Notch Signal Activates Hypoxia Pathway through HES1-Dependent SRC/Signal Transducers and Activators of Transcription 3 Pathway

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Abstract

We report a Notch signal–induced pathway that leads to transcriptional activation of HIF1-α gene. HeLa/rtTAA/TRE-N1-IC cell line capable of doxycycline-induced expression of human Notch1-IC was established. The induction of Notch signaling activates HIF1-α and its target gene expression in HeLa/rtTAA/TRE-N1-IC cells. Notch signaling enhanced signal transducers and activators of transcription 3 (STAT3) phosphorylation required for HIF1-α expression. SRC kinase was found to be responsible for the enhanced STAT3 phosphorylation in response to Notch signaling. Activation of SRC/STAT3 pathway by Notch signaling was dependent on the expression of Notch effector HES1 transcription factor. The induction of HES1 enhanced STAT3 phosphorylation at Tyr 705 as well as SRC phosphorylation at Tyr 416 in inducible HeLa/rtTAA/TRE-HES1 cells, which express HES1 in response to doxycycline treatment. However, the treatment of Trichostatin A that interferes with HES1 transcriptional regulation did not affect STAT3 phosphorylation, and the expression of dominant negative HES1 failed to interfere with HES1-dependnent SRC/STAT3 pathway. These observations have led us to the conclusion that HES1-dependent activation of SRC/STAT3 pathway is independent of HES1 transcription regulation. This study first reports HES1-dependent SRC/STAT3 pathway that provides a functional link between Notch signaling and hypoxia pathway. (Mol Cancer Res 2009;7(10):1663–71)

Received 5/7/09; revised 7/14/09; accepted 7/20/09; published OnlineFirst 10/6/09.
Grant support: Korea Research Foundation (KRF-N01090253) and Korea Science and Engineering Foundation (Korea-Japan Bilateral Joint Project).
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This work was done in partial fulfillment of the requirement for PhD. degree (of J.S.) from Korea Advanced Institute of Science and Technology.
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doi:10.1158/1541-7786.MCR-09-0191

Introduction

Notch signaling is highly conserved through evolution and is crucial for cell death, proliferation, differentiation, and development. Notch signaling is initiated by interactions between Notch receptors (Notch1-4) and Notch ligands (Jagged1-2 and Delta1-4). In response to ligand binding to its cognate receptor, Notch receptor undergoes proteolytic cleavage, resulting in the release of Notch intracellular domain (Notch1-IC). Notch1-IC then enters the nucleus and forms a complex with RBP-jκ (also known as CSL) and histone acetyltransferase (CBP/p300) to activate the expression of Notch target genes such as *HES* and *HERP/HEY* family genes (1). HES family proteins are transcriptional repressors that act as Notch effectors by negatively regulating the expression of downstream target genes (2).

Hypoxia pathway is associated with oxygen homeostasis to maintain the appropriate level of ATP for the correct cellular metabolism. Hypoxia pathway is also a physiologic process that regulates cell proliferation and differentiation of various stem and precursor cells (3, 4). Transcription factor HIF1-α is an important mediator for oxygen homeostasis (5). During normoxia, HIF1-α is hydroxylated at specific proline residues by prolyl hydroxylases that act as oxygen sensors leading to the rapid ubiquitination followed by proteosomal degradation (6, 7). Under hypoxia, HIF1-α expression is elevated as a result of reduced ubiquitination and decreased proteosomal degradation. Stabilized HIF1-α is subsequently targeted into the nucleus where it binds with HIF1-β to form a heterodimeric HIF1 dimer. HIF1-α then binds, in conjugation with HIF1-β, to specific hypoxia response elements (HRE) in the promoters and enhancers of HIF1 target genes. Among the best characterized HIF1-α downstream target genes are genes involved in angiogenesis, erythropoiesis, and glycolysis, namely *differentiated embryo-chondrocyte expressed gene 1* (*DEC1*), *vascular endothelial growth factor* (*VEGF*) gene, *erythropoietin* (*EPO*) gene, and *phosphoglycerate kinase 1* (*PGK1*) gene (8-11).

Growing evidence implies functional links between Notch signaling and hypoxia pathway. For instance, hypoxia pathway amplifies Notch signaling from neighboring cells by inducing the expression of Notch ligands (12, 13) or activating transcription of Notch downstream target genes (4). The biochemical mechanism by which hypoxia pathway activates Notch signaling involves HIF1-α interaction with Notch1-IC and the recruitment of HIF1-α to Notch1-IC responsive promoters (4). Recent studies have also shown that Notch signaling is

involved in vasculogenesis and angiogenesis (14, 15). Therefore, we sought to answer whether Notch signaling affects hypoxia pathway. There are studies that support our rationales for the involvement of Notch signaling in hypoxia pathway. First, the expression of HIF1-α and its downstream target genes requires the phosphorylation of signal transducer and activator of transcription 3 (STAT3; refs. 16-18). Second, Notch signaling activates STAT3 phosphorylation (19, 20).

STAT3 was first identified as a DNA binding factor that is activated by IFN or IL6 cytokine (21-23). STAT3 is now believed to be a pleiotropic transcription factor that is crucial for cell proliferation, angiogenesis, and invasion during tumorigenesis. STAT3 activation is mediated through hormones, cytokines, and nonreceptor tyrosine kinases (24). STAT3 as well as other STAT family proteins contain an SH2 domain and a conserved tyrosine residue near the carboxyl terminus that is phosphorylated upon activation (25). Tyr 705 residue has been documented as the essential activating phosphorylation site in STAT3 (26). Thus far, Janus-activated kinase (JAK; refs. 25, 27), FES (28), SRC (29), and BRK (30) family nonreceptor tyrosine kinases have been reported for their abilities to phosphorylate STAT3.

The present study first reports HES1-dependent SRC/STAT3 pathway that provides a functional link between Notch signaling and hypoxia pathway.

Results

Notch Signaling Stimulates HIF1-α *Gene Expression*

To study the interplay between Notch signaling and hypoxia pathway, we established an inducible HeLa/rtTAA/TRE-N1-IC cell line expressing human Notch1-IC under transcriptional control of tetracycline operator sequence. Doxycycline treatment (1 μg/mL) induced subsequent transcriptional upregulation of Notch1-IC gene along with Notch downstream target gene *HES1* in HeLa/rtTAA/TRE-N1-IC cells (Fig. 1). To evaluate the role of Notch signaling on HIF1-α expression, we analyzed transcriptional expression of HIF1-α after the induction of Notch signaling in HeLa/rtTAA/TRE-N1-IC cells. Northern blot analysis revealed that Notch signaling upregulates transcription of *HIF1-α* gene under hypoxic conditions. To maintain hypoxic condition, cells were incubated in hypoxia chamber or treated with 200 μmol/L $CoCl_2$ for 24 hours. $CoCl_2$ was chosen as a hypoxia-mimicking agent to maintain the hypoxic signal throughout the experiments (31, 32). Transcription of HIF1-α gene was upregulated by the induction of Notch signaling under hypoxic conditions in HeLa/rtTAA/TRE-N1-IC cells (Fig. 2A). Increased expression of HIF1-α protein by the induction of Notch signaling in inducible HeLa cells was also shown by Western blot analysis (Fig. 2B). Intracellular expression of HIF1-α by the induction of Notch signaling under hypoxic condition was confirmed by immunofluorescence microscopy (Fig. 2C). In response to the induction of Notch signaling, transcription of HIF1-α downstream target genes was also upregulated. Notch signaling significantly increased luciferase reporter activity driven by HRE in HeLa/rtTAA/TRE-N1-IC cells under hypoxic condition (Fig. 2D). Notch signal also enhanced transcription of HIF1-α responsive VEGF and DEC1 genes under hypoxic condition as ascertained by Northern blot analysis (Fig. 2E). Notch signaling also activates HIF1-α transcriptional activity under hypoxic condition in 293T cells (Supplementary Fig. S1).

Notch Signaling Enhances STAT3 Phosphorylation

In Fig. 3, we showed that the induction of Notch signaling enhances STAT3 phosphorylation at Tyr 705 resulting in transcriptional upregulation of HIF1-α and its target genes. Neither hypoxic condition nor Notch signaling affected cellular level of STAT3 protein in HeLa/rtTAA/TRE-N1-IC cells. However, Notch signaling resulted in the increased STAT3 phosphorylation as well as HIF1-α expression in inducible HeLa cells under hypoxic condition (Fig. 3A and B). The positive role of Notch signaling on STAT3 phosphorylation was also supported by immunofluorescence microscopic observation. Phosphorylated STAT3 was detected in cells expressing ectopically expressed Notch1-IC, but not in neighboring cells (Fig. 3C). Recombinant adenovirus expressing dominant negative STAT3 (AdSTAT3-DN), in which activating phosphorylation site at Tyr 705 was replaced by Phe (33), was used to confirm the role of STAT3 phosphorylation on the activation of HIF1-α expression by Notch signaling. AdSTAT3-DN suppressed HIF1-α expression as well as STAT3 phosphorylation in HeLa/rtTAA/TRE-N1-IC cells (Fig. 3D).

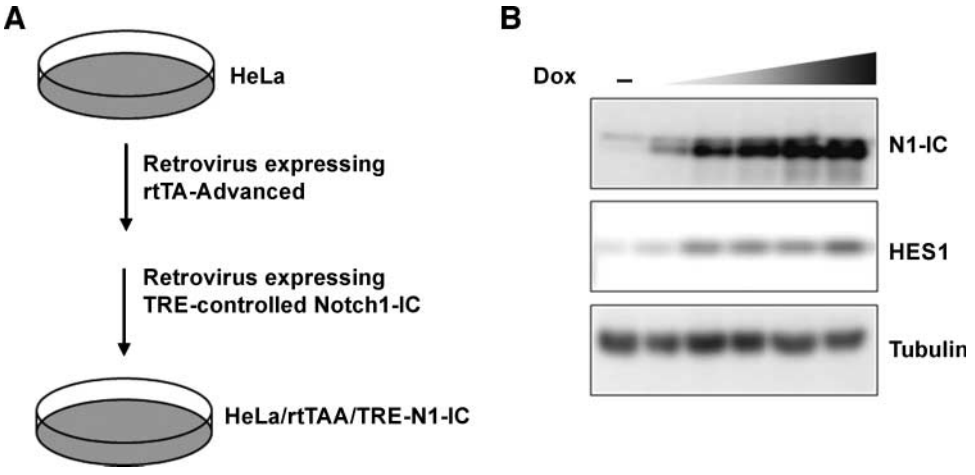

FIGURE 1. Establishment of HeLa/rtTAA/TRE-N1-IC cell line. **A.** An inducible HeLa cell line capable of doxycycline-controlled expression of human Notch1-IC was established as described in Materials and Methods. **B.** HeLa/rtTAA/TRE-N1-IC cells (5×10^5) were treated with concentrations of doxycycline (*Dox*; 0.01, 0.02, 0.1, 0.5, or 1 μg/mL). The induction of Notch1-IC and its downstream target gene HES1 expression were examined by immunoblot analysis.

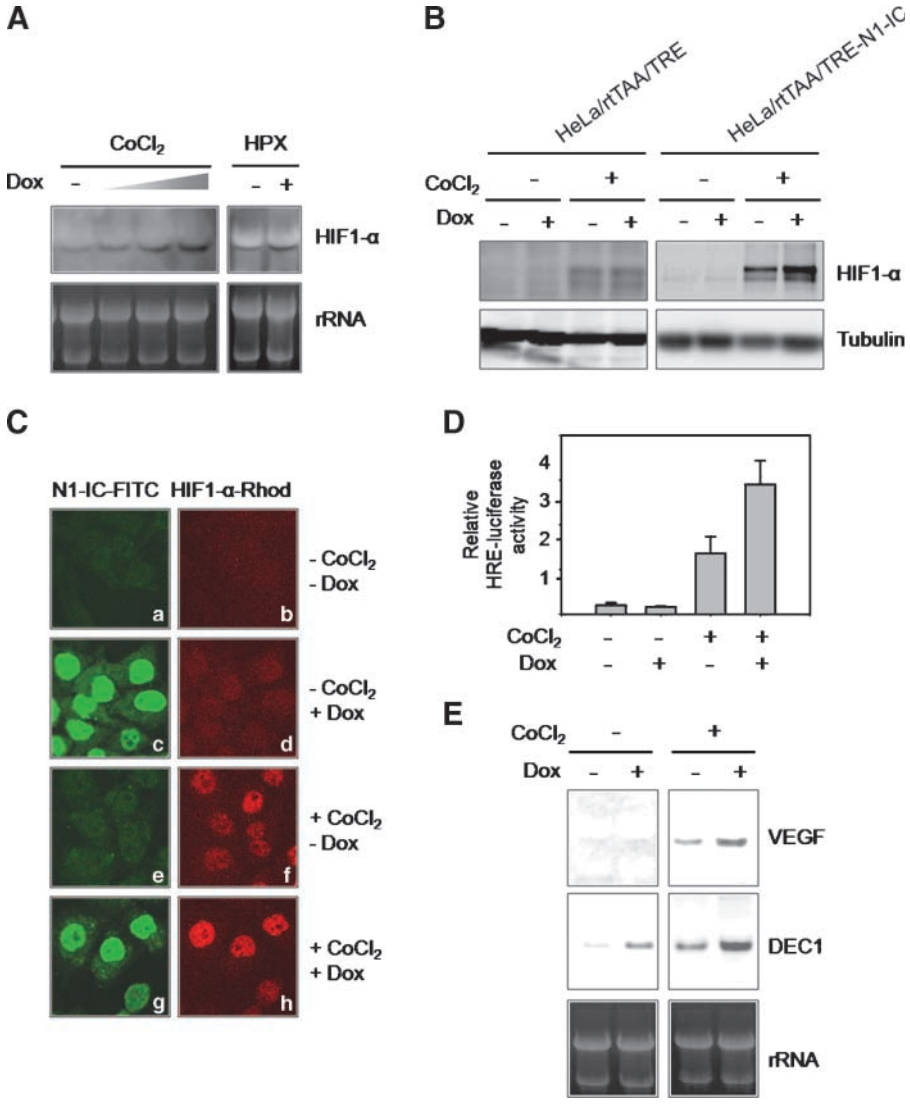

FIGURE 2. Notch signaling activates HIF1-α and its downstream target gene expression. **A.** HeLa/rtTAA/TRE-N1-IC cells (5×10^5) were treated with 0.02, 0.1, or 1 μg/mL doxycycline for 24 h. Cells were further incubated in hypoxia chamber or treated with 200 μmol/L $CoCl_2$ for 24 h. Transcription of HIF1-α gene was analyzed by Northern hybridization. **B.** At 24 h after the subsequent induction of Notch signaling by 1 μg/mL doxycycline (*Dox*) treatment, control HeLa/rtTAA/TRE and inducible HeLa/rtTAA/TRE-N1-IC cells were incubated in the presence of 200 μmol/L $CoCl_2$. After additional incubation for 24 h, cells were harvested and analyzed for intracellular expression of HIF1-α by immunoblotting. **C.** HeLa/rtTAA/TRE-N1-IC cells were treated with 1 μg/mL doxycycline for 24 h followed by the additional treatment with 200 μmol/L $CoCl_2$ for 24 h. Immunofluorescence microscopy was carried out to detect the expression of Notch1-IC or HIF1-α. **D.** HeLa/rtTAA/TRE-N1-IC cells (5×10^5) were transfected with pHRE-luc luciferase reporter vector containing HRE. At 3 h after transfection, Notch signaling was induced by the treatment of 1 μg/mL doxycycline for 24 h. Cells were further incubated in the presence of 200 μmol/L $CoCl_2$ for 24 h. Cells were then harvested and the reporter luciferase activities driven by HRE were determined. **E.** After the induction of Notch1-IC expression for 24 h, HeLa/rtTAA/TRE-N1-IC cells were treated with 200 μmol/L $CoCl_2$ for 24 h. Transcription of HIF1-α downstream target genes DEC1 and VEGF was examined by Northern hybridization.

SRC Tyrosine Kinase Is Responsible for Notch Signal–Induced STAT3 Phosphorylation

Data in Fig. 4 show that Notch signal–induced STAT3 phosphorylation is mediated by SRC tyrosine kinase. Treatment of HeLa/rtTAA/TRE-N1-IC cells with JAK inhibitor AG490 did not block Notch signal–induced STAT3 phosphorylation in HeLa/rtTAA/TRE-N1-IC cells under hypoxic condition. Contrarily, SRC inhibitor PP1 and nonspecific protein tyrosine kinase inhibitor Genistein abolished Notch signal–induced STAT3 phosphorylation. None of these protein tyrosine kinase inhibitors influenced the level of STAT3 expression in HeLa/rtTAA/TRE-N1-IC (Fig. 4A). Another SRC inhibitor SU6656 (34) also blocked Notch signal–induced STAT3 phosphorylation (Supplementary Fig. S2). Moreover, we found that Notch signaling stimulates the activating phosphorylation of endogenous SRC at Tyr 416 but does not affect the inhibitory phosphorylation at Tyr 527 in HeLa/rtTAA/TRE-N1-IC cells under hypoxic condition (Fig. 4B).

Notch Signal–Induced Activation of SRC/STAT3 Pathway Is Dependent on HES1

Because HES1 is a well-known Notch effector in mammals, we explored whether HES1 mediates Notch signal–induced activation of SRC/STAT3 pathway. HeLa cells were cotransfected with expression vector for Notch1-IC, HES1, or ΔRAM-Notch1-IC lacking transcriptional capability to activate HES1 promoter (35), together with luciferase reporter vector containing STAT3 binding element (2× SIE). Both Notch1-IC and HES1 expression transactivate luciferase reporter gene, whereas ΔRAM-Notch1-IC expression failed to transactivate luciferase reporter gene (Fig. 5A). The role of HES1 on Notch signal–induced activation of SRC/STAT3 pathway was examined in HeLa/rtTAA/TRE-HES1 cells capable of doxycycline-induced expression of human HES1. Induction of HES1 expression enhanced HIF1-α expression as well as STAT3 phosphorylation (Fig. 5B). Moreover, the induction of HES1 expression stimulated the phosphorylation of SRC at Tyr 416 in HeLa/rtTAA/TRE-HES1 cells (Fig. 5C and D).

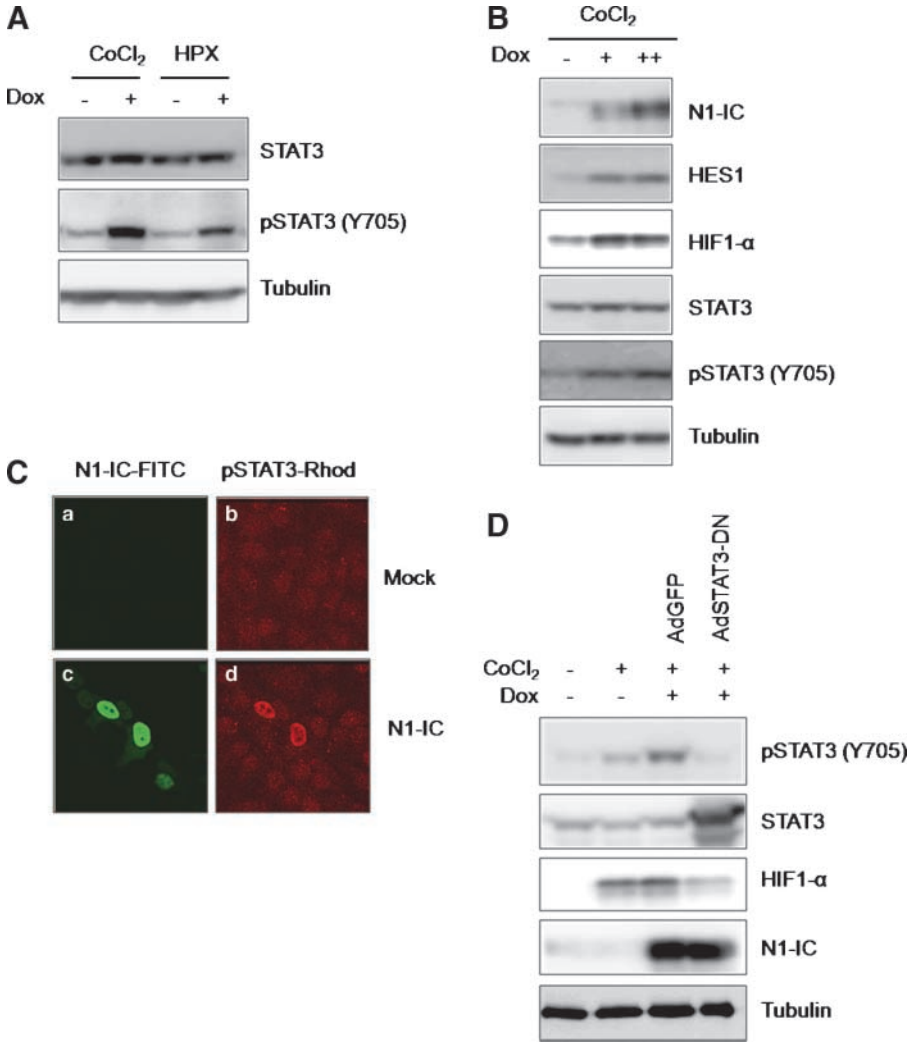

FIGURE 3. Notch signaling activates STAT3 phosphorylation. **A.** HeLa/rtTAA/TRE-N1-IC cells (5×10^5) were either treated with 1 μg/mL doxycycline or left untreated for 24 h. Cells were further incubated for 24 h in hypoxia chamber or treated with 200 μmol/L $CoCl_2$. Phosphorylation status of STAT3 was examined by immunoblot analysis using phospho-specific antibody that specifically recognizes phosphorylated STAT3 at Tyr 705. **B.** Effects of Notch signaling on cellular level of STAT3, phosphorylated STAT3, HES1, or HIF1-α in HeLa/rtTAA/TRE-N1-IC cells were examined by immunoblotting using respective antibodies. Notch signaling was induced by 0.1 or 1 μg/mL doxycycline treatment for 24 h. **C.** HeLa cells (5×10^5) were transfected with pFL2-N1-IC vector (1 μg). After incubation for 24 h, transfected HeLa cells were treated with 200 μmol/L $CoCl_2$ for 24 h. Cells were then fixed and probed with anti-Notch1 and anti–phosphorylated-STAT3 antibodies. The effect of Notch1-IC expression on the phosphorylation of STAT3 at Tyr 705 in transfected HeLa cells was visualized by immunofluorescence microscopy. **D.** HeLa/rtTAA/TRE-N1-IC cells (5×10^5) were infected with either recombinant adenovirus AdSTAT3-DN or control adenovirus AdGFP (multiplicity of infection of 5 each). Infected cells were treated with 1 μg/mL doxycycline for 24 h and postincubated for 24 h in the presence of 200 μmol/L $CoCl_2$. Immunoblot analysis was done to assess cellular level of Notch1-IC, STAT3, phosphorylated STAT3 or HIF1-α.

HES1-Dependent Activation of SRC/STAT3 Pathway Is Irrelevant to Transcriptional Regulation Function of HES1

Because HES1 is a transcriptional repressor, we need to answer whether or not the stimulation of SRC/STAT3 path by the induction of HES1 expression is associated with its transcriptional regulation function. We evaluated the effects of histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) known to abolish HES1-mediated transcriptional repression (36), on the activation of STAT3 phosphorylation in HeLa/rtTAA/TRE-HES1 cells. STAT3 phosphorylation was not influenced by TSA treatment (Fig. 6A). The expression of dominant negative HES1 also failed to interfere with STAT3 phosphorylation (Fig. 6B), implying that the activation of SRC/STAT3 pathway by HES1 is irrelevant to its transcriptional regulation function.

Discussion

Cellular level of HIF1-α is dependent on AKT signaling, which activates HIF1-α protein synthesis and pVHL-dependent ubiquitination of HIF1-α. We examined the role of Notch signaling on AKT pathway and found that levels of cellular AKT and AKT phosphorylation were not elevated by the induction of Notch signaling in HeLa/rtTAA/TRE-N1-IC (data not shown). We also observed that Notch signaling does not affect the ubiquitination of HIF1-α (data not shown). Crucial roles of STAT3 phosphorylation on transcription of HIF1-α gene and its downstream target genes have been described (24, 37). In response to hypoxia, STAT3 is activated by phosphorylation and enhances transcription of HIF1-α gene and its target genes (16). The present study showing the stimulation of STAT3 phosphorylation following the induction of Notch signaling (Fig. 3) implies that Notch signaling activates hypoxia pathway by enhancing STAT3 phosphorylation. In addition, the data representing the abrogation of Notch signal–induced STAT3 phosphorylation by SRC tyrosine kinase inhibitors implicate that SRC tyrosine kinase is responsible for the increased STAT3 phosphorylation in response to Notch signaling. SRC family kinases contain SH3, SH2, and SH1 domains followed by a short COOH-terminal regulation region. The phosphorylation of SRC at Tyr 416 in the activation loop produces an open structure where unlatched SH2 domain adopts an active conformation (38). Previous studies

have shown that COOH-terminal SRC kinase, a negative regulator of SRC tyrosine kinase, participates in the inhibitory phosphorylation of Src at Tyr 527 (39). However, we could rule out the possibility that Notch signaling modulates SRC activity through inhibitory phosphorylation by COOH-terminal SRC kinase, because the induction of Notch signaling in HeLa/rtTAA/TRE/N1-IC cells neither changed COOH-terminal SRC kinase mRNA level (Supplementary Fig. S3) nor the inhibitory phosphorylation of SRC at Tyr 527 (Fig. 4B).

How Notch1-IC in the nucleus can activate cytoplasmic SRC kinase is the most intriguing question about SRC/STAT3-mediated activation of hypoxia pathway by Notch signaling. Our data in Fig. 5 show that Notch signaling activates SRC in a manner dependent on HES1 in inducible HeLa/rtTAA/TRE-HES1 cells. HES1 is present both in the nucleus and cytoplasm. In particular, HES1 is expressed exclusively in the cytoplasm during tumorigenesis (40) and differentiation (41). HES proteins have been known as Notch downstream genes encoding HES factors. Among HES factors, HES1 has been best characterized for its *in vivo* targets (2). HES1 is a basic helix-loop-helix transcription regulator that represses transcription of numerous HES1 target genes in many mammalian progenitor cells. The list of HES1 target genes includes calcipressin, acid-glucosidase, CD4, E2F1, Mash1, p27, p21, p57, Mdm2, and HES1 itself (42). HES1 represses transcription of target genes by directly binding to the specific DNA sequences. For example, HES1 binds to the N-box–related DNA sequences in the promoter regions of Mash1 (43), p27 (44), and E2F1 (45). HES1 is also known to interact with various cellular proteins in both cultured cell system and animal models. HES1 protein interacts with other HES and HERP family proteins to form homodimers and heterodimers. Transcription factors such as Mash1, c-*myc*, RBP-jκ, and STAT3 and transcription cofactors such as HDAC and CBP have been reported for their abilities to interact with HES1 (42).

However, our data imply that transcriptional regulation function of HES1 is irrelevant to the activation of HES1-dependent SRC/STAT3 pathway, because STAT3 activation in HeLa/rtTAA/TRE-HES1 cells was not affected by the treatment of HDAC inhibitor, TSA, which interferes with transcriptional regulation function of HES1 (Fig. 6A; ref. 36). Dispensable role of HES1 transcriptional regulation function on the activation of SRC/STAT3 pathway was further supported by the fact that STAT3 phosphorylation was not affected by dominant negative HES1, which inactivates transcriptional function of HES1 by forming a heterodimer (Fig. 6B; ref. 45). The mechanism through which HES1 stimulates SRC phosphorylation at Tyr 416 in transcription-independent manner remains to be determined. Kumakura (19) have reported cellular interaction of HES1 protein with both STAT3 and JAK tyrosine kinase in cultured mammalian cells and proposed that HES1 facilitates complex formation between JAK tyrosine kinase and STAT3, promoting STAT3 phosphorylation by JAK. It is reasonable to anticipate that HES1 interacts with SRC during the activation of SRC/STAT3 pathway in response to Notch signaling. However, our study of cellular interaction between HES1 and SRC in 293T and HeLa cells did not support the intermolecular association between these two proteins (data not shown). Therefore, HES1 does not likely to participate in the activation of SRC kinase activity through its interaction with SRC.

The present study proposes HES1-dependent SRC/STAT3 signal path leading to the activation of hypoxia pathway in response to Notch signaling. How HES1 modulates SRC activity demands further investigation.

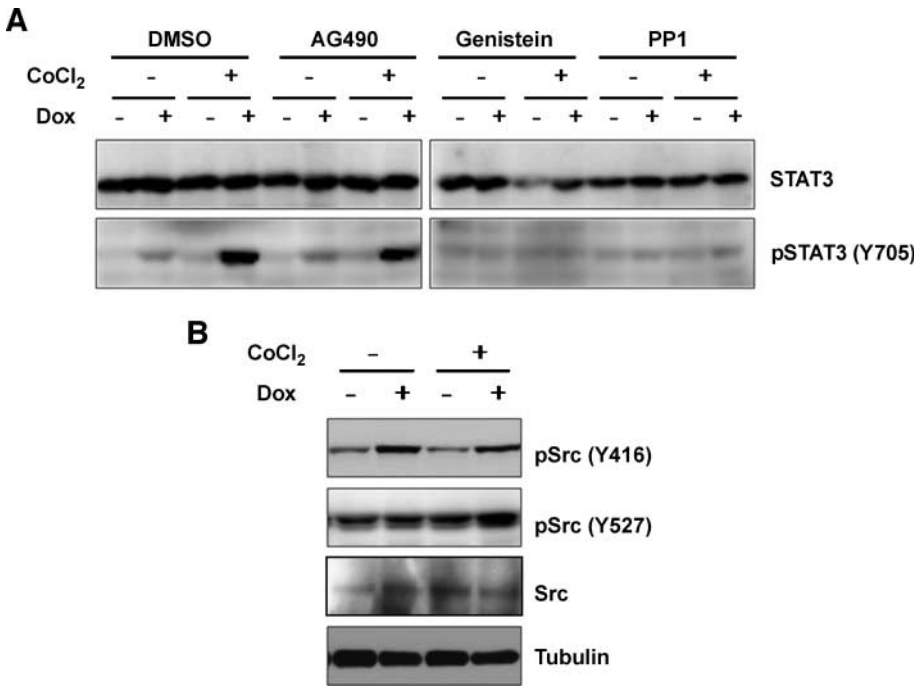

FIGURE 4. SRC tyrosine kinase is responsible for Notch signal–induced STAT3 phosphorylation. **A.** HeLa/rtTAA/N1-IC cells (5×10^5) were treated with 40 μmol/L AG490, 100 μmol/L Genistein, or 200 μmol/L PP1 for 12 h. Cells were then treated with 1 μg/mL doxycycline for 24 h. Cells were further incubated in the presence or absence of 200 μmol/L $CoCl_2$ for 24 h. Effects of tyrosine kinase inhibitors on STAT3 phosphorylation were examined by immunoblot analysis using antibodies against STAT3 and phosphorylated STAT3 at Tyr 705. **B.** After the induction of Notch signaling, HeLa/rtTAA/TRE-N1-IC cells were further treated 200 μmol/L $CoCl_2$ for 24 h and were examined for the activating phosphorylation of SRC at Tyr 416 and the inhibitory phosphorylation at Tyr 527.

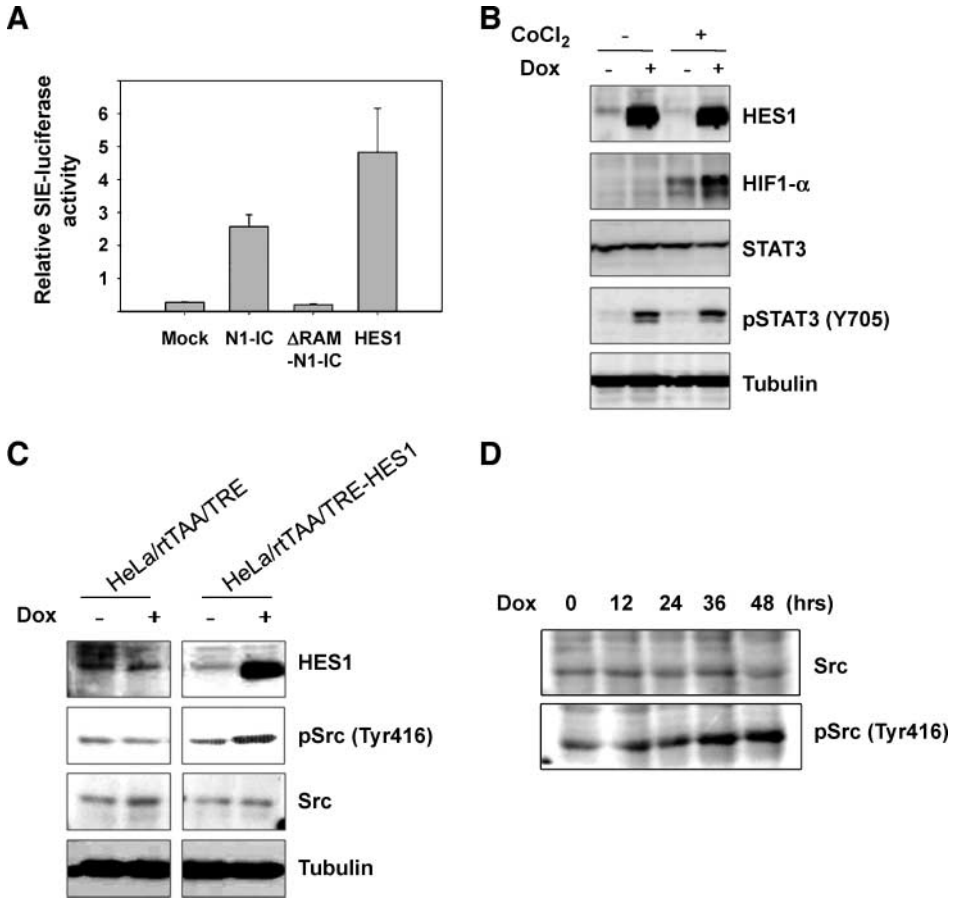

FIGURE 5. Activation of SRC/STAT3 pathway by Notch signaling is dependent on HES1. **A.** HeLa cells (5×10^5) were cotransfected with expression vector for HES1, Notch1-IC, or ΔRAM-Notch1-IC (0.5 μg each) along with luciferase reporter vector (0.5 μg) containing 2× SIE (STAT3 responsive element). After incubation for 24 h, reporter luciferase activities were measured. **B.** HeLa/rtTAA/TRE-HES1 cells were treated with 1 μg/mL doxycycline for 24 h and incubated for 24 h in the presence of 200 μmol/L $CoCl_2$. Effects of HES1 induction on intracellular level of STAT3, phosphorylated STAT3 at Tyr 705, or HIF1-α in HeLa/rtTAA/HES1 cells were examined by immunoblot analysis using respective antibodies. **C.** HeLa/rtTAA/TRE or HeLa/rtTAA/TRE-HES1 cells were treated with 1 μg/mL doxycycline for 48 h. Cells were examined for the activation phosphorylation of SRC at Tyr 416 by immunoblot analysis. **D.** HES1 expression was induced in HeLa/rtTAA/TRE-HES1 cells by treating with doxycycline (1 μg/mL) for the specified amount of time. The activation of SRC phosphorylation at Tyr 416 after the induction of HES1 expression was monitored.

Materials and Methods

Antibodies and Reagents

Antibodies against Notch1 (C-20), HES1 (H-140), STAT3 (H-190), and phosphorylated STAT3 at Tyr 705 (B-7) were purchased from Santa Cruz. Monoclonal antibody against HIF1-α was from BD Transduction Laboratories. Antibodies directed against SRC and phosphorylated SRC at Tyr 416 or Tyr 527 were from Cell Signaling. Doxycycline was supplied by Fluka. SRC inhibitor PP1 was from Calbiochem, whereas JAK2 inhibitor AG490 and general protein tyrosine kinase inhibitor Genistein were from Sigma. HDAC inhibitor TSA was also supplied by Sigma.

Vectors

pFLAG-CMV2-N1-IC (pFL2-N1-IC) vector encoding full-length human Notch1-IC (residues 1762-2556) and pFLAG-CMV2-ΔRAM-N1-IC (pFL2-ΔRAM-N1-IC) vector encoding Notch1-IC deletion mutant lacking RAM domain (residues 1877-2556) were generated as described previously (35). Recombinant adenoviral vector encoding dominant negative STAT3 mutant (AdSTAT3-DN) was provided by Dr. Y. Fujio (Osaka University, Osaka, Japan; ref. 33). Flag-tagged dominant negative HES1 expression vector (pcDNA3.1-dnHES1) was a gift from Dr. Anders Strom (Karolinska Institute, Huddinge, Sweden; ref. 45).

Inducible HeLa Cell Lines

HeLa/rtTAA/TRE-N1-IC cell line capable of doxycycline-induced expression of human Notch1-IC was established by using Tet-On Advanced Inducible Gene Expression System (Takara-Clontech). The full-length rtTA-Advanced (rtTAA) gene was excised by *Eco*RI/*Bam*HI digestion and subcloned into *Eco*RI/*Bam*HI sites of retroviral pcz-CFG5-IEGZ vector (46) to construct pCFG-rtTAA vector. Notch1-IC gene was subcloned into pRevTRE (Takara-Clontech), a doxycycline-regulated retroviral transfer vector, to generate pRevTRE-N1-IC vector. HeLa cell line expressing rtTAA was generated by retrovirus-mediated gene transfer. Briefly, 293T cells were cotransfected with pCFG-rtTAA, pGP (Takara), and pVpack-VSV-G vectors (Stratagene). At 48 h after transfection, the culture medium containing viral particles was collected and used to infect HeLa cells. Infected HeLa cells were cultured in the presence of Zeocin (0.3 mg/mL) to select cells expressing rtTAA. To prepare recombinant retrovirus containing tetracycline response element (TRE)–regulated Notch1-IC gene, 293T cells were cotransfected with pRevTRE-N1-IC, pGP, and pVpack-VSV-G vectors. Culture medium containing retrovirus particles was collected and used to infect HeLa cells expressing rtTAA. HeLa/rtTAA/TRE-N1-IC cells were selected in the presence of 0.3 mg/mL hygromycin. The full-length HES1 gene fragment was excised from pCI-HES1 (47) by *Bam*HI/*Xmn*I digestion and subcloned into *Bam*HI/*Hpa*I site

of pRevTRE. Inducible HeLa/rtTAA/TRE-HES1 cell line was established by the same procedure except that pRevTRE-HES1 was used instead of pRevTRE-N1-IC. To construct retroviral transfer vector pLPCX-dnHES1, dnHES1 gene in pcDNA3.1-dnHES1 was excised by B*am*HI/X*ho*I and subcloned into E*co*RI site of pLPCX (Clontech) by blunt end ligation. HeLa/rtTAA/TRE-N1-IC/dnHES1 or HeLa/rtTAA/TRE-HES1/dnHES1 cell line was established by the same procedure as described above.

Northern Blot Analysis

Notch1-IC expression in HeLa/rtTAA/TRE-N1-IC cells was induced by 1 μg/mL doxycycline treatment. At 24 h after the induction of Notch signaling, cells (1×10^6) were grown in hypoxia chamber or treated with 200 μmol/L $CoCl_2$ for 24 h. Cells were harvested and total RNAs were extracted using Trizol Reagent (Molecular Research Center). The hybridization probes were generated using PCR DIG-labeling kit (Roche). Specific primer for *VEGF* gene (forward 5′-TGA ACT TTC TGC TGT CTT GGG T-3′, reverse 5′-AAG GCC CAC AGG GAT TTT CTT-3′), *DEC1* gene (forward 5′-ACG GAG ACC TAC CAG GGA TGT AC-3′, reverse 5′-TTG GCC AGA TAC TGA AGC ACC T-3′), or *HIF1-α* gene (forward 5′-TGC TTG CCA AAA GAG GTG GA-3′, reverse 5′-ATT TTT CGT TGG GTG AGG GG-3′) was used for PCR to produce DIG-labeled probes. Whole-cell RNAs were hybridized with DIG-labeled probes and assayed (Roche Molecular Biochemicals).

Luciferase Assay

The role of Notch signaling on HIF1-α transcriptional activity in HeLa/rtTAA/TRE-N1-IC cells was examined by luciferase reporter assay. For HIF1-α reporter assay, HeLa/rtTAA/TRE-N1-IC

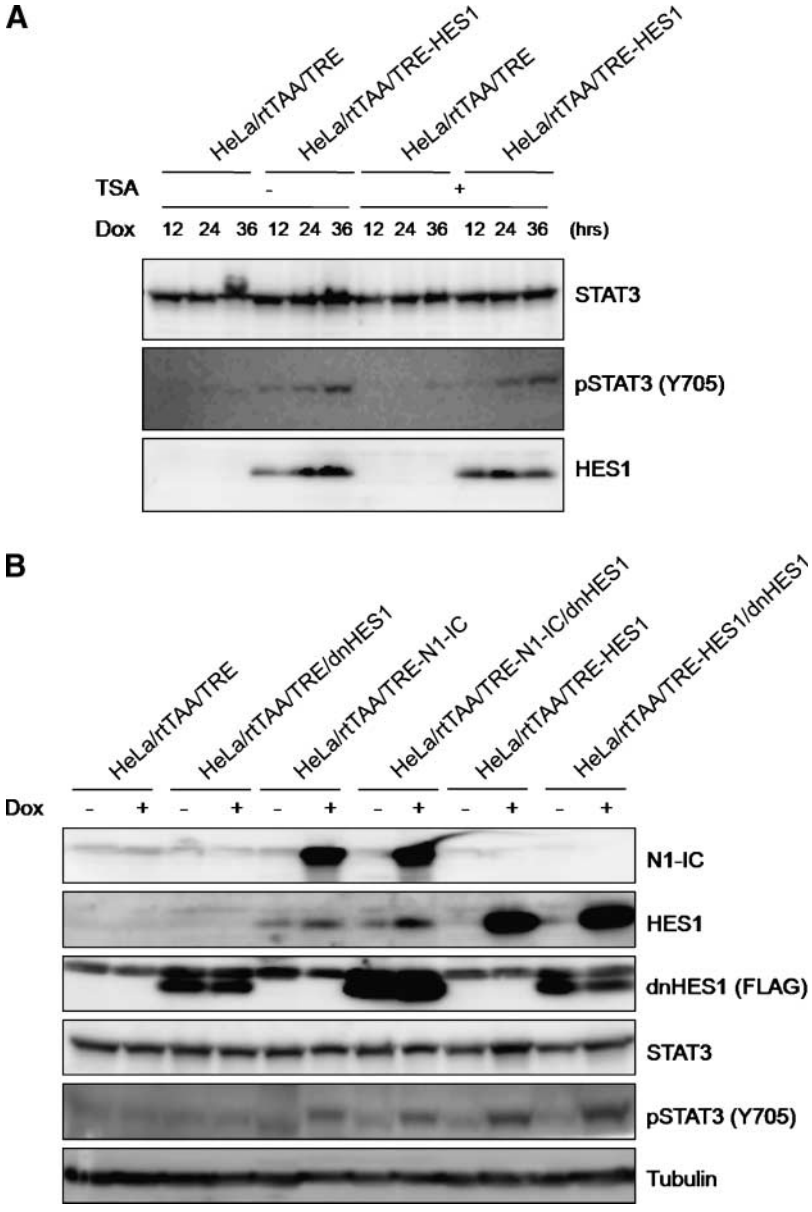

FIGURE 6. HES1-dependent activation of SRC/STAT3 pathway is mediated by transcription-independent mechanism. **A.** TSA does not affect HES1-dependent STAT3 activation. HeLa/rtTAA/TRE-HES1 cells were treated with 1 μg/mL doxycycline and 20 nmol/L of TSA for the stated amount of time. Cells were examined for cellular level of STAT3, phosphorylated STAT3 at Tyr 705, or HES1 by immunoblot analysis. **B.** HES1 transcriptional regulation function is irrelevant to HES1-mediated activation of SRC/STAT3 pathway. HeLa/rtTAA/TRE-N1-IC/dnHES1 or HeLa/rtTAA/TRE-HES1/dnHES1 cell line stably expressing dominant negative HES1 (*dnHES1*) was established. After induction of Notch1-IC or HES1 by doxycycline (1 μg/mL) treatment for 48 h, cellular level of STAT3 or phosphorylated STAT3 at Tyr 705 was analyzed by immunoblotting in respective cell lines.

cells (5×10^5) were transfected with pHRE-luc (100 μg) luciferase reporter vector containing HRE. At 3 h after transfection, cells were treated with 1 μg/mL doxycycline for 24 h. After additional incubation for 24 h in the presence of 200 μmol/L $CoCl_2$, cells were harvested and lysed in Passive Lysis Buffer (Takara-Clontech). The luciferase activities driven by HRE were analyzed using Luciferase Assay System (Takara-Clontech). The values of luciferase activities were normalized for transfection efficiencies by measuring β-galactosidase activities using Luminescent β-galactosidase detection kit II (Takara-Clontech). The role of Notch1 effector HES1 on STAT3 transactivation was evaluated in cotransfected HeLa cells. HeLa cells were cotransfected with luciferase reporter vector containing 2× SIE (STAT3 responsive element; ref. 48) together with pFL2-N1-IC, pFL2-ΔRAM-N1-IC, or pCI-HES1 expression vector.

Immunofluorescence Microscopy

HeLa/rtTAA/TRE-N1-IC cells (5×10^5) were treated with 1 μg/mL doxycycline for 24 h to induce Notch1-IC expression. Cells were treated with 200 μmol/L $CoCl_2$ or left untreated for 24 h. Cells were fixed with 4% formaldehyde, permeabilized with PBS containing 0.5% Triton X-100 at 4°C, and blocked with 2% bovine serum albumin in PBS containing 2% Tween 20. Cells were immunostained with primary antibodies against Notch1 and HIF1-α in PBS containing 2% Tween 20, containing 2% bovine serum albumin for 10 h at 4°C. The antigen-primary antibody complex was detected using tetramethylrhodamine isothiocyanate–labeled goat anti-mouse secondary antibody and FITC-labeled goat anti-rabbit secondary antibody. Microscopic observation was done under a confocal laser scanning microscope (Carl Zeiss). HeLa cells (5×10^5) were transfected with pFL2-N1-IC expression vector (1 μg), fixed, and stained with antibodies directed against Notch1 and phosphorylated STAT3 at Tyr 705.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Dong Soo Im for providing pHRE-luc plasmid and Dr. Y. Eugene Chin for the gift of p2X SIE-luc plasmid.

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