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Inhibition of Egr-1 expression reverses transformation of prostate cancer cells *in vitro* and *in vivo*

Véronique Baron*[,1,5], Giorgia De Gregorio[2,5], Anja Krones-Herzig[1], Thierry Virolle[3], Antonella Calogero[2], Rafael Urcis[1] and Dan Mercola[1,4]

[1]*Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, CA 92121, USA;* [2]*Instituto di Ricovero e Cura a Carattere Scientifico, Neuromed, Pozzilli, 86077, Italy;* [3]*The Burnham Institute, La Jolla Cancer Research Center, 10901 North Torrey Pines Road, La Jolla, CA, USA;* [4]*Cancer Center, University of California San Diego, La Jolla, CA 92093, USA*

Transcription factor early growth response-1 (Egr-1) is a crucial regulator of cell growth, differentiation and survival. Several observations suggest that Egr-1 is growth promoting in prostate cancer cells and that blocking its function may impede cancer progression. To test this hypothesis, we developed phosphorothioate antisense oligonucleotides that efficiently inhibit Egr-1 expression without altering the expression of other family members Egr-2, Egr-3 and Egr-4. In TRAMP mouse-derived prostate cancer cell lines, our optimal antisense oligonucleotide decreased the expression of the Egr-1 target gene transforming growth factor-β1 whereas a control oligonucleotide had no effect, indicating that the antisense blocked Egr-1 function as a transcription factor. The antisense oligonucleotide deregulated cell cycle progression and decreased proliferation of the three TRAMP cell lines by an average of 54 $\pm$ 3%. Both colony formation and growth in soft agar were inhibited by the antisense oligonucleotide. When TRAMP mice were treated systemically for 10 weeks, the incidence of palpable tumors at 32 weeks of age in untreated mice or mice injected with the control scramble oligonucleotide was 87%, whereas incidence of tumors in antisense-Egr-1-treated mice was significantly reduced to 37% ($P = 0.026$). Thus, Egr-1 plays a functional role in the transformed phenotype and may represent a valid target for prostate cancer therapy.
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Introduction

Prostate cancer is the most commonly diagnosed cancer in men and the second cause of cancer-related death among men. Advanced prostate cancer is resistant to hormone therapy, radiation and conventional chemotherapy. Therefore, finding new strategies for treatment remains a crucial issue. Although prostate cancer is fairly well characterized at the histopathological level, the molecular mechanisms leading to cell transformation are still poorly understood.

Recent discoveries in independent laboratories show that the transcription factor early growth response-1 (Egr-1) is present at much higher levels in all the human prostate tumors tested so far, as opposed to normal cells (Thigpen *et al*., 1996; Eid *et al*., 1998). Egr-1 is a nuclear phosphoprotein that was first identified based on its extremely rapid induction by mitogens and differentiation factors (Lim *et al*., 1987; Milbrandt, 1987; Christy *et al*., 1988; Lemaire *et al*., 1988; Sukhatme *et al*., 1988). This protein of 59 kDa is the prototype member of a family of transcription factors, which includes at least four members (Egr-1 to Egr-4). Egr proteins contain a highly conserved DNA-binding domain composed of three zinc fingers. Egr-1 also contains a nuclear localization signal, two activator domains and one repressor domain regulating its function (Gashler *et al*., 1993). Moreover, Egr-1 binds to regulatory proteins called nerve growth factor-I A binding protein (NAB) 1 and 2 (NGF-I A binding protein) that repress its transcriptional activity (Russo *et al*., 1995; Svaren *et al*., 1996).

Egr-1 is induced by many different stimuli ranging from growth factors and cytokines to stress signals such as UV, ionizing radiation and apoptosis-promoting factors and injury. Thus, Egr-1 is involved in a variety of cell processes including growth, differentiation, neurite outgrowth, wound healing and apoptosis (Liu *et al*., 1998; O'Donovan *et al*., 1999; Adamson and Mercola, 2002).

The hypothesis that Egr-1 plays a role in prostate cancer progression is supported by the observation that the mRNA encoding Egr-1 is expressed at much higher levels in prostate adenocarcinoma compared to normal tissues and correlates with higher levels of protein (Thigpen *et al*., 1996; Eid *et al*., 1998). Moreover, the levels of protein expression correlate with Gleason scores and inversely correlate with the degree of differentiation of carcinoma cells (Eid *et al*., 1998). NAB2, which represses the transcriptional activity of Egr-1, seems to be downregulated in primary prostate

*Correspondence: Véronique Baron and Dan Mercola; Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, CA 92121, USA; E-mail: danmercola@skcc.org; vbaron@skcc.org
[5]These two authors contributed equally to the work
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carcinomas (Abdulkadir *et al*., 2001a). Both upregulation of Egr-1 and loss of its repressor NAB2 may play roles in determining the level of Egr-1 activity in human prostate cancer. Interestingly, the human gene encoding NAB2 is localized to chromosomal region 12q13.3-14.1, which is supposed to contain a tumor suppressor for prostate cancer (Svaren *et al*., 1996; Berube *et al*., 1994). Finally, crossbreeding of Egr-1$^{-/-}$ mice with transgenic mouse models of prostate cancer such as CR2-T-Ag mice and TRAMP mice actually showed significantly impaired prostate tumor growth in mice lacking Egr-1 (Abdulkadir *et al*., 2001b). Thus, Egr-1 deficiency delays the progression of prostate carcinoma in these mice.The molecular basis of the putative oncogenic role of Egr-1 in the prostate is unknown, although it is interesting to note that a number of genes that are regulated by Egr-1, such as transforming growth factor (TGF)-β1, PDGF-A, IGF-II and others, have been proposed as growth promoters for prostate epithelial cells (Adamson and Mercola, 2002).

As a whole, these observations strongly point to Egr-1 as a potential target for gene therapy of prostate carcinoma. However, there is no evidence showing that blocking Egr-1 expression in prostate cancer cells will decrease proliferation or reverse the transformed phenotype.

To test this, we have developed a series of antisense oligonucleotides that efficiently and specifically block Egr-1 expression at the mRNA as well as at the protein level. We show that in three cell lines derived from the tumor of a TRAMP mouse (Foster *et al*., 1997) inhibition of Egr-1 expression decreased proliferation and reverted the transformed phenotype. The autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP) model was generated by expression of SV40 large-T antigen driven by a prostate-specific probasin promoter (Greenberg *et al*., 1995). These mice spontaneously develop prostate tumors (arising at a 100% frequency) that are very similar to human tumors. Systemic treatment of TRAMP mice with our test antisense oligonucleotide decreased tumor incidence. These data suggest that antisense oligonucleotides that block Egr-1 function represent promising therapeutic tools for the treatment of prostate cancer.

Results

Design and obtaining of antisense oligonucleotides

We have selected a series of 10 oligonucleotides corresponding to regions equally distributed along the coding sequence of Egr-1, following published guidelines (Bost *et al*., 2000; Stein, 2001). The oligonucleotides are 20-nucleotide-long phosphorothioate analogs and were chosen based on the following criteria: (i) the 10 sequences differ by 4 or more nucleotides from Egr-1 family members, namely Egr-2, Egr-3 and Egr-4, and (ii) sequences are identical in the murine and human genes. In addition, a search was conducted using NIH/BLAST to discard sequences shared with unrelated genes.

Pilot experiments were performed using HT1080-E9 fibrosarcoma cells expressing supraphysiological levels of Egr-1 that are readily detectable (Huang *et al*., 1995). The elevated levels of Egr-1 in these cells provide a critical test for the efficiency of the antisense oligonucleotides. Cells were transfected with increasing concentrations of antisense oligonucleotides or a control oligonucleotide, or with carrier alone. The effect of the oligonucleotides was assessed approximately 24 h after transfection by Western analysis. As shown in Figure 1a, the candidate oligonucleotides inhibited Egr-1 expression with various efficiencies. Oligonucleotides E3 and E4 did not alter Egr-1 expression. Oligonucleotide E9 was the first oligonucleotide that we tested and was used here as a positive control and an internal standard. Antisense oligonucleotide E5 was the most efficient in this experiment, showing almost complete inhibition at the highest concentration tested, i.e., 0.4 μM.

Results with all oligonucleotides at a concentration of 0.4 μM were quantified and are summarized in Figure 1b. The percent of steady-state Egr-1 expressed is given relative to the position of the complementary sequence along the Egr-1 transcript. The nucleotide positions are given in correlation with the specific preparation of antisense oligonucleotides, which are indicated in brackets (E1–E10).

Out of ten oligonucleotides tested two (E3 and E4) did not alter steady-state Egr-1 expression, two (E1 and E2) inhibited expression by only 50% and six (E5 to E10) efficiently inhibited expression (70–80% of inhibition in these cells). Among these, we used mainly the antisense oligonucleotide designated as E5 (corresponding to nucleotides 675–694 of the murine sequence) in the experiments that follow.

Additional experiments were conducted to determine the duration of oligonucleotide effects. Egr-1 levels were tested 24 and 72 h following transfection by Western analysis (Figure 1c). Inhibition of Egr-1 expression by two antisense oligonucleotides E5 and E6 lasted for at least 72 h, indicating that these phosphorothioate oligonucleotides are fairly stable in intact cells. A complete inhibition of Egr-1 expression was observed in murine embryo fibroblasts, human fetal kidney 293 T cells, normal murine mammary cells and in murine prostate cancer cells (data not shown).

Characterization of the antisense oligonucleotides in TRAMP-C cells

Our hypothesis is that reducing Egr-1 expression in cultured prostate cancer cells will inhibit cell growth and revert the phenotype from transformed to 'normal'. To test this, we have used murine cell lines derived from a TRAMP mouse (Foster *et al*., 1997). TRAMP-C cell lines have different tumorigenic capacities and are thought to represent different stages of transformation. In TRAMP-C2 cells, E5 oligonucleotide almost completely inhibited Egr-1 expression at a concentration of 0.2 μM, whereas control oligonucleotide at this concentration had no effect (Figure 2a). In other experiments, cells were transfected either with the control or with E5

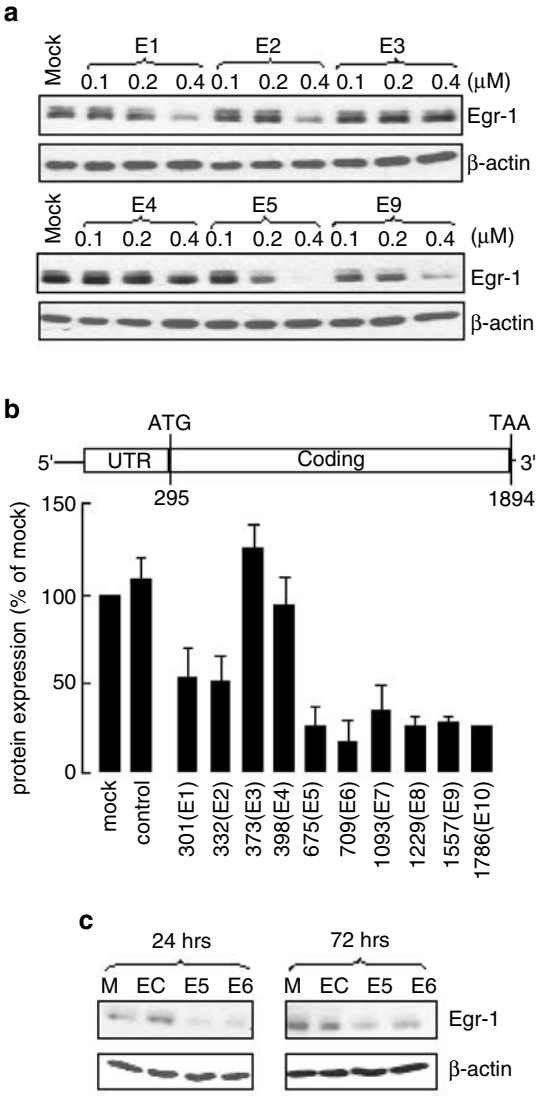

Figure 1 Screening and characterization of the antisense oligonucleotides. (**a**) HT1080-E9 cells were transfected with carrier alone (mock) or with 0.1, 0.2 and 0.4 μM of the indicated oligonucleotides. Cells were lysed and protein expression was detected by Western analysis using antibodies to Egr-1. Equal loading was verified by reprobing the membrane with antibodies to β-actin. A representative experiment is shown. (**b**) Cells were transfected with carrier alone (mock), with control or with antisense oligonucleotides at a concentration of 0.4 μM. Egr-1 protein expression was analysed by Western blot. Autoradiograms were quantified using a Kodak™ DC120-Zoom digital camera and Kodak 1D image analysis software (Eastman Kodak Company, Rochester, NY, USA). Results (means $\pm$ S.E. of at least three separate determinations) are expressed relative to Egr-1 expression in mock-treated cells. Position of each oligonucleotide within the nucleotide sequence of the murine gene is given in correlation with the specific preparation of antisense oligonucleotide, designated E1–E10 (in brackets). (**c**) Cells were transfected with 0.3 μM control oligonucleotide (EC), E5 and E6 antisense oligonucleotides or carrier alone (M). Cells were lysed 24 and 72 h after the start of transfection. Samples were analysed by Western blot using antibodies to Egr-1. Membranes were reprobed with antibodies to β-actin

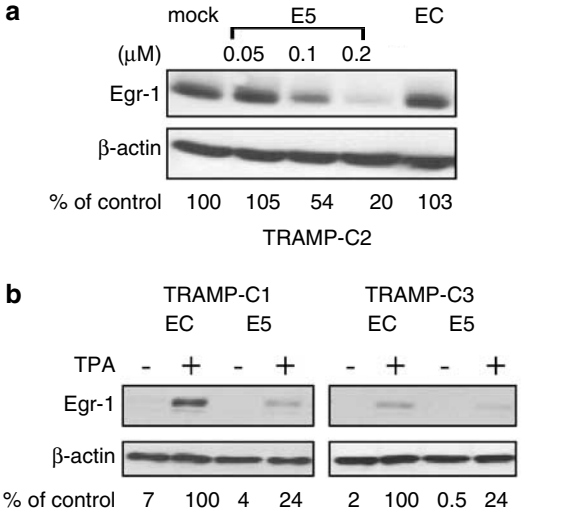

Figure 2 Analysis of Egr-1 expression in TRAMP-C cells. (**a**) TRAMP-C2 cells were transfected with increasing concentrations of oligonucleotide E5, with 0.2 μM of control oligonucleotide (EC) or carrier alone (mock). After 24 h cells were lysed and samples were analysed by Western blot using antibodies to Egr-1. Membranes were reprobed with antibodies to β-actin to control for equal loading. (**b**) TRAMP-C cells were transfected with 0.2 μM control or E5 oligonucleotides the day before the experiment. Cells were treated with carrier alone or with TPA (50 ng/ml) for 3 h before lysis. Samples were submitted to Western analysis using antibodies to Egr-1. Membranes were reprobed with antibodies to β-actin

oligonucleotide. The day after transfection, cells were stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 3 h to induce Egr-1. As shown, antisense oligonucleotide E5 also inhibited TPA-induced expression of Egr-1 (Figure 2b).

The specificity of action of antisense oligonucleotides was examined by comparing their effects on Egr family members. Figure 3a shows that both antisense oligonucleotides E5 and E6 efficiently suppressed Egr-1 expression but did not consistantly alter the expression of other family members Egr-2 and Egr-3. Moreover, they did not alter the expression of the other related transcription factor WT-1 (data not shown). Expression of Egr-4 could not be tested by Western analysis because of the lack of available antibody. However, reverse transcription–polymerase chain reaction (RT–PCR) experiments confirmed that the mRNA encoding Egr-1 was downregulated by antisense oligonucleotide E5 but the mRNA encoding Egr-4 was unchanged (Figure 3b). Thus, the antisense oligonucleotides developed here do not crossreact with other Egr family members and do not randomly alter protein expression.

Antisense oligonucleotide E5 inhibits the expression of TGF-β

To assess whether the antisense oligonucleotide also inhibits Egr-1 function, we measured levels of mRNA expression for two Egr-1-regulated gene products in parallel with Egr-1 itself. The described targets for Egr-1 include TGF-β1 (Dey *et al*., 1994; Liu *et al*., 1996), and tumor suppressor PTEN (Virolle *et al*., 2001). As shown by RT–PCR experiments (Figure 4a), antisense oligonucleotide E5 decreased the expression of Egr-1 mRNA

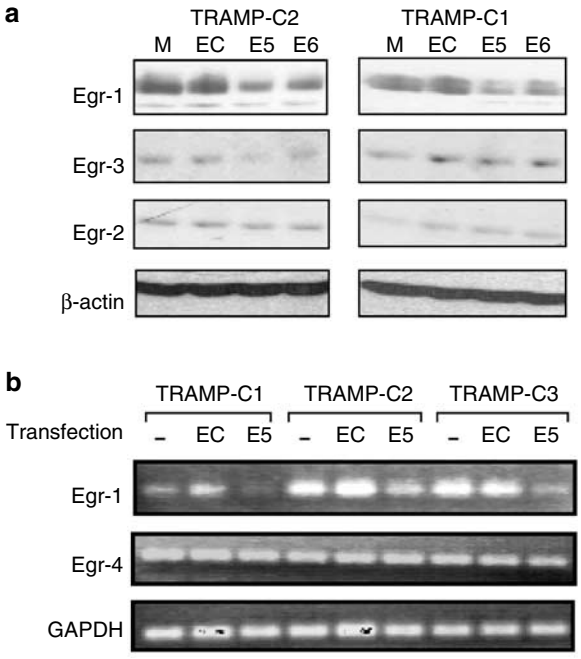

Figure 3 Effect of antisense oligonucleotides on the expression of other Egr family members. TRAMP-C cells were transfected with carrier alone (M), control oligonucleotide (EC) or with E5 or E6 antisense oligonucleotides as indicated. (**a**) Protein expression was determined by Western analysis. Membranes were probed successively with antibodies to Egr-3, Egr-1, Egr-2 and β-actin. A representative autoradiogram is shown. (**b**) mRNA expression was analysed by RT–PCR from total RNA using probes specific for Egr-1, Egr-4 and GAPDH. PCR fragments were submitted to 2% agarose gel electrophoresis containing ethidium bromide and visualized under a UV lamp

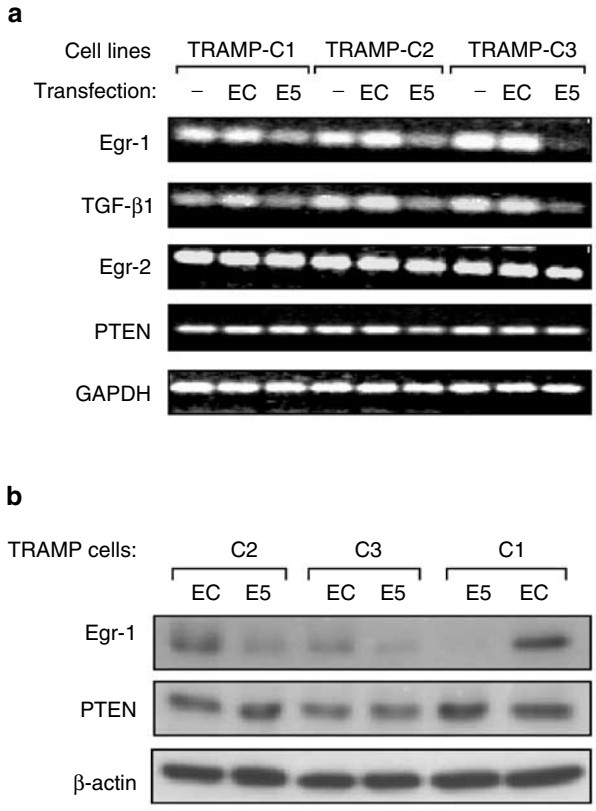

Figure 4 Effect of antisense oligonucleotide E5 on the expression of Egr-1 target genes. (**a**) mRNA expression in transfected TRAMP-C cells was analysed by RT–PCR from total RNA using probes specific for Egr-1, TGF-β1, Egr-2, PTEN or GAPDH as a control. PCR fragments were submitted to 2% agarose gel electrophoresis containing ethidium bromide and visualized under a UV lamp. (**b**) Protein expression in transfected TRAMP-C cells was analysed by Western blot using antibodies to Egr-1. Membranes were reprobed successively with antibodies to PTEN and β-actin

in all three TRAMP-C cell lines. Expression of TGF-β1 mRNA was concomitantly downregulated, whereas the expression of PTEN was not altered. RNA expression levels in TRAMP-C2 cells were also measured by quantitative real-time PCR (Q-PCR). When compared to control, Q-PCR ratios of the E5-treated cells were 0.668, 0.687 and 0.988 for Egr-1, TGF-β1 and PTEN, respectively (a value of 1.0 reflects similar levels in control and antisense-treated cells). These ratios indicate that mRNA levels of Egr-1 and TGF-β1 were concomitantly decreased in E5-treated cells, whereas the level of PTEN mRNA was not altered. The latter result was confirmed by examination of PTEN protein levels, which remained unchanged (Figure 4b). We conclude that the antisense oligonucleotide E5 inhibits Egr-1 transcriptional activity towards TGF-β1. Interestingly, PTEN expression is not regulated by Egr-1 in these cells.

Antisense oligonucleotides inhibit TRAMP-C cells proliferation

In order to test the role of Egr-1 in the growth regulation of TRAMP-C1, TRAMP-C2 and TRAMP-C3, cells were transfected with control or E5 oligonucleotides and initial cell numbers were determined by counting after transfection ($t = 3$ h). Subsequent growth was measured by direct cell counting over a period of 4 days.

Figure 5a shows that oligonucleotide E5 inhibited the proliferation of the three cell lines. Inhibition rates were calculated from the integrated growth curves ($I\% = 100(1 - A_{as}/A_c)$) as determined in three complete replicate experiments for each cell line, where A_{as} is the growth curve of antisense-treated cells and A_c is the growth curve of control-treated cells. The resulting average values are 47.7 ± 4.4, 58.3 ± 5.8, $53.7 \pm 3.3\%$ ($n = 3$) for TRAMP-C1, TRAMP-C2 and TRAMP-C3, respectively, indicating that Egr-1 is required for the normal growth of TRAMP-C cells *in vitro*.

Indication that inhibition of cell growth was not because of a nonspecific toxic effect of the oligonucleotide was provided by the observation that two different oligonucleotides, E5 and E6, gave similar results (Figure 5b). We monitored antisense-induced inhibition of Egr-1 expression by Western analysis at 28 h following transfection (Figure 5c), which confirmed that antisense-treated but not control-treated cells exhibited suppressed Egr-1-protein levels. Thus, inhibition of cell

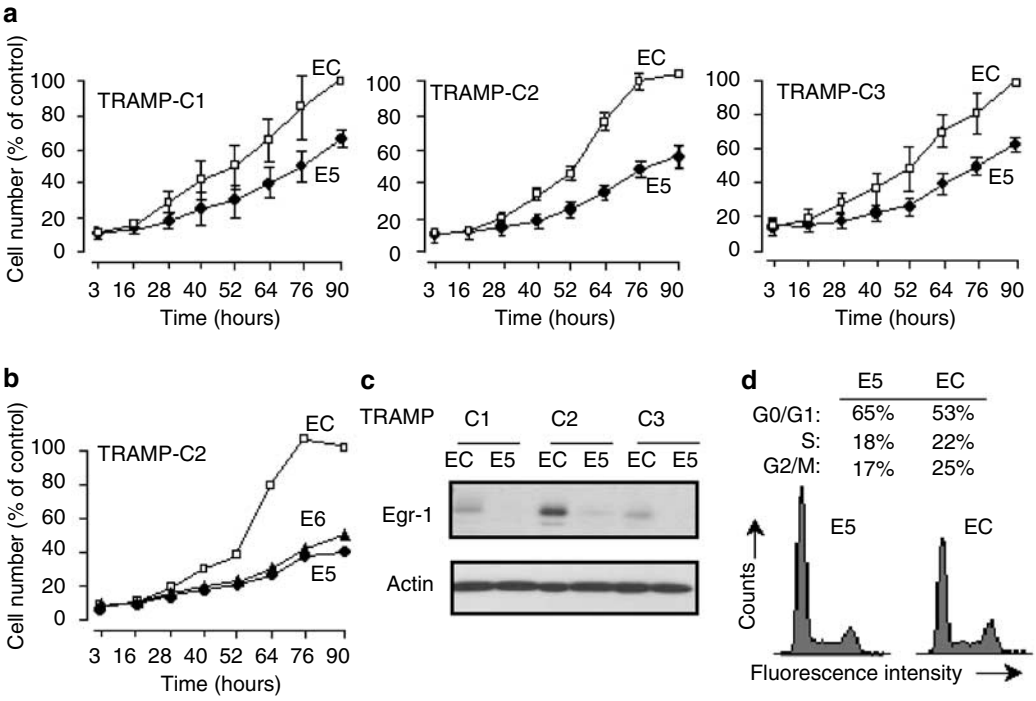

Figure 5 Effect of the antisense oligonucleotides on cell growth and cell cycle progression. (**a**) TRAMP-C cells were transfected with 0.1 μM control or antisense oligonucleotide E5. They were counted as described in Materials and methods using a Coulter counter. Results represent means $\pm$ S.E. from three separate experiments and are expressed relative to control oligonucleotide-treated cells at the maximum of the growth curve, that is, at $t = 90$ h. (**b**) TRAMP-C2 cells were transfected with 0.1 μM control, antisense oligonucleotide E5 or antisense oligonucleotide E6. Results are expressed relative to control oligonucleotide-treated cells at the maximum of the growth curve ($t = 90$ h). (**c**) A fraction of transfected cells was lysed at $t = 28$ h and analysed by Western blot for Egr-1 expression. Membranes were reprobed with antibodies to β-actin. A representative blot from one of the three experiments is shown. (**d**) TRAMP-C2 cells were transfected with control or E5 antisense oligonucleotide (0.2 μM). At 48 h after transfection they were suspended by trypsin digestion and fixed in 70% ethanol. They were stained with propidium iodine as described in Methods and subjected to flow cytometry for DNA analysis

growth is likely to be because of inhibition of Egr-1 expression.

Finally, cell cycle analysis by flow cytometry of transfected TRAMP-C2 cells indicated that the antisense oligonucleotide E5 deregulated progression through the cell cycle, with more cells being in the G0/G1 phase when treated with the antisense oligonucleotide compared to control oligonucleotide-treated cells (Figure 5d). We conclude that expression of Egr-1 is correlated with accelerated cell cycle.

Antisense oligonucleotide E5 inhibits colony formation and growth in soft agar of TRAMP-C1 and TRAMP-C2 cells

Colony formation and growth in soft agar are hallmarks of transformed cells *in vitro*. Thus, TRAMP-C1 and TRAMP-C2 that are rapidly tumorigenic when grafted into athymic or autologous C57Bl/6 hosts (TRAMP background) gave positive results in colony formation assay and growth in soft agar. In contrast, TRAMP-C3 cells are poorly tumorigenic and do not form tumors when grafted into these mice (Foster *et al*., 1997). Consistently, we found that these cells did not form colonies *in vitro* and did not grow in soft agar (data not shown).

In colony formation assays, TRAMP-C1 and TRAMP-C2 cells were transfected with control or E5 oligonucleotides. They were plated at low density to allow single cell growth for 1 week. After staining, colony numbers were counted in each culture dish. Figure 6a shows a picture of a representative dish for each condition and each cell line. Figure 6b shows the quantification standard error ($\pm$S.E.) of three separate experiments, each performed in triplicate wells.

It is apparent (Figure 6b) that antisense oligonucleotide E5 treatment inhibited colony formation in both cell lines compared to cells treated with control oligonucleotides. When counted in tissue culture dishes, the colony number of TRAMP-C1 treated with control oligonucleotide was 30.6 ± 2.1, compared to 18.5 ± 2.5 following treatment with E5, which is significant ($P < 0.005$). The colony number of TRAMP-C2 treated with the control oligonucleotide was 61.7 ± 4.2, compared to 20.1 ± 2 when treated with E5, which is also significant ($P < 0.0005$).

Growth in soft agar reflects anchorage independence, which is a requisite for transformation.

Cells transfected with control or E5 oligonucleotides were seeded in a top layer of 0.35% agar as described in Materials and Methods. After 2 weeks, cells were stained with a vital dye to visualize colonies. Figure 6c

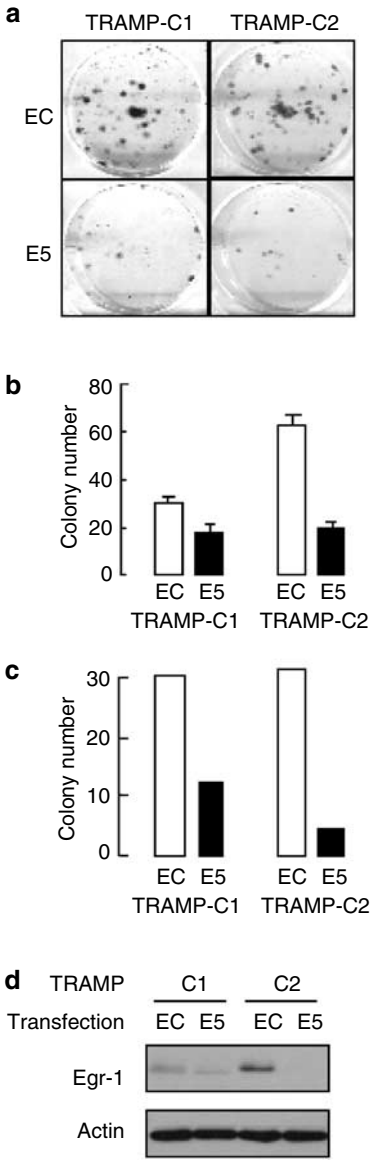

Figure 6 Effect of antisense oligonucleotide E5 on colony formation and growth in soft agar. (**a**) TRAMP-C cells were transfected with control or E5 antisense oligonucleotide (0.1 μM). After 1 day, they were replated at a density of 200 cells/plate in 10% FBS-containing medium. Cells were stained with crystal violet after a week. A representative picture of colony staining is shown. (**b**) Colonies were counted and the actual number of colony/plate was plotted (means $\pm$ S.E. of three separate experiments, each performed in three–six replicate wells). (**c**) Transfected cells were seeded into a 0.35% agar layer on top of a 0.5% agar bottom. After 2 weeks, cells were stained with nitro-blue tetrazolium. Colonies were counted and the total colony number from each experiment was plotted (mean from two separate experiments, each performed in three–six replicate wells). (**d**) A fraction of transfected cells was lysed the day after transfection and analysed by Western blot for Egr-1 expression. Membranes were reprobed with antibodies to β-actin

shows the colony numbers from two separate experiments. Treatment with antisense oligonucleotide E5 led to a decrease in the number of colonies growing as well as the average size of the colonies in both TRAMP-C1 and TRAMP-C2 cells. For each experiment we monitored by Western analysis that the antisense oligonucleotide actually inhibited Egr-1 protein expression (Figure 6d).

We conclude that antisense oligonucleotide E5 suppresses two important features of cellular transformation, that is, colony formation and anchorage independence for growth.

Antisense oligonucleotide E5 decreases tumor incidence in TRAMP mice

PCR-confirmed male TRAMP mice were divided into three treatment groups: saline buffer alone (PBS), mismatch control oligonucleotide or antisense oligonucleotide E5. The average age of the mice at the start of the treatment was 21.8 ± 0.3 ($n = 23$) when prostate cancer is already developing (Dey *et al*., 1994). Mice received systemic intraperitoneal injections (i.p.) three times a week with vehicle alone (seven mice), three-base mismatch control oligonucleotide (eight mice) or E5 antisense oligonucleotide (eight mice), at a dose of 25 mg/kg. Animals were killed when showing signs of illness or when tumors became palpable at which time necropsies were carried out. In order to have an age-matched group, random mice of the same generation in the other groups were killed together with tumor-bearing animals. Thus, the average age at sacrifice for the treated animals was 31 ± 0.7 weeks for all three groups (31.9 ± 0.9; 32.4 ± 0.9; 31.1 ± 1.7 in the saline buffer, control and antisense oligonucleotide group, respectively). The average length of treatment was 69 ± 5 days (~ 10 weeks) for all groups.

Figure 7a shows that the incidence of tumor in antisense-treated mice was lower than that of control mice. All seven mice of the saline buffer group and six out of eight mice in the control oligonucleotide-treated group developed tumors characterized by grossly enlarged prostate glands with extensive and typically bilateral involvement of the vesicular glands (data not shown). In contrast, only three out of eight mice in the E5 antisense-treated group developed tumors.

Figure 7b displays the tumor size for each animal, with the medians indicated as horizontal lines (median = 2, 2.05 and 0 g for saline buffer, control and E5 oligonucleotides, respectively). The average weight at death was 31.9 ± 0.6 g, in which mice with tumor weighed 32.8 ± 0.4 g and mice without tumor weighed 28.3 ± 1. The average weight of tumors was 4.1 ± 0.5 g for all tumor-bearing mice.

The differences between groups were analysed by the Fisher Exact Test, which displays how different treatments have produced different outcomes. Its null hypothesis is that treatments do not affect outcomes – that the two are independent.

The difference in the incidence of tumors between the oligonucleotide control-treated group and the saline buffer group is not significant (6/8 *vs* 7/7; $P = 0.467$). In contrast, when the incidence of tumor for the antisense Egr-1-treated group is compared to the saline buffer group (3/8 *vs* 7/7), the difference is significant, $P = 0.026$. When results are analysed taking the three treatment

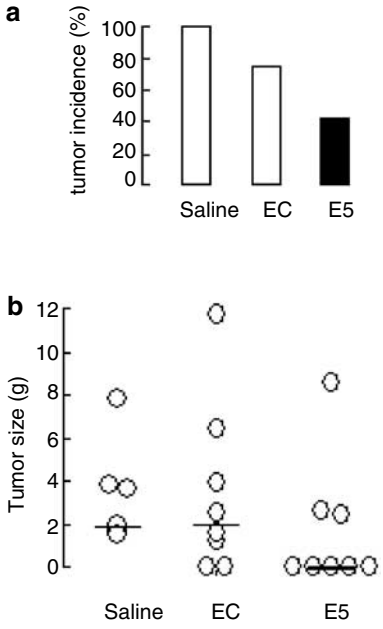

Figure 7 Inhibition of tumor incidence in TRAMP mice. TRAMP mice were treated with saline injections ($n=7$), with control scramble oligonucleotide ($n=8$) or with E5 antisense oligonucleotide ($n=8$). Animals were sacrificed after 10 weeks of treatment (average age of 32 weeks). (**a**) Percentage of tumor incidence in the three treatment groups. (**b**) Tumor size for each mouse. Medians are indicated by horizontal bars

groups altogether, the outcome is also significant, with $P=0.037$ (7/7 *vs* 6/8 *vs* 3/8). Thus, the control oligonucleotide has no effect on the incidence of tumor whereas E5 antisense oligonucleotide significantly delays tumor incidence in TRAMP mice.

As a whole, we conclude that treatment with antisense oligonucleotides that block Egr-1 expression may impede prostate cancer progression both *in vitro* and *in vivo*. Our data suggest that Egr-1 plays an important growth-promoting role in prostate carcinoma development and that antisense oligonucleotides represent interesting potential therapeutic tools.

Discussion

Identification of key players in the process of cellular transformation is a crucial step towards our understanding of cancer progression and towards the development of new cancer therapies. In prostate cancer tissues, transcription factor Egr-1 is overexpressed (Thigpen *et al*., 1996; Eid *et al*., 1998), while its repressor protein NAB2 is downregulated (Abdulkadir *et al*., 2001a). In addition, Egr-1 deficiency in mice models of prostate cancer delays progression, suggesting that Egr-1 could promote tumor growth (Abdulkadir *et al*., 2001b). In this study, we show that blocking Egr-1 expression with specific antisense oligonucleotides inhibits three hallmarks of transformed cells, i.e., cell proliferation, capacity to form colonies and anchorage-dependent growth, adding weight to the idea that Egr-1 supports prostate cancer progression.

Antisense oligonucleotides to Egr-1 have been very valuable for studying its role in the regulation of gene expression (Hu and Levin, 1994; Cogswell *et al*., 1997; Santiago *et al*., 1999; Arai *et al*., 2000; Virolle *et al*., 2001, Nishi *et al*., 2002). In addition, they have been used to study Egr-1 involvement in macrophage differentiation (Nguyen *et al*., 1993), in growth arrest and apoptosis of melanoma cells (Muthukkumar *et al*., 1995; Ahmed *et al*., 1996) or in mediating the survival of B-cell lymphoma (Muthukkumar *et al*., 1997). In the present work we describe the development of high-affinity antisense suitable for use *in vivo*.

Our observation that these Egr-1 antisense oligonucleotides inhibit prostate cell growth is in agreement with the known involvement of Egr-1 in proliferative responses. Indeed, Egr-1 regulates the growth of hematopoietic and epithelial cells, of astrocytes and smooth muscle cells (Irving *et al*., 1989; Perez-Castillo *et al*., 1993; Hu and Levin, 1994; Kinane *et al*., 1994; Hofer *et al*., 1996; Santiago *et al*., 1999; Gousseva *et al*., 2001). Evidence for a mechanism of action came from studies showing that overexpression of Egr-1 in renal epithelial cells leads to upregulated transcription of the Gαi-2 proto-oncogene, which endorses cell growth and oncogenic transformation (Kinane *et al*., 1994). Egr-1 contributes to the regulation of genes that are critical for progression through the cell cycle such as cyclin D1 (Yan *et al*., 1997) and thymidine kinase (Molnar *et al*., 1994). In TRAMP-C2 prostate cancer cells, the oncogenic protein Gα12 and cyclin D2 are both upregulated by Egr-1 (unpublished observations). On the other hand, several genes involved in the inhibition of proliferation, notably $p19^{Ink4d}$ and cyclin G2, are downregulated by Egr-1 (unpublished observations). Our observation that antisense Egr-1 slows down cell cycle progression is consistent with this property.

Microarray analysis suggest that on a global level the effects of Egr-1 on tumor progression also result from altered expression patterns of various growth factors such as IGF-II and PDGF-A (Svaren *et al*., 2000). These have been implicated in accelerating the progression of prostate tumors through autocrine or paracrine stimulation and induction of angiogenesis (Russell *et al*., 1998). In addition, Egr-1 augments the production of VEGF by hypoxia transcription factor HIF-1α (Kroon *et al*., 2001), which is likely to play a role in Egr-1-related cancer progression since it is an autocrine stimulator of prostate cancer proliferation (Jackson *et al*., 2002). Similar remarks hold for the induction of TGF-β1 by Egr-1. This cytokine promotes prostate cancer progression albeit in most other epithelial cells it is a potent growth suppressor.

In cultured TRAMP-C cells though, TGF-β1 does not appear to play a direct role in mediating the effect of Egr-1 on cell growth. TGF-β1 added to TRAMP-C cell lines did not alter cell growth, nor did it alter the effect of the antisense oligonucleotide (data not shown). Observation that Egr-1 antisense inhibits TGF-β1 expression suggests that Egr-1 is transcriptionally active

in TRAMP-C cells and that introduction of antisense oligonucleotides impedes its activity and decreases the production of at least one potential contributor to angiogenesis.

Surprisingly, we did not see a regulation of the newly identified Egr-1 target PTEN (Virolle *et al*., 2001). Since PTEN acts as a tumor suppressor, it is striking that it is not induced by Egr-1 in TRAMP-C cells. Indeed, induction of PTEN would be expected to impair proliferation and/or promote apoptosis, which are both antagonist to an oncogenic effect. Loss of Egr-1 inducibility was observed in several prostate cell lines (unpublished results). This may be because of hypermethylation of PTEN gene promoter, which prevents Egr-1 from binding (unpublished results). PTEN inactivation because of hypermethylation occurs in about 50% of prostate cancer cases (Whang *et al*., 1998) and may play a critical role in cancer progression since lack of PTEN expression correlates with high Gleason score and advanced stage (McMenamin *et al*., 1999). Thus, the gene that could potentially rescue prostate cancer cells from transformation and is actually a target for Egr-1 proves to be deficient in prostate cancer cells. It is seen that two effectors of Egr-1 (TGF-β1 and PTEN) with described growth-suppressive properties are unlikely to have these roles in prostate cancer. Indeed, TGF-β is known to stimulate the growth of prostate cancer cells. These observations may be relevant to the growth-promoting role of Egr-1 in prostate carcinoma.

Antisense oligonucleotides are showing their effectiveness in preclinical studies. Some have reached phase I and II clinical trials for cancer therapy and show much less toxicity than originally feared (Galderisi *et al*., 1999). More specifically, antisense oligonucleotides that inhibit Egr-1 expression have been used in preclinical studies to prevent inflammation and thrombosis after lung transplantation. They were shown to decrease Egr-1-induced genes and strongly increase survival of the transplanted animals (Okada *et al*., 2001).

Here we sought to develop optimized sequences with improved efficacy and specificity suitable for use *in vivo*. We observed that antisense oligonucleotide E5 functions *in vitro* with an EC_{50} of $\sim 0.1\,\mu$M. More importantly, these antisense oligonucleotides can be used in murine as well as human cells. Although still preliminary, our results indicate that antisense oligonucleotides decreased incidence of prostate cancer in TRAMP mice. We conclude that they may constitute a simple and effective tool that warrants further investigation as a novel prostate cancer therapy.

Methods

Cell lines and culture

TRAMP-C1, TRAMP-C2 and TRAMP-C3 (Foster *et al*., 1997) were grown in RPMI-1640 Medium (Invitrogen, Carlsbad, CA, USA), supplemented with 5% Fetal Bovine Serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Irvine Scientific, Santa Ana, CA, USA). Fibrosarcoma HT1080-E9 cells (Huang *et al*., 1995) were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were maintained in a humidified incubator at 37°C and 6% CO_2.

Oligonucleotides

The ten trial antisense oligonucleotides were prepared by Trilink Inc. (San Diego, CA, USA) with all phosphorothioate backbone chemistry. They were provided after sequence verification and double HPLC purification. Dry oligonucleotides were resuspended as 10 μM stock solutions in pure sterile water and frozen as aliquots.

Position within the mouse gene (ATG = 295; accession number NM007913) of each antisense oligonucleotide is shown in Figure 1b. Sequences for the control oligonucleotides are given below. Sequences of the antisense oligonucleotides will be available upon request. Oligonucleotides were named E1–E10 along the gene.

EC (scramble) 5′-TTC TTG CAT CTG TCA-3′ and (mismatch) 5′-AGC GGA CAC TCT AGG CGA TG-3′.

Transfection

Transfection of TRAMP-C cells: cells were seeded at a density of 2×10^5 cells/well in six-well tissue culture plates the day before transfection in order to achieve 70–80% confluence. Transfection was performed using Lipofectamine Plus® Reagent (Invitrogen, Carlsbad, CA, USA) following the instructions provided by the manufacturer, in a final volume of 1 ml RPMI medium without additives, for 3 h at 37°C. Cells were washed once and maintained in complete medium until the experiment.

Transfection of HT1080-E9 cells: cells were plated at a density of 10^5 cells/well of a 12-well tissue culture plate the day before transfection. Transfection was performed using Lipofectin® reagent (Invitrogen) following instructions in a final volume of 0.5 ml. After 15 h, cells were washed and maintained in a complete medium.

Western blot analysis of protein expression

Cells were chilled on ice and washed twice with ice-cold Phosphate Buffered saline (PBS: 43 mM K_2HPO_4; 9 mM Na_2HPO_4; 120 mM NaCl; pH 7.4). They were solubilized on ice in lysis buffer containing 50 mM HEPES pH 7.5; 150 mM NaCl; 100 mM NaF; 10 mM EDTA, 10 mM $Na_4P_2O_7$; 1% (v/v) Triton X-100; 0.5% Deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS) and a Protease Inhibitor Cocktail (Sigma Aldrich Inc., St Louis, MO, USA). Lysates were then clarified by centrifugation at 13 000 *g* for 10 min at 4°C. Protein concentration was determined using the BCA™ protein assay reagent (Pierce, Rockford, IL, USA). Cleared lysates were resuspended in sample buffer containing 70 mM Tris-HCl; 10% (v/v) glycerol; 2% (w/v) SDS; 0.01% (w/v) Bromophenol Blue, 1.5% (v/v) 2-mercaptoethanol. Samples were subjected to electrophoresis on a 10% acrylamide gel and transferred to Immobilon-P® membranes (Millipore, Bedford, MA, USA) using standard procedures. Membranes were blocked in saline buffer (25 mM Tris-HCl pH 7.4; 140 mM NaCl; 0.1% (v/v) Tween-20) containing 5% (w/v) nonfat milk for 2 h at 22°C before the addition of the antibodies for an overnight incubation at 4°C. Several washes were performed in saline buffer and peroxydase-conjugated antibodies against mouse or rabbit immunoglobulins (Amersham Biosciences, Piscataway, NJ, USA) were added at a dilution of 1/6000 for 45 min at 22°C. After washing, the membranes were soaked in Western blotting Luminol Reagent™ (Santa Cruz Biotechnology Inc.,

Santa Cruz, CA, USA) followed by autoradiography. When appropriate, membranes were stripped using Restore™ Stripping Buffer (Pierce, Rockford, IL, USA) for 15 min at 22°C and reprobed with the indicated antibodies.

Antibodies

Antibodies to Egr-1 (sc-189), Egr-2 (sc-190) and Egr-3 (sc-191) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). They were used at a concentration of 0.07 and 0.1 μg/ml, respectively (in a total volume of 12 ml). Antibodies to β-actin (clone AC-15) were from Sigma Aldrich Inc. (St Louis, MO, USA) and were used at a concentration of 0.22 μg/ml in a total volume of 12 ml.

RT–PCR analysis of mRNA expression

Cells were transfected with control or antisense oligonucleotide, or with carrier alone. The day after transfection, total RNA was extracted using the RNeasy mini-kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. A measure of 400 ng of total RNA was used as a template for cDNA synthesis (SuperScript II from Invitrogen).

PCR amplification was performed with Taq PCR Master Mix kit (Qiagen) with the following specific primers. Egr-1: 5′-GGGAGAGGCAGGAAAGACATAA-3′ and 5′-TCTGAGATCTTCCATCTGACCTAAGA-3′; Egr-4: 5′-TACAGCGGCAGCTTCTTCATC-3′ and 5′-TGCCAGACATGAGGTTGAAGAG-3′; GAPDH: 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′; Egr-2: 5′-TGCACGAAAGGCCCTATCC-3′ and 5′-TTCTGCCGAAGGTGGATCTT-3′; TGF-β1; 5′-CACCGGAGAGCCCTGGATA-3′ and 5′-TGTACAGCTGCCGCACACA-3′; PTEN: 5′-TGAAGACCATAACCCACCACAG-3′ and 5′-TTACACCAGTCCGTCCCTTTCC-3′ (GAPDH, glyceraldehyde-3-phosphate dehydrogenase).

Conditions for the PCR reaction were as follows: one cycle of 5 min at 94°C; 35 cycles of 30 s at 94°C; 30 s at 60°C and 30 s at 72°C; one cycle of 7 min at 72°C. PCR products were analysed by electrophoresis through 2% agarose gels containing ethidium bromide.

In some experiments RNA extracted from the transfected cells were analysed by (Q-PCR) using the 7900 Sequence Detection System from Applied Biosystems (Foster City, CA, USA), according to the manufacturer's instructions. A measure of 500 ng of total RNA was used as template for cDNA synthesis as described above. Q-PCR primer sequences were selected for each cDNA with the aid of PRIMER EXPRESS software. GAPDH: 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′; EGR-1: 5′-GGGAGAGGCAGGAAAGACATAA-3′ and 5′-TCTGAGATCTTCCATCTGACCTAAGA-3′; TGF-β1: 5′-CACCGGAGAGCCCTGGATA-3′ and 5′-TGTACAGCTGCCGCACACA-3′; PTEN: 5′-TGAAGACCATAACCCACCACAG-3′ and 5′-TTACACCAGTCCGTCCCTTTCC-3′.

Q-PCR reactions and quantitative measurements were performed with the SYBR-Green PCR-Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instruction (User Bulletin # 2; Abi Prism 7700 Sequence Detection System). The results were normalized to the relative amounts of GAPDH.

Cell proliferation

Cells were plated at a density of 5×10^4 cells/12-well tissue culture dishes the day before transfection. They were transfected with 0.1 μM oligonucleotides for 3 h. To determine cell numbers, cells were washed twice with PBS, digested by trypsin-EDTA, resuspended in 1 ml of 10% serum-containing medium and transferred to a suspension vial in a final volume of 10 ml PBS. Cells were counted using a COULTER™ Multisizer II instrument (Beckman Coulter Inc., Hialeah, FL, USA) gated for the appropriate cell size and corrected for particulate debris. Each experiment was performed in duplicates and each vial was counted at least twice. Initial cell numbers were checked by counting after transfection ($t = 3$ h).

Flow cytometry analysis of cell cycle

TRAMP-C2 cells were transfected with control or antisense oligonucleotides (0.2 μM). After 48 h, cells were treated with trypsin-EDTA, washed, resuspended in medium, counted and then fixed in 70% ethanol at 4°C for 2 h. The cells were washed and resuspended at a concentration of 10^6 cells/0.5 ml in PBS containing 0.1% Triton X-100, 50 μg/ml DNase-free RNase A and 50 μg/ml propidium iodine. They were incubated in the dark for 30 min at room temperature. The red fluorescence of single events was recorded using an argon ion laser at 488 nm excitation wavelength (FACSCalibur flow cytometer, Becton Dickinson Corp., San Jose, CA, USA). Cell Quest™ Software was used for cell cycle histogram determination and data analysis.

Colony formation assay

TRAMP-C cells were transfected with control or antisense oligonucleotide (0.1 μM). The following day, cells were digested by trypsin-EDTA, resuspended in complete medium and a portion of the cells was counted. The remaining cells were plated at a density of 200 cells/well in a six-well tissue culture plate in complete medium containing 0.1 μM control or antisense oligonucleotide. A week later, cells were rinsed in PBS and stained with 2% crystal violet (w/v in methanol), dried, and used for photography and colony counting.

Growth in soft agar

TRAMP-C cells were transfected with control or antisense oligonucleotide (0.1 μM). The day after, cells were trypsinized and seeded in 0.35% (w/v) microbiology grade agarose (Fisher Scientific, Pittsburgh, PA, USA) prepared in complete medium. This top layer was poured onto a first layer of sterile 0.5% (w/v) agarose prepared in complete medium that had been allowed to solidify in six-well plates. After 2 weeks of incubation at 37°C in 5% CO_2, colonies were stained with 0.5 mg/ml of nitro-blue tetrazolium (Sigma Aldrich Inc.), which is a vital dye. After 24 h the plates were photographed and grown colonies were counted.

Animal experiment

A breeding colony of TRAMP mice (background C57BL/6) is maintained in the Animal Care Facility at the Sydney Kimmel Cancer Center, based on a pair of mice kindly provided by Norman Greenberg (Baylor College of Medicine, Houston, TX, USA). PCR assay for monitoring the presence of the large-T antigen in new litters is routinely carried out. Our IACUC approved the protocol and all procedures were performed following Standard Operating Procedures in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Three groups of PCR-confirmed male TRAMP mice were defined for this study. Treatment was started at an average age of 22 weeks (21.81 ± 0.33, $n = 23$) when prostate cancer is already developing (Greenberg *et al*., 1995). The mice received IP injections

three times a week with vehicle alone (seven mice), three-base mismatch control oligonucleotide (eight mice) or E5 antisense oligonucleotide (eight mice), at a dose of 25 mg/kg. Animals were killed when they are 32-week-old. Necropsies with gross microscopic examination were carried out. Significance of results was calculated using the Fisher Exact Test.

Abbreviations

Egr-1, early growth response-1; TGF, transforming growth factor; NAB, nerve growth factor-I A binding protein; PTEN, phosphatase and TENsin homolog deleted on chromosome 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; RT–PCR, reverse transcription–polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; s.e., standard error.

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