

# Inhibition of Egr-1 by siRNA in prostate carcinoma cell lines is associated with decreased expression of AP-1 and NF- $\kappa$ B

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**Abstract.** Previous studies suggest that the effects of Egr-1 on tumorigenesis are critically dependent on the levels of Egr-1 and the cellular context. For this reason, we examined the effects of blocking the Egr-1 activity by a short interfering RNA (siRNA) against Egr-1 on the expression of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) signaling in the PC-3 and LNCaP prostate carcinoma cell lines. We observed that the reduction in expression of Egr-1 in PC-3 and LNCaP cells by effects of the siRNA vector resulted in lower cell viability and increased apoptosis at 24 and 120 h after transfection. This reduced cell viability correlated well with a reduced activity of the NF- $\kappa$ B and AP-1 factors. We analyzed the expression and activity of these factors and found that p65 and I $\kappa$ B but not p50 were reduced in the siRNA-treated cells. Similarly, we found that c-Fos but not c-Jun was reduced in the siRNA treated cells. These effects were translated to reduced transcriptional activity of NF- $\kappa$ B over cellular inhibitor of apoptosis (cIAP) and p21 genes, as assayed by RT-PCR and of AP-1 over a luciferase reporter activated by AP-1 response elements. Egr-1 was also found to interact directly with p65 and I $\kappa$ B members of the NF- $\kappa$ B family, thereby was able to regulate directly their activity by post-transcriptional effects. These results suggest that Egr-1 may promote prostate cancer development by modulating the activity of factors NF- $\kappa$ B and AP-1, which are involved in cell proliferation and apoptosis.

## Introduction

The transcription factor, Egr-1, is rapidly induced by growth factors to transduce the proliferative signal. The induction of

Egr-1 by external stimuli is generally transient but appears to be sustained in some prostate tumor cell lines and tumors, suggesting that Egr-1 stimulates tumor cell growth and that could have an important function because its expression level increases with the degree of malignancy as measured by the Gleason grade of the tumor (1). This seems to be specific to prostate tumor cells, because in mammary and lung tumors, as well as most normal tissues, Egr-1 expression is low (2-6). In addition Egr-1 overexpression is correlated with the loss of its corepressor NAB2 in primary prostate carcinoma (7,8). This disruption of the balance between Egr-1 and NAB2 expression results in a high Egr-1 transcriptional activity in prostate carcinoma cells (1). In contrast, in breast, lung and brain tumor, Egr-1 expression is often absent or reduced and when re-expressed, results in growth suppression (2,3,9,10). Another contradiction is that after stress stimuli to some cell types, Egr-1 is required for programmed cell death or apoptosis in both normal and tumor cells. Egr-1 also plays a role in tumor progression, through the hypoxic signal generated in growing tumors. Egr-1 is highly induced under these conditions and its activities stimulate angiogenesis and improved survival of tumor cells (6,11-13).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) are transcription factors frequently associated with inflammatory response and cancer (14-16). Both factors are well-characterized ubiquitously expressed transcription factors that play important roles in the response to cellular stress situations. In unstimulated resting cells, NF- $\kappa$ B and AP-1 are inactive (14,16,18). A prominent portion of the classical NF- $\kappa$ B target gene program includes chemokines and cytokines, which promote inflammation and angiogenesis (17-19). In addition to the role of NF- $\kappa$ B in directly promoting cancer cell survival, these factors provide selective advantages for tumor growth in the microenvironment via stimulation of inflammatory cell infiltration and angiogenesis (20-22). These transcription factors are induced by a great variety of stimuli and conditions that represent internal or external stress situations for the cells. These include pathological stimuli, such as viruses, bacteria, oxidative stress, hypoxia, and inflammatory mediators as well as internal cellular stress (e.g., endoplasmic reticulum overload) (19,20). Recently we showed that overexpression of Egr-1 in prostate cancer cell lines up-regulated the activity of both, AP-1 and NF- $\kappa$ B (21).

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**Key words:** prostate carcinoma, early growth response gene, short interfering RNA, activator protein-1, nuclear factor- $\kappa$ B, cellular inhibitor of apoptosis 2, p21



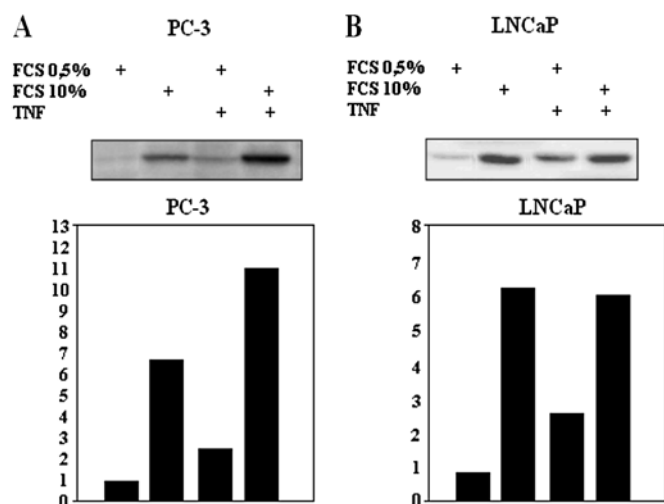


Figure 1. Analysis of Egr-1 expression in (A) PC-3 and (B) LNCaP prostate carcinoma cells treated with FCS 0.5%, FCS 10% and TNF- $\alpha$  (20 ng/ml). (A) LNCaP cells were treated with, FCS 0.5%, FCS 10% for 48 h, followed by TNF- $\alpha$  (20 ng/ml) induction for another 6 h. The cells were lysed and samples were analyzed by Western blot analysis using an anti-Egr-1 antibody (membranes were reprobbed with antibodies to  $\beta$ -actin to control for equal loading). The intensity of each band was quantified by densitometry. (B) PC-3 cells were treated with, FCS 0.5%, FCS 10% for 24 h, following by TNF- $\alpha$  (20 ng/ml) induction for another 3 h. The cells were lysed and samples were analyzed by Western blot analysis using an anti-Egr-1 antibody (membranes were reprobbed with antibodies to  $\beta$ -actin to control for equal loading). The intensity of each band was quantified by densitometry. One of three similar experiments is shown.

Hughes Medical Institute, Yale University School of Medicine, New Haven, CT). The tandem sequences used to construct the different multimer plasmids were as follows: four copies of the AP-1, 5'-TCGATTGAGTCAGGGTAA-3' and the two copies of the NF- $\kappa$ B binding site of the human Ig  $\kappa$  light chain enhancer 5'-GGGACTTTCC-3'.

To assay for luciferase activity, transfected cells in duplicate wells were cultured for 24 h before being stimulated with or without TNF- $\alpha$  (20 ng/ml) for a defined length of time. Cells were harvested, washed twice in PBS and treated with lysis buffer (Luciferase Assay, Promega) for 5-10 min on ice. Lysates were spun down for 1 min, and the total supernatants were analyzed using Luciferase Reagent (Promega) and measured in a luminometer (MicroLumat LB-96P, Berthold) for 5 sec. Background measurement was subtracted from each duplicate, and experimental values are expressed either as recorded light units, luciferase activity or as relative activity compared to extracts from unstimulated cells (34,36).

## Results

NF- $\kappa$ B and AP-1 nuclear transcriptional factors regulate expression of multiple genes involved in tumor growth, metastasis and angiogenesis; however, the relative contribution of each factor to cancer initiation and progression has not been established. Whereas activation of both NF- $\kappa$ B and AP-1 has been implicated in prostate cancer development and growth, we tested the relative effects of blocking the nuclear factor Egr-1 and how this blocking would affect the expression levels of AP-1 and NF- $\kappa$ B in prostate carcinoma cell lines PC-3 and LNCaP.

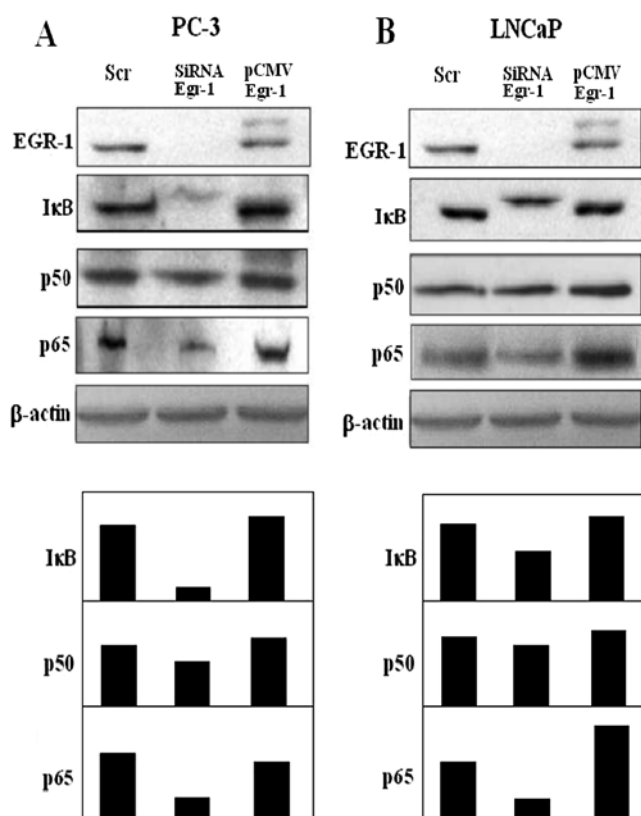


Figure 2. Down-regulation of Egr-1 by siRNA against Egr-1 decrease the protein levels of various members of the nuclear factor- $\kappa$ B. Cells were transiently transfected with either, siRNA-scrambled, siRNA-Egr-1 or pCMV-Egr-1 (expression plasmid) and cultured with, either FCS 0.5 or 10% for 48 h followed by TNF- $\alpha$  (20 ng/ml) induction for another 6 h. The cells were lysed and samples were analyzed by Western blot analysis using anti-Egr-1 antibody (membranes were reprobbed with antibodies to  $\beta$ -actin to control for equal loading). The intensity of each band was quantified by densitometry. Western blot analysis of the NF- $\kappa$ B family members, p50, p65 and the inhibitor I $\kappa$ B in (A) PC-3 and (B) LNCaP cells. The intensity of each band was quantified by densitometry. One of three experiments is shown.

*Egr-1 activity in prostate carcinoma cell lines PC-3 and LNCaP treated with various stimuli.* Egr-1 is among the early response nuclear factor that plays an important role in the regulation of several genes. To determine the activity of Egr-1 in PC-3 and LNCaP prostate carcinoma cell lines (Fig. 1), we treated the cells with FCS 0.5%, FCS 10% and TNF- $\alpha$ . The protein expression of Egr-1 was assessed using Western blot analysis (Fig. 1). The cells were treated with various stimuli. FCS 10% and TNF- $\alpha$  strongly activated Egr-1 expression. In contrast, FCS 0.5% induced only a marginal activation of Egr-1 compared with untreated cells.

*Effects of blocking Egr-1 production by an siRNA on the expression of NF- $\kappa$ B and AP-1.* To test whether blocking of Egr-1 could directly affect NF- $\kappa$ B and AP-1 expression, we performed Western blot analysis experiments in two prostate carcinoma cell lines PC-3 and LNCaP, using the siRNA-Egr-1 (Figs. 2 and 3). A direct inhibitory effect of siRNA-Egr-1 on NF- $\kappa$ B (Fig. 2) and AP-1 (Fig. 3) expression was observed, which was independent of whether cells had been pre-treated with or without TNF- $\alpha$  (20 ng/ml). To identify the protein components of these complexes, we performed Western blot

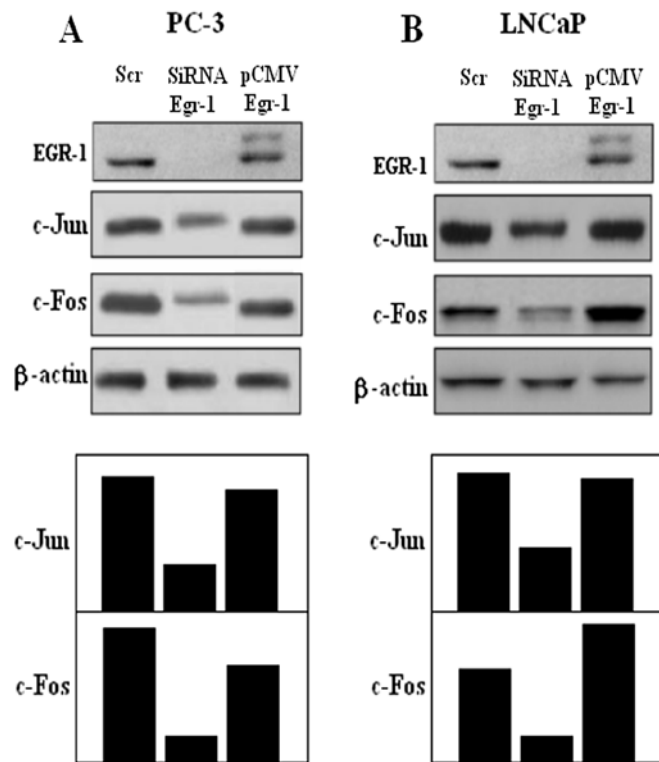


Figure 3. Down-regulation of Egr-1 by siRNA against Egr-1 decreases the protein levels of various members of the nuclear factor AP-1. Cells were transiently transfected with either, siRNA-scrambled, siRNA-Egr-1 or pCMV-Egr-1 (expression plasmid) and cultured with, either FCS 0.5% or 10% for 48 h following by TNF- $\alpha$  (20 ng/ml) induction for another 6 h. The cells were lysed and samples were analyzed by Western blot analysis using anti-Egr-1 antibody (membranes were reprobed with antibodies to  $\beta$ -actin to control for equal loading). The intensity of each band was quantified by densitometry. Western blot analysis of the AP-1 members, c-Jun and c-Fos in (A) PC-3 cells and (B) LNCaP cells. The intensity of each band was quantified by densitometry. One of three experiments is shown.

analysis experiments using anti-Egr-1, anti-I $\kappa$ B, anti-p50, anti-p65 antibodies; an anti  $\beta$ -actin antibody was used to control for the amount of protein loaded in each lane. Incubation of protein extracts with Egr-1 antibody showed that siRNA-Egr-1 completely blocked the formation of Egr-1; in the same way, anti-I $\kappa$ B and anti-p65 antibodies showed a decrease in its activity (Fig. 2). Similarly, the expression of Egr-1 was completely inhibited in the assay for the expression of the AP-1 family members. However, the expression of c-Jun was more affected in PC-3 cells transfected with the siRNA-Egr-1 than in LNCaP cells. In contrast, the decreasing effect of c-Fos was strongly observed in both cell lines, PC-3 and LNCaP transfected with siRNA-Egr-1.

The sum of the results suggest that blocking Egr-1 expression by an siRNA affected some of the complexes of NF- $\kappa$ B or AP-1, but not the stability of the inhibitor of NF- $\kappa$ B, as shown in Figs. 2 and 3.

**Effect of blocking Egr-1 expression on the transcriptional activity of AP-1 and NF- $\kappa$ B in PC-3 and LNCaP prostate carcinoma cell lines.** To address the effect of blocking the expression of Egr-1 on the transcriptional activity of NF- $\kappa$ B and AP-1 we examined whether blocking of Egr-1 by an siRNA resulted in transcriptional or not activation of NF- $\kappa$ B and AP-1

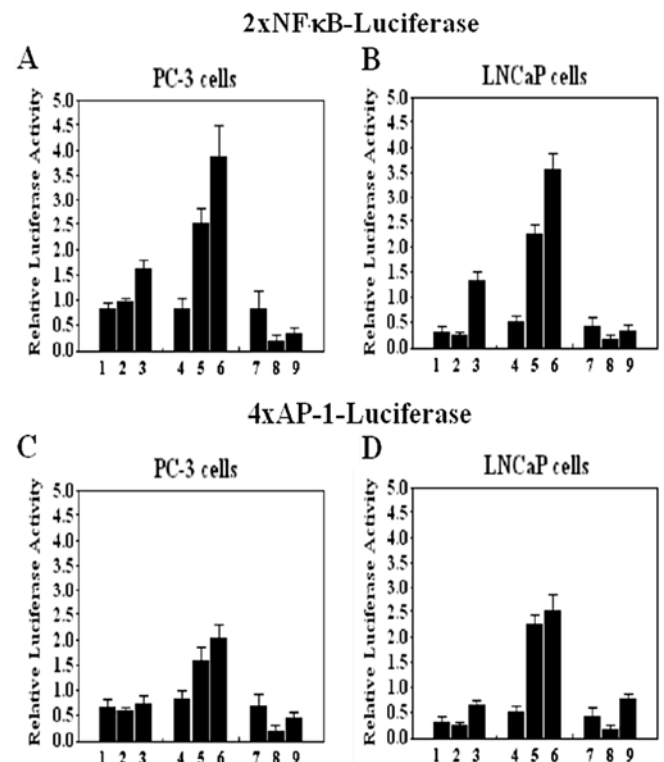


Figure 4. Blocking of Egr-1 by siRNA-Egr-1 altered the transcriptional activity of the nuclear factor NF- $\kappa$ B and AP-1, as demonstrated in a luciferase assay. PC-3 and LNCaP cells were cotransfected with, siRNA-scrambled, or pCMV (empty vector), or pCMV-Egr-1 expression vector or siRNA-Egr-1 and co-transfected with 2xNF- $\kappa$ B-pGL2-luciferase or 4xAP-1-pGL2-luciferase reporter constructs. The cells were cultured with different concentrations of FCS (0.5 and 10%) for 48 h following by TNF- $\alpha$  (20 ng/ml) induction for another 6 h. (A, B, C and D) 1, Scr-siRNA; 2, pCMV+Scr-siRNA; 3, pCMV+Scr-siRNA+TNF- $\alpha$ ; 4, Scr-siRNA; 5, pCMV-Egr-1+Scr-siRNA; 6, pCMV-Egr-1+Scr-siRNA+TNF- $\alpha$ ; 7, Scr-siRNA; 8, pCMV-Egr-1-siRNA; 9, pCMV-Egr-1-siRNA+TNF- $\alpha$ . One of three individual experiments is shown.

by an *in vitro* luciferase assay. Thus, we assayed the activity of the consensus region for AP-1 and NF- $\kappa$ B (34,35), using the pGL-2 luciferase gene reporter construct which contains a tandem repeat of the 4xAP-1 and 2xNF- $\kappa$ B-binding sites, and transfected the reporter constructs into the prostate carcinoma cell lines PC-3 and LNCaP (Fig. 4A and B). As shown in Fig. 4, transient cotransfection of pGL-2-2xNF- $\kappa$ B-Luc with scrambled siRNA (column 1) or pCMV + Scr-siRNA (column 2) did not increase the luciferase activity, and an additive increase was observed in PC-3 cells treated with TNF- $\alpha$  (column 3). In column 4 we observed that scrambled siRNA, did not increase the luciferase activity. In contrast, overexpression of Egr-1 (columns 5 and 6) with or without TNF- $\alpha$ , strongly induced luciferase activity. The effect of the siRNA on the activity of NF- $\kappa$ B, including the cells treated with TNF- $\alpha$  (20 ng/ml) is shown in Fig.4A (columns 7, 8 and 9), suggesting a key role of Egr-1 in the stability of the NF- $\kappa$ B complex.

We performed a similar assay with the nuclear factor AP-1 (Fig. 4B). Similarly to NF- $\kappa$ B, the AP-1-Luc reporter construct, did not increase or reduce the luciferase activity (columns 1, 2 and 3). Overexpression of Egr-1 (columns 5 and 6) moderately increased the activity of AP-1 in both PC-3 and LNCaP cells, but TNF- $\alpha$  failed to further increase the levels of luciferase activity, compared with the same columns in the NF- $\kappa$ B assay.

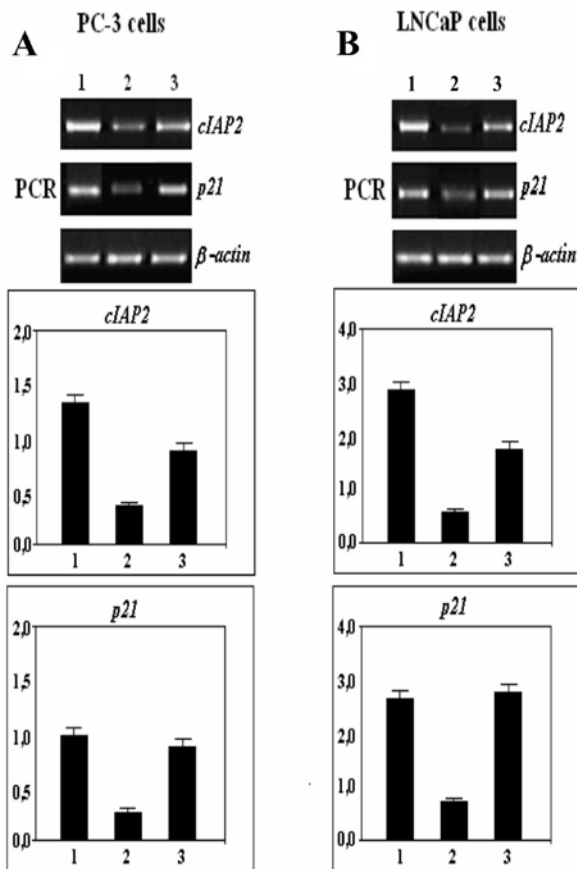


Figure 5. Egr-1 knockdown by siRNA inhibits the expression of cIAP2 and p21 in the (A) PC-3 and (B) LNCaP prostate carcinoma cell lines. Both cells were transfected with either: 1, pCMV-Egr-1; 2, siRNA targeting Egr-1+TNF- $\alpha$  (20 ng/ml); or 3, pCMV-Egr-1+TNF- $\alpha$  (20 ng/ml). Post-transfection of 24 h expression levels of targets were analyzed at the gene level using a semi-quantitative PCR assay. Results are representative of two individual experiments.

In columns 8 and 9, the activity of luciferase returned to the controls levels.

**Analysis of the expression of cIAP2 and p21 by RT-PCR with total-RNA.** To evaluate the mechanisms by which blocking of Egr-1 expression modulates the activity of two proteins involved in the cell cycle and apoptosis signaling pathway, we examined the expression of p21 and cIAP2 protein that was thought to directly bind caspases and inhibit their activation (Fig. 5). RT-PCR analysis indicated a decreased expression of the IAP2 and p21 proteins in both, PC-3 and LNCaP cell lines transfected with the siRNA-Egr-1, suggesting a cooperative role of the NF- $\kappa$ B and AP-1 members of the nuclear factor with Egr-1 (Fig. 5). Surprisingly, the presence of TNF- $\alpha$  could not interfere with the decreasing effect of blocking Egr-1 on the cIAP2 expression levels compared with the effect observed in cells transfected with pCMV-Egr-1. In addition, cIAP1/2 is involved in the activation of the TNF- $\alpha$ -induced canonical NF- $\kappa$ B pathway (36,37).

## Discussion

NF- $\kappa$ B and AP-1 are transcription factors frequently associated with inflammatory response and cancer (14-20). Our

previous study demonstrated overexpression of Egr-1 in PC-3 and LNCaP prostate carcinoma cell lines caused pronounced increase in protein levels of NF- $\kappa$ B and AP-1 (38). In this study, we observed that depletion of the endogenous expression of Egr-1 (over 90%) by siRNA decreased PC-3 and LNCaP cell proliferation, cell growth and induced transcriptional arrest of the members of the related NF- $\kappa$ B and AP-1 nuclear factors. In addition, its depletion reduced the levels of p21 and cIAP2 significantly. We also provide evidence that endogenous Egr-1 stimulates c-Jun, c-Fos, p50 and I $\kappa$ B synthesis with or without a stimulating factor, such as TNF- $\alpha$  or exogenous Egr-1 stimulation. However, after transient transfection of cells with siRNA-Egr-1, the protein levels of c-Jun, c-Fos and p65 were significantly reduced. Similarly, the levels of cIAP2 were reduced. In addition to the decrease in cIAP2 levels, we also observed a steep decrease in the level of other nuclear proteins involved in cell cycle progression, such as p21. The phenotype of this inhibition is seen as the cell cycle arrest at the G1 to S-phase transition. This study complements and extends our previous study (38) in which a 5-10-fold increase in Egr-1 mRNA, NF- $\kappa$ B and AP-1 and protein levels in cell culture studies were observed. This compares to our finding that siRNA mediated silencing resulted in >98% reduction in Egr-1 mRNA and 91% reduction in Egr-1 protein *in vitro*, which led to dramatic changes in the cellular phenotype. As we have shown in this report and that of others, PC-3 and LNCaP cells constitutively produce a significant amount of Egr-1 (39-41). Interestingly, strong stimulating signaling, such as TNF- $\alpha$  is also attenuated without the endogenous expression of Egr-1, which has been inhibited by an siRNA against Egr-1, suggesting Egr-1 activated intracellular signaling may synergistically enhance other protein-induced signals (30,31).

Thus, the results presented in this report clearly demonstrate that endogenous production of Egr-1 plays a significant role in the survival of prostate carcinoma cells such as PC-3 and LNCaP, and enhances proliferation without any extrinsic source of Egr-1. The most significant finding of our study is that knockdown of endogenous Egr-1 expression in PC-3 and LNCaP cells reduces NF- $\kappa$ B and AP-1 activity as well as the mRNA levels of p21 and cIAP2. In PC-3 and LNCaP cells, AP-1 and NF- $\kappa$ B are constitutively activated and are known to exert a significant effect on cell survival, resistance to anticancer drug-induced apoptosis and metastatic potential (42,43). Whether constitutive activation of NF- $\kappa$ B and AP-1 is a cause of Egr-1 production or whether constitutive production of Egr-1 elevates NF- $\kappa$ B and AP-1 activity is not clear. However, at least in PC-3 and LNCaP cells, it was recently elucidated that overexpression of Egr-1 results in increased NF- $\kappa$ B and AP-1 activity during normal and stressed conditions (38). Our results show that indeed, without an external source of Egr-1, constitutive activation of NF- $\kappa$ B and AP-1 is associated with the expression of Egr-1, as knockdown of Egr-1 expression caused a significant inhibition of NF- $\kappa$ B and AP-1 activity and reduction in p21 and cIAP2 mRNA expression. This corroborates our previous studies that have suggested that forced expression of Egr-1 in PC-3 and LNCaP cells causes constitutive activation of NF- $\kappa$ B and AP-1 (38). The external stimulation of Egr-1 may be advantageous to proliferation, survival, motility and invasion, and resistance to cytotoxic drugs, when surviving in an ectopic environment, such as



during seeding and growth in distant organs, such as the bone and lungs. The ability to produce Egr-1 in an autocrine fashion may be a critical determinant. Interestingly, c-Jun silencing also down-regulated cyclin p21, a known AP-1 target gene, which was previously shown to be expressed at higher mRNA and protein levels in PC-3 and LNCaP cells.

In the present study, we have demonstrated that an siRNA against the mRNA of Egr-1 decreased the expression of Egr-1, NF- $\kappa$ B, cIAP2, p21 and AP-1 protein levels, through blocking the transcription process of Egr-1 mRNA. The cascade of events that can explain the effect of blocking Egr-1 on the expression activity of the members of NF- $\kappa$ B, such as p65 or the members of the AP-1 such as c-Jun and c-Fos, are related to the protein interaction of Egr-1 with some of the above mentioned members of the Jun and Fos family proteins. The reduction observed in the mRNA expression of cIAP2, could explain the role of both cIAP1 and cIAP2 in the regulation of TNF- $\alpha$ -mediated NF- $\kappa$ B activation. Our results suggest that suppression of Egr-1 using siRNA may help sensitize PC-3 and LNCaP cells to a wide variety of chemotherapeutic agents and may increase the survival of patients with end-stage disease.

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