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# Cellular Adhesion Regulates p53 Protein Levels in Primary Human Keratinocytes<sup>1</sup>

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## Abstract

To gain insight into p53 tissue-specific regulatory pathways and biological activities, we investigated mechanisms that may account for the elevated levels of p53 protein in human foreskin keratinocytes, relative to levels in dermal fibroblasts *in vitro*. Here, we report that the loss of cell anchorage resulted in an approximately 5-fold decrease in p53 levels in keratinocytes, which was reversible upon reattachment of cells to a substratum. In contrast, fibroblasts did not exhibit such adhesion-dependent regulation of p53 protein. Furthermore, p53 function was attenuated in keratinocytes relative to fibroblasts. These results link p53 to cell adhesion pathways and may provide a molecular basis for epigenetic differences in the maintenance of genomic stability among normal cell types.

## Introduction

Tumor cells are intrinsically more genetically unstable than normal cells, and mutation of the *p53* tumor suppressor gene has provided a genetic link between genomic instability and the development of cancer (1). Cell populations that are deficient in p53 fail to arrest or undergo apoptosis in response to DNA damage and exhibit an elevated frequency of genomic rearrangements, relative to the frequency in normal cells (1). Although the accumulation of wt<sup>3</sup> p53 is a typical response to DNA damage in many cells, increased levels of wt p53 are normally associated with some physiological conditions. The elevated expression of wt p53 protein in embryonic (2) and germ (3) cell types provides compelling evidence that the levels and function of p53 protein may be directly controlled by developmental or tissue-specific cellular programs. In support of this hypothesis, the physical association between p53 and the Wilm's tumor gene product (4), a protein that has a specific role in kidney development, stabilizes and partially inactivates p53 (5). Increased steady-state levels of p53 protein also occur in proliferating primary epithelial cell populations *in vitro* and indicate that novel p53 regulatory pathways may also exist in these cell types (6-9). Here, we observed the nuclear accumulation of p53 protein in cycling primary human epidermal keratinocytes *in vitro* and found that this may, in part, be due to the activation of cell adhesion pathways during keratinocyte proliferation. These results indicate that p53 expression and function may be regulated by tissue-specific cellular processes and have implications for the mechanisms driving early events in the pathogenesis of both familial and sporadic cancers of epithelial origin.

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<sup>3</sup> The abbreviations used are: wt, wild type; NHEK, normal human epidermal keratinocyte; NHF, normal human fibroblast.

## Materials and Methods

**Culture Conditions.** All primary cell populations were prepared in this laboratory. NHEKs were typically used at population doublings 9-15. NHFs were maintained in 10% fetal bovine serum and DMEM (Life Technologies, Inc.). NHEKs were propagated in KGM (Clonetics) without antibiotic or amphotericin. NHEKs were harvested with 0.05% trypsin and diluted with Trypsin Neutralizing Solution (Clonetics) to inactivate the enzyme. Cells were pelleted and resuspended at a density of 750,000 cells in 8 ml of medium per 100-mm dish for both adherent and nonadherent conditions. Plates for suspension culture were coated three times with 3 ml of 10 mg/ml poly-2-hydroxyethylmethacrylate (Aldrich) dissolved in absolute ethanol at 37°C. For nonenzymatic dissociation, cells were rinsed in PBS and harvested over 30 min in Cell Dissociation Buffer (Life Technologies). The initiation of the harvest was treated as time zero for all time course experiments. Cytochalasin D (Sigma) was resuspended in DMSO at a concentration of 1 mg/ml.

**Flow Cytometric Analysis.** Cell cycle progression was analyzed using a flow cytometric assay as described previously (1). Briefly, 10  $\mu$ M bromodeoxyuridine was added 3 h prior to a given time point for the assessment of DNA synthesis. Cells were harvested, resuspended in 1.5 ml of cold PBS, and fixed with 3 ml of cold 95% ethanol, and nuclei were isolated and incubated with anti-FITC bromodeoxyuridine antibody (Becton Dickinson). Samples were analyzed on a Becton Dickinson FACScan. The percentages of nuclei in G<sub>1</sub>, S, and G<sub>2</sub>-M phases of the cell cycle were determined from an ungated population of cells.

**Western Blot Analysis.** Cell lysates and Western blots were prepared as described previously (10). Monoclonal antibodies for p53 (DO-1) and MDM2 (IF2; Oncogene Science) were used at concentrations of 0.2  $\mu$ g/ml and 1  $\mu$ g/ml, respectively. A monoclonal antibody to  $\beta$ -actin (Sigma) was diluted 1:50,000 and was used to standardize for equal loading. Detection was performed using enhanced chemiluminescence according to the manufacturer's instructions (ECL; Amersham), and densitometry was performed on films using NIH Image for Macintosh.

**Northern Blot Analysis.** Total RNA was isolated with TRIzol solution (Life Technologies, Inc.) according to the manufacturer's specifications. RNA was electrophoretically separated on a 1% 3-[N-morpholino]propane-sulfonic acid-formaldehyde agarose gel and transferred to Hybond-N+ membrane (Amersham) in 10 $\times$  SSC. DNA fragments for hybridizations were labeled with [<sup>32</sup>P]dCTP using the Rediprime labeling kit (Amersham). Filters were incubated with labeled probe for 2 h in Rapid Hyb solution (Amersham) at 65°C, and two 15-min washes in 0.3 $\times$  SSC-0.1% SDS at 65°C were subsequently performed.

**Immunohistochemistry.** NHEKs and NHFs were plated in eight-well chamber slides. After 48 h, cells were fixed for 2 min in an equal mixture of acetone and methanol at -20°C and rehydrated with PBS prior to immunohistochemistry. The monoclonal antibody to p53, DO-1, was used at a concentration of 1  $\mu$ g/ml and incubated with cells for 1 h at room temperature. Detection was performed through immunoperoxidase staining according to the protocols in the Vectastain Elite ABC kit (Vector Laboratories). Cells were then counterstained in hematoxylin and mounted.

## Results

Previous studies have noted abundant nuclear p53 protein in basal keratinocytes of stratified squamous cell cultures (7). To determine whether p53 levels in human keratinocytes in monolayer were elevated, relative to the stromal cell type (dermal fibroblasts), protein lysates were prepared from 10 different primary keratinocyte cell

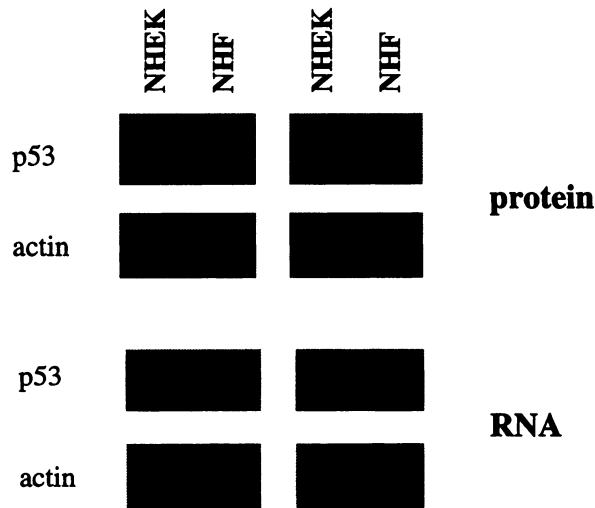


Fig. 1. p53 protein levels of primary human keratinocytes *in vitro* exceed levels in dermal fibroblasts through posttranscriptional mechanisms. For Western blot analysis, protein lysates from keratinocyte (NHEK) and fibroblast (NHF) populations were generated from two different individuals. Lanes contain 20  $\mu$ g of protein and are paired according to the individual from which the cell populations were isolated. Filters were incubated with the monoclonal antibody, DO-1, which recognizes both mutant and wt conformations of the p53 protein and a monoclonal antibody for  $\beta$ -actin as a control for loading. For Northern blot analysis, 5  $\mu$ g of total RNA were separated on a 3-[*N*-morpholino] propane-sulfonic acid-formaldehyde agarose gel. The filter was hybridized with  $^{32}$ P-labeled cDNA fragments of p53 and  $\beta$ -actin as a control for loading. p53 expression levels in NHEKs relative to NHFs in paired populations were 2.8 and 2.0. Signal was quantitated using a PhosphorImager (Molecular Dynamics).

populations (NHEKs) and compared to five primary fibroblast populations (NHFs). In all cases, p53 protein in cycling keratinocytes exceeded levels in cycling fibroblasts by 5–10-fold. These results were not due to genetic variation because cell populations (NHFs and NHEKs) obtained from the same individual (in three matched sets) exhibited these cell type-specific differences in p53 protein (Fig. 1). The basis for increased p53 protein in primary keratinocytes was due to both transcriptional and posttranscriptional mechanisms. The p53 message levels were 2–3-fold greater in NHEKs than NHFs (Fig. 1), and the half-life of p53 in cultured keratinocytes was extended to approximately 3 h, as has been observed previously (8).

In some cells, the accumulation and stabilization of wt p53 protein mediates cell cycle arrest and has been reported to do so by transcriptional activation of other cellular proteins such as p21, an inhibitor of cell cycle-dependent kinases (11, 12). Thus, the continuous growth of adherent NHEKs in the presence of high levels of p53 suggested an alternative mode of p53 regulation and function in these cells. To evaluate the regulatory control of p53 in NHEKs *in vitro*, we exposed the cells to DNA damage, which typically increases the steady-state levels and transcriptional activity of wt p53 protein. Because adherent NHEKs already exhibit high levels of p53, we wanted to determine whether ionizing radiation could additionally elevate these levels. NHEKs and NHFs from the same individual were exposed to 400 rad of  $\gamma$ -irradiation, and p53 protein levels were compared by Western blot at 2, 4, and 8 h posttreatment (Fig. 2A). In NHFs, accumulation of p53 protein was evident within 2 h following treatment and remained elevated. In contrast, treated NHEKs that were obtained from the same individual did not display a change in p53 protein levels. However, basal levels of p53 protein in NHEKs were still greater than induced levels in NHFs.

To determine whether p53 was transcriptionally active in NHEKs, the expression of endogenous p53-regulated genes, p21 and MDM2, was also examined by Western (Fig. 2) and Northern (data not shown) blots. Despite the fact that p53 levels exceeded those in NHFs by as

much as 10-fold, p21 and MDM2 mRNA did not differ greatly among the three keratinocyte and two fibroblast cell populations tested. MDM2 mRNA levels were essentially equivalent among these five cell populations, whereas p21 message did differ between NHFs and NHEKs by 1–3.5-fold. Exposure of both NHEKs and NHFs to ionizing radiation resulted in increased mRNA as well as protein levels (Fig. 2) of these p53-regulated genes by 4 h following treatment. The level of p21 mRNA increased by 2-fold in  $\gamma$ -irradiated NHEKs and by 6-fold in  $\gamma$ -irradiated NHFs. Thus, with regard to transcription of these genes, p53 protein appeared to be largely inactive in NHEKs unless provided with a stimulus such as ionizing radiation.

The endogenous expression of p21 and MDM2 mRNA in proliferating NHEKs or those exposed to DNA damage indicated that the high levels of p53 did not correlate with p53 transcriptional control of these genes. However, p53 has been also shown to inhibit cell cycle progression following DNA damage through mechanisms that may not be evident in transcriptional assays. For example, p53-dependent apoptosis is a response to DNA damage that is characteristic of specific cell types (1). To determine the cell cycle profiles in response to DNA damage, flow cytometry was performed on NHFs and NHEKs that were treated with 400 rad of ionizing radiation (data not shown). NHFs exhibited delays in the G<sub>1</sub> and G<sub>2</sub> phases of the cell cycle 24 h after exposure to ionizing radiation and remained arrested without evidence of apoptosis. S-phase was typically reduced from 20–30% to less than 2%. In contrast, S-phase in NHEKs, which is normally comprised of 30% of the cell population, could remain as high as 20%. The subdiploid (<2N) G<sub>1</sub> content was always less than 5% in both cell types 24 h following treatment. These results indicated that S-phase may be decreased in irradiated NHEKs but not as efficiently as in treated NHFs.

Tumor cells that retain wt p53 use different mechanisms to modulate p53 growth-inhibitory properties. Cytoplasmic sequestration is one way in which some tumor types are thought to attenuate wt p53 function (13). To determine whether subcellular distribution was important in regulating p53 protein function in NHEKs, immunohis-

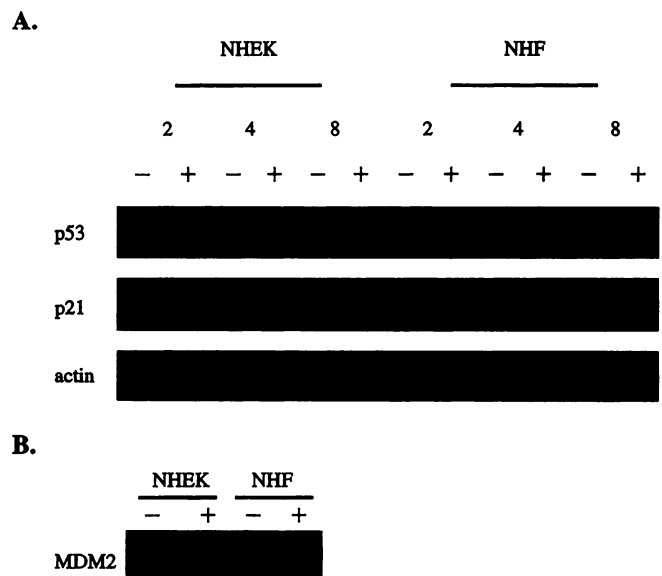


Fig. 2. Steady-state levels of p53 protein but not p21 are maximally induced in adherent keratinocytes. Keratinocytes and dermal fibroblasts from a single individual were exposed to 400 rad of ionizing radiation. Cells were collected following treatment at the time points indicated. A, protein lysates were analyzed by Western blot for p53 and p21 protein levels. Actin was used as a control for loading. B, Western blot analysis of 20  $\mu$ g of protein prepared from the cell populations in A to compare MDM2 protein levels in cycling cells (Lanes -) and cells harvested 4 h after exposure to 400 rad of ionizing radiation (Lanes +).

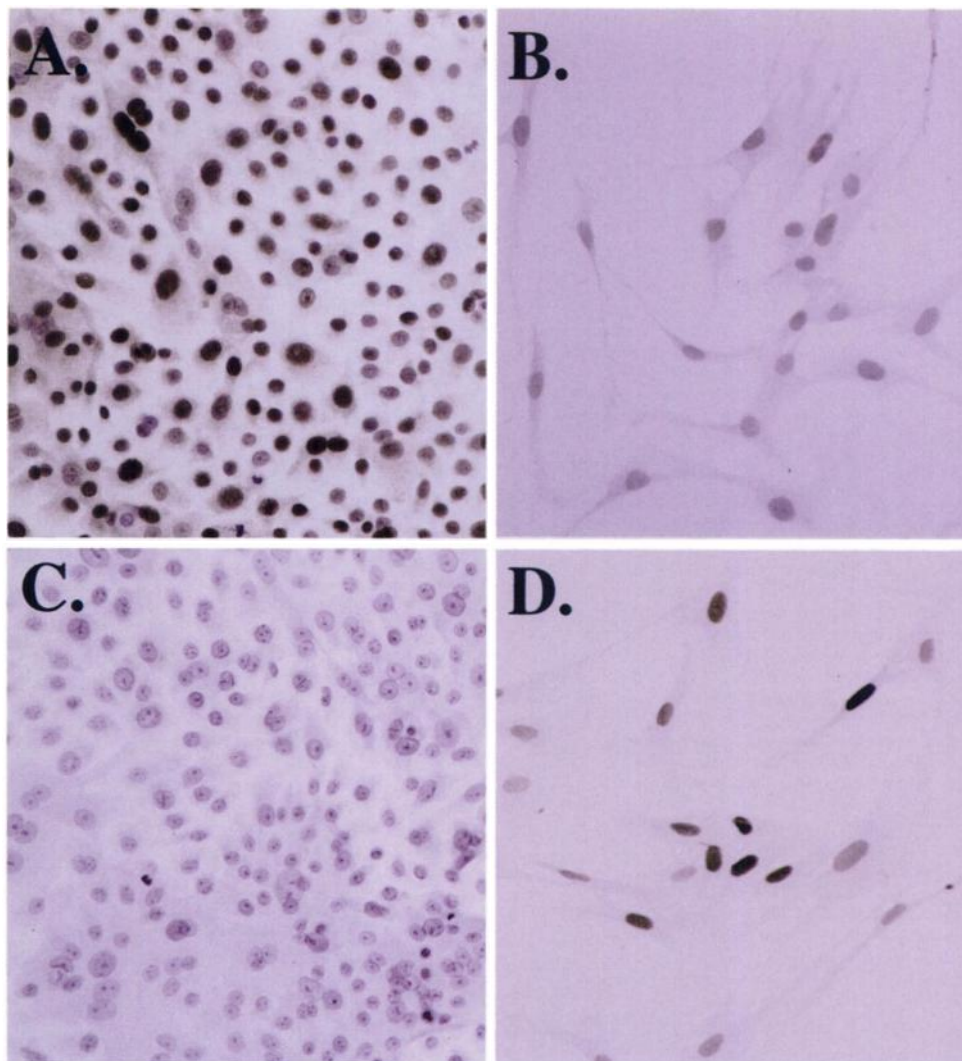


Fig. 3. Subcellular localization of p53 in primary human keratinocytes by immunohistochemistry. Fixed NHEKs (A and C) and NHFs (B and D) were incubated with DO-1 in A, B, and D. Primary antibody was eliminated in C for a negative control. NHFs grown for 3 days in the presence of  $99 \mu\text{M}$  *N*-phosphonoacetyl-L-aspartate, a reagent which stabilizes p53 protein (1), were used as a positive control for nuclear staining of p53 in D. Detection of the primary antibody was performed with the avidin-biotin-horseradish peroxidase complex of the Vectastain Elite ABC Kit (Vector Laboratories). Cells were then counterstained with hematoxylin and mounted.

tochemistry was performed on both NHEKs and NHFs. In keratinocyte cell populations tested, p53 was localized to the nucleus and was detectable with immunoperoxidase staining, indicating that aberrant localization was not responsible for the keratinocyte regulation of p53 activity (Fig. 3A). NHFs had minimal nuclear p53 (Fig. 3B), which was up-regulated in cells following exposure to a DNA-damaging agent (Ref. 1; Fig. 3D). These results are consistent with the observation that nuclear p53 protein was detectable by immunohistochemistry in cultured primary mammary epithelial cells but was much weaker in their stromal counterparts (6). Although p53 was found to be nuclear in NHEKs, its growth-inhibitory functions may in part be limited in NHEKs by increased levels of MDM2 protein (Fig. 2B), a cellular protein that physically associates with p53 and negatively regulates its activity (14, 15).

High levels of p53 protein have previously been associated with proliferating keratinocytes in culture (9), but little is known about the pathways that mediate regulation of p53 protein in this cell type. Two lines of evidence prompted us to consider that accumulation of p53 protein in NHEKs may be due to cellular adhesion processes that are activated upon introduction of these cells to tissue culture. First, cellular proliferation is tightly regulated by signaling that is mediated through the processes of cellular adhesion to a substratum (16). The activation of specific transmembrane proteins, the integrins, mediate these responses and collaborate with growth factors to provide the mitogenic stimulus for cells to enter the cell cycle (16). Second, levels

of p53 have been shown to decrease during differentiation of keratinocytes *in vitro* by high levels of calcium (9). The differentiation of keratinocytes *in vivo* is a process largely driven by changes in adhesive properties to extracellular matrix components, and in fact, it can be reproduced *in vitro* by releasing adherent keratinocytes into suspension culture (17). Therefore, to directly test the role of adhesion on p53 protein levels, NHEKs were subjected to periods of incubation in the absence of a substratum and subsequently reintroduced to adherent conditions. Cells were suspended by trypsinization and reintroduced in culture to plates that had been coated with poly-2-hydroxyethyl-methacrylate, a polymer that inhibits reattachment of cells to plastic (17). A dramatic 5-fold reduction in p53 protein levels in NHEKs was immediately evident upon harvesting of the cells (Fig. 4A). Low p53 levels were observed in cells for as long as they were maintained in suspension culture, with further decrease apparent after 24 h (Fig. 4A). This result is consistent with recent observations made in primary human ectocervical keratinocytes (18). In contrast, p53 protein levels in a human fibroblast population were not altered when cells were placed in suspension (Fig. 4A). To determine whether the decrease in p53 was reversible, NHEKs were placed in suspension for different time periods, allowed to reattach to plastic, and collected 24 h later. NHEKs that had been in suspension for as long as 8 h still retained the ability to accumulate 60% of the original p53 protein levels when replated (Fig. 4A). We noted that 5 h on plastic was sufficient for p53 protein to accumulate to 80% of the original levels in NHEKs fol-

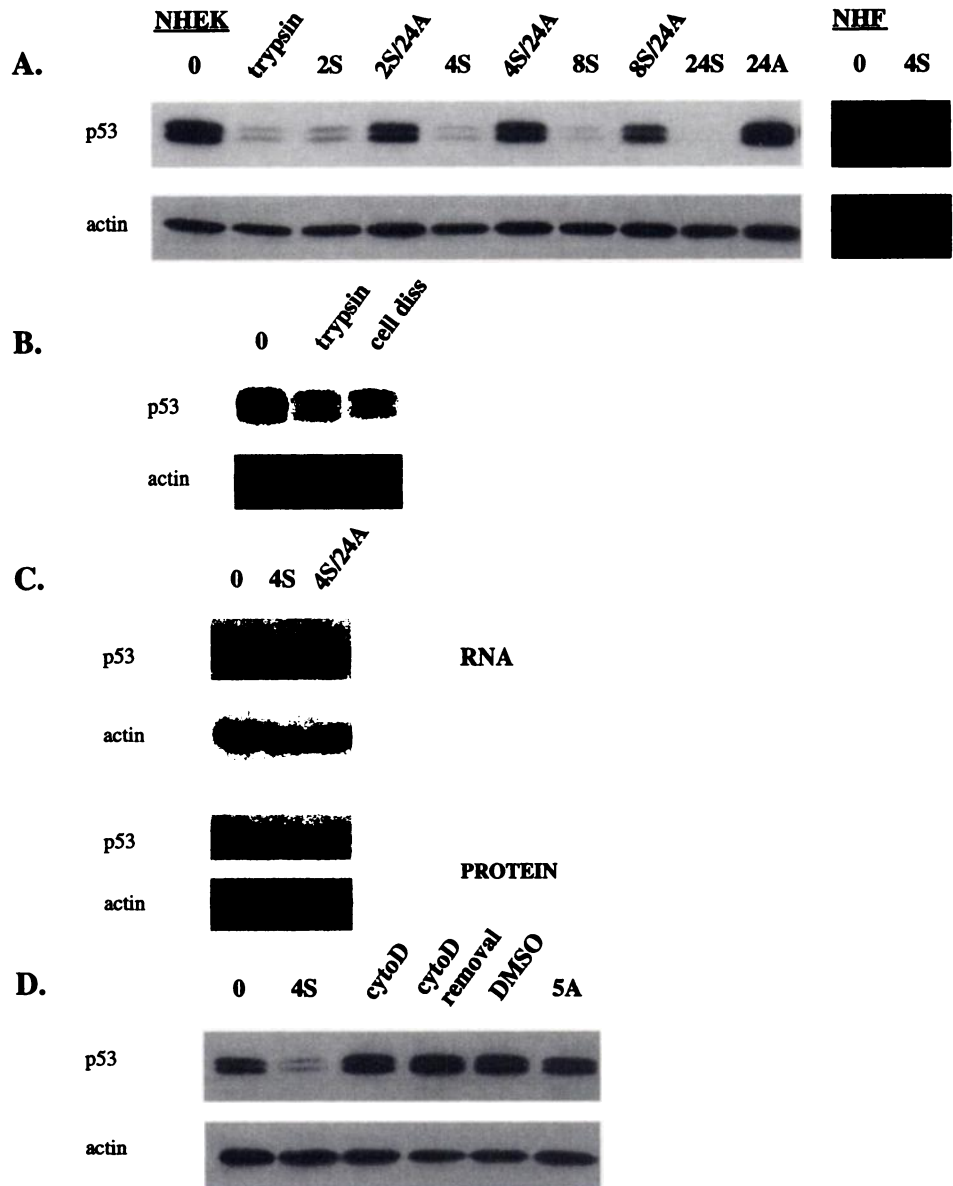


Fig. 4. p53 protein levels reversibly decrease in keratinocytes upon loss of adhesion to a substratum. **A.** Western blot analysis (as in Fig. 1) of adherent (**A**) and suspended (**S**) NHEKs. The doublet for p53 represents two polymorphic alleles of the gene in this individual. Lanes contain protein from cells in suspension for 2, 4, 8, or 24 h (**S**) or protein from cells replated for 24 h following suspension for 2, 4, and 8 h (**S/A**). The corresponding 24-h time point was not obtained because cells were no longer adherent. Plated cells were harvested at time zero by cell scraping (**0**) or trypsin or after 24 h on plastic (**24A**). All replated cells were harvested by cell scraping. Tissue specificity is illustrated by comparison of p53 protein levels in plated NHFs (**0**) and NHFs in suspension for 4 h (**4S**). **B.** Western blot analysis of p53 protein levels in cells trypsinized or harvested by nonenzymatic dissociation (*cell diss*; Life Technologies, Inc.) and placed in suspension for 4 h. p53 protein levels are compared to those in plated NHEKs (**0**). **C.** Northern blot analysis of 10  $\mu\text{g}$  of total RNA prepared from adherent (**0**) or suspended NHEKs (**4S**) and cells plated for 24 h following a 4-h incubation in non-adherent culture (**4S/24A**). p53 protein levels in the same cells were determined by Western blot analysis below. The relative expression levels of p53 message between lanes was 1. **D.** Western blot analysis of NHEKs placed in suspension for 4 h (**4S**), plated for 5 h (**5A**), or plated in the presence of 5  $\mu\text{M}$  cytochalasin D (*cytoD*) or DMSO after a 30-min preincubation. DMSO was used as a solvent control for cytochalasin D. All replated cells were collected by cell scraping after 5 h. In addition, p53 protein levels were assayed in cells treated with cytochalasin D for 5 h and replaced with fresh medium for 24 h (*cytoD removal*).

lowing 4 h in suspension culture. To test whether the decrease in p53 levels was a direct result of trypsinization or loss of adhesion in general, a second method of cell detachment was used. The decrease in p53 protein levels was reproduced in cells that were dispersed with a nonenzymatic cell dissociation buffer (Fig. 4B). Finally, p53 protein levels decreased through posttranscriptional mechanisms because p53 message levels did not differ between adherent and nonadherent keratinocytes (Fig. 4C).

The process of cell adhesion involves two phases: attachment to a substratum, followed by cellular spreading, which is mediated in large part by the actin cytoskeleton. Cytochalasin D inhibits actin polymerization, which disrupts the cytoskeleton, and some molecular events that are associated with adhesion-dependent signaling (19). To determine whether p53 protein levels were dependent on cellular attachment or cellular spreading, NHEKs were replated following a 30-min preincubation at 37°C with 5  $\mu\text{M}$  cytochalasin D. Under these conditions, the cells retained a rounded morphology but adhered to the dish. p53 levels at 5 h in cytochalasin D were comparable to those in fully spread cells, indicating that accumulation of p53 protein was independent of actin cytoskeletal reorganization (Fig. 4D) but dependent upon attachment.

## Discussion

Here, we have demonstrated that increased p53 protein levels are paradoxically associated with proliferating human keratinocytes *in vitro*. The p53 protein appears to be impaired in terms of its transcriptional properties in proliferating cells but may be functionally activated through experimentally induced DNA damage. Increased levels of p53 protein were in part regulated by cellular adhesion pathways activated upon the introduction of primary keratinocytes to culture conditions. These results indicate that the p53 pathway in epithelial cell types is influenced by anchorage-dependent processes and that checkpoint control may be a property altered through epigenetic mechanisms including tissue specific gene expression.

Our results indicate that the levels and function of p53 protein in proliferating primary human keratinocytes can be regulated by specific extracellular signals. These studies provide a link between the extracellular matrix and the expression of an important tumor suppressor gene. In this regard, the tumor suppressor protein, adenomatous polyposis coli, by interacting with  $\beta$ -catenin, is an additional example of a link between cell adhesion and tumor suppression (20, 21). A variety of transmembrane proteins, including the integrins,

mediate cellular functions of keratinocytes requiring specific interactions with the extracellular matrix and other cell types (17, 22). However, activation of processes mediated by adhesion molecules is often the result of a cooperation between different signaling pathways, and therefore, the accumulation of p53 protein may be dependent on the presence of specific growth factors as well (16).

The absence of wt p53 function has been demonstrated to profoundly disrupt processes critical to the maintenance of genomic stability in mammalian cells (1). Therefore, the relative inefficiency of checkpoint control exhibited by normal epithelial cells *in vitro* may reflect a predisposition to undergo genetic change, and that genomic instability in normal cells and cancer-prone syndromes may be a phenotype evident only in the context of specific interactions of the cell with its environment. The attenuation of cell cycle checkpoint control in primary keratinocytes may be facilitated through the simultaneous overexpression of MDM2, a negative regulator of p53 function (14), which has been previously implicated in neoplastic progression of human sarcomas (15). Although this cellular environment may be favorable for the acquisition of genetic changes, the actual source of DNA damage remains unclear and will be a major determinant in whether neoplastic progression proceeds.

It will be important to determine which, if any, physiological processes are mirrored in this *in vitro* system. Processes such as wound healing, which require epithelial cell proliferation, may parallel culture conditions. In fact, some chronic disease states that involve persistent epithelial injury and subsequent tissue reorganization are prone to malignant progression (23). Furthermore, although we and others have observed the absence or infrequent nuclear staining of p53 by immunohistochemistry in the basal layer of normal skin (24), alterations in adhesion signaling may be an additional mechanism to consider for strong nuclear staining of p53 observed in the basal layer of epithelium in some chronic skin disorders (24). Therefore, the accumulation of wt p53 protein may be a critical marker for alternative protein interactions rather than evidence of DNA damage and consequently may perform an unknown function in epithelial cells undergoing tumorigenesis or other proliferative processes. In support of a required function early in tumorigenesis, chemically induced skin papillomas in mice formed less efficiently in a p53 null background (25). In addition, the spontaneous formation of papillomas in transgenic animals expressing oncogenes from a tissue-specific promoter did not occur in the p53 null background (26). It is also intriguing that, in the transgenic animals with wt p53, papillomas appeared most frequently in areas where wound healing was occurring, such as at the ear tag and in other areas subjected to constant scratching and biting of the skin (26). Why this molecular arrangement is unique to epithelial cell types remains to be determined. It has been previously suggested either that, in these mouse models of tumorigenesis, wt p53 may control expression of growth-promoting genes or that the level of DNA damage incurred during these processes in the absence of p53 may simply be prohibitive to cell growth.

Because cellular adhesion mediates processes required throughout the lifetime of an organism, anchorage-dependent expression of p53 protein in keratinocytes provides a powerful pathway in which to explore diverse mediators of p53 function.

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