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Effect of p53 Gene Transfer on the Cell Proliferation and Cell Cycle Progression in a Human Oral Cancer Cell Line with p53 Mutation

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To investigate the effect of p53 gene transfer on the cell proliferation and cell cycle progression in an oral cancer cell line containing p53 mutation, we introduced a recombinant plasmid (pMSG-p53X) encoding wt p53 into SCC-9 line. A stable clonal cell line, SCC-9-p53X, was derived that conditionally expressed wt p53 protein following exposure to dexamethasone. Induction of wt p53 expression may play an important role in the suppression of tumorigenic phenotypes in cancer cells with p53 mutations by decreased expression and/or activities of key G_1-phase cell cycle regulatory proteins. To investigate this possibility, we determined the change of tumorigenic phenotype, the cellular levels of key G_1-phase cell cycle regulatory proteins $p21^{WAF1/CIP1}$, p16, p27, cyclins (D1 and E), cdks (cdk2, cdk4 and cdk6) and PCNA proteins, and the activities of cdks in the SCC-9-p53X cells before and after exposure to dexamethasone. Dexamethasone exposure in the SCC-9-p53X cells caused (i) a significant decrease in the cell proliferation, level of the DNA replication protein, PCNA, and anchorage-independent growth, (ii) an inhibition in the activities of cdk2, cdk4, and cdk6 kinases, and (iii) a decrease in the levels of cdk2 and cdk6 proteins. However, dexamethasone failed to induce these changes in the nontransfected SCC-9 cells. These results demonstrate that in human cancer cells containing p53 mutation, the levels of cdk proteins and their kinase activities of the G_1 phase are notably reduced by expression of wt p53 gene thereby making them to repress of tumorigenic phenotype.

Key words : p53 mutation, p53 gene-transfer, tumorigenic phenotype, cell cycle regulators, oral squamous cell carcinoma cell line

Introduction

The gene for the nuclear phosphoprotein p53 is the most commonly mutated gene known in human cancers (Greenblatt *et al.*, 1994; Lane, 1994). In fact, p53 mutations are found in epithelial, mesenchymal, haemopoietic and lymphoid neoplasms and in tumors of the central nervous system (Stratton, 1996). Wild-type (wt) p53 acts as a transcription factor, but may perform other biochemical functions as well and may regulate multiple cellular processes, including cell cycle progression. DNA repair, apoptosis, and differentiation (Ko and Prives, 1996). Certain kinds of cellular stress, such as DNA damage and hypoxia, cause increased p53 protein levels through protein stabilization and initiation of p53-dependent biological response pathways. p53 orchestrates these responses by activating transcription of target genes containing specific DNA binding sites for p53.

Eukaryotic cells have evolved to respond to a large array of positive and negative signals with an intracellular and extracellular origin. These signals are eventually integrated by a conserved protein engine consisting of holoenzymes with kinase activity, which trigger crucial transitions during the cell cycle. These kinase holoenzymes are subject to a careful control through signaling transduction pathways and feedback loops which ensure that each event is performed correctly and in proper sequence (Hartwell and Weinert, 1989). A series of kinase holoenzymes are composed of a regulatory subunit, called cyclin, and a catalytic subunit, named cyclin-dependent kinase (cdk). These protein complexes are formed and activated at specific stages of the cell division cycle and their activities are required for progression through S phase and mitosis. Therefore perturbations of some of these proteins may play a role in cancer (Mori *et al.*, 1994; Nobori *et al.*, 1994; Bartkova *et al.*, 1995). In addition to positive regulation by

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cyclins, cdk activity is regulated by phosphorylation or dephosphorylation of cdk at specific residues, as well as by association with inhibitory proteins (Hunter, 1993). These inhibitor proteins bind to and inactivate the cdks. Some inhibitor proteins are absent from transformed cells (Xiong *et al.*, 1993; Kamb *et al.*, 1994; Yeudall *et al.*, 1994). One of cdk inhibitors, $p21^{WAF1/CIP1}$ negatively regulates the related G_1 cyclin/cdk activities (Xiong *et al.*, 1993) and can also inhibit DNA polymerase directly (Waga *et al.*, 1994). Induction of $p21^{WAF1/CIP1}$ may lead the cells to G_1 arrest. Induction of $p21^{WAF1/CIP1}$ gene expression has been initially implicated in p53-mediated growth arrest and apoptosis (El-Deiry *et al.*, 1993) and senescence (Noda *et al.*, 1994). Hence the deregulation of $p21^{WAF1/CIP1}$ expression may allow cell growth and division to become insensitive to external cues. Transactivation of the $p21^{WAF1/CIP1}$ gene is to a large extent responsible for p53-induced cell cycle arrest. The p21 protein causes G_1 cell cycle arrest by binding and blocking the function of cyclin D-cdk4/6 complexes (El-Deiry *et al.*, 1993; Sherr, 1996).

G_1 arrest is important in optimizing the extent of DNA repair after exposure to DNA-damaging agents by delaying the onset of DNA synthesis, hence allowing more time for repair. This cell cycle arrest permits the repair of damaged DNA through unscheduled DNA synthesis prior to DNA replication or cell mitosis (Weinert and Hartwell, 1988) or, if the damage is too great, p53 protein can trigger apoptosis (Symonds *et al.*, 1994). Indeed, p53-knockout mice demonstrate increased susceptibility to spontaneous tumors early in life (Donehower *et al.*, 1992) and embryo fibroblasts from these mice transform spontaneously *in vitro*, though is not required during embryogenesis. Similarly, members of Li-Fraumeni syndrome-families who have inherited one non-functional p53 allele develop a range of cancers analogous to those found in knockout mice (Frebourg and Friend, 1992). Therefore, p53 mutations can lead to the genetic instability and uncontrolled cell proliferation characteristic of human cancer (Greenblatt *et al.*, 1994). Therefore inactivation of p53, and hence absence of G_1 arrest and reduction of the opportunity to repair DNA, renders tumor cells more prone to the acquisition of mutations in the genome. Transactivation of the GADD45 gene, the protein product of which binds PCNA and inhibits S phase entry, may contribute to the p53-dependent cell cycle arrest pathway (Smith *et al.*, 1994).

Restoration of normal p53 function in cells with p53 mutations by gene replacement leads to G_1 cell cycle arrest or induction of apoptosis (Liu *et al.*, 1994) and suppression of the tumorigenic potential and tumor growth inhibition (Kock *et al.*, 1996). Additionally, the wt p53 protein is effective in the downregulation of the tumor phenotype of human leukemia cells, though the mechanism by which downregulation occurred remained unknown. However, in some cases malignant cells may eventually resume growth, some at the same rate as untreated cells, after completion of gene transfer (Wills *et al.*, 1994; Eastham *et al.*, 1995).

We previously reported that inactivation of the p53 gene by either mutation or human papillomavirus (HPV) infection was extremely frequent in human oral squamous cell carcinoma cell lines (Min *et al.*, 1994). Thus induction of wt p53 expression by gene replacement may play an important role in the suppression of tumorigenic phenotypes in oral cancer cells with p53 mutations by decreased expression and/or activities of key G_1-phase cell cycle regulatory proteins. To investigate this possibility, we determined the change of tumorigenic phenotype, the cellular levels of key G_1-phase cell cycle regulatory proteins, and the activities of cdks in a human oral squamous cell carcinoma cell line SCC-9 before and after transfection with a recombinant plasmid (pMSG-p53X) encoding wt p53.

Materials and Methods

Cell cultures

The human oral cancer cell line SCC-9 was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). This cell line contained mutation in the highly conserved open reading frames of the p53 gene as follows: the SCC-9 had a deletion of 32 base pairs between codons 274 and 285 (Min *et al.*, 1994). The cancer cell line SCC-9 and transformant SCC-9-p53X were cultured in Dulbecco,s modified minimum essential medium (DMEM)/Ham,s F12 (GibcoBRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 0.4 μg/ml hydrocortisone at 5% CO_2 in a humidified atmosphere at 37°C.

Plasmids

Wild-type p53 cDNA, cloned into pBR322, was

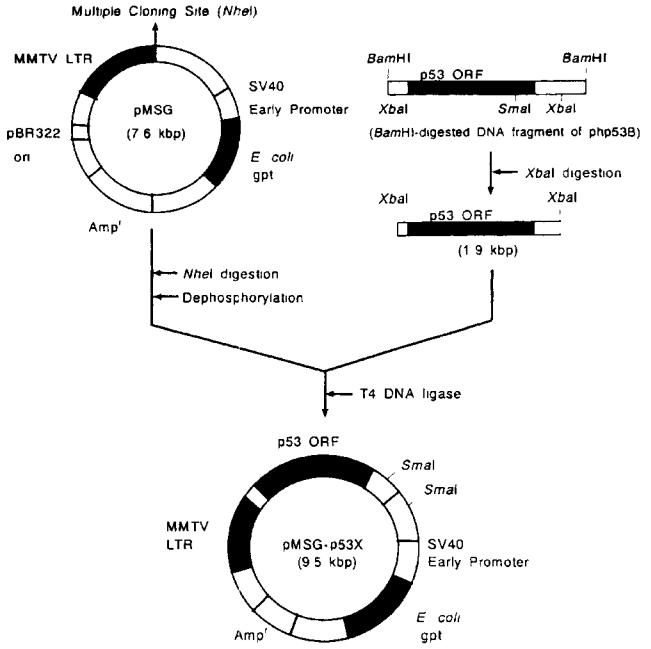

Fig. 1. Schematic diagram for construction of wt p53-expressing plasmid. A plasmid php53B consisting of wt p53 cDNA cloned into pBR322 was digested with *Bam*HI and *Xba*I, and a 1.9-kb fragment, which includes the entire protein-coding region, was recovered. The fragment was cloned into the *Nhe*I site of the glucocorticoid-inducible eukaryotic expression vector pMSG in the sense orientations, relative to the MMTV promoter, to form plasmid pMSG-p53X. The plasmid contains the *E. coli gpt* gene, which is expressed from the SV40 early promoter and therefore provides a marker for selecting stable transformants in eukaryotic cells.

obtained from the plasmid php53B (ATCC 57254). As seen in Fig. 1, we excised the 1.9-kb *Xba*I-*Xba*I fragment containing the entire protein coding region from the 2.2-kb *Bam*HI-digested DNA fragment of the plasmid php53B. The fragment was ligated into the unique *Nhe*I restriction site downstream of the mouse mammary tumor virus long terminal repeat (MMTV LTR) promoter in the glucocorticoid-inducible eukaryotic expression vector pMSG (Pharmacia Biotech, Uppsala, Sweden). This expression vector also contained the *E. coli* xanthine-guanine phosphoribosyltransferase (gpt) gene, which was expressed from the SV40 early promoter and therefore provided a marker for selecting stable transformants in eukaryotic cells. The orientation of the p53 insert relative to the MMTV promoter was ascertained by restriction mapping with restriction enzyme *Sma*I. Recombinant plasmid that expressed wt p53 gene was named pMSG-p53X.

Transfection

Approximately 70% confluent monolayer cultures grown in 35-mm Petri dishes were transfected with pMSG-p53X by using the Lipofectamine™ reagent (GibcoBRL). For each 35-mm Petri dish, a mixture of 2 μg/100 μl of plasmid DNA and 8 μg/100 μl of Lipofectamine™ reagent was added to the culture medium. The cells were incubated for 24 h at 37°C. The medium was then replaced with fresh culture medium and the cultures were incubated for additional 48 h. To select cells transfected with the pMSG-p53X plasmid, the cells were incubated in gpt^+ selection medium (culture medium containing 0.0015% hypoxanthine, 0.001% thymidine, 0.025% xanthine, 0.0025% mycophenolic acid and 0.0002% aminopterin) for 21 days before subculture. One gpt^+-resistent cell colony transfected with pMSG-p53X was isolated, subcultured, and named SCC-9-p53X. One μM of dexamethasone was then treated for induction of wt p53 protein in all subsequent assays.

Dot blot hybridization analysis

To determine the presence of plasmid DNA in the transfected cells, high-molecular-weight cellular DNA was extracted from cells by phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation. The obtained DNAs (2 μg) were heat denatured and spotted on Hybond-N (Amersham Corp., Arlington Heights, IL) under vacuum. Probe used for dot blot hybridization analysis was synthesized using template (a 2.25-kbp fragment, nucleotide 871-3121) representing the *E. coli gpt* gene. Probe was labeled with [^{32}P]dCTP (Amersham Corp.) using megaprime labeling system (Amersham Corp.). Hybridization and washes were done as recommended by the membrane manufacturer.

Growth characteristics

Confluent cell monolayers in 100-mm Petri dishes were trypsinized and counted. The cells were suspended in DMEM/Ham,s F12 supplemented with 10% FBS and 0.4 μg/ml hydrocortisone, and 2.5×10^4 cells were plated onto 35-mm Petri dishes. Media were changed every 2 days. The number of viable cells after trypan blue exclusion was counted after 2, 4, 6, 8, 10, and 12 days of incubation at 37°C. There were four cultures in each group at each time.

Anchorage independence

Anchorage-independent growth was assayed for

their ability to grow on semi-solid agar by the modification method of Macpherson (1970). Two ml of a mixture of 2X medium and 1.0% Noble agar (Difco Laboratories, Detroit, MI) was poured into 60-mm Petri dish and allowed to gel. The basal layer was overlaid with 1,000 viable cells suspended in equal volume of 2X medium containing 40% FBS and 0.6% Noble agar. The Petri dishes were then incubated at 37°C for 3 weeks. Colonies larger than 50 μm in diameter were examined and counted with the aid of an inverted microscope.

Cell cycle analysis

Approximately 70% confluent monolayer cells in 100-mm Petri dish were harvested by trypsinization and washed with phosphate buffered saline. Cells were then fixed in cold 70% ethanol for 45 min at 4°C, pelleted, resuspended in TSP solution (0.1% Triton X-100, 0.1% sodium citrate and 0.005% propidium iodide) containing 1 μg/ml RNase A and incubated for 30 min at room temperature. The state of the cell cycle was then analyzed on FACScan flow cytometer (Beckton-Dickenson, San Jose, CA). There were four cultures in each group.

Western analysis

Approximately 80% confluent cells were lysed, and the cell extracts were processed for Western analysis to determine the intracellular levels of p53, $p21^{WAF1/CIP1}$, p16, p27, cyclin D1, cyclin E, cdk2, cdk4, and cdk6 proteins using Western-Light kit (Tropix, Inc., Bedford, MA) as described previously (Min *et al.*, 1995). Monoclonal antibodies to p53 (PAb 1801), cyclin D1 (17A6-4), and cyclin E (HE 12) were obtained from Oncogene Sciences (Uniondale, NY), and monoclonal antibody against $p21^{WAF1/CIP1}$ (187) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to p16 (C-20), p27 (C-19), cdk2 (M2), cdk4 (C-22), and cdk6 (C-21) were also purchased from Santa Cruz Biotechnology, Inc. After probing with the respective antibodies, the membrane was stained with 1X Ponceau S stain for 10 min to reveal total protein amount that was loaded per lane.

Determination of cdk2, cdk4, and cdk6 kinase activities

Cells were lysed with kinase-lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM EDTA, and 1% Triton X-100. Three hundred μg proteins in the cell lysates were incubated with the anti-human cdk2 (M2) polyclonal antibody (Santa Cruz Biotechnology, Inc.), anti-mouse cdk4 (C-22) polyclonal antibody (Santa Cruz Biotechnology, Inc.) or anti-human cdk6 (C-21) polyclonal antibody (Santa Cruz Biotechnology, Inc.), and further incubated with Protein G agarose (Oncogene Sciences). The immunocomplexes were washed with RIPA buffer and resuspended in 12 μl of RIPA buffer. The kinase activities of cdk2, cdk4, and cdk6 were determined using histone H1 (Calbiochem, San Diego, CA), MEK-1 (FL; Santa Cruz Biotechnology, Inc.), and MEK-1 (FL; Santa Cruz Biotechnology, Inc.) as a substrate to quantitatively determine cdk2, cdk4, and cdk6 activities, respectively. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 2X SDS-PAGE sample buffer to the incubation mixture. Proteins were separated on a 15% polyacrylamide-SDS gel and the gel was dried and band was detected by exposing to Hyperfilm-MP (Amersham Corp.) at -70°C. The bands were scanned and densitometric values were obtained using Fuji Bio-Imaging Analyzer (model, BAS 1500; Japan). The values of each lane were subsequently divided by that of the SCC-9 to determine the relative intensity of the successive culture of SCC-9-p53X cells exposed to dexamethasone for induction of wt p53.

Results

Effect of wt p53 gene transfer on the levels of p53 and PCNA proteins, and cell proliferation in SCC-9-p53X cells before and after dexamethasone treatment

To determine whether the transfectant SCC-9-p53X cells contained pMSG-p53X DNA, we studied the presence of plasmid DNA by dot hybridization analysis using the *E. coli gpt* gene-specific probe. The SCC-9-p53X cells contained pMSG-p53X DNA (Fig. 2). The levels of p53 protein in the SCC-9-p53X cells after dexamethasone treatment were notably higher than that of the SCC-9-p53X cells before dexamethasone treatment. However, the SCC-9 cells did not show an increase in the p53 protein level after dexamethasone treatment (Fig. 7A).

To determine the effect of induction of wt p53 protein by gene transfer on proliferation of cancer

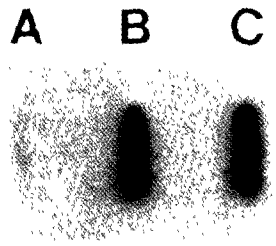

Fig. 2. Dot blot hybridization analysis of high molecular weight cellular DNA from the nontransfected and transfected SCC-9 cells. **A**, nontransfected SCC-9 cells; **B**, SCC-9-p53X cells before dexamethasone treatment; **C**, SCC-9-p53X cells after 1 μM of dexamethasone treatment for 5 days. Two μg of DNAs were spotted on a nylon filter and hybridized to [^{32}P]-labeled probe (a 2.25-kbp fragment, nucleotide 871-3121) representing the *E. coli gpt* gene.

cells with p53 mutation, we cultured the SCC-9 and SCC-9-p53X cells before and after dexamethasone treatment. As shown in Fig. 3A, induction of wt p53 protein expression by dexamethasone treatment in SCC-9-p53X cells caused a significant decrease in the cell proliferation compared to SCC-9 cells before and after dexamethasone treatment. Furthermore, levels of the DNA replication protein, PCNA, in the SCC-9-p53X cells after dexamethasone treatment were notably decreased compared to SCC-9 and SCC-9-p53X cells before dexamethasone treatment. However, the SCC-9 cells did not show a decrease in the PCNA protein level after dexamethasone treatment (Fig. 3B). These results indicate that induction of wt p53 protein by gene transfer represses proliferation of oral cancer cells with p53 mutation.

Effect of the induction of wt p53 protein on the anchorage independence in SCC-9-p53X cells before and after dexamethasone treatment

Nontransfected SCC-9 cells grew in the soft agar with colony-forming efficiencies of 2.52%, indicating the ability of anchorage independence of these cells. The SCC-9-P53X cells produced fewer numbers of anchorage-independent foci in soft agar than that of nontransfected SCC-9 cells. This effect was seen even in the absence of the inducing agent dexamethasone but was significantly greater in the presence of dexamethasone (Table 1).

Effect of the induction of wt p53 protein on the cdk2, cdk4, and cdk6 kinase activities in SCC-9-p 53X cells before and after dexamethasone treatment

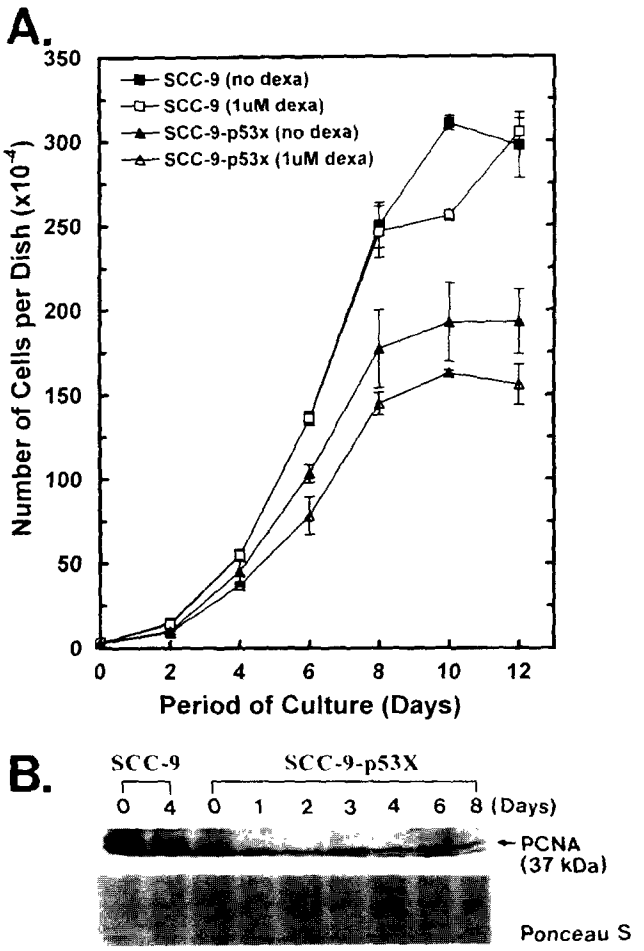

Fig. 3. Effect of wt p53 protein expression by gene transfer on the proliferation of SCC-9-p53X cells. (**A**) Growth curves of nontransfected and transfected SCC-9 cells. Cells were plated at a density of 2.5×10^4 cells in 35-mm Petri dishes. SCC-9 and SCC-9-p53X cells were exposed to dexamethasone (1 μM) for indicated days. The number of viable cells after trypan blue exclusion was counted after 2, 4, 6, 8, 10, and 12 days of incubation. There were four cultures in each group at each time. Values represent averages from four experiments and standard deviations. (**B**) Western blot analysis for the intracellular PCNA levels in the SCC-9 and SCC-9-p53X cells before and after dexamethasone treatment.

Table 1. Characteristics of the SCC-9 cells transfected with recombinant plasmid encoding wt p53 (pMSG-p53X)

Cell lines	Transfection	Induction	Soft agar colony formation (%)*
SCC-9	-	-	2.52±0.48**
SCC-9-p53X	+	-	1.86±0.34
SCC-9-p53X	+	+	1.14±0.39

*Cell suspensions were plated on 0.3% soft agar medium containing 20% fetal bovine serum, and colonies larger than 50 μm in diameter were counted after 21 days incubation at 37°C from 60-mm Petri dishes receiving 1,000 cells each.

**The values represent the average standard deviation of the values obtained from 5 separate experiments.

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To investigate whether induction of wt p53 protein by gene transfer affected the activities of key cell cycle regulatory proteins in oral cancer cells with p53 mutation, we studied the activities of cdk2, cdk4, and cdk6 kinases by immunoprecipitating cell lysates with their antibodies before and after exposing SCC-9 and SCC-9-p53X cells to dexamethasone as described in "Materials and Methods". The cdk2 kinase activity was notably inhibited in SCC-9-p53X cells after dexamethasone treatment for more than 3 days (Fig. 4C and F). These results are consistent with our results that p53 protein is induced significantly in SCC-9-p53X cells after exposure to 1 μM dexamethasone for 3 days and the increased level was persisted through experimental periods. Similarly, cdk4 and cdk6 kinase activities in SCC-9-p53X cells after dexamethasone treatment was also inhibited (Fig. 4A, B, D and E). However, the SCC-9 cells did not show an inhibition in the cdk2, cdk4 and cdk6 kinase activities after dexamethasone treatment for 4 days. These results indicate that the cdk2, cdk4 and cdk6 kinase activities are notably inhibited in the SCC-9-p53X cells expressing wt p53 protein by gene transfer.

Effect of the induction of wt p53 protein on the levels of cdk2, cdk4, cdk6, cyclin D1, and cyclin E in SCC-9-p53X cells before and after dexamethasone treatment

We determined the levels of cdk2, cdk4, cdk6, cyclin D1, and cyclin E proteins by Western analysis in the SCC-9-p53X cells to find whether changes in the levels of these proteins after dexamethasone treatment occur to cause a reduction in cdk activities. Fig. 5A and Figure 6 show that the levels of cdk4 and G_1 cyclins (cyclin D1 and cyclin E) did not alter after dexamethasone treatment in the SCC-9 and SCC-9-p53X cells, respectively. The cdk6 and cdk2 protein levels showed a slight decrease in the SCC-9-p53X cells after dexamethasone treatment compared to that of the SCC-9-p53X cells after dexamethasone

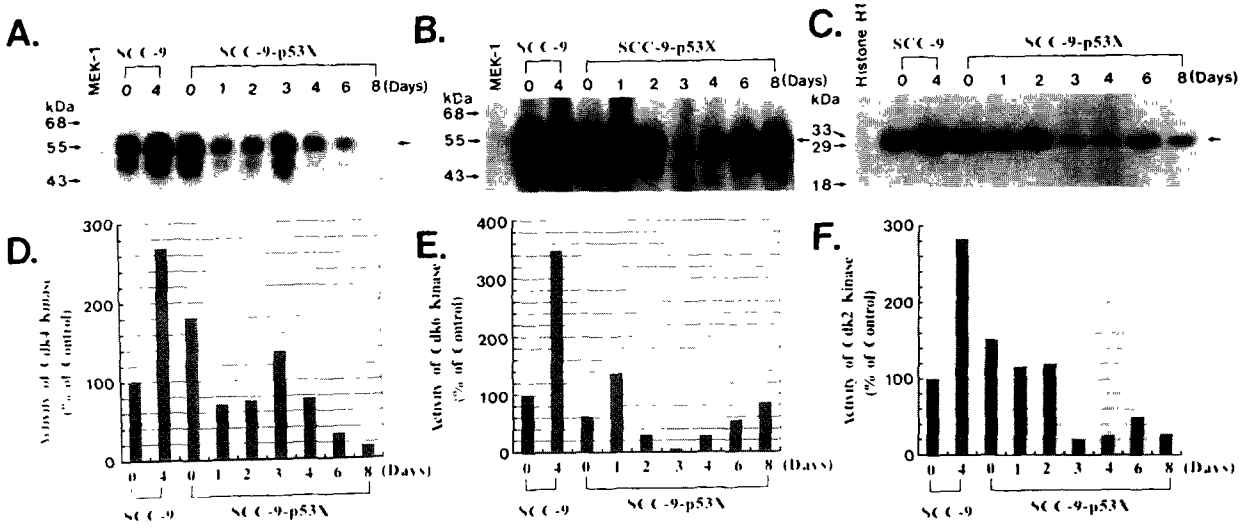

Fig. 4. Effect of wt p53 protein expression by gene transfer on the activities of cdk4 (**A**), cdk6 (**B**), and cdk2 (**C**) kinases in the SCC-9 and SCC-9-p53X cells and its histogram representation of the cdk4 (**D**), cdk6 (**E**) and cdk2 (**F**) kinase activities after densitometric analysis. Autoradiogram of the MEK-1, MEK-1, and histone H1 proteins incubated with anti-cdk4, anti-cdk6, and anti-cdk2 immunoprecipitates, respectively. The phosphorylated proteins are marked with arrows. Activities of cdks in cell lysates were determined as described in "Materials and Methods". Quantification of the signals for cdk activities was performed by densitometry. Kinase activity is represented as the percentage of activity detectable in the SCC-9 cells before dexamethasone treatment as 100%.

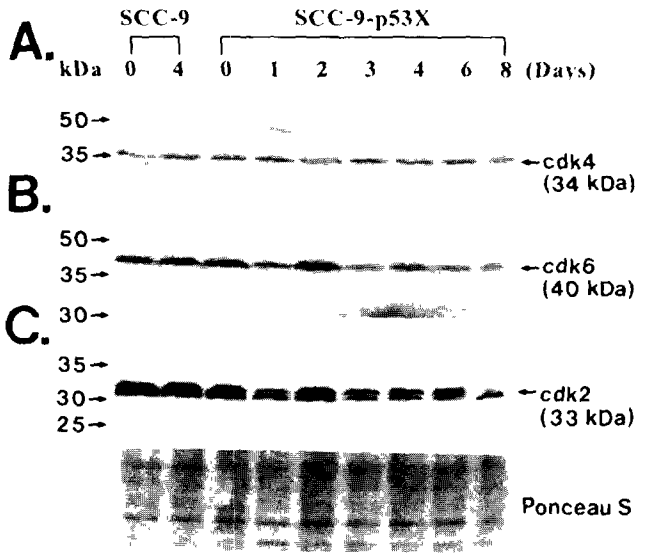

Fig. 5. Western blot analysis for the intracellular cdk4 (**A**), cdk6 (**B**) and cdk2 (**C**) protein levels in the SCC-9 and SCC-9-p53X cells before and after dexamethasone treatment. The membrane was stained with Ponceau S stain to reveal the total protein loaded per each lane. A structural protein stained with Ponceau S serves as the internal control to account for the loading error (lower panel). SCC-9-p53X cells were exposed to dexamethasone (1 μM) for indicated days to induce wt p53 protein expression and SCC-9 cells also exposed to dexamethasone for 4 days.

treatment. The SCC-9 cells did not show significant decrease in the cdk6 and cdk2 protein levels after dexamethasone treatment for 4 days (Fig. 5B and C). We speculate that the altered levels of the cdk6 and cdk2 proteins seen after dexamethasone treatment in the SCC-9-p53X cells might, in part, contribute to the reduced cdk6 and cdk2 kinase activities that was observed in the SCC-9-p53X cells after dexamethasone treatment.

Effect of the induction of wt p53 protein on the levels of $p21^{WAF1/CIP1}$, p16, and p27 in SCC-9-p53X cells before and after dexamethasone treatment

It has been shown that the $p21^{WAF1/CIP1}$ is a universal inhibitor of kinase activity of cdks and regulates the kinase activity by directly binding to the cdk-cyclin protein complex (Dulic *et al.*, 1994; Peter and Herskowitz, 1994). Since we noted a reduction in the kinase activities of cdks in the SCC-9-p53X cells after dexamethasone treatment, we determined the levels of cell cycle inhibitory proteins. The SCC-9-p53X cells did not show an increase in the p16 and $p21^{WAF1/CIP1}$ levels after dexamethasone treatment (Fig. 7B and C). Particularly, the level of p27 protein in these cells after dexamethasone treatment was somewhat lower than that in untreated cells. However, the SCC-9 cells showed a similar level of p27 protein after dexamethasone treatment (Fig. 7D). These results indicate that the induction of wt p53 protein by gene-transfer in oral cancer cells with p53 mutations do not affect the levels of cell cycle

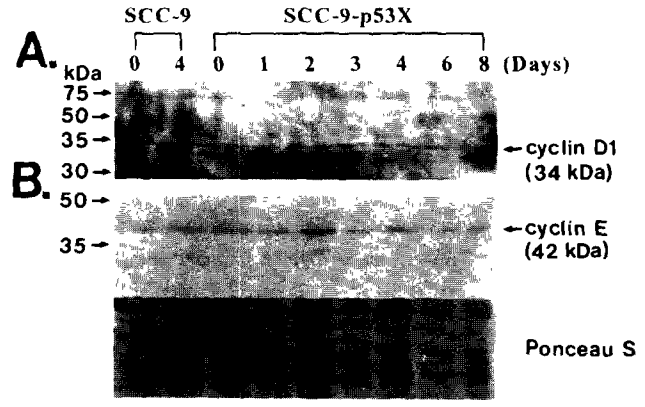

Fig. 6. Western blot analysis for the intracellular cyclin D1 (**A**) and cyclin E (**B**) protein levels in the SCC-9 and SCC-9-p53X cells before and after dexamethasone treatment. The membrane was stained with Ponceau S stain to reveal the total protein loaded per each lane. A structural protein stained with Ponceau S serves as the internal control to account for the loading error (lower panel). SCC-9-p53X cells were exposed to dexamethasone (1 μM) for indicated days to induce wt p53 protein expression and SCC-9 cells also exposed to dexamethasone for 4 days.

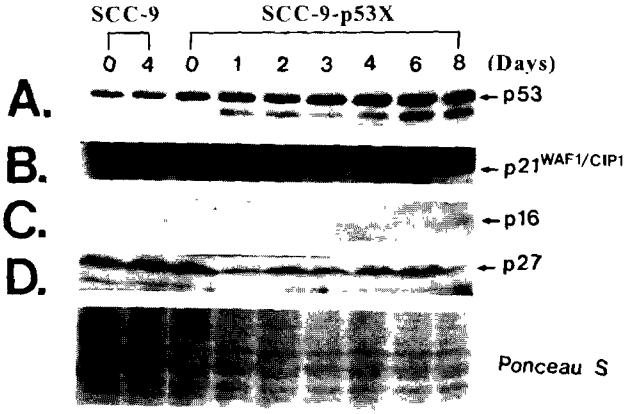

Fig. 7. Western blot analysis for the intracellular p53 (**A**), $p21^{WAF1/CIP1}$ (**B**), p16 (C), and p27 (**D**) protein levels in the SCC-9 and SCC-9-p53X cells before and after dexamethasone treatment. The membrane was stained with Ponceau S stain to reveal the total protein loaded per each lane. A structural protein stained with Ponceau S serves as the internal control to account for the loading error (lower panel). SCC-9-p53X cells were exposed to dexamethasone (1 μM) for indicated days to induce wt p53 protein expression and SCC-9 cells also exposed to dexamethasone for 4 days.

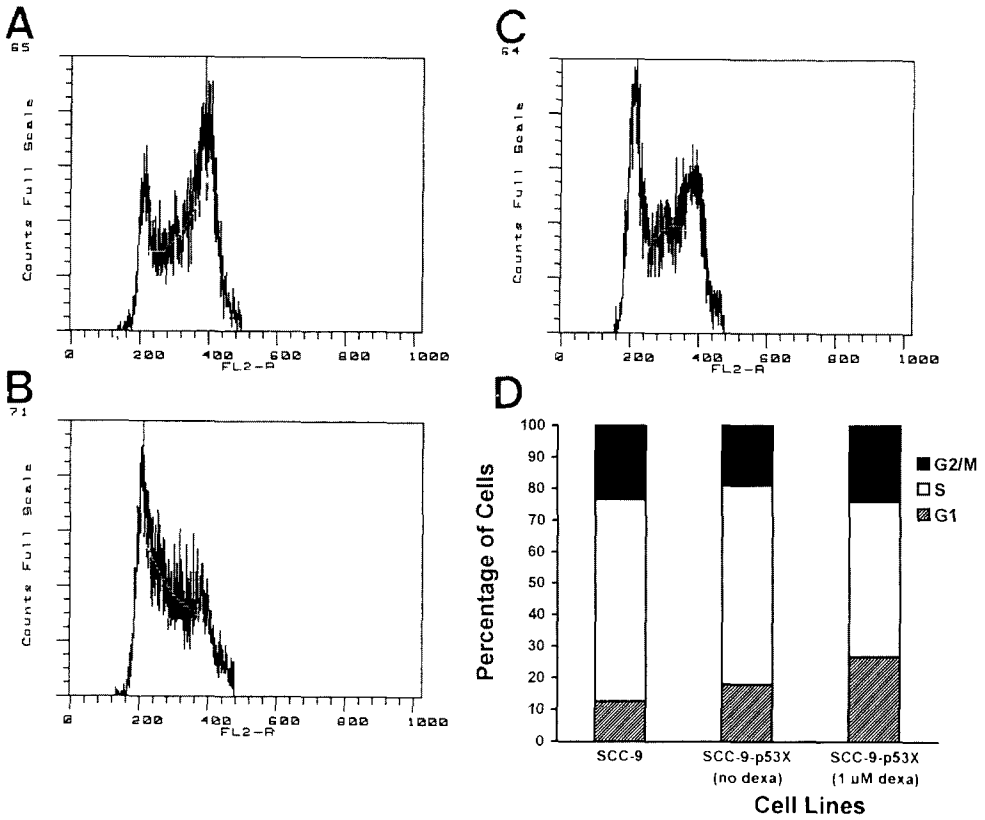

Fig. 8. Cell cycle progression in nontransfected and transfected SCC-9 cells. Actively proliferating cells were labeled with propidium iodide and processed as described in "Materials and Methods". A, nontransfected SCC-9 cells; B, SCC-9-p53X cells before dexamethasone treatment; C, SCC-9-p53X cells after 1 μM of dexamethasone treatment for 5 days; D, quantitative analysis of cell cycle progression in these cells. The number of SCC-9-p53X cells in G_1, and in S phases was significantly enhanced and decreased by dexamethasone treatment for 4 days, respectively.

inhibitory proteins.

Effect of the induction of wt p53 protein on cell cycle progression in SCC-9-p53X cells before and after dexamethasone treatment

To establish a cause and effect relationship between induction of wt p53 protein and the ability of a cell to arrest in G_1, actively proliferating (about 70% confluency) SCC-9-p53X cells before and after dexamethasone treatment processed for FACScan analysis as described in "Materials and Methods". Fig. 8 showed that significant arrest of cell cycle at G_1 phase occurred in the SCC-9-p53X cells after dexamethasone treatment. Though the arrest of cell cycle at G_1 phase was less pronounced, an increase in the number of cells arresting G_1 phase in the absence of the inducing agent was noted in this transfectant relative to parental SCC-9 cells (Fig. 8). These results clearly show that progression of cell cycle is altered differently in the SCC-9 cells after wt p53 expression.

Discussion

We established and characterized a cell line derived from SCC-9 cells with p53 mutation, SCC-9-p53X, that contained wt p53-expressing plasmid by transfection. To establish a cause and effect relationship between expression of wt p53 and the ability of cells to proliferate, we transfected the SCC-9 cells with selectable expression vectors containing wt p53 cDNA. Unfortunately, there are no absolutely regulated promoters yet available in mammalian cell systems. Nevertheless, we utilized a hormone-inducible MMTV promoter to drive the transcription of p53 so that its constitutive expression could be minimized (Sardet *et al.*, 1989).

Since the expression of p53 protein is closely associated with the cell cycle progression, we investigated the correlation of p53 expression by gene transfer with change of tumorigenic phenotype, and intracellular levels and/or activities of key cell cycle regulators in the nontransfected SCC-9 and

SCC-9-p53X cells. Inasmuch as growth of the nontransfected SCC-9 cells was not affected by exposure to 1 μM dexamethasone (Fig. 2), we used 1 μM dexamethasone as the inducer for the expression of wt p53 gene from the transfected line of SCC-9 cells, SCC-9-p53X. Our data showed that the level of p53 protein notably increased when the SCC-9-p53X cells were treated with inducing agent dexamethasone for more than 3 days compared to those in the dexamethasone-treated SCC-9 and SCC-9-p53X cells before dexamethasone treatment, and that the cell proliferation and anchorage independence significantly inhibited in this cell line. Also, the activities of cdks in the SCC-9-p53X cells after dexamethasone treatment was notably decreased compared to the dexamethasone-treated SCC-9 and SCC-9-p53X cells before dexamethasone treatment. These results indicate that induction of wt p53 protein expression by gene transfer causes the cells to inhibit proliferation of the oral cancer cells with p53 mutation by decreasing the activities of cdks. These results are in agreement with previous reports which showed that the restoration of normal p53 function by gene replacement in cells with p53 mutation inhibits the growth of human cancer cells (Mercer *et al.*, 1990; Köck *et al.*, 1996). However, it is possible that the different effects can arise from due to different tumor cell types with p53 mutations and multiple defects in tumor suppressor genes.

The signaling pathways that regulates cell cycle progression seem to be primarily associated with the G_1 phase of the cell cycle (Pardee, 1989). Several cellular proteins, namely p53, Rb, cdk2, cdk4, cdk6, cyclin D, cyclin E, $p21^{WAF1/CIP1}$, and the PCNA have been reported to play important role in the regulation of G_1 phase of cell cycle (Goodrich *et al.*, 1991; Koff *et al.*, 1992; Kuerbitz *et al.*, 1992; Sherr, 1993). Detailed function of p53 protein in the normal regulation of G_1 phase of the cell cycle has been investigated, but their regulatory functions are not completely known. Since the cell cycle phases in dividing mammalian cells are finely regulated by the sequential assembly and activation of key cell cycle regulators namely the cyclins and cdks (Hartwell and Kastan, 1994; Peter and Herskowitz, 1994), the decrease of cdk activities in oral cancer cells expressing wt p53 protein by gene transfer can result in a growth inhibitory effect.

The cyclin-cdk complexes are formed and activated at specific stages of the cell division cycle and their activities are required for progression through S phase and mitosis. Therefore, we determined the levels of cdks and cyclins in the SCC-9 and SCC-9-p53X cells. As expected, expression of cdk2 and cdk6 proteins notably reduced when the SCC-9-p53X cells were treated with dexamethasone for more than 3 days compared to those in the dexamethasone-treated SCC-9 and SCC-9-p53X cells before dexamethasone treatment, consistent with our results demonstrating induction of p53 protein after dexamethasone treatment for more than 3 days. However, the levels of G_1 phase cyclins (cyclin D1 and cyclin E) of the tested cells were similar to each other. Since cdk activity is regulated by association with inhibitory proteins as well as by phosphorylation or dephosphorylation of cdk at specific residues (Hunter, 1993), we also investigated the levels of key cell cycle regulatory proteins. The $p21^{WAF1/CIP1}$, whose expression is induced by wt p53 protein (El-Deiry *et al.*, 1993; 1994), plays a major role in arresting cell cycle progression beyond G_1 phase by inhibiting the activities of cdks (Dulic *et al.*, 1994; El-Deiry *et al.*, 1993; Kuerbitz *et al.*, 1992). The levels of inhibitory proteins of the tested cells, however, were similar to each other. These results indicate that in human cancer cells containing p53 mutation, the levels of cdk proteins and their kinase activities of the G_1 phase are notably reduced by induction of wt p53 gene expression thereby making them to repress of tumorigenic phenotype. The mechanism by which the transformed phenotype was inhibited by the wt p53 gene transfer is largely unknown but the transformed characteristics of SCC-9 cells may be closely associated with the continuous expression of functions of wt p53 protein.

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References

Bartkova, J., Lukas, J., Strauss, M. and Bartek, J.: Cyclin D1 oncoprotein aberrantly accumulates in malignancies of diverse histogenesis. Oncogene 10: 775-778, 1995.

Donehower, L., Harvey, M., Slagle, B.L., McArthur, M. J., Montgomery, C.A.J., Butel, J.S. and Bradley, A.: Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature 356: 215-221, 1992.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I.: p 53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G_1 arrest. Cell 76: 1013-1023, 1994.

Eastham, J.A., Hall, S.J., Sehgal, I., Wang, J., Timme, T.L., Yang, G., Connell-Crowley, L., Elledge, S.J., Zhang, W.W., Harper, J.W. and Thompson, T.C.: *In vivo* gene therapy with p53 or p21 adenovirus for prostate cancer. Cancer Res. 55: 5151, 1995.

El-Deiry, W.S., Harper, J.W., O'Conner, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, Y., Wiman, K.G., Mercer, W.E., Kastan, M.B., Kohn, K. W., Elledge, S.J., Kinzler, K.W. and Vogelstein B.: WAF1/CIP1 is induced in p53-mediated G_1-arrest and apoptosis. Cancer Res. 54: 1169-1174, 1994.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein B.: WAFI, a potential mediator of p53 tumor suppression. Cell 75: 817-825, 1993.

Frebourg, T. and Friend, S.H.: Cancer risks from germline p53 mutations. J. Clin. Invest. 90: 1637-1641, 1992.

Goodrich, D.W., Ping Wang, N., Qian, Y.-W., Lee, EY.-HP. and Lee, W.-H.: The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. Cell 67: 293-302, 1991.

Greenblatt., M.S., Bennett, W.P., Hollsten, M. and Harris, C.C.: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. 54, 4855-4878, 1994.

Hartwell, L.H. and Kastan, M.B.: Cell cycle control and cancer. Science 266: 1821-1828, 1994.

Hartwell, L.H. and Weinert, T.A.: Checkpoints: controls that ensure the order of cell cycle events. Science 629-634, 1989.

Hunter, T.: Braking the cycle. Cell 75: 839-847, 1993.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R. S., Johnson, B.E. and Skolinick, M.H.: A cell cycle regulator potentially involved in genesis of many tumor types. Science 264: 436-440, 1994.

Ko, L.J. and Prves, C.: p53: Puzzle and paradigm. Genes Dev. 10: 1054-1072, 1996.

Kock, H., Harris, M.P., Anderson, S.C., Machemer, T., Hancock, W., Sutjipto, S., Wills, K.N., Gregory, R.J., Shepard, H.M., Westphal, M. and Maneval, D.C.: Adenovirus-mediated p53 gene transfer suppresses growth of human glioblastoma cells *in vitro* and *in vivo*. Int. J. Cancer 67: 808-815, 1996.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, R.B. and Roberts, J.M.: Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. Science 257: 1689-1694, 1992.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V. and Kastan, M.B.: Wild type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Nat. Acad. Sci. USA 89: 7491-7495, 1992.

Lane, D.P., Lu, X., Hupp, T. and Hall, P.A.: The role of p53 in the apoptotic response. Phil. Trans. Roy. Soc. Lond. B. 345: 277-280, 1994.

Liu, T.J., Zhang, W.W., Taylor, D.L., Roth, J.A., Goepfert, H. and Clayman, G.L.: Growth suppression of human head and neck cancer cells by the introduction of a wildtype p53 gene via a recombinant adenovirus. Cancer Res. 54: 3662-3667, 1994.

Macpherson, I.: The characteristics of animal cells transformed *in vitro*. Adv. Cancer Res. 13: 169-213, 1970.

Mercer, W.E., Shields, M.T., Amin, M., Sauve, G.J., Appella, E., Romano, J.W. and Ullrich, S.J.: Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA 87: 6166-6170, 1990.

Min, B.-M., Baek, J.-H., Shin, K.-H., Gujuluva, C.N., Cherrick, H.M. and Park, N.-H.: Inactivation of the p53 gene by either mutation or HPV infection is extremely frequent in human oral squamous cell carcinoma cell lines. Oral Oncol., Eur. J. Cancer 30B: 338-345, 1994.

Min, B.-M., Woo, K.M., Baek, J.-H., Lee, G. and Park, N.-H.: Malignant transformation of HPV-immortalized human oral keratinocytes by chemical carcinogens. Int. J. Oncol. 7: 249-256, 1995.

Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, N. and Nakamura, Y.: Frequent somatic mutation of the MTS1/CDK41 (Multiple Tumor Suppressor/Cyclin-dependent Kinase 4 Inhibitor) Gene in esophageal squamous cell carcinoma. Cancer Res. 54: 3396-3397, 1994.

Nobori, T., Miura, K., Wu, D.J., Lois, A., Takabayashi, K. and Carson, D.A.: Deletion of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368: 753-756, 1994.

Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M. and Smith, J.R.: Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res. 211: 90-98, 1994.

Pardee, A.B.: G_1 events and regulation of cell proliferation. Science 246: 603-608, 1989.

Peter, M. and Herskowitz, I.: Joining the complex: Cyclin-dependent kinase inhibitory proteins and the cell cycle. Cell 79: 181-184, 1994.

Sardet, C., Franchi, A. and Pouyssegur, J.: Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter. Cell 56: 271-280, 1989.

Sherr, C.J.: Mammalian G_1 cyclins. Cell 73: 1059-1065, 1993.

Sherr, C.J.: Cancer cell cycles. Science 274: 1672-1677, 1996.

Smith, M.L., Chen, I.-T., Zhan, Q., Bae, I., Chen, C.-Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M. and Fornace Jr, A.J.: Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266: 1376-1380, 1994.

Stratton, M.R.: Molecular Biology for Oncologists, 2nd ed., pp 92-102, Chapman & Hall, London, 1996.

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T. and Dyke, T.V.: p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. Cell 78: 703-711, 1994.

Waga, S., Hannon, G.J., Beach, D. and Stillman, B.: The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369: 574-578, 1994.

Weinert, T.A. and Hartwell, L.H.: The RAD 9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science 241: 317-322, 1988.

Wills, K.N., Maneval, D.C., Menzel, P., Harris, M.P., Sutjipto, S., Vailancourt, M.T., Huang, W.M., Johnson, D.E., Anderson, S.C. and Wen, S.F.: Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. Hum. Gene Ther. 5: 1079-1088, 1994.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D.: p21 is a universal inhibitor of cyclin kinases. Nature 366: 701-704, 1993.

Yeudall, W.A., Crawford, R., Ensley, J.F. and Robbins, K.: MTS1/CDK41 is altered in cell lines derived from primary and metastatic oral squamous cell carcinoma. Carcinogenesis 15: 2683-2686, 1994.