

Keratinocyte Survival, Differentiation, and Death: Many Roads Lead to Mitogen-Activated Protein Kinase

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The epidermis is a dynamic and continually renewing surface that provides and maintains a life-sustaining interface with the environment. The epidermal keratinocyte, the major cell type of the epidermis, undergoes a complex and carefully choreographed program of differentiation. This process requires a balance between keratinocyte proliferation, differentiation, and apoptosis. This overview will concentrate on cascades that regulate the balance between keratinocyte cell proliferation and survival, and apoptosis and cell differentiation,

with a particular emphasis on the role of the mitogen-activated protein kinase cascades. A summary of the literature suggests that extracellular regulated kinases function to promote keratinocyte proliferation and survival, whereas p38 mitogen-activated protein kinase functions to promote differentiation and apoptosis. Key words: apoptosis/caspase/epidermis/gene expression/keratinocyte differentiation/MAPK. JID Symposium Proceedings 7:36–40, 2002

In recent decades study of keratinocyte differentiation has yielded substantial new insights. Early efforts identified and cataloged many of the morphologic properties of keratinocytes. Progress was limited, however, by the lack of a suitable *in vitro* system for the study of keratinocyte differentiation. The 1970s heralded the arrival of various cell culture models that permitted keratinocytes to be grown in mass culture (Rheinwald and Green, 1975; Elgjo *et al*, 1976; Bettger *et al*, 1981; Boyce and Ham, 1983). This, in turn, accelerated progress in identifying major structural proteins that participate in formation of differentiated structures. The evolution of culture technology coupled with the revolution in gene analysis technologies led to the identification of a host of proteins that form differentiated structures in keratinocytes (Green, 1980; Fuchs and Byrne, 1994; Eckert *et al*, 1997). The ability to isolate the genes encoding these proteins subsequently identified DNA sequences that regulate gene expression. In recent years, the knowledge built during the 1970s and 1980s has led to a substantial interest in understanding how keratinocyte differentiation is regulated and in elucidating the signaling mechanisms that control gene expression.

stacking of multiple keratinocyte layers above the basal zone, forming the spinous, granular, and cornified layers (Eckert *et al*, 1997). Each layer expresses specific gene products that are required for the differentiation process, and expression of each gene product requires specific regulatory inputs. In general, cultured keratinocytes, and keratinocytes in epidermis, undergo differentiation (Gandarillas, 2000), a slow process that involves substantial changes in the intracellular pattern of protein expression. During this process, keratinocytes enlarge, flatten, and are ultimately released from the epithelial surface (Eckert *et al*, 1997). In addition to differentiation, however, there is another pathway available – programmed cell death. The programmed cell death (apoptotic) pathway is best characterized as resulting from exposure to harmful stimuli such as ultraviolet (UV) radiation. In contrast to differentiation, apoptosis is executed on a time scale of hours and results in elimination of individual cells from the tissue. In UVB-exposed epidermis, apoptosis is associated with sunburn cell formation. Differentiation and apoptosis are distinct processes that share significant overlap with respect to marker gene expression. Moreover, the selection of a particular pathway depends upon environmental factors (Mitra *et al*, 1997; Gandarillas *et al*, 1999; Takahashi *et al*, 2000). For example, treatment of cultured keratinocytes with ionophore results in differentiation that is associated with activation of caspase-14 (Lippens *et al*, 2000) but not other caspases (Takahashi *et al*, 2000). In contrast, treating keratinocytes with UVB produces annexin V- and propidium iodide-, and caspase 3 positive cells, indicative of an apoptotic process (Takahashi *et al*, 2000). In addition the death receptor signaling pathway, including Fas and Fas ligand, has now been shown to play a role in the UVB-dependent keratinocyte apoptosis (Wehrli *et al*, 2000). The net sum of these findings suggests that apoptosis and differentiation are distinct processes that share many overlapping features and common markers (Gandarillas, 2000).

There is at present an active search, which will continue in the next decades, to identify signaling pathways that regulate cell survival and death. To achieve a balance between differentiation,

KERATINOCYTE SURVIVAL, DIFFERENTIATION, AND DEATH – A COMPLICATED RELATIONSHIP

Keratinocytes begin as proliferation-competent basal layer cells that are characterized by expression of specific basal-cell-associated marker proteins. These cells, in turn, give rise to daughter cells that terminally differentiate. Cell differentiation results in the

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apoptosis, and proliferation, keratinocytes utilize multiple countervailing mechanisms – some of which promote survival, whereas others promote extinction. These studies have identified regulatory cascades that play a central role in regulating keratinocyte differentiation. This review will examine the contribution of several regulatory cascades on these processes, with a particular emphasis on the role of the mitogen-activated protein kinase (MAPK) cascades (Davis, 1995).

MAPK CASCADES

At least three major MAPK pathways, the ERK1/2, JNK, and p38 MAPK cascades, have been described (**Fig 1**). Each cascade includes a MEK kinase (MEKK), which activates a MAPK/ERK kinase (MEK), which activates a MAPK (Robinson and Cobb, 1997; Ichijo, 1999). Each pathway, in turn, differentially regulates downstream targets including transcription factors. The activated transcription factors, in turn, bind to DNA elements and modulate gene expression. **Figure 1** shows a greatly simplified schematic of these pathways in which each pathway is represented in a linear format. In fact, a great many studies describe extensive crosstalk among these cascades wherein a particular kinase in one pathway influences a kinase in another pathway. For example, a MEKK1, MEK3, p38 MAPK pathway regulates involucrin gene expression (Efimova *et al*, 1998), and MEKK1 and MEK1 regulate SPRR1B expression (Vuong *et al*, 2000). These types of observations illustrate that these cascades are wired into highly interdependent regulatory networks, and further suggest that the ultimate regulatory outcome (proliferation, survival, apoptosis, differentiation) is likely to be dependent upon the balance between regulatory inputs.

PRO-DIFFERENTIATION PATHWAYS

The MAPKs (**Fig 1**) are central players in regulating keratinocyte differentiation. Recent studies suggest that a variety of pro-differentiation signaling kinases channel differentiation-promoting input into the MAPK cascades. Apoptosis signaling kinase (ASK1) is a MAPK kinase kinase (MEKK) involved in stress-induced signaling (Matsuzawa and Ichijo, 2001). ASK1 has been suggested to have a role in regulating keratinocyte differentiation, as it is expressed in the suprabasal epidermal layers (Sayama *et al*, 2001). Moreover, expression of constitutively active ASK1 in keratino-

cytes activates JNK and p38 MAPK-associated signaling (Sayama *et al*, 2001). Differentiation-associated marker gene expression (transglutaminase, loricrin, involucrin) is also increased when ASK1 is overexpressed (Sayama *et al*, 2001). This kinase appears to be linked to p38 MAPK, as inhibition of p38 activity stops these ASK1-dependent responses.

Involucrin, a marker of early keratinocyte differentiation, is expressed in the spinous and granular cell layers (Rice and Green, 1979; Eckert *et al*, 1997). Studies designed to identify mechanisms that regulate hINV gene expression located two elements in the hINV promoter, the distal- and proximal-regulatory regions, that mediate gene activation (Welter *et al*, 1996; Efimova *et al*, 1998). Extensive studies of the DNA elements in these regions identify AP1, Sp1, and C/EBP as the transcription factors responsible for basal and differentiation-associated gene expression (Welter *et al*, 1996; Banks *et al*, 1998; 1999; Dashti *et al*, 2001a; 2001b). Signal transduction analyses, using dominant-negative kinases and kinase inhibitors, show that activation is mediated by a cascade that includes protein kinase c (PKC) (Efimova and Eckert, 2000), Ras, MEKK1 (Efimova *et al*, 1998), various MEK isoforms (Efimova *et al*, 1998; Dashti *et al*, 2001a; 2001b), and p38 MAPK (Dashti *et al*, 2001a; 2001b). Induction of gene expression is strictly associated with activation of the novel PKC isoforms (Efimova and Eckert, 2000). This pro-differentiation role for the novel PKC isoforms is consistent with other studies showing that nPKC isoforms promote differentiation and/or apoptosis (Ohba *et al*, 1998; Li *et al*, 1999). In contrast, the classical and atypical PKC isoforms do not appear to be involved (Li *et al*, 1999; Efimova and Eckert, 2000), and recent studies indicate that the classical PKC isoforms antagonize the calcium-dependent activation of hINV gene expression (Deucher *et al*, 2002). These studies also indicate that Ras activity is required for expression and that the Ras signal is conveyed downstream to MEKK1 (Efimova *et al*, 1998). The MEKK1 signal is then transferred downstream via activation of MEK1, MEK3, MEK6, and/or MEK7 (Efimova *et al*, 1998; Dashti *et al*, 2001a; 2001b). The specific MEK isoform involved in the regulation may depend upon the source of the incoming differentiation signal, as each MEK isoform differentially regulates MAPK activation. In addition, the differentiation-positive signal is associated with reduced ERK1/2 activity (Efimova and Eckert, unpublished). p38 α and p38 δ are the major p38 MAPK isoforms that mediate the activation of gene expression (Efimova *et al*, 1998; Dashti *et al*, 2001a; 2001b).

MAPK cascades have also been reported to regulate expression of other markers of differentiation, including SPRR1B (Vuong *et al*, 2000). A pathway that includes PKC δ , Ras, MEKK1, MEK, and AP1 regulates this gene. A specific MAPK isoform has not been implicated. The human cystatin A gene has also been studied. Cystatin A expression is positively regulated by a pathway that includes Ras, Raf1, MEK1, ERK1/2 pathway (Takahashi *et al*, 2001). These findings are particularly interesting, as they imply a role for Ras in promoting differentiation, although Ras can also function in a positive manner to promote cancer progression (Denning *et al*, 1993). PKC activity is also required for increased transglutaminase activity (Dlugosz and Yuspa, 1994) and this response is mediated by novel PKC isoforms (Ueda *et al*, 1996).

PKC η , which is known to promote keratinocyte differentiation via MAPK cascade activation (Efimova and Eckert, 2000), also appears to work by a second mechanism – that of forming a complex with cyclinE/cdk2/p21 to reduce cdk2 activity (Ishino *et al*, 1998; Kashiwagi *et al*, 2000). This may provide a complementary mechanism, permitting PKC η to reduce proliferation while concomitantly enhancing differentiation. In addition, PKC η interacts with Fyn, an Src kinase family member that is required for normal keratinocyte differentiation (Cabodi *et al*, 2000). Fyn appears to reduce keratinocyte proliferation by downregulating epidermal growth factor receptor (EGFR) signaling. PKC η activity is necessary for Fyn activation and the two proteins are associated (Cabodi *et al*, 2000). This result suggests that PKC η

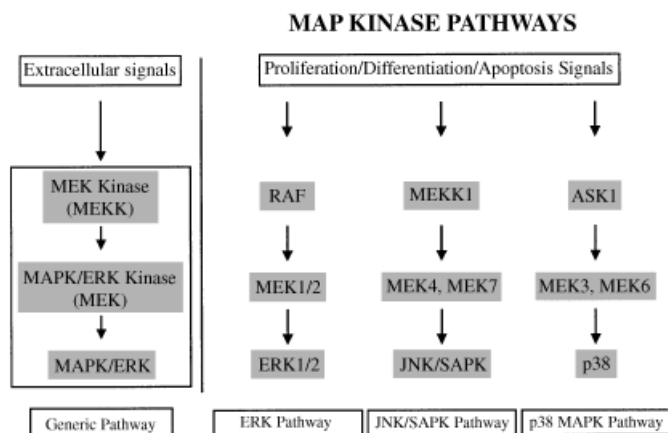


Figure 1. The MAPK cascades. Incoming signals that regulate proliferation, differentiation, and apoptosis are shuttled through three major MAPK cascades. These are named based on the target of a three-kinase module that includes a MAPK kinase kinase, a MAPK kinase, and a MAPK. The MAPKs are ERK1/2, JNK1/2, and p38. For simplicity, these pathways are presented as linear cascades. There is substantial interconnection among the cascades at all levels, however.

suppresses keratinocyte proliferation via a mechanism that involves Fyn-dependent downregulation of EGFR function.

In general, the above studies suggest that pro-differentiation stimuli act via novel PKCs and various MAPK kinase kinases, including MEKK1 and ASK1, to enhance keratinocyte differentiation. In addition, these stimuli also appear to suppress EGFR activity.

PRO-APOPTOSIS PATHWAYS

UVB light is an important apoptotic stimulus that has been extensively studied. UVB exposure produces a number of signaling-related changes in cell function. Efforts to understand the UVB-dependent death process has led to the identification of specific agents that inhibit cell death. Several of these findings are summarized below. UVB exposure on HaCaT cells is associated with sustained activation of p38 MAPK, mitochondrial cytochrome c release, and caspase-3 activation (Assefa *et al*, 2000). Inhibition of p38 MAPK activity inhibits these responses. A related study in HaCaT cells reports UVB-associated p38 MAPK and JNK activation (Shimizu *et al*, 1999). Moreover, SB203580 inhibits the apoptotic response, further suggesting a pro-apoptotic role for p38 MAPK (Shimizu *et al*, 1999). In normal human keratinocytes, UVB treatment increases reactive oxygen species associated events and activation of p38 and ERK1/2. The ERK1/2 activation is transient, whereas the p38 activation is sustained (Peus *et al*, 1999; 2000). Inhibition of ERK1/2, using the MEK1/2 inhibitor PD98059, results in enhanced apoptosis (Peus *et al*, 1999). UVB treatment also increases p38 phosphorylation in SV40 transformed human keratinocytes (Nakamura *et al*, 2001). Taken together, these studies suggest that ERK1/2 is pro-survival and antiapoptotic, and p38 activation is pro-apoptotic.

Upstream signaling kinases have also been implicated in regulation of cell death. UVB treatment of keratinocytes results in the release of the catalytic fragment of PKC δ into the soluble phase (Denning *et al*, 1998). This is associated with increased soluble PKC δ catalytic activity and caspase activation. Caspase activation may regulate PKC δ cleavage, as caspase inhibitors block the UVB-dependent formation of soluble PKC δ , and apoptosis (Denning *et al*, 1998). Moreover, in normal keratinocytes, PKC δ overexpression coupled with phorbol ester treatment results in localization of PKC δ to the mitochondria followed by alteration of mitochondrial membrane potential and cell death. This response requires PKC δ activity, as it is attenuated by PKC inhibitors (Li *et al*, 1999). Vitamin-D-associated apoptosis is also associated with activation of MEKK1 and p38 MAPK, and loss of MEK1 and ERK1/2 activity (McGuire *et al*, 2001).

PRO-SURVIVAL PATHWAYS

Several agents that target MAPK cascade function are important in promoting keratinocyte survival. EGFR is a transmembrane tyrosine kinase that activates a variety of intracellular signaling cascades. In keratinocytes, EGFR promotes survival via effects on MAPK cascade activity (Mendelsohn and Baselga, 2000; Prenzel *et al*, 2001). Culturing keratinocytes in suspension medium or treating with UVB radiation results in cell death. Treatment with EGF counters this tendency. EGFR stimulates keratinocyte survival in suspension culture via a mechanism that requires MEK1/2 activity, as shown by inhibition of the survival response by PD98059, an MEK1/2 inhibitor, or by expression of dominant-negative MEK1 (Jost *et al*, 2001a; 2001b). Keratinocyte apoptosis is also stimulated by UVB treatment, and, perhaps paradoxically, is associated with activation of EGFR. EGFR, in turn, activates ERK1/2 and p38 MAPK (Peus *et al*, 2000). Inhibition of EGFR activation by the EGFR-specific inhibitor PD153035 results in enhanced UVB-dependent apoptosis (Peus *et al*, 2000). Moreover, treatment with this inhibitor results in reduced ERK1/2 activity, indicating that ERK is required for the survival response (Jost

et al, 1999; Peus *et al*, 2000). Thus, EGFR activation may represent an effort by the cell to promote survival in the face of the apoptotic stimulus.

The nuclear factor κ B (NF κ B) cascade has also been implicated in keratinocyte survival. NF κ B interacts with I κ B α , an inhibitor of NF κ B activity, to regulate the level of active NF κ B (Joyce *et al*, 2001). Expression of the super-repressor form of I κ B α in transgenic mouse epidermis results in hyperplasia and increased sensitivity to UVB-dependent apoptosis (van Hogerlinden *et al*, 1999). Other survival agents also function via NF κ B. Interferon- γ (IFN γ)/phorbol ester cotreatment results in enhanced survival of UVB-challenged keratinocytes (Qin *et al*, 1999), and this survival is impaired when dominant-negative I κ B α is expressed. In addition, IFN γ /phorbol ester treatment inhibits caspase-3, caspase-8, and PARP activation in response to UVB treatment (Chaturvedi *et al*, 1999; Qin *et al*, 1999).

Proteins associated with adhesion and adherens junction formation can also function in cell survival via regulation of MAPK function. An example is β 1-integrin. Inhibition of β 1-integrin function by expression of dominant-negative β 1-integrin in cultured keratinocytes results in reduced MAPK activity and cell exit from the stem cell compartment (Zhu *et al*, 1999). Overexpression of wild-type β 1-integrin restores normal regulation. Expression of dominant-negative MEKK1 decreases adhesiveness and stem cell number similar to β 1-integrin knockout, suggesting that the MAPK cascade is downstream of the β 1-integrin signal. These studies suggest that β 1-integrin and MAPK cooperate to maintain the epidermal stem cell compartment (Zhu *et al*, 1999; Haase *et al*, 2001). Forced β 1-integrin expression in suprabasal epidermal layers results in increased MAPK activity in basal and suprabasal keratinocytes, and enhanced cell proliferation (Haase *et al*, 2001).

Other cell adhesion/junction proteins also interface with the MAPK cascades. α -Catenin ablation in mouse epidermis leads to hyperproliferation coupled with the appearance of subbasal mitoses and multinucleated cells, and is associated with MAPK activation (Vasioukhin *et al*, 2001). This result suggests that α -catenin expression may be required to maintain cells in the nonproliferative state. In HaCaT cells, E-cadherins stimulate MAPK activity through the ligand-independent activation of EGFR (Pecce and Gutkind, 2000). Inhibition of adherens junction formation by anti-E-cadherin antibody inhibits MAPK activation, as does AG1478, an EGFR inhibitor. PKC δ expression induces serine phosphorylation of α 6 β 4-integrin, promotes its loss from the hemidesmosome complex, and reduces keratinocyte attachment (Alt *et al*, 2001). These studies point to a close relationship between adhesion protein status and the status of the intracellular signaling cascades.

In addition to mediating apoptotic responses, MAPKs are also involved in processes that promote cell transformation. As noted above, UVB treatment of keratinocytes induces apoptosis. Persistent UVB exposure, however, leads to DNA damage and tumor formation. MAPK cascades have been implicated in the process. Cyclooxygenase-2 (COX-2) is an important enzyme in the synthesis of prostaglandins, which stimulate inflammation and cell proliferation. UVB treatment of cultured keratinocytes leads to increased COX-2 expression (Tang *et al*, 2001). The activation is due to phosphorylation of CREB and ATF1 transcription factors that then bind to the COX-2 promoter. CREB and ATF1 phosphorylation and binding to DNA require p38 MAPK activity, as evidenced by inhibition of these responses by SB202190. Urokinase-type plasminogen activator (uPA) is an enzyme involved in cell mobility and invasiveness (Santibanez *et al*, 2000). Inhibition of ERK1/2 activity by antisense oligonucleotides or by inhibition of MEK1 using PD98059 reduces basal uPA and eliminates the transforming growth factor β 1 induction of uPA, a response that also requires Ras activity (Santibanez *et al*, 2000). IFN γ also increases COX-2 expression. This response is mediated via activation of EGFR and involves subsequent activation of ERK1/2 to increase COX-2 transcription (Matsuura *et al*, 1999).

Matrix protein breakdown is another feature associated with cancer cell survival and movement. Matrix metalloproteinases (MMP) play an important role in conferring this ability on cells (Stetler-Stevenson *et al*, 1993). In HaCaT cells, tumor necrosis factor α treatment stimulates ERK1/2, JNK, and p38 activity, which leads to increased MMP-1, MMP-9, and MMP-13 expression (Johansson *et al*, 2000). Blocking p38 with SB203580 inhibits these responses. Blocking MEK1 using PD98059 stops the MMP-1 and MMP-9 increase, but not the MMP-13 increase. EGFR activation also increases cell motility and MMP-9 production via an MEK1- and ERK1/2-dependent mechanism (Zeigler *et al*, 1999). Thus, ERK1/2 and p38 signaling are important in generating the invasive phenotype.

SUMMARY—MAPK-DEPENDENT REGULATION IN KERATINOCYTES

Although exceptions can be cited, the above results suggest several general regulatory themes. First, there appears to be a consistent role for the ERK1/2 kinases in enhancing keratinocyte proliferation and survival (Fig 2). ERK1/2 appears to mediate pro-proliferation and pro-survival information from a range of different agents, including growth factors and cellular adhesion proteins. Second, agents that promote differentiation and apoptosis appear to signal these processes via a p38 MAPK-dependent mechanism (Efimova *et al*, 1998). This is a particularly interesting finding, as early studies, in other systems, suggested that p38 MAPK was strictly involved in mediating stress responses. Third, kinases that promote both differentiation and apoptosis appear to share overlapping pathways of regulation. For example, ASK1 is known to promote apoptosis in a variety of cell types, and in keratinocytes it promotes expression of markers of differentiation (Sayama *et al*, 2001). Also, the novel PKC isoforms can promote both differentiation and/or apoptosis (Ohba *et al*, 1998; Li *et al*, 1999; Efimova and Eckert, 2000; Vuong *et al*, 2000). The fact that these pathways are regulated by similar input suggests that there may also be common end responses. Thus, it may be difficult to distinguish one process from another. Caspase status may be useful for distinguishing these processes, as recent studies suggest that specific caspase isoforms are expressed during differentiation (caspase-14) versus apoptosis (killer caspases) (Eckhart *et al*, 2000a; 2000b; Lippens *et al*, 2000; Kuechle *et al*, 2001). Fourth, cell fate is likely to depend upon the balance of input from the pro-survival and pro-differentiation/apoptosis cascades. This conclusion is based on studies pitting pro-apoptosis/pro-differentiation agents against pro-survival agents. These studies suggest that the net out-

come is a response to the strength of the inputs. In general, these experiments indicate that the ERK cascade is battling the p38 MAPK cascade, suggesting that the balance of flow through the cascades is likely to determine the final fate of the cell.

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MAPK FUNCTION IN KERATINOCYTES

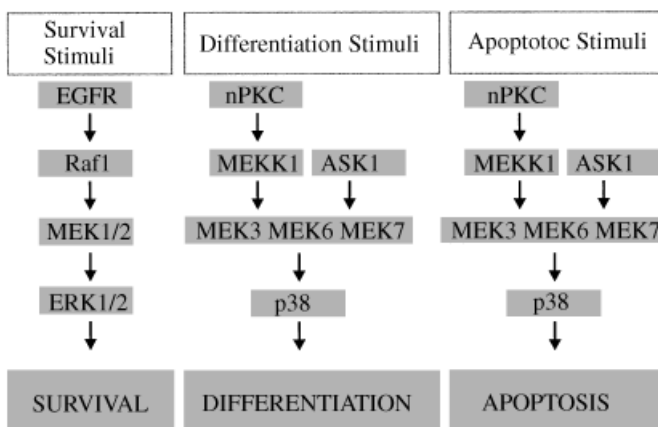


Figure 2. MAPK signaling in keratinocytes. Summary of the role of MAPK cascades in regulating keratinocyte function. In general, the ERK1/2 cascade promotes survival/proliferation, whereas the p38 MAPK cascade mediates pro-differentiation and pro-apoptosis responses.

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