Egr1 promotes growth and survival of prostate cancer cells: identification of novel Egr1 target genes

Thierry Virolle, Anja Krones-Herzig†, Veronique Baron†, Giorgia De Gregorio†^, Eileen D Adamson* and Dan Mercola†

The Burnham Institute, La Jolla Cancer Research Center, 10901 North Torrey Pines Road, La Jolla, California 92037, USA;
†Sidney Kimmel Cancer Center, San Diego, California 92121, USA and Cancer Center, University of California at San Diego, La Jolla, CA 92093;
^Istituto di Ricovero e Cura a Carattere Scientifico, Neuromed, Pozzilli, 86077, Italy

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*Corresponding Author
Thierry Virolle,
Biologie et Physiopathologie de la peau
INSERM
Faculté de Médecine de NICE
av de Valombrose
NICE 06107
FRANCE
Phone: 011 33 4 93 37 76 48
Fax: 011 33 4 93 81 14 04
virolle@unice.fr
Abstract
In the majority of aggressive tumorigenic prostate cancer cells, the transcription factor Egr1 is over-expressed. We provide new insights of Egr1 involvement in proliferation and survival of TRAMP C2 prostate cancer cells by the identification of several new target genes controlling growth, cell cycle progression and apoptosis such as cyclin D2, P19ink4d and Fas. Egr1 regulation of these genes, identified by Affymetrix microarray, was confirmed by real-time PCR, immunoblot and chromatin immunoprecipitation assays. Furthermore we also showed that Egr1 is responsible for cyclin D2 over-expression in tumorigenic DU145 human prostate cells. The regulation of these genes by Egr1 was demonstrated using Egr1 antisense oligonucleotides that further implicated Egr1 in resistance to apoptotic signals. One mechanism was illustrated by the ability of Egr1 to inhibit CD95 (Fas/Apo) expression, leading to insensitivity to FasL. The results provide a mechanistic basis for the oncogenic role of Egr1 in TRAMP C2 prostate cancer cells.

Introduction
Prostate cancer is the most common malignancy in men and a frequent cause of cancer death. The mortality of this disease is due to metastasis to the bone and lymph nodes. Prostate cancer progression is thought to proceed from multiple defined steps through prostatic intra-epithelial neoplasia (PIN), invasive cancer, and progression to androgen-independent and refractory terminal phase (44, 50). A large fraction of early onset, and up to 5-10% of all prostate cancer patients, may have an inherited germline mutation that has facilitated the onset of carcinogenesis. However, in the majority of cases, no inherited gene defects are involved, and cancer arises as a result of a series of acquired somatic genetic changes affecting many genes on several chromosomes. Although the molecular mechanism of prostate cancer progression remains largely unknown, a few genes such as E-cadherin, α-catenin, TGF-β and insulin-like growth factors I and II (IGFs), have been shown to be aberrantly expressed and are markers of prostate cancer (34, 69). To clearly understand the multistep progression of this disease many other genes remain to be identified.

One of the over-expressed genes found in prostate cancer tissue is the transcription factor Early growth response gene 1 (Egr1) (18, 62). This gene could have an important function because its expression level increases with the degree of malignancy as measured by the Gleason grade of the tumor (18). This seems to be specific to prostate tumor cells because in
mammary and lung tumors, as well as most normal tissues, Egr1 expression is low. Egr1 over-expression is correlated with the loss of its co-repressor NAB2 in primary prostate carcinoma. This disruption of the balance between Egr1 and NAB2 expression results in a high Egr1 transcriptional activity in prostate carcinoma cells (1). A recent study based on the cross breeding of Egr1−/− mice with TRAMP mice showed significantly delayed prostate tumor formation in the Egr1-deficient TRAMP mouse compared to TRAMP-Egr1+/− mice (2). The TRAMP mouse is a well known model of prostate cancer (20) in which tumors progress to metastases in a window from 8 to 24 weeks of age. Although Egr1 loss did not appear to prevent tumor initiation, Egr1 deficiency delayed the progression of prostate tumors in these mice. Significantly, several gene products associated with aggressive prostate cancer such as TGF-β and insulin-like growth factor II (37, 60) have been identified as regulated by Egr1. These observations strongly suggest that Egr1 is involved in prostate cancer progression despite its known role as a tumor-suppressor in several other types of human cancers (29).

In this present study on the role of Egr1, we have used the tumorigenic C2 prostate cancer cell line which was established from a prostate tumor from a single TRAMP mouse tumor. These tumorigenic cells express a high constitutive level of Egr1 protein. Transcriptional regulation by Egr1 was assessed using Affymetrix array technology. The unique step used here was to perform a microarray analysis using cells rendered deficient in Egr1 as the comparison sample for the identification of Egr1 target genes, in prostate cancer cells. The results provide new insight into the involvement of endogenous Egr1 in proliferation and survival of prostate cancer cells by the identification of several new target genes specifically controlling growth, cell cycle progression and the apoptosis pathway.

Results and Discussion

AS antisense oligodeoxynucleotide efficiently inhibits Egr1 expression

To examine the functional significance of Egr1 over-expression in prostate cancer cells, we inhibited its expression using an antisense oligonucleotide (AS) in TRAMP C2 prostate cancer cells. To assess the efficiency and the specificity of AS, we performed western blot analyses of the protein expression of Egr1 and other Egr family members, Egr2, Egr3 and wt1, 24 h after transfection of the antisense and control oligonucleotides (Fig. 1A). As seen in Figure 1A, the antisense oligonucleotide strongly decreased Egr1 expression, while there was no effect on Egr2 and wt1 expression. Egr3 seems to be slightly increased when Egr1 was
inhibited. In contrast, the control oligonucleotide (ctl) did not alter the protein expression pattern of the cells. These results demonstrated that a 24 h treatment with a low concentration, 0.1 µM, of the AS oligonucleotide efficiently and specifically inhibited Egr1 expression. To examine the time course of Egr1 inhibition in C2 cells, proteins were extracted each day for 6 days following AS transfection of antisense and control oligonucleotide-treated cells. Egr1 expression in the presence of AS was undetectable from day 1 to day 3, became detectable on day 4, and was fully restored on day 5 to day 6 (Fig 1 B, top panel). As expected, the use of the control oligonucleotide (ctl) did not change Egr1 expression level (Fig 1B, bottom panel). These results show that AS is stable enough over 3 days to allow almost complete and specific inhibition of Egr1 expression for a prolonged period following a single treatment.

_Egr1 contributes to the control of proliferation_

To determine the involvement of Egr1 in the proliferation rate of C2 cells, the growth of the cells in which Egr1 expression was inhibited by AS oligonucleotide (C2-AS), was compared to the control corresponding to C2 cells transfected with control oligonucleotide (C2-ctl). Briefly the cells were transfected at day 0 with either AS or ctl and the proliferation rate was directly assessed every day until day 6 by cell counting (Fig 2A). As seen in Figure 2A, the proliferation rate of C2-AS cells was strongly reduced during the first three days after transfection and started to rise again on day four. Between day 4 and 5 the slope of the proliferation curve was approximately equal to the slope of the control (C2-ctl cells) indicating that the cells recovered their expected proliferation rate (Fig 2A). The proliferation time course was well correlated to the pattern of Egr1 inhibition seen in Figure 1B. Indeed, as long as Egr1 expression was inhibited, the proliferation rate of C2 cells was markedly reduced and then resumed as soon as Egr1 expression recovered. In addition, comparison between C2-AS and C2-ctl cells in a colony forming assay showed 74% fewer colonies in C2-AS (average of 32 colonies +/-6 for C2-ctl versus 8,3 colonies +/-3 for C2-AS), suggesting that the tumorigenicity of the cells may decrease when Egr1 is inhibited (Fig 2B). Furthermore, cell cycle analysis by FACS, performed at day 2 after transfection, showed fewer cells (about 11% less) in G1 phase of C2-ctl cells than C2-AS cells (data not shown). The sum of results strongly argue in favor of a role for Egr1 in the control of growth and cell cycle progression in prostate cancer cells.
Identification of Egr1 target genes by Affymetrix microarray hybridization

To determine the genes that are involved in Egr1-mediated transformation, comparative analyses of mRNA populations from C2 cells one day after transfection with AS or with ctl oligonucleotide were performed using Affymetrix microarray hybridization. Affymetrix analysis revealed a large number of genes (at least 180) involved in the control of proliferation, death and malignant progression. Most of these had not previously been identified as part of an Egr1 signaling pathway. Although many genes are direct Egr-1 target genes, other could be indirectly regulated by Egr-1 or modulated after the change of the physiological behavior of the cells due to the inhibition of Egr-1 expression. However it is important to consider that those genes could be as important as the direct target genes to maintain, potentiate or regulate Egr-1 effect. Genes displaying the highest Affymetrix expression changes upon treatment with AS are listed in Table 1.

To confirm the Affymetrix microarray analysis results, the expression of some genes listed in Table 1 was independently tested by quantitative real time RT-PCR. In these experiments, total RNA extracts from Egr1 expressing and non-expressing C2 cells were used as templates. The fold induction/repression calculated from real time RT-PCR assays compared to the corresponding ratio determined in the Affymetrix analysis (Table 2), produced remarkable concordance. The induction or repression of specific target genes by Egr1 was in the same direction in all cases examined and commonly exhibited a similar degree of change. Indeed, the Pearson Correlation Coefficient of the Affymetrix and real-time PCR results was 0.78 which is significant ($p = 0.008$, Chi Square). These results confirm the reliability of the Affymetrix analysis.

Examination of Table 1 reveals several candidate genes already identified as Egr1 targets, such as transforming growth factor beta 1 (37, 60) and CD95 (15). Expression of other genes such as transcription factor LRG-21/Atf3 and cyclin D2 is known to be correlated with an increase of Egr1 expression (22, 35). Furthermore, several genes identified here, have been directly linked to human prostate cancer. Indeed, inhibitor kappa B alpha (IκBα) was shown in several prostate cancer cell lines to inhibit growth, angiogenesis and metastasis by inhibition of NF-κB activity (31). Mad by interacting with Max, is known to prevent the transforming effect of Myc by inhibition of the Myc/Max association (8). In addition, Myc is often found to be over-expressed in prostate cancer cells (48). Accordingly, Egr1 could
promote Myc-induced transformation by down-regulation of Mad expression. Apolipoprotein D secretion is associated with steroid-induced inhibition of cell proliferation in the LNCaP human prostate cancer cell line (59). Expression of this protein is low in prostate cancer cells and can be modulated by steroid hormones and other factors involved in the control of cell proliferation (59). Caspase 7 and CD95 (Fas/APO) are known to be involved in the apoptotic response in various prostate cancer cell lines (9, 40). Interestingly all these genes which behave as tumor suppressors are down-regulated by Egr1 in C2 prostate cancer cells. On the other hand, genes like IGFBP-4, which stimulates cell proliferation in ALVA31 and M12 human tumor prostate cells (16), and TGF-β1, which is strongly expressed in prostate cancer cells (71, 72), are up-regulated by Egr1.

In summary, the genes that are involved in cell cycle progression, malignant transformation or inhibition of apoptosis are all up-regulated by Egr1 while those involved in growth inhibition and apoptosis are repressed (Table 1). Hence constitutive expression of Egr1 in prostate cancer affects the balance between survival and tumor suppression.

**Characterization of Egr1 regulation**

As seen in Figure 1B, efficient inhibition of Egr1 expression occurred for three days after AS transfection. Thus, a similar time course of expression should be expected for Egr1 target genes. Therefore, mRNA expression of cyclin D2 and G-alpha-12 protein, both known to stimulate growth and cell cycle progression (4, 68), p19ink4d and cyclin G2, which inhibit cell cycle progression (26, 27), were measured daily from day 0 to day 6 by real time quantitative RT-PCR. Cyclin D2 and G-alpha-12 mRNAs expression were drastically inhibited from day 1 to day 3 when Egr1 is inhibited and resumed as soon as Egr1 expression was normal (days 4 to 6) (Fig. 3). Similarly, synthesis of cyclin G2 and p19ink4d mRNAs were increased until day 3 when Egr1 expression was low (Fig. 3) and normal expression was restored on day 4. These results were not observed upon treatment with the ctl oligonucleotide, demonstrating that Egr1 expression is absolutely required for full mRNA expression of cyclin D2 and G-alpha-12 and to repress p19ink4d and cyclin G2 mRNA synthesis. The duration of this regulation (at least 3 days) demonstrates that no other transcription factor compensates for the lack of Egr1 function in these cells.

To test whether mRNA regulation mediated by Egr1 is reflected at the protein level, immunoblotting analysis was performed on proteins extracted from C2-AS and C2-ctl cells.
from day 0 to day 6 after transfection. In these experiments cyclin D2 and p19\textsubscript{ink4d} protein expression level was assessed. The results showed a time-dependent repression of cyclin D2 and an increase of p19\textsubscript{ink4d} protein expression (days 1 to 3) (Fig. 4A) in antisense treated cells, which matched the time course of their mRNA expression patterns (Fig. 3). These results confirm that Egr1 inhibition by antisense is efficient enough to modulate Egr1 target gene expression at the protein level. In addition, the cyclin D2 and p19\textsubscript{ink4d} time-dependent protein expression patterns (from day 0 to day 6) are also highly correlated to the difference found in the cell cycle analysis and in the proliferation rate (Fig. 2A) between C2-AS and C2-ctl cells. This finding corresponds to their activities in the regulation of cell cycle progression. Thus Cyclin Ds are required for cell cycle progression and over-expression of INK4 family proteins is responsible for G1 phase arrest (54, 55). Interestingly, cyclin D2 is found to be upregulated by Egr1 while p19\textsubscript{ink4d} expression, a cyclin D2-dependent kinase inhibitor (5), is repressed. Therefore Egr1, by reciprocally regulating the levels of p19\textsubscript{ink4d} and cyclin D2, would stimulate cell cycle progression and play a pro-survival role in prostate cancer cells.

To test the generality of these results, we examined p19\textsubscript{ink4d}, cyclin D2 and G-alpha-12 protein expression by real time RT-PCR in the human prostate cancer cell line, DU145, transfected either with AS or ctl oligonucleotide. As in C2 cells, Egr1 expression is constitutively high in DU145 and strongly inhibited by the antisense oligonucleotide (Fig. 4C, left panel). In DU145, Egr-1 regulation of these genes appeared to be the same that the regulation observed in the C2 mouse model (Fig. 4B). Furthermore Cyclin D2 protein expression is also strongly repressed during the inhibition of Egr1 expression, indicating that Egr1 is required to maintain cyclin D2 protein expression level in DU145 as well as in mouse TRAMP C2 cells (Fig. 4C, left panel). In order to examine Egr1 and cyclin D2 expression during human prostate cancer progression, we tested three additional cell lines, normal 267B1 prostate epithelial cells, low tumorigenic P69 cells and aggressively tumorigenic DU145 human prostate cells. While Egr1 expression is similar in normal human prostate 267B and P69 cell lines, it is over-expressed in DU145. Thus cyclin D2 expression correlates with Egr1 expression in these cell lines and are strongly expressed only in the aggressive tumorigenic DU145 cells (Fig. 4C, right panel). These results support the relevance of C2 cells as a model to identify new Egr1 target genes in prostate cancer.

\textit{Egr1 desensitizes the cells to Fas L induced apoptosis}

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Egr1 may also play a role in promoting prostate cancer by affecting prostate cell survival (30) or apoptosis (65) and this was tested next. C2-AS and C2-ctl cells were UVC irradiated and dead cells were counted by trypan blue staining 24 and 48 h later. While less than 20% of the C2-ctl cells were dead 24 h following irradiation, almost 50% of C2-AS cells were dead (Fig. 5A). Furthermore, at 48 h following irradiation, less than 50% of control cells versus 95% for C2-AS cells had died (Fig. 5A). These differences demonstrate a critical role for Egr1 in response to stress. Indeed, endogenous expression of Egr1 is not only required for full proliferation of C2 cells but also to decrease sensitivity to radiation, a widely observed phenomenon of human prostate cancer cells (28).

Affymetrix analysis (Table 1) revealed several genes that are downregulated by Egr1, such as caspase 7 (6, 40), Bcl-2-binding protein homolog Nip3 (10) and CD95 (Fas antigen) (9), a gene widely involved in apoptosis pathways. CD95, a member of tumor necrosis factor receptor family, is referred as “death receptor” because of its ability to transduce death signals. On the other hand, the gene PS-2short (upregulated by Egr1, see table 1) is involved in inhibition of Fas mediated apoptosis (66, 67), therefore supporting a role for Egr1 as anti-apoptotic agent in prostate cancer cells.

Egr1 regulation of CD95, although confirmed at the mRNA level by real time PCR (Table 2), was also tested for protein expression in C2-ctl and C2-AS cells treated or not by UVC irradiation. In C2-ctl cells, UVC treatment lead to a significant increase of Egr1 expression, which was strongly inhibited by AS (Fig. 5B, C2-AS). CD95 expression appeared to be undetectable in C2-ctl-treated cells but was clearly expressed in C2-AS-treated cells (Fig. 5B). After UVC treatment CD95 expression was strongly increased in C2-AS while it was only slightly expressed in C2-ctl (Fig. 5B). These results confirm at the protein level the efficient inhibition of CD95 expression by Egr1. This mechanism of repression is all the more relevant since it is still effective even after a strong stress stimulus.

In order to assess whether this difference in basal CD95 expression could be reflected as responses to Fas L mediated apoptosis, we treated C2-ctl and C2-AS cells for 9 and 18h with Fas L, and counted the percentage of dead cells by Trypan Blue staining. As expected from CD95 protein expression profile (Fig. 5B), C2-AS were more sensitive to Fas L mediated apoptosis. Indeed, at 9 h after treatment, 52% of cells were dead in C2-AS versus 18.5% in C2-ctl cell cultures (Fig. 5C). This difference in the resistance to cell death between C2-AS
and C2-ctl cells, although lower, was still present after 18h treatment, with 96% of dead cells compared to 60%, respectively (Fig. 5C). Therefore high constitutive Egr1 expression delays apoptosis of prostate cancer cells mediated by Fas L, in part by down-regulating CD95 expression. The significance of the CD95 signaling pathway in prostate apoptosis has also been demonstrated in the normal rat prostate following castration (14). In addition, further studies have demonstrated the involvement of CD95 in sensitizing prostate cancer cells to undergo apoptosis after chemotherapeutic agent or irradiation treatments (12, 33). These results illustrate well a “desensitizer role” of Egr1 in the cell death response and suggest that sensitization to Fas mediated apoptosis by the inhibition of Egr1 expression could become an attractive therapeutic mechanism. Furthermore this experiment presents corroborating evidence that the modification of gene expression by Egr1 is a major player in the pathological responses of prostate cancer cells.

\[ p19^{\text{ink4a}}, \text{Mad, CD95 and cyclin D2 are directly transcriptionally regulated by Egr1} \]

Gene chip and real time PCR technologies are powerful and sensitive enough to accurately evaluate the differential expression between two mRNA populations, but do not determine if the regulation by Egr1 occurs directly or indirectly. Therefore, we performed chromatin cross-linking and immunoprecipitation assays (ChIP) to screen upstream regulatory sequences of five examples of putative Egr1 target genes indicated by the Affymetrix analysis. For this experiment untransfected, AS and Ctl oligonucleotides transfected C2 cells were used as template. After chromatin cross-linking in living cells, Egr1 became covalently fixed to its DNA target. These captured target DNA fragments were then recovered by specific Egr1 immunoprecipitation and purification. Non-immune serum immunoprecipitation was used as the negative control and C2 genomic DNA was used to assess amplification efficiency of each primers pair. Primers were designed to specifically recognize 5’ regulatory sequences of \[ p19^{\text{ink4a}}, \text{Mad, CD95, cyclin G2 and cyclin D2} \], in order to detect their presence in the captured DNA fragments by polymerase chain reaction. 5’ regulatory sequence analysis of each of these genes showed several putative Egr-1 and Sp-1 binding sites. \[ p19^{\text{ink4a}}, \text{Mad, CD95 and cyclin D2} \] yielded an amplified product from untransfected (Mock) (Fig. 6A) and Ctl oligonucleotide transfected template (Fig. 6B), that showed the same migration pattern as the genomic control input while Cyclin G2 was not detected (Fig. 6 A & B). Since no amplification was found for the control non-immune serum template (Fig. 6A) and the AS oligonucleotide transfected template (Fig. 6B), these results indicate that the successfully
amplified fragments were bound by Egr1 in vivo and therefore indicate the direct regulation of p19<sup>ink4d</sup>, Mad, CD95 and cyclin D2 by Egr1. Furthermore, to rule out the possibility that these genes could be regulated in consequence of the inhibition of the proliferation we performed a kinetic of regulation at early time in parallel of TGF-β1 a well known Egr-1 target gene (73). Since AS oligonucleotide is efficient at 5 hours after transfection (data not shown), we performed the kinetic analysis at 5 h, 10 h and 15 h. As for TGF-β1, the modulation of cyclin D2 and p19<sup>ink4d</sup> expression occurred at 5 hours after AS addition corresponding to the onset of Egr-1 efficient inhibition (Fig. 6C). Taken together these results indicate that many of the Egr1 target genes identified in our study may be regulated directly by Egr1.

Conclusions

Our study provides new insight on the activities and mechanisms of Egr1 in prostate cancer cells. We propose that Egr1 promotes cell growth and desensitization to death by regulating a set of genes known to be very important in cell cycle progression, growth and apoptosis. Therefore, constitutive Egr1 expression observed here in prostate cancer cells is likely to promote both tumor cell growth and progression. We suggest that our results extend the findings of Milbrandt and co-workers (2) in that they indicate the mechanistic basis of the role of Egr1 in cancer growth as well as progression. Our study confirms for the first time in prostate, the growth enhancer role of Egr1 previously observed in other cellular systems such vascular smooth muscle and rat kidney tumor cells (19, 49). However, these roles are tissue specific because in breast cancer, fibrosarcoma and glioblastoma, Egr1 behaves as a tumor suppressor gene (7, 29) that can be required for maximal sensitivity to irradiation (3, 65). Further comparisons of the identity and the regulation of Egr1 target genes from these different tissues will explain this functional discrepancy.

Materials and Methods

Cell culture and transfection condition

C2 TRAMP cells were grown as described elsewhere (20). The cells were seeded into 35 mm dishes at a density of 100,000 cells per well one day before transfection. The transfection was performed as described by the manufacturer with the GenePorter reagent (16 µl) (Gene Therapy Systems, INC, San Diego, CA) and 0.1 µM of antisense oligonucleotide (AS or ctl).
Sequences of the antisense (AS) and mismatch control oligonucleotide (ctl) are 5’-AGC GGC CAG TAT AGG TGA-3’ and 5’-AGC GGA CAC TCT AGG CCA-3’ respectively. The sequence of Ctl oligonucleotide corresponds to AS sequence with 4 bases mutated (underlined).

**Proliferation assay, cell death measurement**

One day before transfection the cells were seeded in duplicate into 35 mm dishes at a density of 70,000 cells per dish. At day 0 cells were transfected as described above. 4 h later the cells were harvested for counting and for protein and total mRNA extraction. This procedure was repeated each day after transfection according to a time course from day 0 to day 6.

The day after transfection, the cells were ultraviolet-C (UVC) irradiated (40J/m²) in a Stratalinker (Stratagene, La Jolla, CA) or treated with 100 ng/ml of Fas L recombinant protein (Oncogene Research Products, Darmstadt, Germany). One or two days after UVC irradiation or 9 h and 18 h after Fas L treatment, detached and trypsinized cells were pooled and incubated with 0.2% trypan blue to determine the percentage of dead cells.

**Colony forming assay**

C2 cells were transfected as described above. After 16 h the cells were counted and seeded into 6 well plates (200 cells/well) in RPMI medium with 0.1 µM of antisense oligonucleotide. After 8 days incubation at 37° C, the colonies were stained with 2% crystal violet.

**Oligonucleotide micro-array analysis**

The protocol recommended by Affymetrix (http://www.affymetrix.com) was used for mRNA quality control and gene expression analysis from C2 cells transfected either with AS or ctl oligonucleotides. The probes were hybridized to Affymetrix MGU75Av2 arrays representing approximately 12,000 mouse transcripts. Detailed protocols for data analysis and documentation of the sensitivity, reproducibility and other aspects of the quantitative microarray analysis using Affymetrix technology were used as reported (39).

**Quantitative real time one step RT-PCR and western blot**

mRNA expression level was quantified by real time one step RT-PCR using the LightCycler-RNA-Amplification Kit SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. A standard curve from several dilutions of a sample of total RNA was established to calculate the relative amount of each gene. Values
were then normalized to the relative amounts of glyceraldehyde-3-phosphate dehydrogenase determined from a similar standard curve. Each gene was amplified using the appropriate specific primers (sequences available upon request).

For the western blot analysis, proteins were blocked and reacted with antibodies to Egr1 (C19, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), mouse and human cyclin D2 (sc-593 and sc-181, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), p19\textsuperscript{ink4d} (sc-1063, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) or CD95 (Anti-mouse Fas/TNFRSF6 (CD95) Antibody, R&D systems, Inc, Minneapolis, MN).

**Chromatin immunoprecipitation assay**

In order to crosslink protein on DNA targets the cells were incubated in 1% formaldehyde at 4°C during 30 min. After extraction as described elsewhere (13), the chromatin was fragmented by sonication to obtain an average size of 1.5 Kb DNA fragment. The DNA fragments mix was then immunoprecipitated using a specific Egr-1 antibody and a non-immune serum as a negative control. After crosslink reversal as described elsewhere (13), the screening for identification of the regulatory sequence of captured Egr1 target genes was performed by PCR using the following primers located in the 5' regulatory sequences of the genes: p19\textsuperscript{ink4d} Forward 5'-ctggtcgctgcacgctgac-3' ; Reverse 5'-agtggataccggtggactgt-3' (respectively –599 and -1 from the ATG), cyclin D2 Forward 5'-ggcgagctgaggagagccg-3' ; Reverse 5'-ctccatagccagccggcca-3' (respectively –269 and +6 from the ATG), cyclin G2 Forward 5'-ccagcatcccccaagctact-3' ; Reverse 5'-cttcatctgcagcaaatacacc-3' (respectively –601 and +6 from the ATG), Mad Forward 5'-aagcggccggtggcccgc-3' ; Reverse 5'-gctgtcgccatcctgcacc-3' (respectively –48 and +11 from the ATG), CD95 Forward 5'-cagtggtagctagctgggtt-3' ; Reverse 5'-gacagccagatcagccagcat-3' (respectively –272 and +345 from the ATG),

Genomic DNA input was used as a control for the amplification efficiency of each primers pair. Non-immune immunoprecipitated DNA and DNA immunoprecipitated from AS transfected C2 cells were used as negative controls. The amplified products were resolved on 2.7 % agarose gel.

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FIGURE LEGENDS

Figure 1. Inhibition of Egr1 expression by E5 oligonucleotide antisense.
(A) C2 cells were transfected with the control oligonucleotide (ctl), the antisense oligonucleotide (AS) or carrier alone (M) for 4 h. After 24 h the cells were lysed and samples were analyzed by western-blotting with antibodies to Egr1. Membranes were reprobed successively with antibodies to Egr-3, Egr-2, WT-1 and β-actin as internal control.
(B) Proteins were extracted every day for 6 days following AS (C2-AS) and ctl (C2-ctl) transfection. Samples were analyzed by western-blotting with antibody to Egr1, and antibody to β-actin to control for protein loading.

Figure 2. Effect of Egr1 inhibition on proliferation.
(A) Proliferation assay. C2 cells were transfected with ctl (C2-ctl) or AS (C2-AS) antisense oligonucleotide and submitted to proliferation assay for 7 days. Each day from day 0 (D0) to day 6 (D6), the number of cells of C2-ctl (line) and C2-AS (dashed line) was counted and plotted as the mean of three separate experiments.
(B) Colony forming assay. C2 cells were transfected with 0.1 µM ctl or AS antisense oligonucleotide for 4 h. After 16 h, 200 cells were placed in each well of 6-well plates in RPMI medium containing 0.1 µM antisense oligonucleotides. After 8 days, the colonies were stained with 2% crystal violet.

Figure 3. Time course of mRNA expression.
Cyclin D2, G-alpha-12 protein, cyclin G2 and p19ink4d mRNA expression, were determined by one step real-time RT-PCR. Expression levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase expression and the ratio between each day versus day 0 was calculated as fold induction. All reactions were performed in duplicate from two different samples corresponding to C2 cells transfected with ctl (black boxes) and AS (grey boxes) antisense oligonucleotide.

Figure 4. Time course of protein expression.
(A) Time course regulation of cyclin D2 and p19ink4d protein expression. Protein extracts from C2-ctl and C2-AS cells were analyzed as described in Fig. 1A and B by western-blotting with antibodies to Egr1, cyclin D2, p19ink4d using β-actin as a loading control. (B) Cyclin D2, G-alpha-12 protein, and p19ink4d mRNA expression, were determined by one step real-time RT-PCR.
PCR in human prostate DU145 cells. Expression levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase expression and the ratio between AS condition versus Ctl oligonucleotide was calculated as fold induction. (C) Protein extracts from DU145 (lanes 1, 2, 3, 6), 267B (lane 4), P69 (lane 5) cells were analyzed by western-blotting with antibodies to Egr1, cyclin D2 and β-actin.

Figure 5. Inhibition of Egr1 expression increases sensitivity to apoptotic stimuli.
(A) C2-ctl and C2-AS were exposed or not to ultraviolet-C radiation (UVC 40J/m²). One and two days later dead cells were determined by trypan blue staining. The blue staining dead cell count is shown as a percentage of the total cells and the absolute number of dead and alive cells is reported within the bar chart. (B) C2-ctl and C2-AS were exposed (lane 2 and 4) or not (lane 1 and 3) to ultraviolet-C radiation (UVC 40J/m²). Twentyfour hours later proteins were extracted and subjected to analysis by western-blotting with antibodies to Egr1 or CD95. β-actin level were used as a loading control. (C) Fas L mediated apoptosis. C2-ctl and C2-AS were treated or untreated with 100 ng/ml Fas L for 9 h and 18 h as described in experimental procedures section. Dead cells were determined by trypan blue staining and reported as described above.

Figure 6. Egr1 binds directly to p19ink4d, Mad, CD95 and Cyclin D2 regulatory sequences. C2 cells were transfected (B) or not (A) with AS and Ctl oligonucleotides. The cells were chromatin crosslinked and then immunoprecipitated with specific Egr1 antibody or nonimmune control antibody. The detection of each gene in the captured fragment mix, was performed by PCR as described in experimental procedures. (A) The top, middle and bottom panels show respectively, PCR products from the genomic DNA input, Egr1-specific immunoprecipitation samples and the non-immune control from untransfected C2 cells (Mock). (B) The top and bottom panels show respectively, PCR products from Egr1-specific immunoprecipitation samples from C2 transfected with Ctl and AS oligonucleotides. (C) Cyclin D2, p19ink4d and TGF-β1 mRNA expression, were determined by one step real-time RT-PCR. Expression levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase expression and the ratio between 5h, 10h, 15h versus 0h was calculated as fold induction.

<table>
<thead>
<tr>
<th>Name Affymetrix ratio</th>
<th>Gene function</th>
<th>Link with prostate cancer</th>
<th>Known as Egr-1 target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thierry Virolle</td>
<td>22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1. Affymetrix analysis of genes regulated in C2 cells that express Egr1 constitutively compared with antisense treated cells.

For each gene, the fold induction (Affymetrix ratio), its function (Gene function), any reported involvement in human prostate cancer (Link with prostate cancer) and data on its regulation by Egr1 (Known as Egr-1 target gene) is given.
Table 2. Comparison of Affymetrix array with real-time RT-PCR ratio for mRNA levels.

Changes in the expression level of several Egr-1 target genes given in Table 1 were independently tested using quantitative RT-PCR analysis of RNA from C2-ctl and C2-AS treated cells. The results were normalized to GAPDH and expressed as the ratio of C2-AS over C2-ctl values. All reactions were performed in triplicate from two different experiments and the resulting standard errors are also given. Positive and negative values mean respectively up-regulation and down regulation in response to Egr-1 inhibition (positive values indicate a down-regulation by Egr1).
<table>
<thead>
<tr>
<th>μM</th>
<th>Ctl</th>
<th>AS</th>
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</thead>
<tbody>
<tr>
<td>M</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
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</tbody>
</table>

- Egr-1
- Egr-3
- Egr-2
- WT-1
- β-actin

**B**

<table>
<thead>
<tr>
<th>Time after AS addition (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>Egr-1</td>
</tr>
<tr>
<td>β-actin</td>
</tr>
</tbody>
</table>

C2-ctl
Number of cells (10^4)

Time after AS addition (day)

C2-ctl
C2-AS

B

C2-ctl
C2-AS
[Image 1] A: Time after AS addition (day)

0 1 2 3 4 5 6 0 1 2 3 4 5 6
Egr-1  cyclin D2  p19ink4d
β-actin  C2-AS  C2-ctl

[Image 2] B: mRNA expression ratio

G-alpha-12 protein
p19ink4d  cyclin D2

[Image 3] C: DU145  M Ctl AS  267B  P69  DU145
Egr-1  cyclin D2  β-actin
1 2 3 4 5 6
DNA input

α-Egr-1

α-NI

ChIP

M

M AD Cyclin G2 CDk5 Cyclin D2

DNA input

α-Egr-1

α-NI

ChIP

M

M AD Cyclin G2 CDk5 Cyclin D2

C2-Ctl

C2-AS

ChIP

C2-AS

C2-ctl

C2-AS

C2-ctl

C2-AS

C2-ctl

C2-AS

C2-ctl

C2-AS

C2-ctl

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