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Regulatory role for Krüppel-like Zinc-finger Protein Gli-similar 1 (Glis1) in PMA-treated and psoriatic epidermis

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Abstract

In this study, we analyze the expression and potential function of the Krüppel-like zinc-finger protein Gli-similar protein 1 (Glis1) in normal and inflammatory skin and in the differentiation of epidermal keratinocytes. Glis1 mRNA is not expressed in normal human epidermis but is significantly induced in psoriatic epidermis and in mouse skin upon treatment with the tumor promoter phorbol-12-myristate-13-acetate (PMA). The expression of Glis1 is restricted to the suprabasal layers. These observations suggest that Glis1 expression is associated with hyperplastic, inflammatory epidermis. Consistent with these findings, Glis1 mRNA is not expressed in undifferentiated or differentiated normal human epidermal keratinocytes (NHEK) in culture but is dramatically induced after the addition of PMA or interferon γ. A similar induction of Glis1 mRNA by PMA treatment was observed in the immortalized epidermal keratinocyte cell line NHEK-HPV whereas PMA did not induce Glis1 in HaCaT cells or in several squamous cell carcinoma cell lines. To obtain insight into its function, Glis1 and a C-terminal deletion mutant Glis1 ΔC were expressed in NHEK-HPV cells and changes in epidermal differentiation and gene expression examined. Microarray analysis revealed that Glis1 ΔC promoted PMA-induced epidermal differentiation as indicated by increased expression of many differentiation-specific genes. This, in association with its induction in psoriasis, suggests that transcriptional factor Glis1 is involved in the regulation of aberrant differentiation observed in psoriatic epidermis.

Keywords

Gli; zinc finger protein; psoriasis; phorbol ester; differentiation; epidermal keratinocyte

Introduction

Hedgehog signaling plays a critical role in embryonic development and various physiological processes in the adult and has been implicated in a number of human diseases (Bale, 2002; Ruiz i Altaba *et al*, 2002; Toftgard, 2000; Villavicencio *et al*, 2000; Wicking *et al*, 2001). Sonic hedgehog (Shh) signaling involves binding of Shh to the transmembrane receptor patched (Ptch). This relieves the inhibition of downstream transcriptional events by the transmembrane protein smoothened (Smo). The Gli1-3 proteins constitute a subfamily of Krüppel-like zinc finger transcription factors that mediate the regulation of gene expression downstream of Shh/Ptch/Smo (Ruiz i Altaba *et al*, 2002; Villavicencio *et al*, 2000).

Recent studies have demonstrated that Shh/Ptch/Gli signaling plays an important role in skin biology and skin carcinogenesis (Chiang *et al*, 1999; Dahmane *et al*, 1997; Gailani *et al*,

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1997; Grachtchouk *et al*, 2000; Green *et al*, 1998; Ikram *et al*, 2004; Mill *et al*, 2003; Oro *et al*, 2003; St-Jacques *et al*, 1998). The Shh signaling cascade, largely through the activation of Gli2, is a key regulatory pathway in hair follicle development (Chiang *et al*, 1999; Ellis *et al*, 2003; Mill *et al*, 2003; St-Jacques *et al*, 1998; Wang *et al*, 2000). Shh signaling also has an important function in the regulation of cellular proliferation. Inappropriate activation of Shh/Ptch/Gli cascade has been linked to the development of various human cancers, including basal cell carcinoma (BCC) (Dahmane *et al*, 1997; GailaniBale, 1997; Grachtchouk *et al*, 2000; Matise *et al*, 1999; Nilsson *et al*, 2000; Ruiz i Altaba *et al*, 2002).

Recently, we identified three novel proteins, referred to as Gli-similar 1-3 (Glis1-3), that are closely related to Gli proteins (Kim *et al*, 2002; Kim *et al*, 2003; Lamar *et al*, 2001; Nakashima *et al*, 2002; Zhang *et al*, 2001a; Zhang *et al*, 2001b). Glis1-3 contain five Cys_2-His_2-type zinc finger motifs that exhibit high homology with those of Gli. However, Glis and Gli proteins exhibit little sequence homology outside their zinc finger domain. Deletion analysis demonstrated that Glis proteins contain both repressor and activation domains suggesting that they may function as positive as well as negative regulators of gene transcription (Kim *et al*, 2002; Kim *et al*, 2003; Zhang *et al*, 2001b). During embryonic development Glis1-3 are expressed in a spatial and temporal manner suggesting roles in the regulation of different steps in organogenesis (Kim *et al*, 2003; Lamar *et al*, 2001; Nakashima *et al*, 2002; Zhang *et al*, 2001b). During embryonic development, Glis1 (also named GliH1) expression was most prominent in craniofacial regions, branchial arches, somites, and kidney and was also detected in the dermal papilla (Kim *et al*, 2002; Nakashima *et al*, 2002).

In this study, we began to examine the potential role of Glis1 in the epidermis. We show that in contrast to normal epidermis, Glis1 mRNA is expressed in the epidermis of human psoriatic and PMA-treated mouse skin. In addition, Glis1 expression is induced in normal human epidermal keratinocytes (NHEK) and immortalized NHEK-HPV cells by two inflammatory mediators interferon gamma (IFNγ) and PMA, both of which are strong inducers of epidermal differentiation (Hennings *et al*, 1987; Saunders *et al*, 1994). To obtain insight into the role of Glis1 in epidermal differentiation, the effect of Glis1 on gene expression was examined by microarray analysis. These findings indicated that Glis1 promotes the expression of a number of differentiation-specific genes. Our observations provide evidence for a regulatory role for Glis1 in the aberrant differentiation observed in inflammatory skin.

Results

Glis1 is induced in psoriatic epidermis. To study whether Glis1 has a regulatory function in the epidermis we examined the expression of Glis1 mRNA in normal human skin, skin from psoriatic patients, and basal cell carcinomas. As shown in Fig. 1A, *in situ* hybridization analysis was unable to detect any Glis1 mRNA in normal human epidermis. In addition, Glis1 mRNA was undetectable in sections from basal cell carcinomas (not shown). In contrast, Glis1 was highly induced in the epidermis of psoriatic patients. Glis1 mRNA was undetectable in the dermal layer of either normal or psoriatic skin. Psoriatic epidermis is hyperplastic and is characterized by an infiltration of neutrophilic inflammatory cells (Fujimoto *et al*, 1997;Peters *et al*, 2000;Van de Kerkhof *et al*, 1996). The thickness of the stratum spinosum and expression of a number of early differentiation markers, including involucrin, cornifin, and transglutaminase I, are greatly increased (de Rie *et al*, 2004;Fujimoto *et al*, 1997;Michel *et al*, 1992). In psoriatic epidermis, Glis1 mRNA was expressed in the suprabasal layers and was absent in the basal cell layers (Fig. 1G), suggesting that undifferentiated keratinocytes do not express Glis1 mRNA. Thus, Glis1 mRNA is induced when cells transit from the basal into the suprabasal layers and begin to differentiate. The expression of Glis1 was compared with that of the differentiation marker involucrin, that is expressed in the upper stratum spinosum and stratum granulosum of normal epidermis and is highly induced in the suprabasal layers of

psoriatic epidermis (Fig. 1F). These observations demonstrate that the expression of Glis1 mRNA is greatly enhanced in psoriatic skin but that, unlike involucrin and many other differentiation markers, its expression appears to be restricted to hyperplastic, inflammatory skin. The pattern of Glis1 expression and its association with hyperplastic, inflammatory skin suggests that Glis1 does not play a role normal differentiation but has might be implicated in the regulation of aberrant differentiation observed in psoriatic epidermis.

Induction of Glis1 expression by PMA in mouse skin. As in psoriatic epidermis, PMA-treated mouse skin exhibits epidermal hyperplasia and neutrophil-dominant, inflammatory cell infiltration (Stanley *et al*, 1991). As in normal human skin, *in situ* hybridization analysis showed that Glis1 was not detectable in normal mouse epidermis (Fig 2B). However, Glis1 mRNA expression was induced in PMA-treated mouse skin (Fig. 2D). Expression of Glis1 mRNA was associated largely with the suprabasal layers of the epidermis. The sense probe did not detect any signal in PMA-treated skin (Fig. 2E). The induction of Glis1 mRNA by PMA was supported by RT-PCR analysis. RT-PCR using RNA extracted from normal mouse skin and mouse skin treated for 1 to 7 days with PMA confirmed that Glis1 mRNA was not expressed in untreated mouse skin, but induced in PMA-treated mouse skin (Fig. 2F).

PMA and IFNγ induce Glis1 mRNA expression in NHEK cells. We next examined the expression of Glis1 mRNA during differentiation of cultured human epidermal keratinocytes (NHEK). NHEK cells growing in the exponential phase do not express or express low levels of differentiation markers. When cultures reach confluence, cells become committed to terminal differentiation and expression of various differentiation markers are induced (Jetten *et al*, 1997). Glis1 was undetectable in exponential phase cultures of NHEK cells as well as in confluent cultures maintained in medium with high calcium (Fig. 3A, lanes 1 and 2). Differentiated NHEK cells grown at air-liquid interface also did not express Glis1 (Fig. 4A, lane 3). The tumor promoter PMA and IFNγ are strong inducers of differentiation in NHEK cells (Hennings *et al*, 1987; Saunders *et al*, 1996). As shown in Fig. 3A (lanes 4 and 5), Glis1 mRNA expression was increased significantly in NHEK cells treated with PMA or IFNγ. The lack of Glis1 expression during normal (confluence-induced) epidermal differentiation and the induction of Glis1 during the differentiation process initiated by PMA and IFNγtwo proinflammatory agents, appear to mirror the pattern of Glis1 expression observed in psoriatic human skin and PMA-treated mouse skin (Reynolds *et al*, 1998).

We then compared Glis1 expression in two immortalized epidermal keratinocyte cell lines NHEK-HPV and HaCaT, and in the squamous cell carcinoma cell lines SCC13 and SQCC/Y1. NHEK-HPV are human epidermal keratinocytes immortalized by HPV-E6 (Harvat *et al*, 2001; Saunders and Jetten, 1994) and retain the ability to undergo differentiation upon PMA treatment and at confluence. In the exponential phase NHEK-HPV cells did not express Glis1 mRNA; however, Glis1 expression was induced upon PMA treatment (Fig. 3B). In contrast, PMA did not induce Glis1 mRNA expression in HaCaT, SQCC/Y1, and SCC13 cells which do not undergo differentiation after PMA treatment. The induction of Glis1 mRNA by PMA in NHEK-HPV cells occurred in a time-dependent manner (Fig. 3C). An increase in Glis1 mRNA expression was observed as early as 16 h after the addition of PMA. This induction paralleled that of the increase in the level of cornifin protein, a marker of epidermal differentiation (Marvin *et al*, 1992). The induction of Glis1 mRNA expression by PMA and IFNγin NHEK and NHEK-HPV cells was confirmed by Real Time QRT-PCR analysis (Fig. 3D).

Expression of Glis1 and Glis1ΔC in NHEK-HPV cells. To study the role of Glis1 in epidermal differentiation, the effect of Glis1 and Glis1ΔC on the differentiation of NHEK-HPV cells was examined. Using pLXIN retroviral vectors NHEK-HPV cell lines were established that stably expressed Flag-Glis1(full-length) fusion protein (Flag-Glis1) and a

mutant Glis1 containing a C-terminal deletion (Flag-Glis1ΔC) (Fig. 4A). These cell lines are referred to as NHEK-HPV-Flag-Glis1 and NHEK-HPV-Flag-Glis1ΔC, respectively. The cell line NHEK-HPV(empty) containing the empty vector pLXIN-Flag, was used as control. In addition, three corresponding HaCaT cell lines were established using the same retroviruses.

The expression of Flag-Glis1 fusion protein was examined by Western blot analysis using anti-Flag M2 antibody. In protein extracts from NHEK-HPV-Flag-Glis1 and HaCaT-Flag-Glis1 cells the anti-Flag antibody recognized the Flag-Glis1 protein with the expected size of 100 kD. However, the antibody reacted with several other smaller proteins (Fig. 4A; lane 2 and 4, respectively). These smaller Flag-Glis1 proteins may be generated by proteolytic processing of Glis1 at two different sites at the carboxyl terminus. Protease inhibitors included during the isolation of cellular protein did not prevent this cleavage. We believe that Glis1 might be specifically processed within the cell. Transient expression of Flag-Glis1 in NHEK-HPV cells showed similar processing of Glis1 (Fig. 4A, lane 6). In extracts from NHEK-HPV-Flag-Glis1ΔC and HaCaT-Flag-Glis1ΔC cells the antibody recognized only the Flag-Glis1ΔC fusion protein of the expected size of 50 kD suggesting that it is not further cleaved. The latter is in agreement with the conclusion that the processing of full-length Glis1 involves the carboxyl terminal half of Glis1.

The morphology of the NHEK-HPV-Flag-Glis1 cells was very similar to that of the parental NHEK-HPV or NHEK-HPV(empty) cells (Fig. 4B, C). Cultures consisted of migratory cells that formed more loose colonies. NHEK-HPV-Flag-Glis1ΔC cells formed more compact colonies with fewer migrating cells (Fig. 4D). These cells were also more adhesive than parental NHEK-HPV cells (not shown). No differences in cellular morphology were observed between HaCaT-Flag-Glis1ΔC, HaCaT-Flag-Glis1, and HaCaT(empty) cells (not shown).

Subcellular localization of Glis1. The activity of Gli and Ci proteins is greatly dependent on their nuclear localization and controlled by suppressor-of-fused (SUFU), proteolytic processing, protein kinases, and nuclear export signals (Dunaeva *et al*, 2003; Kogerman *et al*, 1999; Ruiz i Altaba *et al*, 2002; Villavicencio *et al*, 2000). We, therefore, examined the subcellular localization of Glis1 in stably-transfected cells. In more than 90% of NHEK-HPV-Flag-Glis1 cells, Glis1 protein localized predominantly to cytoplasm (Fig. 5A). This cytoplasmic staining likely represents full-length Glis1 and proteolytically cleaved Glis1 based on the results shown in Fig. 4. In contrast, in NHEK-HPV-Flag-Glis1ΔC cells Glis1ΔC was predominantly localized in the nucleus (Fig. 5C). These results suggest that the C-terminal region of Glis1 contains a domain that retains Glis1 in the cytoplasm.

Identification of Glis1-regulated genes. To investigate changes in gene expression resulting from the expression of Glis1 and Glis1ΔC, we performed microarray analysis using a human oligo-chip representing approximately 17,000 human genes. No significant differences in gene expression were detected between PMA-treated NHEK-HPV(empty) and PMA-treated NHEK-HPV-Flag-Glis1 cells (not shown). In contrast, microarray analysis with PMA-treated NHEK-HPV(empty) and PMA-treated NHEK-HPV-Flag-Glis1ΔC cells identified more than 54 genes that were down-regulated and 23 genes that were up-regulated in NHEK-HPV-Flag-Glis1ΔC cells (Table I). The expression of insulin-like growth factor binding protein 2 (IGFBP2), which is involved in growth inhibition and induction of apoptosis, showed the largest increase in NHEK-HPV-Glis1ΔC cells. Interestingly, among the genes that were significantly increased in NHEK-HPV-Flag-Glis1ΔC compared to NHEK-HPV(empty) cells included many well-established markers of epidermal differentiation, including S100A9, KLK7, SPRR (cornifin), involucrin, and transglutaminase 1. These observations are consistent with the interpretation that Glis1 enhances epidermal differentiation. Genes down-regulated by Glis1 include the notch-1 receptor Jagged 1, the Wnt receptor Frizzled-10, the TGFβ family member inhibin βA (activin A), and the activin receptor ACVR1.

The differential expression of several genes was validated by Northern blot analysis. This analysis confirmed the enhanced expression of the differentiation markers S100A9, KLK7, and transglutaminase type I and the down-regulation of Jagged-1 and inhibin βA (Fig. 6).

Glis1 expression promotes epidermal differentiation. To analyze the action of Glis1 further, we compared the induction of differentiation in NHEK-HPV(empty), NHEK-HPV-Flag-Glis1, and NHEK-HPV-Flag-Glis1ΔC cells upon treatment with PMA. The expression of several proteins known to be differentially regulated during epidermal differentiation were examined by Western blot analysis (Fig. 7A). Previous studies reported that the p53-family member p63 is down-regulated during squamous differentiation *in vivo* and *in vitro* (Koster *et al*, 2002;Pellegrini *et al*, 2001). The basal level of p63 protein in undifferentiated NHEK-HPV (empty), NHEK-HPV-Flag-Glis1, and NHEK-HPV-Flag-Glis1ΔC cells was not significantly different in the three cell lines. In addition, PMA decreased p63 expression in all three cell lines in a similar way (Fig. 7A). The differentiation markers involucrin and cornifin are up-regulated by PMA in all three NHEK-HPV cell lines; however, their level is increased to a significant greater extent in NHEK-HPV-Flag-Glis1ΔC than in NHEK-HPV(empty) or NHEK-HPV-Flag-Glis1 cells. These findings are in agreement with the results obtained by microarray analysis (Table I) and are consistent with the hypothesis that Glis1 promotes epidermal differentiation. Treatment of HaCaT(empty), HaCaT-Flag-Glis1□□ HaCaT-Flag-Glis1ΔC cells with PMA caused a small reduction in p63 while cornifin expression was very low in all three cell lines and remained unchanged after PM treatment (Fig. 7B) indicating that Glis1 expression has little effect on the induction of differentiation in these cells.

Discussion

The Shh/Gli signaling pathway has been reported to play an important role in skin biology and in skin disease (Chiang *et al*, 1999; Dahmane *et al*, 1997; Gailani and Bale, 1997; Grachtchouk *et al*, 2000; Green *et al*, 1998; Ikram *et al*, 2004; Mill *et al*, 2003; Oro and Higgins, 2003; St-Jacques *et al*, 1998). Gli1 and Gli2 are frequently highly induced in basal cell carcinomas and critical in the development of these tumors (Grachtchouk *et al*, 2000; Green *et al*, 1998; Nilsson *et al*, 2000). In this study, we demonstrate that Glis1 mRNA is not expressed in normal human epidermis nor in basal cell carcinomas. These observations suggest that Glis1 does not play a role in the development of this type of skin cancer. In contrast to normal skin, Glis1 mRNA was induced in the epidermis of human psoriatic skin. Psoriasis is one of the most common chronic inflammatory skin diseases and is characterized by local infiltration of T lymphocytes, neutrophils, and monocytes, and epidermal hyperproliferation (de Rie *et al*, 2004; Peters *et al*, 2000). The latter results in a significant increase in the thickness of the epidermis, the stratum spinosum in particular, and a greatly enhanced and aberrant expression of many differentiation-specific genes. In psoriatic epidermis, Glis1 mRNA is not detectable in the most basal layers but only in the suprabasal layers, particularly throughout the lower stratum spinosum. Comparison of Glis1 mRNA expression with that of the early differentiation marker involucrin shows that its induction precedes the appearance of involucrin protein. PMA-treated skin exhibits several similarities with psoriatic skin (Marks *et al*, 1993; Stanley *et al*, 1991). Treatment of mouse skin with PMA causes inflammation, enhances the thickness of the epidermis, and causes an increase in the expression of many differentiation markers. As in the epidermis of psoriatic patients, expression of Glis1 mRNA is induced in PMA-treated mouse skin as shown by *in situ* hybridization and confirmed by RT-PCR. In PMA-treated mouse skin Glis1 mRNA was predominantly localized to the suprabasal layers as observed for psoriatic epidermis.

The expression pattern of Glis1 in cultured human epidermal keratinocytes mirrors that seen in normal and PMA-treated skin. Glis1 mRNA was undetectable in NHEK cells growing in the exponential phase or in cells grown at an air-liquid interface. High Ca^{2+}, which is not a

very effective inducer of differentiation in NHEK, did not induce Glis1 expression. In contrast, treatment with PMA and IFNγ, both of which are proinflammatory mediators and strong inducers of growth arrest and differentiation in NHEK cells (Hennings *et al*, 1987; Saunders *et al*, 1996), significantly induced Glis1 mRNA levels. PMA treatment did not induce Glis1 expression in squamous carcinoma cells that have lost the ability to differentiate.

Epidermal differentiation is a multi-step process that starts with the transition of epidermal stem cells into transient amplifying cells, keratinocytes with a diminished lifespan (Watt, 2001). These cells subsequently undergo terminal differentiation that is associated with loss of proliferative potential and the expression of differentiation-specific genes. The expression of differentiation markers is carefully controlled and induced at specific steps during differentiation. The pattern of Glis1 expression observed in skin and cultured NHEK cells indicates that Glis1 is not required for normal epidermal differentiation. Instead, Glis1 appears to have a specific regulatory function in aberrant epidermal differentiation observed psoriatic and PMA-treated epidermis where it might be involved in the up-regulation of the expression of a number of squamous differentiation-specific markers or inflammatory genes. Previous studies have provided indications for additional functions of Glis1 in skin. During embryonic development, Glis1 mRNA is expressed in dermal papilla cells (Nakashima *et al*, 2002; Zhang *et al*, 2001b). These cells have been reported to play a critical role in hair follicle development (Oro and Higgins, 2003; St-Jacques *et al*, 1998). However, hair follicle development in mice deficient in Glis1 appears undisturbed suggesting that Glis1 is not required for this process (Nakashima *et al*, 2002). What the function is of Glis1 in dermal papilla cells has yet to be established.

To study the potential role of Glis1 in the regulation of epidermal differentiation, we examined the effects of Glis1 and Glis1ΔC expression in an immortalized epidermal keratinocyte cell line NHEK-HPV. Treatment of NHEK-HPV(empty), NHEK-HPV-Glis1, and NHEK-HPV-Glis1ΔC cells with PMA induces differentiation in all three cell lines and no significant differences were observed in the repression of p63, an early event in epidermal differentiation (Koster *et al*, 2002; Pellegrini *et al*, 2001). However, the induction of the differentiation markers involucrin and cornifin was significantly elevated in NHEK-HPV-Glis1ΔC cells compared to NHEK-HPV(empty) and NHEK-HPV-Glis1. These observations are consistent with the hypothesis that Glis1 does not act at the level of the commitment to differentiation but promotes the expression of the differentiated phenotype.

Analysis of the expression of Glis1 protein showed that Glis1 was proteolytically cleaved, that Glis1 and Glis1ΔC have a different subcellular localization. Previous studies have shown that the regulation of Gli protein activity involves activation by Shh, proteolytic processing, and translocation to the nucleus (Dunaeva *et al*, 2003; Kogerman *et al*, 1999; Ruiz i Altaba, 1999; Villavicencio *et al*, 2000). Mouse Gli2 contains a repressor domain near its amino-terminus that is cleaved off to generate a transcriptionally active form and overexpression of the latter induces tumor formation in mouse skin (Grachtchouk *et al*, 2000; Sheng *et al*, 2002). Transient transfection of Glis1 or Glis1ΔC inhibits Gli1-mediated transcriptional activation suggesting that both Glis1 proteins function as repressors (unpublished observations). The lack of a cellular response in NHEK-HPV-Glis1 cells might be due to the observation that Glis1 is largely localized to the cytoplasm. Removal of the carboxyl-terminus results in the translocation of Glis1ΔC to the nucleus and enables it to induce changes in gene transcription.

To obtain greater insight into the possible role of Glis1 in the regulation of gene expression during differentiation of epidermal keratinocytes, we compared the pattern of gene expression between PMA-treated NHEK-HPV(empty) and NHEK-HPV-Glis1ΔC cells by microarray analysis. This analysis identified more than 22 genes that were down-regulated and 54 genes

that were up-regulated in NHEK-HPV-Glis1ΔC compared to NHEK-HPV(empty) cells (Table I). Many of these genes encode proteins for which specific functions in epidermal differentiation have been reported. The notch-1 receptor Jagged 1 and the TGFβ family member inhibin βA (activin A) belong to the group of genes down-regulated in NHEK-HPV-Glis1ΔC cells. Jagged 1 has been reported to be expressed in the basal layer of the epidermis and down-regulated in psoriatic epidermis (Thelu *et al*, 2002). In contrast, inhibin βA which plays a positive role in cell proliferation, is increased in psoriasis (Hubner *et al*, 1996). Genes expressed at higher levels in NHEK-HPV-Glis1ΔC cells include the cross-linked envelope precursors involucrin and small proline-rich proteins (SPRRs or cornifins), the crosslinking enzyme transglutaminase I that catalyzes the crosslinking between these structural proteins, and sulfotransferase Sult2B1b which catalyzes the formation of cholesterol sulfate. These observations are in agreement with the hypothesis that expression of Glis1ΔC promotes differentiation.

It is interesting to note that many of these genes, including involucrin, transglutaminase 1, several of the kallikrein serine proteases, the protease inhibitor cystatin E, epidermal fatty acid binding protein, beta-glucocerebrosidase, and S100 calcium-activated signaling proteins have been reported to be significantly induced in psoriatic epidermis (Eckert *et al*, 2004; Fujimoto *et al*, 1997; Kuwae *et al*, 2002; Michel *et al*, 1992; Peters *et al*, 2000; Zeeuwen *et al*, 2002) (see * in Table I). S100A9 and S100A8 are not or minimally expressed in normal epidermis but are highly expressed in psoriatic epidermis (Eckert *et al*, 2004). In addition, many of these genes are induced in PMA-treated epidermis (Michel *et al*, 1992; Owens *et al*, 1996). The enhanced expression of several differentiation-specific genes in epidermal keratinocytes overexpressing Glis1□C, in combination with the observations that Glis1 is induced in psoriatic and PMA-treated epidermis, suggests that there may be a link between these events. Since Glis1 functions as a transcriptional regulator (Kim *et al*, 2002), it is tempting to hypothesize that the expression of at least a subset of genes induced in psoriasis might be regulated by Glis1. Glis1 regulates gene transcription by binding to Glis DNA response elements (Glis-RE; consensus sequence ACCACCCA) in the promoter regulatory region of target genes (Kim *et al*, 2002). Although Glis1ΔC appears to act as a repressor, it might enhance the expression of differentiation-specific genes by inhibiting the expression of another transcriptional repressor. Further study is needed to determine whether Glis1 regulates these genes directly or indirectly.

In summary, in this study we demonstrate that Glis1 is not expressed in normal epidermis but is induced in psoriatic and PMA-treated skin and in PMA- and IFNγ-treated epidermal keratinocytes. Expression of Glis1ΔC in epidermal keratinocytes NHEK-HPV results in enhanced induction of a great number of differentiation-specific genes after treatment with PMA. Our observations indicate that expression of Glis1 promotes epidermal differentiation and suggest a regulatory role for Glis1 in aberrant epidermal differentiation and remodeling in psoriatic and PMA-treated skin.

Materials and Methods

Cell lines and cell culture. Normal human epidermal keratinocytes (NHEK) were obtained from Cambrex (Walkersville, MD). NHEK-HPV cells is an immortalized cell line derived by stable integration of the plasmid pHPV-18 (Harvat and Jetten, 2001). NHEK, SCC13, SQCC/Y1 cells, HaCaT cells, and NHEK-HPV cells were grown in KGM (Gibco BRL, Gaithersburg, MD) as previously described (Saunders and Jetten, 1994).

Retroviral vectors. The entire coding sequence of mouse Glis1 and a C-terminal truncated mouse Glis1, in which the region immediately following the zinc finger domain (^{518}S) up to the C-terminus was deleted, were amplified by polymerase chain reaction (PCR) and subcloned into pCMV3xFlag 7.1 (Sigma, St. Louis, MO) generating pCMV-Flag-Glis1 and pCMV-Flag-

Glis1ΔC, respectively. Flag-Glis1 and Flag-Glis1ΔC were re-amplified and then subcloned into the retroviral vector pLXIN (BD Biosciences Clontech, Palo Alto, CA). Retroviral infection and neomycin selection was carried out as described (Stapleton *et al*, 2005). The established cell lines are referred to as NHEK-HPV(empty), NHEK-HPV-Flag-Glis1, NHEK-HPV-Flag-Glis1ΔC, HaCaT(empty), HaCaT-Flag-Glis1, and HaCaT-Flag-Glis1ΔC.

Western blot analysis. Cells were lysed in lysis buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin). The proteins were analysed by Western blot analysis using ECL Western Blotting Kit (Amersham Pharmacia Biotech., Piscataway, NJ) as described. Rabbit anti-cornifin antibody, mouse anti-involucrin antibody (Sigma), and mouse anti-p63 antibody (Santa Cruz Biotech., Santa Cruz, CA) were used as previously described (Daniely *et al*, 2004; Fujimoto *et al*, 1997). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were purchased from Chemicon (Temecula, CA). Antibodies were diluted in PBS containing 5% milk and 0.01% Tween 20.

Reverse Transcriptase-PCR Analysis. Total RNA was prepared with TriReagent (Sigma) or RNeasy mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScriptIII first strand cDNA synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Semi-quantitative PCR was carried out using the following primer pair for mouse and human Glis1: forward primer 5'-TCAGCAACTCCAGCGACCGC and reverse primer 5'-GGAGCCAGGA-TACACACCTG. Primers for β-actin were used in controls.

Northern Blot Analysis. Total RNA (20 μg) was isolated using TriReagent (Sigma, St. Louis, MO) and examined by Northern blot analysis as described previously (Nakajima *et al*, 2004) with radiolabeled probes for transglutaminase I, kallikrein 7, S100A9, Jagged-1, and inhibin βA.

***In situ* hybridization analysis.** Adult mouse skin and human skin were fixed in 4% paraformaldehyde, embedded in paraffin, and sections (5 μm thick) prepared for *in situ* hybridization analysis as described (Fujimoto *et al*, 1997). *In situ* hybridization analyses with antisense and sense Glis1 probes were carried out as described previously. Sections were stained with hematoxylin and eosin (H&E).

Microarray analysis. Microarray analysis was carried out by the NIEHS Microarray Group (NMG). Gene expression analysis was conducted using Agilent human oligo arrays (Agilent Technologies, Palo Alto, CA), the Agilent Low RNA Input Fluorescent Linear Amplification Kit protocol, the Agilent *In Situ* Hybridization Kit protocol, and the Agilent Feature Extraction software (v7.1). Images and GEML files were deposited into Rosetta Resolver (version 3.2, build 3.2.2.0.33) (Rosetta Biosoftware, Kirkland, WA). The resultant ratio profiles were combined into ratio experiments as described (Stoughton, 2002). Intensity plots were generated for each ratio experiment and genes were considered “signature genes” if the p value was less than 0.001. RNA preparations from two independent experiments were used and microarray hybridizations were performed in duplicate.

Subcellular localization. Cells overexpressing 3XFlag-Glis1 or 3XFlag-Glis1ΔC were fixed in 4% paraformaldehyde/PBS for 15 min and then treated with 0.3% Triton X-100/PBS for 15 min. Cells were subsequently incubated with anti-Flag M2 monoclonal Antibody (Sigma) for 1h. After washing, cells were incubated with Alexa Fluor 488-conjugated anti-mouse antibody (Molecular Probes, OR) for 1h. After several washings, Alexa Fluor 488 fluorescence was examined in a Zeiss confocal microscope LSM 510 (Zeiss, Thornwood, NY) at excitation and emission frequencies of 488 and 505 nm, respectively.

Immunohistochemical staining. Paraffin-embedded sections of normal and psoriatic skin were stained immunohistochemically with an anti-involucrin antibody using a streptavidin-biotin-peroxidase staining kit (Biomedical Technologies Inc., Stoughton, MA) following the manufacturer's instructions.

Animal and human skin samples. PMA-treated mouse skin samples were kindly provided by Dr. Sung-Jen Wei (NIEHS, NIH). Animal studies were carried out as previously reported (Wei *et al*, 2003). Normal human skin samples were obtained as excess tissue from unrelated surgical procedures. Psoriasis samples used were obtained from the pathology archives, Department of Dermatology, Okayama University Medical School.

Real Time QRT-PCR analyses. The Real Time QRT-PCR reactions were carried out in triplicate on a 7300 Real Time PCR system using the TaqMan® One-Step RT-PCR mix and pre-designed Assays-on-Demand™ primers/probe set for human Glis1 (Hs00377690_m1) from Applied Biosystems. All results were normalized to an internal control, the 18S transcript.

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Abbreviations:

Glis, Gli-similar; PMA, phorbol-12-myristate-13-acetate; NHEK, normal human epidermal keratinocytes; BCC, basal cell carcinoma; Shh, sonic hedgehog; ptch, patched; IFN, interferon.

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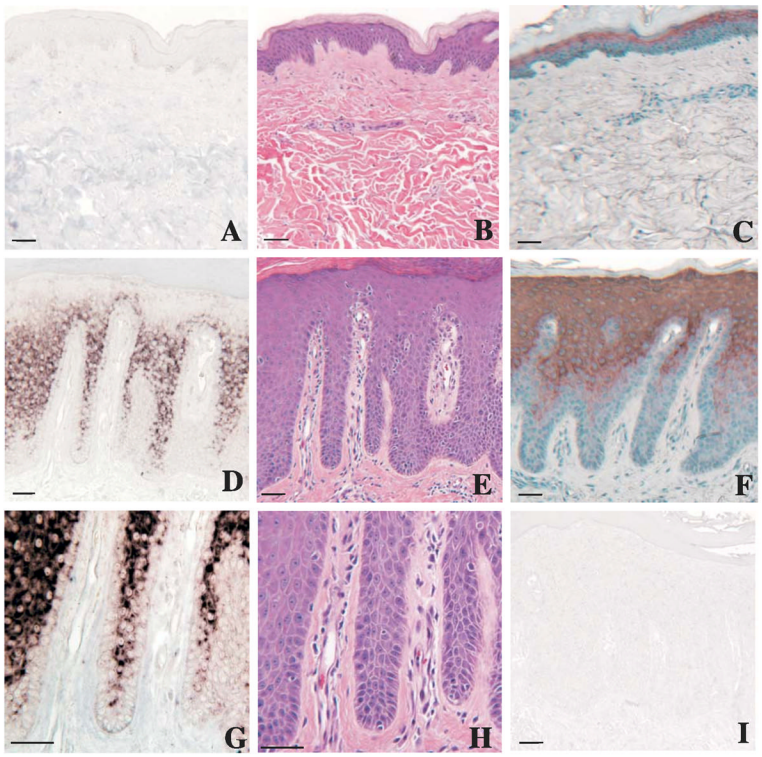

Figure 1.

Glis1 mRNA is not expressed in normal epidermis but induced in the suprabasal layers of psoriatic skin. Paraffin sections of normal human skin were hybridized with a Glis1 antisense probe (A) and adjacent sections were stained with H&E (B) or an anti-involucrin antibody (C). Sections of psoriatic skin were hybridized with an antisense (D, G) or sense (I) probe for Glis1 and adjacent sections were stained with H&E (E, H) or an anti-involucrin antibody (F). (G) and (H) are higher magnifications of D and E, respectively. Bars indicate 50 □m.

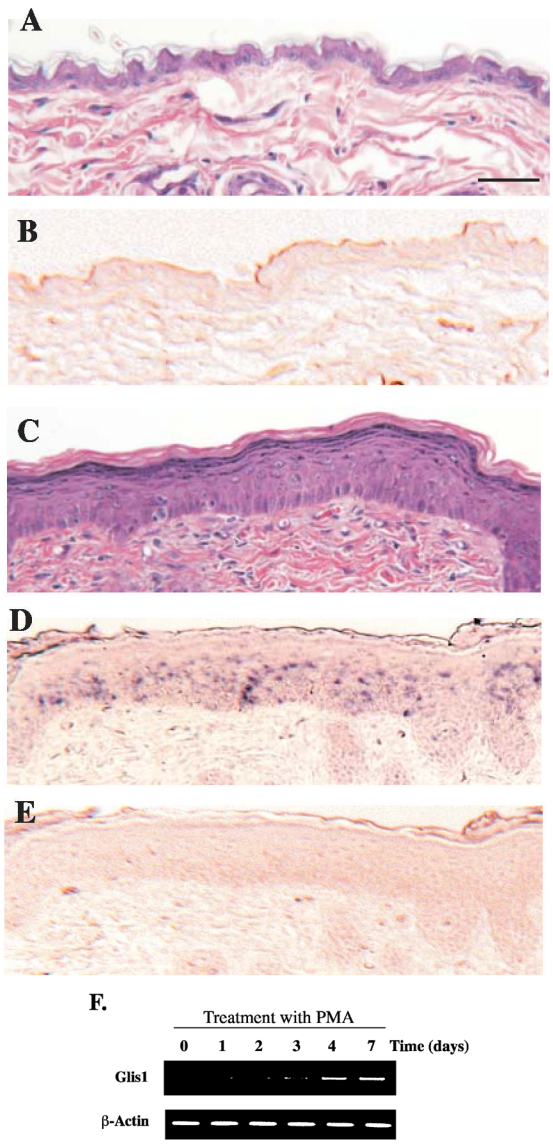

Figure 2.

Induction of Glis1 mRNA expression in PMA-treated mouse skin. Mouse skin was treated with PMA or vehicle (control) once a day during a 7-day period as described in Materials and Methods. A-E. Paraffin sections of skin were obtained and subsequently examined by *in situ* hybridization analysis with sense or antisense Glis1 probes. (A) H&E staining of a section of control mouse skin; (B) control skin section hybridized with a Glis1 antisense probe; (C) H&E staining of a representative section of PMA-treated skin; (D) a representative section of PMA-treated skin hybridized with a Glis1 antisense probes; (E) section of PMA-treated skin hybridized with a Glis1 sense probe. Bar indicates 50 □m. (F) At the times indicated skin was isolated, RNA extracted and the level of Glis1 and β-actin (control) mRNA determined by RT-PCR as described in Materials and Methods.

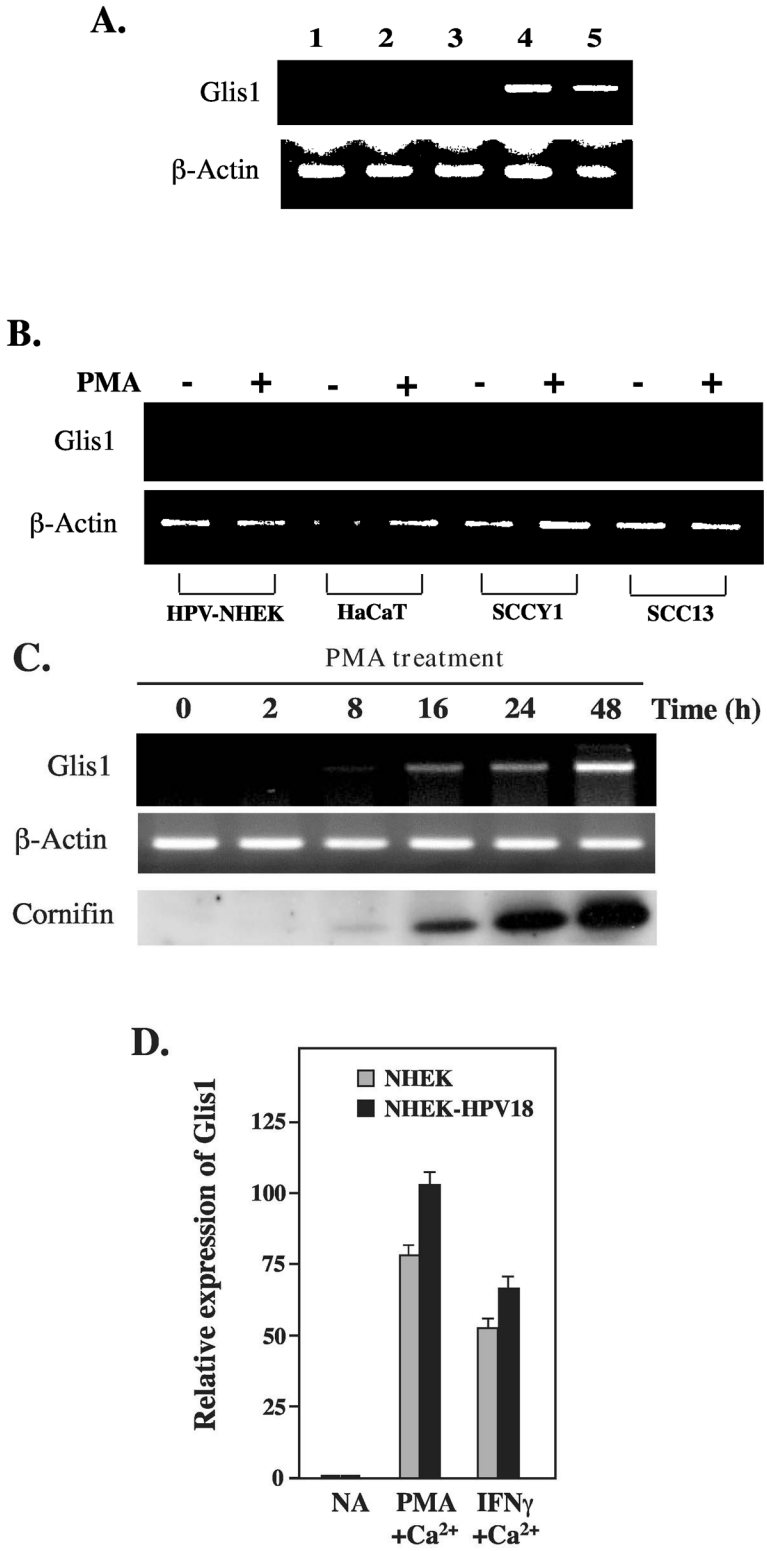

Figure 3.

(A) Induction of Glis1 mRNA expression in NHEK cells in response to PMA and IFNγ. NHEK cells grown under different conditions were harvested and RNA isolated. Glis1 and β-actin

(control) mRNA levels were determined by RT-PCR as described in Materials and Methods. RNA from exponential phase culture of NHEK cells; lane 2: RNA from confluent NHEK cells grown in the presence of 1.5 mM $CaCl_2$; lane 3: RNA from cells grown at an air-liquid interface; lane 4: RNA from cells treated for 24 h with PMA (20 nM) and 1.5 mM calcium; lane 5: RNA from cells treated for 24 h with IFNγ (100 U/ml). (B) PMA induces Glis1 mRNA expression in NHEK-HPV but not in HaCaT and squamous cell carcinoma cell lines SCC13 and SQCC/Y1. Cells were treated with 20 nM PMA in the presence of 1.5 mM $CaCl_2$. After 24 h, RNA was isolated and the level of Glis1 and β-actin (control) mRNA determined by semi-quantitative RT-PCR. (D) Time course of the induction of Glis1 mRNA in NHEK-HPV cells by PMA. Cells were treated with 20 nM PMA in the presence of 1.5 mM $CaCl_2$. At the times indicated, cells were harvested and RNA and protein isolated. Glis1 and β-actin (control) mRNA levels were determined by RT-PCR. Protein samples were used for Western blot analysis to determine the level of cornifin expression. (D) Induction of Glis1 mRNA expression by PMA and IFNγ in NHEK and NHEK-HPV cells. Real-time QRT-PCR analysis was performed as described in Materials and Methods.

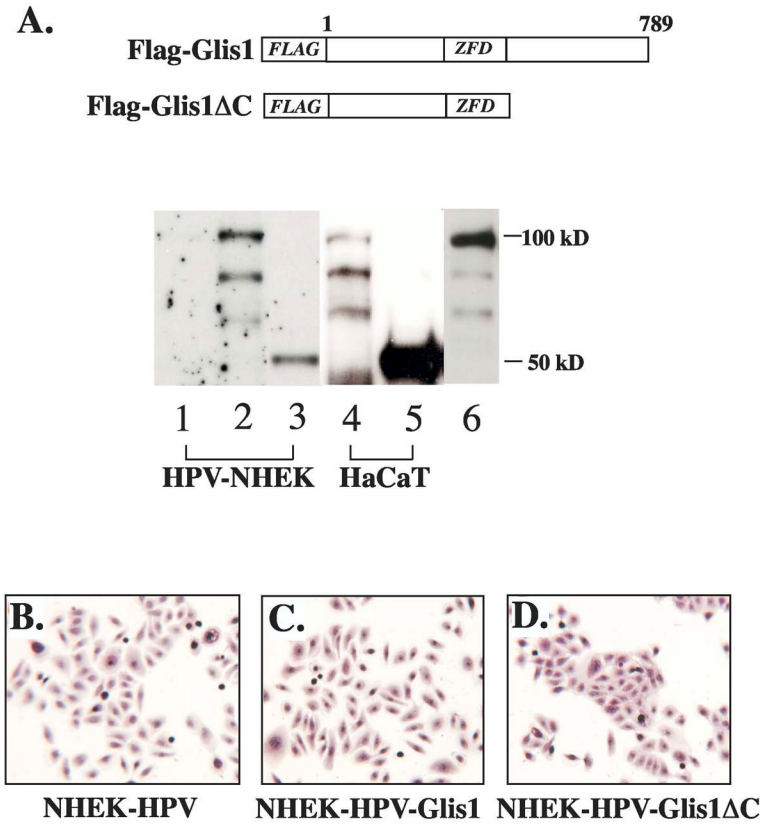

Figure 4.

A. Analysis of Glis1 protein in NHEK-HPV and HaCaT cells expressing Flag-Glis1 and Flag-Glis1ΔC. NHEK-HPV and HaCaT cells were infected with retroviruses containing pLXIN (empty), pLXIN-Flag-Glis1, or pLXIN-Flag-Glis1ΔC and stable cell lines established as described in Materials and Methods. Protein extracts (30 μg) were examined by Western blot analysis using anti-Flag M2 antibody. Lane 1: NHEK-HPV(empty); lane 2: NHEK-HPV-Flag-Glis1; lane 3: NHEK-HPV-Flag-Glis1ΔC; lane 4: HaCaT-Flag-Glis1FL; lane 5: HaCaT-Flag-Glis1ΔC; lane 6: NHEK-HPV cells transiently transfected with pCMV-3xFlag-Glis1 construct. A schematic view of the two fusion proteins is shown above the Western blot. B-D. Comparison of the cell morphology of NHEK-HPV(empty) (B), NHEK-HPV-Flag-Glis1 (C), and NHEK-HPV-Flag-Glis1ΔC (D) cells.

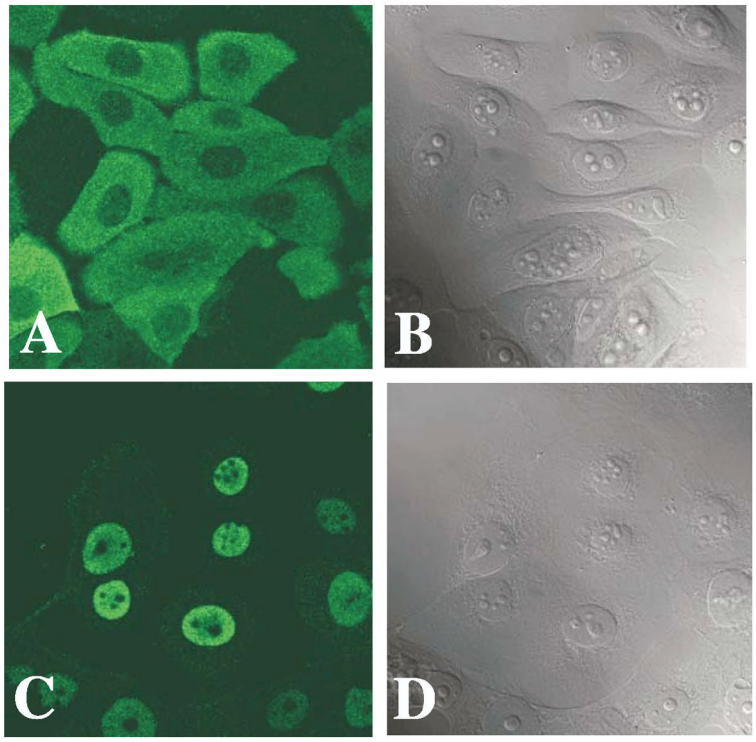

Figure 5.
Subcellular localization of Glis1 and Glis1ΔC in NHEK-HPV cells. Cells were stained by mouse anti-Flag M2 antibody and Alexa 488-conjugated anti-mouse IgG and subsequently examined by confocal microscopy (A,C). Right panels are phase contrast images. A,B. NHEK-HPV-Flag-Glis1; C,D. NHEK-HPV-Flag-Glis1ΔC.

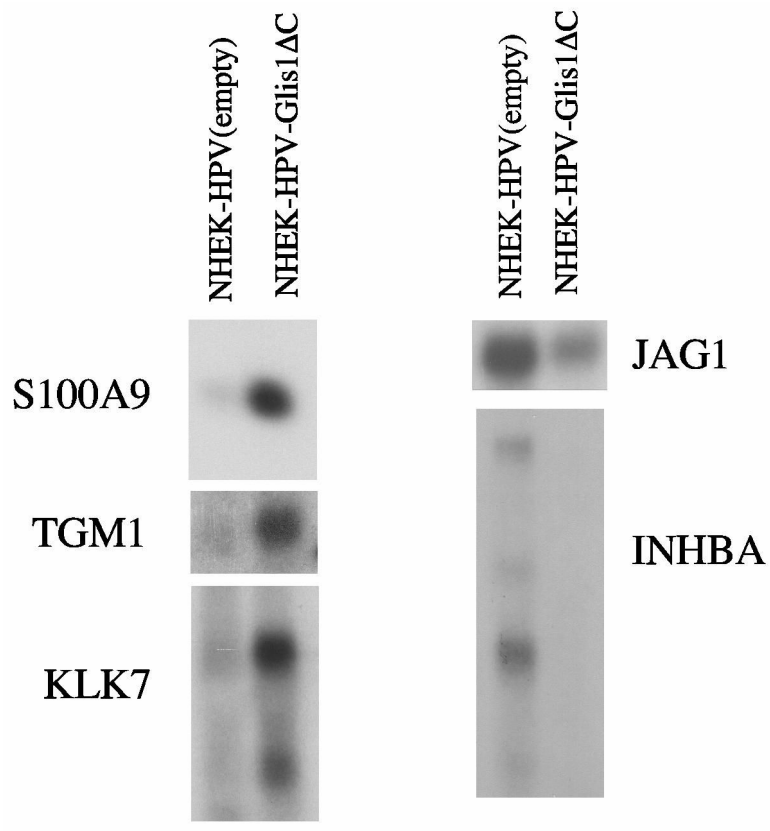

Figure 6.
Validation of several differentially expressed genes identified by microarray analysis. RNA isolated from PMA-treated NHEK-HPV(empty), NHEK-HPV-Flag-Glis1, and NHEK-HPV-Flag-Glis1ΔC cells were examined by Northern blot analysis using radiolabeled cDNA probes for S100A9, transglutaminase type I (TGM1), kallikrein 7 (KLK7), Jagged-1 (JAG1), and inhibin βA (INHBA) as described in Materials and Methods.

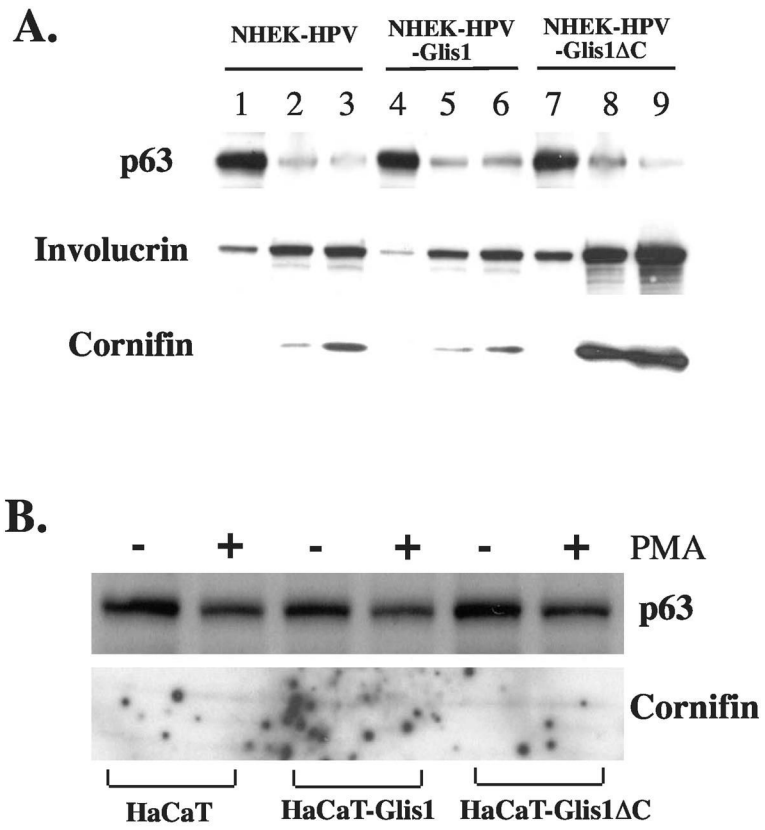

Figure 7.

A. Glis1ΔC enhances PMA-induced differentiation in NHEK-HPV cells. NHEK-HPV (empty), NHEK-HPV-Glis1, and NHEK-HPV-Glis1ΔC cells were treated with vehicle (control; lanes 1, 4, 7), 2 nM PMA (lanes 2, 5, 8) or 20 nM PMA in the presence of 1.5 mM $CaCl_2$ (lanes 3, 6, 9). After 24 h treatment, cells were collected and proteins examined by Western blot analysis using antibodies for p63, involucrin and cornifin. B. Glis1ΔC does not induce differentiation in HaCaT cells. Cells were treated for 24 h with vehicle or 50 nM PMA and 1.5 mM $CaCl_2$. Proteins were examined by Western blot analysis for p63 and cornifin expression.

Table I

Gene	Genbank #	Description	Fold-increase	Function
IGFBP2	P18065	Insulin-like growth factor binding protein 2	13.59	Proliferation/apoptosis
KLK6	Q92876	Kallikrein 6	8.66	Serine proteinase
I_929176	P22466	Preprogalanin	6.42	Neuropeptide*
KLK10	O43240	Kallikrein 10	6.33	Serine proteinase
S100A9	P06702	S100 calcium-binding protein A9	6.29	Ca2+-activated signaling protein*
KLK7	P49862	Kallikrein 7	5.89	Stratum corneum serine protease*
KLK8	NM_144507.1	Kallikrein 8	5.59	Serine protease*
IVL	P07476	Involucrin	5.38	Structural component of stratum corneum*
KLK5	Q9Y337	Kallikrein 5	5.43	Stratum corneum serine protease
G1P2	P05161	Interferon-inducible protein 15	4.77	Ubiquitin-like protein
TXNIP	BAB18859.1	Vitamin D-3 upregulated protein-1	4.29	Inhibitor of thioredoxin
NMU	P48645	Neuromedin U	3.89	Agonist of G protein-coupled receptors
TGM1	P22735	Transglutaminase 1	3.78	Crosslinking enzyme*
SLPI	P03973	Secretory leukocyte protease inhibitor	3.7	Antileukoproteinase*
CST6	Q15828	Cystatin E (cystatin M)	3.66	Protease inhibitor*
KRT13	P13646	Keratin 13	3.59	Intermediate filament protein
IFI27	P40305	IFNa-inducible protein 27	3.44	Membrane protein*
S100A8	NM_002964.3	S100 calcium binding protein A8	3.38	Ca2+-activated signaling protein*
AK3L1	Q9UIJ7	Adenylate kinase 3 like 1	3.28	Unknown
NID2	NM_007361.1	Nidogen 2	3.22	Basement membrane protein/cell adhesion
HSPA1A	NM_005345.4	Heat shock 70 kDa protein 1A	3.16	Protein folding*
CLIC3	NM_004669.1	Chloride intracellular channel 3	3.09	Chloride ion transport
HSPA8	P11142	Heat shock 70kD protein 8	3.08	Protein folding*
TIMP3	P35625	Tissue inhibitor of MP3	3	Protease inhibitor*
RUVBL1	Q9Y265	ATP-dependent helicase	2.88	Nuclear protein with ATPase-helicase motifs
CDH22	Q9UJ99	Cadherin-like 2	2.8	Ca2+-dependent cell adhesion
SPRR1B	NM_003125.1	Small proline-rich protein 1B (cornifin)	2.63	Structural component of stratum corneum*
CXCL14	AAH03513.1	Chemokine C-X-C motif ligand 14	2.62	Chemokine/senescence
KRT6B	NM_005555.2	Keratin 6B	2.53	Epidermal differentiation and wound response*
FGFR3	NM_000142.2	Fibroblast growth factor receptor 3	2.52	Receptor for FGF
HAIK1	Q9C075	Intermediate filament protein	2.42	Intermediate filament protein
DDX9	NM_001357.2	DEAD box protein 9	2.42	RNA helicase A
EXO1	AAC69879.1	Exonuclease 1	2.4	Nuclease
FABP5	Q01469	Epidermal fatty acid binding protein	2.36	Transport fatty acids/keratinocyte differentiation*
EPN3	NM_017957.1	Epsin 3	2.35	Involved in wound healing
DEFB1	Q09753	Defensin beta 1	2.19	Host defense*
WDR3	Q9UNX4	WD repeat domain 3	2.34	Unknown
MKI67	P46013	Ki-67 antigen	2.17	Nuclear protein/proliferation*
TUBA6	NM_032704.1	Keratinocyte alpha-tubulin	2.14	Tubulin family member
ID3	CAA01342.1	Inhibitor of DNA binding 3	2.02	Regulation of proliferation
PHB	P35232	Prohibitin	2.09	Suppressor of cell proliferation
BIRC5	O15392	Survivin	1.98	Inhibitor of apoptosis/involved in G2-M transition
CTSC	P53634	Cathepsin C	1.98	Cysteine proteinase
CGREF1	Q99674	Cell growth regulator with EF-hand domain 1	1.97	Negatively regulator of cell proliferation
KREMEN2	NM_172229.1	Kringle containing transmembrane protein 2	1.91	Receptor for Dkk1
GBA	P04062	Beta-glucocerebrosidase	1.77	Hydrolysis of glucosylceramide*
SULT2B1	AAC78554.1	Sulfotransferase 2B1	1.59	Cholesterol sulfonation*
S100A4	P26447	S100 calcium-binding protein A4	1.53	Ca2+-activated signaling protein
S100A7	P31151	S100 calcium-binding protein A7 (psoriasin)	1.53	Ca2+-activated signaling protein*
ANGPTL4	NM_016109.1	ANGPTL4	1.5	Lipid metabolism
EPPK1	P58107	Epiplakin 1	1.49	Cytolinker
SPRR2C	NM_006518.1	Small proline-rich protein (cornifin)	1.49	Structural component of stratum corneum
PPL	O60437	Periplakin	1.42	Component of (hemi)desmosomes
Gene	Genbank #	Description	Fold-Decrease	Function
ACVR1	Q04771	Activin A receptor type 1	−2.23	Receptor for activin
DDIT3	NM_004083.2	DNA damage inducible transcript 3	−2.27	Transcription factor
ZFP36L1	Q07352	Zinc finger protein	−2.35	Tristetraprolin family member
ALDH1A3	P47895	Aldehyde dehydrogenase 6	−2.37	Retinoic acid metabolism
FOXA2	NM_021784.3	Forkhead box A2	−2.55	Transcription factor
HIPK2	AAL37371.1	Homeodomain interacting protein kinase 2	−2.58	Signaling

Gene	Genbank #	Description	Fold-increase	Function
FZD10	Q9ULW2	Frizzled-10	−2.6	Receptor for Wnt
SERPINE1	P05121	Plasminogen activator inhibitor 1	−2.77	Protease
VEGFC	P49767	Vascular endothelial growth factor C	−2.79	Angiogenesis
NDRG1	Q92597	N-myc downstream regulated gene 1	−2.79	Proliferation
PCK2	Q16822	Phosphoenolpyruvate carboxykinase 2	−3.06	Gluconeogenesis
HSPA5	P11021	Heat shock 70kD protein 5	−3.07	Protein folding
JAG1	NM_000214.1	Jagged 1	−3.32	Receptor for notch
CDKN1A	P38936	Cyclin-dependent kinase inhibitor 1A (p21)	−3.33	Cell cycle control
NR2F1	NM_005654.2	Nuclear receptor COUPTF1	−3.35	Transcription factor
TNFRSF6B	NM_032957.1	TNF receptor superfamily member 6b	−3.38	Receptor for TNF
TRA1	P14625	Tumor rejection antigen 1	−3.69	Chaperone/protein folding
NUCB2	P80303	Nucleobindin 2	−3.78	Ca2+-binding protein
MMP1	P03956	Matrix metalloproteinase 1	−3.97	Protease
LAMB3	Q13751	Laminin beta 3	−4.52	Extracellular matrix
HNT	Q9P121	Neurotrimin	−5.02	Adhesion
PLAB	Q99988	Macrophage inhibitory cytokine 1	−5.18	Apoptosis/cancer
INHBA	P08476	Inhibin beta A (activin A)	−11.48	Proliferation/differentiation