Abstract

Acquired resistance to TGFβ is a key step in the early stages of tumorigenesis. Mutations in TGFβ signaling components are rare, and little is known about the development of resistance in breast cancer. On the other hand, an activated Notch pathway is known to play a substantial role in promoting breast cancer development. Here, we present evidence of crosstalk between these two pathways through HEYL. HEYL, a basic helix–loop–helix transcription factor and a direct target of Notch signaling, is specifically overexpressed in breast cancer. HEYL represses TGFβ activity by binding to TGFβ-activated Smads. HeyL−/− mice have defective mammary gland development with fewer terminal end buds. On the other hand, HeyL transgenic mice show accelerated mammary gland epithelial proliferation and 24% of multiparous mice develop mammary gland cancer. Therefore, repression of TGFβ signaling by Notch acting through HEYL may promote initiation of breast cancer. Cancer Res; 74(22): 6509–18. ©2014 AACR.

Introduction

TGFβ is a multifunctional cytokine that exerts pleiotropic effects on virtually all known cell types through a seemingly simple signal transduction pathway. TGFβ binds to its transmembrane receptors, which leads to phosphorylation of two receptor-regulated Smads (R-Smads), Smad2 and Smad3. The activated R-Smads then bind to Smad4, forming a Smad complex that associates with other transcriptional cofactors to regulate gene expression (1, 2). TGFβ is able to suppress tumor initiation at early stages of cancer development. However, cancer cells usually develop various ways to evade the growth-inhibitory effect of TGFβ. Indeed, loss of TGFβ sensitivity is a hallmark of tumor initiation (3). Although mutations of TGFβ signaling components are common in gastrointestinal cancers (4), such mutations are rarely found in breast cancer. How breast cancer cells acquire resistance to TGFβ-mediated growth inhibition is largely unknown.

In contrast with the TGFβ pathway, activation of the Notch pathway has been frequently shown to promote breast cancer development (5). Upon binding of Notch ligand to its receptor, the intracellular domain of the Notch receptor is released from the cell membrane through the action of a γ-secretase complex and translocates to the nucleus, where it forms a complex with RBP-J to induce the expression of genes that promote cell growth and inhibit cellular differentiation (6, 7). Given the fact that these two pathways have opposing effects on cell growth and mammary gland tumor development, it seems plausible that activation of the Notch pathway in breast cancer can counteract the inhibitory effects of TGFβ signaling. The Notch pathway has been reported to either synergize or antagonize TGFβ signaling depending on the cellular context, but the detailed mechanism of their crosstalk is not well established (8, 9). Moreover, the significance of their crosstalk in breast cancer is not known.

HeyL, Hey1, and Hey2 are the members of the HEY (hairy/enhancer-of-split related with YRPW motif) family (10). All three of these genes and the related HES (hairy and enhancer of split) family members are basic helix–loop–helix (bHLH) transcription factors and have been shown to be the direct targets of the Notch pathway (11–14). Hey2-null mice die in the early postnatal period and have a variety of cardiovascular defects, including atrioventricular valve and ventricular septal defect, tetralogy of Fallot, and congestive heart failure (15–17). Hey1- or HeyL-null mice do not show detectable developmental abnormality, while combined loss of Hey1 and HeyL causes similar cardiovascular defects (18).
To date, the function of HEYL in breast cancer has not been studied. Here, we show that HEYL can inhibit TGFβ signaling by binding to Smad proteins, thereby rendering the cells resistant to the effect of TGFβ.

Materials and Methods

Cell culture and reagents

HaCaT, HS578T, Cos1, MDA-MB-231, and HepG2 were grown in DMEM with 10% FBS, penicillin, and streptomycin. SUM159 cells were grown in Ham's F-12 with 5% FBS, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, penicillin, and streptomycin. MCF-10A cells were cultured in DMEM/nutrient mixture F-12 supplemented with 5% horse serum, 20 ng/mL recombinant EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, penicillin, and streptomycin.

Antibodies used were anti-Myc-tag (9B11) mouse Ab (Cell Signaling Technology), anti-Smad3 rabbit Ab (Cell Signaling Technology), anti-Flag M2 mouse Ab (Sigma), anti-Ki-67 Ab (Thermo Scientific), anti-HEYL mouse Ab (Abnova), and anti-HEYL rabbit Ab.

Details of the construction of HEYL expression vectors and infection of cells with viral supernatants are provided in Supplementary Methods.

Generation of anti-HEYL rabbit antibody and IHC staining

A synthetic peptide corresponding to a peptide sequence (EPSGSDGESDPID) in the N-terminus of HEYL was used to generate polyclonal antibodies in rabbits. Details of the preparation and tests to validate its specificity are provided in Supplementary Methods.

Generation and characterization of HeyL knockout and transgenic mice

HeyL knockout mice were generated as previously described (18, 19). Exons 2–4 of HeyL were deleted. The mammary glands of 13-week-old HeyL knockout mice and wild-type littermates were excised and processed for whole-mount staining. IHC staining and frozen chunks were embedded in the optimal cutting temperature (OCT).

To generate HeyL transgenic mice, the full-length murine HeyL cDNA was inserted into the EcoRI site of MKbpA vector (provided by Dr. Jeffrey Rosen at Baylor College of Medicine). The resulting vector was cut using BssHII restriction endonuclease, and the microinjection of the fragment containing the transgene into single-cell embryos isolated from FVB/N mice was performed by the Transgenic Mouse Core Facility at National Cancer Institute. The establishment of founder mice was confirmed by Southern blotting. The FVB/N founder mice were then crossed with FVB/N wild type mice for 4-5 generations. The genotype of HeyL transgenic mice and the wild type littermates was verified by PCR using genomic DNA isolated from mouse tails. The HeyL transgenic mice and the wild type littermates were euthanized at 13 week old, 10 days of pregnancy, 10 days of lactation and 30 days after involution. The inguinal mammary fat pads were excised and processed for whole mount staining. IHC staining and frozen chunks embedded in OCT.

Whole mount staining, RT-PCR, and immunostaining of mouse mammary glands

For whole-mount staining, the mammary glands were placed between two glass slides and fixed with 10% formalin overnight. Details are provided in Supplementary Methods.

Figure 1. HEYL is overexpressed in breast cancer. A, HEYL IHC staining of representative sections of normal duct, ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC) is shown. Note the absence of staining in the normal duct located in the tumor (arrow). Difference in HEYL IHC staining was determined between the groups using two-sided Fisher exact t test. B, HEYL mRNA expression in normal breast organoids (enriched epithelium following enzymatic digestion of reduction mammoplasty tissue) and malignant samples is shown. Expression difference was compared between the groups using one-sided Wilcoxon rank-sum test. C, differential expression of HEYL in 62 matched normal and breast cancers (BRCA) in TCGA database was determined using two-tailed Student t test; *** P < 0.001.
Treatment of HeyL knockout mice with TGFβ type 1 receptor inhibitor, SB 535334
SB 535334 powder (Selleck) was dissolved in 100% ethanol to 18 mg/mL. The solution was further diluted 1:10 in 100 mg/mL cyclodextrin. Of note, 100 μL final solution (10 mg/kg) was intraperitoneally injected into 7- to 8-week-old HeyL knockout mice daily. The treatment lasted for 4 to 5 weeks. The mammary glands were then removed for analysis.

Immunoprecipitation and Western blotting
Transfected Cos1 cells in T25 flasks were lysed in 250 μL 1× lysis buffer (1% Triton-X, 150 nmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA and EGTA, and 0.5% NP-40) with protease

Figure 2. HEYL antagonizes TGFβ-mediated signaling. A, left, 0.25 μg p3TP-Luc vector alone or with 0.5 μg HEYL expression vector was transfected into HepG2 cells. On the next day, 4 ng/mL TGFβ was added for 16 hours. Right, varying amounts of HEYL (0.1, 0.25, 0.5 μg) were cotransfected with 0.25 μg 3TP-Luc and 0.5 μg Smad3 expression vectors. Luciferase activity was measured 48 hours after transfection. B, p15-promoter luciferase vector, p15-Luc, was used in the same luciferase assay as in Fig. 2A. C, HaCaT control or stably expressing HEYL cells were treated with 4 ng/mL TGFβ for 0, 4, 8, and 18 hours. mRNA levels of PAI and p15 were measured in HaCaT, and PAI and Smad7 were measured in MCF-10A by qRT-PCR. D, MCF-10A control and stably expressing MCF10A-HEYL cells were treated with 0.5 ng/mL TGFβ for 0, 2, 4, and 8 hours. mRNA levels of PAI and Smad7 were measured by qRT-PCR. For all experiments, error bars represent SD (n = 3). Difference between the groups was determined using two-tailed unpaired t test; * P < 0.05; ** P < 0.01.
Figure 3. HEYL interacts directly with Smad3. A, Myc-tagged-HEYL alone, or along with Flag-tagged-Smad3, was transfected into Cos1 cells and cells were treated with or without 4 ng/mL TGFβ for 3 hours. The cell lysates were immunoprecipitated with the either anti-Myc antibody or control IgG and blotted with the anti-Flag or anti-Myc antibody. B, bimolecular complementation assay in Cos1 cells showing green fluorescence at 36 hours after transfection. The N-terminal half of GFP fused with full-length HEYL (FL), basic domain deletion of HEYL (Δ Basic), helix-loop-helix domain deletion of HEYL (Δ HLH), or HoxB7 was coexpressed with the C-terminal of GFP fused with Smad3 in Cos1 cells. Cell nuclei were stained with DAPI. C, confocal microscopy imaging showing the colocalization of HEYL (red) and Smad3 (green) in MDA-MB-231 cells. D, left, flag-tagged Smad3 was coexpressed with control or various Myc-tagged constructs of HEYL in Cos1 cells. Right, Myc-tagged full-length HEYL was coexpressed with control or various Flag-tagged constructs of Smad3 in Cos1 cells. The cell lysates were immunoprecipitated with anti-Flag antibody and blotted with the anti-Myc or anti-Flag antibody. E, flag-tagged Smad4 was coexpressed with control or various Myc-tagged constructs of HEYL in Cos1 cells. The immunoprecipitation and blotting were performed as in Fig. 3D. F, Smad4 expression vector was used in a luciferase assay as described in Fig. 2A. Difference between the groups was determined using two-tailed unpaired t test; * P < 0.05. G, HepG2 cells were transfected with 0.25 μg 3TP-Luc construct and 0.5 μg of various constructs of HEYL. The cells were treated with 4 ng/mL TGFβ for 16 hours. Luciferase activity was measured as described in Fig. 2A. Difference between the groups was determined using two-tailed unpaired t test; ** P < 0.01.
inhibitor on ice for 30 minutes. Details are provided in Supplementary Methods.

qRT-PCR
Details of oligonucleotide primers and methods are provided in Supplementary Methods.

Luciferase assay
HepG2 or HS578T cells on 12- or 24-well plates were transfected with an equal amount of corresponding vectors using Lipofectamine 2000 (Invitrogen). Of note, 4 ng/mL TGFβ (Peprotech) was added into cell media and incubated for 16 hours. The luciferase reading was measured 48 hours after transfection and normalized to β-galactosidase activities. The luciferase assays were repeated three times.

BrdUrd cell proliferation assay
Five thousand MCF-10A control or HEYL-expressing cells were seeded on a 96-well plate and treated the next day with TGFβ for 12 hours. Bromodeoxyuridine (BrdUrd) was added into cell media and incubated for 4 hours. Additional details are provided in Supplementary Methods.

Bimolecular fluorescence complementation assay
The fusion protein expression vectors were transfected into Cos1 cells on 24-well plates. Thirty-six hours later, the cells were fixed with formalin, and the nuclei were stained using DAPI. The fluorescence was then observed under a microscope.

In vitro binding assay
35S labeled full-length, basic domain deletion and helix-loop–helix domain deletion HEYL proteins were synthesized in vitro using Coupled Transcription/Translation System (Promega) and incubated with equal amount of GST or GST-Smad3 fusion protein bound to glutathione-Sepharose beads. The associated proteins were revealed by SDS-PAGE and autoradiography.

Results
HEYL was first discovered to be highly expressed in colon and breast cancer endothelial cells. We and another group isolated endothelial cells from normal and tumor samples using beads conjugated with endothelial-specific antibody. SAGE (Serial Analysis of Gene Expression) analysis of the purified endothelial cells showed that HEYL expression was...
western blotting con HeyL murine positive by immuno cells stably expressing HEYL, but not vector controls, were positive by immunofluorescent staining (Supplementary Fig. S1B). We also confirmed that in immunoanfluorescent analysis, this antibody showed positive staining with HEYL- and HES1-transfected cells (Supplementary Fig. S1C). IHC analysis of breast cancer tissue arrays indicated that, in addition to the positive staining in tumor endothelial cells, HEYL is also abundantly expressed in tumor epithelial cells. Compared with normal breast tissues that showed negative staining (0/13), significantly strong nuclear immunostaining was detected in 45% (50/111; \( P = 0.002 \)) of invasive ductal carcinomas and in 46% (6/13; \( P = 0.015 \)) of invasive lobular carcinomas (representative photomicrographs shown in Fig. 1A). qRT-PCR showed high HEYL mRNA expression (cutoff value: 5.0 U) in 50% (18/36) of primary and in 34.5% (10/29) metastatic breast cancers but not in normal breast tissue organoids (0/8; Fig. 1B). We also compared HEYL expression in 62 matched normal and primary breast tumors (abbreviated as BRCA) in The Cancer Genome Atlas (TCGA) database, and found that HEYL expression is significantly higher in tumors (\( P < 0.0001 \)) compared with normal breast tissue (Fig. 1C).

**HEYL** is a known direct target of the Notch pathway as shown by studies in cell culture and mouse models (12, 13, 22). Taking into account the cell type-specific action of the Notch pathway, we tested whether Notch can activate HEYL expression in breast epithelial cells. Expression of the Notch1 intracellular domain (N1IC), which mimics Notch pathway activation upon ligand binding, in the breast cancer cell line HS578T resulted in increasing levels of HEYL expression (Supplementary Fig. S2A). Cotransfection of the N1IC vector and a HEYL promoter-driven luciferase vector resulted in a significant increase in luciferase activity (Supplementary Fig. S2B). Knockdown of the expression of RBP-J, a critical mediator of the canonical Notch pathway, in MDA-MB-231 cells reduced HEYL expression (Supplementary Fig. S2C). Consistent with our findings, it was reported that soluble Notch receptor interfering with the Notch ligand-receptor binding can repress HEYL expression in MDA-MB-231 cells, and HEYL was the only Notch target gene that was associated with the expression of the Notch ligand Jagged1 in breast cancer clinical samples (23). These data suggest that HEYL may potentially mediate part of Notch oncogenic activities in breast cancer.

Because very few experimental investigations have been performed on the functions of HEYL, we performed bioinformatics analysis of multiple public databases to retrieve biochemical and genetic data that might provide heretofore unknown links of HEYL to key signaling pathways. A genome-wide mass spectrometry-based immune-precipitation proteomics study indicated that HEYL can bind to Smad3, a finding corroborated by a large-scale yeast-2-hybrid study (24, 25). Taking into account the contradictory growth promoting effects of Notch and growth inhibitory effects of TGFβ signaling in early breast cancer development, we examined the possibility that HEYL could inhibit the TGFβ pathway. Using a TGFβ-responsive reporter vector, p3TP-Luc, as seen before, TGFβ treatment or Smad3 overexpression significantly increased the luciferase activity; however, this transactivation was strongly inhibited by HEYL (Fig. 2A). We observed a similar transcription repression when a second TGFβ-responsive reporter vector containing the P15 (CDKN2B) gene promoter was used in the same assay (Fig. 2B). Consistent with findings in the luciferase assays, TGFβ treatment of immortalized human keratinocyte cells, HaCaT, resulted in dynamic upregulation of endogenous mRNA levels of PAI-1 and P15 at different time points; similarly, this transcriptional induction was repressed in the presence of HEYL (Fig. 2C). We performed the same experiment on MCF10A breast cell line and measured PAI-1 and Smad7 expression, (but not of P15, because both alleles of this gene are deleted in MCF10A cells). Again, HEYL repressed the expression of PAI-1 and Smad7 induced by TGFβ (Fig. 2C). As shown in a BrdUrd incorporation assay, MCF-10A human breast epithelial cells expressing HEYL proliferated faster compared with control cells upon TGFβ treatment (Fig. 2D).

Next, we studied TGFβ responses in breast cancer cells that were depleted of HEYL. HEYL expression was significantly reduced in MDA-MB-231 breast cancer cells infected with retroviral vectors and stably expressing two different HEYL shRNAs (Fig. 2E). MDA-MB-231 breast cancer cells have an intact TGFβ signaling pathway, but growth of these cells, for unknown reasons, is not inhibited upon TGFβ treatment. Because P15 is not expressed in MDA-MB-231 cells, we examined alterations of endogenous mRNA expression of PAI-1 and Smad7 upon TGFβ treatment. As expected, TGFβ treatment activated expression of PAI-1 and Smad7 in MDA-MB-231 cells, and their expression levels were higher in the HEYL knockdown cells (Fig. 2F). Collectively, these results

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**Figure 5.** Abnormal mammary gland development in HEYL transgenic mouse. A, the MMTV-HEYL transgenic construct contains the MMTV-LTR promoter and murine HEYL cDNA that are linked by the rabbit β-globin exon II (E2), intron II (I2), and exon III (E3). Poly A, polyadenylation signal; HEYL, transgene and endogenous HEYL expression in mouse mammary glands of wild-type (□) and HEYL transgenic mice (●) at different stages were measured by RT-PCR. Western blotting confirmed higher HEYL expression in pregnant transgenic mice. B, whole-mount analysis of wild-type and HEYL transgenic mice at 13 weeks old or 30 days after involution. C, quantitative measurement of mammary gland side branching of wild-type and HEYL transgenic mice at 13 weeks old or 30 days after involution. Differences between the groups were compared using two-tailed unpaired t test; \( ^{*} \), \( P < 0.01 \); \( N = 4 \) each. D, H&E staining of mammary glands at 30 days after involution. E, Ki-67 staining of the mammary glands. Significant differences between the groups were determined using two-tailed paired t test; \( ^{*} \), \( P < 0.05 \); \( ^{**} \), \( N = 4 \)

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support the notion that HEYL expression renders cells less sensitive to TGFβ.

Direct interaction with Smad3 is one mechanism through which HEYL might interfere with TGFβ signaling as shown by bioinformatics analysis of databases. Using coimmunoprecipitation assays in Cos1, we detected a strong protein–protein interaction between HEYL and Smad3; addition of TGFβ did not increase the strength of this interaction (Fig. 3A). To confirm the interaction between HEYL and Smad3 in vivo, we performed the bimolecular fluorescence complementation assay in Cos1 cells. Two fusion proteins, one containing the N-terminal half of GFP fused with HEYL and the other containing the C-terminal half of GFP fused with Smad3, were coexpressed in Cos1 cells. The emission of green fluorescence in the nuclei of these cells indicated that the interaction between HEYL and Smad3 had pulled the two halves of GFP into close proximity. On the other hand, a HOXB7 fusion protein, used as a negative control, showed very faint fluorescence when coexpressed with the C-terminal half of GFP–Smad3 fusion protein. Deleting the basic domain of HEYL, but not the helix–loop–helix domain, lowered the fluorescence intensity to the levels of the control, suggesting that the basic domain of HEYL may mediate its interaction with Smad3 (Fig. 3B and schematic of HEYL domains). We also detected the interaction of endogenous Smad3 and HEYL in MDA-MB-231 cells by coimmuno-fluorescent staining and confocal microscopy imaging (Fig. 3C). Moreover, GST pulldown assays indicated that their interaction was direct and that deletion of the basic domain significantly decreased the interaction (Supplementary Fig. S3).

To map the domains of HEYL and Smad3 that are involved in the interaction, immunoprecipitation assays were performed in Cos1 cells using a series of constructs with various domain deletions of HEYL and Smad3 (domains of HEYL and Smad3 shown in Fig. 3B). We found that the deletion of the basic domain of HEYL abolished its interaction with Smad3, whereas the MH2 domain of Smad3 alone can mediate the interaction. Therefore, the basic domain of HEYL can interact with the MH2 domain of Smad3 (Fig. 3D). The MH2 domain is highly conserved in the Smad protein family. This raises the possibility that HEYL can interact with other Smads as well. Immunoprecipitation assays showed that HEYL, in fact, did interact with Smad4. Although HEYL interacting with Smad3 through its basic domain only, HEYL interaction with Smad4 required both the basic and helix–loop–helix domain (Fig. 3E). In addition, HEYL was able to repress the transactivation of p3TP-Luc induced by Smad4, suggesting that the binding of HEYL to Smad4 also resulted in the inhibition of Smad4 activity (Fig. 3F).

To test whether the interaction between HEYL and Smad3 was necessary and sufficient for the inhibition of TGFβ signaling, we used (SBE)4-Luc, another TGFβ-responsive reporter vector consisting of tandem synthetic Smad-binding elements transfected into HepG2 cells. Unlike other TGFβ-responsive luciferase vectors, (SBE)4-Luc can be activated by Smad3/4 complex in the absence of additional transcriptional cofactors (26). TGFβ treatment or Smad3 overexpression also transactivates its luciferase activity. However, HEYL repressed the transactivation of (SBE)4-Luc induced by either TGFβ or Smad3 (Supplementary Fig. S4). Therefore, the direct interaction of HEYL with Smad3 alone seems to be sufficient to inhibit TGFβ signaling. In line with our finding that the basic and helix–loop–helix domain of HEYL mediated interaction with either Smad3 or Smad4, the repressive effect of HEYL on the TGFβ pathway was abrogated when either domain was deleted, strongly suggesting that the binding of HEYL to Smad proteins was necessary for HEYL to inhibit the TGFβ pathway (Fig. 3G).

Our data indicate a counteractive effect of Notch and TGFβ on breast epithelial cell growth. In fact, in both Notch2 knockout mice and TGFβ mammary gland–specific transgenic mice, the phenotypes of mammary gland development are remarkably similar, reduced epithelial cell growth and fewer terminal end buds (27, 28) were observed. If HEYL dampens TGFβ signaling, HEYL knockout mice (18, 18) are predicted to show mammary gland defects similar to TGFβ transgenic mice. To test this hypothesis, we examined the mammary glands of virgin wild-type and HEYL knockout littermates at 13 weeks old. We found that the mammary glands of HEYL knockout mice have much less side branching and very few terminal ducts (Fig. 4A and B). The epithelial cells of HEYL knockout mice show reduced cell proliferation with significantly lower Ki-67–positive stained cells (Fig. 4C and D). To test the involvement of TGFβ in the generation of this phenotype, we treated HEYL knockout mice with a specific TGFβ type I receptor inhibitor, SB539334. In response to the treatment, the mammary gland ducts developed more side branches and terminal ducts (Fig. 4E and F), suggesting that enhanced TGFβ signaling inhibits mammary gland development in HEYL knockout mice.

To further probe HEYL’s function in vivo, we generated transgenic mice that specifically express HEYL in the mammary gland under the control of the MMTV-LTR promoter (Fig. 5A). HEYL transgenic mice showed robust transgene HEYL expression in mammary glands at different development stages, and low level of endogenous HEYL expression in mammary glands in both wild-type littermates and transgenic mice (Fig. 5A). Although endogenous HEYL expression did not change significantly at different stages of mammary gland development, the HEYL transgene expression level increased at the pregnancy and involution stages, consistent with the fact that MMTV-LTR promoter can be activated by progestins and corticosteroids. Increased HEYL protein expression level in pregnant transgenic mice was also confirmed by Western blotting (Fig. 5A). Comparing mammary gland development between wild-type littermates and HEYL transgenic virgin mice at 13 weeks old, HEYL transgenic virgin mice showed more mammary gland duct side-branching in the whole-mount analysis (Fig. 5B and C), which was also confirmed by histologic examination (Supplementary Fig. S5). Strong ductal cell proliferation in HEYL transgenic mice was evidenced by increased Ki-67 staining (Fig. 5E). During pregnancy, wild-type mice developed well-differentiated secretory epithelium containing lipid filled secretory vesicles, whereas HEYL transgenic mice showed limited epithelial differentiation and very few secreted vesicles (Supplementary Fig. S6). Thirty days after weaning, the wild-type mouse mammary gland returned back to normal, leaving...
that mediate HEYL’s interaction with Smads are highly conserved among the members of HERP family. In fact, our unpublished data indicate that HEY1 also binds to Smads. Thus, the Notch pathway tightly represses TGFβ signaling through the binding of several of its targets to Smad proteins, indicating a closely interactive, but functionally antagonistic, network between the two pathways (model in Supplementary Fig. S10). Consistent with the in vitro data, Heyl knockout mice have enhanced TGFβ signaling and less mammary gland development. In addition, our in vivo mouse model shows that Heyl transgenic mice have enhanced cell proliferation during mammary gland development and develop mammary gland hyperplasia and mammary tumors. Similar phenotypes are seen between the abnormal mammary gland development in type II TGFβ receptor knockout mice or transgenic mice expressing dominant negative type II TGFβ receptor (28, 29, 30). Thus, Heyl transgenic mice can recapitulate most of the phenotypes of mice with reduced TGFβ signaling.

The early protection provided by the TGFβ pathway plays a central role in suppressing the formation of most types of cancers. Previous studies have identified an oncogene SnoN that interferes with TGFβ signaling in breast cancer in a manner similar to HEYL. However, HEYL and SnoN are regulated in a dissimilar fashion. SnoN expression is upregulated by TGFβ, whereas HEYL expression is induced by the Notch pathway (31). Moreover, HEYL is frequently overexpressed in human breast cancer but not in normal human breast tissues, whereas SnoN expression is present in normal breast epithelial cells, but is variable in breast cancer in terms of subcellular localization and expression level (32).

Extensive reports indicate that the Notch pathway promotes breast cancer development. However, the interplay between the Notch and TGFβ pathways in breast cancer is unclear. In this paper, we have provided several lines of evidence to show that HEYL is a novel negative regulator of the TGFβ pathway. The identification of HEYL as a direct target of the Notch pathway that associates with Smad proteins and inhibits TGFβ signaling provides a new insight into the tumor-promoting capabilities of the Notch pathway.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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**Figure 6.** Mammary gland hyperplasia and tumor development in Heyl transgenic mouse. A, H&E staining of mammary glands from wild-type and Heyl transgenic mice at 12 months of age. B, a and b, H&E staining of a mammary tumor and its adjacent hyperplasic lesion. c, a second representative mammary tumor of Heyl transgenic mouse.

Discussion

In this paper, we provide the first comprehensive report of the role of HEYL in breast cancer. We report that HEYL, a direct target gene of the Notch pathway, is found overexpressed in breast cancer. HEYL inhibits TGFβ signaling through direct binding to Smads. The basic and helix-loop-helix domains that mediate HEYL’s interaction with Smads are highly conserved among the members of HERP family. In fact, our unpublished data indicate that HEY1 also binds to Smads. Thus, the Notch pathway tightly represses TGFβ signaling through the binding of several of its targets to Smad proteins, indicating a closely interactive, but functionally antagonistic, network between the two pathways (model in Supplementary Fig. S10). Consistent with the in vitro data, Heyl knockout mice have enhanced TGFβ signaling and less mammary gland development. In addition, our in vivo mouse model shows that Heyl transgenic mice have enhanced cell proliferation during mammary gland development and develop mammary gland hyperplasia and mammary tumors. Similar phenotypes are seen between the abnormal mammary gland development in type II TGFβ receptor knockout mice or transgenic mice expressing dominant negative type II TGFβ receptor (28, 29, 30). Thus, Heyl transgenic mice can recapitulate most of the phenotypes of mice with reduced TGFβ signaling.

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The Notch Pathway Inhibits TGFβ Signaling in Breast Cancer through HEYL-Mediated Crosstalk

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