Notch and TGF-β pathways cooperatively regulate receptor protein tyrosine phosphatase-κ (PTPRK) gene expression in human primary keratinocytes

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ABSTRACT Receptor protein tyrosine phosphatase-κ (PTPRK) specifically and directly dephosphorylates epidermal growth factor receptor (EGFR), thereby limiting EGFR function in primary human keratinocytes. PTPRK expression is increased by the TGF-β/Smad3 pathway and cell–cell contact. Because the Notch receptor pathway is responsive to cell–cell contact and regulates keratinocyte growth and differentiation, we investigated the interplay between Notch and TGF-β pathways in regulation of PTPRK expression in human keratinocytes. Suppression of Notch signaling by γ-secretase inhibitors substantially reduced cell contact induction of PTPRK gene expression. In sparse keratinocyte cultures, addition of soluble Notch-activating ligand jagged one peptide (Jag1) induced PTPRK. Of interest, cell contact–induced expression of TGF-β1 and TGF-β receptor inhibitor SB431542 inhibited contact-induced expression of PTPRK. Furthermore, inhibition of Notch signaling, via knockdown of Notch1 or by γ-secretase inhibitors, significantly reduced TGF-β–induced PTPRK gene expression, indicating that Notch and TGF-β pathways function together to regulate PTPRK. Of importance, the combination of Jag1 plus TGF-β results in greater PTPRK expression and lower EGFR tyrosine phosphorylation than either ligand alone. These data indicate that Notch and TGF-β act in concert to stimulate induction of PTPRK, which suppresses EGFR activation in human keratinocytes.

INTRODUCTION
The Notch signaling pathway is evolutionarily conserved and known to participate in diverse functions such as cell fate determination, stem cell maintenance, cell proliferation, and apoptosis during both embryogenesis and self-renewal of adult tissues (Artavanis-Tsakonas, 1988; Leong and Karsan, 2006). Mammals have four Notch receptors (Notch1–4) and five Notch ligands–three Delta-like ligands (DLL1, DLL3, and DLL4) and two ligands of the Jagged family (Jag1 and Jag2). Because both Notch receptors and ligands are transmembrane proteins, cell–cell interaction is a prerequisite for Notch signaling. Activation of Notch signaling is initiated by binding of Notch ligand to Notch receptors on adjacent cells. This interaction induces two consecutive proteolytic cleavages by a disintegrin and metalloproteinase (ADAM) family metalloproteinase and a γ-secretase complex, respectively. Consequently, Notch intracellular domain (NICD) is released from the plasma membrane and can enter the nucleus to form a complex with the DNA-binding protein RBP-Jκ and coactivator Mastermind/Mastermind-like to activate transcription of target genes (Bray, 2006; Kopan and Iliagan, 2009).

Keratinocytes form the stratified epithelium of skin epidermis. Keratinocytes proliferate in the lowest (basal) epidermal layer and then undergo maturation as they migrate upward. Terminally differentiated keratinocytes are sloughed off at the surface of the skin. Normal cellular homeostasis of the epidermis requires fine balance between keratinocyte proliferation and differentiation (Watt, 2002).
The epidermal growth factor receptor (EGFR) signaling pathway is a potent regulator of keratinocyte proliferation (Pastore et al., 2008). Ligand binding induces EGFR tyrosine phosphorylation and activation of downstream signaling pathways; EGFR tyrosine phosphorylation is synonymous with receptor activation. Receptor protein tyrosine phosphatase-κ (PTPRK) specifically dephosphorylates EGFR, thereby acting as a major negative regulator of EGFR signaling. Overexpression of PTPRK suppresses both basal and ligand-induced EGFR tyrosine phosphorylation and inhibits cell growth (Xu et al., 2005). Levels of PTPRK correlate with keratinocyte proliferation both in vitro and in vivo (Xu et al., 2005; Xu et al., 2006). In human epidermis, PTPRK expression is lower in basal layer keratinocytes than in nonproliferating keratinocytes in suprabasal layers (Xu et al., 2006). In low-density, highly proliferative keratinocyte cultures, PTPRK expression is lower than with confluent nonproliferative cultures (Xu et al., 2005). The mechanisms by which cell–cell contact up-regulates PTPRK expression is not known.

In addition to cell–cell contact, transforming growth factor-β (TGF-β) is a potent inhibitor of keratinocyte proliferation (Moses et al., 1990). We previously reported that TGF-β induces PTPRK expression, which suppresses EGFR activity in keratinocytes (Xu et al., 2010). Induction of PTPRK by TGF-β requires binding of Smad3 to the proximal promoter region of the PTPRK gene (Xu et al., 2010). Blokzijl et al. (2003) reported physical interaction between the activated form of Notch (NICD) and Smad3. In addition, Notch and TGF-β pathways coordinately regulate expression of target genes such as Hes-1. On the basis of these data, we investigated cross-talk between Notch and TGF-β pathways in regulation of PTPRK gene expression in human primary keratinocytes.

RESULTS
Expression of Notch receptors and ligands in primary human keratinocytes
We initially quantified the relative expression of Notch receptor and ligand family members in human keratinocytes. As shown in Figure 1A, the most abundant Notch receptor is Notch1, followed by Notch2, whereas Notch3 and Notch4 are almost undetectable. The most abundant Notch ligand is Jag1, which is expressed 10-fold higher than Jag2 and DLL1 (Figure 1B). Expression levels of DLL3 and DLL4 are negligible in human primary keratinocytes.

Confluency up-regulates PTPRK and Notch target Hes-1 gene expression in primary human keratinocytes
In tissue culture, proliferation of primary human keratinocytes ceases when cells reach confluency and achieve cell–cell contact, a condition that is required to initiate Notch signaling (Kopan and Ilagan, 2009). Hes-1 is a validated Notch target gene (Bray, 2006; Kopan and Ilagan, 2009) and can serve as an endogenous Notch reporter to reflect Notch pathway activity. We found that Hes-1 mRNA was significantly up-regulated in confluent primary human keratinocytes compared with subconfluent cells (Figure 2A). Like Hes-1, PTPRK expression was also up-regulated by increased cell confluency (Figure 2B).

Involvement of Notch pathway in cell contact–induced PTPRK expression
To investigate the role of Notch signaling in PTPRK gene expression, we inhibited Notch signaling by specific γ-secretase inhibitors. These inhibitors specifically block Notch processing to its transcriptionally active forms (Wolfe, 2009). Treatment of human primary keratinocytes with two different γ-secretase inhibitors significantly reduced confluency-dependent Notch activity as measured by Hes-1 gene expression (Figure 3A). γ-Secretase inhibitors also completely inhibited confluence-dependent increase of PTPRK mRNA (Figure 3B) and protein (Figure 3C). These data suggest that the Notch pathway is involved in increased expression of PTPRK in response to confluency.

Activation of Notch pathway promotes PTPRK expression
We next activated the Notch pathway with a synthetic peptide derived from Notch1 ligand Jag1 (Nickoloff et al., 2002) and then determined PTPRK gene expression. Addition of Jag1 peptide to subconfluent primary human keratinocytes, which mimics engagement of Notch receptor and ligand in cell–cell contact, activated the Notch pathway, as indicated by formation of the Notch intracellular domain (Figure 4A) and up-regulation of Notch target gene Hes-1 in a time-dependent manner (Figure 4B). Concomitantly, Jag1 peptide significantly induced PTPRK mRNA (Figure 4C) and protein (Figure 4D) levels. A scrambled Jag1 peptide had no effect on PTPRK expression (unpublished data).

Inhibition of Notch signaling suppresses TGF-β–induced PTPRK expression
We previously reported that PTPRK transcription is directly regulated by the TGF-β pathway (Xu et al., 2010), and emerging evidence indicates that TGF-β and Notch can act in concert to regulate transcription of target genes (Blokzijl et al., 2003; Samon et al., 2008; Guo and Wang, 2009). Therefore we next investigated the relationship between Notch and TGF-β pathways in the regulation of PTPRK expression. Of interest, we found that increased cell–cell

FIGURE 1: Expression of Notch receptors and ligands in primary human keratinocytes. Total RNA was isolated from cultured primary human keratinocytes, and mRNA for Notch receptors and ligands was quantified by real time reverse transcriptase-PCR (RT-PCR). Housekeeping gene 36B4 mRNA was used as internal control for normalization. Data are means ± SEM; N = 4.
contact induces expression of TGF-β1 by twofold in human keratinocytes (Figure 5A), and SB431542, a TGF-β type I receptor kinase inhibitor, suppresses confluence-induced PTPRK mRNA (Figure 5B) and protein (Figure 5C). In addition, we found that Smad3 and NICD, which mediate the actions of the TGF-β and Notch pathways, respectively, formed a stable complex that coimmunoprecipitated in human primary keratinocytes (Figure 5D).

As shown in Figure 6, γ-secretase inhibitors X and XXI completely blocked TGF-β1–induced Hes-1 (Figure 6A) and PTPRK expression (Figure 6B). Notch1 is the major Notch receptor expressed in primary human keratinocytes (Figure 1A) and appears to be the major functional Notch receptor in skin (Krebs et al., 2000, 2003; Pan et al., 2004). Knockdown of Notch1 by lentivirus-mediated short hairpin RNA (shRNA; Figure 6C) inhibited TGF-β–induced PTPRK expression (Figure 6D). Taken together, these data indicate that up-regulation of PTPRK by TGF-β requires input from the Notch signaling pathway.

**DISCUSSION**

Notch signaling and biological functions are highly cell type and context dependent. In the skin, signaling through Notch receptors regulates differentiation, proliferation, and survival of keratinocytes.
Notch ligand Jag1 induces Notch target Hes-1 gene and PTPRK gene expression in primary human keratinocytes. Subconfluent keratinocytes were treated with 25 μM Jag1 peptide or scrambled Jag1 peptide (Ctrl). (A) One hour after treatments, equal amounts of whole-cell protein lysate were collected and analyzed by Western blot for NICD. β-Actin was used as loading control. Representative Western blot from three independent experiments. (B) After treatment for the indicated times, total RNA was isolated and Hes-1 mRNA was used as internal control for normalization (*p < 0.05). (C) After treatment for the indicated times, equal amounts of whole-cell protein lysates were analyzed by Western blot probed with PTPRK antibody. β-Actin was used as loading control. Immunoreactive bands were quantified by chemiluminescence using a STORM Molecular Imager. Data are means ± SEM, N = 4; *p < 0.05. Inset shows representative Western blots.

In primary human keratinocytes, Notch signaling is activated by cell–cell contact at confluence, a condition in which PTPRK expression is substantially up-regulated (Xu et al., 2005). Notch activation mainly occurs in postmitotic cells in the suprabasal layers of the epidermis (Okuyama et al., 2004), a pattern of expression similar to that of PTPRK in human skin (Xu et al., 2006). PTPRK transcription is directly regulated by TGF-β through binding of Smad3/4 to the proximal promoter region of the PTPRK gene (Xu et al., 2010). Induction of PTPRK by TGF-β, with subsequent reduction of EGFR tyrosine phosphorylation, significantly contributes to growth inhibition of keratinocytes by TGF-β (Xu et al., 2005). Emerging evidence indicates that TGF-β and Notch act in concert to regulate transcription of target genes (Guo and Wang, 2009). For example, TGF-β effector Smad3 and Notch intracellular domain physically interact to coordinately regulate transcription of Hes-1 and Foxp3 (Blokzijl et al., 2003; Samon et al., 2008). In this study, we demonstrate that PTPRK gene expression in human keratinocytes is coordinately regulated by Notch and TGF-β pathways (Figure 8).

We found that γ-secretase inhibition or knockdown of Notch1 reduced TGF-β-induced PTPRK expression (Figure 6). Complete inhibition was seen with blocking γ-secretase activity. However, inhibition with Notch1 knockdown was incomplete. There are several possible reasons for this incomplete inhibition. First, knockdown of Notch1 was not complete; therefore remaining low levels of Notch1 protein could contribute to the transcriptional regulation of PTPRK by TGF-β. Second, Notch2, although expressed at lower levels than Notch1, may partially compensate for Notch1 knockdown. Third, it is possible that other undetermined pathways/proteins may contribute to the residue PTPRK induction after Notch1 knockdown. Cross-talk between different signaling pathways contributes to proper function and homeostasis in eukaryotic cells. Recent studies revealed cross-talk between the Notch signaling pathway and other signaling molecules, including Sonic hedgehog (Shh), β-catenin, and the p53 family member p63 (Lefort and Dotto, 2004). It has been reported that EGFR negatively regulates Notch1 gene expression in primary human keratinocytes (Kolev et al., 2008). Our results indicate that the Notch pathway can also negatively regulate EGFR function via up-regulation of its negative regulator, PTPRK.

Evidence indicates that the Notch pathway may have tumor suppressor functions in skin (Nicolas et al., 2003; Radtke and Raj, 2003). Deletion of Notch1 or overexpression of a dominant-negative inhibitor of downstream effector Mastermind-like 1 in epidermis increases spontaneous squamous cell carcinoma formation (Nicolas et al., 2003; Prowell et al., 2006). In addition, deletion of Notch1 in epidermis increases its susceptibility to developing tumors in response to oncogenic Ras (Nicolas et al., 2003; Lefort et al., 2007).
In mice with conditional epidermal inactivation of Notch1, chemical injury induces cutaneous lesions that resemble both basal cell carcinoma and squamous cell carcinoma (SCC; Nicolas et al., 2003). Impaired Notch signaling has been linked to cutaneous SCC formation (Proweller et al., 2006). Although the prodifferentiation and tumor-suppressive functions of Notch signaling in keratinocytes are well established (Dotto, 2008; Watt et al., 2008), the underlying mechanisms remain to be elucidated.

EGFR signaling has a key role in the positive control of keratinocyte growth potential and carcinogenesis (Kalyankrishna and Grandis, 2006). Keratinocytes depend on EGFR function for proliferation, and elevated levels of PTPRK suppress keratinocyte proliferation by inhibiting EGFR function (Xu et al., 2005). PTPRK has been shown to function as a tumor suppressor in several types of cancer (Flavell et al., 2008; Agarwal et al., 2013; Stevenson et al., 2013; Sun et al., 2013). It is tempting to speculate that tumor suppressor activity of the Notch pathway is linked to its ability to up-regulate expression of PTPRK and thereby limit activation of the EGFR pathway in skin (Figure 8).

Proper epidermal function requires finely tuned balance between keratinocyte proliferation and differentiation. This balance requires complex coordination among multiple signaling pathways. EGFR, Notch, and TGF-β pathways all have been demonstrated to be critical components of epidermal homeostasis (Okuyama et al., 2008; Shirakata, 2010). Our results suggest that PTPRK may serve as a key common element that connects these critical pathways.

**MATERIALS AND METHODS**

**Materials**

Adult human primary epidermal keratinocytes were purchased from Cascade Biologics (Portland, OR). HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA). Jag1 peptide and Jag1 scrambled peptide were purchased from Anaspec (Fremont, CA). γ-Secretase inhibitors X and XXI were purchased from Calbiochem (La Jolla, CA). TGF-β1 and EGF were purchased from R&D Systems (Minneapolis, MN). PTPRK antibody has been described before (Xu et al., 2005). Activated Notch 1 (NICD) antibody and phospho-EGFR (pY1068) antibody were purchased from Cell Signaling Technology (Beverly, MA). Total EGFR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Smad3 antibody was purchased from Abcam (Cambridge, England). NICD adenovirus is a gift from Tatsuya Iso (Gunma University Graduate School of Medicine, Maebashi, Japan).

![FIGURE 5: Involvement of TGF-β pathway in confluence-induced PTPRK expression in primary human keratinocytes. (A) Total RNA was isolated from keratinocytes at different confluency (30–40% confluency for subconfluent and 95–100% for confluent). TGF-β1 mRNA was quantified by real-time RT-PCR analysis. 36B4 mRNA was used as internal control for normalization (N = 3, *p < 0.05). (B) Confluent human primary keratinocytes were treated with TGF-β type I receptor kinase inhibitor SB431542 (10 μM) or vehicle (dimethyl sulfoxide) for 2 d. Total RNA was isolated, and PTPRK and 36B4 (internal control for normalization) mRNA were quantified by real time RT-PCR (N = 4, *p < 0.05). (C) Equal amounts of whole-cell protein lysates were analyzed by Western blot probed with PTPRK antibodies. Immunoreactive bands were quantified by chemiluminescence using a STORM Molecular Imager. Data are means ± SEM; N = 4, *p < 0.05. Inset, representative Western blots (D) Coimmunoprecipitation of NICD and Smad3. Human primary keratinocytes were infected with NICD adenovirus for 2 d and then treated with TGF-β for 1 h; whole-cell lysate were immunoprecipitated with control immunoglobulin G or Smad3 antibody. Cell lysates and immunoprecipitates were subjected to Western blot analysis probed with NICD and Smad3 antibodies as indicated. Representative Western blots from three independent experiments.](image_url)
Cell culture
Adult human primary epidermal keratinocytes were cultured in keratinocyte medium (EpiLife; Cascade Biologics) with human keratinocyte growth supplement (Cascade Biologics) under 5% CO₂ at 37°C. HEK293 cells were cultured in DMEM with 1.5 g/ml sodium bicarbonate supplemented with 10% fetal bovine serum under 5% CO₂ at 37°C.

Lentivirus-mediated shRNA interference knockdown of Notch1 in primary human keratinocytes
The MISSION TurboGFP nontargeting shRNA control vector and shRNA constructs targeting Notch1 (5′-CCGGCGCTGCTGGA-CAAGATCATCTCGAGATTGTCTTGGTCCAGGCGGTTTTT-3′) were purchased from Sigma-Aldrich (St. Louis, MO). Lentivirus was produced in 293FT cells after transfection of the shRNA vectors and helper plasmids using the SuperFect Transfection Reagent as described by the manufacturer (Qiagen, Chatsworth, CA). Two days after transfection, medium from 293FT cells was collected and used to infect human primary keratinocytes to knock down endogenous Notch1.

Real-time reverse transcriptase-PCR
Total cellular RNA was purified using a Miniprep RNA isolation kit according to the manufacturer’s instructions (Qiagen). Reverse transcription of total RNA was carried out using a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a 7300 sequence detector (Applied Biosystems) using the Taqman SybrGreen PCR master mix kit (Applied Biosystems). All liquid-handling procedures were performed with a calibrated robotic workstation (Biomek 2000; Beckman Coulter, Hialeah, FL) to ensure accuracy and reproducibility. Target-gene mRNA levels (number of molecules/10 ng total RNA) were quantified based on a standard curve and normalized to endogenous housekeeping gene 36B4 mRNA levels. Real Time PCR Primers were produced by the Custom Oligonucleotide Synthesis Service (Applied Biosystems). The sequences for real-time PCR primers used in this study are as follows: human PTPRK: forward, 5′-ACA GAG TGG TGA AAA TAG CAG GAA-3′; reverse, 5′-TGA CAA CTG AGA GGA GGA TGA-3′; human Hes-1: forward, 5′-TTG GAG CCT TCC AGG TGG TA-3′; reverse, 5′-GCC CCG TTG GGA ATG AG-3′; human TGF-β1: forward, 5′-TGA CAA GTT CAA GCA GAG TAC ACA CA-3′; reverse, 5′-AGA GCA ACA CGG GTT CAG GTA-3′; and human 36B4 forward, 5′-ATG CAG CAG ATC CGC ATG T-3′; reverse, 5′-TTG CGC ATC ATG GTG TTC TT-3′. The following real-time PCR primers were purchased from Applied Biosystems with ordering information (corresponding assay IDs): human Notch1 (Hs00413187_m1), human Notch2 (Hs00225747_m1), human Notch3 (Hs00166432_m1), human Notch4 (Hs00270200_m1), human JAG1 (Hs00164982_m1), human JAG2 (Hs00171432_m1), human DLL1 (Hs00194509_m1), human DLL3 (Hs00213561_m1), and human DLL4 (Hs00184092_m1).

Notch1 mRNA and 36B4 mRNA (internal control for normalization) were quantified by real-time RT-PCR. N = 3, *p < 0.05. Whole-cell protein lysates were analyzed for Notch1 by Western blot. β-Actin was used as loading control. Inset, representative Western blot of Notch1 protein levels, which correlates with Notch1 mRNA levels.

(D) Keratinocytes were treated with vehicle (Ctrl) or TGF-β1 (2.5 ng/ml) for 24 h. Total RNA was isolated, and PTPRK mRNA and 36B4 (internal control for normalization) mRNA levels were quantified by real-time RT-PCR. N = 5, *p < 0.05 vs. TGF-β1 NT shRNA.

FIGURE 6: Inhibition of Notch signaling suppresses TGF-β1 induction of Notch target Hes-1 and PTPRK gene expression in primary human keratinocytes. (A, B) Subconfluent primary human keratinocytes were treated with γ-secretase inhibitor GSI X (3 μM) or GSI XXI (3 μM) in the presence of vehicle (Ctrl) or TGF-β1 (2.5 ng/ml) for 24 h. Total RNA was isolated and (A) Hes-1 (N = 3, *p < 0.05 TGF-β1 alone vs. TGF-β1 plus GSI X or TGF-β1 plus GSI XXI) and (B) PTPRK (N = 5, *p < 0.05 TGF-β1 alone vs. TGF-β1 plus GSI X or TGF-β1 plus GSI XXI) mRNA levels were quantified by real-time RT-PCR. 36B4 mRNA levels were used as internal control for normalization. (C) Total RNA was isolated, and Keratinocytes were infected with lentiviruses that expressed nontargeting (NT) shRNA or Notch1 targeting shRNA for 48 h. (C) Total RNA was isolated, and

Nor not 1 mRNA and 36B4 mRNA (internal control for normalization) were quantified by real-time RT-PCR. N = 3, *p < 0.05. Whole-cell protein lysates were analyzed for Notch1 by Western blot. β-Actin was used as loading control. Inset, representative Western blot of Notch1 protein levels, which correlates with Notch1 mRNA levels.

(D) Keratinocytes were treated with vehicle (Ctrl) or TGF-β1 (2.5 ng/ml) for 24 h. Total RNA was isolated, and PTPRK mRNA and 36B4 (internal control for normalization) mRNA levels were quantified by real-time RT-PCR. N = 5, *p < 0.05 vs. TGF-β1 NT shRNA.
Human primary keratinocytes were washed twice with ice-cold phosphate-buffered saline, scraped from the dishes in ice-cold whole-cell extraction buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 75 mM NaCl, 2.5 mM MgCl$_2$, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20 mM β-glycerophosphate, 0.1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Equal amounts of whole-cell lysate were subjected to SDS–PAGE and transferred to Immobilon-P filter paper (Millipore, Bedford, MA). Immunoreactive proteins were visualized by enhanced chemifluorescence according to the manufacturer’s protocol (GE Healthcare, Piscataway, NJ).

Western blots. (C) Subconfluent keratinocytes were infected with empty or NICD adenovirus for 24 h and then treated with vehicle (Ctrl) or TGF-β1 (2.5 ng/ml) for 24 h, followed by treatment with vehicle (Ctrl) or EGF (20 ng/ml) for 30 min. Equal amounts of whole-cell protein lysates were analyzed by Western blot probed with pY1068 EGFR and total EGFR antibodies. β-Actin was used as loading control. Immunoreactive bands were quantified by chemifluorescence using a STORM Molecular Imager. Data are means ± SEM; N = 3; *p < 0.05 vs. Ctrl and **p < 0.05 for NICD+TGF-β1 vs. NICD or TGF-β1. Inset, representative Western blots.
ACKNOWLEDGMENTS

We thank Dustin Baker for technical support and Diane Fiolek for graphic preparation and administrative support. We thank Tatsuya Iso for the gift of NICD adenovirus. This work was supported by National Institutes of Health Grant SRO1 ES012920 to Y.X. and G.J.F.

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