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p21$^{Waf1/Cip1}$ REGULATES PROLIFERATION AND APOPTOSIS IN AIRWAY EPITHELIAL CELLS AND ALTERNATIVE FORMS HAVE ALTERED BINDING ACTIVITIES

Renald Blundell, David J. Harrison, and Shirley O'Dea □ *Pathology Department, University of Edinburgh, Scotland*

□ *p21$^{Waf1/Cip1}$ plays central roles in proliferation, differentiation, and apoptosis. Alterations in the expression and subcellular localisation of p21 occur during several lung diseases but the roles of p21 in the lung epithelium are unknown. The effects of p21 on proliferation and apoptosis in mouse airway epithelial cells (AECs) were examined using p21-null mice. AECs isolated from p21-null mice had increased proliferation and apoptotic rates compared to AECs from wild-type mice. Alterations in the subcellular localization of the cell cycle regulatory proteins p27, PCNA, and p53 were also evident in p21$^{-/-}$ cells. The nuclear and cytoplasmic forms of p21 present in AECs were also examined. Full-length p21 (20 kDa) was detected in nuclear fractions but a C-terminal truncated form (17 kDa) of p21 was present in cytoplasmic fractions. The binding activities of truncated p21 were altered compared to full-length p21. Although the latter was complexed with PCNA, Cdk2, Cdk4, Cdk6, cyclin D3, and cyclin E, truncated p21 was bound only to Cdk4 and cyclin D3. In conclusion, p21 regulates proliferation and protects against apoptosis in AECs. In addition, different forms of p21 are present in AECs and the subcellular localization of these forms reflects differences in p21 activity.*

Keywords *airway, epithelium, lung, p21, p27, p57*

p21$^{Waf1/Cip1}$ (referred to hereafter as p21) is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs) that also includes p27^{Kip1} and p57^{Kip2}. These proteins interact directly with the cyclin/cyclin-dependent kinase (CDK) complexes that drive both the G1/S and G2/M phases of the cell cycle [1]. Initially thought to be inhibitors of cyclin/CDK complexes, it has more recently been proposed that low levels of p21 and p27 in fact facilitate cyclin D/CDK interactions in the cytoplasm and their subsequent translocation to the nucleus [2, 3]. Once in the nucleus, cyclin

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The current address of Renald Blundell is Department of Physiology and Biochemistry, University of Malta, Malta.

The current address of Shirley O'Dea is Institute of Immunology, National University of Ireland Maynooth, Ireland.

Address correspondence to Shirley O'Dea, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland. E-mail: shirley.odea@may.ie

D/CDK complexes sequester low levels of p21 and p27, allowing cyclin E/CDK complexes to remain active and permitting cell cycle progression. Only when p21 and p27 levels are sufficiently high will they interact with and inhibit cyclin E/CDK complexes also. In contrast, however, it has also been reported that cyclin D3/CDK4 complexes containing p21 or p27 are inactive both in vivo and in vitro and that neither p21 nor p27 are required for formation of cyclin D3/CDK4 complexes [4]. Thus, the precise roles of Cip/Kip proteins in cell cycle regulation remain unclear. Furthermore, the extent to which the functions of these proteins overlap and can compensate for each other is also unknown.

In addition to their interactions with cyclin/CDK complexes, Cip/Kip proteins interact with a wide range of other proteins both in the cytoplasm and in the nucleus. In the cytoplasm, p21 interacts with stress-activated protein (SAP), ASK kinases, and caspase 3 to modulate apoptosis [2]. p21 is generally considered to inhibit apoptosis [5], particularly when localized to the cytoplasm [6]. However, several reports also describe induction of apoptosis by p21, usually in the absence of functional p53 (reviewed in reference 7). Interactions between p21 and calmodulin may also be important in translocation of p21 complexes to the nucleus [8]. In the nucleus, p21 interacts directly with a number of cell cycle regulatory proteins and transcription factors including PCNA, GADD45, STAT3, c-Myc, CK2, and C/EBP-α [2]. There are fewer reports of direct interactions of p27 and p57 with proteins other than cyclin/CDK complexes. p27 is known to bind to the nuclear pore–associated protein mNPAP60 [9] and the exportin Jab1/CSN5 [10]. p57 binds to and stabilizes the muscle-specific transcription factor MyoD, suggesting an important role for p57 in lineage-specific differentiation [11]. More recently, p57 has also been found to interact directly with p53 [12]. The effects of this interaction on the functions of either protein are as yet unclear. The p21 gene appears to be more readily inducible than p27 or p57 by a wide rage of molecules, including vitamin D3, retinoic acid, interferon (IFN)-γ, epithelial growth factor (EGF), and interleukin (IL)-6 (reviewed in reference 13). p21 may therefore be the principal Cip/Kip protein responsible for regulation of responses of adult cells to local environmental cues and may exert a protective function against stress [14].

In the lung epithelium, expression of the individual Cip/Kip proteins varies with maturation stage and also in a number of disease states. p57 levels are highest during lung development, particularly in the airways [15], and subsequently decline postnatally during maturation and aging [16, 17]. p27 is expressed in quiescent adult airway and alveolar epithelial cells and its expression may be linked to differentiation stage [18]. p21 expression is widespread in developing lungs [15], but levels decline in normal adult lung cells [18]. Somewhat surprisingly, mutations in Cip/Kip

genes are rare in human tumours and only p27 has been strongly linked with lung cancer. Decreased p27 protein levels are associated with non–small cell lung cancer and poor patient outcome [19], whereas conversely, increased levels occur frequently in small cell lung cancer [20]. Interestingly, alterations in expression levels and subcellular localization of p21 have been noted in epithelial cells during several lung diseases. Elevated protein levels and nuclear localization of p21 have been demonstrated in airway epithelial cells of patients with severe asthma [21]. Cytoplasmic localization of p21 may correlate with suppression of cell proliferation in bronchial squamous cell carcinoma, whereas nuclear localization appears to coincide with high cell proliferation [22]. Increased p21 expression has also been reported in lung epithelial cells during idiopathic pulmonary fibrosis [23] and immune complex alveolitis [24]. However, despite being key regulators of the cell cycle, the roles of Cip/Kip proteins in normal and abnormal processes within the lung epithelium have not been investigated.

We have utilized p21-null mice to explore the roles of p21 in airway epithelial cells. p27-null mice display increased body size [25] and p57-null mice display severe developmental defects, increased apoptosis, and delayed differentiation [26], although no specific lung effects have been observed in either knockout animal. In contrast, p21 knockout mice appear to develop and grow normally up to about 16 months when they develop spontaneous malignancies [27, 28]. As with p27- and p57-null animals, no effects of p21 loss on lung cell function have been reported in vivo or in vitro. However, mice lacking both p21 and p57 show altered lung development with failure to form normal air sacs, although the defective cell types have not been identified, indicating that these proteins may compensate for each other in the single knockout animals [15]. Interestingly, the bronchial tree is unaffected in the double knockout animals. p21 chimeric mice also appear normal [29]. In contrast to most of the in vivo studies, the effects of p21 loss are more readily apparent in in vitro studies. Cultured murine embryonic fibroblasts from $p21^{-/-}$ mice have been found to be impaired in their ability to arrest in G1 in response to DNA damage and at later passages achieve higher saturation densities than wild type control cells [27]. We have found that cultured $p21^{-/-}$ mouse airway epithelial cells (AECs) have increased proliferation and apoptotic rates compared to wild-type AECs, demonstrating that p21 plays a central role in these processes in lung epithelial cells and that these activities cannot be compensated for by other Cip/Kip proteins. We have also examined the significance of the subcellular localization of p21 in AECs and identified a novel truncated form of mouse p21 in cytoplasmic fractions. This truncated p21 protein has modified binding activity compared to full-length mouse p21.

MATERIALS AND METHODS

Animals and Cell Culture

p21-null mice were kindly provided by Philip Leder, Harvard Medical School, Boston, USA. C57 black 6 mice were used as control animals. AECs were isolated as described previously [30], seeded onto multiwell slides precoated with 50 ng/mL fibronectin and cultured in Dulbecco modified Eagle medium (DMEM):Hams F12 medium (1:1) supplemented with 5% fetal calf serum and 2 mML-glutamine at 37°C, 5% CO_2. Because AECs are isolated and seeded in clumps, counting of cells is not feasible. In order to consistently seed wells at equal densities, an aliquot of cells was taken prior to seeding and absorbance reading was obtained using the reagent from the CellTiter Assay used in the MTS ((3-(4-5-dimethylthinzol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) proliferation assay described below. Cell suspensions were diluted and seeded accordingly.

Histology

Formalin-fixed, paraffin-embedded tissue sections were dewaxed in xylene, rehydrated, and stained with hematoxylin and eosin.

Immunofluorescence

Formalin-fixed, paraffin-embedded tissue sections were dewaxed and rehydrated. Cultured cells were fixed in prechilled methanol for 5 minutes at − 20°C. Primary antibodies were incubated for 2 hours at room temperature. Antibodies used were as follows: p21 (DAKO, clone SX118), p27 (Sigma, clone DCS-72), p57 (Santa Cruz, clone M30), PCNA (Sigma, clone PC10), and p53 (Vector Laboratories, clone CM5p). Tissue sections were then incubated with biotinylated secondary antibodies (DAKO) followed by StreptABcomplex (DAKO) and stained with Vector Red substrate (Vector Laboratories). Cultured cells were incubated with appropriate Alexa Fluor fluorescent secondary antibodies (Molecular Probes) and counterstained with DAPI nuclear stain.

MTS Proliferation Assay

CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) colorimetric assay was used according to manufacturer's instructions to determine cell proliferation rates. Briefly, 20 µL reagent was added to each test well containing 100 µL fresh culture medium. Cells were incubated for 1 hour at 37°C and supernatant absorbance at 490 nm was determined.

BrdU Incorporation Assay

At days 1, 3, or 5 of culture, cells were incubated with 10 μM BrdU for 6 hours, rinsed with phosphate-buffered saline (PBS), and fixed with 70% ethanol at 4°C for 1 hour. Cells were then rinsed with PBS and incubated with 5 M HCl for 45 minutes followed by 1% hydrogen peroxide for 10 minutes. Cells were then incubated with rat anti-BrdU-HRP (horseradish peroxidase) primary antibody (Oxford Biotech) and binding was visualized using Diaminobenzidine (DAB) solution. An average of 1000 cells were counted using a HOME microscope.

Feulgen Stain for Apoptosis

Cells were fixed in Bouins fixative (17:1:2 methanol:glacial acetic acid:40% formalin) for 12 hours at 4°C, incubated in 5 M HCl for 45 minutes, rinsed with H_2O, and incubated in Schiff's reagent for 1 hour. Stain was developed by rinsing with H_2O and cells were then counterstained with Light Green. Apoptotic nuclei were counted using a HOME microscope.

Protein Extracts

Cells were lysed in RIPA buffer for total protein extracts. Lysates were centrifuged at 15,000 rpm for 10 minutes at 4°C and supernatants were collected. For cytoplasmic extracts, 1×10^6 cells were lysed in 100 μL cytoplasmic lysis buffer (50 mM sodium fluoride, 5 mM tetra sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glyceropyrophosphate, 0.5% NP-40, 2 mM EDTA, 20 mM disodium hydrogen phosphate, 20 mM monosodium dihydrogen phosphate, and protease inhibitor (1 cocktail tablet [Roche] per 10 mL). Lysates were centrifuged at 6500 rpm, 4°C for 10 minutes and the supernatants (cytoplasmic extracts) were collected. 100 μl nuclear lysis buffer (as for cytoplasmic buffer plus 0.3 M sodium chloride) was added to the remaining cell pellets, which were then incubated on ice for 10 minutes. The resulting lysate was centrifuged at 15,000 rpm, 4°C for 10 minutes, and the supernatant (nuclear extract) was collected.

Immunoprecipitation

Lysates were immunoprecipitated using an 'IMMUNOcatcher' kit (CytoSignal) according to the manufacturer's instructions. Primary antibodies specific for PCNA (clone PC10, Sigma), Cdk2 (polyclonal, Sigma), Cdk4 (clone DCS-31, Sigma), Cdk6 (clone DCS-90, Sigma), cyclin D3 (clone DCS-22, Sigma), and cyclin E (polyclonal, Sigma) were incubated with cell lysates and complexes were precipitated using protein A/G resin.

Western Blotting

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Blots were incubated with primary antibodies directed against either the C-terminal end of human p21 but which also recognizes mouse p21 (C-19, Santa Cruz) or full-length mouse p21 (F-5, Santa Cruz) followed by HRP-labeled secondary antibody. Chemiluminescence detection was then carried out.

RESULTS

Histological Analysis of $p21^{-/-}$ Lungs

The gross morphology of $p21^{-/-}$ lungs was similar to that of wild-type lungs. However, a slight flattening of the epithelial cells lining both large and small airways of $p21^{-/-}$ mice compared to those of wild-type airways was apparent (Figure 1). This indicates that p21 may play a role in the differentiation of airway epithelial cells.

Expression of Cip/Kip Proteins in Airways of $p21^{-/-}$ and Wild-Type Mice

To determine whether compensatory up-regulation of p27 and p57 occurs in $p21^{-/-}$ airways in the absence of p21, lung sections were immunostained for Cip/Kip proteins (Figure 2). p21 was confirmed absent in

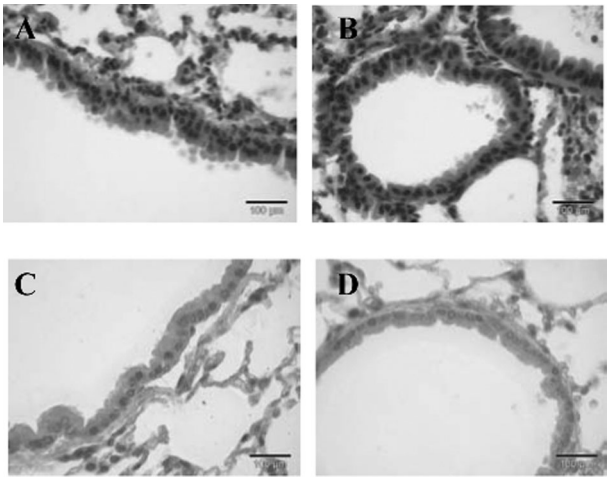

FIGURE 1 Histology of wild-type (*A*) large and (*B*) small airways and $p21^{-/-}$ (*C*) large and (*D*) small airways. H&E staining shows airway epithelial cells are slightly flattened in $p21^{-/-}$ lungs compared with wild-type.

$p21^{-/-}$ lung tissue whereas in wild-type lungs, p21 was localized mainly to the cytoplasm of airway cells, with very occasional nuclear localization evident. Strong cytoplasmic immunostaining for p27 was observed in wild-type airways with a similar pattern occurring in $p21^{-/-}$ lungs. Immunostaining for p57 was weaker than that for p27 but again, a similar cytoplasmic staining pattern was present in both wild-type and $p21^{-/-}$ lungs, suggesting that compensatory up-regulation of p27 and p57 protein expression does not occur in $p21^{-/-}$ airway cells. However, it remains possible that the

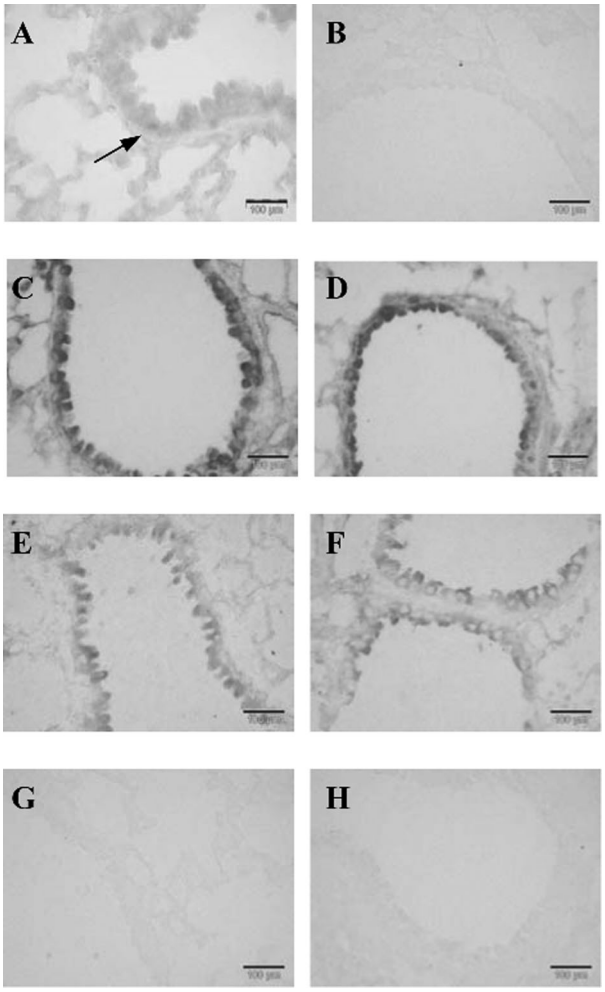

FIGURE 2 Immunostaining of wild-type and $p21^{-/-}$ airways for Cip/Kip proteins. (*A*) Positive cytoplasmic and nuclear (*arrow*) immunostaining for p21 in wild-type airways. (*B*) Negative immunostaining for p21 in $p21^{-/-}$ airways. p27 immunostaining in (*C*) wild-type and $p21^{-/-}$ (*D*) airways. p57 immunostaining in (*E*) wild-type and (*F*) $p21^{-/-}$ airways. (*G*) Isotype control for p21 and p27. (*H*) Isotype control for p57.

activities of p27 and p57 are altered in $p21^{-/-}$ animals at a level other than up-regulation of protein expression or alternatively that proteins other than the Cip/Kip family can compensate for loss of p21 activity.

Comparison of Proliferation Rates in Wild-Type and $p21^{-/-}$ Cultured AECs

Findings from previous studies suggest that under normal conditions, $p21^{-/-}$ animals retain a normal phenotype. The effects of p21 loss only become apparent when $p21^{-/-}$ cells are subjected to stress such as in vitro culture or DNA damage [27], or older ages in the case of $p21^{-/-}$ animals [28]. In these circumstances, information about the specific activities of p21 can be obtained. Therefore, in order to more closely examine the role of p21 in airways, AECs were isolated and cultured. Proliferation assays were carried out to compare proliferation rates between wild-type and $p21^{-/-}$ cells in vitro. In the MTS assay, a tetrazolium compound is metabolically reduced by dehydrogenase enzymes in metabolically active cells. AECs isolated from wild-type mice increased approximately 5-fold over the 5 days of culture. In contrast, cells isolated from $p21^{-/-}$ cells increased approximately 17-fold during the same culture period (Figure 3*A*). An alternative

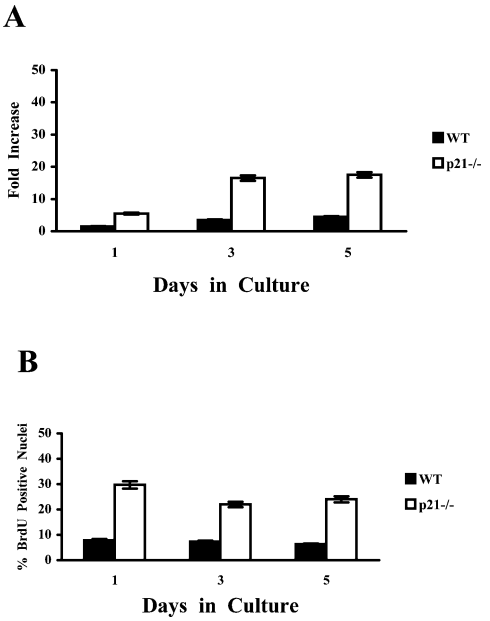

FIGURE 3 $p21^{-/-}$ proliferation assays. (*A*) MTS proliferation assay was used to determine the fold increase in cells over 5 days of culture. (*B*) BrdU incorporation assay. Percentage BrdU positive nuclei were counted over 5 days of culture.

proliferation assay, BrdU incorporation, was also carried out to ensure that potential metabolic differences between wild-type and $p21^{-/-}$ cells did not account for the differences seen in the MTS assay. Similar results were obtained with the BrdU assay. Approximately 5% to 10% wild-type cells were in S-phase of the cell cycle at each time point, as determined by BrdU-positive nuclei, wereas 20% to 30% $p21^{-/-}$ cells were in S phase at equivalent time points during culture (Figure 3*B*). These results indicate that p21 plays a key regulatory role in the cell cycle of AECs and demonstrates that p27 and p57 cannot replace these specific functions.

Apoptosis in Cultured AECs

p21 plays a central role in regulation of apoptosis, although its effects appear to vary with cell type. It was found that apoptosis rates in $p21^{-/-}$ AEC cultures were approximately twice those of wild-type AEC cultures (Figure 4). Therefore, p21 appears to play a role in the protection of airway epithelial cells against apoptosis and this function cannot be replicated by p27 or p57 in these cells.

Subcellular Localization of p27, PCNA, and p53 in $p21^{-/-}$ AECs

Further investigations into the regulatory role of p21 in cell cycle progression and apoptosis were carried out by comparing the subcellular localization of key proteins involved in these processes in wild-type and $p21^{-/-}$ cells. The incidence of both cytoplasmic and nuclear p27 was increased in the absence of p21 during culture (Figure 5*A*). In the case of PCNA, cytoplasmic and nuclear levels were similar in wild-type and $p21^{-/-}$ cells at days 1 and 3 of culture but remained elevated at culture day 5 in $p21^{-/-}$ cells, whereas they became reduced in wild-type cells (Figure 5*B*). Similarly, both cytoplasmic and nuclear p53 remained elevated in $p21^{-/-}$ cells at day 5 of culture compared to wild-type cells (Figure 5*C*). p21 therefore influences the subcellular localization of p27, PCNA, and p53 and this presumably

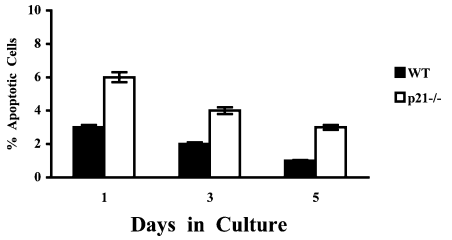

FIGURE 4 Apoptosis in $p21^{-/-}$ cells. Feulgen staining was used to determine the percentage of apoptotic cells present in cultured cells.

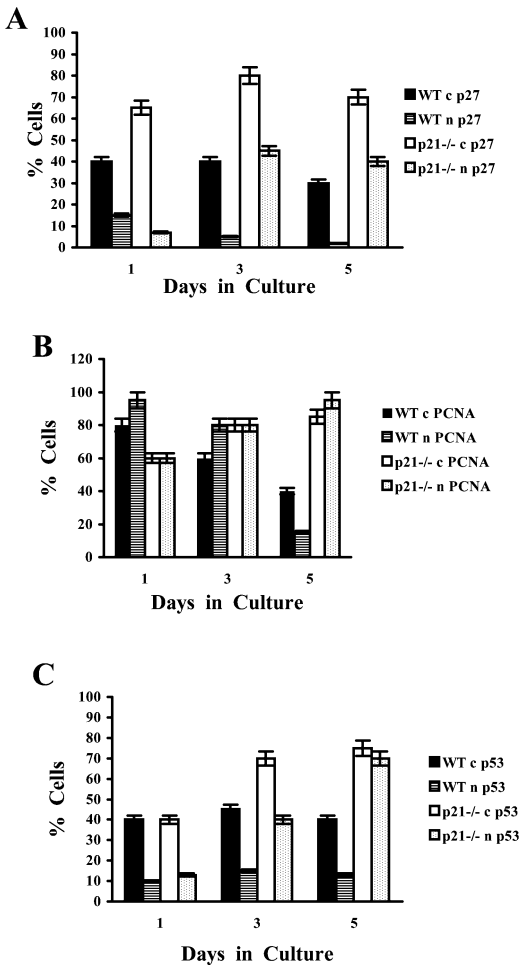

FIGURE 5 Subcellular localization of (*A*) p27, (*B*) PCNA, and (*C*) p53 in wild-type and $p21^{-/-}$ cells over 5 days of culture.

reflects altered activity of these proteins. Alterations in the activities of PCNA and p53 in the absence of p21 and the sustained levels of expression of these proteins in $p21^{-/-}$ cells at day 5 of culture are likely to be responsible, at least in part, for the increased rates of proliferation and apoptosis observed in $p21^{-/-}$ AECs.

Subcellular Localization of p21 in AECs

The above studies demonstrate that p21 plays key roles in airway cell proliferation and apoptosis and that these activities are not fully compensated for by other Cip/Kip family members in cultured AECs. In order to

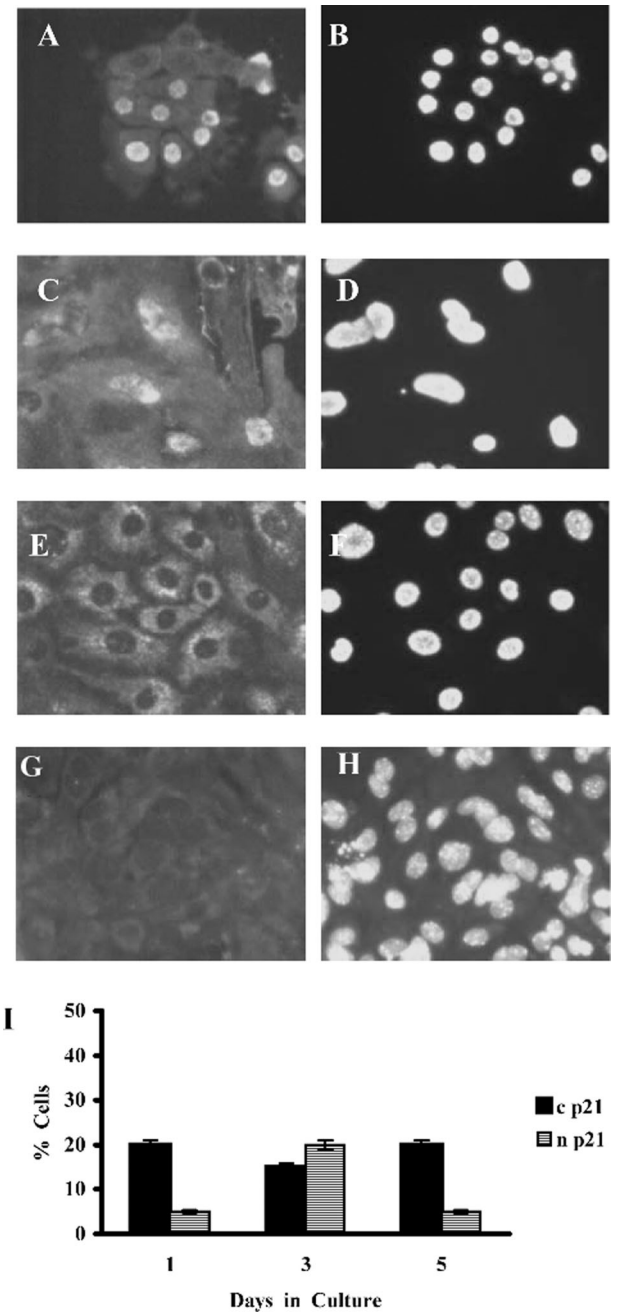

FIGURE 6 Subcellular localization of p21 during culture. Nuclear and cytoplasmic localization of p21 in cultured primary AECs at (*A*) day 1, (*C*) day 3, and (*E*) day 5. (*G*) Isotype control. Panels (*B, D, F, H*) show respective DAPI counterstains. × 63 magnification. (*I*) Percentage of cells with cytoplasmic or nuclear localization of p21.

more closely examine the roles of p21 in these processes, the fluctuations in the subcellular localization of p21 in wild-type AECs were examined over time in culture (Figure 6). At culture day 1, p21 was localized predominantly to the cytoplasm, with approximately 5% cells displaying nuclear localization. There was an increase in nuclear p21 to 20% on day 3, with a slight reduction in cytoplasmic p21. On day 5, the localization was similar to that of day 1. At all time points, a proportion of cells appeared to contain either cytoplasmic or nuclear p21, whereas a subpopulation appeared to contain both forms. There is no clear correlation between the subcellular localization of p21 at each time point and the other parameters examined in wild-type cells, including proliferation, apoptosis, and subcellular localization of p27, PCNA, and p53.

Comparison of Cytoplasmic and Nuclear p21 Proteins in AECs

In order to further examine the role of p21 in cellular processes and to determine the relevance of the alternative subcellular localisations of p21 in AECs, the cytoplasmic and nuclear forms of p21 in cultured AECs were compared by Western blotting. Cytoplasmic and nuclear protein extracts were prepared from AECs after 3 days in culture. The extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blots were probed using an antibody raised against full-length p21. A 20-kDa protein corresponding to full-length p21 [23] was detected in the nuclear extracts whereas a 17-kDa protein was detected in cytoplasmic extracts (Figure 7*A*). Total protein extracts from AECs after 3 days in culture were then immunoprecipitated with an antibody raised against the C-terminus of p21. Only the 20-kDa band was detected with this antibody, indicating that the 17-kDa band represents a cleaved form of p21 that is truncated at its C-terminal end (Figure 7*B*).

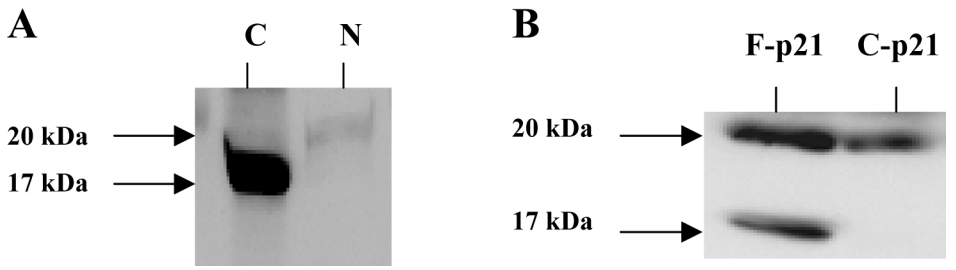

FIGURE 7 (*A*) Cytoplasmic and nuclear protein extracts from AECs at day 3 of culture were blotted against an antibody raised against full-length p21 (F-p21). Cytoplasmic p21 was a 17-kDa protein whereas nuclear p21 was a 20-kDa protein. (*B*) Total protein extracts from AECs at day 3 of culture were probed with either F-p21 or an antibody raised against the C-terminal of p21 (C-p21). The 20-kDa and 17-kDa forms of p21 were detected with F-p21. Only the 20-kDa protein was observed with C-p21. Molecular weights were determined by comparison with molecular weight standards run simultaneously.

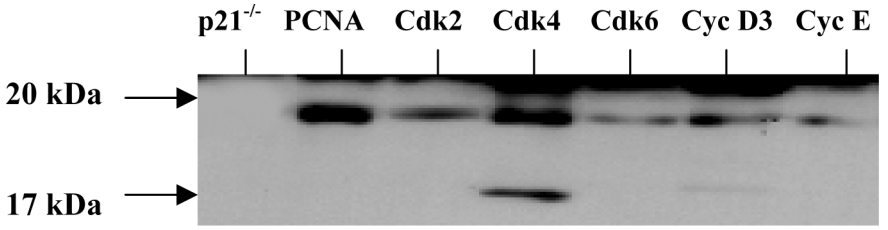

FIGURE 8 Total protein extracts from AECs at day 3 of culture were immunoprecipitated using number of antibodies directed against cell cycle–related proteins, blotted, and then probed using F-p21. Full-length p21 was complexed with PCNA, Cdk2, Cdk4, Cdk6, cyclin D3, and cyclin E, whereas truncated p21 was complexed with Cdk4 and cyclin D3.

Complexes of Cytoplasmic and Nuclear p21$^{Waf1/Cip1}$

In order to examine the activities of the full-length and truncated forms of p21 proteins present in AECs, immunoprecipitations were carried out to identify what cell cycle regulatory proteins, if any, were bound to each form of p21. Total cell extracts from AECs after 3 days in culture were immunoprecipitated with antibodies directed against PCNA, Cdk2, Cdk4, Cdk6, cyclin D3, and cyclin E. The immunoprecipitated proteins were run on SDS-PAGE gels, blotted, and probed using the antibody raised against the full-length p21 protein. AECs cultured from p21-null mice were used as a negative control. Full-length p21 was found to form complexes with PCNA, Cdk2, Cdk4, Cdk6, cyclin D3, and cyclin E in AECs whereas the truncated form of p21 interacted only with Cdk4 and cyclin D3 (Figure 8).

DISCUSSION

Although p21 plays a central role in regulation of cell proliferation, differentiation, and apoptosis, the mechanisms by which p21 regulates these processes are unclear. The activities of p21 appear to vary depending on a number of factors, including cell type, subcellular localization, level of expression, and post-translational modifications. In this study, p21-null mice were used to examine the role of p21 in AECs. Despite its key role in cell fate decisions, the only reported consequence of p21 deletion in vivo, in otherwise normal healthy animals, is an increase in the rate of spontaneous malignancies in older animals [28]. This suggests that p21 activities are largely redundant. However, p21-specific effects become apparent when p21-deficient animals and cells are subjected to various kinds of stress. In a mouse model of chronic renal failure induced by partial renal ablation, p21-null animals exhibit a more hyperplastic reaction that their wild-type counterparts, with up-regulation of PCNA in renal cells, resulting in the failure to progress to end-stage renal disease [31]. Similarly, when cells are isolated

from $p21^{-/-}$ animals and cultured in vitro, effects of p21 loss on cell cycle progression and apoptosis become apparent [27]. These p21 knockout studies confirm the suggestion that p21 exerts a protective function against stress [14].

The roles of p21 in lung cells were examined in this study. A slight alteration in the morphology of airway epithelial cells in $p21^{-/-}$ mice towards a more flattened appearance was the only observable effect of p21 loss in vivo. p21 and p27 have been associated with differentiation of a number of cell types, including keratinocytes [32] and muscle cells [33]. It is possible that p21 plays a minor role in regulation of differentiation of healthy lung epithelial cells or else that p27 and p57, or other proteins, are capable of substituting to a large degree for more significant p21-related differentiation effects. Although up-regulation of p27 and p57 was not observed in lung tissue in the absence of p21, changes in their activities at the subcellular level may occur to achieve these effects. Alternatively, if the primary role of p21 is during times of stress, effects of its loss, such as up-regulation of p27 and p57, may only be apparent in injured lungs and may not be evident in uninjured lungs as examined here. In support of this, levels of p27 were raised in $p21^{-/-}$ cultured AECs indicating firstly, that a major role for p21 in AECs is to respond to stress and secondly, that p27 may indeed substitute for the loss of p21 under stressful circumstances. Animal model studies of lung injury and disease using p21-null mice would allow further dissection of the activities of the Cip/Kip proteins in vivo.

A closer examination of the effects of p21 loss was possible using cultured AECs isolated from $p21^{-/-}$ mice. In culture, $p21^{-/-}$ cells exhibit increased rates of proliferation and apoptosis, demonstrating that p21 regulates the rate of proliferation of AECs and also protects against apoptosis. Sustained high levels of PCNA in $p21^{-/-}$ cultured cells indicate that regulation of cell cycle regulatory proteins is altered in the absence of p21. The greatest difference in PCNA expression levels was seen at day 5 of culture, suggesting that other cell cycle regulatory proteins are also involved in the induction of the high proliferation rates observed at the earlier time points. Increased levels of p53 expression are also likely to be involved in the increased rates of apoptosis in $p21^{-/-}$ cells. The effects of p21 loss on proliferation and apoptosis demonstrate that p27 and p57 cannot fully compensate for p21 in these cells, despite the increase in p27 protein expression.

No correlation was found between the subcellular localization of p21 in wild-type cells and proliferation, apoptosis, and expression and subcellular localization of p27, PCNA, and p53. The signaling pathways involved in these processes are complex and are influenced by a wide range of factors. Additional experiments will be required to dissect the roles of individual proteins such as p21. In order to more closely examine the functions of p21 in AECs, the binding activities of cytoplasmic and nuclear p21 were determined and

two distinct forms of p21 were identified. We are not aware of other reports describing truncated mouse p21. Multiple forms of p21 have been reported in human cells [34–42]. Alternative splice variants and post-translational modifications such as hypophosphorylation and proteolytic cleavage produce forms of p21 with varying sizes and biological activities, although most studies have been carried out using cell lines rather than normal cells. In agreement with several of the human studies, the 17-kDa form of p21 present in normal AECs is truncated at the C-terminal end and is localized to the cytoplasm. As expected, we found that full-length mouse p21 forms complexes with PCNA, Cdk2, Cdk4, Cdk6, cyclin D3, and cyclin E in AECs. In contrast, truncated mouse p21 only binds cyclin D3 and Cdk4. Such complexes have not previously been described in either mouse or human cells. Poon and Hunter showed that a 19-kDa C-terminal truncated form of p21 could be induced in normal fibroblasts following exposure to high-dose ultraviolet (UV) irradiation [39]. The truncated protein also accounted for the majority of p21 protein in several untreated human carcinoma cell lines. The 19-kDa p21 was detected in both nuclear and cytoplasmic fractions, but a higher proportion was present in the cytoplasmic fraction and unlike full-length p21, was not complexed to Cdk2 or PCNA. Caspase-mediated proteolysis has been directly implicated in the production of p21 fragments of varying sizes. A 16-kDa fragment of p21 was detected in a tumor necrosis factor (TNF)-sensitive human cervical carcinoma cell line during apoptosis induced by TNF treatment but localization and activity of the fragment were not reported [40]. p53-dependent production of a 14-kDa p21 fragment occurred in human myeoblastic leukemic cells but not in normal human lung fibroblasts in response to γ-irradiation [35]. The 14-kDa fragment was associated with cyclin A and Cdk2, but did not complex with PCNA. Two reports describe truncated p21 in the A549 human lung carcinoma cell line. The 20-kDa C-terminal truncated form of p21 observed by Tchou and coworkers appeared during phorbol ester–induced G_2/M arrest but its subcellular localization was not reported [38]. Cytoplasmic localization of a caspase-cleaved 15-kDa p21 fragment was also reported [36]. This cleaved form of p21 failed to arrest cells in G_1 phase and failed to bind to PCNA but did bind Cdk2. We are currently investigating if the 17-kDa form of p21 described here in AECs occurs as a result of caspase cleavage and how its appearance correlates with phase of the cell cycle.

This is also the first report of truncated p21 interacting with either cyclin D3 or Cdk4. The demonstration that cyclin D3/Cdk4 complexes can form and become active in the absence of both p21 and p27 suggests that the primary role of p21 in cyclin/Cdk function is as regulator of subcellular localization and activity rather than facilitator of assembly [43]. Our results support this hypothesis. Cleavage of the nuclear localization signal which is also located at the C terminal end of p21 would prevent translocation

of cyclin D3/Cdk4 complexes to the nucleus and their subsequent initiation of cell cycle progression. This may be an important point of cell cycle regulation in epithelial cells that are exposed to conflicting environmental signals such as mitogenic stimuli and lack of substrate attachment. The D-type cyclins (D1, D2, and D3), which are up-regulated in response to mitogenic signals, are rate-limiting controllers of G_1 phase progression [44]. Individual D-type cyclins appear to be expressed within cells in varying proportions in a lineage-specific manner but the functional significance of each type is not clear. The role of CKI/cyclin D/Cdk4 and -Cdk6 complexes in regulation of cell cycle progression is controversial. Once thought to be inactive, it was later proposed that p21/cyclin D/Cdk complexes are in fact active in the presence of low levels of p21 and are only inhibited when p21 levels are high [45]. More recently, it has been suggested that cyclin D/Cdk4 and cyclin D/Cdk6 complexes sequester CKIs in the nucleus, thus permitting cyclin E/Cdk2 complexes to drive the cell cycle [1]. Only when CKI levels become high enough will they bind to and inhibit cyclin E/cdk2 complexes and prevent cell cycle progression. The findings here suggest that modifications of p21 in lung epithelial cells result in retention of p21 and some of its binding partners to the cytoplasm, thus modulating activity of cell cycle regulatory proteins such as cyclin D3 and Cdk4. Dysregulation of this mechanism in airway diseases such as asthma and cancer where there is an increased localization of p21 to the nucleus may reflect the increased proliferation of AECs in these conditions.

In summary, p21 regulates AEC proliferation and protects against apoptosis. Alternative forms of p21 in the cytoplasm and nuclei of AECs have altered binding activities, which probably reflect differences in the functions of p21 in these subcellular compartments.

REFERENCES

1. Sherr CJ, Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999;13:1501–1512.
2. Dotto GP: $p21^{Waf1/Cip1}$: more than a break to the cell cycle? Biochim Biophys Acta. 2000;1471: M43–M56.
3. Coqueret O: New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 2003;13:65–70.
4. Bagui TK, Jackson RJ, Agrawal D, Pledger WJ: Analysis of cyclin D3-cdk4 complexes in fibroblasts expressing and lacking p27(kip1) and p21(cip1). Mol Cell Biol. 2000;20:8748–8757.
5. Gorospe M, Wang X, Guyton KZ, Holbrook NJ: Protective role of p21(Waf1/Cip1) against prostaglandin A2-mediated apoptosis of human colorectal carcinoma cells. Mol Cell Biol. 1996;16:6654–6660.
6. Asada M, Yamada T, Ichijo H, Delia D, Miyazono K, Fukumuro K, Mizutani S: Apoptosis inhibitory activity of cytoplasmic p21(Cip1/Waf1) in monocytic differentiation. EMBO J. 1999;18:1223–1234.
7. Gartel AL, Tyner AL: The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther. 2002;1:639–649.
8. Taules M, Rodriguez-Vilarrupla A, Rius E, Estanyol JM, Casanovas O, Sacks DB, Perez-paya E, Bachs O, Angell N: Calmodulin binds to $p21^{Cip1}$ and is involved in the regulation of its nuclear localization. J Biol Chem. 1999;274:24445–24448.

9. Muller D, Thieke K, Burgin A, Dickmanns A, Eilers M: Cyclin E-mediated elimination of p27 requires its interaction with the nuclear pore-associated protein mNPAP60. EMBO J. 2000;19:2168–2180.
10. Tomoda K, Kubota Y, Arata Y, Mori S, Maeda M, Tanaka T, Yoshida M, Yoneda-Kato N, Kato JY: The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex. J Biol Chem. 2002;277:2302–2310.
11. Reynaud EG, Leibovitch MP, Tintignac LAJ, Pelpel K, Guillier M, Leibovitch SA: Stabilization of MyoD by direct binding to $p57^{Kip2}$. J Biol Chem. 2000;275:18767–18776.
12. Kikuchi J, Furukawa Y, Hayashi N: Identification of novel p53-binding proteins by biomolecular interaction analysis combined with tandem mass spectrometry. Mol Biotechnol. 2003;23:203–212.
13. Gartel AL, Tyner AL: Transcriptional regulation of the $p21^{(Waf1/Cip1)}$ gene. Exp Cell Res. 1999;246:280–289.
14. Gorospe M, Wang X, Holbrook NJ: Functional role of p21 during the cellular response to stress. Gene Expr. 1999;7:377–385.
15. Zhang P, Wong C, Liu D, Finegold M, Harper WJ, Elledge SJ: $p21^{Cip1}$ and $p57^{Kip2}$ control muscle differentiation at the myogenin step. Genes Dev. 1999;13:213–224.
16. Westbury J, Watkins M, Ferguson-Smith AC, Smith J: Dynamic temporal and spatial regulation of the cdk inhibitor $p57^{kip2}$ during embryo morphogenesis. Mech Dev. 2001;109:83–89.
17. Park CW, Chung JH: Age-dependent changes of $p57^{Kip2}$ and $p21^{Cip1/Waf1}$ expression in skeletal muscle and lung of mice. Biochim Biophys Acta. 2001;1520:163–168.
18. Shoji M, Dobashi Y, Morinaga S, Jiang SX, Kameya T: Tumor extension and cell proliferation in adenocarcinomas of the lung. Am J Pathol. 1999;154:909–918.
19. Tsukamoto S, Sugio K, Sakada T, Ushijima C, Yamazaki K, Sugimachi K: Reduced expression of cell-cycle regulator $p27^{Kip1}$ correlates with a shortened survival in non-small cell lung cancer. Lung Cancer. 2001;34:83–90.
20. Masuda A, Osada H, Yatabe Y, Kozaki K, Tatematsu Y, Takahashi T, Hida T, Takahashi T, Takahashi T: Protective function of $p27^{Kip1}$ against apoptosis in small cell lung cancer cells in unfavourable environments. Am J Pathol. 2001;158:87–96.
21. Puddicombe SM, Torres-Lozano C, Richter A, Bucchieri F, Lordan JL, Howarth PH, Vrugt B, Albers R, Djukanovic R, Holgate ST, Wilson SJ, Davies DE: Increased expression of p21(waf) cyclin-dependent kinase inhibitor in asthmatic bronchial epithelium. Am J Respir Cell Mol Biol. 2003;28:61–68.
22. Hirano T, Kakihana M, Tsuji K, Shibanuma H, Ohira T, Ikeda N, Kawate N, Konaka C, Ebihara Y, Kato H: The relationship between p21/waf1 expression patterns and cell proliferation during the tumorigenesis of the bronchus. Int J Oncol. 1999;15:253–258.
23. Kuwano K, Kunitake R, Kawasaki M, Nomoto Y, Hagimoto N, Nakanishi Y, Hara N: P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1996;154(2 Pt 1):477–483.
24. Kuwano K, Hagimoto N, Nomoto Y, Kawasaki M, Kunitake R, Fujita M, Miyazaki H, Hara N: P53 and p21 (Waf1/Cip1) mRNA expression associated with DNA damage and repair in acute immune complex alveolitis in mice. Lab Invest. 1997;76:161–169.
25. Yan Y, Frisen J, Lee MH, Massague J, Barbacid M: Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. Genes Dev. 1997;11:973–983.
26. Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K: Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell. 1996;85:707–720.
27. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P: Mice lacking $p21^{Cip1/Waf1}$ undergo normal development, but are defective in G1 checkpoint control. Cell. 1995;82:675–684.
28. Martin-Caballero J, Flores JM, Garcia-Palencia P, Serrano M: Tumor susceptibility of $p21^{Waf1/Cip1}$-deficient mice. Cancer Res. 2001;61:6234–6238.
29. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ: Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature. 1995;377:552–557.
30. McBride S, Tatrai E, Blundell R, Kovacikova Z, Cardozo L, Adamis Z, Smith T, Harrison D: Characterisation of lectin binding patterns of mouse bronchiolar and rat alveolar epithelial cells in culture. Histochem J. 2000;32:33–40.

31. Megyesi J, Price PM, Tamayo E, Safirstein RL: The lack of a functional $p21^{Waf1/Cip1}$ gene ameliorates progression to chronic renal failure. Proc Natl Acad Sci USA. 1999;96:10830–10835.
32. Harvat BL, Wang A, Seth P, Jetten AM: Up-regulation of $p27^{Kip1}$, $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$ is associated with, but not sufficient for, induction of squamous differentiation. J Cell Sci. 1998;111: 1185–1196.
33. Wei Q, Paterson BM: Regulation of MyoD function in the dividing myoblast. FEBS Lett. 2001;490:171–178.
34. Esposito F, Cuccovillo F, Russo L, Casella F, Russo T, Cimino F: A new $p21^{Waf1/Cip1}$ isoform is an early event of cell response to oxidative stress. Cell Death Differ. 1998;5:940–945.
35. Gervais JLM, Sethi P, Zhang H: Cleavage of CDK inhibitor $p21^{Cip1/Waf1}$ by caspases is an early event during DNA damage-induced apoptosis. J Biol. Chem. 2000;273:19207–19212.
36. Zhang Y, Fujita N, Tsuruo T: Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. Oncogene. 1999:18:1131–1138.
37. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 1993; 75:805–816.
38. Tchou, W, Rom WN, Tchou-Wong, K: Novel form of $p21^{WAF1/CIP1/SDI1}$protein in phorbol ester-induced G_2/M arrest. J Biol Chem. 1996;271:29556–29560.
39. Poon RYC, Hunter T: Expression of a novel form of $p21^{Cip1/Waf1}$ in UV-irradiated and transformed cells. Oncogene. 1998;16:1333–1343.
40. Donato NJ, Perez M: Tumor necrosis factor-induced apoptosis stimulates p53 accumulation and p21WAF1 proteolysis in ME-180 cells. J Biol Chem. 1998;273:5067–5072.
41. Chai F, Truong-Tran AQ, Evdokiou A, Young GP, Zalewski PD: Intracellular zinc depletion induces caspase activation and p21 Waf1/Cip1 cleavage in human epithelial cell lines. J Infect Dis. 2000;182 (Suppl 1):S85–S92.
42. Jin YH, Yoo KJ, Lee YH, Lee SK: Caspase 3-mediated cleavage of p21 Waf1/Cip1 associated with the cyclin A-cyclin-dependent kinase 2 complex is a prerequisite for apoptosis in SK-HEP-1 cells. J Biol Chem. 2000;275:30256–30263.
43. Bagui TK, Jackson RJ, Agrawal D, Pledger WJ: Analysis of cyclin D3-cdk4 complexes in fibroblasts expressing and lacking p27(kip1) and p21(cip1). Mol Cell Biol. 2000;20:8748–8757.
44. Sherr CJ: D-type cyclins. Trends Biochem Sci. 1995;20:187–190.
45. LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E: New functional activities for the p21 family of CDK inhibitors. Genes Dev. 1997;11:847–862.