

# Enhanced GBX2 Expression Stimulates Growth of Human Prostate Cancer Cells via Transcriptional Up-Regulation of the Interleukin 6 Gene<sup>1</sup>

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## ABSTRACT

Previous studies demonstrated that the *GBX2* homeobox gene is consistently overexpressed in cultured human prostate cancer cell lines. In this study, the human *GBX2* cDNA was cloned and a quantitative reverse transcription-PCR method used to demonstrate that *GBX2* mRNA expression is enhanced in approximately 70% of human prostate cancer tissues compared with normal human prostate tissues. Purified recombinant *GBX2* protein binds specifically to an ATTA motif within the promoter of the interleukin 6 (*IL-6*) gene. Using an antisense approach, down-regulation of the expression of *GBX2* correlated with decreased expression of *IL-6* and an inhibition of tumorigenicity of PC3 human prostate cancer cells. In addition, *in vitro* growth of the antisense clones was partially restored by exogenous addition of recombinant *IL-6* protein to the culture media. These data demonstrated that enhanced *GBX2* expression results in a stimulation of malignant growth of prostate cancer cells and that part of this stimulation involves up-regulation in the transcription of the *IL-6* gene.

## INTRODUCTION

The homeobox family of transcription factors contributes to the control of cell identity and differentiation, and aberrant expression leads to transformation or disease (1). This class of genes contains a common DNA sequence motif termed a homeobox domain composed of conserved nucleotides encoding a 60-amino acid residue polypeptide sequence with DNA binding ability (2). In humans, as well as in mice, there are 39 class I homeobox genes that are organized in four clusters designed as

A, B, C, and D (2). In humans, these clusters are located on chromosomes 7, 17, 12, and 2, respectively (2).

We have previously demonstrated that the homeobox gene *GBX2* is consistently overexpressed in prostate cancer cells compared with normal prostate epithelial cells and that down-regulation of the *GBX2* expression inhibits the clonogenic ability and tumorigenicity of prostate cancer cells (3, 4). These results suggest that enhanced expression of the *GBX2* protein contribute to the malignant transformation of human prostate cancer cells. Homeobox genes, including *GBX2*, encode transcription factors of the helix-turn-helix motif that recognize and bind to the specific DNA sequences (2). Through this binding, the homeobox genes either positively or negatively regulate the expression of target genes. *IL-6*<sup>3</sup> is a glycoprotein consisting of 212 amino acids encoded by the *IL-6* gene localized to chromosome 7p21-14 (5). *IL-6* is a pleiotropic cytokine that plays a central role in host defense mechanisms by regulating immune responses, hematopoiesis, and the induction of acute phase reaction (5). The expression of *IL-6* and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients (6, 7). *IL-6* stimulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor (8). In two separated studies, Okamoto *et al.* (9) demonstrated that *IL-6* functions as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for the human DU145 and PC3 androgen-insensitive prostate cancer cells. Chung *et al.* (7), however, demonstrated that *IL-6* functions as a paracrine growth inhibitor for the LNCaP cells and an autocrine growth stimulator for the DU145 and PC3 cells. Furthermore, *IL-6* has been demonstrated as a candidate mediator of human prostate cancer morbidity (10). In this report, we identify *IL-6* as an important downstream target of *GBX2* and imply it as one of the mediators of a molecular pathway initiated by *GBX2* that stimulates the growth of prostate cancer cells.

## MATERIALS AND METHODS

**Tissue Specimens, Cell Culture, Transfection, Selections, and Proliferation.** Normal and cancer tissues were obtained from radical prostatectomy specimens from the Prostate Tissue Resource of the Johns Hopkins SPORE (CA 58236). PC3 and TSU-pr1 human prostate cancer cells were maintained in RPMI 1640 medium with 10% fetal bovine serum as described previously (3). The cDNA of the *GBX2* homeobox

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<sup>3</sup> The abbreviations used are: *IL-6*, interleukin 6; *GST*, glutathione *S*-transferase.

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Human: 1  MSAAFPPSLMMMQRPLGSSTAFSIDSLLIGSPQPSPGQFVYTGYPMPFYPYRPVVLPPPPP 60
Mouse: 1  MSAAFPPSLMMMQRPLGSSTAFSIDSLLIGSPQSPGHFVYTGYPMPFYPYRPVVLPPPPP 60
Human: 61  PPPALPQAALQPALPPAHPHHQIPSLPTGFCSSLAQGMALTSTLMATLPGGFSASPQHQE 120
Mouse: 61  PPPALPQAALQPALPPAHPHHQIPSLPTGFCSSLAQGMALTSTLMATLPGGFSASPQHQE 120
Human: 121 AAAARKFAPQPLPGGNGFDKAEALQADAEDGKFLAKEGSLLAFAAAETVQASLVGAVRG 180
Mouse: 121 AAAARKFAPQPLPGGNGFDKAEALQADAEDGKFLAKEGSLLAFAAAEAVQASLVGAVRG 180
Human: 181 QGKDESKVEDDPKGKEESFSLESVDVYSSDDNLTGQAAHKEEDPGHALEETPPSSGAAGS 240
Mouse: 181 QGKDESKVEDDPKGKEESFSLESVDVYSSDDNLTGQAAHKEEDPGHALEETPPSSGAAGS 240
Human: 241 TTSTGKNRRRRTAFTSEQLLELEKEFHCKKYLSTERSQIAHALKLVSEVQVKIWFQNRRA 300
Mouse: 241 TTSTGKNRRRRTAFTSEQLLELEKEFHCKKYLSTERSQIAHALKLVSEVQVKIWFQNRRA 300
Human: 301 KWKRVKAGNANSKTGEPSSRNPKIVVPIPVHVSRAIRSQHQLEQARP 348
Mouse: 301 KWKRVKAGNANSKTGEPSSRNPKIVVPIPVHVSRAIRSQHQLEQARP 348

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Fig. 1 Comparison of human GBX2 with mouse Gbx2 (one letter amino acid codes). \*, the difference between human GBX2 and mouse Gbx2 cDNA sequences.

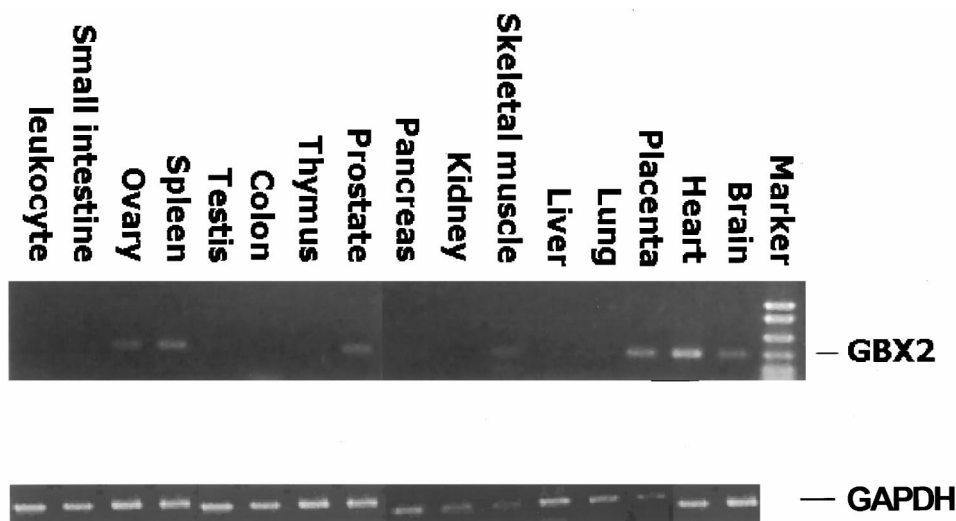


Fig. 2 GBX2 mRNA expression in human tissues analyzed by reverse transcription-PCR. Multiple tissue cDNA panels purchased from Clontech (Palo Alto, CA) were amplified using primers specific for GBX2 (forward, 5'-CACCACGTCTACGGGCAAGAAC-3'; reverse, 5'-AGCTGCTGATGCTGACTTCTGA-3'). The PCR was performed on the cDNAs with a denaturing temperature of 94°C for 45 s, an annealing temperature of 65°C for 30 s, and an extension temperature of 72°C for 1 min for 35 cycles. PCR products were separated by electrophoresis on a 1.5% agarose gel, and DNA fragments were visualized and photographed under UV light by ethidium bromide staining. Human GAPDH primers were used to confirm RNA integrity as described previously (4).

domain was cloned into the pCDNA3.1 vector in the antisense orientation and transfected into PC3 cells as described previously (4). Single transfectant colonies were isolated and maintained in the presence of G418 as described previously (4). The cell proliferation assays were performed as described previously (8).

**Cloning and Sequence Analysis of the Human GBX2 cDNA.** GBX2-specific primers (forward: 5'-GACTTTTCGCCTCTCGCTGGCCTCTA; reverse: 5'-GTTGCTTCAAACA-CAGTGGAGTCCAC) were designed according to the published sequences (11) to amplify the entire coding region of the GBX2 cDNA from GBX2-overexpressing TSU-pr1 human prostate cancer cells. The PCR product was cloned into pCRII

vector (Invitrogen, Carlsbad, CA) and sequenced in both strands.

**Quantitative PCR Analysis.** GBX2 RNA expression analysis was performed by a quantitative competitive PCR method using a synthetic competitor that differed from the GBX2 cDNA by having a small internal deletion as described previously (4).

**Construction of GBX2 Expression Vector, Expression of GST-GBX2 Fusion Protein, and Purification of GBX2 Protein in a Bacteria System.** The full length of the GBX2 cDNA coding region was ligated to bacteria GST gene fusion vector pGEX-6P (Pharmacia Biotech, Piscataway, NJ) in the appropriate reading frame and was expressed as a GST fusion

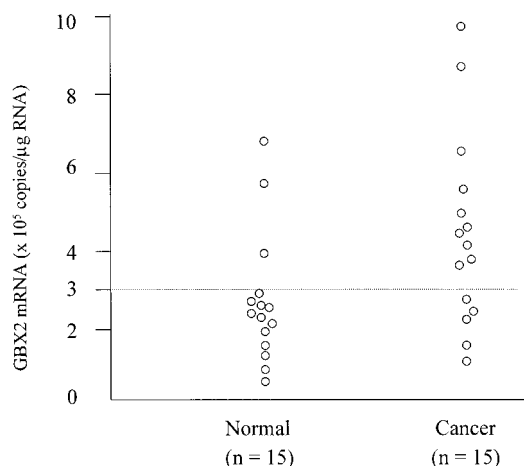


Fig. 3 GBX2 mRNA expression in normal and cancer prostate tissues. The mRNA concentrations were estimated using quantitative competitive PCR as described in "Materials and Methods."

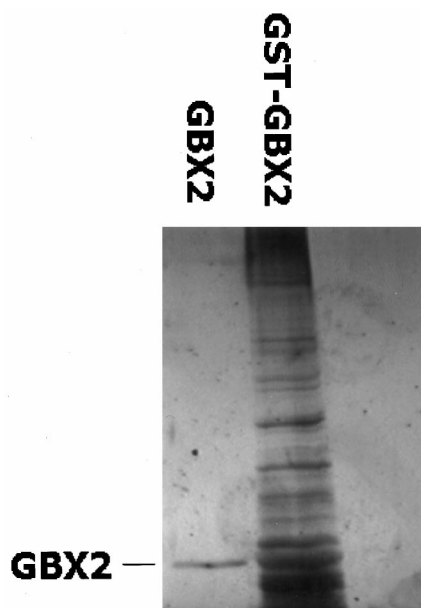


Fig. 4 GST-GBX2 fusion protein expression and digestion by PreScission protease. Lane 1, purified GBX2 protein; Lane 2, sonicate of *E. coli* BL21 cells containing a GST-GBX2 fusion protein.

protein in *Escherichia coli*. The resulting recombinant pGEX-6p-GBX2 was identified. The GBX2-coding sequences in the proper orientation and translation frame relative to the GST was confirmed by sequencing. The propagation of the recombinant plasmids and the expression of the GST fusion protein were hosted in *E. coli* BL21. The GST-GBX2 fusion protein was purified using glutathione Sepharose-4B Redipack columns. The GBX2 recombinant protein was then cleaved and purified by using PreScission protease according to the manufacturer's instructions (Pharmacia Biotech).

**Electrophoretic Mobility Shift Assay.** A double-stranded oligonucleotide with the sequence of 5'-TACAAAT-

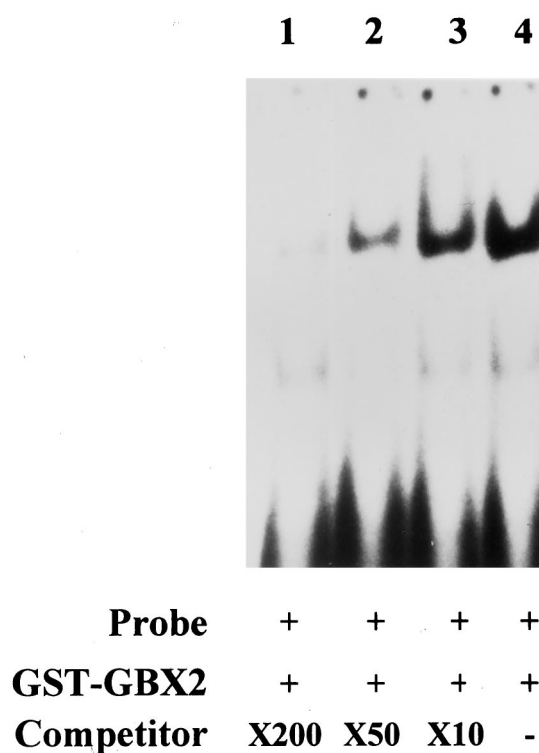


Fig. 5 Electrophoretic mobility shift assay. GST-GBX2 fusion protein was incubated with labeled probe containing the ATTA motif of the IL-6 promoter. Competition analysis was performed with 200-fold (Lane 1), 50-fold (Lane 2), 10-fold (Lane 3) excess of unlabeled competitor probe or no competitor (Lane 4) in the reaction mixture containing the labeled probe and GST-GBX2 fusion protein.

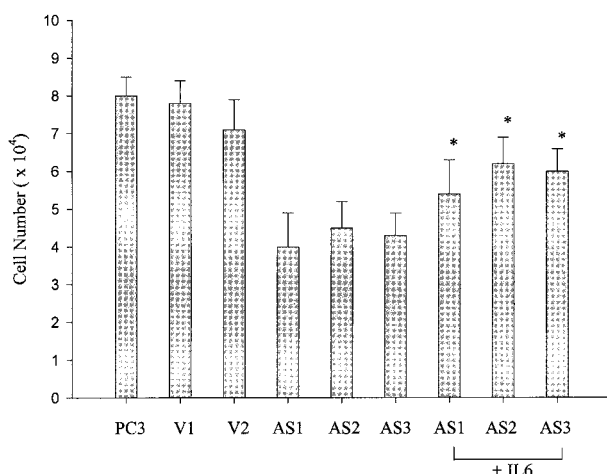
Table 1 GBX2 and IL-6 expression in PC3 parental and GBX2 antisense clones

Cell clones	GBX2		Tumor weight (mg) at excision
	(No. of copies/ $\mu$ g RNA)	IL-6 (pg/ $10^6$ cells/24 h)	
PC3	$1 \times 10^5$	$1465 \pm 105$	$500 \pm 200$ (4) <sup>a</sup>
PC3-V1	$1 \times 10^5$	$1564 \pm 95$	$400 \pm 150$ (4)
PC3-V2	$1 \times 10^5$	$1378 \pm 110$	$450 \pm 150$ (4)
PC3-AS1	$2 \times 10^4$	$190 \pm 15^b$	$150 \pm 100$ (4) <sup>b</sup>
PC3-AS2	$1 \times 10^4$	$48 \pm 25^b$	$0$ (4) <sup>b</sup>
PC3-AS3	$1.4 \times 10^4$	$85 \pm 35^b$	$100 \pm 100$ (4) <sup>b</sup>

<sup>a</sup> Numbers in parentheses represent the number of injection sites per group.

<sup>b</sup>  $P < 0.05$ .

TAACTGG, which corresponds to the nucleotide sequence number -447 to -434 of the human *IL-6* gene (12), was synthesized and end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP. For the gel-shift assay, 50 ng of GST-GBX2 fusion protein and 1 ng of the labeled probe were mixed and incubated in a final volume of 10  $\mu$ l at room temperature for 20 min and then ice for 10 min. For competition analysis, 10-, 50-, and 200-fold molar excesses of cold oligonucleotides were used. The reaction mixture was separated on 5% polyacrylamide/bisacrylamide (29:1, w/w) gels



**Fig. 6** Effect of IL-6 on growth of PC3 cells transfected with antisense GBX2 cDNA.  $2 \times 10^4$  cells were grown in RPMI 1640 with 10% FCS. Cells were downshifted to serum-free medium after 24 h later, and IL-6 was added at a concentration of 100 ng/ml to the GBX2 antisense clones. Five days later, the number of cells was counted. Bars, SD of triplicate samples. Statistical analyses were performed by comparison of the antisense clones treated with IL-6 to their respective antisense controls by *t* test using paired two sample for means. \* $P < 0.05$ .

at 25 mA at room temperature using  $0.5 \times$  Tris-borate EDTA as electrophoretic running buffer.

**ELISA Assay.** Levels of IL-6 in the culture medium were determined by ELISA with the use of polyclonal rabbit antihuman IL-6 as a solid-phase antibody and monoclonal mouse antihuman IL-6 as a second antibody as described by the manufacturer's protocol (R & D Systems, Minneapolis, MN). IL-6 was expressed as a secretion rate into the culture medium in the units of picograms/ml/24 h/ $1 \times 10^6$  cells.

## RESULTS

**Cloning and Sequence Analysis of the Human GBX2 cDNA.** GBX2-specific primers were used to amplify the entire coding region of the GBX2 cDNA from GBX2-overexpressing TSU-pr1 human prostate cancer cells (4). The PCR product was cloned into the pCRII vector and sequenced (Fig. 1). The nucleotide sequences of the GBX2 (GenBank accession no. AF118452) obtained differ slightly (*i.e.*, additional three nucleotides) from the human GBX2 cDNA sequences reported by Lin *et al.* (11). The differences, located within the coding region, result in a predicted protein of 348 amino acids compared with the 347-amino acid GBX2 protein predicted by Lin (11). The predicted 348-amino acid GBX2 sequence is 98% identical to the mouse Gbx2 sequences. The GBX2 cDNA encodes a homeodomain that is identical to the mouse Gbx2 (13). The NH<sub>2</sub> terminus of the GBX2 protein encodes eight proline residues in a row (amino acid positions 56–63). The predicted protein is  $M_r$  36,000 in size without considering any posttranslational modifications.

The expression of the GBX2 gene in normal human prostate as well as several other normal human tissues was examined by reverse transcription-PCR using GBX2-specific primers (4). The GBX2 gene is differentially expressed in selected normal

human tissues, including prostate, brain, heart, placenta, spleen, ovary, and skeletal muscle, but not in the lung, liver, kidney, pancreas, thymus, colon, testis, small intestine, and leukocyte (Fig. 2). These results suggest that GBX2 is not a constitutively expressed housekeeping gene in normal cells, but is involved in tissue-specific differentiation.

**Expression of GBX2 mRNA Is Enhanced in Prostate Cancer versus Normal.** We previously demonstrated that the homeobox gene GBX2 is consistently overexpressed in cultured prostate cancer cells *in vitro* compared with cultured normal prostate epithelial cells. To test whether the expression of the GBX2 is also up-regulated in prostate cancer tissues obtained directly from patients, we examined the levels of the expression of the GBX2 mRNA by quantitative competitive reverse transcription-PCR analysis. GBX2 mRNA was detected in all of the samples examined ranging from  $1 \times 10^5$  to  $1 \times 10^6$  copies/ $\mu$ g RNA (Fig. 3). Using the cutoff value of  $3 \times 10^5$  copies/ $\mu$ g RNA, only 20% of the normal prostate tissues have high GBX2 mRNA expression. In contrast, 67% of the cancer tissues have high GBX2 mRNA expression (*i.e.*,  $>3 \times 10^5$  copies/ $\mu$ g RNA). These results demonstrated that GBX2 mRNA expression is up-regulated in a significant subset of primary human prostatic cancers compared with normal prostate tissues.

**GBX2 Protein Binds the IL-6 Promoter.** Sequence analysis indicated that an ATTA core sequence, an essential motif for GBX2 protein binding (14), is present in the IL-6 promoter region. To test the physical interaction between the putative GBX2 protein and IL-6 promoter, we performed a gel-shift mobility assay by using a purified GST-GBX2 fusion protein. To do this, the full length of the GBX2 cDNA coding region was ligated to the bacteria GST gene fusion vector pGEX-6P in the appropriate reading frame and was expressed as a GST fusion protein in *E. coli*. The GST-GBX2 fusion protein was purified, and the GBX2 recombinant protein was then cleaved and purified by using PreScission protease (Fig. 4). The size of the purified GBX2 recombinant protein is  $M_r$  36,000, which is consistent with the predicted protein of  $M_r$  36,000. When this GBX2 recombinant protein was incubated with an oligonucleotide probe, which corresponds to the -447 to -434 of the IL-6 gene containing the ATTA binding motif (12), there was a shift in mobility of this probe during electrophoresis (Fig. 5). Thus gel shift demonstrated that the recombinant GBX2 protein binds to this IL-6 oligonucleotide. An addition of cold competitor oligonucleotide completely blocked the complex formation (Fig. 5), indicating that GBX2 protein specifically binds to the IL-6 promoter region.

**Down-Regulation of the Expression of GBX2 in PC3 Human Prostate Cancer Cells Is Correlated with Decreased Expression of IL-6 *in Vitro* and Tumorigenicity *in Vivo*.** The androgen-independent PC3 human prostate cancer cell line has been previously demonstrated to have enhanced GBX2 (3, 4) and IL-6 expression (8, 9). To test whether down-regulation of the expression of GBX2 mRNA is correlated with decreased expression of IL-6, IL-6 protein expression in the PC3 cells transfected with GBX2 antisense cDNA was measured using ELISA assay and the results were correlated with the GBX2 mRNA expression levels. Several stable antisense transfectants were obtained and analyzed. The GBX2 mRNA level was analyzed by quantitative competitive PCR as described previ-

ously (4). The GBX2 mRNA expression in the GBX2 antisense clones was decreased by 5–10-fold compared with parental PC3 cells, likewise, the IL-6 protein expression in the GBX2 antisense clones was decreased by more than 10-fold compared with the parental or vector control PC3 cells (Table 1). This decreased IL-6 protein expression is correlated with down-regulation of the IL-6 mRNA expression as examined by reverse transcription-PCR using IL-6-specific primers (data not shown). This decrease in IL-6 production was associated with a decreased growth of these cells *in vitro*, Fig. 6. We have previously demonstrated that all animals that received injection of the PC3 cells transfected with GBX2 antisense clones resulted in a more than 70% suppression of their tumor weight compared with the parental PC3 cells or vector controls (4). These results indicate that down-regulation of the expression of GBX2 mRNA is correlated with decreased expression of IL-6 and decreased tumorigenicity (Table 1).

**Exogenous IL-6 Stimulates the Growth of PC3 Cells Transfected with Antisense GBX2.** Down-regulation of the expression of GBX2 correlates with decreased expression of IL-6 and decreased growth *in vitro*. To test whether IL-6 directly stimulates the growth of GBX2 down-regulated human PC3 prostate cancer cells, human recombinant IL-6 was added into the cell culture of PC3 cells transfected with antisense GBX2. As shown in Fig. 6, exogenous IL-6 stimulates, but not fully restores, the growth of PC-3 cells transfected with GBX2 antisense cDNA. These results suggest that IL-6 production is only one of the mediators of the growth stimulation of prostate cancer cells regulated by GBX2.

## DISCUSSION

We have demonstrated that IL-6 is an autocrine growth factor regulated by the expression of the *GBX2* homeobox gene in human prostate cancer cells and that *GBX2* is overexpressed in approximately 70% of human prostate cancer compared with the normal prostate. In addition, down-regulation of the expression of the *GBX2* inhibits prostate cancer cell growth *in vitro* and tumorigenicity *in vivo*.

Qiu *et al.* (15) demonstrated that the IL-6 ligand/IL-6 receptor complex transmits its signals through ErbB2 to a mitogen-activated protein kinase pathway in prostate cancer cells by inducing tyrosine phosphorylation of ErbB2. The addition of exogenous IL-6 stimulated, but did not fully restore, the growth of the GBX2 antisense clones to that of the parental PC3 cells, suggesting that IL-6 is only one of the several growth regulatory pathways regulated by GBX2. In fact, Chen *et al.* (16) demonstrated that the signal pathway activated by IL-6 is synergistic with epidermal growth factor in human prostate cancer cells. Therefore, we are presently examining the GBX2 effect on the other regulatory pathways such as the epidermal growth factor pathway.

Currently, our working hypothesis is that *GBX2*, encoding a transcription factor of helix-turn-helix motif, acts as a master-controlling gene that stimulates prostate cancer growth by activation of a cascade of its downstream target genes such as *IL-6*. Understanding this molecular pathway is critical to identify the

key regulator(s) in this complex regulatory network that stimulates the growth of prostate cancer cells. This information will be useful for the development of not only a prognostic indicator of prostate cancer cell progression but also therapeutic targets for advanced prostate cancer.

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