Transcriptional Regulation of *EGR1* by EGF and the ERK Signaling Pathway in Prostate Cancer Cells

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Abstract

The early growth response gene 1, *EGR1*, is an important transcriptional regulator and acts as the convergent point between a variety of extracellular stimuli and activation of target genes. Unlike other tumor types, prostate tumors express high levels of *EGR1* relative to normal tissues. However, the mechanism of *EGR1* regulation in prostate tumor cells is unknown. As *EGR1* expression and epidermal growth factor (EGF) signaling are frequently upregulated in prostate tumors, we tested the hypothesis that EGF induces *EGR1* expression in prostate cancer cells. Using RT-PCR to quantify *EGR1* transcripts, we found that EGF induced *EGR1* expression in a dose- and time-dependent manner and the ERK pathway inhibitor, PD98059, abrogated the EGF-mediated *EGR1* response in LNCaP and PC3 cells. Analysis of the *EGR1* promoter using deletion constructs identified an EGF-responsive region in the proximal promoter (–771 to –245 bp) containing 3 potential serum response element (SRE) sites. *In vivo* chromatin immunoprecipitation assays demonstrated that Elk-1 binding at the SRE sites of the *EGR1* promoter was enhanced by EGF treatment in PC3 cells. Overexpression of Elk-1 was sufficient to activate the EGF-responsive region of *EGR1* promoter in PC3 cells and, similarly, a dominant-negative Elk-1 suppressed *EGR1* promoter activity. Taken together, these results demonstrate for the first time that *EGR1* expression in PC3 cells is mediated through an EGF-ERK-Elk-1 signaling cascade.

Keywords

prostate cancer; EGF; *EGR1*; Elk-1 Received October 26 2011; accepted October 30 2011

Introduction

EGR1 is an important transcriptional regulator and has been described as a “master switch” linking extracellular stimuli to diverse long-term responses including cell growth, proliferation, and apoptosis.^{1–3} In many tumor types, such as glioblastoma, lymphoma, and carcinomas of the lung and breast, *EGR1* is low or absent,^{4–7} indicating a tumor suppressive role. Indeed, *EGR1* targets include the tumor suppressors PTEN and p53 and the pro-apoptotic factor c-Jun.^{8–10} In some systems, the absence of *EGR1* keeps cell proliferation and growth signals in check by allowing the growth suppressors to act unopposed.

Paradoxically, *EGR1* expression is elevated in prostate tumor tissues relative to normal tissues at both the mRNA and protein levels, and its expression correlates with Gleason score.^{11,12} Loss of expression of an *EGR1* repressor protein, NGF-1A-binding protein (NAB2), may also contribute to elevated *EGR1* levels found in prostate tumors.¹³ Functional studies using antisense *EGR1* RNA reduced cell proliferation, colony formation, and growth in soft agar in prostate cancer cells, suggesting that *EGR1* is necessary for the transformed tumor phenotype.¹⁴ Additionally, anchorage-independent growth and metastasis may be promoted by the direct action of *EGR1* on target genes fibronectin and

TGF β 1 as well as by the frequent deletion or inactivation of the tumor suppressors PTEN and p53 in prostate tumors.^{15,16}

Although the many transcriptional targets of *EGR1* are well described, little is known about the mechanisms regulating *EGR1* gene expression in the context of prostate cancer. In other tissue types, *EGR1* is induced by a variety of mitogenic stimuli including growth factors, serum, and cytokines.^{17–20} However, changes in *EGR1* transcript levels are largely dependent on the particular growth factor and the tissue type. One rational candidate for regulation of *EGR1* in the prostate is epidermal growth factor (EGF). Both EGF and its receptor have been shown to be elevated in prostate tumors^{21–23} and are associated with disease progression.^{24,25} EGF transmits its signal inside the cell primarily through the extracellular regulated kinase (ERK) pathway. One well-described downstream target of ERK is

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the Elk-1 transcription factor.^{26,27} Elk-1 is characterized by its Ets-binding domain, recognizing the canonical sequence 5'-GGA(A/T)-3', and its ability to form a ternary complex with the serum response factor (SRF) on target promoters at the serum response element (SRE).²⁸ The Elk-1 protein structure not only provides docking sites for ERK and other mitogen activated kinases^{29,30} but also contains a phosphorylation site that confers Elk-1 transcriptional activity onto target genes.^{26,31} Some of the best-described targets of Elk-1 are the immediate early genes (IEGs), *c-fos*,^{32,33} *EGR1*,^{34,35} and *JunB*,¹⁷ whose expression is often rapid and transient after stimulation (reviewed by Adamson and Mercola³).

As *EGR1* expression and EGF signaling are frequently upregulated in prostate tumors, we sought to evaluate the potential of EGF to induce *EGR1* expression in a prostate cancer tissue system. An additional goal of this study was to identify the region of the *EGR1* promoter affected by EGF treatment. Here we report EGF induction of *EGR1* gene transcription and the identification of the EGF-responsive region of the *EGR1* promoter in both androgen-responsive (AS) and androgen-independent (AI) prostate cancer cells. Additionally, in AI PC3 cells Elk-1 was identified as a transcription factor that bound and activated the EGF-responsive region. Interestingly, Elk-1 does not appear to mediate the EGF/ERK signaling pathway in the AS LNCaP prostate cancer cells. Overall, these results identify the EGF-ERK pathway as a major regulatory mechanism of *EGR1* in prostate cancer cells.

Results and Discussion

Regulation of *EGR1* by epidermal growth factor via the ERK pathway. To determine whether EGF induced transient expression of *EGR1* after stimulation, LNCaP, CWR, and PC3 cells were treated with EGF for either 1 h or 4 h and samples collected for RNA analysis (Fig. 1A). As expected, each cell line exhibited a strong increase in *EGR1* transcription at 1 h (190-, 31-, and 60-fold induction for LNCaP, CWR, and PC3 cells, respectively) and significantly reduced activation by 4 h (3.0-, 3.3-, and 33-fold activation, respectively) although levels were still significantly greater than control. Similarly, *EGR1* protein levels responded transiently to EGF with the highest abundance of protein present at 1 h after treatment (Suppl. Fig. S1). To demonstrate the sensitivity of *EGR1* expression to EGF levels, a dose response experiment was performed (Fig. 1B). Surprisingly, *EGR1* transcript levels were elevated more than 50-fold relative to control with as little as 0.5 ng/mL of EGF. A maximal response was seen using 5 ng/mL of EGF, and no significant increase in activation was observed between 5 ng/mL and 50 ng/mL. These results were obtained using physiologically relevant concentrations of EGF, as EGF concentrations in prostatic fluid have been reported to range from 40 to 175 ng/mL.³⁶

Overall, these data support a strong but transient EGF-induced *EGR1* expression pattern in prostate cancer cells. Although the most dramatic increases in *EGR1* expression were seen at 1 h after treatment, *EGR1* mRNA levels remained elevated above control for several hours. Other reports have also described high levels of *EGR1* mRNA and protein in tumor tissues.^{11,12,37,38} The variability in expression of *EGR1* in tumor tissues may reflect the observed variable response of *EGR1* transcript levels to EGF treatment of different cell lines. The latter may be associated with hyperactivity of the EGF receptor, as it has been reported that PC3 cells express a constitutively active EGF receptor and thus are less responsive to an EGF stimulus.^{39,40} Conversely, LNCaP cells, which do not express a constitutively active receptor, exhibited dramatic increases in *EGR1* transcript levels, suggesting that these cells may still be highly sensitive to the EGF ligand.

To determine whether the ERK signaling pathway might be regulating the EGF-mediated *EGR1* transcriptional response, the pharmacological MEK inhibitor PD98059 was used. As seen in Figure 1C, PD98059 attenuated the EGF response by 98% in LNCaP cells and 88% in PC3 cells, decreasing the activation of *EGR1* transcription from 231-fold to 3.6-fold activation and from 57-fold to 7-fold activation, respectively. Phosphorylation of the downstream targets ERK and Akt was examined to determine the specificity of the inhibitor in LNCaP cells. As expected, EGF induced phosphorylation of EGF-receptor, and ERK and PD98059 inhibited ERK phosphorylation by nearly 93% (Fig. 1D, lanes 2 and 3, α -pERK). Interestingly, EGF did not induce Akt phosphorylation above basal levels in LNCaP cells (Fig. 1D, lanes 1 and 2, α -pAkt). A significant amount of basal pAkt signal was observed, likely due to the constitutively active PI3K pathway in LNCaP cells, which are PTEN deficient.⁴¹ Together, these results suggest that the activation of *EGR1* occurs through the ERK pathway.

These results are consistent with the role of ERK as the major regulatory mechanism of *EGR1* expression in other tissue types such as fibroblasts, keratinocytes, and hepatocytes.^{17,19,20} This mechanistic role is supported by results of MEK inhibition studies showing that in both LNCaP and PC3 cells, PD98059 treatment attenuated the activation of the *EGR1* promoter by EGF and effectively abrogated the induced increase in *EGR1* transcript levels. For completeness we also considered the PI3K pathway but obtained no evidence to support a significant role for PI3K in activation of *EGR1* expression. Although wortmanin treatment (500 nM) affected *EGR1* transcript levels, it also reduced pERK levels 2-fold (data not shown), suggesting that wortmanin effects on ERK pathway were responsible for its modest suppression of transcript levels. Taken together, these data support our hypothesis that ERK drives EGF-mediated regulation of *EGR1*.

Figure 1. EGF elevates EGR1 transcript levels via the ERK pathway in prostate cancer cells. **(A)** EGF activates EGR1 transcript levels in a time and dose dependent manner. LNCaP, CWR, and PC3 cells were serum deprived overnight and treated with 50 ng/mL EGF for the times indicated. RNA was isolated and assayed using RT-PCR to quantify EGR1 transcript levels. Values were normalized to 18S transcript levels and each experiment was done in triplicate. Data are represented as mean $\pm$ SEM ($n = 3$) fold change values relative to 1 h control for each cell type. **(B)** LNCaP cells were serum deprived overnight and treated with the indicated concentrations of EGF for 1 h. RNA was isolated and assayed as described above. Data are represented as mean $\pm$ SEM ($n = 3$) fold change values relative to 0 ng/mL EGF. **(C)** EGR1 transcriptional regulation is mediated by the ERK pathway in LNCaP and PC3 cells. Cells were pretreated with 50 μ M PD98059 for 1 h followed by treatment with 50 ng/mL EGF for an additional h. EGR1 transcript levels were measured as described above. Data are represented as mean $\pm$ SEM ($n = 3$) fold change values relative to control for each cell type. **(D)** Phosphorylation of ERK, Akt, and EGFR in LNCaP cells. LNCaP cells were serum-deprived overnight and pretreated with 50 μ M PD98059 for 1 h followed by treatment with 50 ng/mL EGF or vehicle (PBS) for 15 min. Protein lysates were separated by SDS-PAGE and probed with phosphospecific antibodies using standard Western blot techniques. Signal was detected with luminol reagent and a FujiFilm LAS-3000 detection system. Akt antibody was used as a loading control.

Analyses of the EGR1 promoter identified several SRE binding sites and the EGF-responsive region. As the SRF/Elk-1 complex (or TCF) is a well-known target of ERK, we hypothesized that Elk-1 might be regulating the EGR1 promoter at the predicted SRE sites. Locations of predicted SRF/Elk-1 binding sites (SREs) were identified within the EGR1 promoter using MatInspector. Predicted sites were identified as core (c), proximal (p), or distal (d) in relation to the EGR1 transcriptional start site within a luciferase promoter construct containing -1,260 to +35 of the human EGR1 promoter⁴² (Fig. 2A). Deletion constructs were created from the full-length EGR1 promoter to identify the EGF-responsive region. LNCaP and PC3 cell lines were

transiently transfected with each of 3 EGR1 promoter deletion constructs and assayed for luciferase activity (Fig. 2B and 2C). Although deletion of the distal region of SRE sites (-771 hEGR1) had no effect on EGF activation of the EGR1 promoter, deletion of the proximal SRE sites (-245 hEGR1) reduced activation levels to 3% and 22% of the full-length (-1,260 hEGR1) promoter in LNCaP and PC3 cells, respectively. These results indicate that the -771 to -245 bp region of the EGR1 promoter is essential to the EGF-mediated response and that the transcription factor(s) mediating that activity must have binding sites located within the EGF-responsive region. Furthermore, the distal sequence between -1,260 and -771 bp and the core region from -245

Figure 2. Transcriptional activation of the hEGR1 promoter is dependent on the proximal SRE sites and the Erk pathway in LNCaP and PC3 cells. **(A)** Schematic representation of the EGR1-luc promoter deletion constructs. Predicted Elk-1 and SRF transcription factor binding sites were identified using MatInspector (Genomatix, Munich, Germany) software. Clusters of core (c), proximal (p), and distal (d) SRF/Elk-1 sites are indicated. **(B)** Transcriptional activation of the hEGR1 promoter is dependent on the -771 to -245 region containing the proximal SRE sites. Cells were transfected with 500 ng of the appropriate luciferase construct, grown for 24 h, serum deprived overnight, and then treated with either vehicle control or 5 ng/mL EGF (LNCaP) or 50 ng/mL (PC3) for 5 h. **(C and D)** LNCaP and PC3 cells, respectively, were cultured as described above but pretreated with or without 50 μ M PD98059 for 1 h prior to transfection with 500 ng of the appropriate luciferase construct. Luciferase activity was measured and normalized to protein concentration. Data are reported as fold activation relative to vehicle treated -1,260 hEGR1 control. The data represents the average fold activation of relative light units $\pm$ SEM of 3 experiments repeated at least twice with similar results.

to +35 bp contributed little to EGF-mediated activity of the *EGR1* promoter.

The importance of the ERK pathway for transcriptional activation of the *EGR1* promoter was verified using the MEK inhibitor PD98059, which reduced *EGR1* promoter activity in both -1,260 hEGR1 and -771 hEGR1 constructs by ~80% in LNCaP cells (Fig. 2D) and 55% and 36% in PC3 cells (Fig. 2E) relative to the activity in the presence of EGF alone. These results are consistent with the analysis of

EGR1 mRNA expression and demonstrate that the MEK inhibitor attenuated the activity of the *EGR1* promoter within the -771 to -245 bp region. This also indicates that EGF activation of *EGR1* is transcriptionally mediated since both endogenous mRNA and an exogenous reporter constructs were stimulated in the presence of EGF. Taken together, these results indicate that the transcription factor mediating the EGF-response in both AS and AI PrCa cells is a downstream target of the ERK pathway and its binding

Figure 3. Chromatin immunoprecipitation analysis of the proximal region of SRE sites within the *EGR1* promoter in PC3 cells. **(A)** Arrows depict primers designed to flank the proximal region of SRE sites (–603 to –315 bp) within the *EGR1* promoter. SRF/Elk-1 sites are depicted as in Figure 3A. **(B)** Chromatin obtained from PC3 (top panel) and LNCaP (bottom panel) cells treated as described in Figure 1 was immunoprecipitated and amplified by PCR as described in the text. Lane 1, DNA marker. Immunoprecipitations in the absence (–) or presence (+) of EGF using 1 μ g of negative control IgG (lanes 2 and 3) or anti-Elk-1 Ab (lanes 4 and 5). Lanes 6 and 7 represent PCR amplification of 1% input chromatin (nonimmunoprecipitated). **(C)** Quantitation of Elk-1 binding at the proximal promoter of *EGR1* in PC3 cells by RT-PCR. Immunoprecipitated and control input DNA was quantified using SYBR-green master mix and the same primers as described in (A). Data were normalized relative to nonimmunoprecipitated input using adjusted Ct values and is represented as percentage of input DNA.

site is located in the proximal region of the *EGR1* promoter containing 3 SRE sites.

Others have also demonstrated that this cluster of SRE sites is functional although in other cell types and using different stimuli.^{20,43,44} However, it is necessary to examine the regulatory mechanism of *EGR1* in prostate cancer cells, as different stimuli appear to activate different promoter regions in other cell systems. Although this study did not determine which of the 3 SRE sites was necessary for the EGF response, Chen *et al.*⁴³ identified the most downstream of the 3 SRE sites, within the core promoter region, to be essential for estrogen-mediated response in MCF-7 cells and determined that both the TCF and SRF sites within this core SRE region were required. Aicher *et al.*⁴⁵ also found that deletion of the SRE within the core region (within –100 bp) abrogated activity of the *EGR1* promoter in L929 fibroblasts and to a lesser extent in 3T3 fibroblasts. Conversely,

in our studies, this core region responded weakly to EGF in prostate tumor cells. The focus of these studies was to identify the EGF responsive region of the *EGR1* promoter. Localization of the EGF responsive regions to the proximal promoter via deletion analysis did not identify the required bases within the SRE, but additional mutagenesis studies could be used to pinpoint which of the 3 potential SRE sites within the proximal promoter are required for EGF induction of *EGR1*. Overall, these studies provide evidence of the variability of *EGR1* regulation in different cell types and demonstrate that the extent of regulation by each SRE site is highly tissue specific.

Given that *EGR1* transcription is activated by the ERK signaling, we focused on the Elk-1 transcription factor as a potential regulator of the *EGR1* promoter. Elk-1, a member of the Ets family of transcription factors, is a well-described target of ERK signaling^{46–48} that regulates the promoters of other immediate early genes such as *c-fos* and *c-jun*.^{49,50} An *in vivo* analysis of the *EGR1* promoter using chromatin immunoprecipitation assays was performed to identify functional Elk-1 binding. Primers flanking the proximal SRE sites, –603 to –315 bp, within the *EGR1* promoter (Fig. 3A) were used to amplify chromatin immunoprecipitated with Elk-1 antibody (Fig. 3B). Chromatin immunoprecipitation of Elk-1 at the EGF-responsive region of the *EGR1* promoter indicated that Elk-1 was bound to native chromatin in LNCaP and PC3 cells. Although EGF had little effect on the binding of Elk-1 to the *EGR1* promoter in LNCaP cells (Fig. 3B, bottom panel), EGF enhanced binding of Elk-1 at the proximal SRE sites an average of 6-fold over control in PC3 cells (Fig. 3B, top panel, lanes 4 and 5) as determined by background correction using Image J analysis. Interestingly, a significant amount of Elk-1 was bound in the absence of EGF stimulation in both cell lines tested (Fig. 3B, lane 4), suggesting that Elk-1 may be constitutively bound in these cells. This differential immunoprecipitation was quantified by RT-PCR analysis of chromatin bound by Elk-1 in PC3 cells, normalized relative to input (nonimmunoprecipitated) chromatin (Fig. 3C). These results confirmed the enhanced binding of Elk-1 in the presence of EGF but also demonstrated a significant amount of bound Elk-1 in control cells.

Others have reported similar unstimulated Elk-1 binding at the *EGR1* promoter in HeLa cells⁴⁹; it is speculated that constitutive binding of Elk-1 to the promoter of target genes is necessary to elicit a rapid transcriptional response of immediate early genes such as *EGR1*. However, in LNCaP cells Elk-1 binding was unaffected by stimulus, suggesting that another transcription factor downstream of ERK signaling (e.g., SAP-1 or SAP-2), may be mediating the EGF response.

To assess whether Elk-1 was sufficient to activate the *EGR1* promoter, co-transfection experiments using the –771 hEGR1-luc reporter construct and Elk-1 expression

Figure 4. Activation of the -771 hEGR1 promoter is enhanced by Elk-1 expression and inhibited by a dominant negative Elk-1 in PC3 cells. Cells were grown and treated with EGF as previously described in Figure 2 above. **(A)** Cells were co-transfected with 250 ng of the -771 hEGR1-luc promoter construct and either 500 ng of full-length Elk-1, pcDNA3 empty vector, or Elk-1ΔDBD, containing a deletion of the Elk-1 DNA-binding domain. **(B)** PC3 cells were co-transfected with 250 ng of the -771 hEGR1-luc promoter construct and either 500 ng of pcDNA3 empty vector or the indicated concentrations of the Elk-En expression construct. Data are reported as fold activation relative to pcDNA3 control. Luciferase activity is represented as the average fold activation of relative light units $\pm$ SEM of 3 experiments repeated at least twice with similar results.

constructs were performed. In PC3 cells, the Elk-1 expression construct enhanced EGF mediated activation of the hEGR1 promoter by nearly 6-fold relative to activation of the empty vector, pcDNA3 (Fig. 4A). Consistent with the ChIP results seen in Figure 3, Elk-1 did not significantly affect the activity of the EGR1-luc promoter in LNCaP cells (data not shown). These differences between cell lines in response to exogenous Elk-1 may be due to high endogenous levels of Elk-1 in LNCaP cells, as we observed that the -771 hEGR1-luc promoter had high levels of activity even in the absence of EGF in LNCaP cells. To determine the necessity of the DNA binding domain, a construct with a deletion of amino acids 2-93 of the Elk-1 DNA-binding domain, Elk-1ΔDBD, was used. Deletion of the DNA binding domain reduced Elk-1-mediated activation by 36% in

PC3 cells, suggesting that Elk-1 binding at the Ets binding site was necessary for full activation of the *EGR1* promoter in response to EGF. However, given that there was only a partial attenuation of the *EGR1* response by the Elk-1 deletion construct without the DNA-binding domain, this suggests that there may be other indirect Elk-1 interactions, possibly affecting binding of SRF at the SRE site. Although the DNA binding domain was deleted in the Δ-DBD construct, the SRF interaction domain was retained, and thus SRF interactions could be maintained. Indeed, SRF has been shown to recruit Elk-1 to the SRE and is often constitutively bound at the SRE site in other immediate early gene promoters, such as *c-fos*.³³

To assess the necessity of Elk-1 expression for EGF induction of *EGR1*, a dominant-negative Elk-1 expression construct (Elk-En), containing a constitutive repression domain of the *Drosophila* Engrailed gene,⁵⁰ was co-transfected with the -771 hEGR1-luc promoter. In the presence of Elk-En, activation of the *EGR1* promoter was attenuated by more than 76% in PC3 cells relative to the pcDNA3 control vector with all concentrations of Elk-En tested (Fig. 4B). This indicates that *EGR1* is an Elk-1 target, as recruiting the repressor Elk-En to binding sites was sufficient to block EGF-mediated activation of *EGR1* expression. These results, along with the results from the Elk-1 overexpression experiment, indicate that Elk-1 is necessary and sufficient for *EGR1* transcriptional activity in PC3 cells.

Elk-En suppresses gene transcription through histone deacetylation and chromatin modification of Elk-1 target genes.⁵¹ As the dominant-negative Elk-En repressor strongly inhibited EGF-mediated activation of the *EGR1* promoter in PC3 cells, these results confirm that *EGR1* is indeed an Elk-1 target gene. Others have also shown that HEK-293 cells stably transfected with Elk-En inhibited *c-fos* and *EGR1* expression.⁵⁰ Taken together, overexpression of Elk-1 in addition to the use of a dominant-negative Elk-1 demonstrated that Elk-1 is both necessary and sufficient for *EGR1* transcriptional activation in PC3 cells.

In LNCaP cells, expression of the dominant-negative Elk-1 in both the presence or absence of EGF blocked EGR1 promoter activity (data not shown). These results are consistent with the ChIP data, which also demonstrated binding of Elk-1 with or without EGF in LNCaP cells. There are several possible explanation for these results. First, as previously discussed, Elk-1 may be constitutively bound in LNCaP cells and thus not affected by co-transfection of additional Elk-1. Second, it may be possible that SRF is the factor that is constitutively bound, and in the absence of stimulus, SRF is still able to recruit Elk-En to the *EGR1* promoter to repress transcriptional activity. Third, Elk-1 is highly homologous with the other TCF family members, SAP1 and SAP2/Net. As SRF can recruit any of the TCF family members,⁵¹ it is possible that in LNCaP cells another TCF is required for transcriptional activation

of the *EGR1* promoter. A recent study has identified *EGR1* as a transcriptional target of SAP1 in thymocytes.⁵² Moreover, the Elk-En construct is known to block binding by SAP-1 and SAP-2 as well as Elk-1 in 293 cells.⁵⁰

Further studies are warranted to determine the mechanistic differences in EGF mediated activation of *EGR1* expression in hormone sensitive (e.g., LNCaP) versus refractory (e.g., PC3) cells. This type of comparison may shed light on mechanisms of prostate cancer progression and may partly explain the varied levels of EGR1 protein in tumor tissues of different grade and stage. This variation may be important as elevated EGF signaling in prostate cancer indicates high risk of progression to a hormone refractory state,^{24,25,53} and so elevated levels of *EGR1* should also indicate a poor prognosis. Certainly most evidence in prostate cancer is consistent with *EGR1*'s role as a growth promoter.^{11,12,37,54-56} Thus, delineating the mechanisms of regulation of *EGR1* by EGF in prostate cancer cells is important, as it will shed light on the dysregulated EGF signaling pathway in hormone refractory tumors.

Materials and Methods

Tissue culture. LNCaP and CWR22rv1 prostate tumor cells were grown in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum and 100 µg/mL penicillin/streptomycin. PC3 tumor cells were grown in DME/F12 (Sigma) supplemented with 10% fetal calf serum and 100 µg/mL penicillin/streptomycin. All cells were maintained in 5% CO₂ at 37 °C until 80% to 90% confluent and then serum-deprived overnight in 0.1% fetal calf serum before treatment. Both treated and untreated cells were grown in parallel until the time of collection.

EGF and MEK inhibitor treatments. Cells were treated with 50 ng/mL EGF for 1 h unless otherwise indicated in the figure legends. For inhibitor studies, cells were pretreated for 1 h with 50 µM PD98059 (EMD Biosciences/Calbiochem, Gibbstown, NJ) prior to treatment with EGF. In all experiments, treatments were in the presence of 0.1% fetal calf serum. Cells were harvested for RNA and protein analysis as described below.

RNA isolation and quantitative PCR. Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO). Following quantification, RNA samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the TaqMan Universal Master Mix and optimized *EGR1* TaqMan probes (Hs00152928_m1). The 18S housekeeping gene (Hs99999901_s1) was used as an internal control, and all reactions were performed in triplicate. The comparative Ct method ($2^{-\Delta\Delta Ct}$)⁵⁷ was used to analyze gene expression

differences between control (untreated) cells and cells treated with EGF alone or with an inhibitor. Test of significance were done using analysis of variance and a Tukey-Kramer *post hoc* test.

Western blot analysis. Subconfluent monolayers of cells were washed in ice-cold PBS and lysed using RIPA buffer (1% NP40, 0.5% Na deoxycholate, 0.1% SDS, and 150 mM NaCl) containing a protease inhibitor cocktail (Pierce, Rockford, IL). Lysates were flash frozen at -80 °C for mechanical lysis and centrifuged to remove cellular debris. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). Fifty micrograms of protein was separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked in 5% nonfat dry milk and incubated in primary antibody (pERK [Thr202/Tyr204], pAkt [Ser473], EGFR [Tyr1068], or Akt, Cell Signaling, Danvers, MA; EGR1, Santa Cruz Biotechnology, Santa Cruz, CA; β-actin, Santa Cruz) at 4 °C overnight, followed by incubation in anti-rabbit HRP-conjugated secondary antibody (GenScript, Piscataway, NJ). Proteins were visualized by chemiluminescent detection using a Fuji LAS 300 detection system.

***EGR1* promoter and Elk-1 expression constructs.** The human *EGR1*-luciferase promoter construct (pEGR1260/LUC; hEGR1) containing -1,260 bp to +35 bp of the human *EGR1* promoter was a kind gift from Dr. Thomas Eling.⁴² The pcDNA-Elk-1 (Elk-1) expression construct was provided by Dr. Frederick Stanley. The pcDNA3 and Elk-1 ΔDBD were provided by Dr. Mark Perrella.⁵⁸ Dr. Andrew Sharrocks provided the dominant-negative Elk-En expression construct, which consists of the full-length Elk-1 (1-428 a.a.) fused to the Drosophila En repression domain (2-298 a.a.).⁵¹

Transfection and luciferase assays. Cells were grown in 12-well plates until 70% to 80% confluency. Just prior to transfection, standard full-serum medium was replaced with serum-free, antibiotic-free medium to enhance transfection efficiency. For each well, a 1:3 ratio of plasmid DNA to lipid reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA) was used. Unless otherwise indicated, 500 ng of reporter construct was used per well. Cells were transfected with the lipid reagent and plasmid for 5 h, after which the transfection medium was replaced with full-serum medium. Cells were grown for 24 h, serum deprived in 0.1% FCS overnight, and then treated with EGF for 5 h with or without 1-h pretreatments with PD98059. All inhibitor experiments used 50 µM PD98059; EGF concentrations used are indicated in figure legends. Cell lysates were harvested using Promega (Madison, WI) PLB lysis buffer and analyzed for luciferase activity by the Promega luciferase assay system and a Turner luminometer. Promoter

activity was indicated by relative light units (RLU) produced by luciferase; RLU values were normalized to protein concentrations for each well.

Creation of hEGR1 deletion constructs. Using sequence analysis, internal restriction sites were found within the hEGR1 promoter. Two SmaI sites, located at -1,170 bp and -771 bp, and 1 SacI site, located at -245, were identified. Restriction digests were prepared using standard conditions, and plasmid DNA was extracted and purified using the QIAquick gel extraction kit (Qiagen, San Diego, CA) followed by an additional sodium acetate precipitation of DNA. DNA quantity and quality were checked by absorbance. Ligation reactions were prepared using T4 DNA ligase (New England Biolabs, Ipswich, MA), and ligated products were transformed and selected using *Escherichia coli* DH5 α competent cells following standard techniques. The content of each plasmid was verified using sequence analysis. The human EGR1 promoter sequence was obtained from the Ensembl website (www.ensembl.org) and analyzed for predicted transcription factor binding sites using Transfac/MatInspector (Genomatix, <http://www.genomatix.de>).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed as per the manufacturer's recommendations, using the Millipore EZ-Magna ChIP kit (Millipore, Billerica, MA). Briefly, LNCaP and PC3 cells were grown to 80% to 90% confluency, serum-deprived overnight in 0.1% fetal calf serum, then treated for 30 min to 1 h with 5 ng/mL (LNCaP) or 50 ng/mL (PC3) of EGF. Proteins and chromatin were cross-linked in a 1% solution of formaldehyde, followed by quenching with glycine. Fixed cells were sonicated using 6 cycles of 20 sec of sonication followed by 60 sec on ice using a Microson Ultrasonic Cell Disruptor. Sheared DNA was analyzed by gel electrophoresis, and chromatin within the range of 200 to 1,000 bp was used for further analysis. Sheared chromatin was suspended in protein G magnetic beads with 1 μ g of the appropriate antibody (anti-IgG, Millipore; anti-Elk-1, Epitomics; Burlingame, CA) overnight at 4 °C with rotation. Beads were washed per the manufacturer's recommendations, and immunoprecipitations were incubated with proteinase K to reverse cross-linking. Eluted chromatin was purified using DNA purification columns, and the promoter region was amplified by PCR as per the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Primers were designed to flank an Elk-1/SRF cluster containing 4 Elk-1 binding sites from -603 to -315 bp of the EGR1 promoter and were comprised of the following sequences: Forward, 5' GCCATATAAGGAGCAGGAAGG; Reverse 5' CTTCTTCCCTCCTCCAGAG. The following standard PCR conditions were used: 94 °C for 2 min, 36 cycles of 94 °C for 20 sec, 56 °C for 30 sec, 72 °C for 30 sec, and a final extension at 72 °C for 2 min. PCR

products were electrophoretically separated and visualized in an ethidium bromide 1.5% agarose gel.

For quantification of genomic DNA by RT-PCR, samples were analyzed using an ABI 7000 thermocycler, and Ct values were measured by the ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA). Each PCR reaction was carried out in triplicate using the SYBR Green master mix. The amount of DNA immunoprecipitated in the presence of EGF with the target antibody was calculated in comparison to total input (nonimmunoprecipitated) DNA using Ct values normalized to the control samples.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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