Cholesterol Depletion Upregulates Involucrin Expression in Epidermal Keratinocytes Through Activation of p38

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Cholesterol has been recently suggested to regulate the early steps of keratinocyte differentiation through lipid rafts. In many cell types, depletion of cholesterol activates signaling proteins like epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), or extracellular signal-regulated kinase (ERK) known to affect cell differentiation. In this study, we explored the effects of cholesterol depletion on the phenotype of cultured keratinocytes, using a treatment with methyl-β-cyclodextrin (MβCD) to extract cholesterol and a treatment with lovastatin to inhibit cholesterol neosynthesis. Analysis of the expression of differentiation marker genes in early differentiating confluent cultures reveals that cholesterol depletion induces downregulation of keratin 14 (K14) and keratin 10 (K10) and upregulation of involucrin. MβCD treatment induces phosphorylation of EGFR, HER2, and ERK, but not HER3. Inhibition of EGFR with PD153035 impairs the MβCD-induced phosphorylation of EGFR, HER2, and ERK, but does not impair the alteration of K14, K10, or involucrin gene expression, indicating that other signaling proteins regulate this phenomenon. p38 has been suggested to regulate the expression of involucrin during keratinocyte differentiation. We found that MβCD treatment induces a prolonged phosphorylation of p38 in general and p38α in particular. An inhibition of p38 with PD169316 impairs the upregulation of involucrin mRNAs by a treatment with MβCD, but not by a p38δ-activating TPA treatment, which might suggest that cholesterol depletion alters involucrin gene expression through activation of p38α/β.

Key words: cholesterol/gene expression/keratinocytes/MAPK/signal transduction
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The epidermis is a stratified squamous epithelium mainly composed of keratinocytes. In the basal epidermal layer that is anchored to the basement membrane, slowly clonogenic stem keratinocytes produce rapidly replicating transit-amplifying cells that migrate outwardly from the basal layer in the spinous, granular, and cornified layers while undergoing a complex differentiation program. The evolution from a basal keratinocyte to a differentiated corneocyte is associated with a tight regulation of the expression of differentiation-related proteins. For example, expression of the basal keratins 5 and 14 (K5, K14) is repressed upon entry of the cells into the spinous layer, where expression of the suprabasal keratins 1 and 10 (K1, K10) is induced, whereas the differentiation marker involucrin appears in the upper spinous and granular layers (Fuchs, 1990).

The mechanisms that guarantee epidermal homeostasis by regulating keratinocyte proliferation, migration, and differentiation have been elucidated in part. Cell-adhesion proteins (e.g., integrins, cadherins), membrane receptors (e.g., HER) and their ligands (e.g., epidermal growth factor (EGF), heregulins), associated intracellular signaling proteins (e.g., protein kinase C, mitogen-activated protein kinase (MAPK)), and calcium ions all intervene in the regulation of keratinocyte physiology (Eckert *et al*, 1997; Watt, 2002).

Lipids are also involved in epidermal differentiation. For example, cholesterol is synthesized in epidermal keratinocytes (Ponec *et al*, 1985), and cholesterol transport occurs in differentiating keratinocytes via specific ATP-binding cassette (ABC) transporters (Kielar *et al*, 2003). Cholesterol has been shown to be involved in the late stages of epidermal differentiation by promoting cornified envelope formation (Schmidt *et al*, 1991) or by being a critical constituent of the epidermal lipid barrier (Menon *et al*, 1992). Cholesterol is enriched together with sphingolipids in discrete membrane microdomains named lipid rafts. Several signaling proteins share an affinity for lipid rafts and their localization within lipid rafts influences their signaling properties (Simons and Toomre, 2000), such as the epidermal growth factor receptor (EGFR) (Ringerike *et al*, 2002; Roepstorff *et al*, 2002), or the human epidermal growth factor receptor 2 (HER2) (Nagy *et al*, 2002). The presence of lipid rafts at the plasma membrane of keratinocytes has been described (Gniadecki *et al*, 2002). Very recently, an enrichment of lipid rafts has been identified

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HER, human epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; TPA, 12-O-tetradecanoyl-phorbol-13-acetate

in transit amplifying keratinocytes compared with stem cells, suggesting an involvement of lipid rafts in regulating the keratinocyte phenotype (Gniadecki and Bang, 2003). Thus, the literature provides many observations that suggest important roles of cholesterol and lipid rafts in the physiology of epidermal keratinocytes.

Physiological roles of plasma membrane cholesterol can be investigated by observing the effects of a depletion of cholesterol. This can be accomplished by incubating cultured cells with methyl-β-cyclodextrin (MβCD), a cyclic polysaccharide that extracts cholesterol from cellular membranes (Klein *et al*, 1995) and thereby disrupts lipid rafts (Kabouridis *et al*, 2000). In several cell types, cholesterol depletion by MβCD has been found to induce phosphorylation of the EGFR and to influence the activity of HER2 (Chen and Resh, 2002; Nagy *et al*, 2002; Pike and Casey, 2002). The physiology of epidermal keratinocytes, however, is modulated by the activity of EGFR (Piepkorn *et al*, 1998; Jost *et al*, 2000) and HER2 (De Potter *et al*, 2001; Stoll *et al*, 2001), which act as receptor tyrosine kinases and mediate several signaling pathways (Schlessinger, 2000; Yarden and Sliwkowski, 2001). Associating with HER2, a third member of the HER family named HER3 has been proposed to play roles in epidermal differentiation (De Potter *et al*, 2001).

Acting through the EGFR, cholesterol depletion has been suggested to activate MAPK effector proteins, namely, the extracellular signal-regulated kinases (ERK) 1 and 2 (Furuchi and Anderson, 1998; Kabouridis *et al*, 2000; Chen and Resh, 2001). Other MAP kinases beside ERK are the p38 kinases, which play a major role in the cellular response to environmental and physiological stresses (Kyriakis and Avruch, 2001). p38 activity can be modulated by a depletion of cholesterol (Hossain *et al*, 2000; Iwabuchi and Nagaoka, 2002). Involvement of MAP kinases in regulating epidermal differentiation has been suggested: keratinocyte proliferation and survival would be influenced by ERK1/2, whereas keratinocyte differentiation and apoptosis would rather be influenced by p38 (Eckert *et al*, 2002). The p38 MAPK family includes the p38α, p38β, p38γ, and p38δ isoforms, but only the α, β, and δ isoforms are expressed in keratinocytes (Dashti *et al*, 2001). In these cells, involucrin expression can be regulated by both p38α and p38δ (Dashti *et al*, 2001; Efimova *et al*, 2002; Efimova *et al*, 2003).

In this study, we investigated the effects of cholesterol depletion on the differentiation of epidermal keratinocytes. We explored the effects of cholesterol depletion on signal transduction within keratinocytes, focusing on the activation state of receptors (EGFR, HER2, and HER3) and MAP kinase effectors (ERK, p38) relevant in keratinocyte differentiation.

Results

Treatment with MβCD induces extraction of cholesterol from keratinocytes In order to analyze the effects of a treatment with MβCD on the concentration of cholesterol within keratinocytes, keratinocyte cultures were subjected to a treatment with 7.5 mM MβCD for 1 h and cell lysates were assayed for cholesterol and protein concentrations. As shown in Fig 1*A*, MβCD treatment induces a weak but significant ($p<0.01$) decrease in the concentration of cholesterol in keratinocytes ($n=7$).

In order to analyze the localization of cholesterol in keratinocytes following treatment with MβCD, cells grown on glass plates were labeled using filipin. Untreated keratinocytes exhibit plasma membrane staining of filipin (Fig 1*B*, *a*). Treatment with 7.5 mM MβCD for 1 h dramatically reduced the labeling of the plasma membrane (Fig 1*B*, *b*), suggesting extraction of plasma membrane cholesterol by MβCD (Klein *et al*, 1995). Conversely to untreated keratinocytes, intracellular perinuclear filipin labeling is visible after treatment with MβCD. MβCD

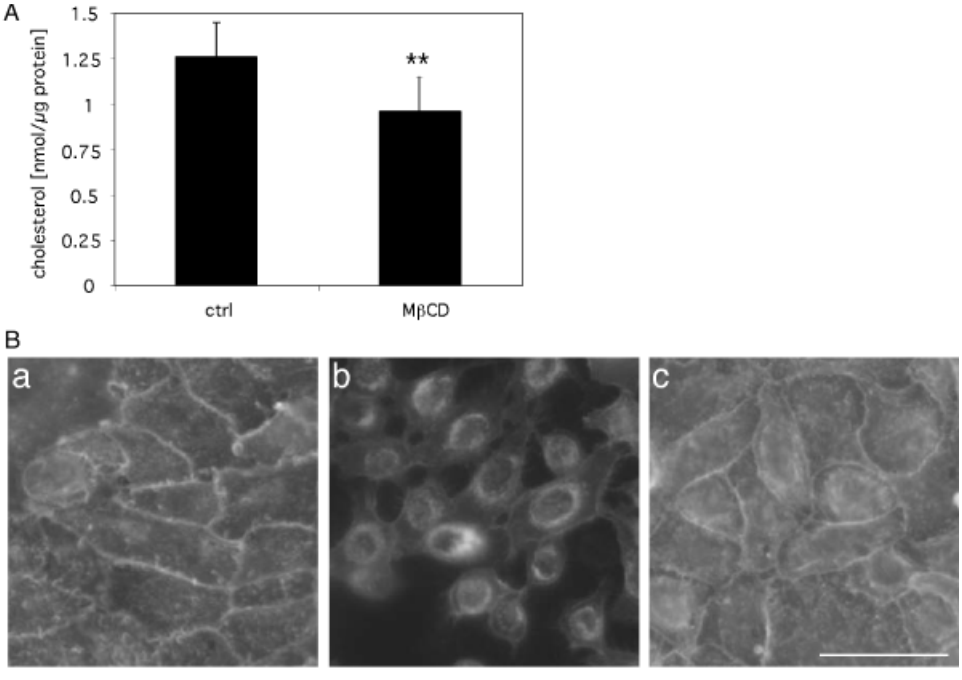

Figure 1
MβCD treatment extracts plasma membrane cholesterol from keratinocytes. (*A*) Keratinocyte cultures were treated for 1 h with 7.5 mM MβCD and assayed for their cholesterol and protein content. Results are expressed as nmol of cholesterol per μg of protein. The data ($n=7$) were compared using the unpaired Student *t* test ($n=7$, $^{**}p<0.01$). (*B*) Filipin labeling was performed on keratinocyte cultures, which were either left untreated (*a*) or treated for 1 h with 7.5 mM MβCD (*b*) or MβCD complexed with cholesterol (*c*). Observation was performed using a microscope equipped for epifluorescence and pictures were taken using identical exposition times for all analyzed samples (bar = 20 μm).

molecules can be complexed with cholesterol prior to incubation with cultured cells, resulting in a suppressed cholesterol-extracting ability of MβCD (Klein *et al*, 1995). Several authors have used cholesterol-complexed MβCD as a control in experiments involving cholesterol extraction from cells by MβCD (Kabouridis *et al*, 2000). Treatment of keratinocytes with MβCD complexed with cholesterol does not alter the filipin staining of the plasma membrane (Fig 1*B*, *c*), which remains similar to untreated cells. This observation confirms that cholesterol-complexed MβCD does not extract cholesterol from the plasma membrane of keratinocytes.

Chronic cholesterol depletion of keratinocytes results in an altered gene expression of K14, K10, and involucrin Next, we sought to know whether cholesterol depletion affects the differentiation process of keratinocytes. We analyzed the mRNA expression of the basal keratin 14 (K14), of the suprabasal keratin 10 (K10), and of involucrin, which is a marker of epidermal differentiation because it is mainly expressed in the upper spinous and granular layers.

In autocrine culture conditions, differentiation markers exhibit a characteristic expression pattern dependent on cell density (Poumay and Pittelkow, 1995; Poumay *et al*, 1999). Subconfluent, confluent, and postconfluent keratinocyte cultures were subjected to the following treatments. A transient cholesterol depletion was performed by incubating keratinocyte cultures with 7.5 mM MβCD for 1 h followed by an incubation for 17 h in culture medium. A chronic depletion of cholesterol was induced by incubating cultures with 7.5 mM MβCD for 1 h followed by inhibition of cholesterol biosynthesis by treatment with 10 μM lovastatin for 17 h.

In untreated keratinocyte cultures, K14 gene expression is observed in subconfluent, confluent, and postconfluent cultures. K10 gene expression is induced in confluent and postconfluent cultures and involucrin gene expression is strongly upregulated in postconfluent cultures (Fig 2*A*). In confluent cells, a combined treatment with MβCD and lovastatin induces an obvious downregulation of K14 and K10 mRNA and a strong upregulation of involucrin mRNA. Treatment of confluent keratinocytes with MβCD results in a minor upregulation of the amount of involucrin mRNA, whereas lovastatin treatment does not induce any detectable modification of the mRNA expression patterns. These results show that the phenotype of keratinocytes in confluent cultures is obviously altered upon cholesterol depletion.

In order to explore whether the alteration of the keratinocyte phenotype at confluence is correlated to the cholesterol-extracting ability of MβCD, we analyzed the effects of a treatment with MβCD complexed with cholesterol and a treatment with lovastatin. This treatment does not result in altered K14, K10, or involucrin mRNA expression (Fig 2*B*). A similar result was observed when keratinocyte cultures were subjected to a treatment with MβCD, a subsequent treatment with cholesterol-complexed MβCD and a treatment with lovastatin. α-Cyclodextrin is less efficient that β-cyclodextrins in extracting membrane cholesterol (Ohtani *et al*, 1989; Nakanishi *et al*, 1992) and a treatment of keratinocytes with α-cyclodextrin and lovastatin does not result in an altered expression of K14, K10, or involucrin (data not shown). These results strongly suggest that the alteration of the keratinocyte phenotype is a consequence of cholesterol depletion.

Since the effects of MβCD have been linked to the treatment duration (Park *et al*, 1998; Lai, 2003), we analyzed the gene expression of K14, K10, and involucrin in confluent keratinocyte cultures subjected to a treatment with MβCD for 15, 30, or 60 min and a treatment with lovastatin for 17 h.

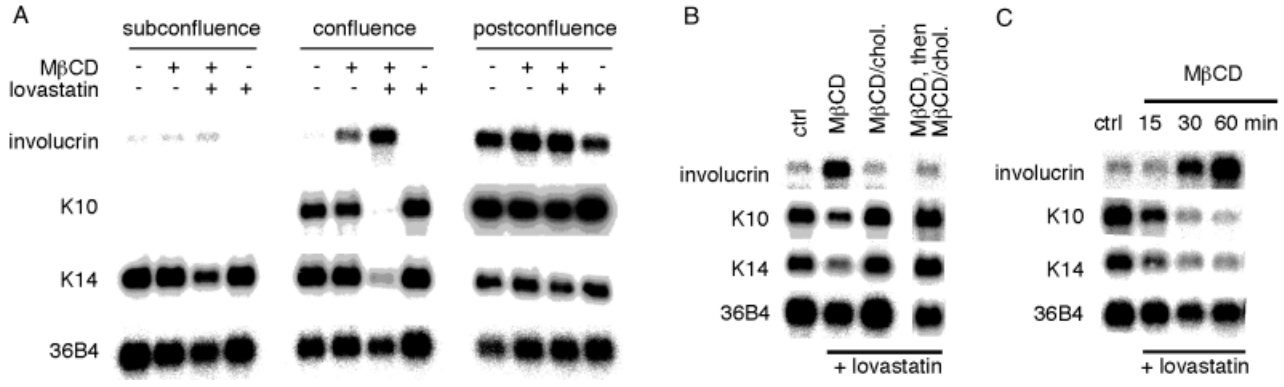

Figure 2
Cholesterol depletion induces downregulation of keratin 14 and keratin 10 mRNA expression and upregulation of involucrin mRNA expression in confluent keratinocyte cultures. (*A*) Experiments were performed on subconfluent, confluent, and postconfluent keratinocyte cultures in autocrine growth conditions. Keratinocyte cultures were subjected to a control incubation with autocrine growth medium for 18 h. Transient cellular cholesterol depletion was achieved by treatment with 7.5 mM MβCD for 1 h followed by incubation for 17 h in culture medium. Chronic depletion of cellular cholesterol was performed by a transient treatment with 7.5 mM MβCD for 1 h followed by inhibition of cholesterol biosynthesis by treatment with 10 μM lovastatin for 17 h. Inhibition of cholesterol neosynthesis without initial cholesterol depletion was accomplished by treatment with 10 μM lovastatin for 17 h. Upon completion of the experiments, keratinocyte cultures were subjected to poly-A RNA extraction. Samples were analyzed by northern blotting using serial hybridizations of the membrane with cDNA probes specific for keratin 14 (K14), keratin 10 (K10), and involucrin followed by autoradiography. Gel loading was analyzed by hybridization of the membrane with a cDNA probe specific for the reference gene 36B4. (*B*) Confluent keratinocyte cultures were treated with MβCD or cholesterol-complexed MβCD for 1 h and were then incubated with lovastatin for 17 h. Other cultures were incubated with MβCD for 1 h, with cholesterol-complexed MβCD for 1 h and with lovastatin for 16 h. Samples were analyzed for expression of K14, K10, and involucrin mRNA as described above. Shown is a representative result of two experiments performed on different keratinocyte strains. (*C*) Confluent keratinocyte cultures were treated with MβCD for 15, 30, or 60 min and with lovastatin for 17 h. Samples were analyzed for expression of K14, K10, and involucrin mRNA as described above.

As shown in Fig 2*C*, the effects of a treatment with MβCD and lovastatin on the gene expression of K14, K10, and involucrin are time dependent.

Cholesterol depletion induces autophosphorylation of EGFR but does not affect ERK phosphorylation, and does not alter the gene expression of K14, K10, and involucrin through activation of EGFR We hypothesized that a treatment with MβCD and lovastatin could induce the activation of signal transduction cascades, which regulate the expression of K14, K10, and involucrin. One candidate pathway involves the EGFR. Indeed, activation of the EGFR by a treatment of keratinocytes with EGF is known to downregulate the expression of K10 (Poumay and Pittelkow, 1995). We verified in confluent cultures that EGF treatment downregulates K10 mRNA (Fig 3*A*). EGF treatment of reconstructed epidermis is known to induce a precocious expression of involucrin in the spinous layer (Gibbs *et al*, 1998; Gibbs *et al*, 2000). We found that EGF treatment of confluent keratinocyte cultures upregulates involucrin mRNA (Fig 3*A*). MβCD treatment could thus alter the gene expression of K10 and involucrin through the activation of EGFR.

To investigate this hypothesis, we analyzed the phosphorylation state of EGFR following a treatment with MβCD. As shown in Fig 3*B*, untreated confluent keratinocyte cultures exhibit undetectable basal amounts of phosphorylated EGFR, whereas a treatment with 7.5 mM MβCD for 1 h obviously induces EGFR phosphorylation. Analysis of the effect of a treatment with cholesterol-charged MβCD reveals no rise in EGFR phosphorylation under these conditions (Fig 3*B*).

PD153035 is a compound that specifically inhibits EGFR tyrosine kinase activity at nanomolar concentrations (Fry *et al*, 1994). Pre-treatment of keratinocytes with PD153035 followed by treatment with MβCD results in a level of EGFR phosphorylation similar to the level in untreated cells (Fig 3*C*). The MβCD-induced activation of EGFR probably results from autophosphorylation since this activation is largely dependent on the tyrosine kinase activity of EGFR.

Activation of EGFR can induce activation of the MAPK/ERK kinase (MEK), which in turn can activate the MAP kinase ERK (Schlessinger, 2000). As shown in Fig 3*B* and *C*, treatment of keratinocytes with MβCD does not affect ERK phosphorylation. In untreated confluent keratinocytes, the levels of phosphorylated ERK are elevated (Fig 3*B* and *C*), which is probably due to stimulation of EGFR by auto- and paracrine EGFR ligands secreted by these cells in serum-free culture conditions (Cook *et al*, 1991; Pittelkow *et al*, 1993; Iordanov *et al*, 2002).

To investigate whether cholesterol depletion alters keratinocyte differentiation through EGFR activation, confluent keratinocyte cultures were incubated with 500 nM PD153035 for 30 min before treatment with MβCD and lovastatin. As shown in Fig 3*D*, inhibition of EGFR tyrosine kinase activity does not impair the alteration of the expression of K14, K10, and involucrin induced by cholesterol depletion, suggesting that another signaling pathway is responsible for this alteration of the keratinocyte phenotype.

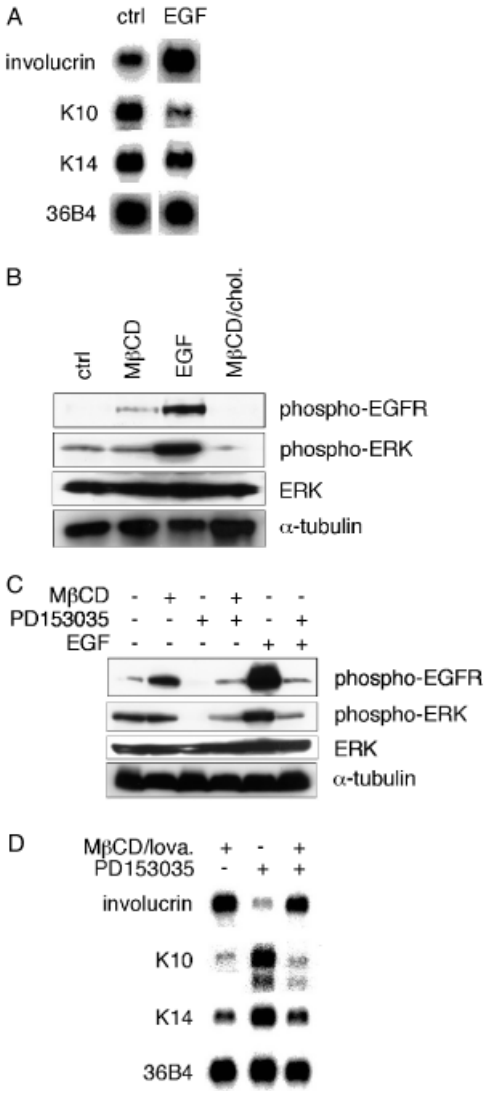

Figure 3
Cholesterol depletion induces autophosphorylation of EGFR but not ERK, and does not alter the gene expression of K14, K10 or involucrin through activation of EGFR. (*A*) Confluent keratinocyte cultures were either left untreated or treated with 10 ng/mL EGF for 18 h. Following poly-A RNA extraction, samples were analyzed by northern blotting as described in Fig 2. (*B*) Keratinocyte cultures were treated with 7.5 mM MβCD or with cholesterol-complexed MβCD for 1 h. Other cultures were treated with 10 ng/mL EGF for 10 min. Cellular lysates were prepared and analyzed by western blotting using antibodies specific for the phosphorylated forms of EGFR and phosphorylated ERK. Gel loading was analyzed by immunodetection of total ERK and of α-tubulin and by amidoblack staining of the blotted membrane (not shown). This experiment was repeated twice using different keratinocyte strains with similar results. (*C*) Keratinocyte cultures were treated with 7.5 mM MβCD for 1 h, with 500 nM PD153035 for 90 min or with 10 ng/mL EGF for 10 min. Other cultures were treated with 500 nM PD153035 for 30 min, then with PD153035 and MβCD for 1 h or with PD153035 and EGF for 10 min. Following treatments, cellular lysates were prepared and analyzed for phosphorylated EGFR and phosphorylated ERK as described for *panel B*. The experiment was repeated three times using different keratinocyte strains with similar results. (*D*) Keratinocyte cultures were treated with MβCD for 1 h and lovastatin for 17 h, or with 500 nM PD153035 for 18 h, or with MβCD, lovastatin, and PD153035. Following poly-A RNA extraction, samples were analyzed by northern blotting as described in Fig 2.

Cholesterol depletion induces EGFR-dependent phosphorylation of HER2, but does not affect the phosphorylation state of HER3 Beside the EGFR, keratinocytes also express HER2 and HER3, which have been suggested to be involved in the regulation of keratinocyte differentiation (De Potter *et al*, 2001; Stoll *et al*, 2001; Piepkorn *et al*, 2003). For instance, heregulin treatment of keratinocytes induces heterodimerization of HER3 with HER2 and transphosphorylation of HER3 by HER2, leading to downregulation of the expression of K10 (De Potter *et al*, 2001). Analysis of the tyrosine phosphorylation of HER3 reveals that MβCD treatment does not induce HER3 phosphorylation above the basal level, though its ligand HRGβ increases phosphorylation (Fig 4*A*). On the other hand, analysis of the tyrosine phosphorylation of HER2 shows that it is barely detectable in untreated keratinocytes, but increased following MβCD treatment (Fig 4*B*).

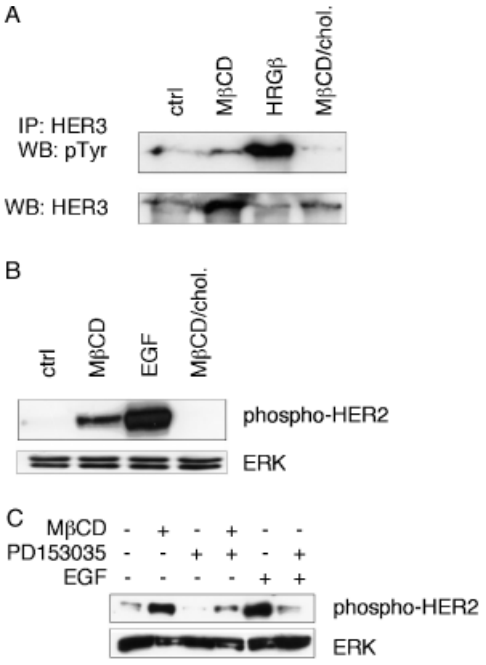

Figure 4
Cholesterol depletion does not affect HER3 phosphorylation, but induces EGFR-dependent phosphorylation of HER2. (*A*) Keratinocyte cultures were treated with 7.5 mM MβCD, 10 ng/mL HRGβ or 7.5 mM cholesterol-complexed MβCD for 1 h. Cellular lysates were prepared and analyzed for phosphorylated HER3 by immunoprecipitation of HER3 and western blotting using phosphotyrosine-specific antibodies. Gel loading was analyzed by immunodetection of total HER3 after stripping the membrane. The experiment was repeated twice using different keratinocyte strains with similar results. (*B*) Keratinocyte cultures were treated with 7.5 mM MβCD or with cholesterol-complexed MβCD for 1 h or with 10 ng per mL EGF for 10 min. Cellular lysates were prepared and analyzed by western blotting using antibodies specific for the phosphorylated form of HER2. Gel loading was analyzed by immunodetection of total ERK. This experiment was repeated twice using different keratinocyte strains with similar results. (*C*) Keratinocyte cultures were treated with 7.5 mM MβCD for 1 h, with 500 nM PD153035 for 90 min or with 10 ng per mL EGF for 10 min. Other cultures were treated with 500 nM PD153035 for 30 min, then with PD153035 and MβCD for 1 h or with PD153035 and EGF for 10 min. Following treatments, cellular lysates were prepared and analyzed for phosphorylated HER2 as described for panel B. The experiment was repeated three times using different keratinocyte strains with similar results.

Treatment with MβCD could induce phosphorylation of HER2 through EGFR activation. To explore this hypothesis, EGFR tyrosine kinase was inhibited by PD153035 prior to a treatment with MβCD, which results in undetectable HER2 phosphorylation (Fig 4*C*), suggesting that EGFR is responsible for HER2 activation upon MβCD treatment. Since EGFR-mediated signaling is probably not involved in the downregulation of K10 expression upon cholesterol depletion (Fig 3*D*), HER2 is probably neither involved in this phenomenon.

Cholesterol depletion induces activation of p38 The MAP kinase p38 is involved in the upregulation of the expression of involucrin during the late stages of keratinocyte differentiation (Efimova *et al*, 2003). Treatment with MβCD could result in an upregulation of the expression of involucrin through activation of p38. Analysis of the phosphorylation state of p38 shows that a basal level of phosphorylated p38 is detectable in untreated keratinocytes (Fig 5*A*). MβCD treatment obviously results in an increased level of phosphorylated p38, but not phosphory-

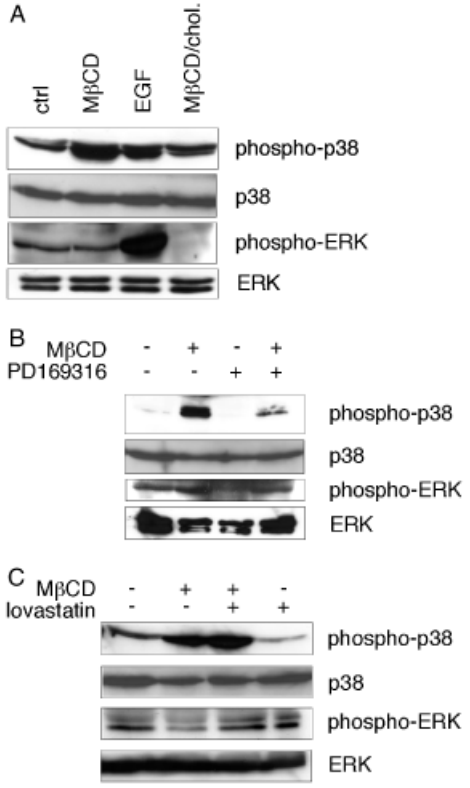

Figure 5
Cholesterol depletion induces prolonged phosphorylation of p38. Following treatments of keratinocyte cultures, cellular lysates were prepared and analyzed by western blotting using antibodies specific for phosphorylated p38 and phosphorylated ERK in *panels A*, *B*, and *C*. Gel loading was analyzed by immunodetection of total p38 and total ERK after membrane stripping. All panels exhibit representative results out of duplicate experiments performed on different keratinocyte strains. (*A*) Keratinocytes were treated with 7.5 mM MβCD or with cholesterol-complexed MβCD for 1 h or with 10 ng per mL EGF for 10 min. (*B*) Keratinocytes were treated with MβCD for 1 h, treated with 15 μM PD169316 for 90 min or treated with 15 μM PD169316 for 30 min and with PD169316 and MβCD for 1 h. (*C*) Keratinocytes were treated with MβCD, with MβCD and lovastatin or with lovastatin as described in Fig 2.

lated ERK. Treatment with EGF also induces some p38 phosphorylation, in accordance with other studies (Cheng *et al*, 2002; Huang *et al*, 2002), while inducing an important increase in the levels of phosphorylated ERK. Treatment of keratinocytes with cholesterol-complexed MβCD does not seem to alter the basal level of p38 phosphorylation. The kinase activity of p38 is inhibited by the compound PD169316 (Kummer *et al*, 1997). We show that the MβCD-induced upregulation of phosphorylated p38 is impaired by a treatment with 15 μM PD169316 (Fig 5*B*), suggesting that MβCD treatment induces autophosphorylation of p38.

Next, we explored whether the activation state of p38 is affected by a treatment with MβCD and lovastatin. As shown in Fig 5*C*, analysis of the phosphorylation of p38 exhibits an obvious upregulation of the amount of phosphorylated p38 (but not phosphorylated ERK) after a treatment with MβCD for 1 h and with lovastatin for 17 h. This observation indicates that cholesterol depletion induces prolonged activation of p38 in keratinocytes.

Cholesterol depletion induces activation of p38α and alters involucrin expression through p38α/β The p38 isoforms activated by cholesterol depletion were then identified by detecting phosphorylated p38α, p38β, and p38δ. As shown in Fig 6*A*, a slightly elevated amount of phosphorylated p38α could be detected in keratinocytes following a treatment with MβCD and lovastatin. This treatment did not seem to affect the amount of phosphorylated p38β or p38δ compared to untreated cells. Similar to previously published observations (Efimova *et al*, 2002), a treatment with TPA clearly elevated the amount of phosphorylated p38δ.

To examine whether p38 activation could be involved in upregulating the involucrin expression upon cholesterol depletion, p38 tyrosine kinase was inhibited by a treatment with PD169316 before a MβCD and a lovastatin treatment. As shown in Fig 6*B*, PD169316 impairs the upregulation of involucrin mRNA levels upon cholesterol depletion. This result strongly suggests that the upregulation of the gene expression of involucrin upon cholesterol depletion is induced by p38 activity.

PD169316 belongs to the pyridinyl imidazole family of p38 inhibitors (Young *et al*, 1997) and has been suggested to specifically inhibit p38α and p38β (Eckert *et al*, 2002; Hendrickx *et al*, 2003). As mentioned above, the MβCD/lovastatin-induced involucrin expression is inhibited by PD169316 (Fig 6*B*), indicating that p38α and/or p38β are involved in this phenomenon. Although it remains to be determined whether PD169316 inhibits p38δ, it is known that the pyridinyl imidazole compound SB203580 inhibits p38α and p38β, but not p38δ (Cohen, 2002). Treatment of keratinocytes with TPA induces expression of involucrin, which is not inhibited by SB203580 (Efimova *et al*, 1998; Eckert *et al*, 2002; Efimova *et al*, 2002), suggesting along with other evidence (Efimova *et al*, 2002; Efimova *et al*, 2003) that this phenomenon does not depend on p38α or p38β but on p38δ. Our results show that the TPA-induced expression of involucrin is also insensitive to PD169316 (Fig 6*C*), indicating that this compound does not inhibit p38δ. Since MβCD/lovastatin-induced involucrin expression is inhibited by PD169316, MβCD/lovastatin treatment probably alters involucrin expression through activation of p38α or p38β, but not p38δ.

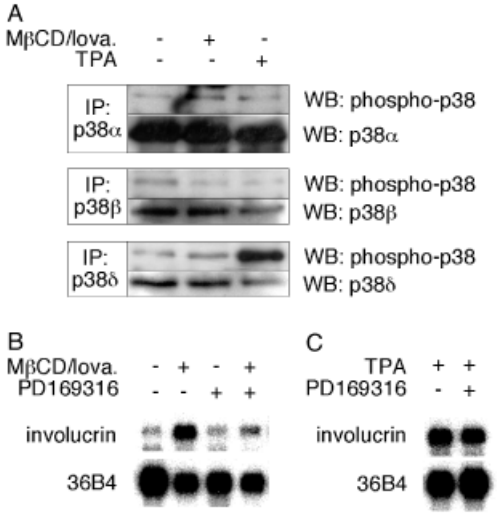

Figure 6
Cholesterol depletion induces phosphorylation of p38α. Inhibition of p38 impairs the upregulation of the gene expression of involucrin induced upon cholesterol depletion. (*A*) Keratinocytes were treated with MβCD and lovastatin as described in Fig 2 or with 10 ng per mL TPA for 18 h. Following treatments, cellular lysates were prepared and analyzed by immunoprecipitation using antibodies specific for p38α, p38β, and p38δ and by western blotting using antibodies specific for phosphorylated p38. Gel loading was analyzed by immunodetection of p38α, p38β, and p38δ. This experiment was repeated twice with similar results. (*B*) Keratinocyte cultures were treated with MβCD for 1 h and lovastatin for 17 h, with 15 μM PD169316 for 18 h, or with MβCD, lovastatin, and PD169316. Following poly-A RNA extraction, samples were analyzed by northern blotting as described in Fig 2. This experiment was repeated twice using different keratinocyte strains with similar results.

Discussion

To gain insight into the role of cholesterol in keratinocyte differentiation, we investigated the effects of cholesterol depletion on this process. We demonstrate that cultured keratinocytes incubated with MβCD are depleted of plasma membrane cholesterol, as shown by a diminished concentration of cellular cholesterol and a less intense filipin staining of the plasma membrane. MβCD treatment of keratinocytes also results in intracellular filipin staining. Since filipin exhibits a relatively low molecular weight (654.8 g per mol), and since MβCD treatment could have disturbed the permeability of the plasma membrane, intracellular cholesterol could have become accessible to filipin. Entry of MβCD into keratinocytes is unlikely since MβCD does not bind nor insert into the plasma membrane in erythrocytes (Ohtani *et al*, 1989). The intracellular localization of filipin observed upon MβCD treatment exhibits a perinuclear pattern, reminding the results described by McGookey who attributes a similar pattern to the localization of lysosomal cholesterol (McGookey *et al*, 1983).

Keratinocyte differentiation is a consequence of a tight regulation of gene expression (Fuchs, 1990). This work

shows for the first time that cholesterol depletion can alter the expression of certain genes involved in keratinocyte differentiation.

Activation of the EGFR by EGF is known to affect the expression of genes in keratinocytes (Poumay and Pittelkow, 1995) whose expression is also altered upon cholesterol depletion. We found indeed that MβCD treatment of keratinocytes induces tyrosine phosphorylation of EGFR. Phosphorylation of EGFR by MβCD has been observed in 3T3 or COS-1 cells (Chen and Resh, 2002; Pike and Casey, 2002). We wondered whether MβCD treatment alters gene expression in keratinocytes through this receptor, but inhibition of EGFR tyrosine kinase activity does not prevent this alteration, suggesting that EGFR-induced pathways are not involved.

We turned our attention to other signaling proteins, which might be involved in regulating gene expression upon MβCD treatment of keratinocytes. The p38 MAP kinases are involved for instance in the upregulation of involucrin expression in keratinocytes, particularly p38α and p38δ (Dashti *et al*, 2001; Efimova *et al*, 2002; Efimova *et al*, 2003). We found that treatment of epidermal keratinocytes with MβCD results in phosphorylation of p38. Furthermore, we demonstrate that inhibition of p38 kinase activity impairs the upregulation of involucrin upon MβCD treatment, suggesting that involucrin gene expression is upregulated through p38 activation.

We also found that p38α seems to be activated upon MβCD treatment. Furthermore, we observed that (conversely to the TPA-induced upregulation of the involucrin expression) the MβCD-induced upregulation of the involucrin expression is inhibited by PD169316. TPA-induced involucrin expression, however, is p38δ-dependent (Efimova *et al*, 2002; Efimova *et al*, 2003), suggesting that cholesterol depletion does not alter involucrin expression through p38δ. This response could proceed through p38α since this p38 isoform seems activated upon cholesterol depletion (Fig 6*A*) and is inhibited by PD169316 (Hendrickx *et al*, 2003).

In keratinocytes, p38α and/or p38β are involved in the response to treatments like UVB irradiation, oxidative stress, tumor necrosis factor α, interleukin 1β (Zhang *et al*, 2001) or osmotic stress (Garmyn *et al*, 2001), thus mediating cell stress responses (Eckert *et al*, 2002). The p38α-dependent alteration of the keratinocyte phenotype by cholesterol depletion that we describe in this study could thus be part of a cell stress response induced by this treatment.

At this moment, we do not have any evidence regarding the possible mechanism(s) by which MβCD treatment can induce phosphorylation of p38 in keratinocytes. p38 can be phosphorylated in keratinocytes by Rho GTPases (Cheng *et al*, 2002). In fibroblasts, apolipoprotein A-I-induced cholesterol efflux from the plasma membrane activates Rho GTPases and activates p38 downstream of these GTPases (Nofer *et al*, 2003). In keratinocytes, MβCD-mediated cholesterol extraction from the plasma membrane could possibly activate p38 through activation of Rho GTPases.

Cholesterol is known to be involved in keratinocyte physiology by promoting cornified envelope formation (Schmidt *et al*, 1991) or by being a critical constituent of the epidermal lipid barrier (Menon *et al*, 1992), thus playing important roles in differentiation. The results presented in this study point to an as yet undescribed involvement of cholesterol in this process since depletion of cholesterol alters the expression of the epidermal differentiation markers K14, K10, and involucrin. Downregulation of K10 and upregulation of involucrin has been interpreted as changes in keratinocyte gene expression that occur during the spinous to granular cell transition in the epidermis (Dlugosz and Yuspa, 1993). Cholesterol depletion induces similar changes in the expression of these genes, which might suggest that cholesterol is involved in regulating this transition.

When investigating the causes of an epidermal pathology, the involvement of a certain molecule is suspected when this molecule exhibits altered properties in the pathological state. For example, elevated levels of cholesterol have been detected in psoriatic patients (Benton *et al*, 1963), and lipid-lowering drugs can induce exacerbation of psoriasis (Fisher *et al*, 1988), suggesting an involvement of cholesterol in this disease. Psoriatic epidermis exhibits altered expression of differentiation marker genes: a K14-specific antibody only stains a small subset of basal keratinocytes (Parent *et al*, 1994), psoriatic skin exhibits diminished K10 expression (van Erp *et al*, 1989), basal and several rows of suprabasal cells are devoid of K10 (Bernerd *et al*, 1992) and involucrin is precociously expressed and present in all but basal epidermal layers (Watanabe *et al*, 1991). Most interestingly, the altered pattern of differentiation gene expression in psoriatic keratinocytes is similar to cholesterol-depleted keratinocytes, which might suggest that altered differentiation in psoriasis could be linked to altered cholesterol levels in keratinocytes.

We show that a treatment with MβCD induces activation of p38 in keratinocytes. Several studies point to an involvement of p38 in psoriasis. Psoriatic keratinocytes exhibit p38-dependent phosphorylation of keratins (Toivola *et al*, 2002). p38 is involved in the regulation of the gene expression of skin-derived antileukoprotease (SKALP)/elafin (Pol *et al*, 2003), which is strongly upregulated in psoriatic keratinocytes (Kuijpers *et al*, 1997). Our unpublished results show that MβCD treatment of keratinocytes strongly upregulates the expression of heparin-binding EGF (HB-EGF), which is overexpressed in lesional psoriatic skin (Stoll and Elder, 1998). MβCD-induced HB-EGF expression is impaired upon inhibition of p38 (our unpublished results). Clinical trials are ongoing with p38 kinase inhibitors for treatment of psoriasis (Lee *et al*, 2000). Thus, a correlation might exist between altered cholesterol levels, p38, and psoriasis.

Taken together, our data demonstrate that cholesterol depletion modulates the phenotype of early differentiating keratinocytes and the activity of signaling proteins known to regulate keratinocyte differentiation, suggesting that cholesterol is involved in the regulation of the early steps of keratinocyte differentiation.

Materials and Methods

Chemicals and antibodies Triton X-100, glycin, paraformaldehyde and methanol-stabilized 37% formaldehyde solution, and

dimethylsulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). MβCD, cholesterol, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), bovine serum albumin (BSA), lovastatin, filipin, amidoblack solution, sodium deoxycholate, and mouse monoclonal anti-α-tubulin antibody were purchased from Sigma-Aldrich (Bornem, Belgium). Protein A-agarose and protein G-agarose were obtained from Roche (Basel, Switzerland). Sodium dodecylsulfate (SDS) and dithiothreitol (DTT) were obtained from Acros (Geel, Belgium). PD153035 was purchased from A.G. Scientific (San Diego, California). AG825 and PD169316 were obtained from Calbiochem (San Diego, California). EGF and heregulin β were purchased from R&D Systems (Minneapolis, Minnesota).

Mouse monoclonal antibodies against phosphotyrosine and rabbit polyclonal phosphospecific antibodies against EGFR (pY^{1173}) and against p38 were obtained from BioSource (Nivelles, Belgium). Rabbit polyclonal anti-phospho-ErbB2-HER-2 (pY^{1248}) antibody, anti-ERK1/2 antibody, and mouse monoclonal anti-phospho-ERK1/2 antibody were obtained from Upstate Biotechnology (Lake Placid, New York). Rabbit polyclonal anti-HER3 antibody was purchased from Novocastra (Newcastle-upon-Tyne, UK). Rabbit polyclonal anti-p38 antibody, goat polyclonal anti-p38β, and goat polyclonal anti-p38δ antibodies were purchased from Cell Signaling (Beverly, Massachusetts). Mouse monoclonal anti-p38α antibody was obtained from Zymed (San Francisco, California). Horseradish peroxydase (HRP)-conjugated goat anti-rabbit and anti-mouse antibodies were obtained from DAKO (Glostrup, Denmark).

Cell culture Normal adult skin samples were obtained at plastic surgery (Dr B. Bienfait, Clinique St. Luc, Namur-Bouge, Belgium) from informed and consenting donors and keratinocytes were isolated by the trypsin float technique (Wille *et al*, 1984). Primary cultures were propagated in complete keratinocyte growth medium (KGM-2) (Clonetics, Verviers, Belgium). Proliferating primary cultures were trypsinized and keratinocytes were plated into secondary cultures at 6×10^3 cells per cm^2. When the cells covered approximately 50% of the culture substratum, keratinocytes were cultured in autocrine growth medium by excluding bovine pituitary extract, insulin, transferrin, epinephrine, and EGF from the KGM-2 medium. Keratinocytes proliferate autonomously in subconfluent autocrine cultures (Cook *et al*, 1991), expressing basal K14 (Poumay and Pittelkow, 1995). Progression of the culture is characterized by a cell-density-dependent growth arrest, commitment to differentiation, and expression of differentiation-specific keratins 1 and 10 (K1, K10) as keratinocytes reach culture confluence (Poumay and Pittelkow, 1995). During the days past confluence of the culture, keratinocytes start to express the late differentiation marker involucrin (Poumay *et al*, 1999). This simple culture method provides an easy way to investigate keratinocyte physiology and its regulation at three defined maturation stades. The attractivity of this autocrine culture setting is accentuated by the absence of exogenous factors, which could otherwise influence keratinocyte physiology in an uncontrollable manner.

Cholesterol depletion Keratinocytes were grown in autocrine conditions and treatments were performed when the cultures were subconfluent (covering 70%–80% of the culture substratum) or confluent, as assessed by morphological observation using phase-contrast microscopy. Four days after reaching confluence, cultures were considered to be postconfluent (Poumay *et al*, 1999). Cholesterol depletion of cellular membranes was accomplished by incubating cells in the presence of MβCD (Klein *et al*, 1995). MβCD was used at a concentration of 1% (wt/vol), corresponding to approximately 7.5 mM. This is a classical working concentration for MβCD and has been previously used for treatment of keratinocytes (Gniadecki *et al*, 2002). MβCD-cholesterol inclusion complexes were prepared according to Klein *et al* (1995). Where indicated, cholesterol neosynthesis was inhibited following MβCD treatment by incubation with 10 μM of the hydroxymethylglutaryl-coenzyme A reductase inhibitor lovastatin (Kita *et al*, 1980), of which a stock solution was prepared in DMSO at 50 mM. Reagents were dissolved (MβCD) or diluted (lovastatin) in culture medium immediately before use and treatments were performed with 0.2-μm filtered reagent solutions for indicated times at 37°C. When experiments included lovastatin treatment, control cultures were incubated with 0.02% (vol/vol) DMSO. For analysis of the effects of chronic cholesterol depletion on keratinocytes, cultures were subjected to combined MβCD treatment for up to 1 h and lovastatin treatment for 17 h. A similar approach was described by Cooper *et al* (2003) who used MβCD and statin treatment to chronically deplete cultured fibroblasts of cholesterol.

All treatments described above were subjected to a cytotoxicity test (ToxiLight, Cambrex, Verviers, Belgium) used according to the manufacturer's instructions. No significant difference in cytotoxicity was detected between treatments, all of which resulted in $>90\%$ of viable cells. All treatments were also subjected to an apoptosis assay (*In Situ* Cell Death Detection Kit, Roche, Basel, Switzerland) used according to the manufacturer's instructions. The proportion of apoptotic cells was $<10\%$ in all analyzed samples.

Cholesterol assay Keratinocyte cultures were subjected to cholesterol depletion, washed with phosphate-buffered saline (PBS) and lysed with PBS containing 1% (vol/vol) Triton X-100. Total cholesterol within the lysates was assayed using the Cholesterol RTU kit (bioMérieux, Marcy-l'Etoile, France) according to the instructions of the manufacturer. The cholesterol concentration was reported to the protein concentration of the lysates, which was assayed using the Dc protein assay kit (Bio-Rad, Nazareth Eke, Belgium).

Filipin staining Filipin has been widely used to localize cellular cholesterol (Porn and Slotte, 1995). Keratinocytes cultured on glass slides were subjected to various treatments, washed with PBS solution, fixed with 4% paraformaldehyde for 30 min, rinsed with PBS, and incubated with filipin (50 μg per mL) for 30 min at room temperature. The cells were then washed in PBS, mounted in Mowiol mounting medium (Molecular Probes, Eugene, Oregon) and examined by epifluorescent microscopy using a UV filter (Olympus AX70). Pictures were acquired using a Zeiss AxioCam in conjunction with Zeiss AxioVision computer software (Carl Zeiss, Jena, Germany).

Poly(A)RNA isolation and northern blot analysis Poly(A) RNA was prepared from keratinocyte cultures and analyzed by northern blotting as described using randomly labelled radioactive probes (Poumay and Pittelkow, 1995). RNA expression of epidermal differentiation markers was analyzed by using cDNAs as probes specific for basal K14 and suprabasal K10 (Roop *et al*, 1988) and involucrin (Eckert and Green, 1986). The membrane was also hybridized with 36B4 cDNA specific for the house-keeping ribosomal phosphoprotein PO (Laborda, 1991) in order to estimate the amount of poly(A) RNA loaded onto the gel and transferred to the membrane. RNA expression was visualized by a Cyclone apparatus (Packard BioScience Company, Meriden, Connecticut) using storage phosphor screens.

Western blot analysis of phosphorylated EGFR, HER2, ERK, and p38 Cellular protein extracts were analyzed by western blotting as described by Poumay and Pittelkow (1995). Keratinocytes were grown in 6 cm Petri dishes (TPP, Trasadingen, Switzerland), and subjected to the indicated treatments. Thereafter, cells were washed with PBS and lysed in twice concentrated Laemmli sample buffer (Laemmli, 1970). Proteins of interest were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham, Piscataway, New Jersey) and detected by incubation with diluted primary rabbit or mouse antibodies. The final detection was performed with 1:1000 dilutions of HRP-conjugated secondary anti-rabbit or anti-mouse antibodies and visualized using the POD Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland) on BioMax films (Kodak, Rochester, Minnesota). For the

detection of other proteins, the membrane was stripped using the Re-Blot reagent from Chemicon (Temecula, California) and re-incubated with other antibodies where indicated. To evaluate the gel loading of equivalent protein levels, tubulin and/or total ERK1/2 (Schmidt *et al*, 2000) were detected and the membrane was stained with amidoblack.

Detection of the phosphorylated forms of HER3, p38α, p38β, and p38δ Keratinocyte cultures were grown in 10 cm Petri dishes, subjected to treatments, then washed and scraped in 1 mL lysis buffer (PBS containing 1% Triton X-100, protease inhibitors (5 μg per mL pepstatin A, 1 μg per mL leupeptin, 0.02 mg per mL aprotinin) and 10 μM sodium orthovanadate as a phosphatase inhibitor). The lysates were centrifuged in a microfuge for 15 min at 13,000 × *g* at 4°C and the resulting supernatants were collected, pre-absorbed with protein A-agarose (HER3) or protein G-agarose (p38α, p38β, or p38δ) for 1 h and used for immunoprecipitation. Lysates were incubated with 5 μL of rabbit polyclonal antibodies against HER3 (or goat polyclonal antibodies against p38α, p38β, or p38δ) for 1 h at 4°C on a test tube rotator. Immune complexes were captured by incubation with protein A-agarose (HER3) or with protein G-agarose (p38α, p38β, or p38δ) for 1 h at 4°C. The agarose beads were then washed five times with PBS containing 0.5% sodium deoxycholate, 1% Triton-X100, and 0.1% SDS. The immune complexes were released from the beads by boiling the samples for 5 min in twice concentrated Laemmli sample buffer. The immunoprecipitates were separated by SDS-PAGE and blotted onto PVDF membranes. Phosphorylated HER3 was detected by incubation with mouse monoclonal antibodies against phosphotyrosine (1:500 dilution). Phosphorylated p38α, p38β, or p38δ was detected by incubation with rabbit polyclonal antibodies against phosphorylated p38 (1:500 dilution). The final detection was performed as described above. To estimate the amount of immunoprecipitated HER3, p38α, p38β, or p38δ, the membrane was stripped and re-incubated with antibodies against HER3, p38α, p38β, or p38δ for the detection of the total levels of HER3, p38α, p38β or p38δ.

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