# p21 WAF1/CIP1 is a Downstream target of ELK-1 Growth / Tumor Suppressor Pathway in Breast and Androgen-independent Prostate Cancers

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## Abstract

African American men have the highest risk of developing prostate cancer and have more than twice the mortality rate compared to Caucasian men in the US. ELK-1 belongs to the ETS family of ternary complex factors that plays a major role in cell proliferation, tumorigenesis, as well as differentiation. We have previously shown ELK-1 to not only suppress growth but also to augment the growth suppressive function of BRCA1 proteins in human breast cancer cells. In the current study we have demonstrated for the first time Elk-1 to inhibit the growth of androgen-independent prostate cancer cells. ELK-1 transfected prostate and breast cancer cells were slow growing and inhibited in their capacity to form colonies in soft agar. The p21WAF1/CIP1 protein was found to be expressed at elevated levels both in ELK-1 transfected breast and prostate cancer cells. The p21WAF1/CIP1 promoter activity was also enhanced in the presence of increasing concentrations of ELK-1 protein. Furthermore, ELK-1 protein by binding to the ETS binding motif in the p21WAF1/CIP1 promoter induced the expression of the protein in a p53-independent manner. This work suggests for the first time ELK-1 to function as a growth /tumor suppressor of androgen independent human prostate and breast cancer cells by activating p21WAF1/CIP1 independent of the presence of p53. These findings can provide a novel mechanism that can bypass the p53 defect in tumors with endogenous p53 mutations thus developing effective therapy for patients with advanced-stage androgen independent prostate and breast cancers.

**Keywords:** Elk-1, Breast cancer, Androgen Receptor, Androgen-independent Prostate Cancer, Growth / Tumor Suppressor, p21 WAF1/CIP1

**Abbreviations:** ELK-1: ETS like gene; AIPC: Androgen-independent prostate cancer; AR: Androgen Receptor; AA: African American; TCF: ternary complex factors; CRPC: Castration resistant prostate cancers; SRE: serum response element; SRF: Serum response factor.

## Introduction

According to the American Cancer Society (ACS), it has been estimated that 191,930 men will develop and 33,330 will die of prostate cancer in 2020 [1]. One of the most affected ethnicities are African Americans (AA), having the highest incidence and mortality rates of prostate cancer. Risk factors that have been seen are increasing age, African ancestry, a family history of the disease, and certain inherited genetic conditions [1]. In support of this data, other studies have also shown that African American men have among the highest incidence of prostate cancer (PCa) worldwide. African American men are more likely to develop PCa at any age, as

well as develop the disease earlier in life than men from other racial and ethnic groups [2]. There are two forms of prostate cancer: androgen-dependent prostate cancer and androgen-independent prostate cancer. Although all prostate cancers begin in an androgen-dependent state, over time, after androgen deprivation therapy is administered, some cells can survive and become androgen-independent prostate cancer (AIPC) by Androgen Receptor (AR) gene amplifications, AR gene mutations, changes in expression of AR co-regulatory proteins, changes in expression of steroid-generating enzymes, and ligand-independent activation of AR [3].

Due to the lack of effective targeted therapies for the androgen-independent form, it is considered the fatal point of the disease [3].

In contrast to prostate cancer, Non-Hispanic White women have a higher incidence of breast cancer; however, African American women have a higher mortality rate [4]. Recent data from the ACS states that the estimated number of invasive breast cancer in the United States for 2020 are 276,480 [5]. These findings prompted us to look for molecular pathways that could potentially be used to treat breast and androgen-independent prostate cancers. ELK-1 gene identified and cloned by Rao, et al. [6] belongs to the ETS family of ternary complex factors (TCFs), i.e., SAP1, NET/ERP/SAP2/ELK-3 [7, 8, 9]. The ELK-1 gene codes for at least two alternately spliced products. ELK-1 and Δ ELK-1, which function as transcriptional activators, are substrates for MAP kinases and JNK protein kinases [10, 11, 12, 13]. The ELK-1 protein is a ternary complex factor (TCF) which, in association with serum response factor (SRF), forms a ternary complex on the serum response element (SRE) of the c-Fos promoter and regulates c-Fos transcription [7]. ELK-1 and Δ ELK-1 proteins were shown to induce apoptosis in breast cancer cells when treated with calcium ionophore [14]. Δ ELK-1 is an alternatively spliced variant of ELK-1, which has lost the SRF interaction domain, negative regulatory DNA binding domain, and part of the ELK-1 DNA binding domain [15]. Δ ELK-1 protein has lost the capacity to form an SRF-dependent ternary complex with SRE and to activate Fos transcription [15]. Δ ELK-1 might function by competing for some of the ELK-1 target sequences, thereby blocking the transcriptional activation of Fos by ELK-1. ELK-1 appears to fall in the category of genes that encode activators and repressors through the mechanism of differential splicing [10]. We have previously also looked at the interaction of ELK-1 with the tumor suppressor BRCA1 and found both BRCA1a and BRCA1b isoforms to interact in vivo with the ETS domain of the ELK-1 protein (16). We have found ELK-1, but not Δ ELK-1 and SAP1a protein, to enhance BRCA1a-mediated growth suppression in MCF-7 breast cancer cells [16, 17]. ELK-1 has been shown to have not only an impact on breast, but prostate cancer as well. ELK-1 was also shown to be required for androgen receptor-dependent growth in various hormone-dependent and castration-resistant prostate cancer models [18]. The amino-terminal domain of AR docks at two sites on ELK-1 to co-activate essential growth genes [18]. A unique feature of prostate cancers is its dependence on androgen, which acts by binding to and activating transcriptional signaling by the androgen receptor (AR) [18]. ELK-1 is an AR-tethering protein that is obligatory for androgen/AR-dependent malignant growth in a variety of well-established prostate cancer/castration-resistant prostate cancer (CRPC) models. With the knowledge that ELK-1 has an impact on androgen-dependent prostate cancer, we wanted to assess how ELK-1 would impact AIPC. Cyclin-dependent kinase inhibitors (CDKIs) are proteins that bind to and inhibit the activity of CDKs. Two major classes of CDK inhibitors are p16 family (p15, p16, p18 and p19) that binds to and inhibits the activities of CDK4 and CDK6, and the p21 family (p21, p27, p28 and p57) which can bind to a broad range of CDK-cyclin complexes and inhibit their activities [20]. CDKIs are capable of suppressing growth, and

studies show that p21 can function as a tumor suppressor protein [19]. p21 is a mediator of p53 tumor suppressor activity and works as an inhibitor of cell cycle progression by inhibiting the activity of cyclin-dependent kinase (CDK)–cyclin complexes and proliferating cell nuclear antigen (PCNA). Recent data suggests a tumorigenic role for p21 in certain contexts that relies on its ability to suppress apoptosis and promote the assembly of type-D cyclins with CDK4 and CDK6 [20, 21] also noted that p21 is a tumor suppressor, but also can behave as an oncogene in certain cellular conditions; however, other studies indicated that knock down of p21 & p27 resulted in an increased rate of tumor growth and aggressive prostate carcinoma phenotype [19]. ELK-1 was also shown to transactivate the p21 gene independent of p53 and EGR-1 genes in HaCaT cells that were exposed to sodium arsenate [22]. The putative role of p21 in growth arrest mediated by p53 makes it an important gene with respect to the understanding of loss of growth control in transformation processes [23]. In another study CARM1, a coactivator-associated arginine methyltransferase 1, along with p300, another co-activator, was shown to cooperate with BRCA1 and p53 to induce expression of p21 WAF1/CIP1 in response to DNA damage [24]. Our previous work also demonstrated BRCA1 isoforms BRCA1a and BRCA1b to transactivate p21 expression in both a p53-dependent and independent manner (25, 26). We have found ELK-1 to be one of the downstream targets of BRCA1 growth suppression pathway in breast cancer cells [16]. Since ELK-1 functions as a growth suppressor, we can hypothesize that it can function as a growth / tumor suppressor by inducing the expression of p21WAF1/CIP1 in breast and prostate cancer cells. In this study, we have investigated whether ETS-like gene ELK-1 functions as a growth/tumor suppressor in MCF-7 breast cancer and PC-3 androgen-independent prostate cancer cells. Our results suggest ELK-1 to function as a potential growth/tumor suppressor of breast and androgen-independent prostate cancer cells by inducing the expression of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 independent of p53.

## Materials And Methods

### Cell Culture

MCF-7 pcDNA3, MCF7 ELK-1#13, MCF7 ELK-1#16, MCF7 ΔELK-1, PC-3 pcDNA3, PC-3 ELK-1 #3, PC-3 ELK-1 #3, PC-3 ELK-1 #6 and PC-3 ΔELK-1 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). The stable cell lines were generated as described previously [16, 27].

### Expression Constructs

The pcDNA3 ELK-1, pcDNA3 ΔELK-1, pSG5-ELK-1, and pSG5-ELK-1 DN (aa 1-82) expression plasmids were cloned as described previously [16].

### Antibodies and Reagents

The antibodies used in this study were primary polyclonal ELK-1 antibody (NEB) and p21 polyclonal antibody (Santa Cruz Biotechnology).

465

## Western Blot Analysis

ELK-1 transfected MCF-7 cells and PC-3 stable cells were maintained in Eagle's Minimum Essential Medium (MEM) and ATCC-formulated F-12K Medium (Catalog No. 30-2004) supplemented with 10% Fetal Bovine Serum (FBS). Cells were lysed in lysis buffer and the extracts were separated on 4-20% SDS-PAGE gradient gel and subjected to Western blotting using ELK-1 polyclonal antibody and p21 antibody as described previously (16,27).

## Colony suppression assay

PC-3 cells were transfected with 20 μg of plasmid DNAs (pcDNA3, pcDNA-ELK-1 or pcDNA3 Δ ELK-1) using the calcium phosphate mammalian transfection system from Promega. The next day, cells were washed twice in PBS and re-fed with fresh medium (F-12K Medium supplemented with 10% FBS). Twenty-four hours later, the cells were trypsinized and plated into six 10 cm petri dishes containing complete medium and 600 μg/mL G418. Cells were fed with medium containing G418 every 3-4 days. Cells were fed approximately 18-21 days after transfection in methanol acetic acid (3: 1) for 1 hour at -20oC and stained with crystal violet as described previously [16].

## Soft agar assay

Soft agar assay was carried out using 0.3% agar / DMEM or Ham's F-12K /10% FBS and plated on a base of 0.5% agar/ DMEM or Ham's F-12K as described previously (31). MCF-7 and PC-3 stable cells were plated at a concentration of 1X104 per 35 mm plate in soft agar containing complete media and kept at 37oC in a $CO_2$ incubator, adding a few drops of media every week. Colonies greater than 80 μm in diameter were scored after 3 weeks. Each soft agar assay was performed in triplicate.

## EMSA

EMSA was carried out as described previously [7,10] but using 32P- labelled ETS binding motifs in the p21 promoter and in vitro translated full-length ELK-1 protein. Rabbit polyclonal antisera against ELK-1 and the appropriate pre-immune serum was used [15]. Nuclear extract from human HeLa cells were obtained from Sigma-Aldrich.

## Transcriptional Activation Assays

COS-7 or MCF-7 or MEF p53-/- cells were co-transfected with 6 μg of WWP-Luc and 200 ng, 400 ng, and 1 μg of ELK-1, ELK-1-DN (aa 1-89) expression plasmids using the Stratagene transfection kit. The cells were harvested after 26 hours of transfection and assayed for luciferase activity. Luciferase activity was measured using a scintillation counter with the Promega kit as described previously [16]. The luciferase activity shown represents relative activity compared to the vector alone. The experiments were repeated at least three times and the experiments represent the mean of three independent experiments.

## Results and Discussion

### p21 WAF/CIP1 protein levels are upregulated in ELK-1 transfected MCF-7 and PC-3 cells.
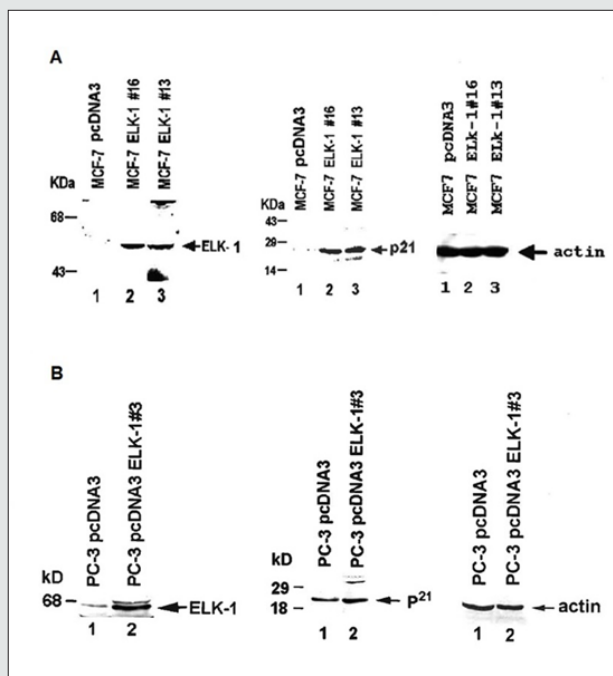


**Figure 1:** Expression of ELK-1 and p21WAF1/CIP1 protein levels in ELK-1 transfected MCF-7 and PC-3 cells (A) Western blot-analysis of total cellular ELK-1 and p21WAF1/CIP1 protein levels in MCF-7 ELK-1 transfectants. Total cellular extracts from MCF-7 pcDNA3 (lane 1), MCF-7 ELK-1#16 (lane 2) and MCF-7 ELK-1#13 (lane 3) were electrophoretically separated, transferred to PVDF membrane and subjected to immunoblot analysis using an ELK-1 antibody (NEB) and p21 antibody (Santa Cruz Biotechnology, Inc.). The apparent molecular weight of the prestained protein standards is shown. The molecular weights of the 62 kD ELK-1 and 21 kD p21 proteins are shown. (B) Total cellular extract (~90μg) from PC-3 pcDNA3 (lane 1); PC-3 ELK-1#13 (lane 2) were subjected to western blot analysis as described in (A). Equal amount of protein was loaded on the gels using actin antibody as a control.

As mentioned earlier, we have previously shown ELK-1, unlike Δ ELK-1 and SAP1a, to function both as a growth suppressor and to enhance BRCA1a/1b-mediated growth suppression of MCF-7 breast cancer cells. To investigate whether ELK-1 induces the expression of p21 WAF1/CIP1 in both MCF-7 and PC-3 cells, we developed stable MCF-7 and PC-3 cell lines expressing ELK-1 and subjected them to Western blot analysis using ELK-1 and p21 antibodies. Our results (Figure 1) showed increased expression of endogenous p21 WAF1/CIP1 protein in both the stable MCF-7 (Figure 1A) and PC-3 cell lines (Figure 1B) that were transfected with ELK-1 compared to cells transfected with pcDNA3 vector alone by western blot analysis. These results suggest that one of the mechanisms by which ELK-1 could function as a growth suppressor of breast and prostate cancer cells could be via induction of p21 WAF1/CIP1 protein expression.

## ELK-1 inhibits the growth of PC-3 prostate cancer cells

ELK-1, unlike Δ ELK-1 protein, was previously shown by us to function as a growth suppressor of human breast cancer cells [16]. To investigate whether ELK-1 suppresses the growth of androgen-independent PC-3 prostate cancer cells, we transfected pcDNA3, ELK-1 and Δ ELK-1 cDNAs into PC-3 prostate cancer cells and analyzed their effect on G418 colony formation. We have used PC-3 cell lines because they are extremely aggressive, are negative for androgen receptor (AR) and prostate-specific antigen (PSA) and do not respond to hormonal therapy [28]. The expression of pcDNA3 ELK-1 resulted in the reduction in the number of G418 colonies formed compared to pcDNA3 vector (Figure 2), unlike Δ ELK-1 (pcDNA3 Δ ELK-1). These results indicate ELK-1, unlike Δ ELK-1, can function as a growth suppressor of androgen-independent human prostate cancer cells.
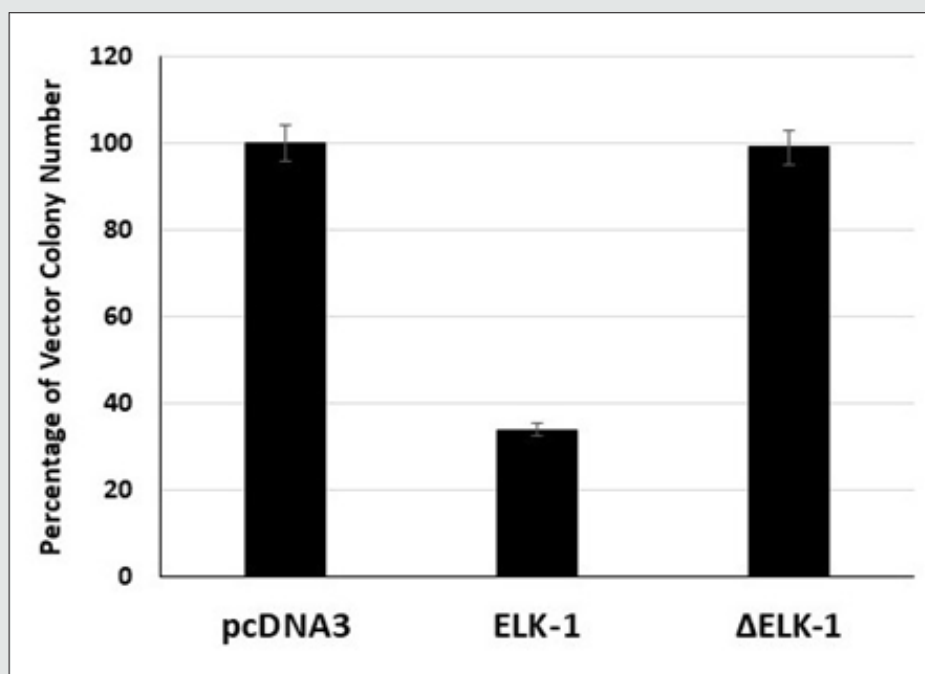


**Figure 2:** Elk-1 inhibits the growth of PC-3 prostate cancer cells. Cells were transfected with pcDNA3 or pcDNA3 ELK-1 or pcDNA3 Δ ELK-1 selected with G418 and the resulting colonies were stained and counted. The number of colonies obtained by pcDNA3 clone was considered as 100%. Each experiment was repeated at least three times and done as described previously [16, 26].

## Overexpression of ELK-1 inhibits in vitro growth in soft agar of MCF-7 and PC-3 cells

Since ELK-1 was found to suppress the growth of MCF-7 and PC-3 cells, we next tested if ELK-1 can inhibit the growth of MCF-7 and PC-3 cells in vitro in soft agar. All the ELK-1 stable MCF-7 and PC-3 transfectants were greatly inhibited in their capacity to grow and form colonies in soft agar, unlike the Δ ELK-1 transfectants (Figure 3A, 3B). These results show ELK-1 to function as a tumor suppressor in vitro in human breast and androgen-independent prostate cancer cells.

## ELK-1 activates the p21 WAF1/CIP1 promoter activity independent of p53

Cell proliferation is closely linked to cell cycle progression, which is regulated by a complex network involving cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs). The CKIs can be divided into two structurally related groups. The INK4 family, which includes p14, p15INK4b, p16INK4a, p18INKc, and P19INK4d that inhibit cyclin D-Cdk4/6 complexes and maintains Rb in its active anti-proliferative state. The other group of CKIs, the CIP1/KIP family, include p21 WAF1/CIP1, p27KIP1, and

p57KIP2, which inhibit kinase activities of cyclin E-Cdk2, and cyclin D-Cdk4/6. Functional disruption of p16INK4a and p21 WAF1/CIP1 occurs in prostate tumors. p21 WAF1/CIP1 has been implicated in growth arrest, cellular senescence, and terminal differentiation. Induction of p21 WAF1/CIP1 expression has been linked to growth inhibition by p53 and p21 WAF1/CIP1. Expression of p21 WAF1/CIP1 has also been found to signal growth arrest independent of p53 in cells undergoing differentiation. The altered growth and increased apoptosis observed in MCF-7-ELK-1 transfectants suggested that p21 WAF1/CIP1 and p16INK4A could be potential downstream targets. p21 WAF1/CIP1 has been reported to be a transcriptional target of overexpressed ETS factor EIAF [29]. In fact, alterations in the p16/Rb cell cycle checkpoint occur frequently in prostate cancers [30]. Since we observed increased levels of p21 WAF1/CIP1 in both stable MCF-7 ELK-1 and PC-3 ELK-1 cells (Figure 2), it suggests that ELK-1 could transcriptionally induce the

expression of p21 WAF1/CIP1. We therefore tested the ability of ELK-1 to activate reporter genes containing the p21 WAF1/CIP1, p15, and p16INK4a promoter luciferase reporters in COS-7 and MCF-7 cells. We observed ELK-1 to activate the p21 WAF1/CIP1 promoter activity at a higher level compared to p15 and p16INK4a (Figure 4A, 4B). Interestingly, we found ELK-1 binding motifs (5'-AGGAAG/C-3') in the mouse and rat p21 WAF1/CIP1 promoters [23]. We next transfected the p21 luciferase reporter with an increasing concentration of full-length ELK-1 into COS-7, MCF-7, and MEF p53-/- cells. There was a dose-dependent increase in the p21 luciferase reporter plasmid expression in COS-7 and MEF p53-/- cells (Figure 5). Using an ELK-1-DN (aa-1-89) expression plasmid in the presence of ELK-1 inhibited the p21 luciferase reporter expression in MCF-7 cells (Figure 5B). These results indicate that ELK-1 transactivates the p21 WAF/CIP promoter by binding to the p21 WAF1/CIP1 promoter and independent of the presence of p53.
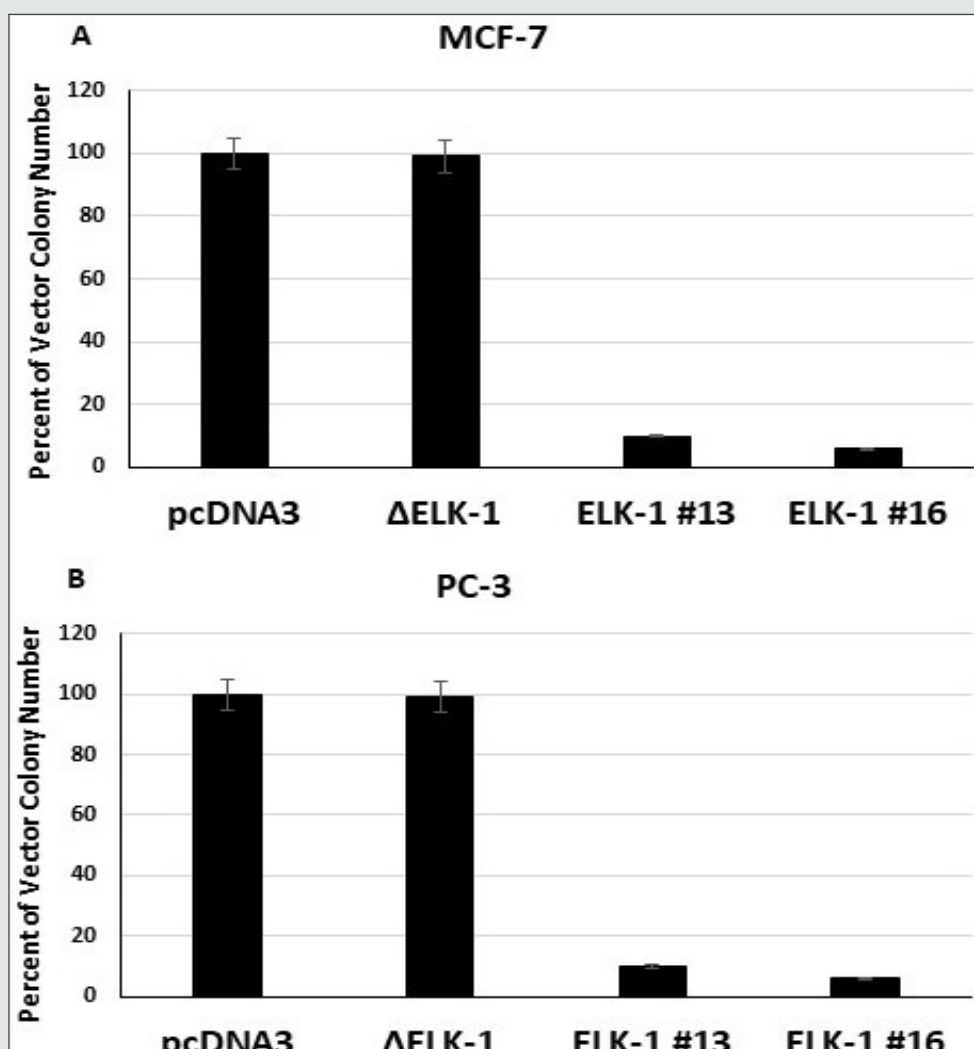


**Figure 3:** Over expression of ELK-1 inhibits in vitro growth in soft agar of MCF-7 cells (A) and PC-3 cells (B).  1 x 104 cells per dish of MCF-7 or PC-3 pcDNA3 vector transfectants or ELK-1 or Δ ELK-1 transfectants were analyzed for anchorage independent growth as described previously (31). The figure shows clonogenicity of both pcDNA3 vector, ELK-1 and ΔELK-1 transfectants. Each experiment was repeated at least three times and the bars represent standard deviation.
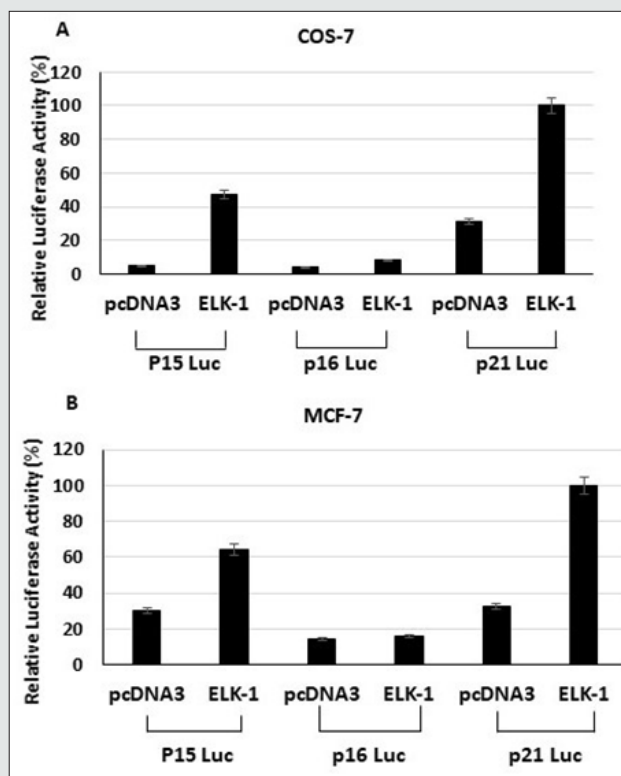
**Figure 4:** ELK-1 activates the p21WAF1/CIP1 promoter activity more than p15 and p16 in Cos-7 (A) and MCF-7 cells (B). Cells were co-transfected with expression vectors for pcDNA3 or pcDNA3-ELK-1 with luciferase reporter plasmid. The cells were harvested 36 hours after transfection and assayed for luciferase activity. The experiments represent mean of three independent experiments.
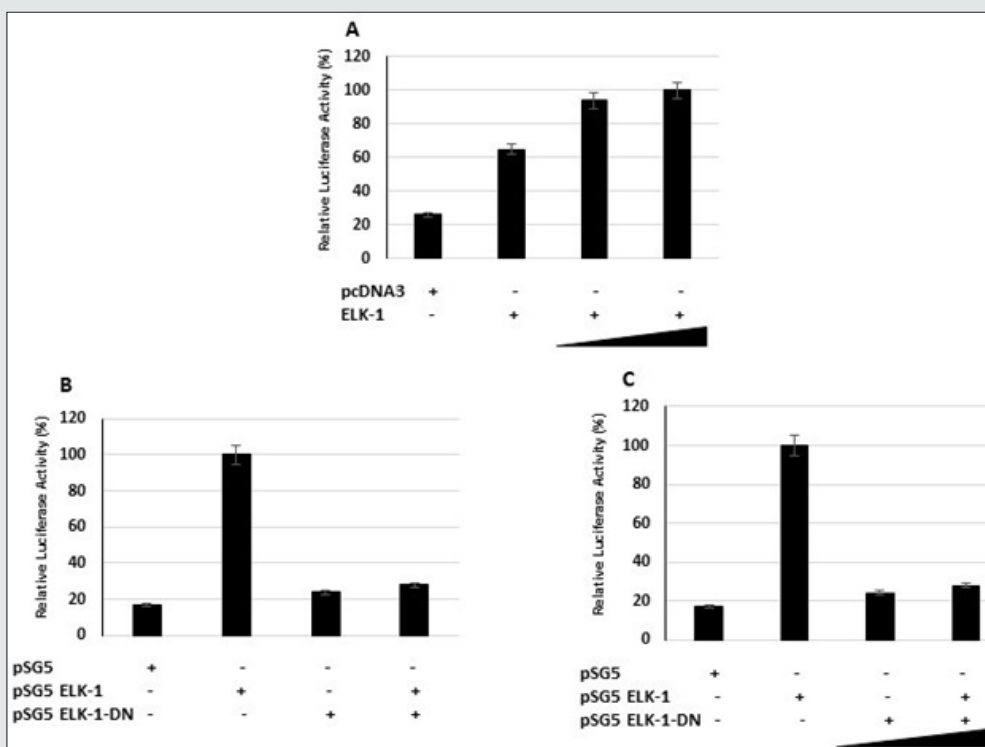


**Figure 5:** Elk-1 transactivates the p21 WAF1/CIP1 promoter in COS-7 (A), MCF-7 (B) and MEF p53-/- cells (C). COS-7 cells or MCF-7 cells or MEF p53-/- cells were co-transfected with 6ug of WWP-Luc and 200ng, 400ng, and 1ug of Elk-1, Elk-1-DN (aa 1-89) expression plasmids and luciferase activity was measured by scintillation counter using the Promega kit. The luciferase activity shown represents relative activity compared to vector alone. The experiments were repeated at least three times.

## ELK-1 binds to the ETS binding motifs in the p21 WAF1/CIP1 promoter

To investigate whether ELK-1 binds to the p21 WAF1/CIP1 promoter sequences, we analyzed the p21 WAF1/CIP1 promoter sequences for the presence of potential ELK-1 binding sites using the GGAA consensus binding sequence determined for the ETS proteins [29]. We next investigated whether ELK-1 binds to the ELK-1 binding motifs in the p21 WAF1/CIP promoter using EMSA. We obtained fragments corresponding to the promoter sequences of p21 WAF1/CIP by PCR. This fragment was 32p-labelled and used for EMSA with invitro translated full length ELK-1 protein as described previously [7,10]. We observed binding of full length in vitro translated ELK-1 to p21 WAF1/CIP1 promoter sequences, which was competed on using ELK-1 antibodies (Figure 6). The same scenario was seen using HeLa cell nuclear extract which was also competed by ELK-1 antibodies (Figure 6). These results further confirm our hypothesis that ELK-1 induces the expression of p21 WAF1/CIP1 by binding to the ETS binding site in the promoter.
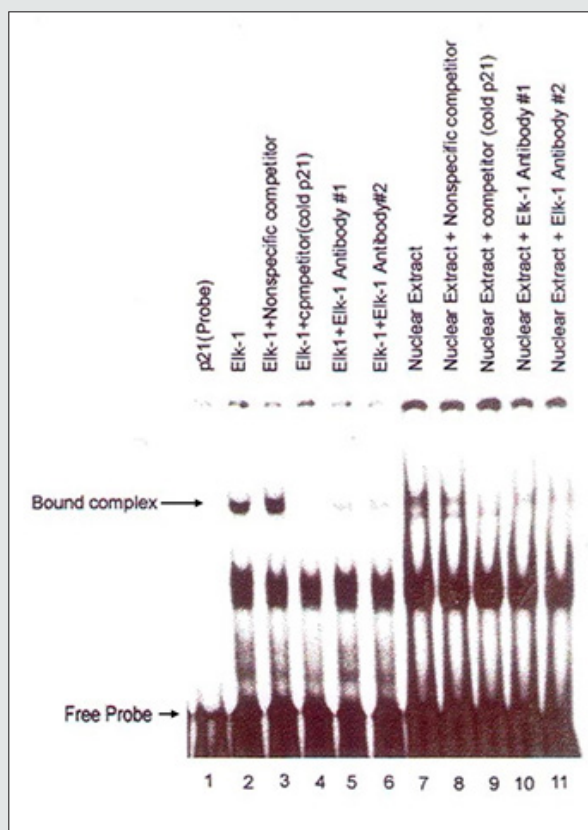


**Figure 6:** ELK-1 binds to the ETS binding motifs in the p21WAF1/CIP1 promoter. EMSA was performed as described previously (7, 10) but using p32-labeled oligonucleotide containing the ETS binding sites (EBS) and invitro translated full length ELK-1 protein or using nuclear extract obtained from Hela cells. The bound complexes as well as free probes are indicated by arrows.

p21WAF1/CIP1 has dual functionality in tumor development and progression depending on the cellular context and its subcellular localization. Cytoplasmic expression of p21 plays a role as an oncogene, whereas nuclear p21 works as a tumor suppressor. Cytoplasmic localization of p21 was associated with advanced disease stage and overall survival in various types of cancers [32,33]. Therefore, identifying specific inhibitors that selectively block the cytoplasmic shuttling of p21 or promote cytoplasmic degeneration could be promising targeting strategies. Small molecule inhibitors of p21 such as sorafenib, a tyrosine kinase inhibitor, was shown to induce proteasomal degradation of p21 independent of p53 in renal cell carcinoma [34]. Challenges lie in devising therapeutic agents that inhibits the oncogenic activities of p21 without interfering with tumor suppressing activities. Further work is needed to elucidate the role of p21 under distinct cellular context independent of p53 and to explore is its potential as therapeutic target. Our results suggest for the first time that ELK-1, unlike Δ ELK-1, to function as a growth /tumor suppressor of androgen-independent human prostate cancer cells and breast cancer cells. We have found p21 WAF1/CIP1 to be one of the potential downstream targets in this pathway. ELK-1 protein, by binding to the ETS binding site in the p21 WAF1/CIP1 promoter, induces the expression of the protein in a p53-independent manner. These studies will help us in the future to develop an effective mechanism-based therapeutic paradigm for patients with advanced-stage androgen-independent prostate and breast cancers. It may also help in developing potential biomarkers for the early detection of these cancers.

## Conflict of Interest

Authors have no conflict of interest to disclose.

## Acknowledgements

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