Posttranslational Mechanisms Contribute to the Suppression of Specific Cyclin:CDK Complexes by All-Trans Retinoic Acid in Human Bronchial Epithelial Cells

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ABSTRACT

Retinoids have demonstrated activity in the chemoprevention of aerodigestive tract cancer. Potentially contributing to their lung cancer chemopreventive effects, retinoids inhibit the growth of human bronchial epithelial (HBE) cells. We observed previously that all-trans retinoic acid (t-RA) arrests the growth of HBE cells in the G0 phase of the cell cycle through activation of retinoic acid receptor-dependent pathways, which enhances the association of E2F-4 with retinoblastoma protein family members, converting E2F into a transcriptional suppressor. In this study, we examined the mechanism by which t-RA blocks cell cycle progression in HBE cells and the possibility that this signaling event is blocked in non-small cell lung cancer (NSCLC) cells that are refractory to the growth inhibitory effects of t-RA. t-RA suppressed the expression and activity of cyclin D1, cyclin E, and cyclin-dependent kinases (CDK)-2 and CDK-4, increased expression of the CDK inhibitor p27, and shifted the retinoblastoma protein to a hypophosphorylated form. Posttranslational mechanisms contributed to the changes in CDK-2, CDK-4, and p27 levels, which, in the case of CDK-4, involved the ubiquitin-proteasome pathway. In contrast, despite retinoic acid receptor transcriptional activation, these signaling events did not occur in a NSCLC cell line that is refractory to growth inhibition by t-RA. These findings provide the first evidence that t-RA activates degradation of CDK-4 through the ubiquitin-proteasome pathway, a novel mechanism by which t-RA causes HBE cells to exit the cell cycle, and blockade of these signaling events may contribute to the development of retinoid resistance in NSCLC cells.

INTRODUCTION

Retinoids (including retinol and stereoisomers of retinoic acid) have demonstrated chemopreventive activity in preclinical and/or clinical models of lung, head and neck, and breast cancer (1–3). The chemopreventive effect of retinoids against epithelial carcinogenesis is a consequence of the crucial role that retinoids play in maintaining normal epithelial cell growth and differentiation. For example, retinoids are potent inhibitors of HBE\textsuperscript{1} cell growth and squamous differentiation (reviewed in Ref. 1). Potentially limiting the lung cancer chemopreventive effect of retinoids, HBE cells develop resistance to the growth-inhibitory effects of retinoids during the process of malignant transformation (4, 5). Recent efforts to develop more effective lung cancer chemoprevention strategies have focused on understanding the basis of retinoid resistance in lung cancer cells.

Retinoids are ligands for nuclear hormone receptors, including RARs and RXRs (reviewed in Ref. 6). Of the naturally occurring retinoids, 9-cis retinoic acid binds to RARs and RXRs, activating retinoid receptor transcriptional properties (6). RXRs function as homodimers and as heterodimers with RARs, other steroid hormone receptors, and several orphan receptors (6). As dimers, these nuclear hormone receptors control gene expression directly by binding to RAREs within gene promoters and indirectly by interacting with other transcription factors. The importance of retinoid receptors in mediating the biological effects of t-RA has been demonstrated (7, 8). We have shown that t-RA inhibits the growth of HBE cells in the G0 phase of the cell cycle through activation of RAR-dependent pathways (9). In contrast, a proportion of NSCLC cells, which are derived from normal HBE cells, are refractory to the growth-inhibitory effects of t-RA (4, 5). Contributing to their retinoid refractoriness, a proportion of NSCLC cell lines have a transcriptional defect specific to retinoid nuclear receptors (10). In these cells, RARs and RXRs are expressed but are not transcriptionally activated by t-RA. In other NSCLC cell lines, retinoid receptors are transcriptionally activated by t-RA, but t-RA does not inhibit cell growth (4), suggesting that retinoid signaling is blocked at a step subsequent to retinoid receptor activation.

Entry of cells into the G0 phase of the cell cycle is regulated by E2F transcription factors (reviewed in Ref. 11). Cells accumulate in the G0 phase after translocation of E2F-4 to the nucleus and association of E2F-4 with a retinoblastoma protein family member RB, p130, or p107, which converts E2F-4 into a transcriptional suppressor (12–15). Consistent with this model, t-RA treatment of HBE cells increased nuclear levels of E2F-4 and caused association of E2F-4 with p107, converting E2F into a transcriptional suppressor (9). Association of E2F with RB family members is inhibited by RB protein phosphorylation, which is activated by specific cyclin:CDK complexes (cyclin D:CDK-4 and cyclin E:CDK-2; reviewed in Ref. 11). CDK activity is regulated by changes in CDK expression, phosphorylation, and association with CDK inhibitors, including p16, p21, and p27 (11). The abundance of cyclin D, cyclin E, p21, and p27 is inhibited by the ubiquitin-proteasome pathway; ubiquitination of lysine residues targets these cell cycle proteins for proteolysis by the 26S proteasome complex (reviewed in Ref. 16). Dysregulation of the ubiquitin-proteasome pathway occurs in certain cancers; increased proteasome-mediated degradation of p27 has been detected in human colorectal tumors (17).

We have sought to identify retinoid signaling events that cause HBE cells to accumulate in the G0 phase of the cell cycle and mechanisms by which NSCLC cells become resistant to the growth-inhibitory effect of t-RA. In this study, t-RA treatment of HBE cells inhibited the expression and activity of cyclin D1, cyclin E, CDK-2, and CDK-4; increased p27 levels; and shifted RB to a hypophosphorylated state. Posttranslational mechanisms contributed to the suppression of CDK-2, CDK-4, and p27, which, in the case of CDK-4, involved the ubiquitin-proteasome pathway. These retinoid signaling events did not occur in a NSCLC cell line that is refractory to the growth-inhibitory effect of t-RA. These findings reveal that the ubiquitination of E2F-4 is a consequence of the crucial role that retinoids play in maintaining normal epithelial cell growth and differentiation. For example, retinoids inhibit the growth of human bronchial epithelial (HBE) cells (11). Potential limitations of this study include the possibility that E2F-4 is not the only transcriptional suppressor involved in the growth-inhibitory effects of t-RA in HBE cells. Further studies are needed to determine the role of E2F-4 in the growth-inhibitory effects of t-RA in HBE cells and the mechanism by which NSCLC cells acquire resistance to the growth-inhibitory effects of t-RA.
uitin-proteasome pathway is involved in a novel mechanism of retinoid signaling that may contribute to the suppression of HBE cell growth and the development of retinoid resistance in NSCLC cells.

MATERIALS AND METHODS

Cell Culture Conditions and Reagents. Normal HBE cells were cultured from bronchial mucosal biopsy samples using fresh surgical specimens as described previously (4). HBE cells were grown in Keratinocyte Serum-Free Medium (Life Technologies, Inc., Gaithersburg, MD) containing EGF (Life Technologies, Inc.) and bovine pituitary extract (Life Technologies, Inc.) at 37°C in 5% CO2. Calu-6 cells were obtained from the American Type Culture Collection and were maintained in RPMI containing 10% fetal bovine serum. For all experiments involving t-RA treatment, cells were first incubated for 24 h in medium without growth factors (0.5% serum for Calu-6 cells and deprived of EGF and bovine pituitary extract for HBE cells) and then treated with 1 μM t-RA (Sigma Chemical Co., St. Louis, MO) in the absence of exogenous growth factors for the indicated time periods. The 26S proteasome inhibitor LLnL, the structurally related but proteasome-inactive compound LLM, and the serine protease inhibitor PMSF were purchased from Sigma.

BrdUrd Incorporation. Cells were treated with 1 μM t-RA or medium alone for 6, 24, and 72 h. Cells were then treated with 10 μg/ml BrdUrd (Sigma Chemical Co.) for 2 h, trypsinized, and fixed onto slides as described previously (9). The cells were incubated with a monoclonal antibody to BrdUrd (mAbBR3; Caltag Laboratories, South San Francisco, CA) for 45 min at 37°C. The Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine were used for detection of BrdUrd incorporation. Cells (103) were counted, and the percentages of cells that incorporated BrdUrd were calculated.

Fig. 1. t-RA inhibits BrdUrd incorporation in HBE cells. The percentages of cells that incorporate BrdUrd were calculated from in situ analysis of cells treated for the indicated time periods with 1 μM t-RA (+) or medium alone. Results represent the means of values from three separate experiments; bars, SD.

Fig. 2. t-RA inhibits the expression of specific cyclins and CDKs in HBE cells. Northern (A) and Western (B) analyses of the indicated mRNAs and proteins were performed on total cellular RNA (20 μg/lane) and cell lysate (50 μg/lane) prepared from HBE cells treated for the indicated time periods with 1 μM t-RA or medium alone. The membranes were stripped and reincubated with the indicated probes and antibodies. Relative amounts of RNA and protein loaded per lane are shown by ethidium bromide staining of the RNA gel and immunoblotting with an antibody to actin. Laser densitometric scanning of the Northern and Western blots is illustrated at the bottom, measuring bands of t-RA-treated cells relative to bands of untreated cells at each time point.

Fig. 3. t-RA increases CDK-4 degradation in HBE cells. After 72 h of treatment with 1 μM t-RA or medium alone, whole-cell lysates were isolated and subjected to an in vitro degradation assay for CDK-4 as described in “Materials and Methods.” After incubation of cell lysates with [35S]methionine-labeled CDK-4 for 2 and 4 h at 37°C, aliquots were subjected to SDS/PAGE, and CDK-4 was quantified by phosphorimage analysis.
t-RA  -  -  -  -  +  +  +  +   
- PMSF LLM LLnL  - PMSF LLM LLnL  

CDK4  
Actin  

Fig. 4. Proteasome inhibitor blocked the suppression of CDK-4 by t-RA. Normal HBE cells were treated with 1 μM t-RA for 72 h, at which time the protease inhibitors LLnL (50 μM), LLM (50 μM), or PMSF (100 μM) were added. After incubation for 8 h, the cells were subjected to Western analysis to determine CDK-4 expression and to examine relative amounts of protein loaded per lane actin expression.

Northern Blot Analysis. After treatment with 1 μM t-RA for 6, 24, and 72 h, total RNA was isolated from cells. Twenty μg of total RNA were electrophoresed on an agarose gel, blotted onto a nylon membrane (Duralon UV; Stratagene, La Jolla, CA), and hybridized to α-32P-labeled cDNAs of cyclin D1, CDK2, CDK4, p21, p27, and RARβ. The human cyclin D1, CDK-2, and CDK-4 cDNAs were gifts from Dr. Stephen Elledge (Baylor College of Medicine); the human p21 and p27 cDNAs were gifts from Dr. Bert Vogelstein (John Hopkins Oncology Center, Baltimore, MD); and the human RARβ cDNA was a gift from Dr. Bill Lamph (Ligand Pharmaceuticals, La Jolla, CA).

Western Blot Analysis. Cells were incubated with 1 μM t-RA or media alone for 6, 24, and 72 h. Whole-cell lysates were prepared using MEGA-RIPA buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaF, 0.5% NP40, 200 mM NaCl, 5 mM EDTA, 5 mM EGTA, 20 mM β-glycerophosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 200 μM Na3VO4]. Thirty μg of protein lysate were electrophoresed using 12% SDS-PAGE (for cyclin D, cyclin E, CDK-2, CDK-4, p27, and p21), 15% SDS-PAGE (for cyclin D1, CDK2, CDK4, p21, p27, and RAR), 30% SDS-PAGE, and autoradiographed (18).

Fig. 5. t-RA increases p27 expression in HBE cells. Northern (A) and Western (B) analyses of the indicated mRNAs and proteins were performed on total cellular RNA (20 μg/lane), and cell lysate (50 μg/lane) was prepared from HBE cells treated for the indicated time periods with 1 μM t-RA or media alone. A separate membrane was incubated with the indicated probes and antibodies. Relative amounts of RNA and protein loaded per lane are shown by ethidium bromide staining of a representative RNA gel and immunoblotting with an antibody to actin. Laser densitometric scanning of the Northern and Western blots is illustrated at the bottom, measuring bands of t-RA-treated cells relative to bands of untreated cells at each time point.
as described by the manufacturer. The integrity of the in vitro translated product was confirmed by SDS-PAGE, followed by autoradiography.

**In Vitro Degradation of CDK-4.** HBE cells were lysed in hypotonic buffer [10 mM Tris (pH 8.3), 1 mM MgCl₂], and briefly sonicated. After centrifugation, supernatant (100 μg of protein) was mixed with 10 μM of 10X ATP-regenerating system [100 mM Tris (pH 7.5), 50 mM CaCl₂, 50 mM MgCl₂, 20 mM ATP, 30 units/ml creatine phosphokinase, and 100 μM phosphocreatine] and 1 μl rabbit reticulocyte lysates containing radiolabeled CDK-4 (19). After incubation for different time periods at 37°C, aliquots were subjected to SDS-PAGE, and the gel was subjected to phosphorimage analysis using Image IC software (Scion Corp.) on a Hewlett-Packard ScanJet 4C.

**RESULTS**

We sought to investigate in HBE cells the effect of t-RA on the expression and activity of cell cycle proteins and the role of these changes in the growth inhibition induced by t-RA. Toward this end, we investigated whether: (a) changes in the expression and activity of cell cycle proteins occurred coincidently with changes in HBE cell growth; and (b) the effect of t-RA on cell cycle proteins was abrogated in retinoid-resistant NSCLC cells.

**t-RA Inhibited HBE Cell Growth.** HBE cells were treated with 10⁻⁸ M t-RA or medium alone, and cellular proliferation was evaluated at 6, 24, and 72 h by in situ analysis of BrdUrd incorporation. The percentages of cells that incorporate BrdUrd at each time point were determined. Cells from three separate bronchial samples were treated with t-RA and analyzed as three separate experiments, from which the mean (± SD) BrdUrd incorporation at each time point was calculated. t-RA inhibited BrdUrd incorporation in a time-dependent manner, with a prominent reduction in cellular proliferation first detected at 72 h (Fig. 1).

**1-RA Decreased the Level of Specific Cyclins and CDKs.** We examined the effect of t-RA on cyclin/CDK complexes known to regulate entry of cells into the G₀ phase of the cell cycle. t-RA decreased, in a time-dependent manner, the mRNA levels of cyclin D₁, but not CDK-2 or CDK-4 (Fig. 2A). We showed previously that cyclin E mRNA levels decrease with t-RA treatment in HBE cells (9). The protein levels of cyclin D₁, cyclin E, CDK-2, and CDK-4 decreased in response to t-RA in a time-dependent manner, with striking reductions in these proteins first detected at 72 h (Fig. 2B).

Because t-RA decreased the protein levels but not the mRNA levels of CDK-2 and CDK-4 (Fig. 2), we investigated the role of posttranslational mechanisms in the suppression of CDK levels by t-RA. We examined the effect of t-RA on CDK proteolytic activity by incubating in vitro translated CDK-4 with lysates of HBE cells treated for 72 h with medium alone or t-RA. Relative to the control lysates, incubation of radiolabeled CDK-4 with lysates from t-RA-treated cells decreased CDK-4 levels in a time-dependent manner (Fig. 3), providing evidence that t-RA increased CDK proteolytic activity. We investigated the contribution of ubiquitin-proteasome pathways to t-RA actions by treatment of HBE cells with LLnL, a specific 26S proteasome inhibitor, LLM, a compound that is structurally related to LLnL but proteasome inactive, or PMSF, a serine protease inhibitor. HBE cells were treated for 72 h with 1 μM t-RA, at which time these protease inhibitors were added. The cells were incubated for 8 h and then subjected to Western blot analysis to determine CDK-4 expression. LLM and PMSF had minimal effects on CDK-4 levels, whereas LLnL completely abrogated the suppression of CDK-4 by t-RA (Fig. 4). These findings support a role for the 26S proteasome in the suppression of CDK-4 levels by t-RA.

**t-RA Increased the Level of the p27 CDK Inhibitor.** We investigated the effect of t-RA on the levels of p27, p21, and p16, the CDK inhibitors known to interact with CDK-2 and/or CDK-4. Although the level of p27 mRNA did not measurably change, p27 protein increased in response to t-RA treatment (Fig. 5). The increase in p27 was first detected at 72 h. In contrast to p27, p21 protein decreased at 72 h (Fig. 5), and p16 protein did not detectably change (data not shown).

**t-RA Decreased CDK Activity and RB Phosphorylation.** In light of the observed changes in cyclins, CDKs, and CDK inhibitors, we investigated the effect of t-RA on CDK activity. HBE cells were treated with t-RA for different time periods and then treated with EGF for 15 min to activate CDKs. Cells were lysed, and CDK-2 and CDK-4 were immunopurified. The immunoprecipitant was incubated with histone H1 and [γ⁻³²P]ATP, and ³²P incorporation into histone H1 was measured. Laser densitometry was performed on the autoradiograph to measure incorporation in samples from t-RA-treated cells relative to that of cells treated with EGF alone. Western analysis of RB (B) was performed on lysates prepared from HBE cells treated for the indicated time points with 1 μM t-RA or media alone. The positions of hyperphosphorylated (ppRB) and hypophosphorylated (pRB) RB are indicated.
cells that express functional retinoid nuclear receptors, hypothesizing that, in these cells, t-RA will not alter the expression of cell cycle proteins that are required for growth arrest. For these studies, we used the Calu-6 NSCLC cell line. Transient transfection assays using a luciferase reporter containing a DR5 RARE showed that t-RA increased retinoid receptor transcriptional activity, and Northern analysis revealed that t-RA increased RAR-β expression (Fig. 7A), demonstrating that retinoid receptors are functionally activated in response to the presence of ligand in Calu-6 cells. BrdUrd incorporation assays revealed that t-RA treatment did not measurably alter the growth of Calu-6 cells (Fig. 7B).

Western analysis was performed to examine the effect of t-RA on the expression of cell cycle proteins in Calu-6 cells (Fig. 8). Cyclin D1, cyclin E, and RB levels were reduced by t-RA at 72 and 120 h. Although the expression of RB decreased, its electrophoretic migration did not change, suggesting that there was no effect on RB phosphorylation. Moreover, the changes in cyclin D1, cyclin E, and RB in Calu-6 cells were less than those observed in HBE cells. Furthermore, t-RA did not detectably change the protein levels of CDK-2, CDK-4, or p27 in Calu-6 cells. These observations in Calu-6 cells provide evidence of a disruption in the linkage of retinoid receptors with cell cycle proteins that are crucial for G0/G1 growth arrest.

**DISCUSSION**

In this study, we investigated the mechanism by which t-RA causes HBE cells to exit the cell cycle. t-RA treatment of HBE cells altered the expression and activity of G0/G1 cyclins, CDKs, and CDK inhibitors. For CDK-2, CDK-4, and p27, t-RA mediated these effects through posttranslational mechanisms that, in the case of CDK-4, involved the ubiquitin-proteasome pathway. Despite retinoid receptor transcriptional activation, these events did not occur in a NSCLC cell
line that is resistant to the growth-inhibitory effects of t-RA. Moreover, our findings demonstrate that retinoid signaling is linked to the G0/G1 cell cycle checkpoint, and this linkage may be crucial to the growth-inhibitory effect of retinoids.

Our findings in HBE cells are both similar to, and different from, the effects of t-RA on other cell types that are growth inhibited by t-RA. Like our findings in HBE cells, the protein levels of cyclins D1 and E are reduced by t-RA in the SV40-immortalized HBE cell line BEAS-2B (19, 20). However, cyclin D1 mRNA levels are not reduced by t-RA in BEAS-2B cells (20). Possibly contributing to this difference, viral antigens inactivate RB family members, reducing the potential of E2F to transcriptionally suppress the activity of the cyclin D1 promoter, which contains an E2F-binding site (21). Similar to our findings in HBE cells, t-RA treatment of MCF-7 breast cancer cells reduced the mRNA and protein levels of cyclin D1, CDK-2, and CDK-4, but t-RA had no effect on p27 levels in MCF-7 cells (22, 23).

In contrast to the suppression of p21 we observed in HBE cells, t-RA enhanced p21 mRNA and protein levels in U937 myeloid leukemia cells (24). In light of the role of p21 in growth inhibition, the reduction in p21 levels we observed in t-RA-treated HBE cells is somewhat paradoxical. One possible explanation is that CDK inhibitors are known to function on a stoichiometric basis with CDKs, and the reduction in p21 could reflect a response to reduced CDK levels in t-RA-treated HBE cells. In this regard, t-RA could alter the expression of certain cell cycle proteins indirectly through its effect on key growth-regulatory pathways. Overall, these findings suggest that t-RA inhibits cell growth through multiple mechanisms that are cell type specific.

Retinoids mediate their initial effects through the transcriptional activation of retinoid nuclear receptors. However, our findings provide evidence that retinoid receptors do not directly regulate the expression of some cell cycle proteins; the mRNA levels of CDK-2, CDK-4, and p27 did not change with t-RA treatment. Consistent with this finding, posttranslational mechanisms involving the ubiquitin-proteasome pathway are known to be important in the regulation of the cell cycle. Ubiquitination of cyclins A, B, D, and E, p21, and p27 targets these proteins for proteolysis by the proteasome complex (16). Whereas posttranslational mechanisms stabilize CDK-4 through its interactions with the heat shock protein-90 complex (25), regulation of CDK levels by the ubiquitin-proteasome pathway has not been demonstrated. Here, we provide the first evidence that CDK-4 levels are inhibited by t-RA through posttranslational mechanisms involving the ubiquitin-proteasome pathway. The characteristics of CDK-4 that target it for regulation through the ubiquitin-proteasome pathway are not clear. The destruction box motif and PEST sequences (rich in proline, glutamic acid, serine, and threonine) found in mitotic and G1 cyclins, respectively, are thought to play a role in cyclin destruction (20, 26, 27). These features are not present in CDKs, which is also true for other proteins regulated by the ubiquitin-proteasome pathway (reviewed in Ref. 28). Posttranslational mechanisms contribute to the effects of t-RA on other pathways that are crucial to the growth regulation of HBE cells. We showed previously that Jun NH2-terminal kinase activity is inhibited by t-RA in HBE cells through posttranslational mechanisms involving mitogen-activated protein kinase (MAPK)-1 (MKP-1), a dual-specificity phosphatase that dephosphorylates Jun NH2-terminal kinase at threonine-183 and tyrosine-185 (29). Retinoid receptor transcriptional activation is required for the sustained activation of MKP-1 expression and suppression of JNK activity (29). These findings provide evidence that although transcriptional mechanisms are required for retinoid receptors to initiate retinoid signaling, downstream signaling events that are important mediators of retinoid actions can occur through posttranslational mechanisms.

In this study, we investigated the basis of retinoid resistance in NSCLC cells expressing functional retinoid nuclear receptors. We found that Calu-6 cells differ from HBE cells in the effect of t-RA on cell cycle proteins that control the G0/G1 cell cycle checkpoint. These findings provide evidence that HBE cells and Calu-6 cells differ in their capacity to undergo cell cycle arrest in response to t-RA. This difference in retinoid sensitivity could be the result of cell type-specific variations between HBE cells and Calu-6 cells, which may be derived from a progenitor other than the HBE cells examined in this study. Alternatively, Calu-6 cells may be retinoid resistant because, during the process of HBE malignant transformation, the signaling pathways that link retinoid receptors with cell cycle proteins are disrupted. A key regulator of G0/G1 cell cycle arrest is the p53 protein, a transcription factor that activates p21 expression. Wild-type p53 protein is not detectable in Calu-6 cells (30), which could contribute to retinoid resistance in these cells. Alternatively, a retinoid signaling defect could occur because of an alteration in the activity of the ubiquitin-proteasome pathway. Increased proteasome-mediated p27 proteolytic activity has been detected in colorectal tumors, resulting in reduced p27 levels (17). Similarly, p27 levels are reduced in some NSCLC biopsies (31, 32). The level of cyclin D1 protein, which is regulated by ubiquitin-mediated proteolysis, is increased in a proportion of lung, head and neck, esophagus, and breast cancers, only some of which express increased cyclin D1 levels as a consequence of cyclin D1 gene amplification (reviewed in Ref. 33). In tumors without evidence of gene amplification, alterations in the ubiquitin-proteasome pathway may contribute to the increased levels of cyclin D1.

Although the mechanisms that initiate ubiquitin-mediated cyclin D1 proteolysis have not been defined, PEST sequences in the COOH terminus of cyclin D1, which contain several phosphorylation sites that may target the protein for ubiquitination, appear to be required (20, 27). One could speculate that, in some tumors, abnormal p27 and cyclin D1 levels could be the result of alterations in the activity of ubiquitination enzymes (E1, E2, and E3) or pathways that regulate the ubiquitination process through phosphorylation of target proteins. To address this possibility, future work will focus on mechanisms that control the ubiquitination of cell cycle proteins in HBE cells, whether these pathways are disrupted in NSCLC cells, and how this impacts retinoid signaling pathways in NSCLC cells. Because increased cyclin D1 and reduced p27 levels are markers of poor prognosis in people with a variety of different solid tumors (17, 31, 32), findings from these studies may reveal novel features of tumors that are clinically relevant.


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