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(71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Stanford, CA 94305 (US).

(72) Inventors: SEITZ, Cornelia, S.; Hautklinik, Uniklinik Wurzburg, Josef-Schneider Strasse 2, D-97080 Wurzburg (DE). KHAVARI, Paul, A.; 1290 Sharon Park Drive #43, Menlo Park, CA 94025 (US).

(74) Agents: GORTHEY, LeeAnn et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

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(54) Title: METHOD OF REGULATING EPITHELIAL GROWTH

(57) Abstract

Methods of treating epithelial hyperproliferative disorders and accelerating wound healing, by modulation of NF-κB activity in the epithelium, are disclosed. Also disclosed are methods of screening for compounds effective to treat epithelial hyperproliferative disorders or enhance wound healing.

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METHOD OF REGULATING EPITHELIAL GROWTH

FIELD OF THE INVENTION

The present invention relates to methods of promoting or inhibiting cell proliferation in the epithelium by modulation of NF-$_K$B activity. More particularly, the invention relates to the treatment of epithelial hyperproliferative diseases, methods of promoting wound healing, and methods of screening for compounds effective for these applications.

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BACKGROUND OF THE INVENTION

NF-$_K$B/Rel proteins are potent inducible gene regulatory factors expressed in a wide variety of tissues. NF-$_K$B subunits function as DNA-binding transcription factors, with mammalian family members that include RelA (p65), RelB, c-Rel, p50 and p52. NF-$_K$B activity is controlled at a number of levels, prominent among these being the regulation of its transition from an inactive preexisting cytoplasmic form to an active nuclear protein.

NF-$_K$B gene regulatory proteins are activated in a range of conditions involving cellular stress and injury (Baeuerle *et al.* 1996, Verma *et al.*, Baldwin *et al.*). Studies of NF-$_K$B in lymphoid tissues have revealed potent effects in stimulating proliferation, preventing apoptosis, activating the immune response, and triggering cellular stress response genes.

Stratified epithelial tissues must respond to such frequent environmental stresses while maintaining a precise balance between cellular proliferation and cell loss via desquamation. In stratified epithelium, proliferative basal cells adherent to the underlying basement membrane undergo cell cycle arrest associated with outward migration and activation of terminal differentiation genes (Jones *et al.*). Abnormalities in this process disrupt epithelial homeostasis and are characteristic of cutaneous neoplasms as well as a wide array of inflammatory skin diseases.

In addition, normal somatic cells do not replicate indefinitely but ultimately undergo cellular senescence, which is characterized by an irreversible withdrawal from the cell cycle that is resistant to mitogenic stimuli. Recent evidence suggests that neoplastic transformation may

require mechanisms that, in addition to avoiding apoptosis, also bypass cellular senescence (Brown *et al.*; Serrano *et al.*; Yeager *et al.*).

The gene regulatory transcription factors mediating control of epithelial growth and differentiation are not fully known. In particular, little has been reported about the expression and function of NF-$_K$B proteins in epithelia.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of inhibiting cellular proliferation in the epithelium. According to the method, a therapeutically effective amount of an activator of NF-$_K$B activity, or of an NF-$_K$B protein subunit, is administered to a subject in need of such treatment, and is effective to inhibit said proliferation. Examples of such activators include tumor necrosis factor alpha (TNF$_\alpha$), phorbol 12-myristate 13-acetate (PMA), interleukin-1 (IL-1), interleukin-2 (IL-2), and bacterial lipopolysaccharide (LPS). Preferred NF-$_K$B protein subunits include the p50 and the p65 subunit. In one embodiment, where the method is used to treat a hyperproliferative skin disorder, the activator is administered, preferably topically, to the epidermis.

In another aspect, the invention provides a method of promoting cellular proliferation in the epithelium. According to the method, a therapeutically effective amount of an inhibitor of NF-$_K$B activity is administered to a subject in need of such treatment, and is effective to promote said proliferation. Examples of such inhibitors include I$_K$Bβ, I$_K$Bα, pyrrolidine dithiocarbamate (PDTC), dimethyl sulfoxide (DMSO), an α-amido-substituted cyclic imide, 2-(2,6-dioxo-3-piperidinyl)-4-azaisoindoline-1,3-dione, serine protease inhibitors, glucocorticoids, and NF-$_K$B antisense compounds,. In one embodiment, where the method is used to accelerate or enhance wound healing, the inhibitor is administered, preferably topically, to the site of the wound.

Also included in the invention is a method of identifying compounds effective to treat an epithelial hyperproliferation disorder. The method includes the steps of measuring the activity of NF-$_K$B in the presence and absence of a test compound, and identifying the test compound as effective if it results in an upregulation or promotion of NF-$_K$B activity.

In a related aspect, the invention includes a method of identifying compounds useful for promoting wound healing. The method includes the steps measuring the activity of NF-$_K$B in the presence and absence of a test compound, and identifying the test compound as useful if it results in a downregulation or inhibition of NF-$_K$B activity.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A, 1B, 1C, 1D are schematics of retroviral expression vectors used in experiments assessing effects of modulating NF-$_\kappa$B activity.

Figures 2A and 2B are computer-generated images of Western Blots showing expression of NF-$_\kappa$B activating (Fig. 2A) and inhibitory (Fig. 2B) proteins.

Figs. 3A-3B show the effects of the above expression vectors on NF-$_\kappa$B directed gene expression of a reporter gene in epithelial cells.

Figs. 4A-H are computer-generated images of the epidermal architecture of human skin transfected with the indicated vectors and grown *in vivo* on SCID mice. Vector CN.50 was used in Figs. 4C and 4D. Vector $I_\kappa B_\alpha M$ was used in Figs. 4B, 4E, 4F, 4G and 4H. Fig. 4A shows skin transduced with lacZ control vector.

Figs. 5A-5B summarize the histological results from images such as are shown in Figs. 4A-H. Fig. 4A shows the % sections with deep hyperplasia, and Fig. 4B shows the % sections with epidermis < 0.03 mm.

Fig. 6 shows schematics of K14-$I_\kappa B_\alpha M$ and K14-p50 transgenes used for targeted expression to murine epidermis.

Figs. 7A-7B show the impact of altering NF-$_\kappa$B function on epithelial cell growth *in vitro*.

Fig. 8 shows DNA synthetic activity as a function of NF-$_\kappa$B subunit expression, expressed as the number of cells incorporating BrdU as a percentage of total.

Fig. 9 shows a cell cycle analysis of NF-$_\kappa$B transduced cells versus control, giving the relative percentage of cells in different cell cycle phases.

Fig. 10 shows the lack of effect of added growth factors on NF-$_\kappa$B induced growth arrest, vs. p50 and lacZ control-transduced cells.

Fig. 11 shows the proportion of transduced and control cells demonstrating senescence-associated β-galactosidase staining.

Fig. 12 shows the proportions of cells transduced with expression vectors for p50, p65, $I_\kappa B_\alpha M$ and lacZ control, respectively, expressing nuclear $p21^{Cip1}$.

Fig. 13 shows percentages of cells transduced with a retroviral expression vector for $p21^{Cip1}$ and GFP control, respectively, demonstrating senescence-associated β-galactosidase expression.

Fig. 14 shows cell cycle distribution for cells transduced as for Fig. 13.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

"Cellular proliferation in the epithelium" refers to cell growth in the mitotically active basal layer. In normal cells, this proliferation ultimately ceases, and cells undergo a transition to growth-arrest and terminal differentiation, associated with outward migration to suprabasal layers.

The term "significant", when used with reference to, e.g., "significantly different", "significantly inhibits" or "significantly increases", refers to a difference in a quantifiable parameter between the two groups being compared that is statistically significant using standard statistical tests. For example, the degree of binding in a protein binding assay may be quantified using standard methods, and the degree of binding under different conditions can be compared for statistically significant differences.

The term "therapeutically effective amount" refers to an amount of compound that is of sufficient quantity to ameliorate a selected disorder, such as a hyperproliferative disorder of the skin. The term "ameliorate" refers to a lessening of the detrimental effect of the disorder in the patient receiving the therapy.

"Treating" a disease refers to administering a therapeutic substance effective to reduce the symptoms of the disease and/or lessen the severity of the disease.

II. Effects of NF-$_\kappa$B Modulation in Epithelium

Several studies reported to date have proposed that inhibition of NF-$_\kappa$B activity can be used to treat a variety of inflammatory and hyperproliferative diseases (see, *e.g.*, Muller *et al.*; Ghosh, 1997; Narayananof *et al.*). For example, induction of NF-$_\kappa$B DNA-binding activity has been associated with the G0 to G1 transition in murine fibroblasts (Baldwin *et al.*). In ras-transformed NIH 3T3 fibroblasts, antisense inhibition of p65 expression decreased tumor cell growth in vivo (Higgins *et al.*), and, in a separate study with 3T3 fibroblasts, the blockade of $I_\kappa B_\alpha$ increased neoplastic growth in vivo (Beauparlant *et al.*, 1994). A similar NF-$_\kappa$B growth-promoting role has been suggested in lymphocytes, as in the cases of lymphoid cells transformed by HTLV-1 (Kitajima *et al.*).

Results disclosed herein, however, demonstrate the opposite effect in epithelium, i.e. that stimulation of NF-$_\kappa$B results in a decrease in the proliferation of epithelial cells, while inhibition of NF-$_\kappa$B increases such proliferation. Specifically, activation of NF-$_\kappa$B activity, *e.g.*, through the expression of a constitutively nuclear p50 NF-$_\kappa$B, led to inhibition of epithelial cell proliferation. Conversely, blockade of NF-$_\kappa$B function, *e.g.*, using a trans-dominant $I_\kappa B_\alpha$ mutant, led to stimulation of epithelial cell proliferation. These effects

were manifested, for example, in transgenic human tissue and transgenic mice as atrophic epithelium and massive epithelial hyperplasia, respectively.

Further experiments showed that blockade of NF-$_\kappa$B function prevents nuclear expression of the cyclin-dependent kinase inhibitor p21/CIP-1/WAF-1, while dominantly active p50 augments the proportion of epithelial cells with nuclear localized p21. The latter effect results in inhibition of cell cycle progression and premature cellular senescence, as discussed below.

Taken together, the data described below indicate that activators or stimulators of NF-$_\kappa$B may be used to reduce or inhibit undesirable epithelial cell proliferation, such as occurs during various epithelial hyperproliferative disorders. Inhibitors or antagonists of NF-$_\kappa$B may be used to upregulate or increase desirable epithelial cell proliferation, such as occurs during wound healing.

III. Inhibitors and Activators of NF-$_\kappa$B Activity

Compounds effective to inhibit or promote the activity of NF-$_\kappa$B are known in the art, and progress continues in this field. See, for example, recent reviews by Beauparlant *et al.*, 1996; Barnes; and Okamoto *et al.*

Presently known inhibitors include, for example, proteinaceous inhibitors, such as $I_\kappa B_\beta$, described in U.S. Patent 5,597,898 (Ghosh, 1997) and $I_\kappa B_\alpha$, described herein and in Hiscott *et al.* Also effective are a class of non-polypeptide cyclic imides disclosed as inhibitors of TNF-$_\alpha$ in U.S. Patent 5,605,914 (Muller, 1997) and in Corral *et al.* An exemplary compound of this group is 2-(2,6-dioxo-3-piperidinyl)-4-azaisoindoline-1,3-dione. Other NF-$_\kappa$B inhibitors known in the art include antisense inhibitors, as described in U.S. Patent 5,591,840 (Narayananof , 1997) and Sharma *et al.*, serine proteinase inhibitors such as N-tosyl-L-phenylalanine cloromethyl ketone and N-$_\alpha$-p-tosyl-L-lysine chloromethyl ketone (see e.g. Jeong *et al.*), sesquiterpene lactones (Bork *et al.*) and glucocorticoids (Barnes). Pharmacological inhibitors include dimethyl sulfoxide (Essani *et al.*) and pyrrolidine diothiocarbamate.

Known activators of NF-$_\kappa$B include subunit proteins, such as the p50 and p65 proteins, as described herein. Other activators known in the art include tumor necrosis factor alpha (TNF-$_\alpha$), phorbol 12-myristate 13-acetate (PMA), hydrogen peroxide (see e.g. Kaul *et al.*), interleukin-1 (IL-1), interleukin-2 (IL-2), neuropeptide substance P (Lieb *et al.*) and bacterial lipopolysaccharide (LPS).

Although exemplary inhibitors and activators are noted above, it is understood that other compounds known to have such activity may also be used in the methods disclosed herein, and that additional suitable compounds may be determined by use of the screening methods described in Section VII, below.

III. Experimental Results

A. Site of Expression of NF-$_\kappa$B Proteins within the Epithelium

To characterize the expression of NF-$_\kappa$B proteins in different layers of stratified epithelium, frozen tissue sections of non-sun exposed adult human abdominal skin were double immunostained with antibodies to p50 and the activated form of RelA/p65, then analyzed via laser confocal microscopy. Results demonstrated that, in cells of the basal epithelial layer, p105/p50 is strongly expressed in the cytoplasm. In contrast, in the non-proliferative cells of the suprabasal layers, this cytoplasmic expression is entirely absent and cells demonstrate nuclear expression. Given the well characterized regulation of NF-$_\kappa$B via control of nuclear translocation (see e.g. Baeuerle *et al.*, 1994, 1996; Verma *et al.*; Ghosh *et al.* 1990), these results are consistent with a key role of NF-$_\kappa$B in the control of epithelial growth and differentiation.

B. Preparation and Expression *in vitro* of Vectors Encoding NF-$_\kappa$B-Activating and -Inhibiting Proteins

Vectors encoding proteins which exert either activating (constitutively nuclear p50 and p65/RelA) or inhibitory (trans-dominant mutant $I_\kappa B_\alpha M$ repressor) effects on NF-$_\kappa$B function were constructed as described in Example 1. These vectors, shown schematically in Figs. 1A-D, were used to directly assess the role of NF-$_\kappa$B in the control of epithelial growth and differentiation. Cells transduced with a particular construct effectively express the protein(s) encoded by that construct, as verified via Western blot (Figs. 2A-2B; see Example 1).

The vectors were expressed in primary cultures of human keratinocyte epithelial cells, and the cells were assessed for expression of the proteins encoded by the transducing vectors, as described in Example 3, below. Data is shown in Figs. 3A-3B, reported as fold induction in reporter gene activity. The LZRS lacZ vector served as a control. As shown in Fig. 3A, p50 combined with p65, as well as either subunit alone, produced consistent activation of $NF_\kappa B$-directed gene expression. $I_\kappa B_\alpha M$, in contrast, blocks phorbol ester induced NF-$_\kappa$B-directed reporter gene expression in a dose-dependent manner (Fig. 3B).

To assess the cellular localization of the p50 NF-$_\kappa$B subunit after gene transfer, the transduced keratinocytes were immunostained with an antibody to the p50/105 NF-$_\kappa$B subunit and visualized by laser confocal fluorescence microscopy. Results showed that expression of the NF-$_\kappa$B activating and inhibitory proteins altered the subcellular distribution pattern of NF-$_\kappa$B subunits. Specifically, expression of NF-$_\kappa$B activating proteins, encoded by vector CN.p50, resulted in nuclear localization of NF-$_\kappa$B immunoreactivity, while expression of NF-$_\kappa$B inhibiting proteins, encoded by vector $I_\kappa B_\alpha M$, resulted in cytoplasmic localization of

NF-$_K$B immunoreactivity.

C. Effect of Altering NF-$_K$B Function in Human Epithelial Tissue *in vivo*.

Keratinocytes expressing p50, $I_KB_\alpha M$ and lacZ control were used to regenerate human epidermis on SCID mice, as described in Example 4. Results are shown in Figs. 4A-4H. Figs. 4C and 4D show skin transduced with CN.50 (Fig. 1A), and Figs. 4B, 4E, 4F, 4G and 4H show skin transduced with $I_KB_\alpha M$ (Fig. 1B). Control skin is shown in Fig. 4A. Immunostaining of each tissue section was performed prior to hematoxylin and eosin staining to confirm human tissue origin using species-specific antibody to a panel of human proteins.

The results reveal striking effects of altering NF-$_K$B function *in vivo*. Tissue transgenic for constitutively nuclear p50 consistently produced markedly atrophic epithelium (Figs. 4C, 4D) while inhibition of NF-$_K$B function was consistently associated with massive epithelial hyperplasia (Figs. 4B, 4E, 4F, 4G and 4H). This hyperplasia was characterized by extensive epidermal thickening as well as formation of invaginations and cystic structures penetrating deeper in the dermis. These changes in tissue architecture *in vivo* provide strong support for the regulation of epithelial growth by NF-$_K$B. Other vectors had no effect on the respective characteristics.

The frequency of histologic abnormalities in the above-described transgenic human epidermis samples was quantitated, as described in Example 4. Atrophic changes were defined as less than 30% thickness of viable epidermis as compared to normal value of 0.1 mm found in lacZ transgenic and unengineered control, as measured with a micrometer. Hyperplastic proliferations were defined as epithelial tissue islands penetrating into underlying dermis to a depth of at least 3 times the thickness of surrounding epidermis. The results are shown in Figs. 5A and 5B, expressed as the percent of individual tissue sections displaying the given histologic abnormality. As can be appreciated from the figures, 89% of sections of skin transfected with CN.p50 had an epidermis thickness of <0.03 mm (Fig. 5B), while 100% of sections of skin transfected with $I_KB_\alpha M$ had deep hyperplasia.

D. Transgenic Mice with Alterations in Epidermal NF-$_K$B Function

Transgenic mice were generated expressing proteins for dominant-negative inhibition, as well as for constitutive activation of NF-$_K$B function. For blockade of epidermal NF-$_K$B function, expression of the dominant negative $I_KB_\alpha M$ mutant was targeted to the epidermis via the keratin 14 promoter, as described in Vassar *et al.* (See schematic in Fig. 6.). $I_KB_\alpha M$ contains substitutions at serines 32 and 36 of I_KB a along with deletion of COOH-terminal PEST sequences and has been shown to abolish nuclear DNA binding activity by any of the 5 NF_KB subunits in a range of mammalian cells (Baeuerle *et al.* 1996; Van Antwerp *et al.*).

Fourteen $I_KB_\alpha M[+]$ transgenic mice were generated, with transgene integration confirmed

by Southern analysis and polymerase chain reaction. The mice expressed $I_\kappa B_\alpha M$ on Western analysis of epidermal tissue extracts, as detected by antibodies to $I_\kappa B_\alpha$ that recognize both the wild type and mutant proteins. $I_\kappa B_\alpha M[+]$ mice also demonstrated markedly increased immunostaining with these antibodies throughout all layers of transgenic epidermis, consistent with the expected resistance to degradation by this mutant protein. $I_\kappa B_\alpha M[+]$ transgenic epidermis displays a complete absence of nuclear NF-$_\kappa$B subunit expression in the suprabasal layers.

All $I_\kappa B_\alpha M[+]$ transgenic mice developed epidermal hyperplasia clinically and histologically within 2 days after birth. This hyperplasia appeared to be due to an increase in the thickness of the suprabasal squamous layer (to approx. 200 μM, as compared to approx. 60μM in control mice). In addition, these mice lacked both clinical and histologic evidence of normal hair formation, exhibited growth retardation and died within 5 to 7 days. No abnormalities in any internal organs were seen on either histologic or macroscopic evaluation.

Transgenic mice expressing constitutively nuclear NF-$_\kappa$B subunits in the basal layer of the epidermis were also generated, using the p50 construct of Fig. 6. Constitutive nuclear expression of p50 in transgenic skin, confirmed by immunohistochemistry, resulted in epidermal hypoplasia at the clinical and histologic levels. The markedly thin epidermis (approx. 25 μM) of the p50[+] mice consisted of as little as 2 viable cell layers, and the mice failed to gain weight normally and died within 5 days after birth. The most severely affected mice demonstrated open eyes at birth accompanied by extreme skin fragility and death within hours after being born.

These results suggest that nuclear translocation of NF-$_\kappa$B subunits to cells of the non-proliferative compartment of the tissue is necessary for proper growth control in stratified epithelium ($I_\kappa B_\alpha M[+]$ mice), and that induced activation of NF-$_\kappa$B in the proliferative basal compartment of the epidermis leads to premature growth inhibition (p50[+] mice).

E. Growth Characteristics of Murine Epidermis Transgenic for Alterations in NF-$_\kappa$B Function

To follow epithelial growth characteristics beyond the time of perinatal mortality observed in both $I_\kappa B_\alpha M[+]$ and P50[+] mice, skin from transgenic mice and nontransgenic littermate controls was grafted onto immune deficient mice, according to a procedure recently shown to preserve growth characteristics of donor skin (Oro *et al.*).

At 14 days postgrafting, grafted $I_\kappa B_\alpha M[+]$ epidermis demonstrated pronounced epidermal hyperplasia clinically and histologically (approx. 150 μM in thickness, vs. approx. 65 μM for control), with epidermal invaginations penetrating deeply into the underlying dermis. The p50[+] epidermis, in contrast, remained hypoplastic clinically and histologically (approx. 20

μM in thickness).

To further examine the basis for these alterations in epidermal homeostasis, DNA synthetic activity was studied in transgenic and control epidermis by BrdU incorporation. CB.17 scid/scid mice bearing $I_\kappa B_\alpha M[+]$, p50[+] and non-transgenic control skin were injected with BrdU (250 mg/kg of body weight) intraperitoneally. Skin biopsy specimens were taken 2 hours later and tissue sections stained with antibody to BrdU. Immunofluorescence micrographs showed increased labeling activity in $I_\kappa B_\alpha M[+]$ skin, extending into cells of the suprabasal layers, and a marked decrease in labeling activity in p50[+] skin, compared with the control.

$I_\kappa B_\alpha M[+]$ epidermis thus demonstrated a marked increase in the proportion of cells actively synthesizing DNA. BrdU positive cells in $I_\kappa B_\alpha M[+]$ transgenic epidermis included those well above the basal layer, indicating a possible failure of the cell cycle arrest that is normally associated with outward migration. p50[+] epidermis, in contrast, demonstrated a near absence of cells incorporating BrdU over the 2 hour time period analyzed, consistent with inhibition of proliferation.

These findings further suggest that functional loss of NF-$_\kappa$B leads to hyperproliferation in stratified epithelium, due to failure of growth arrest, and that premature expression of activated $NF_\kappa B$ subunits in mitotically active basal epithelial cells leads to growth inhibition.

F. Effect of a Pharmacologic Inhibitor of NF-$_\kappa$B on Normal Murine Skin.

In an alternative approach to alter NF-$_\kappa$B function in epidermis, a pharmacologic inhibitor of NF-$_\kappa$B, pyrrolidine dithiocarbamate (PDTC) (Schreck *et al.*), was applied topically in a PBS solution to the skin of normal adult C57BL/6J mice. A 0.01% SDS solution in PBS was applied under occlusion for the same period as an additional control for non-specific reactive hyperplasia to irritant stimuli.

Application of 10 mM PDTC twice daily for one week induced significant epidermal thickening (to approx. 225 μM) over the controls (approx. 70 μM for SDS and 30 μM for the PBS control). These results indicate that topical application of an agent that blocks NF-$_\kappa$B function to normal intact adult skin is associated with epidermal hyperplasia.

G. Effect of NF-$_\kappa$B on Epithelial Morphology and Proliferation *in vitro*.

In this study, normal human keratinocytes were transduced with the vectors described above (Figs. 1A and 1B, plus a lac Z control), incubated in SFM/154 growth media, and assayed for number of viable cells every 24 hours for 4 days. As shown in Figs. 7A-7B, proliferation in cells expressing $I_\kappa B_\alpha M$ was comparable to that of control cells, but was significantly inhibited in cells expressing CN.p50.

Control-transduced cells and cells expressing molecules that are dominant-negative for NF-$_\kappa$B function, including $I_\kappa B_\alpha M$ and $_\Delta$SP, exhibited the normal polygonal shaped cell morphology

and colony growth pattern. In contrast, NF-$_\kappa$B subunits produced cell morphologic changes as early as 24 hours after gene transfer. Cells became flat and enlarged with a vacuolated cytoplasm and lost the pattern of growth in colonies. These morphologic changes are consistent with those seen in epithelial cells undergoing replicative senescence (Saunders *et al.*). Nuclear stains failed to reveal morphologic changes characteristic of apoptosis, and non-viable cells comprised <5% of cells in each vector group at all time points, indicating that these findings were not due to increased cell death.

Cellular DNA synthesis and cell cycle distribution was also examined two days post-transduction with p50, p65 and $I_\kappa B_\alpha M$ retroviral expression vectors. Expression of active p50 and p65 subunits decreased the proportion of cells actively synthesizing DNA in vitro (Fig. 8), as measured by incorporation of bromodeoxyuridine (BrdU). Consistent with this result, cell cycle distribution analysis demonstrated that activated NF-$_\kappa$B subunits produced a greater than 50% decrease in epithelial cells in S-phase (Fig. 9).

H. Effect of Growth Factors on NF-$_\kappa$B Subunit Transduced Cells.

The NF-$_\kappa$B subunit expressing cells described above could be maintained for up to 4 weeks displaying the same morphology and apparent growth arrest. Because cellular senescence is characterized by irreversible growth arrest that is resistant to growth factor growth stimulation (Goldstein; Phillips *et al.*), the cells were next grown either in minimal media lacking growth factors or in media containing both epidermal growth factor (EGF) and keratinocyte growth factor (KGF). Under appropriate conditions, these factors can serve as epithelial cell mitogens in vitro (Gilchrest; Rheinwald *et al.*).

Control cells, transduced with lacZ, grew slowly in minimal media, but proliferate exponentially in the presence of growth factors, as expected (Fig. 10). Growth factors, however, did not overcome the growth arrest of NF-$_\kappa$B subunit expressing cells (Fig. 10), indicating that NF-$_\kappa$B rendered these cells resistant to these mitogenic stimuli.

An additional feature of cells that have undergone senescence is the induction of a senescence-associated β-galactosidase (SA-β-gal) that can be specifically detected in vitro at pH 6.0 (Dimri *et al.*). Accordingly, SA-β-gal was observed in NF-$_\kappa$B expressing cells as soon as 3 days after gene transfer, and the percentage of SA-β-gal positive cells consistently increases over the following 4 days (Fig. 11).

I. Effects of NF-$_\kappa$B on the Cyclin-Dependent Kinase (CDK) Inhibitor Protein $p21^{Cip1}$

Two families of cyclin-dependent kinase (cdk) inhibitors, Ink4 proteins and Cip1/Kip1 proteins, interact with cyclin/cdk targets, by different mechanisms (Sherr *et al.*). Only members of the latter group are upregulated during epithelial differentiation (Missero *et al.*; el-Deiry *et al.*, 1993; Harper *et al.*; Xiong *et al.*). Nuclear $p21^{Cip1}$ expression is seen in cells undergoing

terminal differentiation (Gartel *et al.*), and p21, like NF-$_\kappa$B, is expressed in the nuclei of suprabasal cells in normal stratified epithelial tissues, including skin and gastrointestinal tract (el-Deiry *et al.*, 1995; Inohara *et al.*).

To further elucidate the basis for NF-$_\kappa$B inhibition of cellular growth, the effect of NF-$_\kappa$B on expression of cyclin-dependent kinase inhibitors (CKIs) was studied. Cells were transduced with retroviral expression vectors for NF-$_\kappa$B subunits p50 and p65. Western blot analysis of cell extracts were prepared at 9 and 18 hours post transduction. The analysis showed that F-$_\kappa$B subunit-expressing cells induced high levels of $p21^{Cip1}$ protein (Fig. 12). Such induction was not observed in cells transduced with the transcriptionally inactive $_\Delta$SP p50 deletion mutant, lacZ or $I_\kappa B_\alpha M$

To analyze this effect at the level of individual cells, immunofluorescence staining was performed with antibodies to $p21^{Cip1}$ with cells expressing p50, p65 or lacZ control. Expression of active NF-$_\kappa$B subunits was associated with an augmented proportion of cells with nuclear $p21^{Cip1}$.

NF-$_\kappa$B induction of $p21^{Cip1}$ appeared to be selective in that it was not accompanied by changes in the levels of other CKIs, including $p27^{Kip1}$ or the INK4 family proteins $p15^{INK4B}$ or $p16^{INK4A}$. The induction also occurred without an increase in p53 expression, suggesting a p53-independent mechanism.

This increase in nuclear $p21^{Cip1}$ expression is consistent with a role for $p21^{Cip1}$ in NF-$_\kappa$B-induced epithelial growth arrest. This was confirmed by demonstrating that $p21^{Cip1}$ triggers growth inhibitory and senescence features induced by NF- B. An amphotropic retroviral vector for constitutive $p21^{Cip1}$ expression was produced. After confirming expression by the vector of full length $p21^{Cip1}$ protein and >98% gene transfer efficiency by immunofluorescence, using antibody to $p21^{Cip1}$, transduced cells were analyzed for proliferation kinetics and appearance of SA-β-gal. Similar to NF-$_\kappa$B, $p21^{Cip1}$-expressing cells demonstrated induction of SA-β-gal (Fig. 13). In addition, $p21^{Cip1}$ caused cell cycle arrest, with cell cycle distribution similar to that induced by NF-$_\kappa$B subunits (Fig. 14).

The above results demonstrate that activation of NF-$_\kappa$B inhibits cell cycle progression, and can trigger cell cycle arrest and cellular senescence in association with induction of the cell cycle inhibitor $p21^{Cip1}$.

V. Therapeutic Applications

A. Hyperproliferative disorders

Neoplastic and non-neoplastic hyperproliferative skin disorders present an ever-increasing

burden to health care providers. Increased UV exposure of skin has contributed to a significant increase in the incidence of premalignant lesions (e.g., actinic keratoses). Specifically, the number of cases in the U.S. of superficial squamous and basal cell carcinoma now exceeds 700,000 per year. Further, other localized hyperproliferative conditions, such as warts and psoriasis, are extremely prevalent.

Presently available treatments for hyperproliferative skin disorders limit the options available to the clinician. For example, many current treatments involve the application of cytotoxic agents (e.g., bleomycin and 5-fluorouracil) that are not selective for the hyperproliferative tissue and have significant side effects, including irreparable damage to surrounding skin and systemic absorption. Further, these methods are not always curative.

The present invention provides an alternative to the above-described treatments. Specifically, in one aspect, the invention includes a method of treating a hyperproliferative disorder of the skin, by administering to the subject a therapeutically effective amount of an activator of NF-$_K$B.

The term "hyperproliferative disorder of the skin" refers to malignant as well as non-malignant cell populations which morphologically differ from the surrounding tissue due to excessive growth and/or proliferation of epithelial cells. Hyperproliferative disorders thus include most skin diseases wherein the growth control mechanisms have been disrupted. Examples of hyperproliferative skin disorders include, but are not limited to, human papilloma virus (HPV) infected cells commonly associated with warts, superficial neoplasias of the skin such as melanomas, pre-malignant and malignant carcinomas, actinic keratosis, and psoriasis. Additional conditions amenable to treatment using methods of the invention include atopical dermatitis, contact dermatitis and further eczematous dermatitises, seborrhoeis dermatitis, pemphigus, lichen planus, lupus erythematosus, bullous pemphigoid, angioedemas, vasculitides, epidermolysis bullosa, urticaria, erythema, cutaneous eosinophilia, acne and alopecia areata.

It is contemplated that diseases of epithelial tissues other than the skin (e.g. endothelium, mesothelium) may also be treated using the methods of the invention. Examples include reversible obstructive airway disease, which includes conditions such as asthma (e.g., bronchial asthma, allergic asthma, dust asthma, intrinsic asthma, and extrinsic asthma), certain chronic or inveterate asthma (e.g., late asthma and airway hyper-responsiveness), bronchitis and the like. Further, various eye diseases may be treated, including conical cornea, dystrophia epithelialis corneae, keratoconjunctivitis, vernal conjunctivitis, keratitis, herpetic keratitis, sarcoidosis, corneal leukoma, ocular pemphigus, Mooren's ulcer, Scleritis, Graves' ophthalmopathy, and Vogt-Koyanagi-Harada syndrome. The methods may also be used to treat hyperproliferative vascular diseases such as intimal smooth muscle cell hyperplasia, restenosis and vascular

occlusion.

In addition, as noted above, recent evidence suggests that neoplastic transformation may require mechanisms that, in addition to avoiding apoptosis, also bypass cellular senescence. Activation of NF-$_K$B has been shown, as described above, to induce the cell cycle inhibitor $p21^{Cip1}$ and promote premature senescence. Accordingly, the method may also be used to inhibit neoplastic growth.

B. Promotion of Cell Proliferation

In one aspect, inhibition of NF-$_K$B activity, as described herein, may be used to promote wound healing. Wound healing involves the repair of injured tissue, the regeneration of specialized tissue, and reorganization of new tissue. It consists of three major phases: i) an inflammation phase lasting up to about three days, ii) a cellular proliferation phase lasting from about three to about 12 days, and (c) a remodeling phase lasting from about three days to six months. During the inflammation phase, clotting factors and platelet aggregation act to form a matrix, trapping plasma proteins and blood cells. New connective or granulation tissue, as well as blood vessels, form during the cellular proliferation phase. The granulation tissue is replaced by a network of collagen and elastic fibers (leading to the formation of scar tissue) during the remodeling phase.

It will be appreciated that wound healing can be facilitated if any of the above-described phases can be accelerated. In this context, it is an object of the present invention to provide a method of accelerating or enhancing the healing of wounds to the epithelium by stimulating epithelial cell proliferation. The method includes administering to the subject a therapeutically effective amount of an activator of NF-$_K$B.

In transgenic mouse epidermis overexpressing p-50 (Section E above), a increase in hair growth was also observed. Because the activation of NF-$_K$B has been shown herein to promote cell cycle arrest, and thus accelerate terminal differentiation, in epithelial cells, this would be consistent with increased production of hair, a terminally differentiated epidermal structure. Accordingly, these preliminary results suggest that administration of an NF-$_K$B activator, as described herein, could promote hair growth in the epidermis.

VI. Administration of Compounds Effective to Alter NF-$_K$B Activity

In accordance with the method, a therapeutically effective amount of an activator or inhibitor of NF-$_K$B, in a pharmaceutically acceptable carrier, is administered to a subject in which it is desired to promote or inhited, respectively, NF-$_K$B activity. Any conventional method for delivery of a biologically active compound may be used to deliver a therapeutically effective compound according to the methods of the present invention. The preferred dosage

and formulation typically depends on the type of compound to be delivered.

For example, in the case of NF-$_\kappa$B antisense oligomer compositions useful for wound healing applications, the oligomer may be delivered as described, e.g., in Narayananof *et al.* (1997). The oligomer may be delivered alone, or in composition with a suitable pharmaceutical carrier or coupled with carriers. Examples of suitable carriers include peptides, immunoglobulins and their fragments, liposomes, receptor molecules, ligand molecules such as hormones, enzymes, and any conventional compounds for pharmaceutical administration.

In the case of proteinaceous compounds, such as IL-1, IL-2, and $I_\kappa B\beta$, the compound can be, for example, encapsulated in microspheres or proteinoids. Such compounds may also be delivered transdermally by iontophoresis or transdermal electroporation. Methods for the preparation and administration of therapeutically-active proteins are known to one of skill in the art (see, for example, Banga, 1995).

Delivery of an effective amount of a therapeutic compound may be oral, parenteral, intravenous, transdermal, or by any conventional pharmaceutical route. As activating and inhibiting compounds have been shown herein to be effectively expressed in vivo, administration via gene therapy is also contemplated.

In a preferred embodiment, the compound is applied topically to the site of the hyperproliferative disorder, wound, or site of desired hair growth, to minimize systemic activity of the compound. Such topical applications typically involve suspending the therapeutic compound in a solution, emulsion, cream or ointment with a pharmaceutically acceptable carrier.

For transdermal delivery, the use of a transdermal patch allows for continuous delivery of compound to a selected skin region. Examples of transdermal patch delivery systems are provided by U.S. Patent 4,655,766 (fluid-imbibing osmotically driven system), and U.S. Patent 5,004,610 (rate controlled transdermal delivery system). If desired, permeation enhancing substances, such as fat soluble substances (e.g., aliphatic carboxylic acids, aliphatic alcohols), or water soluble substances (e.g., alkane polyols such as ethylene glycol, 1,3-propanediol, glycerol, propylene glycol, and the like) may be included. In addition, as described in U.S. Patent 5,362,497, a "super water-absorbent resin" may be added to transdermal formulations to further enhance transdermal delivery. Examples of such resins include, but are not limited to, polyacrylates, saponified vinyl acetate-acrylic acid ester copolymers, cross-linked polyvinyl alcohol-maleic anhydride copolymers, saponified polyacrylonitrile graft polymers, starch acrylic acid graft polymers, and the like. Such formulations may be provided as occluded dressings to the region of interest, or may be provided in one or more of the transdermal patch configurations described above.

For delayed release, the activator or inhibitor may be included in a pharmaceutical composition formulated for slow release, such as in microcapsules formed from biocompatible polymers or in liposomal carrier systems according to methods known in the art.

The dosage of therapeutic compound administered is determined in accord with clinical practice, and will vary depending upon such factors as the patient's age, previous medical history, and general medical condition. The dose is determined in part based on the pharmacokinetics of clearance of the administered compound, using standard pharmacokinetics principles known in the art (Gennaro, 1990; Gilman *et al.*, 1995).

IVII. Screening Applications

The present invention also includes methods of identifying compounds effective to treat an epithelial hyperproliferation disorder. One such method includes the steps measuring the activity of NF-$_K$B in the presence and absence of a test compound, and identifying the test compound as effective in the treatment if it results in an upregulation of NF-$_K$B activity. Any of a number of screens of NF-$_K$B activity can be employed by one of skill in the art. An exemplary assay of NF-$_K$B activity is the reporter gene assay described in Example 2, herein.

In another aspect, the invention includes a method of identifying compounds useful for promoting wound healing. The method includes the steps measuring the activity of NF-$_K$B in the presence and absence of a test compound, and identifying the test compound as useful if it results in a downregulation of NF-$_K$B activity. As described above, any of a number of NF-$_K$B activity assays known to those skilled in the art may be employed in such a screen.

A variety of different compounds may be screened using methods of the present invention. They include peptides, macromolecules, small molecules, chemical and/or biological mixtures, and fungal, bacterial, or algal extracts. Such compounds, or molecules, may be either biological, synthetic organic, or even inorganic compounds, and may be obtained from a number of sources, including pharmaceutical companies and specialty suppliers of libraries (*e.g.*, combinatorial libraries) of compounds.

The following examples illustrate but are not intended to limit the present invention.

MATERIALS AND METHODS

Unless otherwise indicated, restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). Nitrocellulose paper was obtained from Schleicher and Schuell (Keene, NH). Materials for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories

(Hercules, CA). Other chemicals were purchased from Sigma (St. Louis, MO) or United States Biochemical (Cleveland, OH).

Production of transgenic animals and skin tissue

Sequences encoding the $I_\kappa B_\alpha M$ mutant (Van Antwerp *et al.*) and the constitutively nuclear p50 XbaI mutant (Blank *et al.*) were subcloned downstream of a 2075 bp human keratin 14 promoter (Vassar *et al.*) construct containing a 5' intron from the D-globin gene and used to produce transgenic mice. Transgene integration was confirmed both by Southern analysis and by polymerase chain reaction of genomic DNA. For the latter, primers specific for the 3' end of the K14 promoter and the 5' end of the expressed cDNA, either the $I_\kappa B_\alpha M$ mutant and the constitutively nuclear p50 XbaI mutant, were used to amplify an 850 base pair fragment. Fourteen $I_\kappa B_\alpha M[+]$ and thirteen CN.p50[+]mice were characterized.

Genetically engineered human epidermis was regenerated on CB.17 *scid/scid* mice from early passage keratinocytes after high efficiency retroviral gene transfer by previously described methods (Choate *et al.*, 1996a; Medalie *et al.*). Briefly, transduced keratinocytes were plated on devitalized human dermis and grown *in vitro* with growth media for 7 to 10 days followed by grafting to the backs of CB.17 *scid/scid* mice. Analysis of grafted human tissue was performed 3 weeks post-grafting.

Immunoblotting and Immunohistochemistry

Whole cell extracts were prepared from cells grown *in vitro* and immunoblotted as described (Choate *et al.*, 1996a,b) after separation by SDS-PAGE on 8%, 10% or 15% gel. Approximately 20μg of protein, as determined by Bradford (Biorad, Hercules, CA), were loaded per lane. Equal loading conditions were confirmed by Coomassie blue staining. Antibodies to $p21^{Cip1}$, $p27^{Kip1}$, $p15^{INK4B}$, $p16^{INK4A}$, p50, p65, p50/105 and $I_\kappa B_\alpha$ were obtained from Santa Cruz Biotech. Blots were incubated simultaneously with polyclonal antiserum to BRG1 (Khavari *et al.*), a constitutively expressed 205 kDa protein that served to control for cell extract quality and protein transfer efficiency. Blots were visualized using the ECL-detection system (Amersham, Arlington Heights, IL).

Immunoblots were performed on transgenic skin tissue extracts as an additional confirmation of transgene expression. Following skin biopsy, tissue was incubated for 1 hour at 37°C with dispase (Becton-Dickinson) (25U/ml) to separate the epidermis from underlying dermis, then epidermal extracts were prepared and analyzed as above.

Immunohistochemical analysis was performed as previously described (Choate *et al.*, 1996a,b) using antibodies to NF-κB subunits and to $p21^{Cip1}$ (Santa Cruz Biotech) as well as to BrdU (Becton-Dickinson). Prior to immunostaining, cells were rinsed with PBS, fixed for 10 minutes in

acetone at room temperature, air-dried and blocked with 5% normal goat serum. For staining, slides were incubated with primary antibodies for 30 minutes, followed by PBS washing and incubation with FITC-conjugated secondary antibodies (Sigma, St. Louis, MO) and mounted with Vectashield mounting media (Vector Inc., Burlingame, CA). Immunohistochemical staining after retroviral transduction of cells grown in vitro was performed as noted below. Where indicated, cells were counterstained 15 seconds with propidium iodide (20μg/ml in PBS) to visualize all nuclei. Slides were then analyzed by fluorescence microscopy. For senescence-associated β-gal (SA-β-gal) staining, cells were washed in PBS, fixed with 2% formaldehyde/ 0.2% glutaraldehyde for 5 minutes at room temperature and stained for β-gal at pH 6.0 as described. (Dimri *et al.*, 1995)

Cell culture and gene transfer

Normal human epithelial cells were isolated from human skin as described (Rheinwald *et al.*) . Cells were grown in a 1:1 mixture of SFM (Gibco) and 154 media (Cascade Biologics), optimal conditions for proliferation. Retroviral expression vectors for ΔSP, IκBαM, p50 and p65 were constructed as described (Seitz *et al.*, 1998) . cDNA sequences corresponding to the coding regions of human p50 (amino acids 1-502, XbaI truncation) (Blank *et al.*), p65 (Nolan *et al.*) and the dominant-negative mutants $I_\kappa B_\alpha M$ (Van Antwerp *et al.*) and $_\Delta$SP (Logeat *et al.*) were subcloned into the EcoRI site of the LZRS retroviral vector (Kinsella *et al.*). The $p21^{Cip1}$ vector was produced by subcloning the full length $p21^{Cip1}$ cDNA into the EcoRI site of the LZRS backbone vector (Kinsella *et al.*) after removal of the EcoRI fragment containing the *lacZ* gene. Amphotropic retrovirus production and gene transfer with test and lacZ and GFP control vectors was performed as previously described (Choate *et al.*, 1996a,b; Kinsella *et al.*) ; >95% gene transfer efficiency was confirmed for each vector by immunofluorescence staining with antibodies to NF-κB subunits, IκB and $p21^{Cip1}$.

Transient transfections were performed by the modified polybrene shock method, as previously described (Freiberg *et al.*). Briefly, 30% confluent normal human keratinocytes were transfected

using 2 μg of p50, p65, $I_\kappa B_\alpha M$ or $_\Delta$SP expression plasmid, 2 μg of NF-κB-luciferase reporter plasmid and 1 μg of RSV-CAT internal control. For assessment of $I_\kappa B_\alpha M$ and $_\Delta$SP dominant negative effects, NF-κB activity was induced for 4 hours with 30ng/ml of PMA prior to reporter gene analysis (Khavari *et al.*).

Analysis of mitotic activity and cell cycle distribution

For analysis of cellular proliferation, cells were transduced in triplicate 35 mm plates for each vector as previously described (Choate *et al.*, 1996a,b). 48 hours following gene transfer, cells were re-plated at low densities of 10^4 cells/35 mm plate. Following this, cells were harvested and

counted in triplicate at 24 hour intervals. Cell morphology was determined at each time point by phase contrast microscopy, and nuclear morphology was evaluated by fluorescence microscopy after staining with propidium iodide. Cell viability was determined by trypan blue exclusion. For mitogenic stimulation, cells were grown in the presence of epidermal growth factor (EGF) and keratinocyte growth factor (KGF), both at a concentration of approximately 10 ng/ml.

For BrdU labeling *in vitro*, cells were grown on glass cover slides and incubated for 2 hours with 10 μM BrdU (Boehringer, Indianapolis, IN), then rinsed with PBS, fixed for 30 minutes in 70% ethanol and air dried. After treatment with 0.07 N NaOH for 2 minutes, the slides were thoroughly rinsed in PBS and stained with anti-BrdU monoclonal antibody (Becton Dickinson, San Jose, CA). Cells were then counterstained with propidium iodide (20μg/ml) for 15 seconds to visualize all cells in a given field. For *in vivo* BrdU labeling, mice were injected intraperitoneally with BrdU (250mg/kg body weight), then sacrificed 2 hours later and tissue sections subjected to immunofluorescence staining with FITC-conjugated antibody to BrdU.

For cell cycle analysis, cells were stained with propidium iodide 72 hours after transduction, then subjected to flow cytometry. Briefly, cells were trypsinized, washed in PBS and incubated for 20 minutes in a solution containing 0.1% sodium citrate (pH 7.8), 0.1% Triton X, 50μg/ml propidium iodide, and 1mg/ml RNAse. Then an equal volume of a solution containing 0.376M NaCl, 0.1% Triton X, 50μg/ml propidium iodide was added and kept at 4°C until subjected to flow cytometry. Data was analyzed using ModFit software as previously described (Missero *et al.*) .

EXAMPLE 1: Construction and Expression of Retroviral Vectors for Activating or Inhibiting NF-$_\kappa$B

Retroviral expression vectors encoding proteins exerting either activating or inhibitory effects on NF-$_\kappa$B function were generated using standard cloning techniques (Ausubel *et al.*, Sambrook *et al.*, 1989). The vectors were made using the MFG-based LZRS retroviral expression vector (Kinsella and Nolan, 1996) and amphotropic retrovirus produced in modified 293 packaging cells as described (Choate *et al.*, 1996a,b; Kinsella *et al.*).

Schematics of the vectors are shown in Figs 1A-D. Ψ+ represents extended retroviral packaging sequence. Vector CN.p50 (Fig. 1A) contains cDNA sequences encoding constitutively nuclear p50 (p105 amino acids 1-502) (Blank *et al.*), while vector $I_\kappa B_\alpha M$ (Fig. 1B) contains cDNA sequences encoding a trans-dominant mutant $I_\kappa B_\alpha M$ repressor (Van Antwerp *et al.*, 1996). A lacZ vector (Fig. 1C; Kinsella *et al.*), a p50 deletion construct (Fig. 1D), as well as mock transduction, served as controls.

The above-described vectors were effectively expressed in primary cultures of human keratinocyte epithelial cells (Rheinwald *et al.*) using a high efficiency gene transfer approach

(Choate *et al.*, 1996a,b). Forty eight hours following transduction, cell extracts were prepared, separated via SDS-PAGE on an 8% polyacrylamide gel, and immunoblotted with antibodies to p50 and I_KB_α. [-] = untransduced.

The results are shown in Figs. 2A and 2B. The blot in Fig. 2A was stained with an anti-p50 antibody. The lanes were as follows: lane 1 - untransduced control, lane 2 - mutant dominant negative p50 transduced, and lane 3 - CN.p50 transduced. The blot in Fig. 2B was stained with an anti-I_KB_α antibody. The lanes were as follows: lane 1 - untransduced control, and lane 2 - $I_KB_\alpha M$ transduced. The results indicate that cells transduced with the indicated constructs effectively express the proteins encoded by those constructs.

EXAMPLE 2: Assessment of NF-$_K$B Activity in Transduced Cells

Additional experiments were performed to assess the cellular localization of the p50 NF-$_K$B subunit after gene transfer. After retroviral transduction with the panel of expression vectors described above, normal human keratinocytes were immunostained with an antibody to the p50/105 NF-$_K$B subunit and visualized by laser confocal fluorescence microscopy. Results, as described above, showed that expression of NF-$_K$B activating proteins (encoded by vector CN.p50) resulted in nuclear localization of NF-$_K$B immunoreactivity, whereas expression of NF-$_K$B inhibiting proteins (encoded by vector $I_KB_\alpha M$) resulted in cytoplasmic localization of NF-$_K$B immunoreactivity.

To determine effect of expression of the NF-$_K$B activating and inhibitory proteins on NF-$_K$B directed reporter gene expression in these cells, keratinocytes were transfected with a plasmid containing 3 copies of NF-$_K$B DNA consensus binding sites driving expression of the luciferase reporter gene (Freiberg *et al.*), along with a CMV-CAT plasmid that served as an internal control of transfection efficiency. Reporter gene activity, summarized in Fig. 3, was assessed 48 hours after transduction with the indicated vectors, normalized for transfection efficiency using a cotransfected RSV-CAT internal control. The results illustrate that $I_KB_\alpha M$ significantly inhibits the ability of NF-$_K$B to induce expression of the reporter gene, while CN.50 causes a slight but insignificant increase in reporter gene expression.

EXAMPLE 3: Effects of Modulating NF-$_K$B Activity *In Vivo* in Human Skin

Primary human keratinocytes transduced with the indicated vectors, as described above, were used to regenerate transgenic human skin on SCID mice *in vivo* as described in Choate *et al.*, 1996a,b, and Medalie *et al.*, 1996. Transduced cells were seeded on devitalized human dermal substrate *in vitro* and left to grow for 7 days prior to direct grafting onto the fasia of SCID mice recipients. Six mice were grafted for each vector in 2 separate sets of experiments. Four weeks after grafting, dressings were removed and the tissue was analyzed. Exemplary

results of histological analyses are shown in Figs. 4A-4H.

Expression of terminal differentiation markers was normal in human skin transgenic for either activating or inhibitory NF-$_\kappa$B subunits and lacZ control. Double immunostaining was performed with species-specific antibodies to human involucrin (Murphy *et al.*) and BPAG2, a basement membrane zone protein to highlight the inferior boundary of the basal epidermal layer (Fairley *et al.*). The results showed that epidermal differentiation markers including keratin 10, involucrin, keratinocyte transglutaminase and filaggrin, however, were expressed in normal suprabasal distribution in transgenic skin of all vector groups.

The frequency of histologic abnormalities in the above-described transgenic human epidermis samples was quantitated as follows. Multiple 5 μM sections were obtained in a stepwise fashion through tissue biopsies that spanned the full 1.5 cm thickness of each transgenic and control regenerated human graft, and representative sections were analyzed. After confirmation of human species origin via immunostaining with antibody to involucrin (Murphy *et al.*, 1984), histologic appearance was analyzed. Atrophic changes were defined as less than 30% thickness of viable epidermis as compared to normal value of 0.1 mm found in lacZ transgenic and unengineered control, as measured with a micrometer. Hyperplastic proliferations were defined as epithelial tissue islands penetrating into underlying dermis to a depth of at least 3 times the thickness of surrounding epidermis.

The results are shown in Figs. 5A and 5B. A total of 9 representative tissue sections were analyzed from all grafted mice for constitutively nuclear p50 subunit (CN.p50), 12 for $I_\kappa B_\alpha M$, and 6 for lacZ and unengineered control. The data are expressed as the % of individual tissue sections displaying the given histologic abnormality. As can be appreciated from the figures, 89% of sections of skin transfected with CN.p50 had an epidermis thickness of < 0.03 mm (Fig. 5B), while 100% of sections of skin transfected with $I_\kappa B_\alpha M$ had deep hyperplasia.

EXAMPLE 4: Effects of NF-$_\kappa$B on Epithelial Proliferation

Normal keratinocytes were transduced in 6 parallel transductions for each vector. The cells were incubated in SFM/154 growth media as described (Choate *et al.*, 1996a,b) and replicate transductions for each vector harvested by trypsinization at 24 hour intervals for 4 days. Cells were stained with trypan blue and counted using phase contrast microscopy. The proportion of viable cells for all vectors was $>95\%$ at all timepoints.

The results are shown in Fig. 7. Cells expressing $I_\kappa B_\alpha M$ proliferated at rates similar to control cells, whereas proliferation of cells expressing constitutively nuclear p50 was significantly inhibited. In additional experiments, asynchronously dividing primary keratinocytes were incubated with BrdU for 3 hours in growth media and stained with

monoclonal antibody to BrdU as described in Missero *et al.*, 1996. Counterstaining with propidium iodide was used to identify all cells present in a given field. Three independent transductions were evaluated for each vector; at least 1000 cells were counted in all cases. These results demonstrate that constitutively nuclear p50 NF-$_\kappa$B is associated with a decreased proportion of cells actively synthesizing DNA.

Cell cycle distribution was assessed by transducing normal primary human keratinocytes with activating and inhibitory subunits for NF-$_\kappa$B function and staining with propidium iodide. The cells were analyzed by flow cytometry, and fraction of cells in G_1, S and G_2/M was calculated using "MODFIT LT" software, as described in Missero *et al.* The results showed that blockade of NF-$_\kappa$B function was associated with an increase in proportion of cells in S phase.

The above-described changes correlated with the proportion of basal keratinocytes expressing the Ki-67 marker of cellular proliferation *in vivo*. Constitutively nuclear p50 was associated with a decrease as opposed to the augmented numbers of Ki-67(+) cells that were seen in $I_\kappa B_\alpha M$ transgenic epidermis compared to lacZ controls.

While the invention has been described with reference to specific methods and embodiments, it is appreciated that various modifications and changes may be made without departing from the invention.

It is claimed:

1. A method of inhibiting cellular proliferation in the epithelium, comprising administering to a subject in need of such treatment an amount of an activator of NF-$_\kappa$B activity, or an NF-$_\kappa$B protein subunit, which is effective to inhibit said proliferation.

2. The method of claim 1, wherein said protein subunit is a p50 or p65 subunit.

3. The method of claim 1, wherein said activator is selected from the group consisting of tumor necrosis factor alpha (TNF$_\alpha$), phorbol 12-myristate 13-acetate (PMA), interleukin-1 (IL-1), interleukin-2 (IL-2), and bacterial lipopolysaccharide (LPS).

4. The method of claim 1, for use in treating a hyperproliferative skin disorder, wherein said activator is administered to the epidermis.

5. The method of claim 4, wherein said activator is administered topically.

6. A method of promoting cellular proliferation in the epithelium, comprising administering to a subject in need of such treatment an amount of an inhibitor of NF-$_\kappa$B activity which is effective to promote said proliferation.

7. The method of claim 6, wherein said inhibitor is selected from the group consisting of I$_\kappa$Bβ, I$_\kappa$B$_\alpha$, pyrrolidine dithiocarbamate (PDTC), 2-(2,6-dioxo-3-piperidinyl)-4-azaisoindoline-1,3-dione, a glucocorticoid, a serine protease inhibitor, and an NF-$_\kappa$B antisense compound.

8. The method of claim 6, for use in accelerating or enhancing wound healing, wherein said inhibitor is administered to the site of the wound.

9. The method of claim 8, wherein said inhibitor is administered topically.

10. A method of identifying compounds effective to treat an epithelial hyperproliferation disorder, comprising

measuring the activity of NF-$_\kappa$B in the presence and absence of a test compound, and

identifying the test compound as effective if it results in an upregulation or promotion of NF-$_\kappa$B activity.

11. A method of identifying compounds useful for promoting wound healing, comprising measuring the activity of NF-$_K$B in the presence and absence of a test compound, and identifying the test compound as useful if it results in a downregulation or inhibition of NF-$_K$B activity.

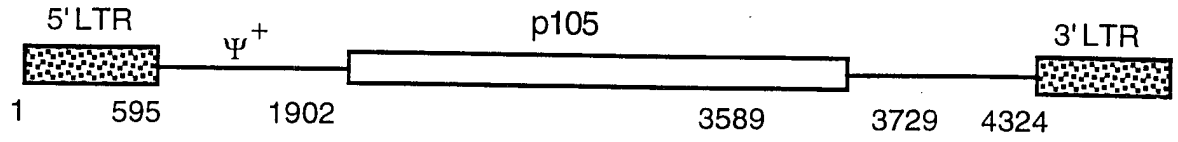


Fig. 1A

CN.p50

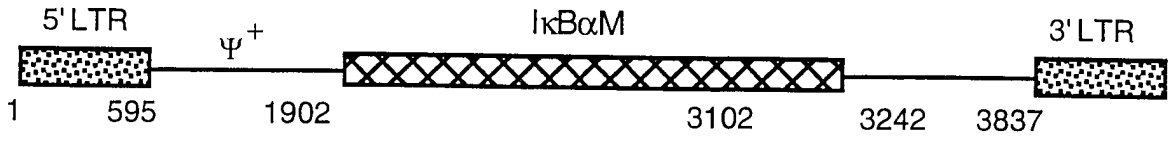


Fig. 1B

IκBαM

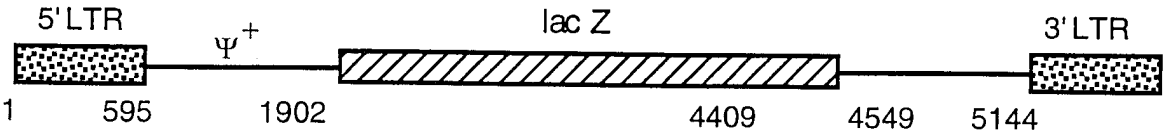


Fig. 1C

lac Z

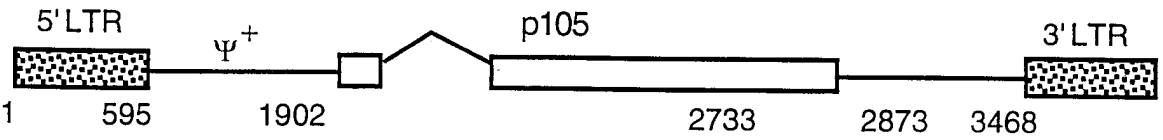


Fig. 1D

DN.p50

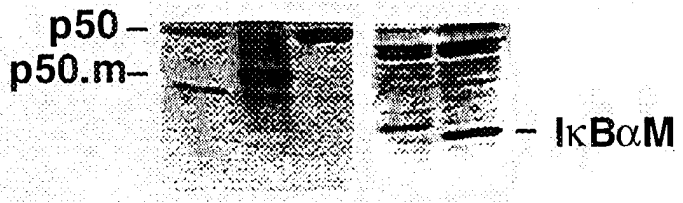


Fig. 2A Fig. 2B

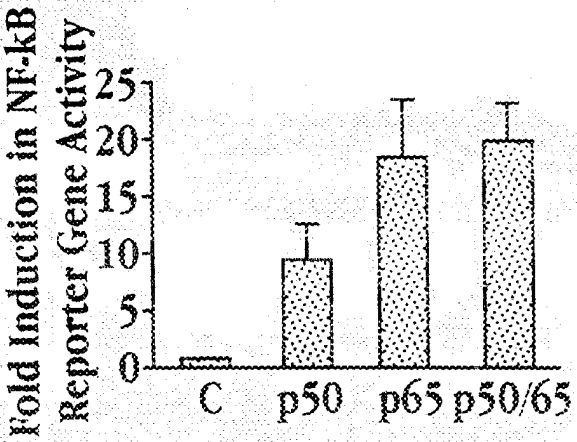


Fig. 3A

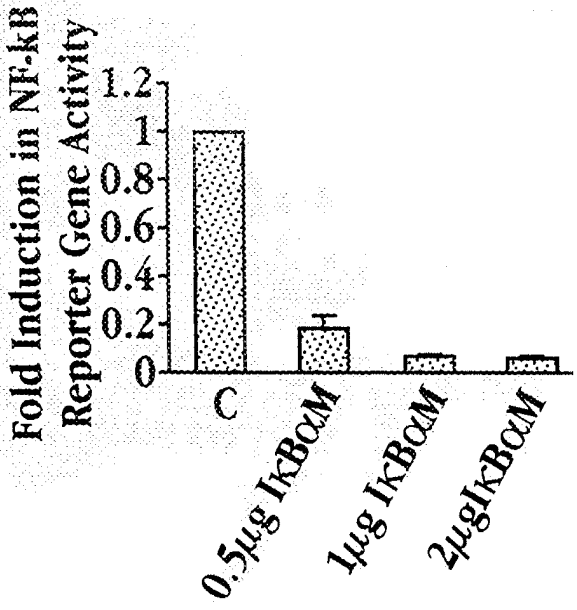


Fig. 3B

Fig. 4D

Fig. 4C

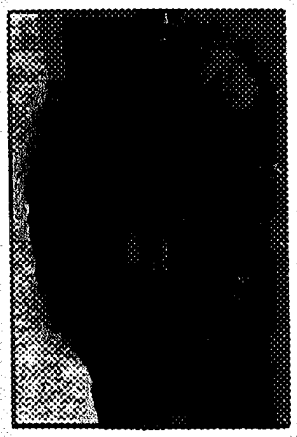


Fig. 4B

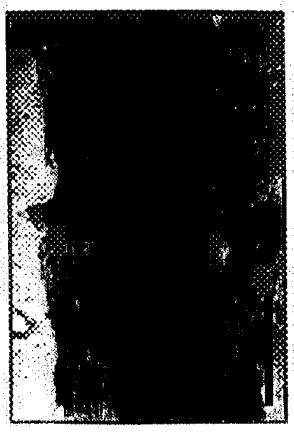


Fig. 4A

Fig. 4H

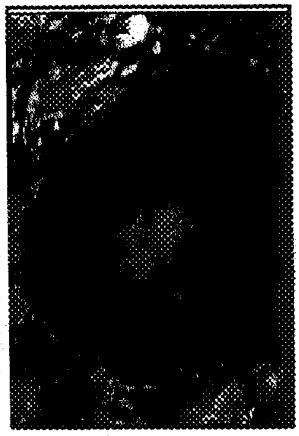


Fig. 4G

Fig. 4F

Fig. 4E

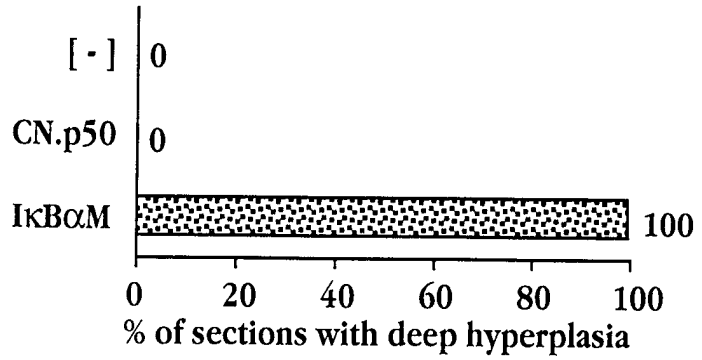


Fig. 5A

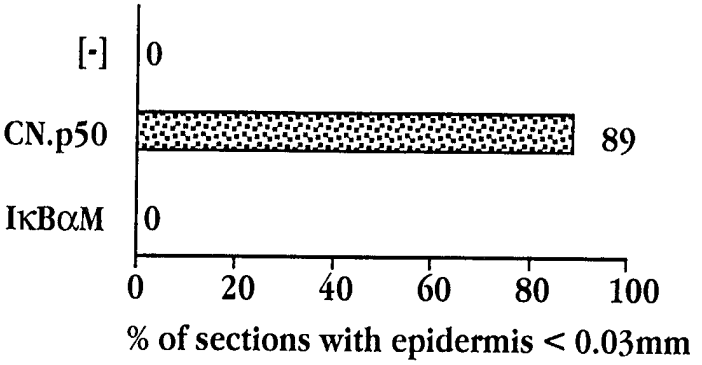
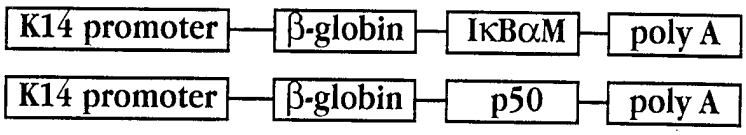


Fig. 5B

Fig. 6



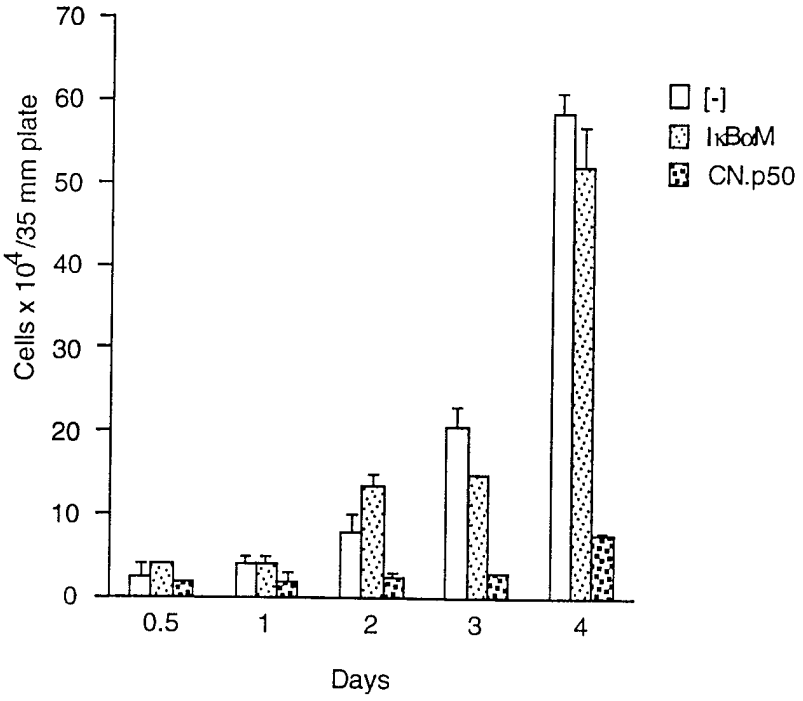

70
60
50
40
30
20
10
0
Cells x 10^4/35 mm plate
0.5
1
2
3
4
Days
[-]
IκBαM
CN.p50

Fig. 7A

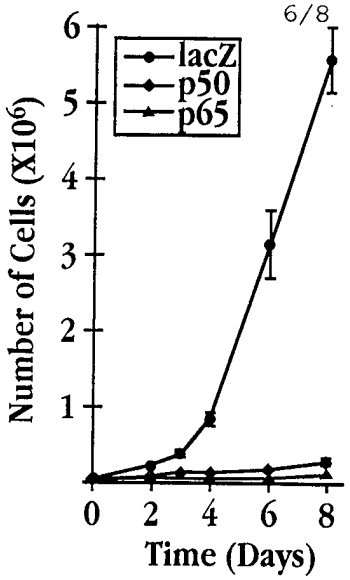


Fig. 7B

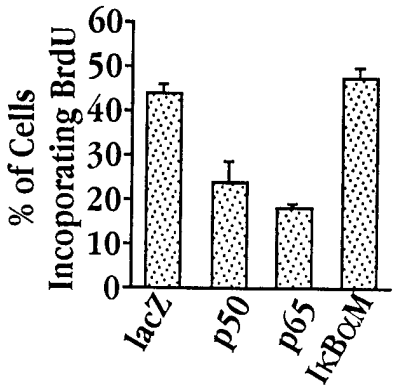


Fig. 8

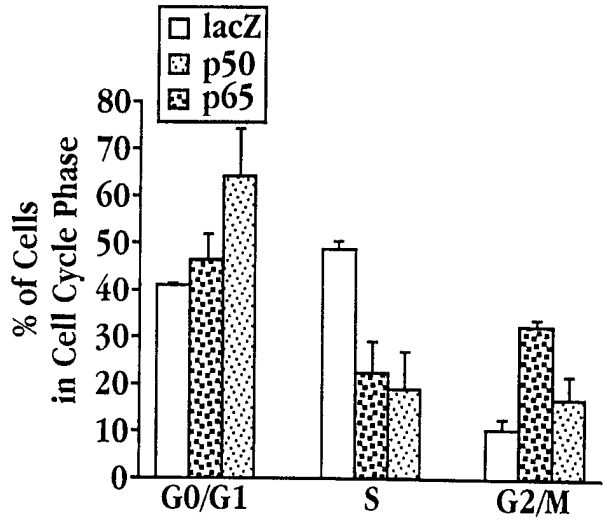


Fig. 9

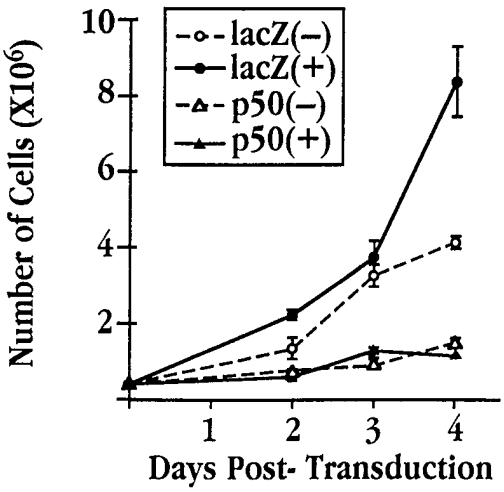


Fig. 10

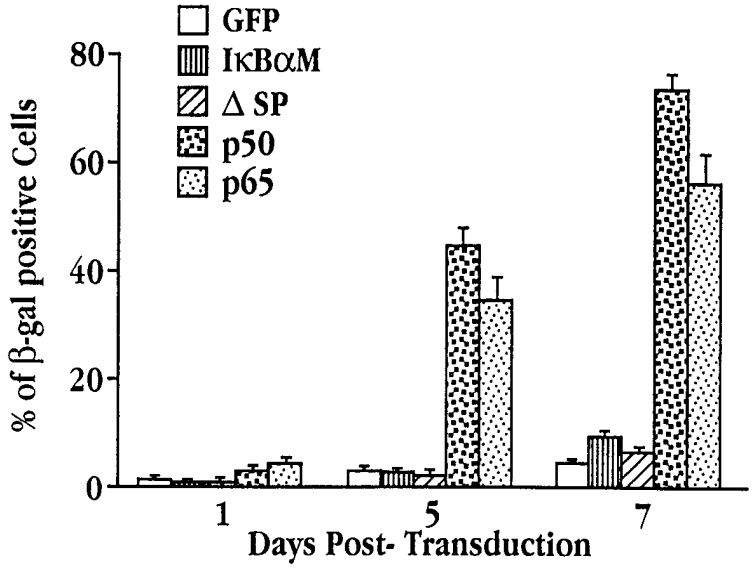


Fig. 11

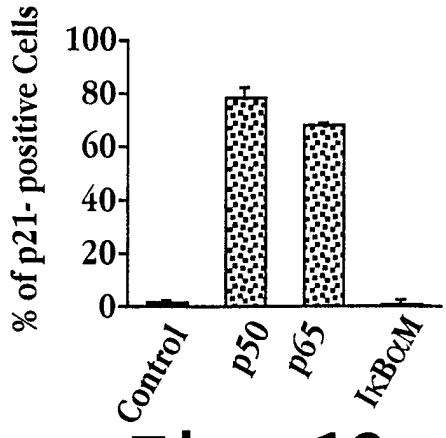


Fig. 12

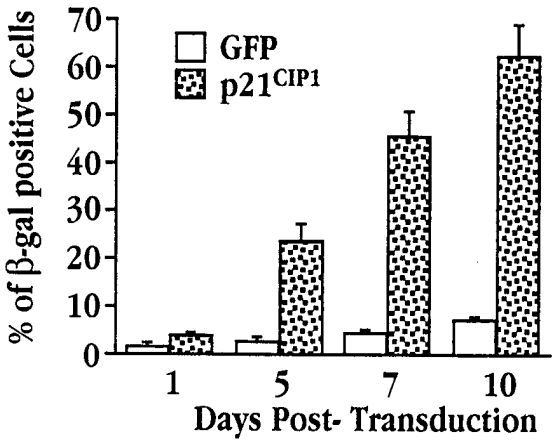

% of β-gal positive Cells
70
60
50
40
30
20
10
0
GFP
p21CIP1
1
5
7
10
Days Post- Transduction

Fig. 13

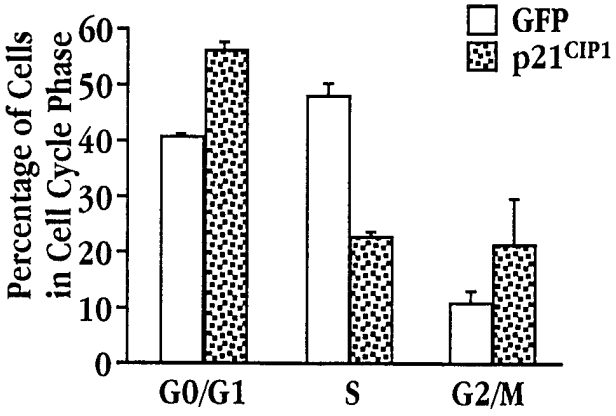

Percentage of Cells in Cell Cycle Phase
60
50
40
30
20
10
0
GFP
p21CIP1
G0/G1
S
G2/M

Fig. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07266

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/06, 38/19, 38/20, 38/55, 48/00

US CL :424/85.1, 85.2; 514/2, 8, 12, 44, 887, 946, 947; 536/24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 85.2; 514/2, 8, 12, 44, 887, 946, 947; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORIKAWA et al. Enhancement of Therapeutic Effects of Recombinant Interleukin 2 on a Transplantable Rat Fibrosarcoma by the Use of a Sustained Release Vehicle, Pluronic Gel. Cancer Research. 01 January 1987, Vol.47, pages 37-41, especially pages 40-41.	1-5
X	LEE et al. Interleukin-1 alpha mediates phorbol ester-induced inflammation and epidermal hyperplasia. The FASEB Journal. 1994, Vol.8, pages 1081-1087, especially pages 1081-1082.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 JUNE 1998	Date of mailing of the international search report 30 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PREMA MERTZ Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07266

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROBERTSON et al. Inhibition of pro-inflammatory cytokine gene expression and papilloma growth during murine multistage carcinogenesis by pentoxifylline. Carcinogenesis. 1996, Vol.17, No.8, pages 1719-1728, especially pages 1719-1728.	1-5
X	US 5,194,248 A (M. HOLICK) 16 March 1993, see entire document, especially column 6, lines 22-68.	6-9
X	US 4,716,030 A (D. MACY) 29 December 1987, see entire document, especially column 5, lines 46-68.	6-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07266

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07266

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS, CAPLUS
search terms:tumor necrosis factor alpha, phorbol 12-myristate 13-acetate (PMA), IL-1, IL-2, LPS, glucocorticoid, inhibitor, activator, administration, therapy, treatment, topical.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-9, drawn to a method of inhibiting cellular proliferation or a method for promoting cellular proliferation, comprising administering an activator or an inhibitor of NF-kappaB activity, respectively.
Group II, claims 10-11, drawn to a method of identifying compounds to treat epithelial hyperproliferation disorder or promoting would healing comprising measuring the activity of NF-kappaB in the presence and absence of the test compound.

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule
13.2, they lack the same or corresponding special technical features for the following reasons:
Pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first-recited method, a method of inhibiting cellular proliferation or a method for promoting cellular proliferation, comprising administering an activator or an inhibitor of NF-kappaB activity, respectively. Further pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that any feature which the subsequently recited method shares with the main invention does not constitute a special technical feature within the meaning of PCT Rule 13.2 and that each of such methods accordingly defines a separate invention.