microRNA-31/factor-inhibiting hypoxia-inducible factor 1 nexus regulates keratinocyte differentiation

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Notch plays a critical role in the transition from proliferation to differentiation in the epidermis and corneal epithelium. Furthermore, aberrant Notch signaling is a feature of diseases like psoriasis, eczema, nonmelanoma skin cancer, and melanoma where differentiation and proliferation are impaired. Whereas much is known about the downstream events following Notch signaling, factors responsible for negatively regulating Notch receptor signaling after ligand activation are incompletely understood. Notch can undergo hydroxylation by factor-inhibiting hypoxia-inducible factor 1 (FIH-1); however, the biological significance of this phenomenon is unclear. Here we show that FIH-1 expression is upregulated in diseased epidermis and corneal epithelium. Elevating FIH-1 levels in primary human epidermal keratinocytes (HEKs) and human corneal epithelial keratinocytes (HCEKs) impairs differentiation in submerged cultures and in a "three-dimensional" organotypic raft model of human epidermis, in part, via a coordinate decrease in Notch signaling. Knockdown of FIH-1 enhances keratinocyte differentiation. Loss of FIH-1 in vivo increased Notch activity in the limbal epithelium, resulting in a more differentiated phenotype. microRNA-31 (miR-31) is an endogenous negative regulator of FIH-1 expression that results in keratinocyte differentiation, mediated by Notch activation. Ectopically expressing miR-31 in an undifferentiated corneal epithelial cell line promotes differentiation and recapitulates a corneal epithelium in a three-dimensional raft culture model. Our results define a previously unknown mechanism for keratinocyte fate decisions where Notch signaling potential is, in part, controlled through a miR-31/FIH-1 nexus.

A major function of stratified squamous epithelia such as the epidermis and corneal epithelium is to provide protection from the external environment. Such protection is achieved through an elaborate program of differentiation culminating in the formation of an outermost layer of specialized cells. A crucial juncture in this process is the transition of proliferating keratinocytes, which maintain the steady-state nature of these self-renewing epithelia, to postmitotic differentiated cells. How the balance between proliferation and differentiation is regulated is a major theme of keratinocyte biology.

Notch signaling is one of the key determinants that functions in epithelial growth/differentiation control (1–3) (and references therein). In Notch signaling, the Notch receptor undergoes a series of activation steps upon ligand binding (e.g., Delta-like and Jagged) culminating in the liberation of the Notch intracellular domain (ICD) from the plasma membrane. Notch ICD enters the nucleus where it becomes transcriptionally active (4–7). Whereas much is known about the downstream events following Notch signaling, factors regulating Notch receptor activation are incompletely understood.

The asparaginyl hydroxylase factor inhibiting HIF-1α (FIH-1) can negatively regulate Notch signaling (8). FIH-1 was originally identified as a protein that interacts with and inhibits the activity of HIF-1α in the C-terminal transactivation domain (CAD) (9) by coupling the oxidative decarboxylation of 2-oxoglutarate to the hydroxylation of HIF-1α (8). Significantly, proteins containing the ankyrin repeat domain (ARD) such as Notch are other substrates for FIH-1 (8). Moreover, the binding affinity of FIH-1 for Notch 1 is appreciably greater than for HIF-1α (8, 10). With respect to Notch 1, FIH-1 hydroxylates the Notch ICD at two asparagine residues (N^{1945} and N^{2012}) (11). This has been shown to negatively regulate Notch activity in a myogenic cell line (10). Notch normally functions to maintain an undifferentiated state in this cell line, thus FIH-1's affect was to accelerate differentiation (10). Other than this study, the biological significance of the interaction of FIH-1 and Notch is unknown. Furthermore, only recently has FIH-1 been recognized to play a role in regulating stratified squamous epithelia. For example, we have recently demonstrated that FIH-1 negatively regulates corneal epithelial glycogen metabolism in a HIF-1α–independent manner, which is a unique function for this hydroxylase (12).

Here we show that FIH-1 expression is minimal in normal epidermis and corneal epithelium but markedly increased in the epidermis of patients with psoriasis and in the corneal epithelium from patients with diabetic keratopathies. We found that increasing FIH-1 in primary human epidermal keratinocytes (HEKs) and in a "three-dimensional" organotypic raft model of human epidermis impairs differentiation via a coordinate decrease in Notch signaling. In contrast, loss of FIH-1 promotes keratinocyte differentiation. Confirming these in vitro findings: (*i*) an increase in expression of Hes 1 and Hey 2, markers of Notch activity (3, 13); (*ii*) a decrease in expression of p63 and K15, markers of undifferentiated limbal keratinocytes (14, 15); and (*iii*) an increase in PAI-2, a marker of keratinocyte differentiation (16), were observed in the limbal epithelium of mice null for FIH-1. We identify an endogenous mechanism for keratinocytes to regulate FIH-1 levels via microRNA-31 (miR-31). Antagomir-based suppression of miR-31 in human corneal epithelial keratinocytes (HCEKs) increases FIH-1 protein, decreases Notch activity, and diminishes differentiation. Ectopically expressing miR-31 in an undifferentiated corneal epithelial cell line promotes differentiation and more importantly, recapitulates a corneal epithelium in a three-dimensional raft culture model. Together these data identify FIH-1 as a negative regulator of epithelial differentiation.

Results and Discussion

FIH-1 Expression Is Elevated in Conditions of Abnormal Epithelial Differentiation. Skin diseases such as psoriasis and basal cell and squamous cell carcinomas have been linked to abnormal Notch

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signaling (17, 18); however, the mechanism(s) through which Notch participates in the etiology of these diseases is not well understood. Given the association of FIH-1 with Notch (10, 11) and previous work demonstrating that Notch plays a positive role in regulating epithelial differentiation (for reviews see refs. 2, 3), we investigated whether FIH-1 expression in psoriasis differed from that of normal epidermis in a manner that was inversely associated with Notch activity. When surveying tissue from patients with psoriasis, we observed: (*i*) a marked increase in FIH-1 expression (Fig. 1*B*) and (*ii*) a marked decrease in Hey 2-stained cells (Figs. 1 *D* and *I*) compared with normal skin (Fig. 1 *A*, *C*, and *I*). Because the corneal epithelium is frequently the target of patients with diabetic complications (19), we surveyed the corneas from patients with diabetic keratopathies and observed a similar reciprocal relationship between FIH-1 and Hey 2 expression, where FIH-1 was increased (Fig. 1*F*) and Hey 2 expression was decreased (Fig. 1 *H* and *J*) in the corneal epithelium from these patients compared with normal corneal epithelium (Fig. 1 *E*, *G*, and *J*). Furthermore, keratin 3 (K3) expression, a marker of corneal epithelial differentiation (20), was also decreased in these patients (Fig. 1*H*). Collectively, these observations indicate that FIH-1 expression is correlated with Notch activity and associated with impaired epidermal and corneal epithelial differentiation.

FIH-1 Impairs Keratinocyte Differentiation, in Part, by Attenuating Notch Signaling. To investigate whether FIH-1 can impair epidermal differentiation, we sustained expression of FIH-1 by retroviral delivery of a FIH-1 ORF (FIH-1-coding DNA sequence; FIH-1–cds) construct to submerged cultures of HEKs. Such cells were switched to high calcium, which is a well-established means of inducing stratification and differentiation in human keratinocytes (21). FIH-1–expressing HEKs elaborated minimal amounts of differentiation markers (K10, desmoglein 1, and loricrin) (22) compared with the control transduced HEKs (Fig. 2*A*). To investigate this FIH-1 attenuation in HEK differentiation and increase in proliferation in a more physiologically relevant manner, we used a three-dimensional organotypic raft model of human epidermis (23). FIH-1 was detected in the nucleus and cytoplasm of cells in all but the uppermost cornified layer of raft cultures generated from FIH-1–transduced HEKs (Fig. S1 *A* and *B*). The FIH-1–transduced HEKs gave rise to rafts that were morphologically and biochemically distinct (Fig. 2 *B*, *D*, *F*, and *H* and Fig. S1*F*) from control-transduced HEKs (Fig. 2 *B*, *C*, *E*, and *G* and Fig. S1*F*). For example, rafts generated from the FIH-1–transduced HEKs, harvested at day 3 (Fig. 2*B*), were biochemically similar to submerged cultures (Fig. 2*A*) with little expression of K10, desmoglein 1, and loricrin. Rafts harvested at days 7 and 12 (Fig. 2*B*) from the FIH-1–transduced HEKs were less differentiated although not as dramatically as the FIH-1–transduced HEKs seen at day 3 (Fig. 2*B*) and in submerged HEKs (Fig. 2*A*). This is most likely due to the complexity of the raft system and more closely reflects diseased states where K10/desmoglein 1 are reduced but still abundantly expressed (24). Morphologically, basal cells in the rafts derived from the FIH-1–transduced HEKs (Fig. 2*D*) contained larger nuclei with multiple nucleoli, and numerous nucleolar organizer regions (NORs) (Fig. 2*D*, *Inset*) compared with basal cells in control rafts (Fig. 2*C*). A greater frequency of mitotic figures and $BrdU^+$ stained cells were observed in the FIH-1–transduced HEK-derived rafts at days 3, 7 (Fig. S1 *C* and *D*), and 12 (Fig. 2 *D* and *F* and Fig. S1 *C* and *D*) compared with control rafts (Fig. 2 *C* and *E* and Fig. S1 *C* and *D*). This more rapidly proliferating phenotype is consistent with the less differentiated state of the raft culture. Interestingly, submerged cultures of FIH-1–expressing HCEKs more rapidly proliferated compared with control transduced HCEKs (Fig. S2). Recently, FIH was shown to enhance in vitro proliferation of LS174 colon adenocarcinoma and A375 melanoma cells (25). Our work shows a unique function for FIH-1 in the positive regulation of keratinocyte proliferation.

Immunohistochemical analysis of the developing rafts (Fig. S1*F*) showed close agreement with the immuoblotting data (Fig. 2*B*). A marked decreased in immunostaining for keratin 10, desmoglein 1, and loricrin were noted in the FIH-1–transduced HEK-derived rafts throughout their development (Fig. S1*F*). However, the spatial expression patterns of these products were not altered compared with the control rafts.

In agreement with the in vivo data on the psoriatic epidermis and the diabetic corneal epithelium (Fig. 1), a correlation between FIH-1 expression and Notch activity was observed in the raft cultures with a decrease in Notch ICD-stained nuclei observed in FIH-1–overexpressing rafts (Fig. 2*H*) compared with control rafts (Fig. 2*G* and Fig. S1*E*). Taken together these data support the idea that our organotypic raft setting reflects the in vivo situation and that FIH-1 limits keratinocyte differentiation.

To address further whether the FIH-1 restriction in the differentiation potential of keratinocytes involved Notch signaling, we assessed Notch downstream target genes and the ability of the Notch ICD to rescue keratinocyte differentiation. In these studies, we used submerged cultures of HEKs and HCEKs, which elaborate a set of differentiation products unique to the corneal epithelium. Keratinocytes were transduced with the FIH-1–cds. We observed decreases in the expression of Hey 2 (Fig. 2*I*) and Notch transcriptional activity (Fig. 2*J*). In concert with this decrease in Notch activity, FIH-1–expressing HEKs failed to increase the expression of differentiation markers K10 and desmoglein 1 in response to a calcium switch (Fig. S3*A*). FIH-1–expressing HCEKs (Fig. S3*B*) did not express K3 and mucin 1 (Muc1), which are markers of corneal epithelial differentiation (20, 26). To confirm that FIH-1–induced impairment of differentiation involved Notch signaling, we transduced the FIH-1–expressing HEKs and HCEKs with an adenovirus encoding Notch 1 ICD. Such treatment likely enabled the excess Notch ICD to escape hydroxylation by FIH-1, which allowed translocation of Notch ICD to the nucleus. This restored the ability of the FIH-1–expressing cells to differentiate in response to a calcium switch as evidenced by the increased expression of differentiation markers (Fig. 2 *K* and *L* and Fig. S3 *C*–*F*). Having observed attenuation in keratinocyte differentiation resulting from increased levels of FIH-1, we reasoned that silencing FIH-1 in keratinocytes grown in low calcium should mimic a calcium switch as evidenced by the expression of differentiation markers.

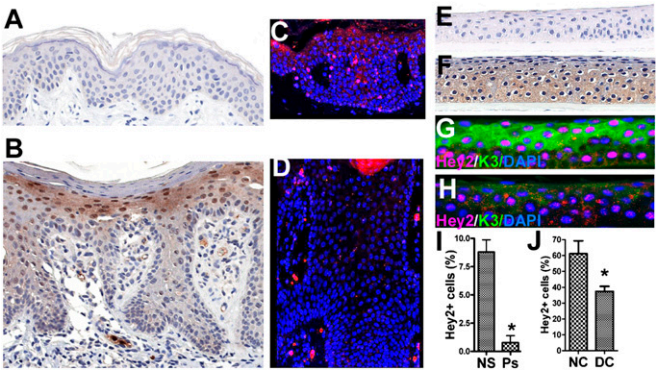

Fig. 1. FIH-1 expression is elevated in conditions of abnormal epithelial differentiation. (*A*–*H*) Paraffin sections of human epidermis (*A*–*D*) and corneal epithelium (*E*–*H*) immunostained with an antibody that recognizes FIH-1 (*A*, *B*, *E*, and *F*), Hey 2 (*C*, *D*, *G*, and *H*), and K3 (*G* and *H*). (*I*) Quantification of Hey 2 staining in psoriatic epidermis. (*J*) Diabetic corneal epithelium. Values are means ± SD $*P < 0.01$.

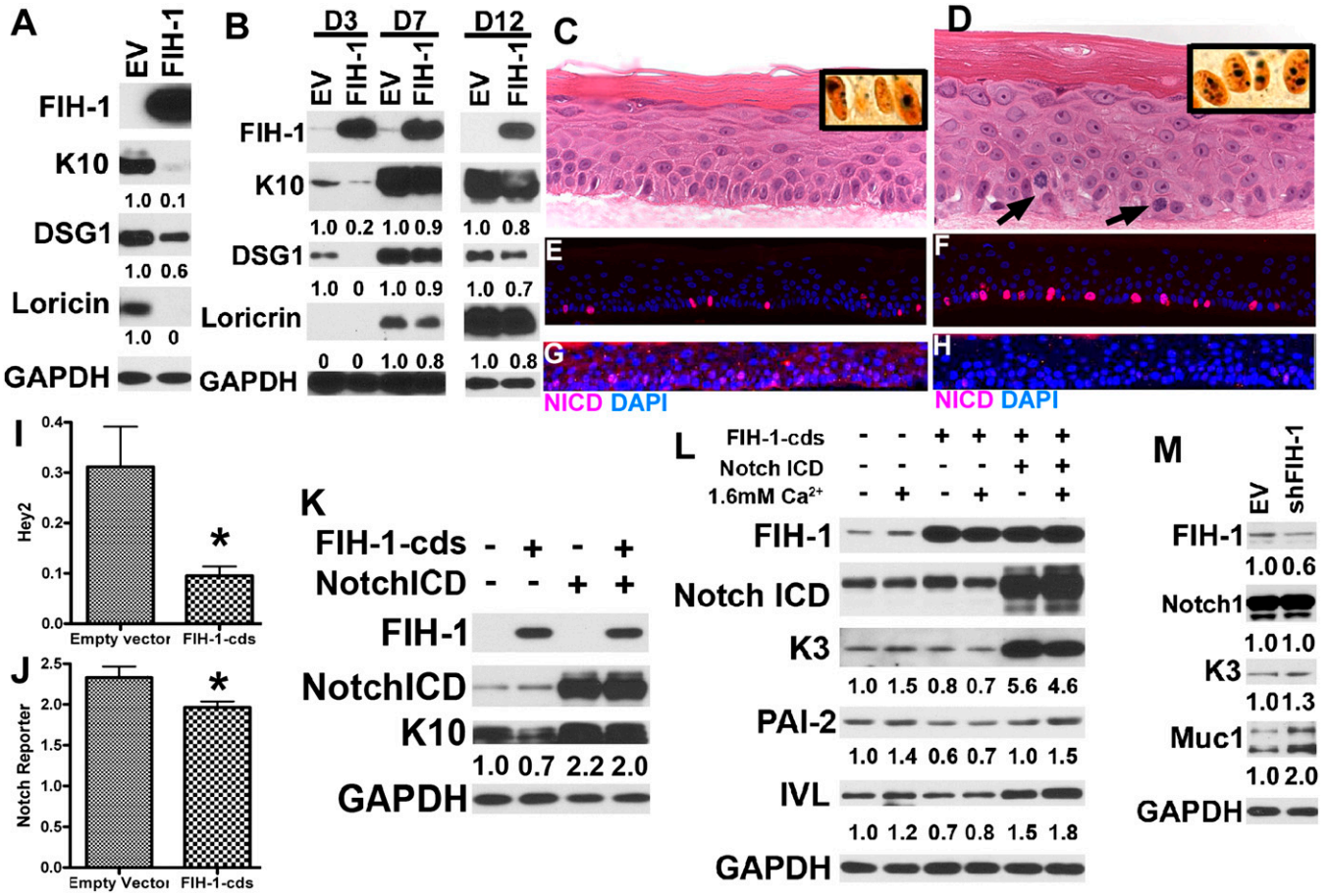

Fig. 2. FIH-1 impairs keratinocyte differentiation, in part, by attenuating Notch signaling. (*A*) Western blot analysis of K10, desmoglein 1 (DSG1), loricrin, and glyceraldeyhyde-3-phosphate dehydrogenase (GAPDH) in submerged cultures of primary human epidermal keratinocytes (HEKs) that were transduced with an empty vector (EV; control) or FIH-1–cds. HEKs were maintained in high calcium to induce differentiation. (*B*) Western blot analysis of K10, DSG1, loricrin, and GAPDH in 3D organotypic raft cultures derived from HEKs retrovirally transduced with an empty vector (EV; control) or FIH-1–cds. Raft cultures were harvested at days 3, 7, and 12. (*C* and *D*) H&E and silver-stained nucleolar organizing region (*Inset*) analyses of 12 d, 3D organotypic raft cultures derived from HEKs transduced with an empty vector (control; *C*) or FIH-1–cds (*D*). Arrows point to mitotic figures present in FIH-1–cds transduced rafts (*D*). (*E* and *F*) Bromodeoxyuridine (BrdU) analysis of 12 d, 3D organotypic raft cultures derived from HEKs transduced with an empty vector (control; *E*) or FIH-1–cds (*F*) showing cells (red stain) in the S phase of DNA synthesis. DAPI (blue) is used as a counterstain. (*G*) Notch intracellular domain (ICD) immunostaining of 12 d, 3D organotypic raft cultures derived from HEKs transduced with an empty vector (control; *G*) or FIH-1–cds (*H*). (*I*) Real time qPCR analysis of Hey 2 levels in HEKs that were retrovirally transduced by either FIH-1–cds or empty vector LZRS in low calcium. Values are means ± SD of three independent experiments. $*P < 0.05$. (*J*) Luciferase assay of Notch activity reporter-transduced HEKs, ectopically expressing either FIH-1 or empty vector. HEKs were maintained in low calcium. Values are means ± SD of three independent experiments. $*P < 0.05$. (*K*). Western blot analysis of FIH-1, Notch ICD, keratin 10 (K10), and GAPDH in submerged cultures of HEKs transduced with an empty vector (control) or FIH-1–cds. HEKs were subsequently transfected with an adeno-Notch ICD in high calcium. (*L*) Western blot analysis of FIH-1, Notch ICD, keratin 3 (K3), plasminogen activator inhibitor-type 2 (PAI-2), involucrin (IVL), and GAPDH in submerged cultures of HCEKs transduced with an empty vector (EV; control) or FIH-1–cds. HCEKs were subsequently transfected with an adeno-Notch ICD and switched to high calcium. (*M*) Western blot analysis of FIH-1, Notch 1, K3, Muc1, and GAPDH in submerged cultures of HCEKs maintained in low calcium and transduced with an empty vector control or a shRNA–FIH-1 (shFIH-1) construct. Numbers below the panels represent the normalized expression signal of proteins.

HCEKs were transduced with a shFIH-1 for 4 d and a significant knockdown of FIH-1 was achieved along with an increase in K3 and Muc1 proteins compared with a control shRNA (Fig. 2*M* and Fig. S3 *G–I*). Collectively, these findings establish that FIH-1 impairs keratinocyte differentiation, in part, by attenuating Notch signaling.

To extend these in vitro findings, we investigated the phenotype of mice with a null mutation in the FIH gene (27), focusing on the limbal epithelium. The limbal epithelium, which is the preferential site of the corneal epithelial stem cells (20, 28), interestingly has high levels of FIH-1 expression compared with the corneal epithelium (12). In concert with a lack of FIH-1 staining in the limbal epithelium (Fig. 3), we observed: (*i*) an increase in Hes 1 staining, (*ii*) an increase in *Hey 2* mRNA (Fig. S4*A*), (*iii*) a decrease in p63 expression, (*iv*) a decrease in K15 staining, and (*v*) an increase in plasminogen inhibitor-type 2 (PAI-2) staining. These in vivo results confirm our in vitro findings that FIH-1 attenuates Notch signaling and negatively regulates keratinocyte differentiation.

miR-31 Targets FIH-1 to Positively Regulate Differentiation, in Part, via Notch Signaling. We have recently demonstrated that miR-31 regulates FIH-1 in corneal epithelial keratinocytes to maintain glycogen metabolism (12). To investigate the relevance of this miR-31/FIH-1 relationship to keratinocyte differentiation, we altered miR-31 levels in submerged cultures of HCEKs (Fig. 4*A*). HCEKs were cultured under low and high calcium conditions and treated with either Antago-31 or an irrelevant antagomir. The expression of FIH-1 and markers of corneal epithelial differentiation were monitored. Antago-31 treatment markedly increased FIH-1 protein in HCEKs (Fig. 4*A*).

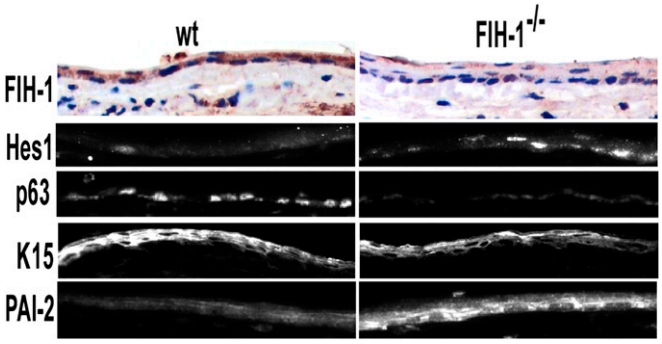

Fig. 3. FIH-1 alters limbal epithelial Notch signaling and differentiation in vivo. Paraffin sections of limbal epithelium from wild-type (WT) and FIH-1 null (FIH-$1^{-/-}$) mice were immunostained with antibodies against FIH-1, Hes 1, keratin 15 (K15), and PAI-2. Frozen sections of limbal epithelium from WT and FIH-1 null (FIH-$1^{-/-}$) mice were immunostained with an antibody against p63.

This resulted in the inability of these cells to express K3 and Muc1 following a calcium switch (Fig. 4*A*). Treating HCEKs with Antago-31 to increase FIH-1 levels also decreased Notch transcriptional activity (Fig. 4*B*) and *Hey 2* transcription (Fig. 4*C*). We did not see a decrease in Hes 1 (Fig. S4*B*). Collectively these data clearly establish a miR-31/FIH-1 nexus that regulates corneal keratinocyte differentiation, in part, via Notch signaling.

miR-31 Down-Regulates FIH-1 to Enhance Differentiation. Having demonstrated that FIH-1 overexpression can attenuate stratified epithelial differentiation (Fig. 2 *B–H*), we reasoned that overexpression of miR-31 should enhance differentiation. To show this, we took advantage of a telomerase immortalized human corneal epithelial cell line (hTCEpi) (29) that constitutively expressed lower levels of miR-31 (Fig. 5*A*) and had higher levels of FIH-1 than primary cultures of HCEKs (Fig. 5*B*). Consequently, raising calcium levels in this immortalized cell line did not markedly enhance differentiation (Fig. 5*C*), as evidenced by the lack of expression of Muc1 and PAI-2. As predicted, lentiviral transduction of hTCEpi cells with miR-31 (hTCEpi–miR-31) reduced the expression of FIH-1 and increased the expression of Muc1 and PAI-2, 24 h after a switch to high calcium (Fig. 5*C*). To see whether we could recapitulate a corneal epithelium, we created three-dimensional organotypic rafts from the hTCEpi–miR-31 cells. Not surprisingly, such rafts expressed lower levels of FIH-1 (Fig. 5*H*) and were more differentiated than controls (hTCEpi-C; Fig. 5 *D*, *F*, and *H*) as evidenced by increased expression of involucrin (IVL) and PAI-2 (Fig. 5*H*) as well as greater numbers of K3- and PAI-2–stained keratinocytes (Fig. 5 *E* and *G*). These results clearly indicate that modulation of miR-31/FIH-1 in this cell line, in essence created a corneal epithelium. To further establish the relationship between miR-31/FIH-1 and differentiation, we overexpressed FIH-1 in the miR-31–transduced hTCEpi cells (Fig. S5). In these cultures, FIH-1 was sufficient to inhibit differentiation induced by miR-31, supporting the idea that FIH-1's effect on differentiation is downstream of miR-31. To confirm the Notch–miR-31 connection in corneal keratinocyte differentiation, we conducted a Notch loss-of-function experiment using DAPT to inhibit Notch signaling (30). miR-31 treatment increased expression of PAI-2, involucrin, and Muc1, whereas DAPT treatment in the miR-31–transduced cells inhibited their expression (Fig. 5*I*). This is strong evidence that miR-31's prodifferentiation effect is dependent on Notch activity.

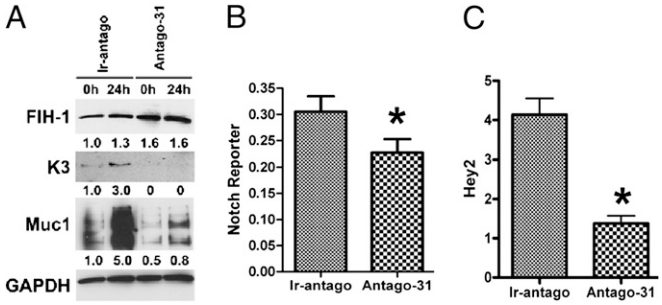

Fig. 4. miR-31 targets FIH-1 to positively regulate differentiation, in part, via Notch signaling. (*A*) Western blot analysis of FIH-1, K3, mucin 1 (Muc1), and GAPDH in submerged cultures of HCEKs grown to confluence in low calcium and treated with an irrelevant antagomir (Ir-antago) or an antagomir to miR-31 (Antago-31) for 48 h and then switched to high calcium. Numbers below the panels represent the normalized expression signal of proteins. (*B*) Luciferase assay of Notch activity reporter-transduced HCEKs, treated with either antago-31 or an Ir-antago. HCEKs were maintained in low calcium. Values are means ± SD of three independent experiments. *$P <$ 0.05. (*C*) Real-time qPCR analysis of Hey 2 levels in HCEKs grown to confluence in low calcium and treated with an Ir-antago or an Antago-31 for 48 h. Values are means ± SD of three independent experiments. *$P < 0.01$.

miR-31/FIH-1 Nexus Contributes to Corneal Epithelial Morphogenesis. This study reveals an unrecognized role for FIH-1 in regulating keratinocyte fate decisions. In the epidermis, miR-31 is primarily expressed in the growing (anagen) hair follicles in mice (31). Interestingly, expression profiling indicated that with respect to the ocular anterior segmental epithelium, miR-31 is a corneal epithelial-preferred miRNA (32). As described previously, FIH-1 protein is abundantly detected in human limbal epithelium but not in human corneal epithelium (12). We propose that low levels of miR-31 and correspondingly high levels of FIH-1, which attenuates Notch signaling, may be one mechanism that maintains the relatively undifferentiated phenotype characteristic of limbal epithelial basal cells (20). Accordingly, the preferential expression of miR-31 in corneal epithelium may, in part, function to regulate the transition from the less differentiated limbal basal cells to the more differentiated corneal epithelial basal cells. It should be noted that other mechanisms may also be involved in mediating the potent roles that FIH-1 plays in keratinocyte proliferation and differentiation.

Materials and Methods

Cell culture conditions, primers, plasmids, antibodies, and other standard methods are described in detail in *SI Materials and Methods*.

Tissues. Blocks of paraffin-embedded specimens from seven (7) individuals with psoriasis and five (5) individuals with diabetic keratopathies were obtained from the Dermatopathology and Ophthalmic Pathology archival tissue repositories of deidentified patients seen at Northwestern University under approval of Northwestern University Institutional Review Board (IRB) and in accordance with the Declaration of Helsinki principles. Tissue sections were diagnosed by dermatopathologists and ophthalmic pathologists. Normal human skin was obtained prospectively from three (3) volunteers under approval of the IRB and in accordance with the Declaration of Helsinki principles. All volunteers gave informed consent for use of their tissues. Normal human corneas ($n = 50$) were obtained from the Midwest Eye Banks (Ann Arbor, MI).

FIH-1 null mice. The FIH-1 null mice were generated by breeding FIH^{flox} mouse with the Ella-Cre transgenic mouse (27). Animal experiments were approved by the Northwestern University Animal Care and Use Committee (NUACUC).

Constructs and oligonucleotides. A cDNA encoding miR-31–resistant FIH-1 (FIH-1–cds) was ligated between BamHI and XhoI sites of the retroviral expression plasmid LZRS. The following constructs were used in this study: Human premicroRNA Expression Construct Lenti-miR-31 (System Biosciences I) and FIH-1 shRNA expression lentiviral vector pGIPZ–shRNA–FIH-1 (Open Biosystems). Antagomirs were synthesized by Dharmacon. Sequences were 5′-$mA_smG_smC_smUmAmUmGmCmCmAmGmCmAmUmCmUmUmG_smC_smC_smU$-Chol 3′ (Antago-31), 5′-$mG_smG_smC_smAmUmUmCmAmCmGmCmGmUm$-$GmCmC_smU_smU_smA$-Chol 3′ (irrelevant-antago). "mN" represents 2′-*O*-methyl-modified oligonucleotide, the subscript "s" represents a phosphorothioate linkage, and "Chol" represents linked cholesterol.

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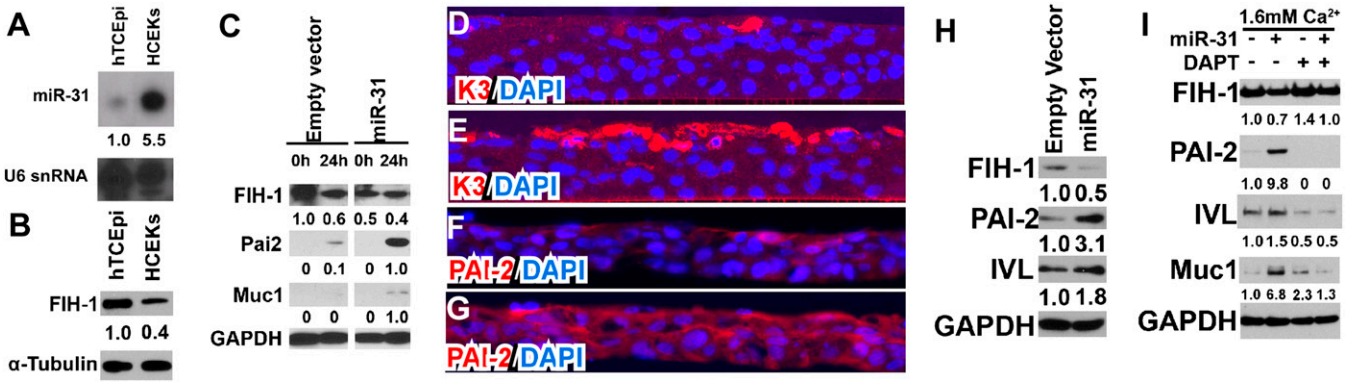

Fig. 5. miR-31 down-regulates FIH-1 to enhance differentiation. (*A*) Northern blot analysis of miR-31 and U6 in submerged cultures of a human limbal-derived cell line (hTCEpi) and primary human corneal epithelial keratinocytes (HCEKs). (*B*) Western blot analysis of FIH-1 and α-tubulin in submerged cultures of hTCEpi cells and HCEKs. (*C*) Western blot analysis of FIH-1, PAI-2, Muc1, and GAPDH in submerged cultures of hTCEpi cells transduced with a lenti–miR-31 or empty vector in low (0.15 mM) calcium. Cells were plated to confluence and then switched to high (1.6 mM) calcium. (*D* and *E*) Immunostaining for K3 (red-stained cells) in three-dimensional organotypic raft cultures derived from hTCEpi cells transduced with a lenti–miR-31 (*E*) or empty vector (*D*). (*F* and *G*) Immunostaining for PAI-2 (red-stained cells) in three-dimensional organotypic raft cultures derived from hTCEpi cells transduced with a lenti–miR-31 (*G*) or empty vector (*F*). DAPI (blue) is used as a counterstain to mark the nuclei. (*H*) Western blot analysis of FIH-1, PAI-2, involucrin, and GAPDH in 3D organotypic raft cultures derived from hTCEpi cells transduced with a lenti–miR-31 or empty vector. (*I*) Western blot analysis of FIH-1, PAI-2, IVL, Muc1, and GAPDH in the empty vector or miR-31–transduced hTCEpi cells maintained in calcium (1.6 mM) for 48 h and treated with or without DAPT (20 μM). Numbers below the panels represent the normalized expression signal of proteins and RNAs.

Three-dimensional organotypic raft cultures. Raft cultures of HEKs were produced by the Northwestern University Skin Disease Research Center's keratinocyte core facility. Briefly, HEKs were grown to confluence in E-medium [DMEM/F12, 5% (vol/vol) FBS] with EGF (5 ng/mL) and switched to an air-medium interface for 3, 7, and 12 d (23). The raft cultures of hTCEpi cells were derived as previously described (33). The hTCEpi cells were grown in a duplex feeder system to confluence in corneal raft medium [DMEM/F12, 10% (vol/vol) FBS, 10 ng/mL EGF (Millipore), 5 mg/mL human recombinant insulin, 500 ng/mL hydrocortisone, 5 mg/mL human Apo-transferrin, 250 ng/mL isoproterenol hydrochloride (Sigma), and antibiotics] and then switched to an air-medium interface for 12 d. Duplicate 3D organotypic raft cultures were prepared for histological and biochemical analysis. All raft experiments were replicated at least three times.

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1. Djalilian AR, et al. (2008) Down-regulation of Notch signaling during corneal epithelial proliferation. *Mol Vis* 14:1041–1049.
2. Dotto GP (2008) Notch tumor suppressor function. *Oncogene* 27:5115–5123.
3. Watt FM, Estrach S, Ambler CA (2008) Epidermal Notch signalling: Differentiation, cancer and adhesion. *Curr Opin Cell Biol* 20:171–179.
4. Blanpain C, Lowry WE, Pasolli HA, Fuchs E (2006) Canonical notch signaling functions as a commitment switch in the epidermal lineage. *Genes Dev* 20:3022–3035.
5. Fortini ME (2009) Notch signaling: The core pathway and its posttranslational regulation. *Dev Cell* 16:633–647.
6. Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: Unfolding the activation mechanism. *Cell* 137:216–233.
7. Nicolas M, et al. (2003) Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* 33:416–421.
8. Cockman ME, Webb JD, Kramer HB, Kessler BM, Ratcliffe PJ (2009) Proteomics-based identification of novel factor inhibiting hypoxia-inducible factor (FIH) substrates indicates widespread asparaginyl hydroxylation of ankyrin repeat domain-containing proteins. *Mol Cell Proteomics* 8:535–546.
9. Mahon PC, Hirota K, Semenza GL (2001) FIH-1: A novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 15:2675–2686.
10. Zheng X, et al. (2008) Interaction with factor inhibiting HIF-1 defines an additional mode of cross-coupling between the Notch and hypoxia signaling pathways. *Proc Natl Acad Sci USA* 105:3368–3373.
11. Coleman ML, et al. (2007) Asparaginyl hydroxylation of the Notch ankyrin repeat domain by factor inhibiting hypoxia-inducible factor. *J Biol Chem* 282:24027–24038.
12. Peng H, et al. (2012) MicroRNA-31 targets FIH-1 to positively regulate corneal epithelial glycogen metabolism. *FASEB J* 26:3140–3147.
13. Jarriault S, et al. (1995) Signalling downstream of activated mammalian Notch. *Nature* 377:355–358.
14. Pellegrini G, et al. (2001) p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 98:3156–3161.
15. Yoshida S, et al. (2006) Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* 47:4780–4786.
16. Williams DL, et al. (1999) Plasminogen activator inhibitor type 2 in human corneal epithelium. *Invest Ophthalmol Vis Sci* 40:1669–1675.
17. Lefort K, et al. (2007) Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. *Genes Dev* 21:562–577.
18. Thélu J, Rossio P, Favier B (2002) Notch signalling is linked to epidermal cell differentiation level in basal cell carcinoma, psoriasis and wound healing. *BMC Dermatol* 2:7.
19. Kaji Y (2005) Prevention of diabetic keratopathy. *Br J Ophthalmol* 89:254–255.
20. Schermer A, Galvin S, Sun T-T (1986) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 103:49–62.
21. Green H (1977) Terminal differentiation of cultured human epidermal cells. *Cell* 11: 405–416.
22. Fuchs E, Byrne C (1994) The epidermis: Rising to the surface. *Curr Opin Genet Dev* 4: 725–736.
23. Getsios S, et al. (2009) Desmoglein 1-dependent suppression of EGFR signaling promotes epidermal differentiation and morphogenesis. *J Cell Biol* 185:1243–1258.
24. Wan H, et al. (2004) Striate palmoplantar keratoderma arising from desmoplakin and desmoglein 1 mutations is associated with contrasting perturbations of desmosomes and the keratin filament network. *Br J Dermatol* 150:878–891.
25. Pelletier J, et al. (2012) The asparaginyl hydroxylase factor-inhibiting HIF is essential for tumor growth through suppression of the p53-p21 axis. *Oncogene* 31:2989–3001.
26. Govindarajan B, Gipson IK (2010) Membrane-tethered mucins have multiple functions on the ocular surface. *Exp Eye Res* 90:655–663.
27. Zhang N, et al. (2010) The asparaginyl hydroxylase factor inhibiting HIF-1alpha is an essential regulator of metabolism. *Cell Metab* 11:364–378.
28. Cotsarelis G, Cheng SZ, Dong G, Sun T-T, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: Implications on epithelial stem cells. *Cell* 57:201–209.
29. Robertson DM, et al. (2005) Characterization of growth and differentiation in a telomerase-immortalized human corneal epithelial cell line. *Invest Ophthalmol Vis Sci* 46:470–478.
30. Kolev V, et al. (2008) EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. *Nat Cell Biol* 10:902–911.
31. Mardaryev AN, et al. (2010) Micro-RNA-31 controls hair cycle-associated changes in gene expression programs of the skin and hair follicle. *FASEB J* 24:3869–3881.
32. Ryan DG, Oliveira-Fernandes M, Lavker RM (2006) MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis* 12:1175–1184.
33. Miyashita H, et al. (2008) A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations. *Tissue Eng Part A* 14:1275–1282.