Notch and TGF-β Pathways Cooperatively Regulate Receptor Protein Tyrosine Phosphatase-kappa (PTPRK) Gene Expression in Human Primary Keratinocytes.

Yiru Xu, Siliang Xue, Jin Zhou, John J. Voorhees, and Gary J. Fisher. Department of Dermatology, University of Michigan Medical School, Ann Arbor, MI 48109.

Running title: Notch and TGF-β Regulation of PTPRK

Address for correspondence:
Yiru Xu, Ph.D.
Department of Dermatology
University of Michigan Medical School
Medical Science I, Room 6447
1150 W. Medical Center Drive
Ann Arbor, MI 48109-5609
Telephone: (734) 763-1469
Facsimile: (734) 647-0076
E-mail: xuyiru@umich.edu

Key words: Notch, TGF-β, protein tyrosine phosphatase, keratinocyte, contact inhibition

Abstract

Receptor protein tyrosine phosphatase-kappa (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. Since 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 gamma-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. Interestingly, 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 gamma secretase inhibitors, significantly reduced TGF-β-induced PTPRK gene expression, indicating that Notch and TGF-β pathways function together to regulate PTPRK. Importantly, 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). Since 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 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 co-activator Mastermind/Mastermind-like to activate transcription of target genes (Bray, 2006; Kopan and Ilagan, 2009).

Keratinocytes form the stratified epithelium of skin epidermis. Keratinocytes proliferate in the lowest (basal) epidermal layer and then undergo maturation as they migrate upwards. 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).

Epidermal growth factor receptor (EGFR) signaling pathway is a potent regulator of keratinocytes proliferation (Pastore *et al.*, 2008). Ligand binding induces EGFR tyrosine phosphorylation and activation of down-stream signaling pathways; EGFR tyrosine phosphorylation is synonymous with receptor activation. Receptor protein tyrosine phosphatase-kappa (PTPRK) specifically dephosphorylates EGFR thereby acting as a major negative regulator of EGFR signaling. Over-expression 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, compared to non-proliferating keratinocytes in suprabasal layers (Xu *et al.*, 2006). In low density, highly proliferative keratinocyte cultures, PTPRK expression is lower, compared to confluent non-proliferative 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 beta (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.* recently 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 (Blokzijl *et al.*, 2003). Based on these data, we have 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 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, while Notch3 and Notch4 are almost undetectable. The most abundant Notch ligand is Jag1, which is expressed 10-fold higher than that of 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 adult 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 gene to reflect Notch pathway activity. We found that Hes-1 mRNA was significantly up-regulated in confluent primary

human keratinocytes compared to that of sub-confluent 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 have been shown to 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 sub-confluent 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 (data not shown).

Inhibition of Notch signaling suppresses TGF-β -induced PTPRK expression

We previously reported that PTPRK transcription is directly regulated by 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. Interestingly, we found that increased cell-cell contact induces expression of TGF-β1 by 2-fold 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 co-immunoprecipitated 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; Krebs *et al.*, 2003; Pan *et al.*, 2004). Knockdown of Notch1 by lentivirus-mediated shRNA (**Figure 6C)** inhibited TGF-β induced PTPRK expression (**Figure 6D**). Taken together, these data indicate that that up-regulation of PTPRK by TGF-β requires input from the Notch signaling pathway.

Notch and TGF-β pathways cooperatively stimulate PTPRK expression

Given that both Notch and TGF-β pathways promote PTPRK expression, we investigated the impact of combined activation of both signaling pathways on PTPRK gene expression. As

shown in **Figure 7**, combined treatment of keratinocyte with Jag1 peptide and TGF-β up-regulated PTPRK mRNA (**Figure 7A**) and protein (**Figure 7B**) levels, to a significantly greater extent than addition of either ligand alone. In addition, this induction of PTPRK by TGF-β and Notch-1 signaling pathways resulted in reduced tyrosine phosphorylation of EGFR (**Figure 7C**).

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 (Rangarajan *et al.*, 2001). Activation of Notch signaling in mouse and human keratinocytes induces growth arrest and the onset of differentiation (Lowell *et al.*, 2000; Rangarajan *et al.*, 2001; Nickoloff *et al.*, 2002; Tsai and Tsao, 2004). Loss-of-function and gain-of-function studies in cell culture and animal models have demonstrated that Notch signaling regulates the balance between keratinocyte proliferation and early-stage differentiation of the epidermis (Rangarajan *et al.*, 2001; Uyttendaele *et al.*, 2004; Blanpain *et al.*, 2006; Moriyama *et al.*, 2008).

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 post-mitotic cells in the supra-basal 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-secratase inhibition or knockdown of Notch1 reduced TGF-β-induced PTPRK expression (**Figure 6**). Complete inhibition was seen with blocking γ-secratase 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 following 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 over-expression of a dominant negative inhibitor of down-stream effector Mastermind-like 1 in epidermis increases

spontaneous squamous cell carcinoma formation (Nicolas *et al.*, 2003; Proweller *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 BCC and SCC (Nicolas *et al.*, 2003). Impaired Notch signaling has been linked to cutaneous SCC formation (Proweller *et al.*, 2006). While the pro-differentiation 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, Inc. (Portland, OR). HEK293 cells were purchased from the ATCC (Manasas, VA). Jag1 peptide and Jag1 scrambled peptide were purchased from Anaspec (Fremont, CA). Gamma-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). Beta-Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Smad3 antibody was purchased from abcam (Cambridge, England). NICD adenovirus is a gift from Dr. Tatsuya Iso of Gunma University Graduate School of Medicine, Maebashi, Japan.

Cell Culture

Adult human primary epidermal keratinocytes were cultured in keratinocyte medium (EpiLife, Cascade Biologics, Inc.) with human keratinocyte growth supplement (Cascade Biologics, Inc.) under 5% CO_2 at 37°C. HEK293 cells were cultured in DMEM with 1.5g/ml sodium bicarbonate, supplemented with 10% fetal bovine serum under 5% CO_2 at 37°C.

Lentivirus-mediated shRNAi knockdown of Notch1 in Primary Human Keratinocytes

The MISSION TurboGFP non-targeting shRNA control vector and shRNA constructs targeting Notch1 (5'-CCGGCGCTGCCTGGACAAGATCAATCTCGAGATTGATCTTGTCCAGGCAGCG-

TTTTT-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 Regeant 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 knockdown endogenous Notch1.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was purified using a Miniprep RNA isolation kit according to the manufacturers' 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, Inc., 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 Primes were

produced by the Custom Oligonucleotide Synthesis Service (Applied Biosystems). The sequences for real-time PCR primers used in this study are as follow: human PTPRK: Fwd 5'-ACA GAG TGG TGA AAA TAG CAG GAA-3', Rev 5'-TGA CAA CTA GGA GAA GGA GGA TGA-3'; human Hes-1: Fwd 5'-TTG GAG GCT TCC AGG TGG TA-3', Rev 5'-GCC CCG TTG GGA ATG AG-3'; Human TGF-β1: Fwd 5'-TGA CAA GTT CAA GCA GAG TAC ACA CA-3', Rev 5'-AGA GCA ACA CGG GTT CAG GTA-3'; Human 36B4 Fwd 5'-ATG CAG CAG ATC CGC ATG T-3', Rev 5-TTG CGC ATC ATG GTG TTC TT-3'. The following real time PCR primers were purchased from Applied Biosystems (Foster City, California) 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), Human DLL-4 (Hs00184092_m1).

Cell Lysate and Western Blot

Human primary keratinocytes were washed twice with ice-cold PBS, scraped from the dishes in ice-cold whole cell extraction (WCE) buffer (25 mM HEPES, pH 7.2, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 20 mM β-glycerophophate, 0.1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Equal amounts of whole cell lysate were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P filter paper (Millipore, Bedford, MA). Immunoreactive proteins were visualized by enhanced chemifluorescence (ECF) according to the manufacturer's protocol (GE Healthcare, Piscataway, NJ).

Acknowledgments

We thank Dustin Baker for technical support and Diane Fiolek for graphic preparation and administrative support. We thank Dr. Tatsuya Iso (Gunma University Graduate School of Medicine) for the gift of NICD adenovirus. This work is supported by National Institutes of Health Grant 5R01 ES012920 to Y.X. and G.J.F.

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Figure 1

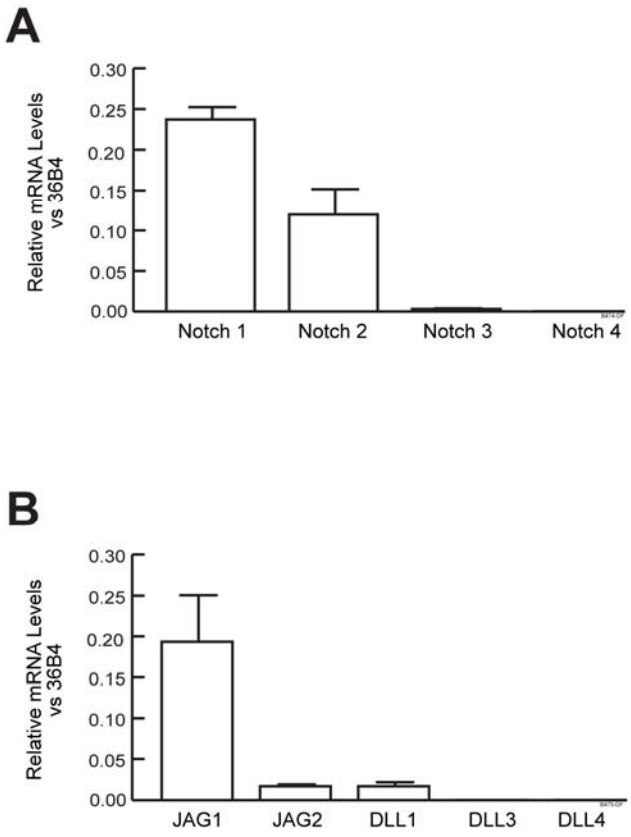

Figure 1. Expression of Notch receptors and ligands in primary human keratinocytes. Total RNA was isolated from cultured primary human keratinocytes, and mRNA for Notch receptors and ligands were quantified by real time RT-PCR. Housekeeping gene 36B4 mRNA was used as internal control for normalization. Data are means±SEM, N=4.

Figure 2

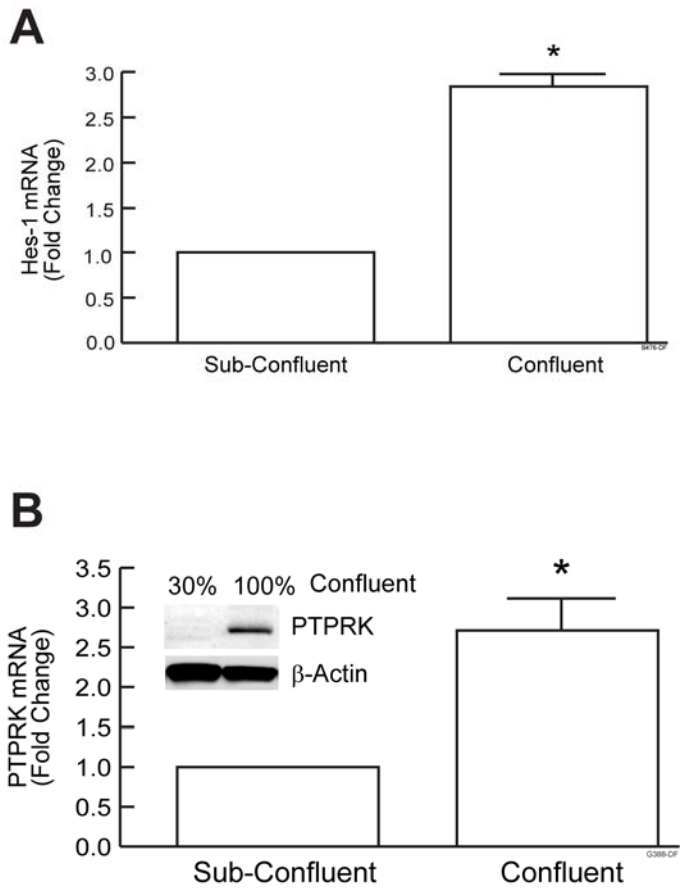

Figure 2. Increased cell-cell contact promotes Notch target Hes-1 gene and PTPRK expression in primary human keratinocytes. Total RNA was isolated from keratinocytes at different confluency (30-40% confluency for sub-confluent and 95%-100% for confluent). **A**) Hes-1 (N=6, *p<0.05); **B**). PTPRK (N=5, *p<0.05) mRNA levels were quantified by real time RT-PCR. 36B4 mRNA levels were used as internal control for normalization. Inset shows a representative Western blot for PTPRK protein expression, which correlates with PTPRK mRNA levels. Beta-actin was used as loading control.

Figure 3

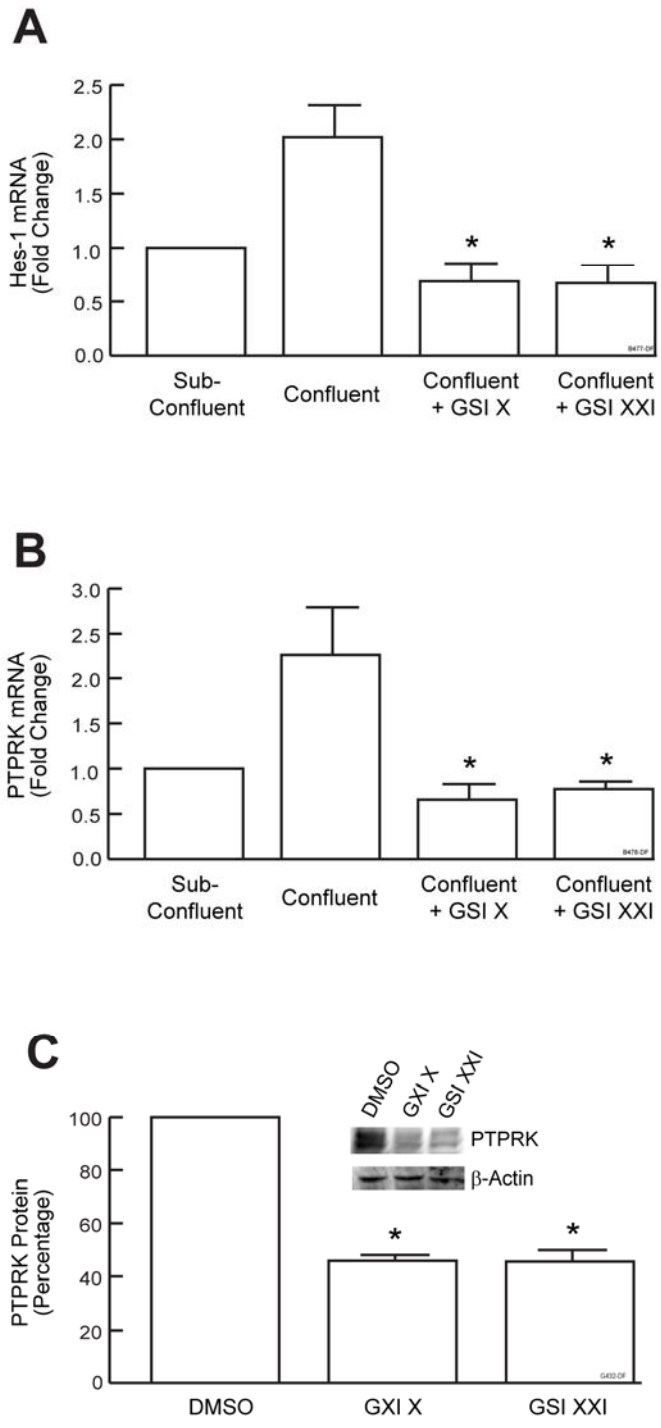
A
Hes-1 mRNA (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Sub-Confluent
Confluent
Confluent + GSI X
Confluent + GSI XXI
B
PTPRK mRNA (Fold Change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Sub-Confluent
Confluent
Confluent + GSI X
Confluent + GSI XXI
C
PTPRK Protein (Percentage)
100
80
60
40
20
0
DMSO
GXI X
GSI XXI
PTPRK
β-Actin
*
*
DMSO
GXI X
GSI XXI

Figure 3. Inhibition of Notch signaling suppresses confluence-induced Notch target Hes-1 and PTPRK gene expression in primary human keratinocytes. Keratinocytes were treated with 3 μM γ-secretase inhibitors GSI X or GSI XXI for 48 hours. **A**) Hes-1 (N = 3, *p<0.05); **B**) PTPRK (N=3, *p<0.05) mRNA levels were quantified by real time RT-PCR. 36B4 mRNA was used as internal control for normalization. **C**) Equal amounts of whole cell protein lysates from confluent keratinocyte cultures were analyzed by Western blot probed with PTPRK antibody. Beta-actin was used as loading control. Immunoreactive bands were quantified by chemifluorescence using a STORM Molecular Imager. Data are means±SEM, N=4; *p< 0.05. Inset shows representative Western blots.

Figure 4

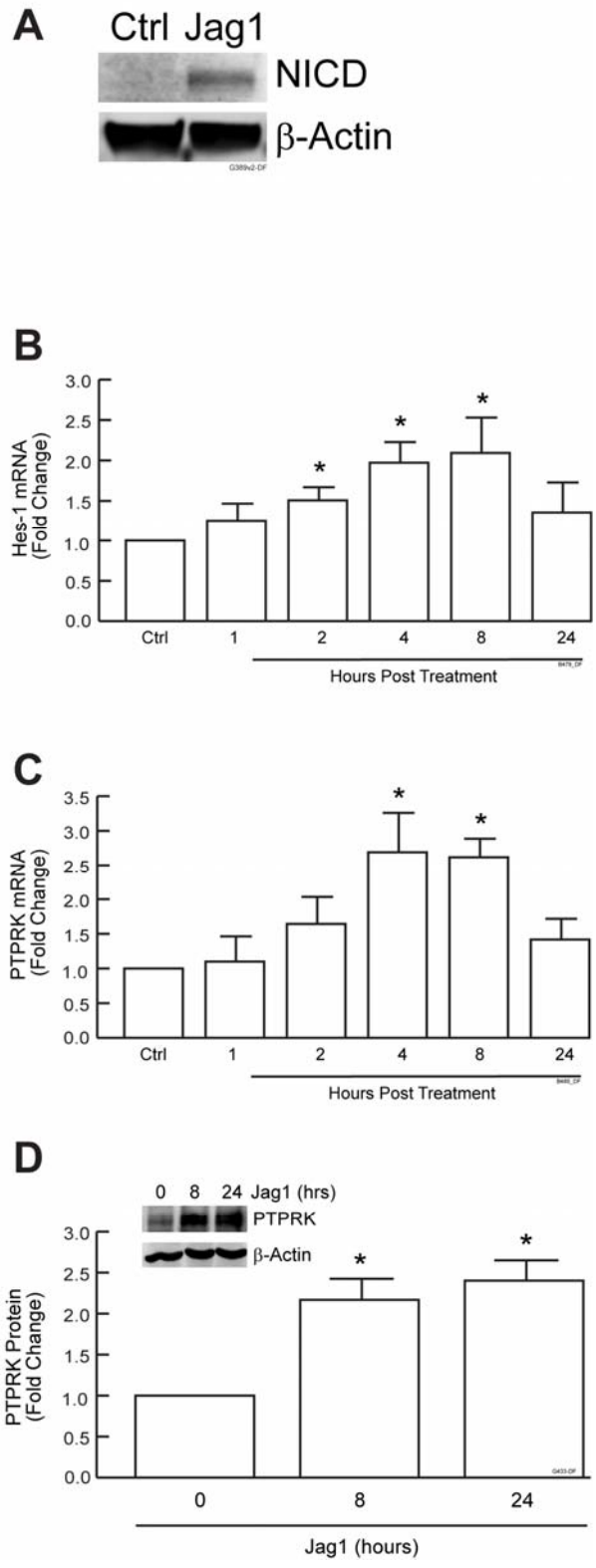
A
Ctrl Jag1
NICD
β-Actin
B
Hes-1 mRNA (Fold Change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
1
2
4
8
24
Hours Post Treatment
C
PTPRK mRNA (Fold Change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
1
2
4
8
24
Hours Post Treatment
D
0 8 24 Jag1 (hrs)
PTPRK
β-Actin
PTPRK Protein (Fold Change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
8
24
Jag1 (hours)

Figure 4. Notch ligand Jag1 induces Notch target Hes-1 gene and PTPRK gene expression in primary human keratinocytes. Sub-confluent 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 Notch intracellular domain (NICD). Beta-actin was used as loading control. A representative Western blot from three independent experiments is shown. B and C) After treatment for the indicated times, total RNA was isolated and Hes-1 (**B**) or PTPRK (**C**) mRNA levels were quantified by real time RT-PCR. 36B4 mRNA was used as internal control for normalization (N=3, *$p<0.05$). **D**) After treatment for the indicated times, equal amounts of whole cell protein lysates were analyzed by Western blot probed with PTPRK antibody. Beta-actin was used as loading control. Immunoreactive bands were quantified by chemifluorescence using a STORM Molecular Imager. Data are means±SEM, N=4; *$p<0.05$. Inset shows representative Western blots.

Figure 5

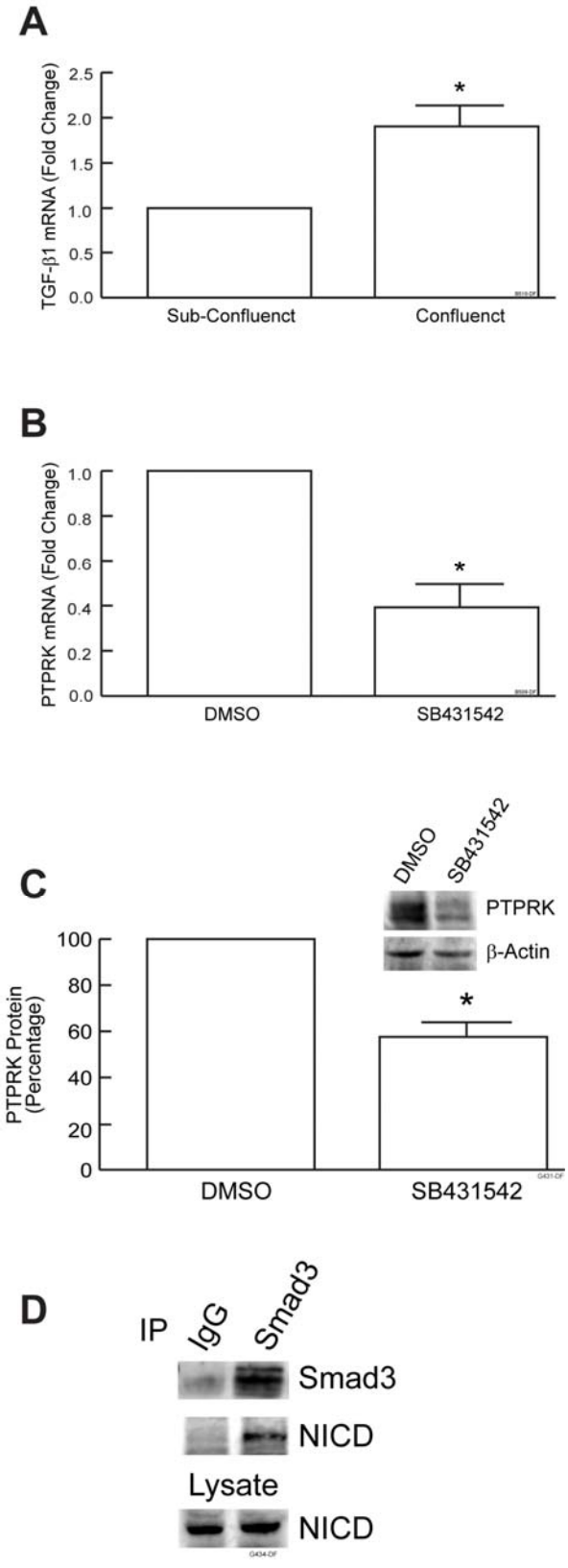
A
TGF-β1 mRNA (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
Sub-Confluenct
Confluenct
B
PTPRK mRNA (Fold Change)
1.0
0.8
0.6
0.4
0.2
0.0
*
DMSO
SB431542
C
DMSO
SB431542
PTPRK
β-Actin
PTPRK Protein (Percentage)
100
80
60
40
20
0
*
DMSO
SB431542
D
IP
IgG
Smad3
Smad3
NICD
Lysate
NICD

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 sub-confluent 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 (DMSO) for 2 days. 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 chemifluorescence using a STORM Molecular Imager. Data are means±SEM, N=4; *p<0.05. Inset shows representative Western blots. **D**). Co-immunoprecipitation of NICD and Smad3. Human primary keratinocytes were infected with NICD adenovirus for 2 day and then treated with TGF-β for 1 hour, whole cell lysate were immunoprecipitated with control IgG 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 were shown.

Figure 6

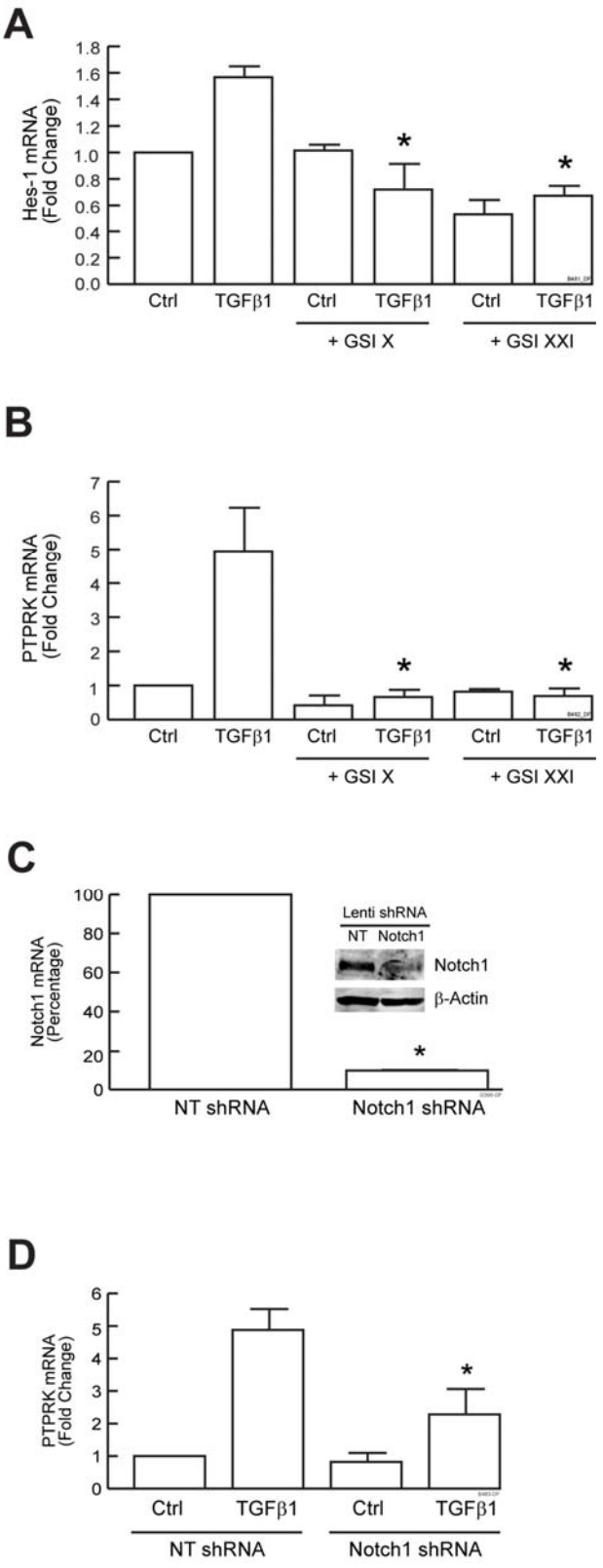
A
Hes-1 mRNA (Fold Change)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Ctrl
TGFβ1
Ctrl
TGFβ1
Ctrl
TGFβ1
+ GSI X
+ GSI XXI
B
PTPRK mRNA (Fold Change)
7
6
5
4
3
2
1
0
*
*
Ctrl
TGFβ1
Ctrl
TGFβ1
Ctrl
TGFβ1
+ GSI X
+ GSI XXI
C
Notch1 mRNA (Percentage)
100
80
60
40
20
0
Lenti shRNA
NT
Notch1
Notch1
β-Actin
*
NT shRNA
Notch1 shRNA
D
PTPRK mRNA (Fold Change)
6
5
4
3
2
1
0
*
Ctrl
TGFβ1
Ctrl
TGFβ1
NT shRNA
Notch1 shRNA

Figure 6. Inhibition of Notch signaling suppresses TGF-β-induction of Notch target Hes-1 and PTPRK gene expression in primary human keratinocytes. A) and **B**) Sub-confluent 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 hours. Total RNA was isolated and **A**) Hes-1 (N=3, *p<0.05 TGF-β alone vs TGF-β1 plus GSI X or TGF-β plus GSI XXI); and **B**) PTPRK (N=5, *p<0.05 TGF-β alone vs TGF-β1 plus GSI X or TGF-β plus GSI XXI) mRNA levels were quantified by real time RT-PCR. 36B4 mRNA levels were used as internal control for normalization. **C**) and **D**) Keratinocytes were infected with lentivirus that expressed non-targeting (NT) shRNA or Notch1 targeting shRNA for 48 hours. **C**) Total RNA was isolated, and 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. Beta-actin was used as loading control. Inset shows a 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 hours. 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 7

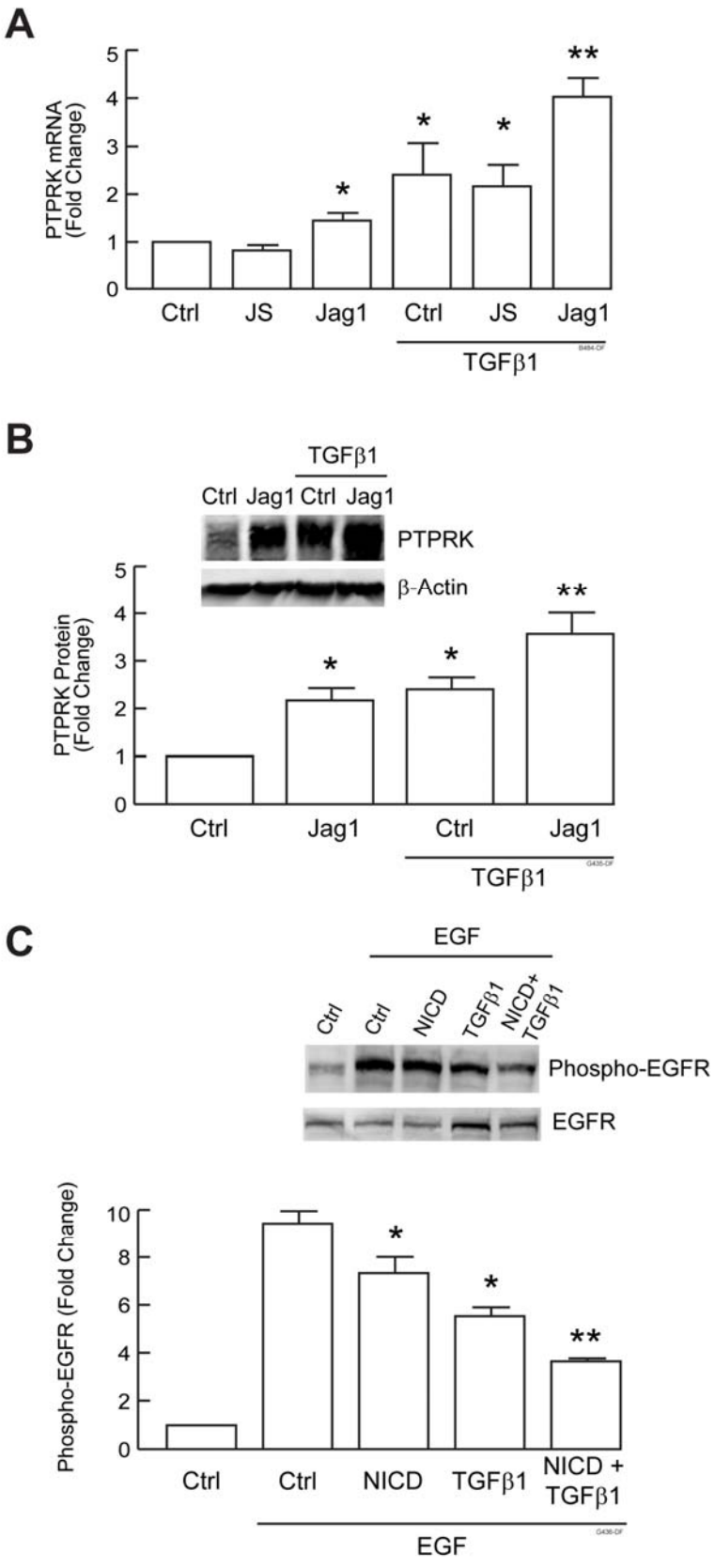
A
PTPRK mRNA (Fold Change)
Ctrl
JS
Jag1
Ctrl
JS
Jag1
TGFβ1
B
TGFβ1
Ctrl Jag1 Ctrl Jag1
PTPRK
β-Actin
PTPRK Protein (Fold Change)
Ctrl
Jag1
Ctrl
Jag1
TGFβ1
C
EGF
Ctrl
Ctrl
NICD
TGFβ1
NICD+ TGFβ1
Phospho-EGFR
EGFR
Phospho-EGFR (Fold Change)
Ctrl
Ctrl
NICD
TGFβ1
NICD + TGFβ1
EGF

Figure 7. Activation of Notch pathway enhances TGF-β-induced PTPRK gene expression in primary human keratinocytes. A) Sub-confluent keratinocytes were treated with vehicle (Ctrl), 10 μM Jag1 peptide, or scrambled Jag1 peptide (JS) alone, or in the presence of TGF-β1 (2.5 ng/ml) for 24 hours. PTPRK and 36B4 (internal control for normalization) mRNA levels were quantified by real time RT-PCR. N=4, *p<0.05 vs Ctrl or JS, **p<0.05 vs TGF-ß1, JAG or JS alone. **B**) Sub-confluent keratinocytes were treated with vehicle (Ctrl), Jag1 peptide (10 μM), with or without (2.5 ng/ml) TGF-β1 for 24 hours. Equal amounts of whole cell protein lysates were analyzed by Western blot probed with PTPRK antibody. Beta-actin was used as loading control. Immunoreactive bands were quantified by chemifluorescence using a STORM Molecular Imager. Data are means±SEM, N=4; *p<0.05 vs Ctrl, **p<0.05 for Jag1+TGF-β1 vs Jag1 or TGF-β1 alone. Inset shows representative Western blots. **C**). Sub-confluent keratinocytes were infected with empty or NICD Adenovirus for 24 hours, then treated with vehicle (Ctrl) or TGF-β1 (2.5 ng/ml) for 24 hours, followed by treatment with vehicle (Ctrl) or EGF (20 ng/ml) for 30 minutes. Equal amounts of whole cell protein lysates were analyzed by Western blot probed with pY1068 EGFR and total EGFR antibodies. Beta-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, **p<0.05 for NICD+TGF-β1 vs NICD or TGF-β1. Inset shows representative Western blots.

Figure 8

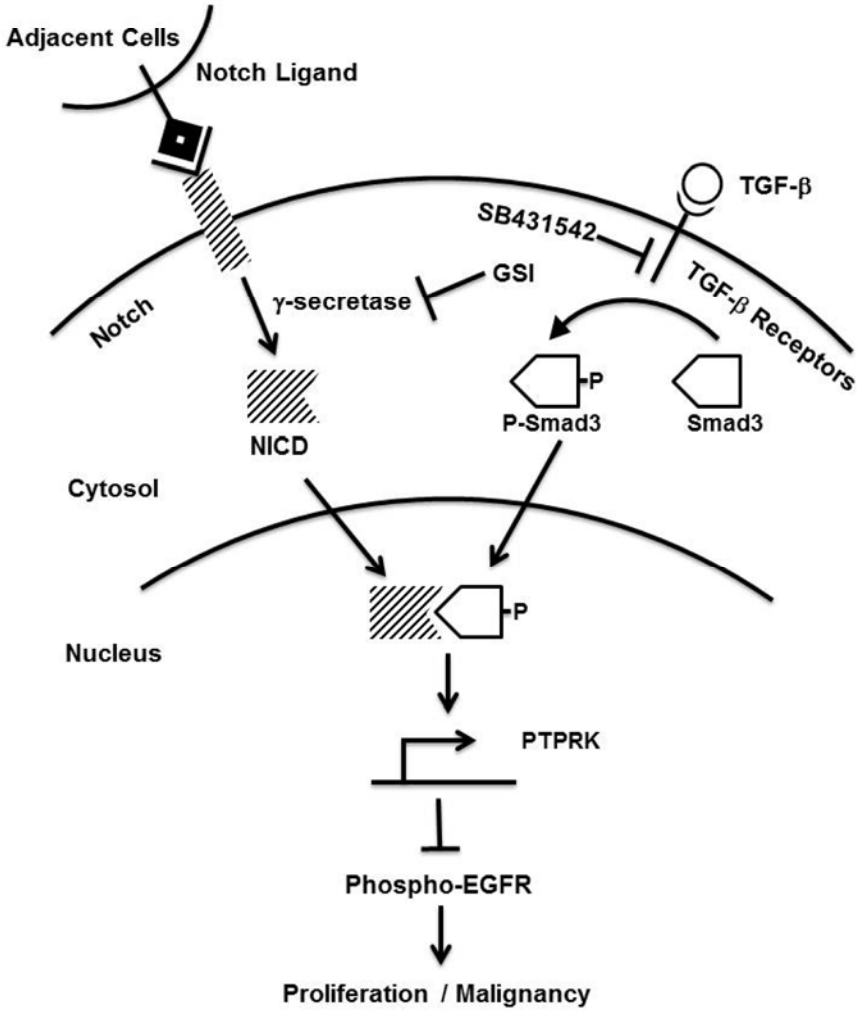

Figure 8. Gene expression of PTPRK and Hes-1 is coordinately regulated by Notch and TGF-β pathways.

Notch activation by ligand on the surface of adjacent cell induces proteolytic processing by γ-secratase, which can be blocked by γ-secratase inhibitor (GSI), leading to formation of Notch

Intracellular Domain protein (NICD). Binding of TGF-β to TGF-β receptors leads to activation/phosphorylation of Smad3, which can be blocked by TGF-β type I receptor kinase inhibitor SB431542. NICD and Smad3 can physically interact with each other and regulate expression of target genes such as PTPRK which negatively regulates EGFR phosphorylation/activation and potential downstream responses.