

Green Tea Polyphenol-Induced Epidermal Keratinocyte Differentiation Is Associated with Coordinated Expression of p57/KIP2 and Caspase 14

Stephen Hsu, Tetsuya Yamamoto, James Borke, Douglas S. Walsh, Baldev Singh, Sushma Rao, Kamatani Takaaki, Nam Nah-Do, Carol Lapp, David Lapp, Emily Foster, Wendy B. Bollag, Jill Lewis, John Wataha, Tokio Osaki, and George Schuster

Department of Oral Biology and Maxillofacial Pathology (S.H., J.B., B.S., S.R., N.N.-D., C.L., E.F., J.L., J.W., G.S.), School of Dentistry, Department of Biochemistry and Molecular Biology (D.L.), and Institute of Molecular Medicine and Genetics (W.B.B.), Medical College of Georgia, Augusta, Georgia; Department of Oral Surgery (T.Y., K.T., T.O.), Kochi Medical School, Kochi, Japan; and Dermatology Service (D.S.W.), Eisenhower Army Medical Center, Fort Gordon, Georgia

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ABSTRACT

Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, exerts chemopreventive effects by selectively inducing apoptosis in tumor cells. In contrast, EGCG accelerates terminal differentiation in normal human epidermal keratinocytes (NHEK) mediated partially by up-regulation of p57/KIP2, a cyclin-dependent kinase inhibitor that confers growth arrest and differentiation. However, it is unclear if EGCG modulates caspase 14, a unique regulator of epithelial cell terminal differentiation associated with cornification. Here, we examined the effect of EGCG on caspase 14 expression in NHEK and correlated the protein and mRNA expression of p57/KIP2 with those of caspase 14 in either normal keratinocytes or p57/KIP2-expressing tumor cells (OSC2, an oral squamous cell carcinoma cell line). Additionally, paraffin-embedded normal and untreated psoriatic (aberrant keratinization) skin

sections from humans were assessed for caspase 14 by immunohistochemistry. In NHEK, EGCG induced the expression of caspase 14 mRNA and protein levels within a 24-h period. The expression of p57/KIP2 in OSC2 cells was adequate to induce caspase 14 in the absence of EGCG; this induction of caspase 14 was down-regulated by transforming growth factor- β 1. In human psoriatic skin samples, caspase 14 staining in the upper epidermis was reduced, especially in nuclear areas. These results suggest that, in addition to p57/KIP2, EGCG-induced terminal differentiation of epidermal keratinocytes involves up-regulation of caspase 14. Further understanding of how EGCG modulates cellular differentiation may be useful in developing green tea preparations for selected clinical applications.

Unique characteristics of green tea polyphenols include their ability to induce growth arrest and apoptosis in tumor cells, especially in epithelial-type cells (Hsu et al., 2002b; Adhami et al., 2003), as well as protecting normal epithelial cells from carcinogens (Mukhtar and Ahmad, 2000; Katiyar, 2003). Among the four major polyphenols present in green tea, (-)-epigallocatechin-3-gallate (EGCG) is the most abundant (Miyazawa, 2000).

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We previously reported that green tea polyphenols, EGCG in particular, activate a pathway for cell differentiation in normal human epidermal keratinocytes (NHEK). Unlike epithelial-derived tumor cells, NHEK undergo an accelerated differentiation that is associated with p57/KIP2 induction when exposed to EGCG (Hsu et al., 2001, 2002a, 2003a). The p57/KIP2 gene product is a p53-independent G1 cyclin/cyclin-dependent kinase inhibitory protein (Lee et al., 1995); the C terminus contains a binding domain for proliferating cell nuclear antigen (Watanabe et al., 1998). Embryonic development in mice requires p57/KIP2 expression, lack of which causes early postnatal death and growth retardation (Takahashi et al., 2000). Conversely, in continuously dividing hu-

ABBREVIATIONS: EGCG, (-)-epigallocatechin-3-gallate; NHEK, normal human epidermal keratinocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TGF- β 1, transforming growth factor- β 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein.

man intestinal cell models, elevations of p57/KIP2 are associated with differentiation (Deschenes et al., 2001). Whereas other cyclin-dependent kinase inhibitors such as p16, p21, and p27 are proapoptotic (Opalka et al., 2002), p57/KIP2 confers growth arrest, differentiation, and cell survival.

In human epidermis, the stratum corneum consists of anucleated keratinocytes with cross-linked proteins and lipids that generate a mechanical barrier (Madison, 2003). This barrier formation relies on the cornification of epidermal keratinocytes, which undergo growth arrest, terminal differentiation, and an epidermal-specific apoptosis referred to by Nickoloff as "planned cell death" (Nickoloff et al., 2002). Abnormalities in any of these programmed features may manifest in skin disorders such as psoriasis or cancer. However, the mechanisms triggering proliferating keratinocytes to enter planned cell death are not completely understood (Martinez et al., 1999; Alonso and Fuchs, 2003).

Caspase 14, identified in 1998 first in mice and later humans (Ahmad et al., 1998), is expressed only in epithelial tissues, especially the differentiating regions of the outer epidermis. Unlike the other caspases, caspase 14 is not involved in the typical apoptotic cascade but is associated with terminal differentiation of NHEK and barrier formation (Eckhart et al., 2000b; Lippens et al., 2000). In addition to the epidermis, caspase 14 expression also is found in the choroid plexus, the retinal pigment epithelium, and thymic Hassall's bodies, tissues that function as barriers (Lippens et al., 2003). A series of elegant experiments showed that epidermal induction of caspase 14 at the transcriptional level occurs during stratum corneum formation, whereas inhibition of cell differentiation results in diminished caspase 14 expression (Eckhart et al., 2000a; Rendl et al., 2002). Thus, caspase 14 is thought to specifically modulate epidermal differentiation, possibly signaling terminal differentiation and cornification of the epidermis (Mikolajczyk et al., 2004). In psoriasis, whereby rapidly migrating keratinocytes result in abnormal cornification, caspase 14 expression is altered (Lippens et al., 2000).

It is unknown whether caspase 14 is involved in EGCG-induced keratinocyte differentiation or EGCG-induced p57/KIP2 expression. We hypothesized that EGCG activates a differentiation pathway in normal epidermal keratinocytes and an apoptotic pathway in tumor cells (Hsu et al., 2003c). Here, we investigated the relationship between EGCG-induced p57/KIP2 and caspase 14 expression using *in vitro* models of NHEK, an oral squamous cell carcinoma line (OSC2), and subclones derived from OSC2 with stable expression of p57/KIP2. Expression patterns of caspase 14 in normal and psoriatic human skin samples were also examined.

Materials and Methods

Chemicals and Antibodies. EGCG and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-human caspase 14 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-human p57/KIP2 antibody was purchased from BD Biosciences Pharmingen (San Diego, CA).

Cell Lines and Cell Culture. NHEK were purchased from Cambrex Corporation (East Rutherford, NJ) and subcultured in KGM-2 provided by the manufacturer. Subculture of the NHEK was per-

formed by detaching the cells in 0.25% trypsin and transfer into new tissue culture flasks. The human oral squamous cell carcinoma lines OSC2 and the subclones (G6, S1, S2, S3, S4, and S5) were maintained in DMEM/Ham's F-12 medium with 10% fetal calf serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 5 μ g/ml hydrocortisone.

Immunocytochemistry of NHEK. To evaluate the intracellular distribution patterns of caspase 14 in NHEK, exponentially growing NHEK were seeded in 8-well chamber slides (Nalge Nunc International, Naperville, IL) 12 h prior to EGCG treatment. At the end of a 24-h treatment, the slides were washed with PBS and fixed in ice-cold 4% paraformaldehyde solution for 10 min. Then, 3% hydrogen peroxide solution and normal goat serum were applied to block endogenous peroxidase activity and nonspecific binding. The caspase 14 antibody was applied for 1 h at 37°C at a dilution of 1:50. A streptavidin detection technique (Biogenex, San Ramon, CA) was used with 3-amino-9-ethylcarbazole as chromogen. Mayer's hematoxylin was used as a counterstain. Negative control slides consisted of cells treated with 1% diluted normal goat serum instead of primary antibody.

Human Skin Sample Collection and Processing for Caspase 14 Expression. Under an approved human use protocol, 4-mm punch biopsies of normal ($n = 3$) and untreated psoriatic skin samples ($n = 6$) were obtained from different patients who provided written, informed consent. Samples were fixed in 10% neutral-buffered formalin, paraffin embedded, sectioned at 5 μ m, and immunostained for caspase 14 presence by immunohistochemistry using a modified avidin-biotin-peroxidase technique described previously (Hsu et al., 2003a). The paraffin sections were deparaffinized in limonene (Sigma-Aldrich) and rehydrated in a descending series of ethanol to water. Endogenous peroxidase activity was blocked by a 5-min incubation in 3% H₂O₂. Nonspecific binding of the antibody to tissue sections was blocked by incubation for 1 h in 10 mg/ml bovine serum albumin. Rabbit anti-caspase 14 (Santa Cruz Biotechnology, Inc.) at a dilution of 1:50 was applied to the sections for 1 h. Control sections were treated with normal goat serum instead of the anti-caspase 14 antibody. After washing in PBS, a 1:200 dilution of the secondary biotin-conjugated goat anti-rabbit immunoglobulin antibody (Vector Laboratories, Burlingame, CA) was applied for 30 min. The sections were washed in PBS and then incubated for 30 min in an avidin-peroxidase complex reagent (ABC reagent; Vector Laboratories). Following three additional PBS washes, the peroxidase molecule in the immobilized avidin-peroxidase complex was used to reduce H₂O₂ in the presence of Vector NovaRED substrate (Vector Laboratories) to produce a red reaction product. In addition, sections were counterstained with Mayer's hematoxylin as an aid to morphological identification. After staining, tissue sections were dehydrated in ascending concentrations of ethanol to xylene and coverslipped with Cytoseal XYL (Stephens Scientific, Riverdale, NJ).

Total RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). To determine the temporal sequence of EGCG-activated p57/KIP2 and caspase 14 expression, total RNA samples were obtained from cells incubated with 50 μ M EGCG for 0, 0.5, 2, 6, 8, 12, 20, and 24 h. To determine whether expression of p57/KIP2 in OSC2 cells up-regulated caspase 14 levels, total RNA was collected from five p57/KIP2-expressing subclones (S1–S5) along with the parental cell line OSC2 and a mock-transfected cell line G6. To determine whether p57/KIP2 mRNA was reduced by TGF- β 1, an inhibitor of gene expression and proteolytic degrader of p57/KIP2 (Urano et al., 1999; Nishimori et al., 2001), the S subclones, along with the OSC2 and G6 cell lines, were treated with 1 ng/ml TGF- β 1 for 24 h followed by RNA preparation. The mRNA species were analyzed by semiquantitative RT-PCR using primers specific for p57/KIP2, caspase 14, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Total RNA was extracted using an RNeasy total RNA isolation system (QIAGEN, Valencia, CA) and quantified using the Ribogreen Assay (Molecular Probes, Eugene, OR). The extracted RNA (1 μ g) was

added to 20 μ l of reverse transcription buffer and 2.5 U MuLV reverse transcriptase (GeneAmp RNA PCR kit; Applied Biosystems, Forest City, CA). This mixture was incubated at 42°C for 15 min and at 99°C for 5 min. The primers for caspase 14 were designed according to the GeneBank sequence for caspase 14 (accession no. NM012114): sense, 5'-ATATGATATGTCAGGTGCCCG-3' and antisense, 5'-TACCGTGAATAAACGTGCAA-3'. The PCR was performed with 20 μ l of the cDNA preparation, which was added to a reaction mixture containing 5 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl₂, 1 μ M sense primer, 1 μ M antisense primer, and 2.5 U AmpliTaq DNA polymerase (PerkinElmer Optoelectronics, Fremont, CA) in a total volume of 100 μ l. The PCR reaction was performed in a thermal cycler (GeneAmp PCR system 2400; PerkinElmer Optoelectronics) with an initial cycle of 5 min denaturing at 96°C, 5 min annealing at 60°C, and a 60-s extension at 72°C, followed by 25 cycles for NHEK samples (30 cycles for tumor cell samples) of 96°C for 30 s, 60°C for 45 s, and 72°C for 90 s. A final extension was performed at 72°C for 10 min. For p57 cDNA amplification, 25 cycles of PCR were carried out for NHEK samples (30 cycles for tumor cell samples) with 1.5 min denaturing, 0.75 min for annealing, and 1.5 min for extension, followed by the final 10 min extension at 72°C. Primers for p57 were synthesized according to a published sequence (Oya and Schulz, 2000) (sense, 5'-GCGGCGATCAAGAAGCTGTC-3'; antisense, 5'-CCGGTTGCTGCTACATGAAC-3'). PCR products for GAPDH (380 base pairs), p57 (269 base pairs), and caspase 14 (484 base pairs) were separated on a 2% agarose gel and visualized by staining with ethidium bromide. For quantitative purposes, all samples were initially amplified for 20, 25, 30, and 35 cycles, producing a linear response for selecting appropriate numbers of amplification cycles for each set of samples. The PCR products were confirmed by sequence analysis.

Western Blotting. To determine the changes in protein levels of p57/KIP2 and caspase 14 upon activation by EGCG, cell lysates were prepared from NHEK exposed to 50 μ M EGCG for 0, 4, 6, 8, 12, 20, and 24 h and analyzed by Western blot as described below. Cell lysates were prepared by homogenization of cells collected by centrifugation in radioimmunoprecipitation assay buffer containing 0.1% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 g/ml each of aprotinin, leupeptin, and pepstatin). The homogenates were centrifuged at 2500g, and supernatants were collected for protein determination (Bio-Rad protein assay; Bio-Rad, Hercules, CA) and Western blot analysis. Equivalent amounts of protein were mixed with 3 \times Laemmli sample buffer containing β -mercaptoethanol, placed in a boiling water bath for 5 min, and fractionated by SDS-polyacrylamide gel electrophoresis on 10% gels. The proteins were transferred onto Immobilon-P membrane (Millipore Corporation, Billerica, MA) and hybridized with antibodies against p57 (1:250), caspase 14 (1:1000), and actin (1:1000). The density of actin bands was used to normalize p57/KIP2 or caspase 14 expression. Detection of proteins was enhanced by chemiluminescence (ECL; Amersham Biosciences Inc., Piscataway, NJ).

Generation of OSC2 Subclones. Wild-type human p57/KIP2 cDNA (hKIP2) was kindly provided by Dr. Stephen Elledge (Baylor College of Medicine, Houston, TX). The original p57 cDNA was cloned into the EcoRI site of pBS KSII+ (5'... EcoRI-hp57ORF-EcoRI-PstI... 3'). The p57 cDNA was subcloned into the HindIII site of the retroviral vector pLNCX2 (under control of the cytomegalovirus promoter; BD Biosciences Clontech, Palo Alto, CA). The green fluorescent protein (GFP) cDNA (BD Biosciences Clontech) was subcloned into the HindIII site of the retroviral vector pLNCX2. The recombinant retroviral particles were generated in RetroPack PT67 cells (BD Biosciences Clontech) by transfection and antibiotic G-418 selection. The transfected PT67 cells were cultured in standard DMEM medium. The viral titer was determined according to the manufacturer's instruction. OSC2 cells were transfected by incubation for 24 h with the virus-containing DMEM medium removed from PT67 culture. The p57/KIP2-expressing OSC2 subclones S1-S5 and

the GFP-expressing OSC2 subclone G6 were selected by 60 μ g/ml G-418.

MTT Assay. To assess the relationship between p57/KIP2 expression and the cellular response to EGCG, five retrovirus-transfected p57/KIP2-expressing OSC2 subclones (S1-S5) were examined by MTT assay to determine the changes in cell viability with EGCG treatment in comparison with the parental cell line (OSC2) and mock-transfected cell line G6 (OSC2 transfected with GFP cDNA) (Hsu et al., 2003c). All cells were exposed to EGCG at 0, 50, 100, or 200 μ M for 48 h prior to the assays. Cells (1×10^4) were seeded in each well of a 96-well plate. Following treatment with EGCG, medium was replaced with 100 μ l of 2% MTT, and the plate was incubated at 37°C for 30 min. One hundred microliters of a solution containing 0.2 M Tris, pH 7.7, and 4% formalin were added to each well. After incubation at room temperature for 5 min, liquid was removed, and the wells were allowed to dry. Each well was rinsed with 200 μ l of deionized water followed by addition of 100 μ l of dimethyl sulfoxide (6.35% 0.1 N NaOH in dimethyl sulfoxide) to each well. Colorimetric measurements were determined by a spectrophotometer at a 562-nm wavelength.

Cell Growth Assay. Growth assays were conducted in triplicate on exponentially growing cells of OSC2, G6, and the S subclones in complete DMEM/Ham's F-12 medium. For each cell line, 1×10^4 cells were initially plated in six-cell culture dishes (6 cm in diameter) at time 0. Cells were counted and assessed with a hemacytometer and Trypan blue exclusion at 24 and 48 h, respectively.

Results

Caspase 14 Expression in EGCG-Treated NHEK. The immunocytochemistry result demonstrated that caspase 14 protein was almost undetectable without EGCG exposure in subconfluent control samples of NHEK (Fig. 1A). Cells incubated with EGCG for 24 h showed large amounts of caspase 14 staining appearing to involve the cytosol and nuclei (Fig. 1B).

Caspase 14 Expression in Normal and Psoriatic Skin. The immunohistochemistry result showed that normal human epidermis exhibited minimal staining in the basal layers and intense homogeneous caspase 14 immunostaining in the upper layers, including nuclear staining (Fig. 1C). In contrast, the psoriasis samples showed a reduction in epidermal caspase 14 immunostaining, especially in the nuclei of the upper layers (Fig. 1D).

EGCG-Modulated Expression of p57/KIP2 and Caspase 14 in NHEK. In preconfluent NHEK culture, EGCG induced p57/KIP2 expression at 2 h, but the largest amount of p57/KIP2 mRNA (approximately 5-fold over the control, normalized to GAPDH) was observed at 6 h, followed by a gradual decline to approximately 3-fold over the control level at 24 h (Fig. 2A). EGCG also increased caspase 14 mRNA within 30 min (Fig. 2A). The RT-PCR products increased 14- to 17-fold over the control level, normalized to GAPDH, from 0.5 to 8 h and climbed to 25-fold of control levels by 12 h before declining to 18-fold over the control level at 24 h (Fig. 2A). The p57/KIP2 induction was transient since p57/KIP2 mRNA declined gradually after it peaked, whereas EGCG-induced caspase 14 mRNA was persistent compared with p57/KIP2 induction.

EGCG Modulated Protein Levels of p57/KIP2 and Caspase 14 in NHEK. p57/KIP2 protein levels were significantly elevated at 4 h and peaked at 8 h, a 14-fold increase relative to control levels normalized to actin (Fig. 2B). Levels of p57/KIP2 protein remained relatively stable during the

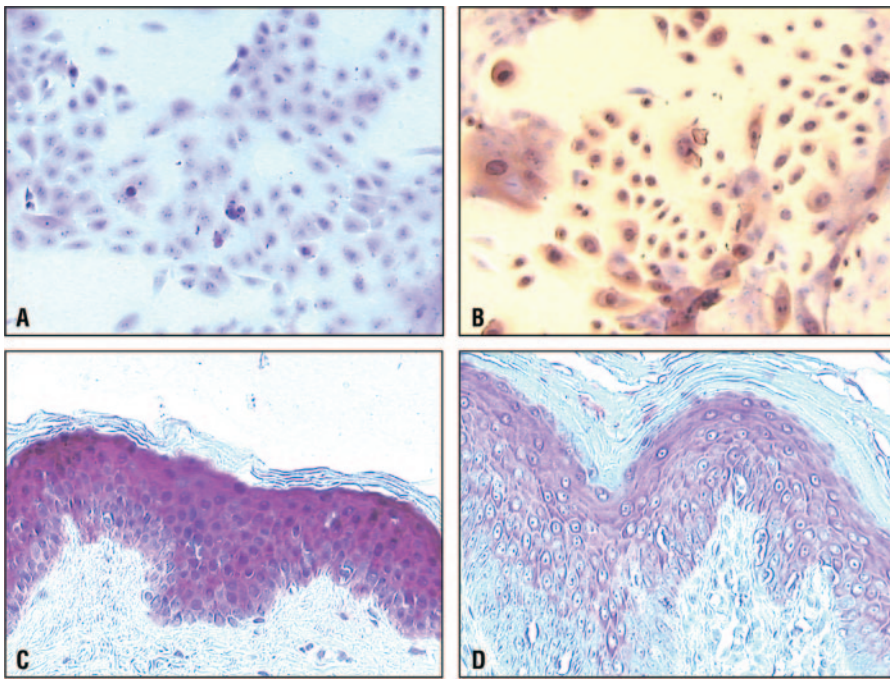


Fig. 1. Caspase 14 expression in EGCG-treated NHEK and normal versus psoriatic human tissue samples. A, NHEK incubated with KGM-2 for 24 h followed by caspase 14 immunostaining. B, NHEK incubated with KGM-2 containing 50 μ M EGCG for 24 h prior to immunostaining for caspase 14. C and D, representative normal and psoriatic skin samples, respectively, immunostained with anti-caspase 14 antibody.

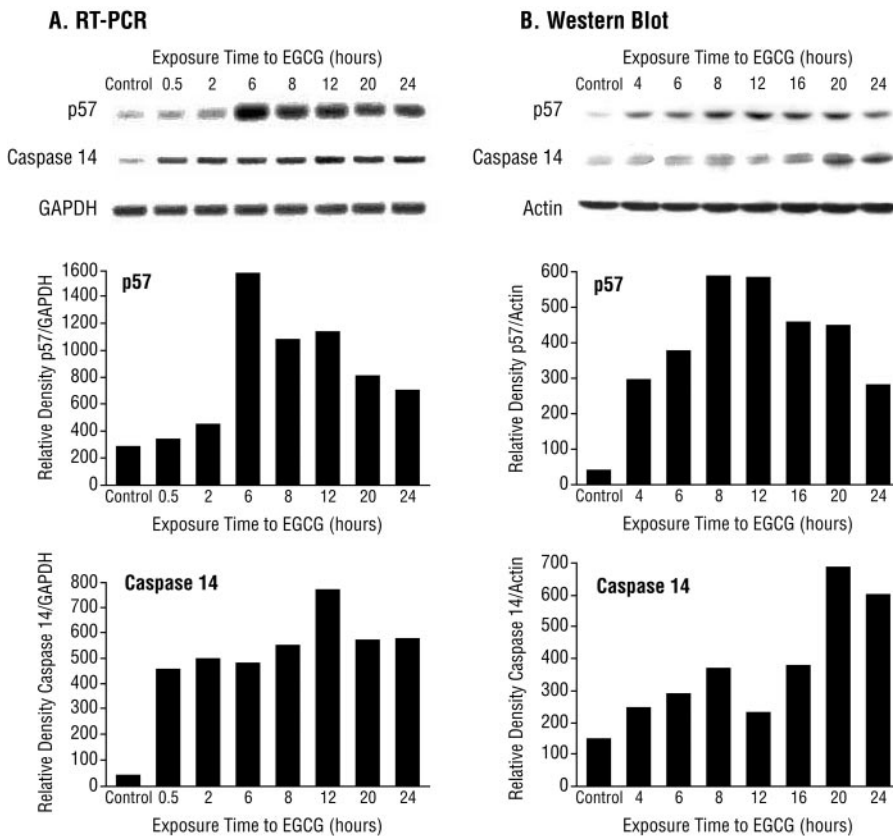


Fig. 2. mRNA and protein levels of p57 and caspase 14 in NHEK exposed to 50 μ M EGCG during a 24-h period. A, semiquantitative RT-PCR conducted on total RNA samples collected from NHEK treated with EGCG for the indicated times. B, representative Western blot of EGCG-treated NHEK protein samples using antibodies against p57/KIP2, caspase 14, and actin. Bar graphs represent the relative density of each band normalized to internal controls (GAPDH for RT-PCR, actin for Western blot). Experiments were conducted three times with similar results.

later hours and declined to approximately 7-fold of control levels at 24 h. Protein levels of caspase 14 gradually increased from 4 h and peaked at 20 h for an increase of 5-fold compared with control, normalized to actin (Fig. 2B). EGCG-induced p57/KIP2 protein accumulation was an earlier event compared with that of caspase 14.

Effects of Retrovirus-Mediated p57/KIP2 Expression on S Subclones in Response to EGCG. Subclones of OSC2

cells expressing either p57/KIP2 (S1–S5) or green fluorescent protein (G6) were established and maintained in DMEM/F-12 culture medium. OSC2 cells and G6 cells exhibited similar degrees of dose-dependent loss of cell viability, whereas p57/KIP2-expressing S1, S3, and S4 cells did not exhibit cytotoxicity in response to 100 μ M EGCG; S1–S4 cells also showed a lower degree of cytotoxicity to 200 μ M EGCG than OSC2 and G6 cells (Fig. 3A). In fact, S1, S3, and S4 cells

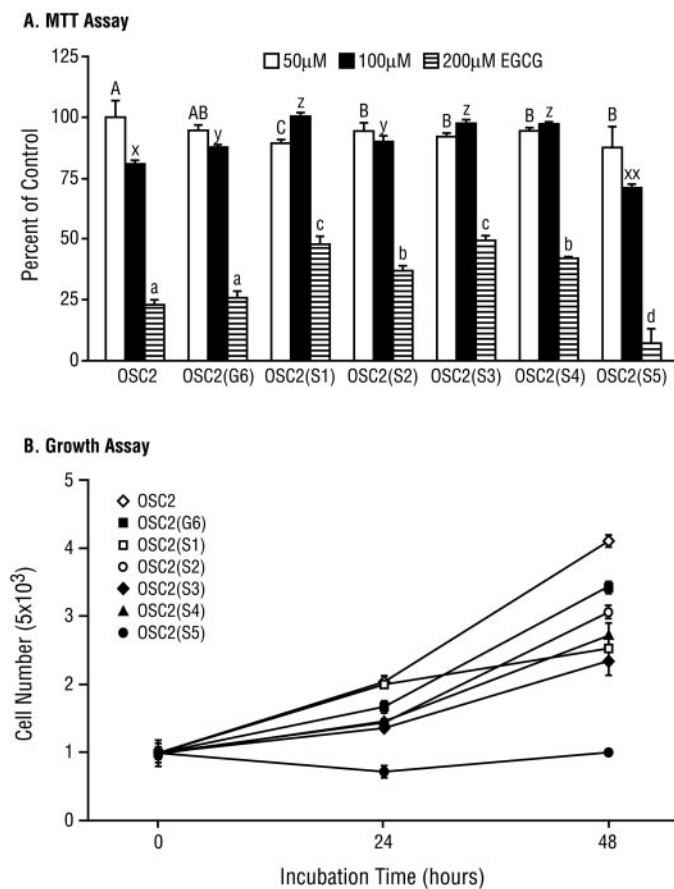


Fig. 3. MTT cell viability and cell growth assays from OSC2 cell line and its p57/KIP2-expressing subclones. A, cell viability changes in p57/KIP2-expressing OSC2 subclones in response to EGCG treatment with data presented as a percentage of control (untreated cells). Open bars represent samples treated with 50 μ M EGCG, solid bars represent samples treated with 100 μ M EGCG, and striped bars represent samples treated with 200 μ M EGCG for 48 h. Experiments were conducted in triplicate; error bars are standard deviations. Letters denote statistical groupings (analysis of variance, Tukey, $\alpha = 0.05$). B, rate of cell growth of OSC2 and its p57/KIP2- or GFP-expressing subclones. Experiments were conducted in triplicate; error bars are standard deviations.

treated with 100 μ M EGCG exhibited increased viability resembling that of aged NHEK exposed to EGCG (Hsu et al., 2003a). The S5 cells were similar to OSC2 and G6 cells in their response to EGCG exposure (Fig. 3A). However, S5 cells exhibited a much lower proliferation rate; the cell number of S5 did not increase during the 48-h period, whereas OSC2 and G6 cell numbers increased more than 3-fold. S1–S4 cells exhibited reduced growth rates in comparison to the parental cells or G6 cells, but greater rate than S5 cells (Fig. 3B).

p57/KIP2 Expression in OSC2 Cells Activated Expression of Caspase 14. The semiquantitative RT-PCR amplification of total RNA samples demonstrated that both OSC2 and G6 cells expressed only minimal levels of p57/KIP2 message, whereas S1, S2, S3, and S5 expressed higher levels (approximately 200%) of p57/KIP2 message than OSC2 or G6, and S4 cells expressed considerable (approximately 150%) p57/KIP2 message (Fig. 4A). When caspase 14 mRNA from these cell lines was amplified by semiquantitative RT-PCR, OSC2 and G6 exhibited only background levels of caspase 14 mRNA. In contrast, the S subclones expressed higher levels of caspase 14 mRNA, indicating activation of caspase 14 gene expression (Fig. 4A).

TGF- β 1 Mediated Down-Regulation of p57/KIP2 and Caspase 14 Expression in S1–S5 Cells. TGF- β 1 is known to induce the growth arrest of epidermal keratinocytes without triggering differentiation, and in fact, treatment with TGF- β 1 inhibits keratinocyte differentiation (reviewed in Hu et al., 1998). In comparison to the untreated cells, TGF- β 1 treatment eliminated p57/KIP2 message in OSC2 and G6 cells within 24 h (Fig. 4B). The S clones also demonstrated reduced p57/KIP2 mRNA upon TGF- β 1 exposure. RT-PCR showed that mRNA levels of caspase 14 were also significantly decreased upon treatment with TGF- β 1 (Fig. 4B).

Discussion

EGCG induces production of involucrin in epidermal keratinocytes, an important protein in keratinocyte differentiation (Balasubramanian et al., 2002). We previously demonstrated that EGCG accelerated NHEK differentiation with increased keratin 1 and filaggrin expression and activation of transglutaminase (Hsu et al., 2003a). Here, we report that EGCG induces caspase 14, a recently described putative regulator for keratinocyte terminal differentiation, within a 24-h period with evidence suggesting it may be induced independently by p57/KIP2, a well established regulator of cell growth and differentiation (Figs. 1 and 2). We also found that epidermal cells in psoriasis, a disease characterized by aberrant keratinization, exhibit reduced caspase 14 expression, particularly in the nuclear regions of the cells (Fig. 1). This observation provided an in vivo human correlate for the relationship between caspase 14 expression and terminal differentiation. Together with our in vitro data, this suggested an alternative approach would be available to psoriasis treatment besides currently licensed modalities, most of which are immunosuppressive with untoward side effects. Collectively, the data demonstrate that EGCG enabled NHEK to accelerate terminal differentiation, marked by a coordinated activation and expression of p57/KIP2 and caspase 14. Since caspase 14 is strictly associated with NHEK terminal differentiation and skin barrier formation (Mikolajczyk et al., 2004), we propose that EGCG directs keratinocytes to enter a pathway leading to terminal differentiation and accelerated skin barrier formation.

Our data suggest that the EGCG-induced differentiation requires activation of both p57/KIP2 and caspase 14. The mRNA levels of p57/KIP2 and caspase 14 in NHEK were elevated by EGCG treatment (Fig. 2A). However, notable differences in the times for protein peaks of p57/KIP2 (6 h) versus caspase 14 (20 h) suggested significant p57/KIP2 protein accumulation prior to that of caspase 14. Conversely, acute induction of caspase 14 mRNA by EGCG was observed despite the delayed onset of caspase 14 protein accumulation (Fig. 2). This delayed peak of caspase 14 protein may due to the time required for post-transcriptional processing of pro-caspase 14, which involves proteolytic cleavage and dimerization (Mikolajczyk et al., 2004). In addition, p57/KIP2 was able to induce caspase 14 independently. Retroviral expression of p57/KIP2 in OSC2 cells induced the endogenous transcription of caspase 14 (Fig. 4A). This p57/KIP2 effect was confirmed by pretreatment of S1–S5 cells with TGF- β 1 to inhibit p57/KIP2 gene expression, resulting in significant reduction of caspase 14 (Fig. 4B). This result is consistent with the ability of TGF- β 1 to inhibit keratinocyte differenti-

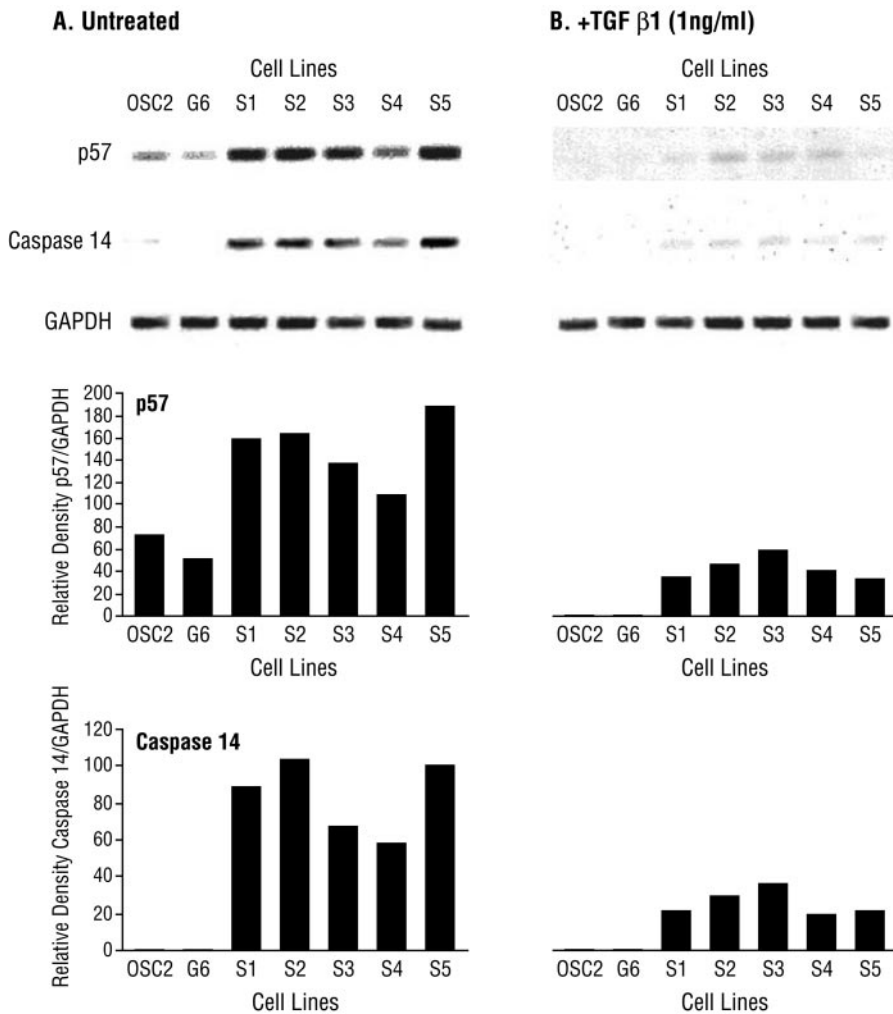


Fig. 4. Changes in mRNA levels of p57 and caspase 14 in OSC2 and its p57/KIP2- or GFP-expressing subclones in the absence or presence of 1 ng/ml TGF- β 1. A, semiquantitative RT-PCR performed on total RNA samples collected from OSC2 cells and its subclones in the absence of TGF- β 1. B, semiquantitative RT-PCR performed on total RNA samples collected from OSC2 cells and its subclones in the presence of 1 ng/ml TGF- β 1. The RT-PCR products were collected after 30 cycles for caspase 14 and 25 cycles for p57/KIP2. Bar graphs represent the relative density of each band normalized to internal controls (GAPDH). Experiments were conducted three times with similar results.

ation (Hu et al., 1998) and suggests that p57/KIP2, induced by EGCG in NHEK, may serve as a regulator for caspase 14 expression. In addition, these results imply an integral relationship between p57/KIP2 and induction of keratinocyte differentiation.

In contrast to NHEK, OSC2 cells, which express basal levels of p57/KIP2 and caspase 14 (Fig. 4), undergo caspase 3-dependent apoptosis when exposed to EGCG (Hsu et al., 2003b). However, when exogenous p57/KIP2 was expressed by OSC2 cells (S-clones), cell growth was inhibited up to 100% (S5 cells, Fig. 3A). The S-clones exhibited different degrees of resistance to EGCG-induced cytotoxicity with the exception of S5 cells, which appeared to lose the ability to repopulate (Fig. 3B). This failure of repopulation, possibly due to the p57/KIP2 cDNA integration, might cause S5 cells to be less viable in response to EGCG possibly due to induction of differentiation. S1–S4 cells exhibited slower growth rates than OSC2 and G6 cells, suggesting that the expression of exogenous p57/KIP2 resulted in different degrees of growth inhibition, but these cell lines retained the ability to repopulate (Fig. 3B). Although the tumor-specific toxic effects of EGCG were not completely prevented by p57/KIP2 expression in S-clones, a protective role of p57/KIP2 was apparent. This is notable because OSC2 is a metastatic squamous carcinoma line with mutated p53 and silenced p16 (Yoneda et al., 1999). As a member of the KIP/CIP family of

proteins involved in cell growth and differentiation (Lee et al., 1995; Deschenes et al., 2001), p57/KIP2 may inhibit mitochondrial-mediated apoptosis by blocking c-Jun N-terminal kinase phosphorylation (Chang et al., 2003), as well as initiation of terminal differentiation by inducing caspase 14. Thus, the protective effect of p57/KIP2 on the S-clones could be closely associated with a cell differentiation pathway. This result is consistent with observations that p57/KIP2 also plays a vital role in both cell differentiation and survival in tissues other than the epidermis (Vattemi et al., 2000; Deschenes et al., 2001). Thus, EGCG-induced p57/KIP2 expression may activate a pathway for cell differentiation.

Recent pathological studies demonstrated that tumor specimens expressed reduced levels of p57/KIP2 protein compared with paired normal tissues, and this reduced expression was often correlated with poor prognosis (Ito et al., 2001, 2002; Nakai et al., 2002), suggesting that the loss of p57/KIP2 expression may contribute to the accelerated growth rate of the more advanced tumors and decreased differentiation. In addition, p57/KIP2 is known to bind to and assist the nuclear translocation of LIM-kinase 1, a downstream effector of the Rho family of GTPases, resulting in the regulation of actin (Yokoo et al., 2003). In this regard, nuclear localization of caspase 14 was observed only in epidermal keratinocytes committed to terminal differentiation, but not in undifferentiated basal cells or psoriatic tissue samples.

This evidence suggests that nuclear translocation of active caspase 14 may guide the cells toward formation of the anucleated stratum corneum. It remains unclear, however, whether p57/KIP2 plays a role in nuclear translocation of caspase 14. Future investigation to better understand the effect of EGCG on epidermal cell modulation will involve the use of animal models such as the “flaky” mouse model with altered keratinization and eventually clinical studies with a focus on treating skin disorders such as psoriasis or improving wound healing.

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References

- Adhami VM, Ahmad N, and Mukhtar H (2003) Molecular targets for green tea in prostate cancer prevention. *J Nutr* **133**:2417S–2424S.
- Ahmad M, Srinivasula SM, Hegde R, Mukattash R, Fernandes-Alnemri T, and Alnemri ES (1998) Identification and characterization of murine caspase-14, a new member of the caspase family. *Cancer Res* **58**:5201–5205.
- Alonso L and Fuchs E (2003) Stem cells of the skin epithelium. *Proc Natl Acad Sci USA* **100** (Suppl 1):11830–11835.
- Balasubramanian S, Efimova T, and Eckert RL (2002) Green tea polyphenol stimulates a Ras, MEK1, MEK3 and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J Biol Chem* **277**:1828–1836.
- Chang TS, Kim MJ, Ryoo K, Park J, Eom SJ, Shim J, Nakayama KI, Nakayama K, Tomita M, Takahashi K, et al. (2003) p57KIP2 modulates stress-activated signaling by inhibiting c Jun NH2-terminal kinase/stress-activated protein kinase. *J Biol Chem* **278**:48092–48098.
- Deschenes C, Vezina A, Beaulieu JF, and Rivard N (2001) Role of p27 (Kip1) in human intestinal cell differentiation. *Gastroenterology* **120**:423–438.
- Eckhart L, Ban J, Fischer H, and Tschachler E (2000a) Caspase-14: analysis of gene structure and mRNA expression during keratinocyte differentiation. *Biochem Biophys Res Commun* **277**:655–659.
- Eckhart L, Declercq W, Ban J, Rendl M, Lengauer B, Mayer C, Lippens S, Vandana-bee P, and Tschachler E (2000b) Terminal differentiation of human keratinocytes and stratum corneum formation is associated with caspase-14 activation. *J Invest Dermatol* **115**:1148–1151.
- Hsu S, Bollag WB, Lewis J, Huang Q, Singh B, Sharawy M, Yamamoto T, and Schuster G (2003a) Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. *J Pharmacol Exp Ther* **306**:29–34.
- Hsu S, Lewis J, Singh B, Schoenlein P, Osaki T, Athar M, Porter AG, and Schuster G (2003b) Green tea polyphenol targets the mitochondria in tumor cells inducing caspase 3-dependent apoptosis. *Anticancer Res* **23**:1533–1540.
- Hsu S, Lewis JB, Borke JL, Singh B, Dickinson DP, Caughman GB, Athar M, Drake L, Aiken AC, Huynh CT, et al. (2001) Chemopreventive effects of green tea polyphenols correlate with reversible induction of p57 expression. *Anticancer Res* **21**:3743–3748.
- Hsu S, Yu FS, Lewis J, Singh B, Borke J, Osaki T, Athar M, and Schuster G (2002a) Induction of p57 is required for cell survival when exposed to green tea polyphenols. *Anticancer Res* **22**:4115–4120.
- Hsu S, Yu FX, Huang Q, Lewis J, Singh B, Dickinson D, Borke J, Sharawy M, Wataha J, Yamamoto T, et al. (2003c) A mechanism-based in vitro anticancer drug screening approach for phenolic phytochemicals. *Assay Drug Dev Technol* **1**:611–618.
- Hsu SD, Singh BB, Lewis JB, Borke JL, Dickinson DP, Drake L, Caughman GB, and Schuster GS (2002b) Chemoprevention of oral cancer by green tea. *Gen Dent* **50**:140–146.
- Hu PP, Datto MB, and Wang X-F (1998) Molecular mechanisms of transforming growth factor- β signaling. *Endocr Rev* **19**:349–363.
- Ito Y, Takeda T, Sakon M, Tsujimoto M, Monden M, and Matsuura N (2001) Expression of p57/Kip2 protein in hepatocellular carcinoma. *Oncology (Basel)* **61**:221–225.
- Ito Y, Takeda T, Sakaki Y, Sakon M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Monden M, and Matsuura N (2002) Expression of p57/Kip2 protein in extrahepatic bile duct carcinoma and intrahepatic cholangiocellular carcinoma. *Liver* **22**:145–149.
- Katiyar SK (2003) Skin photoprotection by green tea: antioxidant and immunomodulatory effects. *Curr Drug Targets Immune Endocr Metabol Disord* **3**:234–242.
- Lee MH, Reynisdottir I, and Massague J (1995) Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* **9**:639–649.
- Lippens S, Kockx M, Knaapen M, Mortier L, Polakowska R, Verheyen A, Garmyn M, Zwijsen A, Formstecher P, Huylebroeck D, et al. (2000) Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing. *Cell Death Diff* **7**:1218–1224.
- Lippens S, Vandenaabee C, Van Damme E, Tschachler E, Vandenaabee P, and Declercq W (2003) Caspase 14 is expressed in the epidermis, the choroid plexus, the retinal pigment epithelium and thymic Hassall's bodies. *Cell Death Diff* **10**:257–259.
- Madison KC (2003) Barrier function of the skin: “la raison d’être” of the epidermis. *J Invest Dermatol* **121**:231–241.
- Martinez LA, Chen Y, Fischer SM, and Conti CJ (1999) Coordinated changes in cell cycle machinery occur during keratinocyte terminal differentiation. *Oncogene* **18**:397–406.
- Mikolajczyk J, Scott FL, Krajewski S, Sutherlin DP, and Salvesen GS (2004) Activation and substrate specificity of caspase-14. *Biochemistry* **43**:10560–10569.
- Miyazawa T (2000) Absorption, metabolism and antioxidative effects of tea catechin in humans. *Biofactors* **13**:55–59.
- Mukhtar H and Ahmad N (2000) Tea polyphenols: prevention of cancer and optimizing health. *Am J Clin Nutr* **71**:1698S–1702S.
- Nakai S, Masaki T, Shiratori Y, Ohgi T, Morishita A, Kurokohchi K, Watanabe S, and Kuriyama S (2002) Expression of p57(KIP2) in hepatocellular carcinoma: relationship between tumor differentiation and patient survival. *Int J Oncol* **20**:769–775.
- Nickoloff BJ, Qin JZ, Chaturvedi V, Bacon P, Panella J, and Denning MF (2002) Life and death signaling pathways contributing to skin cancer. *J Invest Dermatol Symp Proc* **7**:27–35.
- Nishimori S, Tanaka Y, Chiba T, Fujii M, Imamura T, Miyazono K, Ogasawara T, Kawaguchi H, Igarashi T, Fujita T, et al. (2001) Smad-mediated transcription is required for transforming growth factor-beta 1-induced p57(Kip2) proteolysis in osteoblastic cells. *J Biol Chem* **276**:10700–10705.
- Opalka B, Dickopp A, and Kirch HC (2002) Apoptotic genes in cancer therapy. *Cells Tissues Organs* **172**:126–132.
- Oya M and Schulz WA (2000) Decreased expression of p57(KIP2) mRNA in human bladder cancer. *Br J Cancer* **83**:626–631.
- Rendl M, Ban J, Mrass P, Mayer C, Lengauer B, Eckhart L, Declercq W, and Tschachler E (2002) Caspase-14 expression by epidermal keratinocytes is regulated by retinoids in a differentiation-associated manner. *J Invest Dermatol* **119**:1150–1155.
- Takahashi K, Nakayama K, and Nakayama K (2000) Mice lacking a CDK inhibitor, p57Kip2, exhibit skeletal abnormalities and growth retardation. *J Biochem (Tokyo)* **127**:73–83.
- Urano T, Yashiroda H, Muraoka M, Tanaka K, Hosoi T, Inoue S, Ouchi Y, and Toyoshima H (1999) p57(Kip2) is degraded through the proteasome in osteoblasts stimulated to proliferation by transforming growth factor beta1. *J Biol Chem* **274**:12197–12200.
- Vattemi G, Tonin P, Filosto M, Spagnolo M, Rizzuto N, and Tomelleri G (2000) T-cell anti-apoptotic mechanisms in inflammatory myopathies. *J Neuroimmunol* **111**:146–151.
- Watanabe H, Pan ZQ, Schreiber-Agus N, DePinho RA, Hurwitz J, and Xiong Y (1998) Suppression of cell transformation by the cyclin-dependent kinase inhibitor p57KIP2 requires binding to proliferating cell nuclear antigen. *Proc Natl Acad Sci USA* **95**:1392–1397.
- Yokoo T, Toyoshima H, Miura M, Wang Y, Iida KT, Suzuki H, Sone H, Shimano H, Gotoda T, Nishimori S, et al. (2003) p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus. *J Biol Chem* **278**:52919–52923.
- Yoneda K, Yokoyama T, Yamamoto T, Hatabe T, and Osaki T (1999) p53 gene mutations and p21 protein expression induced independently of p53, by TGF-beta and gamma-rays in squamous cell carcinoma cells. *Eur J Cancer* **35**:278–283.

Address correspondence to: Dr. Stephen Hsu, Department of Oral Biology and Maxillofacial Pathology, School of Dentistry, AD1443, Medical College of Georgia, Augusta, GA 30912-1126. E-mail address: shsu@mail.mcg.edu