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Cancer Letters 168 (2001) 165–172

CANCER Letters

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# Cyclin D1 as a proliferative marker regulating retinoblastoma phosphorylation in mouse lung epithelial cells

Cynthia L. Mamay[a], Irene E. Schauer[b], Pamela L. Rice[c], Lori D. Dwyer-Nield[a], Ming You[d], Robert A. Sclafani[b], Alvin M. Malkinson[a,*]

[a]*Department of Pharmaceutical Sciences, University of Colorado Cancer Center and Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262, USA*

[b]*Department of Biochemistry and Molecular Genetics, University of Colorado Cancer Center and Health Sciences Center, Denver, CO 80262, USA*

[c]*Department of Gastroenterology, University of Colorado Cancer Center and Health Sciences Center, Denver, CO 80262, USA*

[d]*Department of Pathology, Medical College of Ohio, Toledo, OH, USA*

Received 29 December 2000; received in revised form 6 March 2001; accepted 7 March 2001

**Abstract**

Elevations in cyclin D1 content increase the phosphorylation status of retinoblastoma (Rb) protein to encourage cell cycle transit. We sought to determine if cyclin D1 content could be used as an index of cell proliferation in mouse lung epithelia following growth manipulations in vitro and in vivo. Rb protein concentration was high in 82-132 and LM2, two fast-growing neoplastic mouse lung epithelial cell lines. The hyperphosphorylated form of Rb predominated in these two cell lines, while Rb in slower-growing cell lines was predominantly hypophosphorylated. Consistent with this, more cyclin D1 protein was expressed in the fast-growing cell lines than in slower-growing cells. We therefore tested whether cyclin D1 content varied with growth status. The amount of cyclin D1 decreased upon serum removal coincident with growth inhibition and then increased upon serum re-addition which stimulated resumption of proliferation. This correlation between cyclin D1 content and growth status also occurred in vivo. Cyclin D1 content increased when lungs underwent compensatory hyperplasia following damage caused by butylated hydroxytoluene administration to mice and in lung tumor extracts as compared with extracts prepared from uninvolved tissue or control lungs. We conclude that elevated cyclin D1 levels account, at least in part, for the hyperphosphorylation of Rb in neoplastic lung cells, and are associated with enhanced lung growth in vitro and in vivo.
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*Keywords*: Cyclin D1; Proliferative marker; Retinoblastoma phosphorylation; Mouse lung epithelial cells

## 1. Introduction

A major site that regulates entrance into the S phase of the cell cycle is the phosphorylation status of retinoblastoma (Rb) protein [1]. Hypophosphorylated Rb can sequester the transcription factor E2-F1, preventing E2-F1 from activating genes that encode products required for DNA replication [2]; phosphorylation of Rb releases E2-F1. Rb is phosphorylated to become pRb by either of two cyclin D-dependent protein kinases, CDK4 and CDK6, and also by CDK2-cyclin E [3]. The activity of these kinases is stimulated by

* Corresponding author. Tel.: +1-303-315-4579; fax: +1-303-315-6281.

*E-mail address:* al.malkinson@uchsc.edu (A.M. Malkinson).

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PII: S0304-3835(01)00480-3

cyclin D1 and inhibited by the INK 4 and CIP/KIP families of cyclin kinase inhibitors [4,5].

Neoplastic growth is typified by aberrations in these G1 controls that decrease the cellular content of hypophosphorylated Rb. Small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) differ from each other in how they inactivate the Rb brake. Homozygous deletion of the *RB* gene is frequent in SCLC [6], while in human NSCLC neither deletion nor mutation of this gene occurs with high frequency [7]. In mouse lung tumors (a model for human adenocarcinoma) [8], Rb mRNA was lower than in normal lung [9,10], while tumor Rb protein content increased in one report [10] but not in another [11]. These differences in immunoblotting assessments may reflect the heterogeneous distribution of Rb protein in these tumors [10]. p16 is frequently altered, however, both in NSCLC and in the mouse model [12,13]. *Cdkn2a*, the gene encoding p16, undergoes homozygous deletion and is considered a tumor suppressor gene for this cancer [12]. While expression of the p18 and p19 members of the INK 4 family was the same among 18 different mouse lung epithelial cell lines, p15, p16α, and p16β were expressed only in slow-growing cells [13]. p16 alleles that remain extant in the tumors may be transcriptionally silenced, probably by methylation at the 5′ upstream promoter [14,15].

Cyclin D1 also plays a role in the growth status of lung epithelial cells. Cyclin D1 is overexpressed in most NSCLC cell lines, whereas elevated cyclin D1 expression in SCLC cell lines is rare [16]. The importance of cyclin D1 in regulating the growth of mouse lungs is indicated by the fact that stable transfection with an antisense oligonucleotide to cyclin D1 reduced the growth rate in vitro and upon SC implantation into mice [17]. Herein we extend the analysis of Rb and cyclin D1 in the mouse lung, and find that Rb hyperphosphorylation is positively correlated with growth rate. Further, the cellular content of cyclin D1 plays a major role in this regulation as shown by its association with growth status following experimental manipulations. This tumor model thus resembles NSCLC where similar changes in Rb and cyclin D1 occur that do not take place in SCLC [14]. As in NSCLC, alterations in both p16 and cyclin D1 characterize mouse lung neoplasia. If *Cdkn2a* is a tumor suppressor gene, it follows that cyclin D1 overexpression may be oncogenic. Cyclin D1 is a useful marker of enhanced proliferation in non-tumorigenic pulmonary lung epithelial cells and in certain types of lung neoplasia.

## 2. Materials and methods

### *2.1. Mouse lung epithelial cell lines*

The cell lines used herein were described extensively in a review [18]; their growth characteristics are briefly summarized in Table 1. C10 and E10 cells are non-tumorigenic but immortalized cell lines that displayed at early passage features of alveolar type 2 cells [19], one of the cell types of origin of lung adenocarcinoma. E9 is a spontaneous transformant of E10 that is characterized by neoplastic properties such as anchorage-independence, tumorigenicity, and a lack of density-dependent inhibition of growth [20]. 82-132 and LM1 cells are derived from solid lung tumors induced by ethyl nitrosourea and urethane, respectively [21]. The LM2 line was derived from a urethane-induced papillary tumor [22] and PCC4 from a spontaneously occurring papillary tumor [21].

### *2.2. Preparation of lung samples from butylated hydroxytoluene-treated mice*

Six-week-old BALB/cByJ male mice were obtained from Jackson Laboratories (Bar Harbor, MI). They were fed Wayne Lab Blox and given water ad libitum, and maintained on hardwood bedding with a 12 h light/dark cycle. Two weeks after arrival they were injected intraperitoneal (i.p.) with butylated hydroxytoluene (BHT) (200 mg/kg body weight) (Sigma, St. Louis, MO) dissolved in

Table 1
Growth properties of mouse lung epithelial cell lines[a]

| Cell line | Tumorigenicity | Growth rate | Anchorage dependence |
|---|---|---|---|
| C10 | − | Slow | + |
| E10 | − | Slow | + |
| LM1 | + | Slow | − |
| E9 | + | Moderate | − |
| LM2 | + | Fast | − |
| 82-132 | + | Fast | − |
| PCC4 | + | Fast | − |

[a] Cell line properties are described in detail in Ref. [18].

Mazola® corn oil or with only the corn oil vehicle as a control, as described previously [23]. The mice were sacrificed by lethal pentobarbital anesthesia and their lungs perfused with 0.9% saline. Homogenates were prepared by Dounce homogenization in 5 × wt./vol. of 20 mM HEPES (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 5 μg/ml aprotinin, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenized samples were separated into soluble and particulate fractions by a 16 000 × $g$ centrifugation for 30 min. Prior to SDS-PAGE analysis, samples were added to a protein solubilizing mixture (500 mM Tris (pH 6.8), 10% SDS, 2 mM β-mercaptoethanol, 20% glycerol, and 0.25% pyronine Y).

### 2.3. Preparation of lung tumor extracts

Tumors were induced in A/J mice with a 1 mg/g dose of urethane, as described previously [24]. Ten months after injection, tumors and portions of normal-appearing lung were dissected and samples were prepared as described above.

### 2.4. Western blotting

Cells were harvested at 70% confluency and washed in phosphate-buffered saline. The cell pellets were solubilized in a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 5 μg/ml final concentration of the protease inhibitors leupeptin, aprotinin, and PMSF. Aliquots were removed and sheared through a syringe needle for protein concentration determination [25]. The remaining cells were resuspended in a protein solubilizing mixture (500 mM Tris (pH 6.8), 10% SDS, 2 mM β-mercaptoethanol, 20% glycerol, and 0.05% Bromophenol Blue) at 1–4 × $10^7$ cells per ml, immediately boiled for 5 min, sheared through a syringe needle to reduce viscosity, aliquotted, and stored at −80°C. Anti-pRb, a kind gift from Dr Wen-Hua Lee (University of Texas Health Science Center, San Antonio, TX), was used to estimate Rb amounts. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG (Biorad, Hercules, CA). To estimate cyclin D1 concentrations, a polyclonal antibody prepared against an 11 amino acid C-terminal epitope on human cyclin D1 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and used at a dilution of 1:1000. The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG from Santa Cruz Inc. (Santa Cruz, CA). Proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) and detected using enhanced chemiluminescence (Amersham, Piscataway, NJ). Ponceau (Sigma, St. Louis, MO) staining of proteins on the membranes was used to ensure equal loading.

### 2.5. Proliferative measurements in vitro

DNA content was determined by fluorescence-activated cell sorter analysis by the University of Colorado Cancer Center Flow Cytometry Core to assess cell cycle distribution.

## 3. Results

### 3.1. Rb and cyclin D1 protein contents in several mouse lung cell lines

Differences among the cell lines were noted for both proteins. The highest amount of Rb protein was in two of the fast-growing tumor-derived cell lines, namely 82-132 and LM2 (Fig. 1A). The two bands migrating at 116 and 110 kDa are thought to represent the phospho (pRb) and dephospho (Rb) forms of Rb, respectively, and their relative amounts varied among

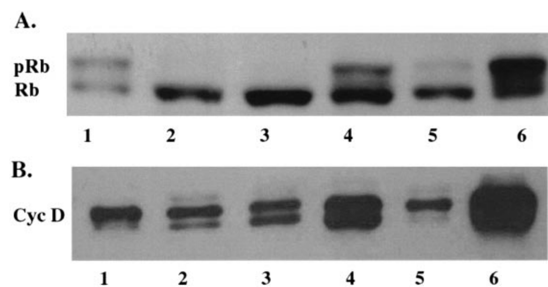


Fig. 1. Immunoblot analysis of cyclin D1 and Rb expression levels in mouse lung cell lines. Extracts were prepared from the indicated cell lines and subjected to SDS-PAGE. Proteins were then transferred to membranes and probed with anti-Rb (A) or anti-cyclin D1 (B) antibodies. Ponceau staining of the membranes was used to verify equal protein loading. Mouse lung cell lines: E9 (lane 1); E10 (lane 2); C10 (lane 3); LM2 (lane 4); LM1 (lane 5); and 82-132 (lane 6). Results shown are a representation typical of each of three independent experiments.

the cell lines. 82-132 cells had more of the pRb protein than Rb protein, E9 and LM2 had roughly equal amounts of pRb and Rb, LM1 had a trace amount of pRb, while the two non-tumorigenic lines, C10 and E10, contained only Rb. The pRb/Rb thus correlates with the growth rates of these cells, with the faster-growing cells (82-132 and LM2) having the highest pRb/Rb ratio and the slowest-growing cells (LM1, E10, and C10) having the lowest pRb/Rb ratio. E9 cells have a proliferative rate that is intermediate to these groups [26] and also have an intermediate pRb/Rb ratio.

Upon immunoblotting for cyclin D1 in the cell lines, one to three bands were seen at 34.6, 37.1, and 39.8 kDa, with the middle band being predominant and the lower band observed only occasionally (Fig. 1B). The cyclin D1 antibody cross-reacts with cyclin D2 which has a highly homologous epitope [27]; Western blots of cyclin D1 using other antibody sources also reveal at least a doublet [28]. The faster-growing cells (82-132 and LM2) contained the most cyclin D1.

A clear trend can thus be seen between the quantities of cyclin D1 and the pRb/Rb ratio. Cells with a relatively high amount of hyperphosphorylated Rb, such as 82-132 and LM2, also have a high cyclin D1 content. Cells that mainly contain dephospho Rb, such as LM1, E10, and C10, have the lowest amounts of cyclin D1.

### *3.2. Manipulation of lung cell growth status to examine the correlation between cyclin D1 content and proliferation*

One in vitro and two in vivo studies were done. The first experiment was to deprive normal E10 cells of serum, a manipulation which is cytostatic for this line, as shown in Table 2. After 24 h without serum, E10 cells were incubated in the presence of serum for an additional 24 h to stimulate growth again. Samples were taken from control (never serum-deprived), serum-deprived, and serum-replenished cells, and cyclin D1 content was examined by immunoblotting. The amount of cyclin D1 dramatically diminished when cells ceased to grow and then increased nearly back to control levels 24 h after the re-addition of serum (Fig. 2).

Two independent experiments were performed to assess pulmonary cyclin D1 content during compensatory hyperplasia in vivo. Upon i.p. injection of BHT into mice, the type 1 pneumocytes that line most of the alveolar septae are damaged [29]. This is a reversible injury and its repair is characterized by massive hyperplasia of type 2 pneumocytes, endothelial cells, and interstitial cells [30]. This can be used as a non-neoplastic model of biochemical changes associated with proliferation [31]. The peak time of cellular accretion is 6 days after BHT administration [32]. Cyclin D1 was undetectable in control quiescent lungs or 4 h after BHT treatment, but gradually increased 1, 3, and 6 days after treatment (Fig. 3A).

Ten months following injection of a 1 mg/g body weight dose of urethane into adult A/J mice, malignant and benign tumors are typically observed [8]. Tumor and normal-appearing tissue (uninvolved tissue) were dissected from five individual mice, along with age-matched A/J control mice that had been injected with only the saline vehicle. The cyclin D1 content of these samples was estimated by immunoblotting. In common with the lung injury study of Fig. 3A, cyclin D1 was undetectable in control lungs. In two independent studies, benign tumors contained slightly more

Table 2
Cell cycle distributions of the E10 cells used in Fig. 2

| | % G0/G1 | % S | % G2/M |
|---|---|---|---|
| Control[a] | 80.9 | 14.1 | 1.8 |
| Serum-deprived | 94.0 | 3.7 | 1.9 |
| Serum-replenished | 81.6 | 13.0 | 1.9 |

[a] See Section 2 for a description of serum manipulations of E10 cells.

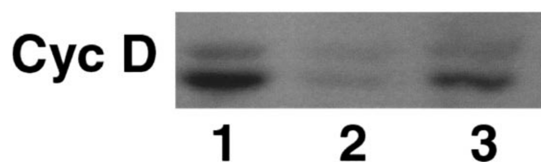


Fig. 2. Immunoblot analysis of cyclin D1 expression in serum-deprived and serum-replenished E10 mouse lung cells. Cellular extracts were prepared following growth manipulations and subjected to SDS-PAGE. Proteins were then transferred to membranes and probed with an anti-cyclin D1 antibody. Ponceau staining of the membranes was used to verify equal protein loading. Control (lane 1); serum-deprived (lane 2); and serum-replenished (lane 3). Results shown are representations of each of two independent experiments.

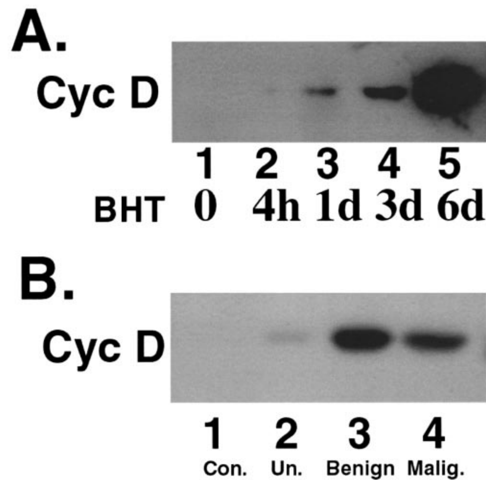


Fig. 3. Immunoblot analysis of cyclin D1 expression in lungs from BHT-treated BALB/cByJ mice and urethane-treated A/J mice. Tumors and portions of normal-appearing lung were dissected, and extracts prepared and subjected to SDS-PAGE. Proteins were then transferred to membranes and probed with an anti-cyclin antibody. Ponceau staining of the membranes was used to verify equal protein loading. (A) Times after BHT administration to mice: 0 h (lane 1); 4 h (lane 2); 1 day (lane 3); 3 days (lane 4); and 6 days (lane 5). (B) Samples collected 10 months after urethane administration to mice: control (lane 1); normal-appearing, uninvolved lung (lane 2); benign tumors (lane 3); and malignant tumors (lane 4). Results shown are a typical representation found in each of two independent experiments.

cyclin D1 than malignant tumors (Fig. 3B), while in three other studies, malignant tumors had slightly more cyclin D1 than benign tumors (data not shown). In all five independent experiments, however, both classes of tumors had much more cyclin D1 than uninvolved tissue and normal lung controls.

Because cyclin D1 content rose with increased growth rate in all of these experiments, we infer that the amount of cyclin D1 is a valid indicator reflecting the growth status of mouse lung epithelium.

## 4. Discussion

Adult mouse lung is proliferatively quiescent unless responding to chemically-induced injury [30], partial ablation [33], or during neoplasia [34]. The signals regulating this transition from stasis to active growth are unknown. Enhanced expression of cyclin D1, a required element in the transit from G1 to S phase, occurred during compensatory hyperplasia following BHT-induced lung damage and in urethane-induced tumors (Fig. 3). Fast-growing 82-132 and LM2 cells expressed elevated amounts of cyclin D1 and Rb proteins. Most neoplastic lung cell lines contained more cyclin D1 than non-tumorigenic E10 and C10 cells (Fig. 1) except for LM1 cells whose growth rate is as slow or slower than that of non-tumorigenic E10 and C10 cells [18]. This apparent growth dependence of cyclin D1 expression was confirmed when lung cell proliferative status was experimentally perturbed. Cyclin D1 levels decreased when E10 cells were made cytostatic by serum deprivation and then rose upon serum replenishment (Fig. 2). Variations in cyclin D1 content were reflected in the phosphorylation status of Rb. Rb was predominantly phosphorylated when cyclin D1 content was high, while dephospho Rb was the main form present when cyclin D1 levels were low (Fig. 1).

Several markers have been used to monitor mouse pulmonary proliferation. These include lung DNA content [32], incorporation of [$^3$H]thymidine or bromodeoxyuridine into DNA [34], the amount of the proliferating cell nuclear antigen subunit of DNA polymerase δ [35], and the activity of thymidine kinase [32]. According to the results presented herein, cyclin D1 is also an effective and convenient marker of lung growth. Cyclin D1 was barely detectable in quiescent non-neoplastic lung extracts, but became abundant during regenerative repair and upon neoplastic conversion. Determining the amount of cyclin D1 could be of value in studies of fetal lung development, early stages of injury/repair, and the development of hyperplastic foci. Interestingly, E9 cells transfected with excess *Gja1*, the gene encoding the Cx43 connexin protein found in gap junctions, displayed both slower growth and a lower cyclin D1 content than their parental E9 cells [36].

Does cyclin D1 itself stimulate proliferation or merely mediate exposure to positive growth factors as a cell cycle component? One experimental approach to questions of causality is genetic manipulation. Transfection with antisense oligonucleotides to cyclin D1 diminished cellular cyclin D1 content and depressed the growth of LM2 cells in vitro and in vivo [17]. This suggests the utility of such antisense strategies for treating lung cancer. Cyclin D1 content is inversely related to that of the INK 4 inhibitors. The

p15, p16α, and p16β genes were deleted in the most rapidly proliferating cells, namely LM2, 82-132, and PCC4, while the slow-growing neoplastic LM1 line and the non-tumorigenic E10 and C10 cell lines not only retained these genes but expressed them [13]. Reducing cyclin D1 while elevating *Cdkn2a* expression might be a particularly effective therapeutic maneuver. It should be noted that *Cdkn2a* has been suggested as a candidate for a lung tumor suppressor gene in mice [37].

*K-ras* mutation occurs in one-third to one-half of human lung adenocarcinoma patients [38] and is the initiating mutation in both NNK [39] and urethane [40] induced mouse lung cancer. The rapidly dividing 82-132 cells contain predominantly pRb and a high cyclin D1 content but lack mutations in *K-ras* [22]. The oncogene that maintains 82-132 cells in a neoplastic state in unknown, and illustrates the point that neoplastic conversion of lung epithelium into an adenocarcinoma can occur through various pathways. It would be interesting to examine the mutational status of the promoter region of the cyclin D1 gene in 82-132 cells.

Dozens of alterations in gene structure or content have been found in mouse and human lung cancer along with many more changes in gene expression [41]. These include genes encoding growth factor receptors [42,43], signal transducing G proteins [36,38], and protein kinase effector molecules [44,45]. Gene therapy studies demonstrate that reversing the consequences of a single neoplastic change has profound effects on lung cell growth. Roth and colleagues [46] have applied antisense K-ras oligonucleotides to a human lung adenocarcinoma cell line that had a *K-ras* mutation and reduced its growth rate and tumorigenicity. Wild-type *p53* genes administered via adenovirus vectors to lung cancer patients also reduced tumor mass [47]. *K-ras* mutation and *p53* deletion are early and late events, respectively, in mouse lung tumor progression [40]. The expression and phosphorylation status of Rb and cyclin D1 described herein in the mouse system are quite similar to those described by Schauer et al. [16] in NSCLC. Reversible changes in gene expression occur during the interval between the onset of the *K-ras* and *p53* genetic lesions. An antisense strategy aimed at a gene whose expression is increased in lung neoplasia, namely cyclin D1, retards the growth of neoplastic cells [17]. We suggest the utility of a combined gene therapy approach involving genes that have undergone a structural change, such as *K-ras*, *p53*, or *Cdkn2a*, and those whose expression has been modified during lung neoplasia, such as cyclin D1.

## Acknowledgements

This work was supported by USPHS grants CA33497 (A.M.M.), CA58187 (R.A.S.), and CA78797 (M.Y.).

## References

[1] M. Hatakeyama, R.A. Weinberg, The role of Rb in cell cycle control, Prog. Cell Cycle Res. 1 (1995) 9–19.
[2] S.J. Weintraub, C.A. Prater, D.C. Dean, Retinoblastoma protein switches the E2F site from positive to negative element, Nature 358 (1992) 259–261.
[3] J.W. Harbour, R.X. Luo, A. Dei Santi, A.A. Postigo, D.C. Dean, Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1, Cell 98 (1999) 859–869.
[4] D.E. Quelle, R.A. Ashmun, G.J. Hannon, P.A. Rehberger, D. Trono, K.H. Richter, C. Walker, D. Beach, C.J. Sherr, M. Serrano, Cloning and characterization of murine p16INK4a and p15INK4b genes, Oncogene 11 (1995) 635–645.
[5] L. Gomez, N.J. Liegeois, P. Zhang, J.A. Engleman, J. Horner, A. Silverman, R. Burde, M.F. Roussell, C.J. Sherr, S.J. Elledge, Cyclin D- and E-dependent kinases and the p57(KIP2) inhibitor: cooperative interactions in vivo, Mol. Cell. Biol. 19 (1999) 353–363.
[6] C.H. Hensel, H. Sieh, A.G. Gazdar, B.E. Johnson, A.Y. Sakaguchi, S.L. Naylor, W. Lee, E.Y.P. Lee, Altered structure and expression of the human retinoblastoma susceptibility gene in small cell lung cancer, Cancer Res. 50 (1990) 3067–3072.
[7] M. Nishio, T. Koshikawa, Y. Yatabe, T. Kuroishi, M. Suyama, M. Nagatake, T. Sugiura, Y. Ariyoshi, T. Mitsudomi, T. Takahashi, Prognostic significance of cyclin D1 and retinoblastoma expression in combination with p53 abnormalities in primary, resected non-small cell lung cancers, Clin. Cancer Res. 3 (1997) 1051–1058.
[8] A.M. Malkinson, Primary lung tumors in mice: an experimentally manipulable model of human adenocarcinoma, Cancer Res. 52 (1992) 2670s–2676s.
[9] F.C. Re, G. Manenti, M.G. Borrello, M.P. Colombo, J.H. Fisher, M.A. Pierotti, G. Della Porta, T.A. Dragani, Multiple molecular alterations in mouse lung tumors, Mol. Carcinog. 5 (1992) 155–160.
[10] C.L.K. Sabourin, Q.-S. Wang, S.L. Ralston, J. Evans, J. Coate, C.R. Herzog, S.L. Jones, C.M. Weghorst, G.J. Kelloff, R.A. Lubet, M. You, G.D. Stoner, Expression of cell cycle proteins

in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced mouse lung tumors, Exp. Lung Res. 24 (1998) 499–522.

[11] S.A. Belinsky, D.S. Swafford, S.K. Middleton, C.H. Kennedy, J. Tesfaigzi, Deletion and differential expression of *p16*$^{INK4a}$ in mouse lung tumors, Carcinogenesis 18 (1997) 115–120.

[12] M. Sanchez-Cespedes, A.L. Reed, M. Buta, L. Wu, W.H. Westra, J.G. Herman, S.C. Yang, J. Jen, D.I. Sidransky, Inactivation of the INK4A/ARF locus frequently coexists with TP53 mutations in non-small cell lung cancer, Oncogene 18 (1999) 5843–5849.

[13] C.R. Herzog, E.V. Soloff, A.L. McDoniels, F.L. Tyson, A.M. Malkinson, A. Haugen-Strano, R.W. Wiseman, M.W. Anderson, M. You, Homozygous codeletion and differential decreased expression of p15 INK4β, p16 INK4a-α and p16 INK4a-β in mouse lung tumor cells, Oncogene 13 (1996) 1885–1891.

[14] S.A. Belinsky, Role of the cytosine DNA-methyltransferase and p16INK4α genes in the development of mouse lung tumors, Exp. Lung Res. 24 (1998) 463–479.

[15] S. Gazzeri, V. Gouyer, C. Vour'ch, C. Brambilla, E. Brambilla, Mechanisms of p16INK4A inactivation in non small-cell lung cancers, Oncogene 16 (1998) 497–504.

[16] I.E. Schauer, S. Siriwardana, T.A. Langan, R.A. Sclafani, Cyclin D1 overexpression vs. retinoblastoma inactivation: implications for growth control evasion in non-small cell and small cell lung cancer, Proc. Natl. Acad. Sci. USA 91 (1994) 7827–7831.

[17] D.S. Schrump, A. Chen, U. Consoli, Inhibition of lung cancer proliferation by antisense cyclin D, Cancer Gene Ther. 3 (1996) 131–135.

[18] A.M. Malkinson, L.D. Dwyer-Nield, P.L. Rice, D. Dinsdale, Mouse lung epithelial cell lines – tools for the study of differentiation and the neoplastic phenotype, Toxicology 123 (1997) 53–100.

[19] J.M. Bentel, A.W.J. Lykke, G.J. Smith, Cloned murine non-malignant, spontaneously transformed, and chemical tumor-derived cell lines related to the type 2 pneumocyte, Cell Biol. Int. Rep. 13 (1989) 729–738.

[20] G.J. Smith, A.W. Lykke, Characterization of a neoplastic epithelial cell strain derived by dexamethasone treatment of cultured normal mouse type 2 pneumocytes, J. Pathol. 147 (1985) 165–172.

[21] K.A. Droms, B.E. Haley, A.M. Malkinson, Decreased incorporation of the photoaffinity probe 8N3-[γ-$^{32}$P]-GTP into a 45 kDa protein in lung tumors, Biochem. Biophys. Res. Commun. 144 (1987) 591–597.

[22] Y.H.L. Pan, E.O. Nuzum, L.A. Hanson, D.G. Beer, *Ki-ras* activation and expression in transformed mouse lung cell lines, Mol. Carcinog. 3 (1990) 279–286.

[23] A.M. Malkinson, Prevention of butylated hydroxytoluene-induced lung damage in mice by cedar terpene administration, Toxicol. Appl. Pharmacol. 49 (1979) 551–560.

[24] A.M. Malkinson, D.S. Beer, A single gene in BALB/cBy J mice with a major effect on susceptibility to urethane-induced pulmonary adenoma, J. Natl. Cancer Inst. 70 (1983) 981–986.

[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 100–103.

[26] C.A. Lange-Carter, J.J. Vuillequez, A.M. Malkinson, 8-Cl-Adenosine mediates 8-Cl-cAMP induced down-regulation of cAMP-dependent protein kinase in normal and neoplastic mouse lung epithelial cells by a cAMP-independent mechanism, Cancer Res. 53 (1993) 393–401.

[27] F.L. Hall, R.T. Williams, L. Wu, F. Wu, D.A. Carbonaro-Hall, J.W. Harper, D. Warburton, Two potentially oncogenic cyclins, cyclin A and cyclin D1, share common properties of subunit configuration, tyrosine phosphorylation and physical association with the Rb protein, Oncogene 8 (1993) 1377–1384.

[28] D.J. Mann, T. Higgins, N.C. Jones, E. Rozengurt, Differential control of cyclins D1 and D3 and the cdk inhibitor p27 KIP1 by diverse signaling pathways in Swiss 3T3 cells, Oncogene 14 (1997) 1759–1766.

[29] A.A. Marino, J.T. Mitchell, Lung damage in mice following intraperitoneal injection of butylated hydroxytoluene, Proc. Soc. Exp. Biol. Med. 140 (1972) 122–125.

[30] I.Y. Adamson, D.H. Bowden, M.G. Cote, H. Witschi, Lung injury induced by butylated hydroxytoluene: cytodynamic and biochemical studies in mice, Lab. Invest. 36 (1977) 26–32.

[31] H.P. Witschi, A.M. Malkinson, J.A. Thompson, Metabolism and pulmonary toxicity of butylated hydroxytoluene (BHT), Pharmacol. Ther. 42 (1989) 89–113.

[32] H.P. Witschi, W. Saheb, Stimulation of DNA synthesis in mouse lung following intraperitoneal injection of butylated hydroxytoluene, Proc. Soc. Exp. Biol. Med. 147 (1974) 690–693.

[33] L.M. Brown, A.M. Malkinson, D.E. Rannels, S.R. Rannels, Compensatory lung growth following partial pneumonectomy enhances lung tumorigenesis induced by 3-methylcholanthrene, Cancer Res. 59 (1999) 5089–5092.

[34] L.G. Thaete, D.G. Beer, A.M. Malkinson, Genetic variation in the proliferation of murine pulmonary type II cells: basal rates and alterations following urethane treatment, Cancer Res. 46 (1986) 5335–5338.

[35] L.G. Thaete, D.J. Ahnen, A.M. Malkinson, Proliferating cell nuclear antigen (PCNA/Cyclin) immunocytochemistry as a labeling index in mouse lung tissues, Cell Tissue Res. 256 (1989) 167–173.

[36] L. Koffler, S. Rushong, I.K. Park, K. Cesen-Cummings, D.C. Thompson, L.D. Dwyer-Nield, P. Rice, C. Mamay, A.M. Malkinson, R.J. Ruch, Growth inhibition in G1 and altered expression of cyclin D1 and p27 kip-1 after forced connexin expression in lung and liver carcinoma cells, J. Cell. Biochem. 79 (2000) 347–354.

[37] C.R. Herzog, S. Noh, L.E. Lantry, K.-L. Guan, M. You, *Cdkn2a* encodes functional variation of p16INK4α but not p19ARF, which confers selection in mouse lung tumorigenesis, Mol. Carcinog. 25 (1999) 92–98.

[38] J.F. Lechner, J.M. Fugaro, *RAS* and *ERBB 2*, in: H.I. Pass, J.B. Mitchell, D.H. Johnson, A.T. Turrisi, J.D. Minna (Eds.), Lung Cancer: Principles and Practice, 2nd Edition, Lippincott, Williams and Wilkins, Philadelphia, PA, 2000, pp. 89–97.

[39] S.A. Belinsky, T.R. Devereux, J.F. Foley, C.M. White, R.R. Maronpot, M.W. Anderson, The role of alveolar type 2 cells in the development and progression of pulmonary tumors in the A/J mouse, Cancer Res. 52 (1992) 3164–3173.

[40] Y. Horio, A. Chen, P. Rice, J.A. Roth, A.M. Malkinson, D.S. Schrump, Ki-ras and p53 mutations are early and late events, respectively, in urethane-induced pulmonary carcinogenesis in A/J mice, Mol. Carcinog. 17 (1996) 217–223.

[41] A.M. Malkinson, Molecular comparison of human and mouse pulmonary adenocarcinomas, Exp. Lung Res. 24 (1998) 541–555.

[42] P.L. Rice, S.E. Porter, K.M. Koski, G. Ramakrishna, A. Chen, D. Schrump, A. Kazlauskas, A.M. Malkinson, Reduced receptor expression for platelet-derived growth factor and epidermal growth factor in dividing mouse lung epithelial cells, Mol. Carcinog. 25 (1999) 285–294.

[43] W. Zumkeller, P.N. Schofield, Growth factors, cytokines and soluble forms of receptor molecules in cancer patients, Anticancer Res. 15 (1995) 343–348.

[44] C.A. Lange-Carter, T. Fossli, T. Jahnsen, A.M. Malkinson, Decreased expression of the type I isozyme of cAMP-dependent protein kinase in tumor cell lines of lung epithelial origin, J. Biol. Chem. 265 (1990) 7814–7818.

[45] E. Rozengurt, Autocrine loops, signal transduction, and cell cycle abnormalities in the molecular biology of lung cancer, Curr. Opin. Oncol. 11 (1999) 116–122.

[46] R. Alemany, S. Ruan, M. Kataoka, P.E. Koch, T. Mikhopadhyay, R. Cristiano, J.A. Roth, W.W. Zhang, Growth inhibitory effect of anti-K-ras adenovirus on lung cancer cells, Cancer Gene Ther. 3 (1996) 296–301.

[47] S.G. Swisher, J.A. Roth, J. Nemunaitis, D.D. Lawrence, B.L. Kemp, C.H. Carasco, D.G. Connors, A.K. El-Nagger, F. Fossella, B.S. Glisson, W.K. Hong, F.R. Khuri, Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer, J. Natl. Cancer Inst. 91 (1999) 763–771.