Retinoid Targeting of Different D-Type Cyclins through Distinct Chemopreventive Mechanisms

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Abstract

D-type cyclins (cyclins D1, D2, and D3) promote G1-S progression and are aberrantly expressed in cancer. We reported previously that all-trans-retinoic acid chemoprevented carcinogenic transformation of human bronchial epithelial (HBE) cells through proteasomal degradation of cyclin D1. Retinoic acid is shown here to activate distinct mechanisms to regulate different D-type cyclins in HBE cells. Retinoic acid increased cyclin D2, decreased cyclin D3 and had no effect on cyclin D1 mRNA expression. Retinoic acid decreased cyclin D1 and cyclin D3 protein expression. Repression of cyclin D3 protein preceded that of cyclin D3 mRNA. Proteasomal inhibition prevented the early cyclin D3 degradation by retinoic acid. Threonine 286 (T286) mutation of cyclin D1 stabilized cyclin D1, but a homologous mutation of cyclin D3 affecting threonine 283 did not affect cyclin D3 stability, despite retinoic acid treatment. Lithium chloride and SB216763, both glycogen synthase kinase 3 (GSK3) inhibitors, inhibited retinoic acid repression of cyclin D1, but not cyclin D3 proteins. Notably, phospho-T286 cyclin D1 expression was inhibited by lithium chloride, implicating GSK3 in these effects. Expression of cyclin D1 and cyclin D3 was deregulated in retinoic acid–resistant HBE cells, directly implicating these species in retinoic acid response. D-type cyclins were independently targeted using small interfering RNAs. Repression of each D-type cyclin suppressed HBE growth. Repression of all D-type cyclins cooperatively suppressed HBE growth. Thus, retinoic acid repressed cyclin D1 and cyclin D3 through distinct mechanisms. GSK3 plays a key role in retinoid regulation of cyclin D1. Taken together, these findings highlight these cyclins as molecular pharmacologic targets for cancer chemoprevention. (Cancer Res 2005; 65(14): 6476-83)

Introduction

Lung cancer is the leading cause of cancer mortality for men and women in the United States. Despite contemporary chemotherapy, radiation therapy, and surgery, only a small subset of lung cancer cases is cured. Innovative approaches to address this major public health problem are needed. Even if all the national antismoking goals were met, lung cancer would remain a substantial medical concern for decades given the large number of former smokers, who remain at risk. Multiple steps occurring over long time periods lead to epigenetic or genetic changes in the lung that cause invasive or metastatic lung cancers (1). Chemoprevention is appealing to reduce lung cancer mortality because this uses pharmacologic agents to prevent, inhibit, or reverse carcinogenesis (2).

D-type cyclins (cyclin D1, cyclin D2, and cyclin D3) are encoded by distinct genes and their protein products have highly homologous cyclin box and PEST sequence domains (3). D-type cyclins serve as cellular sensors and integrators of extracellular signals during the early to mid G1 cell cycle phase (4). These regulate cellular proliferation or differentiation by binding cyclin-dependent kinases, sequestering the cyclin-dependent kinase inhibitors p21 and p27, or by affecting various transcription factors independent of cyclin-dependent kinases, as reviewed in ref. (4). Numerous studies linked deregulation of D-type cyclins with various types of cancers (5). Prior work highlighted the cell cycle regulator, cyclin D1, as a chemopreventive or therapeutic target in the lung and at other organ sites, as reviewed in refs. (6, 7). Overexpression of cyclin D1 is a frequent and early step in lung carcinogenesis (8–11). Cyclin D1 overexpression is often associated with gene amplification (12) as well as allele-specific expression imbalance (11). The finding that aberrant expression of cyclin D1 is often detected in bronchial preneoplasia and in non–small cell lung cancers has implicated this cell cycle regulator as a molecular pharmacologic target in these settings (6). The role of other D-type cyclins in lung carcinogenesis has not yet been extensively explored. However, some studies have implicated the roles of cyclin D2 or cyclin D3 in this process. For example, up-regulation of cyclin D3 mRNA was associated with carcinogen-induced murine lung adenocarcinoma (13). Aberrant methylation of cyclin D2 gene is frequent in lung cancers and is associated with repression of gene expression (14). The current study comprehensively explores retinoid regulation of D-type cyclins in human bronchial epithelial (HBE) cells.

Retinoids, natural and synthetic derivatives of vitamin A are active in cancer therapy and chemoprevention, as reviewed (7). The retinoid role in cancer chemoprevention was highlighted based on in vitro studies, preclinical animal model experiments, epidemiologic evidence, and results of clinical trials that treated certain premalignancies or second malignancies (7). Immortalized HBE (BEAS-2B) cells (15) were treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane that caused malignant transformation (16). All-trans-retinoic acid prevented this carcinogenic change in BEAS-2B HBE cells (16). This chemoprevention was associated with G1 arrest and concomitant suppression of cellular growth (16, 17). Growth suppression was linked to cyclin D1 proteolysis through retinoid-induced proteasomal degradation (17–20). Cyclin D1 proteasomal degradation program was proposed as an important cancer chemopreventive mechanism because the retinoid-mediated G1 cell cycle arrest was expected to permit repair of carcinogenic damage to genomic DNA (7, 17, 18). A key role for cyclin D1 in conferring retinoid response was established using retinoic acid–resistant BEAS-2B-R1 HBE cells that had deregulated cyclin D1 expression (20). An important role...
for cyclin D1 in cancer chemoprevention was shown in an animal model (21) and a retinoid clinical cancer chemoprevention trial (22).

The present study extends prior work by comprehensively investigating which D-type cyclins are retinoid-regulated. These findings reveal that distinct mechanisms are engaged. Derived retinoic acid–resistant BEAS-2B-R1 HBE cells were used to confirm that the retinoid regulation of specific D-type cyclins was deregulated, implicating these species directly in retinoid acid response. Studies were undertaken to reveal a candidate D-type cyclin involved in the retinoid regulation of cyclin D1. Prior work indicated that mutation of threonine 286 (T286) of cyclin D1 stabilized this species, despite retinoic acid treatment (20). This finding was confirmed and extended using a phospho-specific antibody that recognized phosphorylation of cyclin D1 at T286. Pharmacologic inhibitors of glycogen synthase kinase 3 (GSK3) antagonized retinoic acid effects on cyclin D1, but not on cyclin D3. To directly explore the roles of D-type cyclins in regulating HBE growth, each D-type cyclin was specifically targeted using two independent small interfering RNAs (siRNA).

Recent experiments that targeted two or all the D-type cyclins indicated functional redundancy between these species in some of the examined mouse tissues (23, 24). To extend these analyses to HBE cells, many different siRNAs were used to target all D-type cyclins in these cells. Cooperation between these species was observed. Results that will be presented indicate in HBE cells that specific D-cyclins represent candidate molecular targets for lung cancer chemoprevention. This study reports the mechanistic basis for retinoid repression of D-type cyclins in HBE cells and their individual roles in growth regulation of HBE cells. The implications of this work for clinical lung cancer chemoprevention will be discussed.

Materials and Methods

Cell lines, reagents, and plasmids. The parental BEAS-2B and retinoic acid–resistant BEAS-2B-R1 HBE cell lines were each cultured, as previously described (20). The features of retinoic acid–resistant cells have been described in prior work (20, 25). All-trans-retinoic acid (RA), lithium chloride (LiCl), and sodium chloride (NaCl) were purchased (Sigma, St. Louis, MO), as were the proteasome inhibitor, ALLN (Calbiochem, San Diego, CA) and the GSK3 inhibitor, SB216763 (Tocris, Ellisville, MO). Retinoic acid and SB216763 stock solutions were prepared in DMSO. LiCl and NaCl stock solutions were dissolved in H2O. The pRcCMV-cyclin D1-hemagglutinin (HA) and pRcCMV T286A cyclin D1-HA plasmids have been previously described (19). The pRcCMV-cyclin D1-HA plasmid (a gift from Dr. Mark E Ewen, Dana-Farber Cancer Institute, Harvard Medical School) is a cytomegalovirus promoter driven and hemagglutinin (HA)-tagged human cyclin D3 expression vector. The pEGFP-C1 expression plasmid was purchased (Clontech, Palo Alto, CA).

Immunoblot analyses. Cells were lysed with ice-cold radiomimunoprecipitation cell lysis buffer using established techniques (25) or with EBC lysis buffer used for detection of phospho-T286 cyclin D1, as described (26). Immunoblot analyses were done as previously described (25). Densitometric analyses of images were done as described (25). Primary antibodies used included rabbit polyclonal antibodies that recognized cyclin D1, cyclin D3, or β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), or a rabbit polyclonal antibody that recognized phospho-T286 cyclin D1 (26). Murine monoclonal antibodies that individually recognized hemagglutinin-tagged proteins (Babco, Richmond, CA) or enhanced green fluorescent protein (EGFP; BD Biosciences, San Jose, CA) and a goat polyclonal antibody that recognized actin (Santa Cruz Biotechnology) were each purchased. Anti-mouse and anti-rabbit antisera were also purchased (Amersham Biosciences, Piscataway, NJ) as was the anti-goat antisera (Santa Cruz Biotechnology). Northern and reverse transcription PCR assays. Total cellular RNA was isolated and contaminating DNA was removed as previously described (25). Northern hybridization was done using established methods (27). The radiolabeled cDNA probes used for Northern analyses were a 1.1 kb HindIII restriction endonuclease fragment of the pRcCMV-cyclin D1-HA vector, a 1.1 kb HindIII/Abal restriction endonuclease fragment of the pRcCMV-cyclin D3-HA vector or a 1.8 kb human β-actin cDNA (Clontech). Reverse transcription (RT)-PCR assays were done using previously established methods (23). PCR products were detected using the GDS-800 system (Ultra-Violet Products Ltd., Cambridge, United Kingdom) and analyzed by LabWorks 4.0 software (Ultra-Violet Products). The primers used for RT-PCR assays were: forward primer for cyclin D1, 5′-TCACCAACACAGACGGTGGAT-3′; and reverse primer for cyclin D1, 5′-AATCTGGCATCCTCATACAGGTCC-3′; forward primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GAAGGTGAAGGTCGGAGTCA-3′; and reverse primer for glyceraldehyde-3-phosphate dehydrogenase, 5′-GACAGAAGTTGCCGTGCAGAAGG-3′. The specificity of cyclin D2 primers was confirmed by DNA sequencing of the PCR product (data not shown).

Transfection experiments. A mutant cyclin D3 species was engineered to contain a threonine to alanine substitution at residue 283 of human cyclin D3 within the pRcCMV-cyclin D3-HA expression vector. In vitro mutagenesis was done using the QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA). The primer sequences used for these mutagenesis studies were: 5′-GGCCCTACGACGCTAGCTCCAGGC-3′ and 5′-GGCTGTGACATCTGTAGGCGCGC-3′ for the forward primer for cyclin D3, and 5′-GGCTGTGACATCTGTAGGCGCGC-3′ and 5′-GGCTGTGACATCTGTAGGCGCGC-3′ for the reverse primer for cyclin D3. In the transfection experiments, BEAS-2B HBE cells were cotransfected with the pEGFP-C1 vector and the desired D-type cyclin expression vector using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and techniques established by the manufacturer. Enhanced green fluorescent protein expression served as a control for transfection efficiency.

Small interfering RNA experiments. Double-stranded siRNAs with 19-nucleotide duplex RNA and a 2-nucleotide overhang at the 3′ region were each synthesized (Dharmacon, Lafayette, CO). Two distinct siRNAs were individually designed to independently target human cyclin D1, cyclin D2, or cyclin D3 mRNA coding or 3′ untranslated regions. Cyclin D1 mRNA target sequences were 5′-AACCTGAGGAGCCCCAACAC-3′ (cyclin D1-1 siRNA) and 5′-AACAAACAGACATCCGGCA-3′ (cyclin D1-2 siRNA). Cyclin D2 mRNA target sequences were, 5′-AAGTCGGTGCAGAACAC-3′ (cyclin D2-1 siRNA) and 5′-AATAGGGCAGCAAGCTCGG-3′ (cyclin D2-2 siRNA). Cyclin D3 mRNA target sequences were, 5′-AAGATGTGGCTTCTGACACCTC-3′ (cyclin D3-1 siRNA) and 5′-TAGATGTAGCTCTCTCTGACACTC-3′ (cyclin D3-2 siRNA). Firefly luciferase GL2 siRNA (Dharmacon) served as a siRNA duplex control. The target sequence for GL2 siRNA is

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Regulation by retinoic acid treatment of D-type cyclin mRNA expression in BEAS-2B HBE cells. BEAS-2B cells were treated with 4 μM/L retinoic acid for the indicated time periods. Regulated expression was studied by Northern analysis for cyclin D1 and cyclin D3 (A) or by RT-PCR assay for cyclin D2 (B). Actin or glyceraldehyde-3-phosphate dehydrogenase served as loading controls.
Results

Regulation of D-type cyclins. Consistent with prior work, retinoic acid substantially reduced cyclin D1 protein, but not mRNA expression as early as 7 hours after retinoic acid treatment (Figs. 1A and 2A). Both protein and mRNA expression of cyclin D3 decreased after retinoic acid treatment of BEAS-2B HBE cells, but the kinetics and the level of the regulation differed from that observed for cyclin D1 (Figs. 1A and 2A). Retinoic acid rapidly repressed cyclin D3 protein expression by 7 hours (Fig. 2A), but a later decline of cyclin D3 mRNA was observed after retinoic acid treatment (Fig. 1A). This indicated that unlike cyclin D1, retinoic acid regulated cyclin D3 at both mRNA and protein levels. In contrast, cyclin D2 expression was not readily detected in BEAS-2B cells by either immunoblot or Northern analyses, but could be found in human embryonal carcinoma NT2/D1 cells that served as a control for cyclin D2 expression (data not shown). RT-PCR assays were done to detect this species using primers that specifically amplified cyclin D2. Unlike cyclin D1 and cyclin D3, cyclin D2 mRNA expression increased by 3 hours after retinoic acid treatment (Fig. 1B). ALLN, a proteasome inhibitor, antagonized the retinoic acid–mediated decline of both cyclin D1 and cyclin D3 proteins (Fig. 2B), indicating that retinoic acid also triggered the proteasomal degradation of cyclin D3.

Glycogen synthase kinase 3 involvement in cyclin regulation. It was previously reported that T286 of cyclin D1 played a direct role in regulating cyclin D1 stability after retinoic acid treatment (20). Phosphorylation of T286 by GSK3 is known to trigger the rapid turnover of cyclin D1 (28). Because this threonine and neighboring residues are conserved in D-type cyclins, it was next examined whether a homologous threonine residue in cyclin D3 (T283) played a direct role in the retinoid regulation of cyclin D3. Consistent with prior work (20), expression of transfected wild-type cyclin D1 decreased by 42% as compared with controls, but transfected T286A cyclin D1 was stabilized, despite retinoic acid treatment (Fig. 3A). Retinoic acid treatment repressed expression of both the wild-type and the mutant T283A cyclin D3 species by 40% and 44%, respectively (Fig. 3B), indicating that the homologous threonine residue in cyclin D3 was not critical for regulating cyclin D3 stability after retinoic acid treatment.

To investigate whether GSK3 was involved in the retinoic acid–dependent degradation of cyclin D1 or cyclin D3, the effect of GSK3 inhibitors, LiCl and SB216763, was evaluated. As expected, LiCl stabilized β-catenin protein, a known GSK3 target (ref. 29; Fig. 4A). LiCl inhibited the retinoic acid–mediated decline of cyclin D1, but not of cyclin D3 proteins (Fig. 4A). As a control, NaCl treatment did not attenuate retinoic acid–dependent changes in cyclin D1 or cyclin D3 immunoblot expression (Fig. 4A). SB216763 also stabilized β-catenin and inhibited the retinoic acid–dependent decline of cyclin D1, but not cyclin D3 proteins (Fig. 4B). Thus, two
different GSK3 inhibitors exerted similar effects on cyclin D1 by inhibiting retinoid-mediated changes on cyclin D1 stability.

Whether this retinoic acid–mediated decline of cyclin D1 involved cyclin D1 phosphorylation at T286 was next examined. Retinoic acid decreased phosphorylation of cyclin D1 at T286 (Fig. 4D), as confirmed using an antibody that was generated to specifically recognize this phosphorylated T286 residue (Fig. 4C). The repression of this species is likely due to the rapid degradation of total cyclin D1 protein following retinoic acid treatment. LiCl inhibited phosphorylation of cyclin D1 at T286, as expected (Fig. 4D). At the same time, LiCl repressed the retinoic acid–dependent decline of transfected wild-type cyclin D1 (Fig. 4D). These data indicate the involvement of GSK3 in retinoic acid–mediated degradation of cyclin D1. However, retinoic acid–dependent proteolysis of cyclin D3 was independent of the T283 residue of cyclin D3 and GSK3 inhibition.

**Cyclin D1 and cyclin D3 deregulation.** Retinoic acid–dependent regulation of cyclin D1 and cyclin D3 proteins was compared in retinoic acid–sensitive BEAS-2B and retinoic acid–resistant BEAS-2B-R1 HBE cells. Retinoic acid decreased both cyclin D1 and cyclin D3 protein expression by 62% and 89%, respectively, in BEAS-2B cells as compared with controls (Fig. 5A). In contrast, cyclin D1 expression was unaffected and cyclin D3 expression decreased by 52% in BEAS-2B-R1 cells following retinoic acid treatment (Fig. 5A), indicating deregulation of these species in retinoic acid–resistant HBE cells.

**Small interfering RNA targeting of D-type cyclins.** A genetic approach using siRNA was employed to target individual D-type cyclins to explore their functional roles in regulating bronchial epithelial cell growth. Two independent siRNAs targeting different regions of each D-type cyclin were engineered.

Following transfection of BEAS-2B cells with cyclin D1-1 or cyclin D1-2 siRNAs, cyclin D1 protein expression was reduced by 82% and 73%, respectively, as compared with control transfection levels (Fig. 5B). Cyclin D1 siRNA transfection also decreased cyclin D3 protein expression by 73% as compared with controls (Fig. 5B), but did not affect cyclin D2 expression (data not shown). Due to the ability of this siRNA to confer dual repression of cyclin D1 and cyclin D3 protein expression, this siRNA was used for combination siRNA experiments. The cyclin D1-2 siRNA mediated repression of cyclin D1 was specific because cyclin D2 and cyclin D3 expression were each unaffected (data not shown). Repression of cyclin D1 and cyclin D3 expression by cyclin D1-1 siRNA caused a similar degree of growth suppression as did targeting of cyclin D1 by cyclin D1-2 siRNA (Fig. 5B).

After cyclin D2-1 or cyclin D2-2 siRNA transfection, cyclin D2 mRNA expression was reduced by 84% and 44%, respectively (Fig. 5C), as compared with GL2 siRNA–transfected BEAS-2B cells. Cyclin D2-1 siRNA repression of cyclin D2 was specific because transfections did not appreciably affect either cyclin D1 or cyclin D3 protein levels (data not shown). Cyclin D2-2 siRNA treatment also did not appreciably affect cyclin D1 expression, but transiently repressed cyclin D3 protein (data not shown). Cyclin D2-1 and cyclin D2-2-independent siRNA transfections caused 59% and 45% growth suppression in BEAS-2B cells, respectively, as compared with control transfections (Fig. 5C).
Cyclin D3-1 and cyclin D3-2 siRNAs individually reduced cyclin D3 protein expression by 87% and 90%, respectively, after transfection as compared with controls (Fig. 5D). These siRNAs did not affect either cyclin D1 or cyclin D2 expression (data not shown). Repression of cyclin D3 by cyclin D3-1 or cyclin D3-2 siRNAs caused 34% growth suppression of these transfected HBE cells after transfection (Fig. 5D).

**Small interfering RNA targeting of multiple D-type cyclins.** Whether repression of all the D-type cyclins cooperatively suppressed BEAS-2B HBE cell growth was next studied. As shown in Fig. 6A, cyclin D1 and cyclin D3 protein expression was efficiently repressed by cyclin D1-1 siRNA transfection or combined siRNA transfection of BEAS-2B cells. After transfection, cyclin D2 mRNA was repressed to a similar degree as in individually transfected cyclin D2-1 siRNA or combined siRNA-transfected BEAS-2B cells (Fig. 6B). Transfecting BEAS-2B cells with reduced dosage of cyclin D1-1 or cyclin D2-1 siRNAs caused 16% and 21% growth inhibition after transfection, respectively.

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**Figure 5.** Roles of individual D-type cyclins in regulating HBE cell growth. A, BEAS-2B and BEAS-2B-R1 cells were independently treated with 4 μmol/L retinoic acid (+) or DMSO (−) as a vehicle for 7 hours. Cyclin D1 and cyclin D3 expression was each examined by immunoblot analyses. BEAS-2B cells were individually transfected with (B) cyclin D1-1 or cyclin D1-2 siRNAs, (C) cyclin D2-1, or cyclin D2-2 siRNAs or (D) cyclin D3-1 or cyclin D3-2 siRNAs or control GL2 siRNA as described in Materials and Methods. Expression of D-type cyclins was examined by immunoblot analyses for cyclin D1 and cyclin D3 at 96 hours after transfection or by RT-PCR assays for cyclin D2 at 24 hours after transfection. Viable cell numbers were measured 96 hours after cyclin D1-1, cyclin D1-2, cyclin D2-1, and cyclin D2-2 siRNA transfections and 120 hours after cyclin D3-1 or cyclin D3-2 siRNA transfections using a hemocytometer and trypan blue staining. Cell proliferation was expressed as the percentage of viable control cells. Columns, mean; bars, SD; asterisks, statistical significance between the control siRNA–treated group and experimental siRNA–treated groups targeting specific D-type cyclins (**, P < 0.01; ***, P < 0.001).
The respective roles of those species in response to retinoic acid are differentially regulated D-type cyclins and by revealing the study extended this prior work by investigating how retinoic acid repair of carcinogenic damage to genomic DNA (7, 17, 18). This chemopreventive trial of a classical retinoid conducted in mouse lung cancer model established that chemopreventive agents are needed between these species for efficient HBE cell growth. Further support for a cooperative role for different D-type cyclins are needed between these species for efficient HBE cell growth.

DNA damage repair and chemotherapeutic responses of certain cancer cell lines (31–33). Consistent with a clinical role for D-type cyclins "addicted" to cyclin D1 (30). This view is consistent with results from recent studies targeting all D-type cyclins in the mouse (24). Findings indicated that D-type cyclins are not developmentally required, but knock-out mice lacking D-type cyclins display a reduced susceptibility to oncogenic transformation (24). This finding also implicates repression of these species as a potential strategy for cancer chemoprevention.

These findings are relevant to clinical cancer chemoprevention. The frequent aberrant expression of cyclin D1 in bronchial preneoplasia and in lung cancer highlighted this species as a chemopreventive target or biomarker of therapeutic response (6). Overexpression of cyclin D1 seems critical for maintaining cellular transformation, indicating that these altered cells may be "addicted" to cyclin D1 (30). This view is consistent with results of antisense cyclin D1 experiments that could affect both proliferation and chemotherapeutic responses of certain cancer cell lines (31–33). Consistent with a clinical role for D-type cyclins in cancer chemoprevention, studies from a carcinogen-treated mouse lung cancer model established that chemopreventive agents directly affected expression of cyclin D1 and cyclin D2 (21).

Figure 6. Cooperative effects on HBE cell growth. The growth suppression that followed from targeting cyclin D1 in HBE cells was modest. Retinoic acid increased in HBE cells cyclin D2 mRNA expression as early as 3 hours after treatment. Increased expression of cyclin D2 could provide a feedback mechanism to limit retinoid suppression of HBE cell growth. The observed increased cyclin D2 expression in HBE cells may compensate for retinoid-mediated repression of cyclin D1 and cyclin D3. The siRNA targeting of cyclin D2 caused substantial growth suppression, indicating that cyclin D2 is required for bronchial epithelial cell growth even though it is basally expressed at quite low levels in these cells.

To search for potential cooperation between three D-type cyclins, BEAS-2B HBE cells were transfected with multiple siRNAs to target all the D-type cyclins. Cooperative growth suppression was observed in HBE cells, indicating that functional interactions are needed between these species for efficient HBE cell growth. Further support for a cooperative role for different D-type cyclins comes from recent studies targeting all D-type cyclins in the mouse (24). Findings indicated that D-type cyclins are not developmentally required, but knock-out mice lacking D-type cyclins display a reduced susceptibility to oncogenic transformation (24). This finding also implicates repression of these species as a potential strategy for cancer chemoprevention.

Discussion

The classic retinoid, retinoic acid, prevented carcinogenic transformation of HBE cells at least partly through proteasome-dependent degradation of cyclin D1 (17, 18). This chemopreventive mechanism was proposed to cause G1 arrest that would permit repair of carcinogenic damage to genomic DNA (7, 17, 18). This study extended this prior work by investigating how retinoic acid differentially regulated D-type cyclins and by revealing the respective roles of those species in response to retinoic acid treatment and in regulating bronchial epithelial cell growth. Taken together, this study highlighted specific D-type cyclins as candidate pharmacologic targets for lung cancer chemoprevention. These findings also identified repression of cyclin D1 and cyclin D3 as key downstream steps in retinoid chemoprevention. Whether a similar regulation of these D-type cyclins will occur clinically is the subject of current work. Retinoids are useful tools to uncover therapeutic pathways that might be targeted by other agents with optimized pharmacologic properties.

Retinoic acid was found to repress both cyclin D1 and cyclin D3 expression through distinct mechanisms in BEAS-2B HBE cells. Retinoic acid–resistant BEAS-2B-R1 cells were previously used to uncover important retinoic acid target genes (25). Unlike parental BEAS-2B cells, BEAS-2B-R1 cells still grow in the presence of retinoic acid treatment (20). Previous work identified alterations of retinoic acid receptor-β and other retinoic acid target genes in these cells (20, 25). Retinoic acid–mediated reduction of cyclin D1 and cyclin D3 expression was blunted in BEAS-2B-R1 cells, indicating their likely important growth-regulatory roles in HBE cells. The siRNA targeting of each D-type cyclin caused growth suppression of bronchial epithelial cells, confirming that retinoic acid–mediated growth suppression of HBE cells was at least partly due to repression of either cyclin D1 or cyclin D3. Notably, siRNA targeting of both cyclin D1 and cyclin D3 did not exhibit cooperative effects on HBE cell growth. The growth suppression that followed from targeting cyclin D1 in HBE cells was modest.

Retinoic acid increased in HBE cells cyclin D2 mRNA expression as early as 3 hours after treatment. Increased expression of cyclin D2 could provide a feedback mechanism to limit retinoid suppression of HBE cell growth. The observed increased cyclin D2 expression in HBE cells may compensate for retinoid-mediated repression of cyclin D1 and cyclin D3. The siRNA targeting of cyclin D2 caused substantial growth suppression, indicating that cyclin D2 is required for bronchial epithelial cell growth even though it is basally expressed at quite low levels in these cells.

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aerodigestive tract cancers (22, 34) and a proof of principle trial of an epidermal growth factor receptor-tyrosine kinase inhibitor in non–small cell lung cancers confirmed a role for changes in cyclin D1 as a biomarker of clinical response (35). The current study has highlighted repression of cyclin D1 and cyclin D3 as important retinoid chemopreventive mechanisms. The siRNA targeting of each species inhibited HBE cell growth, thereby directly confirming their roles in regulating HBE cell growth. However, these same experiments indicated that targeting these species alone may not be sufficient to fully inhibit HBE cell growth. Targeting of all D-type cyclins was found to be required to signal optimal growth suppression of HBE cells.

Combination therapy with different agents that affect distinct D-type cyclins might confer desired chemopreventive effects. For example, an epidermal growth factor receptor-tyrosine kinase inhibitor antagonized epidermal growth factor–dependent induction of cyclin D1, through a transcriptional mechanism (35). It also repressed cyclin D2 mRNA expression in HBE cells (data not shown). A nonclassical retinoid, the retinoid bexarotene, exerts biological effects by activating the retinoid X receptor pathway. Retinoid X receptor agonists also can trigger proteasomal degradation of cyclin D1 (17, 20) and repress cyclin D3 protein expression (data not shown). Combined treatment with an epidermal growth factor receptor-tyrosine kinase inhibitor (erlotinib) and bexarotene in retinoic acid–resistant BEAS-2B-R1 cells confirmed at least additive growth inhibition and suppression of cyclin D1 expression (36). Notably, a recently completed phase I clinical trial of bexarotene and erlotinib in patients with advanced aerodigestive tract tumors indicated that this targeted combination regimen has clinical activity (37). This study has confirmed and extended prior work by highlighting repression of D-type cyclins as a clinical strategy to treat or chemoprevent lung cancers.

The retinoid repression of cyclin D1 and cyclin D3 proteins was shown in this study to involve the proteasome pathway. The mechanistic basis for repression of these D-type cyclins was explored. The T286 residue present in the PEST domain of cyclin D1 is important in signaling retinoic acid effects because mutation of this residue inhibited the ability of retinoic acid to trigger cyclin D1 ubiquitination and thereby stabilized cyclin D1 expression, despite retinoic acid treatment (19, 20). Phosphorylation of T286 by GSK3 has been implicated in the rapid turnover of cyclin D1 (28). Given this prior work, this study explored the role of this kinase in the retinoid repression of cyclin D1. Inhibition of GSK3 by LiCl or SB216763 each inhibited the retinoic acid–mediated decline of cyclin D1. To examine the mechanistic basis for this, a T286 phosphorylation-specific cyclin D1 antibody was used to confirm that LiCl inhibited phosphorylation at the T286 residue of cyclin D1. These studies indicated that GSK3 is involved in the retinoid regulation of cyclin D1. Future work will investigate the precise mechanisms through which GSK3 is involved.

The homologous residue (T283) within cyclin D3 can be phosphorylated by GSK3 (38). However, inhibition of GSK3 did not affect the retinoic acid decline of cyclin D3 in our study. The fact that mutation of the homologous residue (T283) within cyclin D3 did not affect retinoid repression of cyclin D3 further supports that this residue and GSK3 are not apparently critical for signaling retinoid-mediated degradation of cyclin D3. Previous work established threonine 288 within human cyclin D1 to be important for ubiquitination of cyclin D1 (39). Mutation of this residue to alanine stabilized cyclin D1 protein (40). Mirk phosphorylated cyclin D1 at T288, which enhances its rapid turnover and this is independent of phosphorylation of T286 by GSK3 (40). The homologous residue (T285) exists in cyclin D3. Future work should investigate whether T285 in cyclin D3 and Mirk are involved in the retinoid regulation of cyclin D3. Retinoic acid treatment decreased cyclin D3 mRNA at a later time point than cyclin D3 protein expression. The possibility still exists that retinoic acid could affect cyclin D3 mRNA stability. Future studies should address this possibility.

In summary, this study found that retinoic acid treatment activated distinct mechanisms to regulate different D-type cyclins. GSK3 was found to play a key role in retinoic acid–mediated repression of cyclin D1, but not of cyclin D3. A role for both cyclin D1 and cyclin D3 in regulating HBE cell growth was shown from analyses of retinoic acid–resistant HBE cells. The direct role of different D-type cyclins in regulating HBE cell growth was confirmed by siRNA targeting of individual D-type cyclins. The cooperative effects of these species were also found through combination siRNA experiments that targeted all the D-type cyclins and inhibited growth of HBE cells. Taken together, these findings directly implicate D-type cyclins as candidate molecular pharmacologic targets for lung cancer chemoprevention.

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References


D-Type Cyclins and Chemoprevention

Retinoid Targeting of Different D-Type Cyclins through Distinct Chemopreventive Mechanisms

Yan Ma, Qing Feng, David Sekula, et al.


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