found indispensable for E2-induced reporter activity, which contained the binding sites for c-Myc and ELF-1, respectively. Accumulating evidence does support this scenario that ER indirectly activates gene transcription via binding to other DNA-bound transcription factors [10–12,43]. For example, ERα complexes with c-Myc to mediate the expression of Noxa in breast cancer cells [12]. The association of ER with Sp1 is also reported to modulate c-fos expression [11]. In our data, ERα, by interacting with c-Myc and ELF-1, conferred estrogen responsiveness to vav1 gene (Fig. 5E). Indeed, c-Myc and ELF-1 possess the leucine-rich motif: LXXLL, which can be recognized by ERα [43]. Of course, other coregulators involved in complex with ERα remain to be identified.

Two activation functions (AFs) mediate the transcriptional activation of ER, the N-terminal AF-1, and the AF-2 in ligand binding domain (LBD) [44]. The biological ligand, E2, binds to LBD and induces its conformational change to trigger the activity of AF-2 which can be recognized by coactivators [43]. Previous studies suggest that Tamoxifen, the non-steroidal type I ER antagonist, induces a different conformation from that induced by E2, and thus blocks the binding of coactivators and inhibits AF-2 activity [45,46]. By contrast, ICI 182,780, a steroidal type II ER antagonist, binds competitively to the E2 binding site, blocks ER activity [45,46]. By contrast, ICI 182,780, a steroidal type II ER antagonist, blocks competitively to the E2 binding site, blocks ER

Vav1 has been recognized as a hematopoietic-specific protein and plays important roles in T cell activation. The non-hematopoietic expression of Vav1 has been reported recently in association with several human malignancies, including pancreatic ductal adenocarcinomas [23], lung cancer [22], neuroblastoma [21], melanoma [41], and breast cancer [24–27]. Our present study revealed that Vav1 protein expression was observed in some breast cancer cell lines by Western Blot, especially in ER+ cell lines, though with discrepancy in MCF-7 cell line [27]. It was mutually believed that such discrepancy may result from the resources and passage numbers of the cell line (personal communication with Dr. Katzav). Nevertheless, Vav1 was aberrantly expressed in breast cancer tissue and cell lines.

Several studies are attempted to explore the mechanisms involved in non-hematopoietic expression of Vav1. Epigenetic indication of vav1 expression is proposed. For example, the demethylation of the vav1 gene promoter is detected in primary pancreatic adenocarcinomas [23]; methylation of CpG in 5′-regulatory sequences of the vav1 promoter is addressed in lung cancer cells [30]; and degradation of Vav1 through Cbl ubiquitination is proposed in breast cancer cells [27]. Given a positive correlation between Vav1 and ER expression in breast cancer tissue [27], we were motivated to explore the Vav1 expression along the E2-ER axis. Our data unveiled the transcriptional control of the vav1 gene under the estrogen-ER pathway, and the required isoform, ERα, was the dominant form in breast tissue [42].

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Figure 6. Effect of Vav1 in cell cycle progression. (A) T47D cells were infected with lentivirus particles that expressed Vav1-specific shRNA or shRNA with a scramble sequence (served as a control). The homogenous cells were first synchronized to G0/G1 phase and then treated with DMSO as control or E2 (10−9 mol/L) for 36 h before harvest. The expression of Vav1 and Cyclin D1 were analyzed by Western Blot with indicated antibodies respectively. The density of the bands was quantitated by Quantity One software (Bio-Rad, version 4.4.0, CA, USA). The bar chart represents the normalized protein level of Cyclin D1 to tubulin of three independent experiments. ** indicates P < 0.01 versus corresponding control group analysis (Fig. S1B). The implications from the above experiments are 2-fold, 1) Vav1 expression level significantly influences the cell cycle progression and cell proliferation; and 2) The amount of Vav1 protein contributes the E2-upregulated cell proliferation.

Discussion

Vav1 has been recognized as a hematopoietic-specific protein and plays important roles in T cell activation. The non-hematopoietic expression of Vav1 has been reported recently in association with several human malignancies, including pancreatic ductal adenocarcinomas [23], lung cancer [22], neuroblastoma [21], melanoma [41], and breast cancer [24–27]. Our present study revealed that Vav1 protein expression was observed in some breast cancer cell lines by Western Blot, especially in ER+ cell lines, though with discrepancy in MCF-7 cell line [27]. It was mutually believed that such discrepancy may result from the resources and passage numbers of the cell line (personal communication with Dr. Katzav). Nevertheless, Vav1 was aberrantly expressed in breast cancer tissue and cell lines.

Several studies are attempted to explore the mechanisms involved in non-hematopoietic expression of Vav1. Epigenetic indication of vav1 expression is proposed. For example, the demethylation of the vav1 gene promoter is detected in primary pancreatic adenocarcinomas [23]; methylation of CpG in 5′-regulatory sequences of the vav1 promoter is addressed in lung cancer cells [30]; and degradation of Vav1 through Cbl ubiquitination is proposed in breast cancer cells [27]. Given a positive correlation between Vav1 and ER expression in breast cancer tissue [27], we were motivated to explore the Vav1 expression along the E2-ER axis. Our data unveiled the transcriptional control of the vav1 gene under the estrogen-ER pathway, and the required isoform, ERα, was the dominant form in breast tissue [42].

A plethora of genes are reported to be modulated by estrogen-ER pathway via estrogen response elements. We analyzed the vav1 promoter sequence in silico, and identified two half ERE sites. However, it was not supported by the ChIP analysis, as these sequences did not bind and recruit ERα (Fig. 4B). Rather, promoter sequences stretching from −232 to +71 bp to TSS associated with ERα indirectly. Further, two regions in the vav1 promoter were found indispensable for E2-induced reporter activity, which contained the binding sites for c-Myc and ELF-1, respectively. Accumulating evidence does support this scenario that ER indirectly activates gene transcription via binding to other DNA-bound transcription factors [10–12,43]. For example, ERα complexes with c-Myc to mediate the expression of Noxa in breast cancer cells [12]. The association of ER with Sp1 is also reported to modulate c-fos expression [11]. In our data, ERα, by interacting with c-Myc and ELF-1, conferred estrogen responsiveness to vav1 gene (Fig. 5E). Indeed, c-Myc and ELF-1 possess the leucine-rich motif: LXXLL, which can be recognized by ERα [43]. Of course, other coregulators involved in complex with ERα remain to be identified.

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activation, and leads to a rapid degradation of ER [35]. As both Tamoxifen and ICI 182,780 eliminated E2-induced vav1 expression (Fig. 3), and the Co-IP analysis revealed that the association of ERα with the two cofactors was disrupted by Tamoxifen (Fig. S3D), we speculated that the correct conformation of ERα is required for its complex with the DNA-bound c-Myb and ELF-1 to access vav1 promoter, thus control vav1 transcription.

Elevated expression of Vav1 has been demonstrated to affect cell proliferation in lung cancer and pancreatic cancer cells [22,23]. As a GDP/GTP exchange factor, Vav1 is also involved in CXCL12-promoted invasion of melanoma cells [41]. In this study, we observed that the aberrant expression of Vav1 correlated well with the production of Cyclin D1, a critical mediator of estrogen-stimulated cell cycle progression [47,48], thus contributing to the proliferation of breast cancer cells (Fig. 6, Fig. S1). We could not rule out other mechanisms that Vav1 played in corresponding to the growth and development cancer, as Vav1 might also protect cells from apoptosis by enhancing anti-apoptotic Bcl2 transcription that we reported in leukemia cells [49].

In summary, our data revealed that E2 promoted the expression of vav1 in a dose- and time-dependent manner in ER-positive breast cancer cells. E2-ER indirectly enhanced vav1 transcription, perhaps via the interactions with other transcription factors such as c-Myb and ELF-1. The E2-induced Vav1 level in breast cancer cells was in favor of promoting Cyclin D1 expression and accelerating the cell proliferation, and Vav1 might partially contribute to the pathogenesis and prognosis of breast cancer. This study emphasized the involvement of Vav1 ectopic expression in ER positive breast cancer cells, which reinforced the hypothesis that Vav1 could exert its oncogenic role in human breast cancer.

Supporting Information

Figure S1 Effect of Vav1 overexpression on cell proliferation. T47D cells were transduced with lentivirus particles encoding Vav1 (pCDH-Vav1) or the control vector backbone (pCDH). The expression of Vav1 and Cyclin D1 were analyzed by Western Blot and cell proliferation was determined by WST-1 proliferation assay in these cells. (TIF)

Materials and Methods S1 Overexpression of Vav1 by lentivirus-based transduction and WST-1 cell proliferation assay.

(DOC)

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Author Contributions

Conceived and designed the experiments: MD YC. Performed the experiments: MD XC XZ. Analyzed the data: MD YC. Contributed reagents/materials/analysis tools: YW BL CZ. Wrote the paper: MD YC.

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