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$p21^{WAF1/CIP1}$ Expression Correlates with Calcium-induced Differentiation in Normal Human Oral Keratinocytes

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Primary normal human oral keratinocytes (NHOK) undergo differentiation in the presence of higher calcium level than 0.15 mM *in vitro*. Though detailed mechanisms of calcium-induced differentiation of normal epithelial cells remain unknown, it may be due to cell cycle arrest through induction of cyclin-dependent kinase (Cdk) inhibitors. To investigate this possibility, we cultured the NHOK with passage number 2 in medium containing 0.15, 0.3, 0.6, or 1.2 mM of calcium, and determined the degree of cell differentiation and expression of p53 and $p21^{WAF1/CIP1}$ in the cells. Calcium, in a dose-dependent manner, increased cell differentiation. Cellular p53 protein level was not changed notably after exposure to calcium, while the level of cellular $p21^{WAF1/CIP1}$ was significantly enhanced by the exposure. These data indicate that $p21^{WAF1/CIP1}$ may be involved in calcium-induced differentiation of NHOK through overexpression of this Cdk inhibitory protein in response to a p53-independent differentiation signal.

Key words : oral keratinocytes, differentiation, calcium, $p21^{WAF1/CIP1}$, p53

Introduction

Cell growth and differentiation are two fundamental aspects of multicellular existence, and intertwining these processes is the phenomenon of unlimited growth which is the basis of the neoplastic state. Cancer is now believed to result from unlimited growth of a given cell which is often due to a block in the ability of cells to undergo differentiation and/or apoptosis (Grana and Reddy, 1995). The cell division cycle is typically divided into four phases. The periods associated with DNA synthesis (S phase) and mitosis (M phase) are separated by gaps of varying length called G_1 and G_2. The signalling pathways that regulate cell cycle progression seem to be primarily associated with the G_1 phase of the cell cycle. All eucaryotes appear to be subject to extracellular signals until a specific point in G_1 is reached, called "restriction point" in mammalian cells (Pardee, 1989). In higher eucaryotes, the degree of complexity due to cellular specialization results in a substantial increase in the number of different signals that must be integrated during G_1, where cells can eventually withdraw from the cell cycle to a G_0 state and undergo differentiation, which was supported by reports that differentiation is frequently characterized by G_1 arrest; differentiating cells manifest prolonged G_1 phase of the cell cycle and inhibition of G_1-S transition (Chen *et al.*, 1989; Pardee, 1989). However, the mechanisms responsible for cell cycle arrest in differentiating cells are poorly understood.

Progression through the cell cycle is controlled by activation of a series of kinase holoenzymes composed of a regulatory subunit, called cyclin, and a catalytic subunit, named cyclin-dependent kinases (Cdks). 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. In addition to positive regulation by cyclins, Cdk activity is regulated by phosphorylation or dephosphorylation of Cdk at specific residues, as well as by association with inhibitory proteins (Hunter, 1993). $p21^{WAF1/CIP1}$ negatively regulates the related Cdk activities (El-Deiry *et al.*, 1993) and can also inhibit DNA polymerase directly (Waga *et al.*, 1994). Hence, 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). The wild-type p53

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has been identified as a product of a tumor suppressor gene. The p53 protein functions in a variety of cellular processes, including growth arrest (Martinez *et al.*, 1991), apoptosis (Shaw *et al.*, 1992; Lowe *et al.*, 1993), senescence (Shay *et al.*, 1991; Bond *et al.*, 1994), and differentiation (Shaulsky *et al.*, 1991). Later on, it was demonstrated that $p21^{WAF1/CIP1}$ can be induced independently of p53 (Michieli *et al.*, 1994). The most recent data show that $p21^{WAF1/CIP1}$ expression correlates with terminal differentiation in several cell types (Jiang *et al.*, 1994; Steinman *et al.*, 1994). Therefore, $p21^{WAF1/CIP1}$ may be involved in cell cycle exit by inducing G_1 arrest in response to a p53-independent differentiation signal. On the other hand, it is not certain whether p53 is involved in cell differentiation (Spandau, 1994; Sebag *et al.*, 1994; Missero *et al.*, 1995).

Extracellular calcium levels influence the growth and differentiation of keratinocytes in culture (Hennings *et al.*, 1980; Pillai *et al.*, 1988; Pillai *et al.*, 1990). Low extracellular calcium levels induce their growth while physiological extracellular calcium levels induce their differentiation. Though the mode of calcium-induced differentiation remains speculative, two to three-fold increase in the intracellular calcium level is followed by enhanced extracellular calcium concentration from 0.05 to 1.2 mM (Hennings *et al.*, 1989). Enhanced intracellular calcium level may subsequently increase the formation of desmosomes (Hennings & Holbrook, 1983), the plasma membrane-bound transglutaminase activity, the cornified envelope formation (Lichti & Yuspa, 1988), and the involucrin level (Pillai *et al.*, 1990). Though detailed mechanisms of cell cycle control in calcium-induced differentiation of normal epithelial cells remain unknown, it may be due to cell cycle arrest through induction of $p21^{WAF1/CIP1}$. To investigate this possibility, we cultured the normal oral keratinocytes (NHOK) with passage number 2, exposed the cells to 0.15, 0.3, 0.6, or 1.2 mM of calcium, and determined the degree of cell differentiation and expression of $p21^{WAF1/CIP1}$ and p53 in the cells.

Materials and Methods

Cell Culture

To prepare primary NHOK, excised gingival tissue was washed in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS, Gibco/BRL, Grand Island, NY, U.S.A.). To separate the epithelium from the underlying mucosa, the tissue was incubated in CMF-HBSS containing collagenase (type II; 1.0 mg/ml, Sigma, St. Louis, MO, U.S.A.) and dispase (grade II; 2.4 mg/ml, Boehringer-Mannheim, Indianapolis, IN, U.S.A.) for 90 min at 37°C in an atmosphere of 95% air and 5% CO_2. Separated epithelial sheets were then dissociated into single cells by incubation in trypsin-EDTA with agitation at 37°C for 8 min. The cells were washed with CMF-HBSS, resuspended with keratinocyte growth medium (KGM, Clonetics Corp., San Diego, CA, U.S.A.), and plated at 2×10^5 cells per 60-mm Petri dish. Primary cells were subcultured and the assays were performed using the NHOK with passage number 2.

Light microscopic examination of cells

The cultures were washed twice with CMF-HBSS and fixed *in situ* with 2.5% glutaraldehyde solution for 1 h at room temperature. After washing twice with 0.1 M cacodylate buffer, the microscopic features of the cells were photographed.

Determination of transglutaminase activity and detection of cornified envelope

To determine the effect of calcium on cell differentiation, determination of transglutaminase activity and detection of cornified envelope were assayed. The activity of transglutaminase, an enzyme catalyzing the γ-glutamyl(ε-lysyl) cross-link, was determined according to the method of Schmidt *et al.* (1985). When the cultures were approximately 70% confluent, the media were replaced with fresh KGM containing 0.15, 0.3, 0.6, or 1.2 mM of calcium. After three days of culture, the cells were washed with phosphate buffered saline (PBS), harvested, homogenized by sonication in 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 0.1% Triton X-100, and then centrifuged at 12,000×g for 30 min. The pellet was resuspended in the same buffer and 100 μl of the suspension was incubated with 600 μl of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM calcium chloride, 5 mM dithiothreitol, 540 μg/ml dimethyl casein, 1 mM putrescine, and 2.5 μCi [^{3}H]putrescine (5-40 Ci/mmol, Amersham Corp., Arlington Heights, IL, U.S.A.) for 30 min at 37°C. The reaction was stopped by the addition of 600 μl of 10% TCA, and the ^{3}H-putrescine radioactivity associated with the precipitated casein was quan-

titated using a β-scintillation counter. The specific radioactivity was calculated as cpm putrescine incorporated into casein per μg of protein.

The cornified envelope formation, a marker of epidermal differentiation, was determined according to the method of Cline & Rice (1983). When the cultures were approximately 70% confluent, the media were replaced with fresh KGM containing 0.15, 0.3, 0.6, or 1.2 mM of calcium. After three days of culture, the cells were washed with PBS, harvested, counted, and then centrifuged at 1,000×g for 5 min. The pellet was resuspended in 1% SDS and 20 mM dithiothreitol and the suspension was incubated for 20 min at room temperature. After centrifugation, the pellet was resuspended in a small volume of PBS, and scored for envelopes by phase-contrast microscopy. The percentage of cornified cell envelopes were determined by number of 100× cornified cell envelopes/total number of cells. Average percentage and standard deviation were calculated from three independent experiments.

Cell viability

To determine the effect of calcium on cellviability, cells were plated at 2×10^5 cells per 60-mm Petridish. When the cultures reached 70% confluency, cells were exposed to 0.15, 0.3, 0.6, or 1.2 mM of calcium for 3 days. Cells were harvested by trypsinization to count the number of viable cells after trypan blue exclusion. The percentage of viable cells were determined by number of 100× cells not stained with trypan blue/total number of cells. Average percentage and standard deviation were calculated from four independent experiments.

Western analysis

To investigate the effect of calcium on expression of $p21^{WAF1/CIP1}$ and p53, western blot analysis was performed. Cells grown in 100-mm Petri dishes were lysed in a lysis buffer [10 mM Na_2HPO_4 (pH 7.2), 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide, and 0.004% sodium fluoride] on ice for 15 min. The cell lysate was centrifuged at 15,000×g for 20 min, and the supernatant containing 1 mg/ml of protein was denatured by boiling for 2 min in a sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.001% bromophenol blue]. An aliquot of the denatured supernatant containing 100 μg of protein was electrophoresed in a 15% or 10% SDS-polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA, U.S.A.). After incubation in a blocking buffer (0.2% I-block, phos-

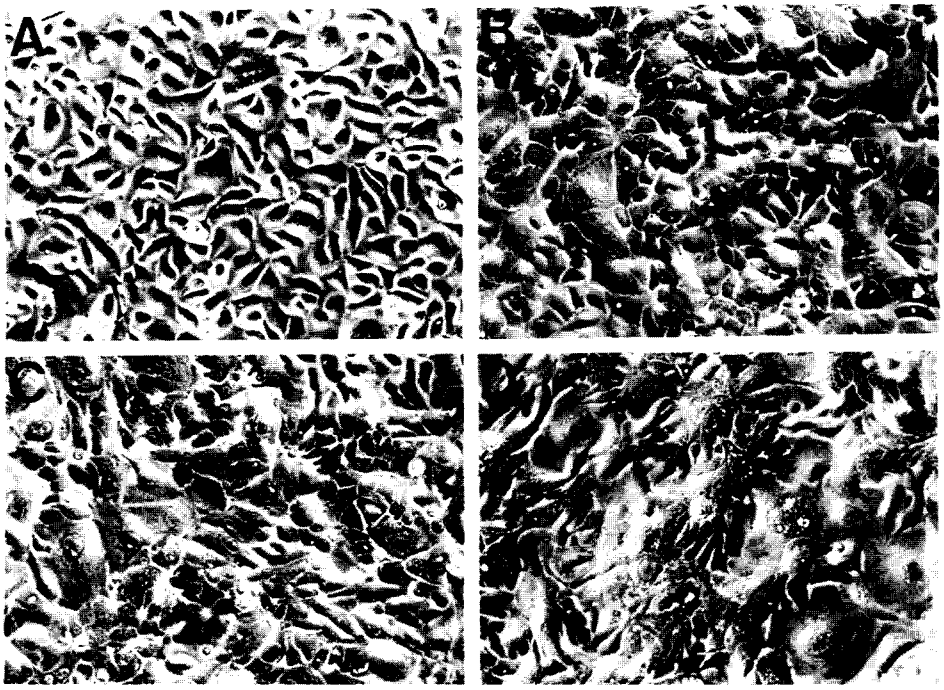

Fig. 1. Microscopic features of NHOK that were cultured in KGM containing 0.15 (A), 0.3 (B), 0.6 (C), or 1.2 mM (D) calcium for 3 days (×150).

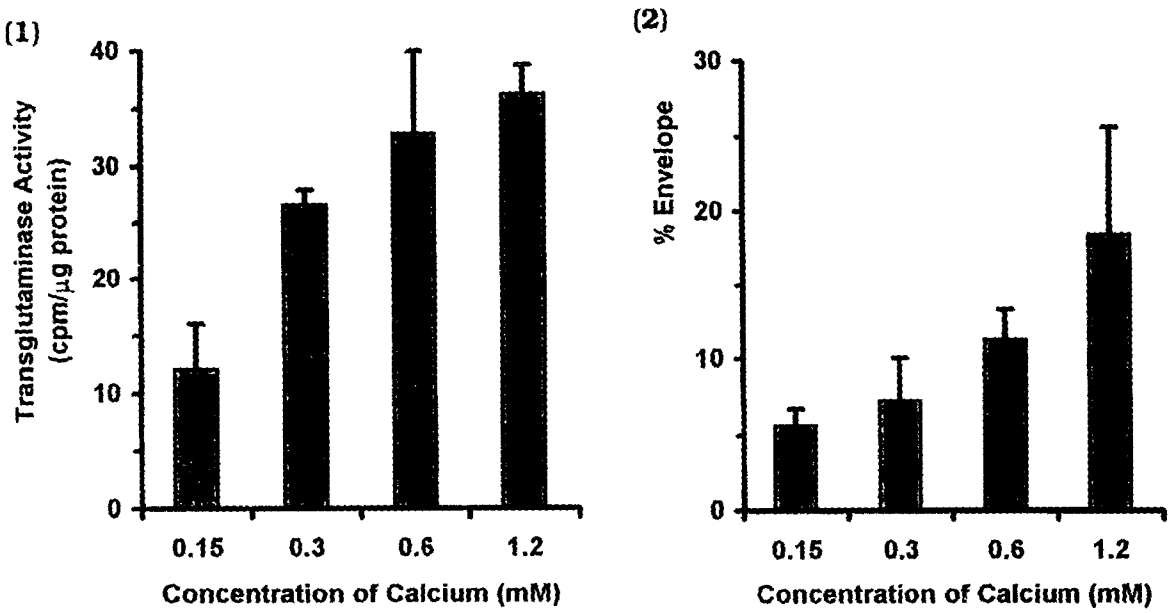

Fig. 2. Calcium-induced keratinocyte differentiation. (1) Effect of different extracellular calcium concentrations on the transglutaminase activity. (2) Effect of extracellular calcium concentrations on cornified cell envelope content. The cells were cultured in KGM containing 0.15, 0.3, 0.6, or 1.2 mM calcium for 3 days, and harvested for assaying the activity of transglutaminase and the percentage of cornified cell envelope. Values represent averages from three independent experiments and standard deviations.

phate buffered saline, and 0.05% Tween 20) for 1 h at room temperature, the membrane was exposed to mouse anti-human $p21^{WAF1/CIP1}$ antibody (187, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or mouse anti-human monoclonal p53 antibody (Ab-2, Oncogene Science, Uniondale, NY, U.S.A.) at room temperature for 1 h. After washing with the blocking buffer, the membrane was treated with anti-mouse IgG-alkaline phosphatase conjugated antibody (Tropix Inc., Bedford, MA, U.S.A.), and again washed four times with the blocking buffer and an assay buffer (0.1 M diethanolamine and 1 mM $MgCl_2$). The membrane was then incubated in Nitroblock reagent, washed again with the assay buffer, incubated in chemiluminescent substrate solution using the Western-Light kit (Tropix Inc.), and exposed to Hyperfilm-MP (Amersham Corp.) at room temperature.

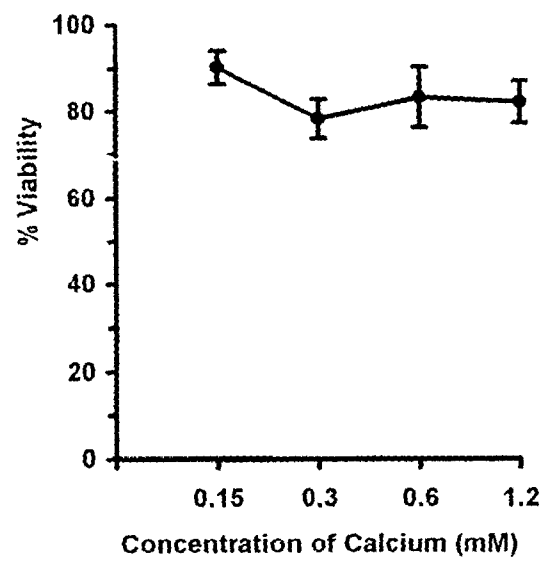

Fig. 3. Effect of different extracellular calcium concentrations on the cell viability. The cells were cultured in KGM containing 0.15, 0.3, 0.6, or 1.2 mM calcium for 3 days, and viable cells were counted in a hemocytometer after trypan blue exclusion. Viability was expressed as percentage of total cell numbers. Values represent averages from four independent experiments and standard deviations.

Results

Effect of calcium on morphology, cell differentiation and cell viability

Fig. 1 shows the micrographic features of NHOK cultured in KGM containing 0.15, 0.3, 0.6, or 1.2 mM of calcium. The 2nd passage of NHOK displayed a typical keratinocyte morpholgy and retained an undifferentiated phenotype with distinct intercellular space (Fig. 1A). In the presence of calcium higher than 0.15 mM, the cells began to differentiate and the morphology of the cells were

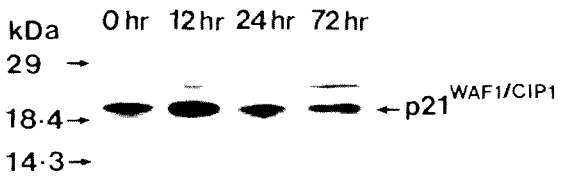

Fig. 4. Western blot analysis of electrophoretically separated *WAF1/CIP1* protein from NHOK after exposure to 1.2 mM of calcium for 0 h, 12 h, 24 h, or 72 h.

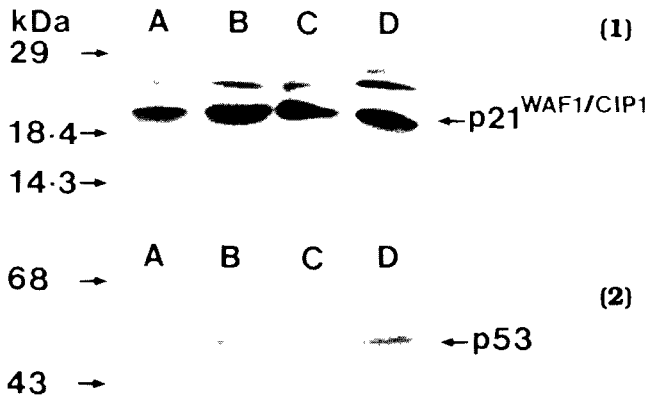

Fig. 5. Levels of *WAF1/CIP1* and p53 proteins in NHOK that were cultured in KGM containing 0.15 (A), 0.3 (B), 0.6 (C), or 1.2 mM (D) calcium for 12 h. (**1**) Western blot analysis of electrophoretically separated *WAF1/CIP1* protein. (**2**) Western blot analysis of electrophoretically separated p53 protein.

notably changed. Calcium also significantly increased the percentage of cornified cells and transglutaminase activity of the cells in a concentration-dependent manner (Fig. 2). As shown in Fig. 3, the cells grown in calcium higher than 0.15 mM produced a slight loss of viability, but the difference was not significant. On the other hand, calcium did not show the characteristics of apoptosis; chromatin condensation and DNA fragmentation did not occur (data not shown). These data showed that increase of the extracellular calcium level induces differentiation of NHOK. However, calcium may not cause apoptotic changes of NHOK.

Effect of calcium on expression of p21$^{WAF1/CIP1}$ and p53 protein

As shown in Fig. 4, p21$^{WAF1/CIP1}$ protein was induced significantly as early as 12 h after exposure of 1.2 mM calcium, and returned to original levels by 72 h. This induction was occurred by as low as 0.3 mM calcium and the increased level was not altered by higher calcium concentration (Fig. 5A). While calcium induced p21$^{WAF1/CIP1}$ protein, p53 protein level was not altered notably after exposure to calcium (Fig. 5B).

Discussion

The present data show that exposure of primary NHOK that normally proliferate in medium containing low level of calcium (0.15 mM) to higher level of calcium than 0.15 mM results in cell differentiation. After exposure to high calcium, NHOK showed altered cell morphology, enhanced cornified envelope formation, and increased transglutaminase activity. Calcium, however, may not cause apoptotic change of the cells during the experimental period, consistent with prior report of Steinman *et al.* (1994). The molecular bases of calcium-induced differentiation of NHOK remain largely unknown, but we attempted to unveil the mechanisms by determining the expression of genes that are associated with the cell cycle arrest in eucaryotic cells. First, inasmuch as p21$^{WAF1/CIP1}$ is induced during differentiation in certain cell types (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Parker *et al.*, 1995) and arrests the cell cycle progression (El-Deiry *et al.*, 1993), we determined the expression of *WAF1/CIP1* from the calcium-treated NHOK. As expected, the exposure of NHOK to calcium notably enhanced the expression of p21$^{WAF1/CIP1}$ in NHOK. p21$^{WAF1/CIP1}$ protein peaked around 12 h, and returned to original levels by 24 h, consistent with previous results demonstrating no induction of *WAF1/CIP1* mRNA at 24 h following Ca^{2+} shift (Weinberg *et al.*, 1994). p21$^{WAF1/CIP1}$ protein induction by calcium occurred days before terminal differentiation. Inhibition of Cdk activity by p21$^{WAF1/CIP1}$ depends on the relative stoichiometric amounts of this inhibitor in the complex (Zhang *et al.*, 1994). Although further examinations shall be done whether differentiated keratinocytes induced by calcium manifest G_1 arrest, these observations suggest that enhanced p21$^{WAF1/CIP1}$ protein level may be involved in cell cycle exit by inducing G_1 arrest in response to calcium, which induces differentiation of oral keratinocytes.

We also determined the expression of p53 because wild-type p53 could induce p21$^{WAF1/CIP1}$ and arrests the cell cycle progression (Martinez *et al.*, 1993). Several reports suggest that p53 could also be important in regulating epidermal differ-

entiation; increasing medium calcium concentration produced marked elevation of p53 mRNA in normal human skin keratinocytes (Sebag *et al.*, 1994), human foreskin keratinocyte cultures overexpressing wild-type p53 exhibited aberrant expression of the differentiation-specific markers involucrin and keratin 1 (Woodworth *et al.*, 1993), and p53 protein was present in the nucleus of the keratinocytes in the granular layer of cholesteatoma epithelium (Shinoda and Huang, 1995). Present study showed that p53 protein level is not altered in calcium-induced differentiation.

Involvement of p53 in differentiation is controversial, especially on induction of $p21^{WAF1/CIP1}$. Induction of $p21^{WAF1/CIP1}$ expression was initially considered to be dependent upon wild-type p53; $p21^{WAF1/CIP1}$ is transcriptionally induced by overexpression of p53 (El-Deiry *et al.*, 1993), or by activation of p53 after DNA damage (El-Deiry *et al.*, 1994), consistent with a role for $p21^{WAF1/CIP1}$ in the p53-dependent G_1 checkpoint. However, recent studies implicate that this assuption must be reevaluated. These include that mitogens transiently stimulate $p21^{WAF1/CIP1}$ expression in quiescent fibroblasts from p53 knock out mice (Michieli *et al.*, 1994), that differentiation is able to proceed in the absence of p53 (Donehower *et al.*, 1992; Jiang *et al.*, 1994), and that $p21^{WAF1/CIP1}$ expression is triggered by multiple differentiation-inducing agents in hematopoietic cells, hepatoma cells, myoblast, and keratinocytes through a p53-independent pathway (Steinman *et al.*, 1994; Missero *et al.*, 1995; Parker *et al.*, 1995). Therefore, it is accepted that p21 protein may function as an inducible growth inhibitor which contributes to differentiation in a p53-independent manner. Our results, together with prior reports, support that $p21^{WAF1/CIP1}$ may be involved in calcium-induced differentiation of NHOK through overexpression of this Cdk inhibitory protein in response to a p53-independent differentiation signal.

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