Human Papilloma Virus (HPV) E7-Mediated Attenuation of Retinoblastoma (Rb) Induces hPygopus2 Expression via Elf-1 in Cervical Cancer


Abstract

The human papillomavirus (HPV) is the etiologic agent of cervical cancer. In this study, we provide evidence for the human Pygopus (hPygo)2 gene as a cellular biomarker for HPV-related disease. In a tumor microarray of cervical progression, hPygo2 levels were greater in high-grade lesions and squamous cell carcinomas than in normal epithelia. Similarly, hPygo2 mRNA and protein levels were greater in HPV-positive cervical cancer cells relative to uninfected primary cells. RNA interference (RNAi)-mediated depletion of HPV-E7 increased whereas E74-like factor (Elf)-1 RNAi decreased association of Retinoblastoma (Rb) tumor suppressor with the hPygo2 promoter in cervical cancer cell lines. Transfection of dominant-active Rb inhibited Elf-1-dependent activation of hPygo2, whereas Elf-1 itself increased hPygo2 expression. Chromatin immunoprecipitation assays showed that Rb repressed hPygo2 by inhibiting Elf-1 at the Ets-binding site in the hPygo2 promoter. These results suggested that abrogation of Rb by E7 resulted in derepression of Elf-1, which in turn stimulated expression of hPygo2. Thus, initiation of hPygo2 expression by Elf-1 was required for proliferation of cervical cancer cells and its expression therefore may act as a surrogate marker for dysplasia. Mol Cancer Res; 11(1); 19–30. ©2012 AACR.

Introduction

The Wnt/β-catenin transcription complex component, Pygopus, was originally identified as a protein required for Wnt-dependent transcriptional activation during embryonic development (1). Pygopus is also overexpressed in and is required for the growth of a number of cancer cell lines of diverse origin (2–6). Because of its use by malignant cells, Pygo is a candidate biomarker both for diagnostic and potentially therapeutic benefit, but our understanding of the mechanisms that augment its expression in disease is limited.

An important factor required for hPygo2 expression in breast and cervical carcinoma cell lines is the E74-like factor (Elf-1; ref. 7), an E26 transformation–specific (ETS) family member. ETS factors have well-established roles in carcinogenesis, regulating oncogenes and tumor suppressors involved in apoptosis, angiogenesis, invasion, and metastasis (8). Elf-1 itself activates genes involved in tumorigenesis (9–12), is overexpressed in several cancers (13–16), and correlates with poor prognosis (17–20). In normal resting T lymphocytes, the pocket region of the Retinoblastoma (Rb) tumor suppressor interacts with the N-terminal LXCXE motif of promoter bound Elf-1 and blocks its transactivation (21).

Paradigmatic of pathogenically mediated Rb deregulation is the action of human papillomavirus (HPV) E7 protein, a key etiologic agent in the initiation of cervical cancer (22). E7 is expressed following infection of proliferation-competent cervical cells with certain high-risk HPV subtypes. Similar to other viral proteins such as papovavirus T antigen and adenovirus E1A (23), E7 is a 98 amino acid nuclear phospho-oncoprotein, which cooperates with the HPV E6 protein to cause immortalization of epithelial cells (24). E7 induces degradation of the Rb protein, resulting in activation of several transcriptional regulators such as E2F and Elf-1, which promote cell-cycle progression (25).

We are interested in understanding the mechanism of expression and requirement of hPygo2 in cancer. In the initiation of cervical carcinoma, HPV genome integration in abnormally proliferating cervical cells raises the probability that they will progress to malignancy because the HPV E2 genomic region is spliced out and the HPV E2 regulatory protein is no longer produced (26). The loss of E2 causes the constitutive expression of E6 and E7. E6 recruits the cellular E3 ubiquitin ligase E6-associated protein and forms a
trimeric complex with p53 (27) leading to proteosomal degradation (28), thereby preventing cell-cycle arrest and/ or inducing apoptosis. The E7 protein, through the LXCXE motif, binds to the pocket region of Rb and drives its continuous proteolytic degradation (29), permitting Elf-1 to initiate expression of target genes. We hypothesized that the increase in hPygo2 expression required for growth of transformed cervical cells may be the result of Elf-1 activation caused by HPV E7-mediated attenuation of Rb.

In this study, we provide evidence that hPygo2 is over-expressed in cervical cancer and is required for growth of transformed HPV-infected human endo- and ectocervical cells. Our data indicated that hPygo2 overexpression in cervical cancer is augmented by abrogation of Rb function by E7 leading to derepression of Elf-1, suggesting a mechanistic link between HPV and the cellular response of the oncogenic hPygo2 transcription factor.

Materials and Methods

Tissue microarray and immunostaining

Cervical tumor microarrays (Cybridi, Lot No. CC10-11-004 (192-194) and US Biomax, Inc., (Cat No. CIN481; Supplementary Table S1) were stained using a Ventana Benchmark Ultra automated clinical immunostainer optimized for each of the antibodies as provided in Supplementary Table S2 (staining protocols as per manufacturer's instruction or as indicated in Supplementary Table S1). Benchmark Ultra automated clinical immunostainer optimized for each of the antibodies as provided in Supplementary Table S2 (staining protocols as per manufacturer's instruction or as indicated in Supplementary Table S1). Cells were processed and stained for immunofluorescence as previously described (3).

ImageJ (Rasband, W.S., ImageJ. U.S. NIH, Bethesda, MD; http://imagej.nih.gov/ij/, 1997–2012) counting software was used to quantify staining intensity in immunohistochemical images. Only cells in which nuclei were visible were stained and were categorized into negative, weak, and strong staining (Supplementary Fig. S1). The percentages of negative, weak, and strong staining cells were calculated for each core representing 5 diagnoses of disease progression [normal, CIN 1, CIN 2, CIN 3, and squamous cell carcinoma (SCC)] for 3 observers.

Cell lines

Normal human endocervical (HEN; ref. 30) and ectocervical (HEC; ref. 31) primary cells and their HPV16 or 18 and cigarette smoke condensate transformed subclones, HEN 16T (32) and HEC 18T (33), were generous gifts from Dr. A. Pater. Primary cell lines were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 2.1 μL/mL bovine pituitary extract (Gibco) and 0.06 μL/mL EGF (Gibco BRL) and cancer cells in DMEM plus 10% heat-inactivated FBS. Detailed information on cell lines is provided in Supplementary Table S4 and Supplementary Fig. S4.

RNA extraction, cDNA generation, and quantitative PCR

Cellular RNA isolation using the Nucleospin RNA II Kit (Macherey-Nagel) and cDNA generation was previously described (34). cDNA samples were subjected to real-time quantitative PCR using RT2 SYBR green master mix (SA Biosciences; ref. 7) and analyzed by the relative quantitative comparative threshold cycle (ΔΔCt) method (3). Primer sequences are listed in Supplementary Table S5.

Protein extraction, SDS-PAGE, and immunoblotting

Cellular protein extraction and separation on SDS-PAGE were transferred and detected as previously described (2). Antibody information is provided in Supplementary Table S2.

RNA interference and rescue assays

Four siRNA oligonucleotides were synthesized by Dharmacoon, including siPy2-X (which targets the 3'-UTR sequence of hPygo2 of endogenous hPygo2 mRNA), siPy2-Z (which targets the coding region of hPygo2), siE7 (34), and a nonspecific negative control, siNTC. We have previously described the Elf-1 targeting siRNA, siElf-1, (7). Oligonucleotide sequences are listed in Supplementary Table S5.

A total of 3 × 10^5 cells per well were seeded in 6-well plates and forward transfected with siRNA oligos at final concentrations of 10 to 25 nmol/L using Lipofectamine RNAiMAX (Invitrogen) as per the manufacturer’s instructions. In rescue assays, cells were additionally transfected with 1 μg/well of pCS2+/ pCS2 + hPygo2 24 hours after the siRNA transfection using Lipofectamine with Plus Reagent (Invitrogen). Cells were harvested 72 hours after seeding.

Fluorescent-activated cell sorting

Trypsinized and PBS washed cells were fixed in 2% paraformaldehyde and permeabilized in 90% methanol/1 × PBS. After washing, cells were incubated in PBS containing 1 μL of 10 mg/mL propidium iodide and 10 μL of 10 mg/mL RNAse at 37°C for 20 minutes in the dark. Samples were analyzed using a FACSCalibur flow cytometer and graphically displayed using ModFit analysis software.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted as previously described (7). Cross-linked cells were sonicated to produce 500 bp genomic DNA fragments. About 300 μg of precleared chromatin were immunoprecipitated with 2 μg of anti-Rb and anti-Elf-1 antibodies. Normal rabbit and mouse IgGs were used as negative immunoprecipitation controls. After washing, reversing cross-links and purifying DNA, hPygo2, CCNA, and HCCS1 promoters were amplified by qPCR (sequences given in Supplementary Table S5). Promoter occupancy was calculated as a percentage relative to input chromatin levels.

Plasmids

pECE-PSM-Rb was generously provided by Dr. Brenda Gallie (35). The pECE-ΔKP vector control for pECE-PSM-Rb was generated by digesting pECE-PSM-Rb with KpnI and re-ligating to remove the transcriptional start site.
pCS2 + Elf-1 (7) contains the human Elf-1 cDNA. pCS2 + was used as the empty vector control for Elf-1 plasmids. hPygo2 promoter constructs: pGL3-1494 antisense, pGL3-1494, pGL3-48, and pGL3-basic were used previously (7) Site-directed mutagenesis to generate the dominant-active (DA) Rb binding–deficient Elf-1 mutant, pCS2 + –DA-Elf-1, using sequences in Wang and colleagues (21), and the Ets-binding site mutant, pGL3-48 mutEBS was conducted using the QuickChange Kit (Stratagene; primers listed in Supplementary Table S5). Plasmids were verified by sequencing.

**Transient transfections**

Cells were seeded 24 hours before transfection using Lipofectamine and Plus reagent in 6 well plates (3.0 × 10⁷ cells/well) using 1 μg of DNA (per well) or 15 cm plates (7.5 × 10⁶ cells) using 5 μg of DNA. Three types of transfections were conducted: (i) 2 expression plasmids (pECE-PSM-Rb, pCS2 + DA-Elf-1, pCS2 + Elf-1) and their empty plasmid counterparts (0.5 μg of each per well); (ii) one luciferase reporter plasmid (pGL3-1494 antisense, pGL3-1494, pGL3-48, pGL3-48 mutEBS, or pGL3-basic) with a plasmid expressing β-galactosidase, pRSV-βgal (0.5 μg of each per well or 2.5 μg of each per plate); and (iii) 2 expression plasmids (0.25 μg of each per well), one luciferase reporter plasmid (0.25 μg per well) and pRSV-βgal (0.25 μg per well). Luciferase assays were conducted and normalized to β-galactosidase activity as previously described (7).

**Image acquisition and analysis**

Films were scanned at a resolution of 600 dpi, set to grayscale, cropped, and used to make films. No software adjustments were made. Densitometry was performed on scanned films to quantify relative protein levels using ImageJ (NIH) software.

**Results**

**hPygo2 protein is overexpressed in CIN3 and SCC**

The relative expression of hPygo2 in HPV-infected cervical tissues was assessed using a microarray of tissue core samples representing various stages of disease progression, stained by immunohistochemistry, with antibodies against HPV L1, hPygo2, PCNA, and Elf-1.

HPV protein was detected in normal ectocervical as well as dysplastic and malignant tissue. The staining intensity using this antibody was higher in nondiseased ectocervical but not in endocervical epithelia (Supplementary Fig. S2A; ref. 36). The proportion of HPV-positive cells did not vary significantly.

hPygo2 protein was detected in both normal and pathologic tissues, with the intensity of staining appreciably higher in cervical intraepithelial neoplasia grade 3 (CIN3) and SCC cores. The proportion of positively stained cells increased significantly from 7% in normal tissues to 93% in severely dysplastic (CIN3) and malignant tissues. The cellular localization of hPygo2 was either solely cytoplasmic or both cytoplasmic and nuclear (Supplementary Fig. S2B).

Both the expression and staining intensity of the proliferation marker proliferating cell nuclear antigen (PCNA) (37) significantly increased with severity of disease, increasing from approximately 20% in normal epithelia and 41.5% in mild dysplasia (CIN1) to 81% and 76% in CIN3 and SCCs, respectively. In positive cores, PCNA protein was primarily localized to the nucleus (Supplementary Fig. S2B). Elf-1 protein was detected in all tissues and tumors. While staining intensity clearly decreased with disease severity, the proportion of positively stained cells remained high in all epithelia (above 40%) with the exception of normal endocervical cores (24%). As expected, Elf-1 was localized to both the cytoplasm and nucleus (13).

The relatively higher levels of hPygo2 in CIN3 and SCCs prompted further examination. We compared the expression of hPygo2 and 2 established diagnostic markers for cervical neoplasia (p16 and Ki-67; ref. 38), in a separate cervical intraepithelial neoplasia tissue array, by immunohistochemistry. The numbers of negative, weak, and strongly staining cells were determined (Fig. 1 and Supplementary Figs. S1 and S3 and Supplementary Table S3). The expression of Ki-67 increased progressively with the highest intensity (P < 0.02), as measured by the percentage of strongly staining cells, in cores representing SCC (Fig. 1A and Supplementary Fig. S3). Expression of p16 was absent from normal and CIN1 cores and slightly increased in CIN2 (Fig. 1B and Supplementary Fig. S3), but staining intensity was significantly higher (P < 0.02) in CIN3 and SCC cores. Using an ordinal logistic random-effects model (Supplementary Table S6), we determined that the intensity of hPygo2 antibody staining (Fig. 1C and Supplementary Fig. S3) increased significantly with disease progression from CIN2 to CIN3 and SCCs (P < 0.05). Taken together, these observations suggested that expression of hPygo2 protein was upregulated in many CIN3 lesions and SCC tumors with respect to less serious disease and normal cervical tissue.

**hPygo2 mRNA and protein expression and subcellular localization in cervical cancer cell lines**

The foregoing observations suggested that increased hPygo2 expression might be a response to HPV integration and E7 upregulation (occurring in CIN3 and beyond). To determine the relationship between hPygo2 and E7, we conducted gene expression analyses in a variety of previously developed normal and transformed cervical cell lines (30–33). The control cell lines included human primary HPV-negative endo- (HEN) and (HEC) ectocervical cells. The experimental cancer cell lines included HPV-16 and HPV-18 immortalized cells transformed with cigarette smoke condensate, HEN-16T and HEC-18T, respectively. All 4 human cell lines have been extensively characterized (Supplementary Table S4 and Supplementary Fig. S5). hPygo2 mRNA expression was slightly higher in HEC cells relative to HEN cells and significantly increased (by at least 9-fold) in HEN 16T and HEC 18T cervical cancer cells (Fig. 2A). The expression of PCNA and Elf-1 followed the same trend yet increased in HEC and cancer cell lines. As...
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S4). These results suggested that hPygo2 mRNA and protein expression by immunofluorescence (Fig. 2C and Supplementary Fig. S4). These results suggested that they are activated following HPV integration.

hPygo2 and Elf-1 are required for cervical cancer cell proliferation

The higher levels of hPygo2 observed in HPV-infected cells and tissues suggested that it may, as in other cancers (2–6, 39), have an important function in cervical cancer. HEN16T cells were therefore transfected with 2 siRNAs directed against hPygo2, siPy2-X, targeting its 3'-UTR and siPy2-Z, targeting the coding region to determine the requirement of hPygo2. Either siPy2-X or siPy2-Z significantly reduced both isoforms of hPygo2 as compared with the nontarget control siRNA (siNTC). When transfected with siNTC, the proportion of cells that accumulated in G1 was 33% but was appreciably higher at 66% (P < 0.01) after siPy2-X treatment and 55% (P < 0.01) after siPy2-Z treatment (Fig. 3A). The increase of cells in G1 was accompanied by a significant decrease in the percentage of cells in S-phase. HEC 18T cells redistributed in a similar manner after hPygo2 siRNA treatment with the largest proportion of cells arresting in G1 (Fig. 3B).

Figure 1. Quantification of protein expression by immunohistochemistry. A cervical intraepithelial neoplasia tissue microarray was stained for (A) Ki-67, (B) p16, and (C) hPygo2. Proportions of negative, weak, and strongly positive staining cells in each set of cores representing each stage of cervical disease progression (horizontal axes) determined by 3 observers. Shown are the means and SDs of each of the calculated values for each core analyzed. The asterisks over the strongly expressing data points indicate a significant difference between these diagnoses and the CIN2 samples.

expected, Rb tumor suppressor levels were lower in the tumorigenic cell lines. The protein levels of Elf-1 and PCNA closely paralleled mRNA levels (Fig. 2B). The 80 kDa (cytoplasmic) and 98 kDa (nuclear) are the predominant forms of Elf-1. Henceforth, we focus on the nuclear isoform as it is the only isoform capable of binding to the hPygo2 promoter. hPygo2 is detected as 2 differentially migrating bands on gel electrophoresis, possibly identifying 2 isoforms, which were observed using a variety of antibodies (data not shown). Both forms also paralleled the corresponding mRNA levels of expression. While not detectable in HEN cells, hPygo2 was highly localized to nuclei in HEN 16T cells using immunofluorescence (Fig. 2C and Supplementary Fig. S4). These results suggested that hPygo2 mRNA and protein levels of expression.

Several studies have shown that both Elf-1 and hPygo2 are required for cervical cancer cell proliferation. siElf-1 increased the proportion of HEN 16T cells in G1 by 27% (P < 0.01) relative to siNTC (Fig. 3E), which was accompanied by a significant decrease in the proportion of cells in S-phase. siElf-1 treatment of HEC 18T cells had a similar effect, increasing the proportion of cells in G1 by 34% (P < 0.01; Fig. 3F).

In both HEN 16T and HEC 18T cells, the reduction of nuclear Elf-1 protein levels by siElf-1 paralleled a significant decrease in hPygo2 protein expression (Fig. 3G and H). Similar to our results with the hPygo2 knockdown, there was an increase in p53 and p21 expression upon depletion of nuclear Elf-1. The siElf-1–induced increase in p53 was attenuated back to control levels by overexpression of hPygo2 (Fig. 3G and H), suggesting that Elf-1–mediated activation of hPygo2 expression was required for cell-cycle progression in these cells.

Rb-dependent regulation of hPygo2 expression via Elf-1 is deactivated by HPV E7 protein

The requirement of Elf-1 and hPygo2 in cervical cancer suggested that they are activated following HPV integration.
A critical effector for HPV pathogenesis is the deregulated expression and activity of its E7 viral oncoprotein. As E7 induces degradation of Rb, we predicted that upregulation of Rb by reducing E7 would be expected to decrease Elf-1 activity, leading to hPygo2 downregulation.

To assess whether E7 was required for increased Elf-1 activity and subsequently hPygo2 expression, we knocked down E7 protein using RNAi. Treatment of HEN 16T cells with an E7-specific siRNA siE7 effectively reduced E7 protein levels compared with the nontarget control siRNA (Fig. 4A). The reduction in E7 was associated with a decrease in hPygo2, but an increase in Rb protein levels, while not affecting levels of Elf-1. It is possible, therefore, that Rb attenuated hPygo2 levels by regulating the activity but not the expression or stability of Elf-1.

Elf-1 is an Ets-related transcription factor that binds to a number of target genes including the hPygo2 promoter whose expression is augmented in cancer (2, 9, 41, 42). In vitro protein interaction assays showed that Rb represses Elf-1 by binding to its transactivation domain in nondividing cells, attenuating its ability to contribute to the oncogenic phenotype (21). As Rb is a major degradation target of HPV-E7, we determined whether knockdown of E7 affected its presence, along with Elf-1, at the hPygo2 promoter using chromatin immunoprecipitation (ChIP) assays. We previously examined a 1,568 bp (−1,494 to +74) segment of the hPygo2 promoter and showed that Elf-1 binds to the −48 to −25 region which contains an Ets-binding site (7).

siNTC treatment of HEN 16T cells, the presence of Elf-1 and Rb was detected at the hPygo2 promoter and at the promoters of a known Elf-1 target gene, hepatocellular carcinoma suppressor 1 (HCCS1; ref. 9; Fig. 4B) and an Rb target gene, cyclin A (CCNA; ref. 43; Fig. 4C). Treating cells with siE7 did not affect Elf-1 binding to the hPygo2 or HCCS1 promoters. There was, however, a significant increase in the presence of Rb at both the hPygo2 (56%, P = 0.038) and the CCNA promoters. Thus, E7 reduced the association of Rb at the hPygo2 promoter while not affecting Elf-1. This finding suggested that activation of hPygo2 by HPV in cervical cancer cells was due to derepression of Elf-1 activity by the repressive action of E7 on Rb.

To further analyze the derepression of hPygo2 by HPV E7, we used a gain-of-function mutant of Rb (PSM-Rb; ref. 35), a dominant acting Rb-independent mutant of Elf-1 (DA-Elf-1; ref. 21) and wild-type Elf-1 and assessed their effect on hPygo2 expression. The PSM-Rb construct has 8 phosphorylation sites substituted by alanines (Supplementary Fig. S5A), making it refractory to cyclin/CDK complexes and thus rendering it constitutively repressive (35). Protein products synthesized using this construct are still able to bind Elf-1 (Supplementary Fig. S5B). The DA-Elf-1 construct has the 3 crucial amino acids in its LXCXE, which are required for binding to Rb, substituted with RXRXH (Supplementary Fig. S5C), rendering it unable to interact with Rb (Supplementary Fig. S5B). We hypothesized that overexpression of PSM-Rb would reduce the positive effect...
of E7 on hPygo2 expression, and in contrast, the DA-Elf-1 mutant would constitutively activate hPygo2 expression.

Our previous work indicated that Elf-1 augmented hPygo2 gene expression by binding to the Ets-binding site (EBS) present within the first 48 bp of the hPygo2 promoter (7). To assess the effect of the Rb and Elf-1 constructs on hPygo2 gene regulation, we overexpressed different combinations of PSM-Rb, Elf-1, and DA-Elf-1 in conjunction with 2 reporter constructs, both of which included the EBS but differed in the length of hPygo2 promoter DNA. These included the minimal hPygo2 promoter containing the first 48 bp (pGL3–48), a large segment containing 1,494 bp (pGL3–1494), and a negative control with no promoter sequence (pGL3 basic; ref. 7).

Overexpression of PSM-Rb in HEN 16T cells led to 2.9-fold ($P = 0.037$) and 7.1-fold ($P = 0.038$) decreases in the pGL3–48 and pGL3–1494 samples, respectively, compared with the empty vector control (Fig. 5A). In contrast, transfection with either Elf-1 or DA-Elf-1 significantly increased hPygo2 reporter activity compared with the control. Elf-1 overexpression caused a 17.6-fold increase ($P = 0.017$) in the minimal promoter reporter and a 3.7-fold increase ($P = 0.002$) in the large hPygo2 construct.

Figure 3. Depletion of hPygo2 and Elf-1 causes activation of p53 and G1 phase cell-cycle arrest. A and B, HEN 16T and HEC 18T cells were treated with either siNTC or hPygo2 siRNAs. Fluorescent-activated cell sorting (FACS) was conducted to show the percentage of cells in each phase of the cell cycle. C and D, HEN 16T and HEC 18T cells were treated with either siNTC or hPygo2 siRNA in combination with either pCS2+ or pCS2+hPygo2 expression vectors. E and F, HEN 16T and HEC 18T cells were treated with either siNTC or Elf-1 siRNA (siElf-1) and subsequently analyzed by FACS. G and H, HEN 16T and HEC 18T cells were treated with either siNTC or siElf-1 in combination with either pCS2+ or pCS2+hPygo2. Full-length blots are presented in Supplementary Fig. S6.
0.047) in the large reporter. DA-Elf-1 overexpression increased the minimal reporter activity by 24.1-fold ($P < 0.01$) and the large reporter activity by 5.3-fold ($P = 0.011$). When PSM-Rb and Elf-1 were overexpressed in combination, neither the minimal promoter reporter plasmid nor the large promoter reporter plasmid activity was significantly higher than that of the empty vector control. However, when PSM-Rb was overexpressed with DA-Elf-1, there was a significant increase in both the minimal reporter activity (7.4-fold, $P = 0.036$) and the large reporter activity (2.3-fold, $P = 0.012$) compared with the control.

Overexpression of the Rb and Elf-1 constructs in HEC 18T cells yielded similar results; Rb reduced hPygo2 promoter activity of both reporters whereas Elf-1 increased it and DA-Elf-1 was able to overcome the repressive effects of PSM-Rb (Fig. 5B). The similar effects of Rb and Elf-1 overexpression on both the pGL3-48 and pGL3-1494 reporters suggested that Rb and Elf-1 modulated hPygo2 transcription through its Ets-binding site in its promoter.

Thus, Rb negatively, whereas Elf-1 positively modulated hPygo2 reporter activity through its Ets-binding site in its promoter. Various combinations of PSM-Rb, Elf-1, and DA-Elf-1 were overexpressed, and the presence of Rb and Elf-1 on chromatin located at the hPygo2 promoter was measured. Interestingly, while overexpression of PSM-Rb significantly reduced Elf-1–dependent hPygo2 promoter activity, it did not alter the level of Elf-1 at the hPygo2 promoter compared with the empty vector control (Fig. 5E).

In the remaining cases, in which either Elf-1 or DA-Elf-1 was overexpressed, there was a consistent increase (3.3- to 4.3-fold) in Elf-1 at the promoter. This increase in Elf-1 was not significantly affected by the presence of PSM-Rb. Similarly, Elf-1 at the HCCS1 gene promoter only increased when it was overexpressed, which was expectedly independent of PSM-Rb overexpression. These results showed that the interaction between Elf-1 and Rb is not required for Elf-1 to bind to the hPygo2 promoter.

Given that Rb did not influence the association of Elf-1 at the hPygo2 promoter, we hypothesized that the reciprocal may be true, that its own presence at the hPygo2 promoter is dependent on Elf-1. While overexpression of PSM-Rb resulted in a 6.5-fold increase ($P = 0.017$) in Rb (both endogenous and exogenous) at the promoter relative to the control (Fig. 5F), the highest levels were achieved when PSM-Rb was overexpressed in combination with Elf-1 (11-fold above control, $P = 0.024$). As expected, when PSM-Rb was overexpressed with (Rb-independent) DA-Elf-1, its presence at the hPygo2 promoter was significantly reduced compared to when it was co-expressed with wild-type Elf-1 (32% reduction, $P = 0.015$). On the other hand, the presence of Rb at the CCNA gene promoter increased binding whenever PSM-Rb was overexpressed and was not affected by DA-Elf-1. These results strongly suggested that Rb associated specifically with chromatin at the hPygo2 promoter through its interaction with Elf-1 (Fig. 5G).

We next assessed whether Elf-1 was required for the presence of Rb at the hPygo2 promoter. Treatment of HEN 16T cells with siElf-1 effectively reduced Elf-1 and hPygo2 protein levels but without affecting Rb protein levels compared with the nontarget control siRNA (Fig. 6A). Treatment with the siNTC control did not alter the presence of Elf-1 and Rb at the hPygo2 promoter. Various combinations of PSM-Rb, Elf-1, and DA-Elf-1 were overexpressed, and the presence of Rb and Elf-1 on chromatin located at the hPygo2 promoter was measured. Interestingly, while overexpression of PSM-Rb significantly reduced Elf-1–dependent hPygo2 promoter activity, it did not alter the level of Elf-1 at the hPygo2 promoter compared with the empty vector control (Fig. 5E). In the remaining cases, in which either Elf-1 or DA-Elf-1 was overexpressed, there was a consistent increase (3.3- to 4.3-fold) in Elf-1 at the promoter. This increase in Elf-1 was not significantly affected by the presence of PSM-Rb. Similarly, Elf-1 at the HCCS1 gene promoter only increased when it was overexpressed, which was expectedly independent of PSM-Rb overexpression. These results showed that the interaction between Elf-1 and Rb is not required for Elf-1 to bind to the hPygo2 promoter.

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![Figure 4](https://example.com/figure4.png)
The hPygo2 promoter and at the promoters of their target genes, HCCS1 and CCNA, respectively (Fig. 6B and C). Treating cells with siElf-1 significantly reduced Elf-1 binding to both hPygo2 (2.5-fold, \( P = 0.024 \)) and HCCS1 (2.4-fold, \( P = 0.031 \)) promoters. The presence of Rb at the hPygo2 promoter was also significantly reduced (2.1-fold, \( P = 0.033 \)), but Rb binding to CCNA was not affected. These results are consistent with aforementioned findings, which showed specific Elf-1–dependent association of Rb with hPygo2.

Given that the presence of the Rb–Elf-1 complex at the hPygo2 promoter depended on Elf-1, we hypothesized that Elf-1 may bind to 1 of the 2 putative EBSs, in the promoter. We used site-directed mutagenesis to change the sequence of both putative EBSs in the pGL3-48 luciferase reporter plasmid thereby generating pGL3-48 mutEBS.

First, HEN 16T cells were transfected with pGL3-basic, pGL3-1494, pGL3-48, or pGL3-48 mutEBS and assayed for luciferase activity and Rb and Elf-1 promoter occupancy in the luciferase plasmids. Luciferase reporter activity levels in the pGL3-1494 and pGL3-48 samples were significantly higher (4.5 \( \times \) \( 10^6 \) RLU, \( P < 0.01 \)) than in the empty vector control, pGL3-basic, (2.2 \( \times \) \( 10^3 \) RLU; Fig. 6D). The reporter activity level of the pGL3-48 mutEBS sample was slightly and nonsignificantly higher (979 RLU, \( P = 0.061 \)) than pGL3-basic.

Second, ChIP assays were used to measure the association of Rb and Elf-1 to the hPygo2 promoter in each of the reporter plasmids (Fig. 6E). Elf-1 promoter occupancy was 74.6-fold higher in pGL3-1494 and 70.9-fold higher in pGL3-48 relative to the empty plasmid control. Rb binding to the hPygo2 promoters in pGL3-1494 (12.4-fold) and pGL3-48 (13.4-fold) was significantly higher than pGL3-basic. Mutating both EBS completely abolished the association of Rb and Elf-1 to that of the level of the empty vector control.

Discussion

The increased expression of hPygo2 in severely dysplastic lesions and cervical tumors and in cervical cancer cell lines, along with its requirement for proliferation of these cells, suggested that hPygo2 may play an important role in cervical cancer pathogenesis. In our microarray analyses, we used 2 different arrays from 2 separate commercial sources, for which little pre-analytic information was derived, causing variability within and between arrays, in immunohistochemical reactivity. While our initial analysis of staining intensity during disease progression suggested that hPygo2 is...
expressed at higher levels in severe disease, we are presently optimizing our staining protocol using controlled fixation techniques and array preparation, toward clinical use.

Activation of the Wnt signaling pathway and components of the pathway have been associated with cervical carcinoma (44), so it is not unreasonable to suggest that the activity of hPygo2 in cervical cancer and in cervical dysplasia which we report in this study, may be linked to, or be a prerequisite for Wnt signal transduction, given its role as a chromatin modifier in this context (45). Our recent findings (Andrews and colleagues, submitted), however, have shown an additional but strong requirement for malignant growth–associated ribosome biogenesis in HeLa cells, a cervical adenocarcinoma–derived cell line. While these concurrent results do not necessarily preclude a role in Wnt signaling in cervical cancer, the association of hPygo2 with cell-cycle progression genes we observed in the present study would also support its role in ribosome biogenesis.

Our findings are consistent with others showing the overexpression and requirement of hPygo2 in a broad range of cancers (2, 3), but they are novel as they showed that the increase in hPygo2 expression begins at a premalignant stage. Because not all high-grade (CIN3) lesions progress to cancer, specific and effective biomarkers are needed to predict when and whether this progression will occur. The finding that hPygo2 expression increases specifically in the CIN3 precancerous stage indicates that hPygo2 may effective biomarker to predict the transition from high-grade dysplasia to cancer.

High-grade lesions have an elevated probability for progressing to cancer because HPV genome integration results in the loss of the E2 regulatory region and subsequent E7 oncoprotein upregulation. Because the increase in hPygo2 expression correlates with the HPV integration event, we hypothesized that the 2 were causally linked. By knocking down E7 expression, we observed a decrease in hPygo2 protein levels, which correlated with an increase in Rb binding to the hPygo2 promoter while not affecting Elf-1 levels or promoter binding. hPygo2 levels were likely increased in cervical carcinoma, therefore, because E7 upregulation caused the degradation of Rb. Furthermore, the reduction of p53 by re-expression of hPygo2 in cells depleted of Elf-1 showed a relationship between these 2 genes in deregulated growth during oncogenesis. This molecular interaction may be part of an important signaling axis for the identification and possible targeting of early stage disease.

Elf-1 protein is continuously expressed and localizes to the cytoplasm, where it becomes activated through phosphorylation and glycosylation. These modifications cause its translocation to the nucleus, where it binds to target gene promoters and activates gene expression. Our immunohistochemical analyses of dysplasia core samples revealed nuclear Elf-1 in CIN3, suggesting that Elf-1 is active, consistent with previous findings (13). In the nucleus, Rb interacts with the LXCXE motif of Elf-1, inhibiting its transcriptional activity. We propose that following HPV integration, E7 displaces Rb from Elf-1 and results in Elf-1–mediated hPygo2 expression.

Initially, Elf-1 was regarded as a lymphoid gene specific transcription factor. However, it is also regarded as an important activator or repressor of genes involved in several processes such as development (41, 46, 47), oncogenesis (48), and viral gene activation (49). The bulk of research examining the role of Elf-1 in cancer has focused on the expression of Elf-1 in relation to other biomarkers, such as VEGF and PCNA or with clinical correlates. Detailed studies of Elf-1 gene targets in cancer include Elf-1 activation of TIE2 (46) and binding to the HCCS1 promoter (13). An efficient way to potentially identify all Elf-1 gene targets in these cells would be to conduct a genome-wide chromatin association analysis and to functionally group these genes to identify in which processes Elf-1 is involved.

Disclosure of Potential Conflicts of Interest
K.R. Kao and C. Popadiuk have Ownership Interest (including patents). No conflicts of interest disclosed. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: Y.R. Tzenov, K.R. Kao, C. Popadiuk
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.R. Tzenov, K.R. Kao, C. Popadiuk
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