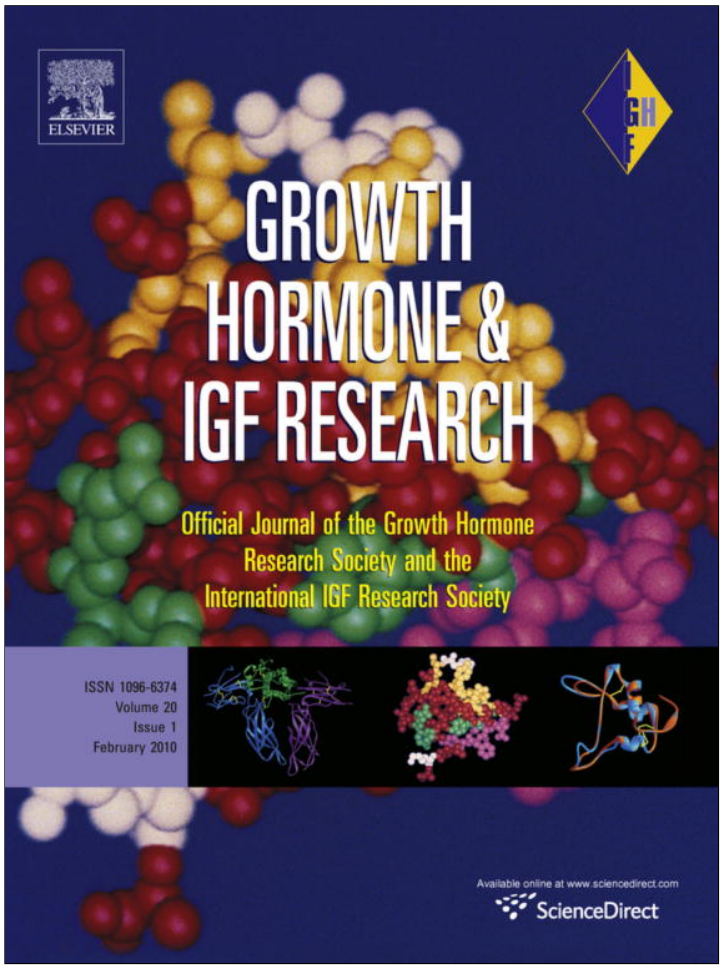

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Transcription factor E2F1 is a potent transactivator of the insulin-like growth factor-I receptor (IGF-IR) gene

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ABSTRACT

Objectives: The insulin-like growth factor-I receptor (IGF-IR) plays an important role in cancer development. The E2F1 transcription factor activates S-phase promoting genes and mediates apoptosis. Microarray analyses of E2F1-induced genes revealed that genes associated with proliferation as well as apoptosis are upregulated by E2F1. Among other candidate genes, DNA microarrays identified the IGF-IR gene as a putative E2F1 target. The aim of this study was to investigate the involvement of E2F1 in regulation of IGF-IR gene transcription.
Methods: To examine the potential regulation of IGF-IR gene expression by E2F1, an E2F1 expression vector was transfected into P69 and M12 prostate cancer cell lines, after which IGF-IR levels were measured by Western blots. Transient transfections were used to evaluate IGF-IR promoter activity and chromatin immunoprecipitation (ChIP) assays were employed to assess E2F1-binding to the IGF-IR promoter.
Results: Results obtained showed that E2F1 expression induced a significant increment in endogenous IGF-IR levels. ChIP assays showed enhanced E2F1-binding to the IGF-IR promoter in E2F1-expressing cells. Transient coexpression of an E2F1 vector along with an IGF-IR promoter-luciferase reporter resulted in a ~140-fold increase in IGF-IR promoter activity. Furthermore, deletion and bioinformatic analyses indicate that the ability of E2F1 to stimulate IGF-IR promoter activity was correlated with the number of E2F1 sites in the promoter region.
Conclusions: In summary, we provide evidence that E2F1 regulates IGF-IR gene transcription in prostate cancer cells *via* a mechanism that involves direct binding to specific elements in the proximal IGF-IR promoter.

1. Introduction

Growth of normal cells is tightly controlled at several critical phases of the cell cycle, collectively referred to as cellular checkpoints. The G_1/S-phase checkpoint is the major cell cycle transition point in which cells are susceptible to mitotic and proliferative signals [1]. The insulin-like growth factors (IGFs) are a family of growth factors, binding proteins, and receptors that are involved in normal growth as well as in a number of pathological states. IGF-I is a progression factor that is required by the cell to traverse the cell cycle and to evade arrest in G_1 [2,3]. IGF-I stimulates cell proliferation, induces differentiation, and inhibits cell death in a wide variety of cell types [2,4–6].

IGF-I activates a ubiquitously expressed cell-surface receptor, the IGF-I receptor (IGF-IR), the product of a single-copy gene located on the long arm of chromosome 15. The IGF-IR signals mitogenic, antiapoptotic, and transforming activities. The IGF-IR includes a tyrosine kinase domain in its cytoplasmic portion and is coupled to several intracellular second messenger pathways [7,8]. The IGF-IR is vital for cell survival, as demonstrated by the lethal phenotype of mice in which the IGF-IR gene was disrupted by homologous recombination [9]. Examination of multiple types of cancer, including breast, ovarian, prostate, colon, hematopoietic, rhabdomyosarcoma, and renal, revealed an abundant expression of IGF-IR [10], suggesting that upregulation of the IGF-IR gene constitutes a common paradigm in cancer. Furthermore, molecular analyses have identified the IGF-IR promoter as a downstream target for various zinc finger transcriptional activators, including Sp1 and KLF6, as well as several tumor suppressors, including p53,

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Wilm's tumor protein-1 (WT1), breast cancer gene-1 (BRCA1), von-Hippel Lindau (VHL), and others [11–13].

The E2F family of transcription factors plays a key role in regulating the expression of genes involved in the G_1/S transition and DNA synthesis [14–16]. The retinoblastoma (Rb) and E2F proteins form a complex (Rb-E2F) which undergoes dissociation upon phosphorylation of Rb, with ensuing activation of E2F-dependent transcription and cell cycle progression [16,17]. E2F binds to DNA and regulates the expression of genes involved in cell cycle progression. E2F family members are divided into distinct subgroups on the basis of structural and functional similarities. E2F1, E2F2 and E2F3a are transcriptional activators, whereas E2F3b, E2F4, E2F5, E2F6, E2F7, and E2F8 are repressors [18]. E2F1 is particularly prominent for its dual function, triggering both proliferation and apoptosis [16]. However, dysregulated expression of E2F1 may cause apoptosis via p53-dependent and p53-independent pathways [19]. These conflicting roles prompted research on E2F1 responsive genes. Microarray analyses of E2F1-induced genes revealed that genes associated with proliferation as well as with apoptosis are usually upregulated by E2F1 [20–22].

Epidemiological, clinical, and experimental data support a major role for the IGF axis in prostate cancer [23–27]. The IGF-IR has been shown to be upregulated at early stages of the disease [28]. However, the transcription factors responsible for IGF-IR gene regulation in prostate cancer are yet to be identified. In view of previous data regarding the mechanisms of transcription of the IGF-IR gene, we hypothesized that the IGF-IR gene may constitute a novel downstream target for E2F1 action. The results obtained indicate that E2F1 is a potent transactivator of the IGF-IR gene. Furthermore, stimulation of the IGF-IR gene by E2F1 was shown to involve specific binding to sequences in the proximal IGF-IR promoter. Control of IGF-IR expression by E2F1 may be important in terms of normal cell cycle progression as well as prostate (and other types of) cancer development.

2. Materials and methods

2.1. Cell cultures

Derivation of the P69 and M12 cell lines has been described [29,30]. Briefly, the P69 cell line was obtained by immortalization of prostate epithelial cells with SV40 T antigen, and the M12 cell line was derived by injection of P69 cells into athymic nude mice and serial reimplantation of tumors into mice. P69 and M12 cells were cultured in RPMI 1640 medium with 10 ng/ml EGF, 0.1 nM dexamethasone, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium. P69 cells are responsive to IGF-I and are rarely tumorigenic whereas M12 cells are highly tumorigenic and metastatic, and exhibit a reduced IGF-I responsiveness [31].

2.2. Plasmids and DNA transfections

For cotransfection experiments, luciferase reporter constructs p(−2350/+640)LUC, p(−476/+640)LUC, and p(−188/+640)LUC (nt 1 corresponds to the transcription start site) were employed. These plasmids include 2350, 476, or 188 nucleotides of the 5′-flanking region, linked to 640 bp of the 5′-untranslated region of the IGF-IR gene [32]. The E2F1 expression vector, pcDNA-I-E2F-1, was previously described [33]. Cells were seeded in six-well plates 24 h before transfection, and transfected with 1 μg of the indicated IGF-IR promoter plasmid, along with 1 μg of the E2F1 expression vector (or empty pcDNA-I vector) and 0.3 μg of a β-galactosidase plasmid (pCMVβ, Clontech, Palo Alto, CA), using the jetPEI reagent (Polyplus Transfection, Illkirch, France). Forty-eight hours after transfection, cells were harvested and luciferase and β-galactosidase activities were measured [34]. To evaluate the effect of E2F1 on endogenous IGF-IR levels, cells were seeded in six-well plates 24 h before transfection, and transfected with 1 μg of E2F1 vector (or empty vector). Forty-eight hours after transfection, cells were harvested for Western immunoblots.

2.3. Western immunoblots

Cells were harvested with phosphate buffered saline (PBS), lysed, and electrophoresed through 10% SDS–PAGE, followed by blotting onto nitrocellulose filters. After blocking with 5% milk, blots were incubated with an IGF-IR β-subunit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with a horseradish peroxidase-conjugated secondary antibody. In addition, blots were incubated with antibodies against E2F1 (Santa Cruz Biotechnology), phospho-IGF-IR (Ser473) (Cell Signaling) and tubulin. Proteins were detected using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.4. Chromatin immunoprecipitation (ChIP) studies

Cells transfected with E2F1 were incubated with formaldehyde (1%) for 10 min. At the end of the incubation, cells were washed and harvested using ice-cold PBS. Pelleted cells were resuspended in 1% SDS-containing buffer, incubated on ice for 10 min, and sonicated for 3 min. Extracts were immunoprecipitated with anti-E2F1 for 18 h at 4 °C. For PCR analysis of E2F1-immunoprecipitated chromatin, a set of primers encompassing the IGF-IR proximal promoter (nt −469 to +288) was employed, using the following primers: sense, 5′-CTTTCCAGCCGCGCTGTTGTTG-3′; antisense, 5′-GGTAAACAAGAGCCCCAGCCTC-3′. PCR was performed using TermalAce™ DNA polymerase (InVitrogen, Carlsbad, CA).

2.5. Identification of consensus E2F1-binding sites in the IGF-IR promoter region

To address the presence of potential E2F1-binding elements in the genomic locus of the IGF-IR promoter, the Genomatix software (MatInspector, Genomatix Software Inc., Ann Arbor, MI) was employed.

2.6. Statistical analysis

Statistical significance was determined using Student's *t*-test. A *p*-value less than 0.05 ($PV < 0.05$) was considered significant.

3. Results

3.1. Effect of E2F1 on IGF-IR expression

To investigate the potential regulation of IGF-IR expression by transcription factor E2F1, we employed the P69 and M12 human prostate cancer cell lines. Cells were transiently transfected with an E2F1 expression vector for 48 h, after which total IGF-IR levels were measured by Western blotting. Results obtained showed an approximately 1.5-fold increase in endogenous IGF-IR levels, in comparison with empty pcDNA-I vector-transfected cells ($p < 0.01$), in P69 cells and an approximately 2.5-fold increase in M12 cells (Fig. 1A and B). Corresponding changes in phospho-IGF-IR levels were also observed, consistent with basal activation of the IGF-IR signaling axis. No change in tubulin levels was observed.

3.2. Effect of E2F1 on IGF-IR promoter activity

To assess whether the E2F1-induced increase in IGF-IR levels was mediated at the level of transcription, transient cotransfection

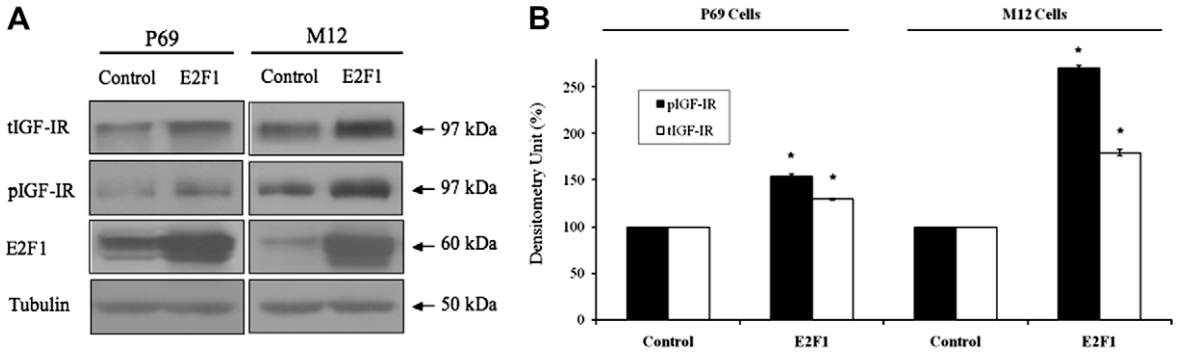


Fig. 1. Regulation of IGF-IR levels by E2F1. (A) P69 and M12 cells were transfected with an E2F1 expression vector (or empty pcDNA-I) for 48 h. At the end of the incubation period, cells were lysed and E2F1 and total (t) and phosphorylated (p) IGF-IR levels were assessed by Western blotting. Results shown are representative of an experiment repeated three times with similar results. Blots were stripped and blotted with anti-tubulin as a loading control. The 97-kDa IGF-IR β-subunit, 60-kDa E2F1, and 50-kDa tubulin bands are denoted. (B) The bar graph denotes the densitometric scanning of the total and phospho-IGF-IR bands normalized to the corresponding tubulin bands. The bars represent the mean ± S.E.M of three independent experiments. $^{*}p < 0.05$ vs. untreated cells.

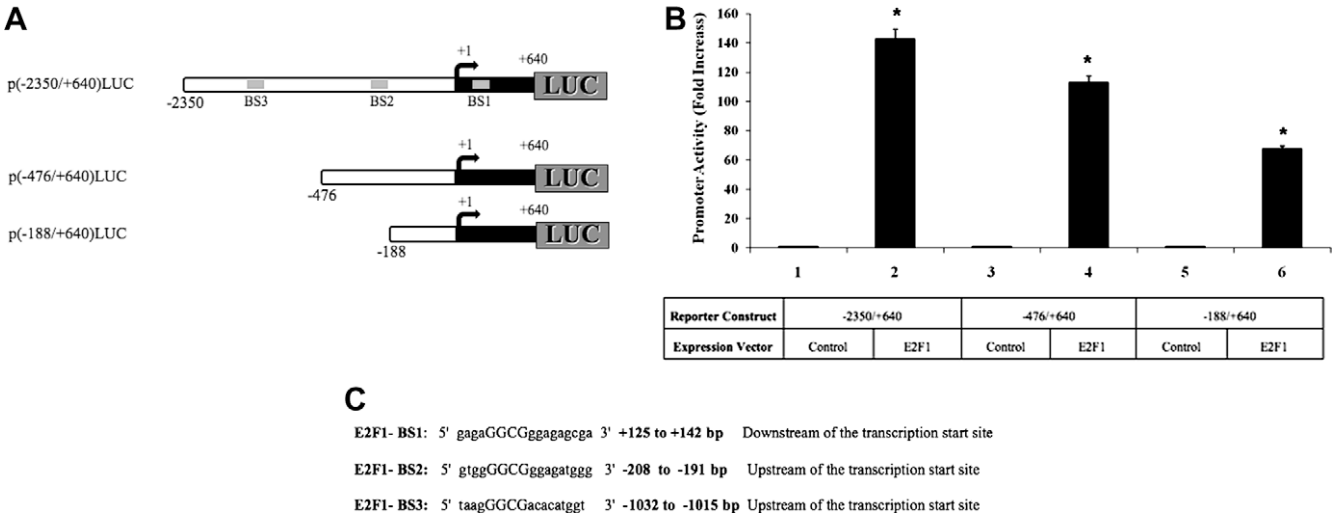
experiments were performed in M12 cells using the full-length IGF-IR promoter-luciferase reporter construct [p(−2350/+640)LUC] (Fig. 2A), along with an E2F1 expression vector and a β-galactosidase plasmid. Luciferase measurements revealed that E2F1 stimulated IGF-IR promoter activity by ∼140-fold (Fig. 2B). The discrepancy between IGF-IR promoter activity and IGF-IR protein values in the previous section can be explained by the very high basal IGF-IR levels in most cancer cells and by the fact that the efficiency of transient transfections is usually 10–30% of the cells. Therefore, it is easy to appreciate changes in luciferase values (not expressed in control, untransfected cells) whereas changes in endogenous IGF-IR protein (expressed in both transfected and untransfected cells) are more difficult to see.

3.3. Bioinformatic analysis of E2F1-binding sites in the IGF-IR promoter region

To explore whether potential E2F1-binding elements reside in the genomic locus of the IGF-IR promoter region, we used the computer software Genomatix. Three putative E2F1-binding sites (E2F1-BS1 to E2F1-BS3) were found within the full-length promoter region. The locations and sequences of the E2F1 *cis* elements are denoted in Fig. 2A and C.

3.4. Deletion analysis of E2F1 stimulation of IGF-IR promoter activity

To identify the IGF-IR promoter region responsible for mediating the effect of E2F1, cotransfections were performed using promoter constructs with sequentially deleted 5′-flanking regions. As shown in Fig. 2A, only two E2F1 sites (E2F1-BS1 and E2F1-BS2) are included in the proximal promoter construct, p(−476/+640)LUC, and only one site (E2F1-BS1) is included in the minimal promoter construct, p(−188/+640)LUC. Deletion analyses showed that the stimulatory effect of E2F1 was reduced in cells transfected with shorter promoter fragments. Thus, E2F1 stimulated activity of the p(−−476/+640)LUC construct (which lacks the fragment between nt −2350 to −476) and p(−188/+640)LUC construct (which lacks the fragment between −476 and −188) by ∼110- and ∼60-



C

E2F1- BS1: 5' gagaGGCGggagagcga 3' **+125 to +142 bp** Downstream of the transcription start site

E2F1- BS2: 5' gtggGGCGggagatggg 3' **-208 to -191 bp** Upstream of the transcription start site

E2F1- BS3: 5' taagGGCGacacatggt 3' **-1032 to -1015 bp** Upstream of the transcription start site

Fig. 2. Effect of E2F1 on IGF-IR promoter activity. (A) Schematic representation of IGF-IR promoter deletion constructs employed in cotransfection experiments. Plasmids p(−2350/+640)LUC, p(−476/+640)LUC, and p(−188/+640)LUC contain, respectively, 2350, 476, and 188 bp of 5′-flanking region (open bars) and 640 bp of 5′-untranslated region (solid bar) of the IGF-IR gene, fused to a luciferase cDNA (LUC). Arrows indicate the transcription start site. The basal promoter activities of the various fragments was reported [32]. Putative E2F1-binding sites (BS1, BS2 and BS3) are indicated. (B) Deletion analysis of E2F1 effect on IGF-IR promoter activity. M12 cells were cotransfected with 1 μg of the indicated reporter, along with 1 μg of the E2F1 vector (or empty pcDNA-I) and 0.3 μg of pCMVβ using the JetPEI reagent. Luciferase values, normalized for β-galactosidase, are expressed as fold-increase of the luciferase activity in the absence of E2F1. A value of 1 was assigned to the promoter activity in cells transfected with an empty pcDNA-I expression vector. Results are mean ± S.E.M. of three independent experiments, performed in duplicate. $^{*}p < 0.01$ vs. cells transfected with empty vector. (C) Sequences of putative E2F-binding sites in the IGF-IR genomic locus. Capitals represent core sequences.

fold, respectively (Fig. 2B). These analyses indicate that the ability of E2F1 to stimulate IGF-IR promoter activity was correlated with the number of E2F1-binding sites present in each of the fragments analyzed.

3.5. ChIP analysis of the physical interactions between E2F1 and the IGF-IR promoter

Finally, the potential physical interactions between E2F1 and the IGF-IR promoter were assessed using ChIP assays. Briefly, E2F1-overexpressing M12 and control cells were treated with 1% formaldehyde for 10 min, after which the cells were lysed and immunoprecipitated with anti-E2F1 or normal mouse serum. The E2F1-precipitated chromatin was amplified by PCR with a set of primers encompassing the proximal promoter region (nt −469 in the 5′-flanking region to nt +288 in the 5′-untranslated region). Results of ChIP assays showed that E2F1-binding to the IGF-IR promoter region was largely enhanced in E2F1-overexpressing cells in comparison to untransfected cells (Fig. 3).

4. Discussion

The involvement of IGF-IR in prostate cancer has been the subject of extensive investigation [35]. The capacity of the IGF-IR to favor a particular biological pathway is dictated by multiple cellular and extracellular factors, many of whom are yet to be identified. Published microarray data suggest that the IGF-IR gene is a target for E2F1 action [36]. In addition, studies have shown that IGF-I causes phosphorylation of Rb and augments E2F1 protein levels in a time-dependent manner [37], suggesting a complex interplay between the IGF system of ligands and receptors and the E2F-Rb regulatory network. This study identifies the IGF-IR gene as a downstream target for E2F1 action in prostate cancer cells.

Using transient transfection assays we demonstrate that E2F1 is a potent inducer of IGF-IR expression. Specifically, the results obtained show that enforced E2F1 expression in cancer cells induced an increment in endogenous IGF-IR levels. Augmented IGF-IR levels were correlated with elevated phospho-IGF-IR values, suggesting activation of the IGF-IR signaling pathway. In addition, results of transfection experiments showed that the effect of E2F1 on IGF-IR levels was mediated at the transcription level. The discrepancy between the increases in IGF-IR protein levels (∼1.5–2.5-fold increase) and IGF-IR promoter activity (∼140-fold increase) is most probably due to the fact that it is difficult to appreciate changes in endogenous IGF-IR levels in cells with high endogenous expression. Of course, we cannot discard the possibility of compensating alterations in IGF-IR levels at the level of translation. Bioinformatic analysis predicted three E2F1-binding sites in the human IGF-IR promoter. Furthermore, we showed that the ability of E2F1 to stimulate IGF-IR promoter activity was correlated with the number of E2F1 sites present in the specific constructs, suggesting a dose effect for E2F1-binding on IGF-IR expression. In addition, *in vivo* analysis of promoter occupancy by ChIP assays revealed that E2F1 is specifically recruited to the IGF-IR promoter in the genomic regions including the E2F1 consensus elements. Combined, these data indicate that transcription factor E2F1 is a novel regulator of the IGF-IR gene.

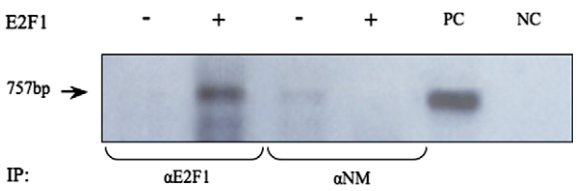


Fig. 3. ChIP analysis of E2F1 interactions at the IGF-IR promoter. M12 cells were transfected with an E2F1 vector (lanes 2 and 4) or with an empty vector (lanes 1 and 3). After 48 h, cells were lysed and immunoprecipitated with an E2F1 antibody (lanes 1 and 2) or with a normal mouse serum (αNM) (lanes 3 and 4), followed by PCR amplification of precipitated chromatin using primers encompassing the IGF-IR promoter. The position of the 757 bp-amplified fragments is indicated. Lane 5 represents the PCR product of M12 DNA (positive control, PC). Lane 6 represents the PCR product without template (negative control, NC).

E2F1 functions as a transcription factor that enhances proliferation by binding to the promoter region of several genes, including those that are involved in cell cycle regulation and DNA replication. Activation of E2F1 has been suggested to constitute a key event in the development of many human cancers [38]. Previous studies have implicated the E2F-Rb pathway in prostate tumorigenesis. Thus, between 17% and 60% of prostate cancers demonstrate loss of heterozygosity of the *Rb* locus [39–42], and both reduced expression of *Rb* mRNA [41] and pRb protein have been reported [39,43]. Although there has been no clear correlation between pRb loss and tumor stage or grade, several studies suggested that mutations in *Rb* can be an early events in prostate cancer [44]. A study by Davis et al. [45] showed that disruption of the Rb-E2F complex by homozygous deletion of *Rb* in a prostate tissue recombination model led to increased E2F activity and activation of E2F-target genes *in vitro* and predisposed prostatic epithelium to hormonal carcinogenesis *in vivo*.

Our results are consistent with a model in which high E2F1 levels in primary prostate cancer may cause an elevation in IGF-IR levels. The elevation in IGF-IR protein may contribute to the proliferative effects associated with initiation of prostate cancer. Increased production of IGF-IR is usually associated with augmented activation of cell-surface receptors by locally produced and/or circulating IGF-I and IGF-II [12,24]. In summary, the present study identifies the IGF-IR gene promoter as a novel downstream target for transcription factor E2F1, and suggests a potential functional link between these important players in the etiology of a subset of prostate and other types of cancer.

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