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Snail inhibits Notch1 intracellular domain mediated transcriptional activation via competing with MAML1

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ABSTRACT

Notch1 intracellular domain (NICD) is the transcription factor which controls cell fate and differentiation in embryonic and tumor cells. Snail has a critical role which increases invasion and metastasis of cancer cell as a transcription factor and epigenetic regulator. Recently, we discovered NICD induced Snail degradation by direct binding interaction with Snail. In this experiment, we found that Snail suppressed transcriptional activity of the protein complex formed with NICD and RBPJk in nucleus. Moreover, Snail decreased transcription of NICD target genes via competing with MAML1, co-activator, in NICD complex. In conclusion, Snail inhibited NICD-mediated transcriptional activation of target genes by physical interaction with NICD.

1. Introduction

Snail is an important oncogenic protein which induces epithelial-mesenchymal transition by acting as a transcription factor and epigenetic regulator [1,2]. Snail is composed of N-terminal SNAG domain for the interaction with other transcription factors, internal serine-rich domain for regulation of its function by protein modification, and C-terminal zinc finger domain (ZF) for DNA binding [1].

Snail acts as a transcriptional repressor of E-cadherin promoter and a transcriptional activator of MMPs through its DNA binding property [1]. Also, it induces methylation of E-cadherin promoter by interaction with HDAC and DNMT and represses E-cadherin expression which results in increase of cell invasiveness [2,3]. Besides, Snail is induced to degrade by interaction with well-known tumor Suppressor p53 [4]. Thus, Snail is a target for inhibiting cell invasion by p53.

Notch1 is a transmembrane receptor protein which controls signal pathway inducing appropriate cell fate by regulating tumor cell differentiation [5]. Notch1 is activated by juxtacrine ligand-receptor interaction and is cleaved into Notch1 intracellular domain (NICD) by gamma-secretase [5]. Then, NICD translocates to nucleus and increases target gene transcription via inhibiting repression of target genes by CSL (CBF1/RBPJk in vertebrates, Suppressor of hairless in Drosophila, Lag-1 in *Caenorhabditis elegans*) through binding interaction with CSL and MAML1, which is a co-activator of NICD [5,6].

NICD consists of a RBP-J kappa-associated module (RAM) domain, an 6 ankyrin/cdc10 repeats (ANK) domain, 2 nuclear localization signals, a transcriptional transactivation domain (TAD), an polyglutamine tract (OPA) domain, and a proline, glutamic acid, serine, and threonine-rich region (PEST) domain [5]. Among them, CSL is known to interact with RAM and ANK domain, and MAML1 is known to interact with ANK domain [7].

NICD increases Snail mRNA expression by acting as a transcriptional factor of Snail promoter, and synergistically increases Snail expression in hypoxic condition [8]. In our recent research, we elucidated NICD interacts with Snail in nucleus and ANK domain of NICD and ZF domain of Snail are necessary for the binding interaction of two proteins [9]. Through the binding between NICD and Snail, NICD induces degradation of Snail and inhibits Snail-induced cell invasiveness [9]. Despite the interaction between NICD and Snail acting as transcriptional factors, the regulation of NICD-mediated transcriptional activation via this interaction has not been studied.

We have studied the role of Snail in regulation mechanism of NICD transcriptional activity and NICD transcription factor complex formation. Our results showed that Snail inhibited the interaction between NICD and its co-activator MAML1 via competitive interaction and therefore Snail repressed the NICD-mediated transcriptional activation.

Abbreviations: NICD, Notch1 intracellular domain; MAML1, mastermind-like protein 1; RBPJk, recombination signal binding protein for immunoglobulin kappa J region; CSL, CBF1/RBPJk in vertebrates/Suppressor of hairless in Drosophila/Lag-1 in *C. elegans*; ANK, 6 ankyrin/cdc10 repeats domain; ZF, zinc finger domain.

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2. Materials and methods

2.1. Cell culture and treatments

Hep3B (human hepatoma cells) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). All other reagents were purchased from Sigma (St. Louis, MO).

2.2. Plasmids

Human *SNAI1*, *Notch1* intracellular domain, *RBPJk*, and *MAML1* cDNAs were obtained from Huh7 cell lines and cloned into pCMV/HA, pCMV/Myc, and/or p3xFlag/CMV expression vectors. Using pCMV/HA-Snail as a template, SNAG domain deleted Snail-construct (ΔSNAG), zinc finger region deleted Snail-construct (ΔZF), and zinc finger region construct (ZF) were generated by PCR with the following primers: 5′-AGAATTCGGAAGCCCTCCGAC-3′ (forward for ΔSNAG), 5′-AGCGGCCGCTCAGCGGGGACATCC-3′ (reverse for ΔSNAG), 5′-AGAATTCCGCGCTCTTTCCTC-3′ (forward for ΔZF), 5′-AGCGGCCGCTCAT CGAGCCTGGAGATCCTTGGC-3′ (reverse for ΔZF), 5′-AGAATTCCGAAGGCCTTCAACTGCAAATAC-3′ (forward for ZF), and 5′-AGCGGCCGCTCAGCGGGGACATCC-3′ (reverse for ZF). All constructs were confirmed by sequencing.

2.3. Antibodies

Rat and mouse monoclonal anti-Snail antibodies (Cell Signaling Technology Inc.), rabbit polyclonal anti-Snail antibody (Abcam) rabbit monoclonal anti-Notch1 antibody (Epitomics Inc., Burlingame, CA), mouse monoclonal anti-Myc antibody (Santa Cruz), mouse monoclonal anti-Flag antibody (Sigma), rabbit polyclonal anti-HA antibody (Abcam), and mouse monoclonal anti-β-actin antibody (Sigma) were used in the study.

2.4. Immunoblot analysis, immunoprecipitation, and immunocytochemistry

Cell lysates and immunoblot analysis were performed as described [10]. Band intensity was determined using ImageMaster 2D Elite software 4.01 (Amersham, Upsala, UK). In HCC tissue analysis, changes in protein expression were evaluated by dividing the intensity of the signal seen in tumor tissue by that seen in non-tumor tissue. For Immunoprecipitation, Hep3B cells were transfected with Flag-Snail, HA-NICD, and Myc-MAML1. After 48 h, cells were lysed in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) and centrifuged at 16,000*g* for 15 min to remove debris. Cleared lysates were subjected to immunoprecipitation with antibodies. For immunocytochemistry, cells were fixed in 4% paraformaldehyde at RT for 15 min and permeablized in 5% Triton-X100 5 min and then stained using primary antibodies. The secondary antibodies were anti-mouse Alexa 647, anti-goat Alexa 594, anti-mouse Alexa 594, and/or anti-rabbit Alexa 488 (Molecular Probes, Carlsbad, CA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). After mounting, cells were visualized with a multi-photon confocal laser scanning microscope (Carl Zeiss, Thornwood, NY).

2.5. Promoter analysis

To analyze promoter activity, luciferase assays were performed with the 4x-CSL, Hes1, and Hes5 promoters (kindly provided by Dr. Kopan). Cells were plated at 1.5 × 105 cells/ml in 6-well plates one day before transfection. Non-liposomal-mediated gene transfer was performed using FuGENE6 (Roche) according to the manufacturer's instructions, using 2 μg of DNA consisting of the reporter construct, expression vectors, or the internal control vector. Thirty hours after transfection, cells were analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay (Promega) following the manufacturer's instructions and quantified with a Luminoskan Ascent Luminometer (Thermo LabSystems).

2.6. Statistical analysis

Data in bar graphs are expressed as the mean and standard deviation of three independent experiments. All results in bar graphs are expressed as the fold ratio relative to untreated or control cells. Statistical analysis was performed using SPSS statistics software (Ver.19, SPSS Inc., Chicago, IL).

3. Results

3.1. Snail inhibits NICD-mediated transcriptional activation in dose-dependent manner

When 4xCSL-Luc vector, which is reporter to measure NICD transcriptional activity, was transfected along with NICD expression vector in Hep3B cells, the transcriptional activation of 4xCSL promoter was increased (Fig. 1A). However, when Snail expression vector was transfected along with NICD, the transcriptional activation of 4xCSL promoter, which was increased by NICD, was decreased by Snail in dose-dependent manner (Fig. 1A). Also, the reporter assay using other promoter of NICD target genes, Hes1 and Hes5 was performed and it confirmed the inhibition of transcriptional activation of those promoters which was decreased by Snail dose-dependently (Fig. 1B and C). In immunoblotting using the same cells sample, NICD expression level remained the same despite increase in Snail transfection (Fig. 1D).

Overexpression of Snail did not change the NICD expression level while it decreased the transcription of NICD target gene promoter. According to these results, Snail takes a role as a transcriptional repressor of NICD target genes.

3.2. Zinc finger domain of Snail is important in repression of NICD transcriptional activity

We constructed Snail deletion mutant expression vectors that deleted SNAG domain, which is required for recruit HDAC1 and DNMT1, or zinc finger domain, which is required for DNA binding (Fig. 2A) [1]. Then, we measured the transcriptional activation of 4xCSL promoter after overexpression with wild type Snail or three truncated Snail each along with NICD.

As a result, NICD-mediated transcriptional activation was approximately inhibited by 75% and 50% by Snail and ΔSNAG and by zinc finger domain, respectively (Fig. 2B). However, in the absence of zinc finger domain, ΔZF, NICD-mediated transcriptional activation was only inhibited by approximately 25% (Fig. 2B). Also, wild type Snail and three truncated Snail did not influence on NICD expression level (Fig. 2C). Conclusively, the Snail ZF domain was important to inhibit NICD function through the direct physical interaction with NICD.

3.3. Snail inhibits NICD-mediated transcriptional activation via competing with MAML1

NICD forms complex with RBPJk and MAML1 in CSL motif of NICD target gene promoter, and performed transcriptional regulation [5]. Therefore, we measured the transcriptional activation of 4xCSL promoter after expression of NICD, RBPJk, MAML1, and Snail separately or co-expression of these factors.

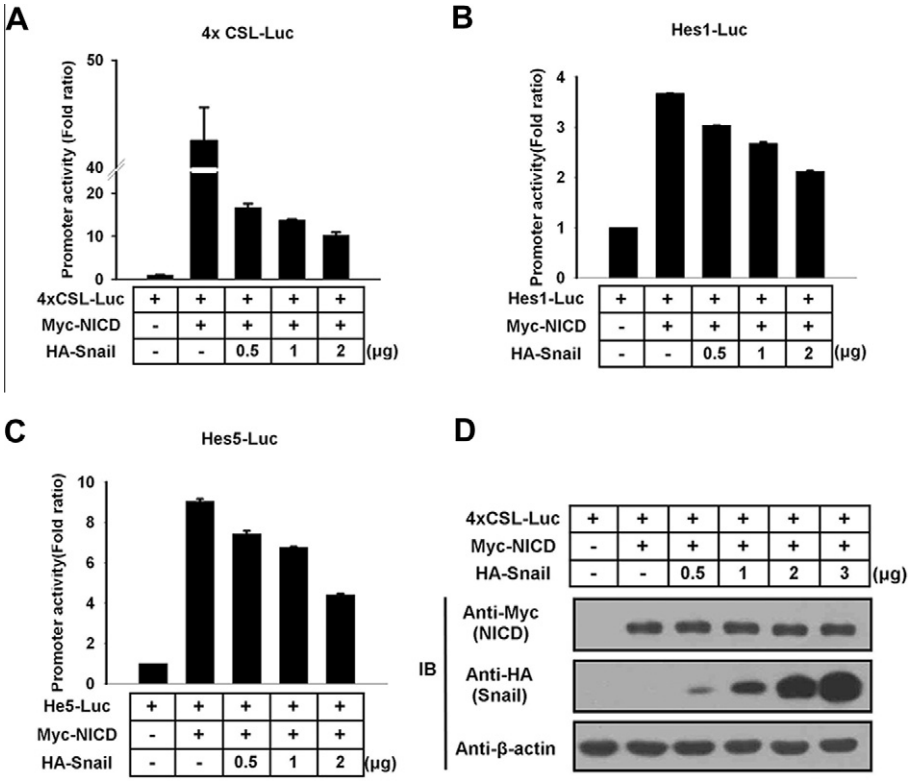

Fig. 1. Snail inhibits NICD-mediated transcriptional activation in dose-dependent manner. Hep3B cells were transfected with Myc-NICD and/or HA-Snail expression vectors and a 4xCSL (A), Hes1 (B), or Hes5 (C) promoter fused to luciferase for 48 h and analyzed for luciferase activity. (D) In the same samples of (A), expression of Myc-NICD and HA-Snail was analyzed by immunoblot. β-actin served as a internal control.

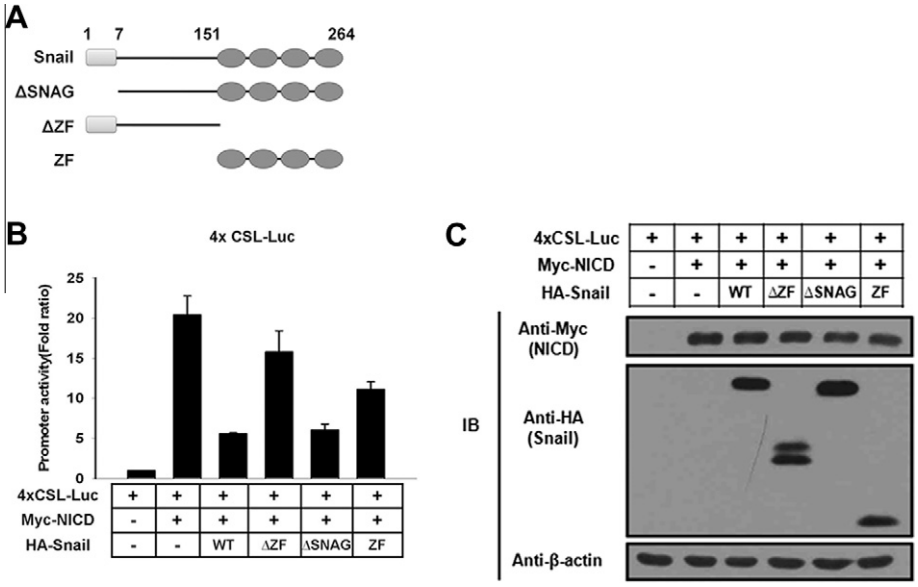

Fig. 2. Snail zinc finger domain is important in the NICD inhibition. (A) Schematic diagram of Snail and deletion mutants. (B) Hep3B cells were transfected with Myc-NICD and/or HA-Snail WT, ΔSNAG, ΔZF, and ZF expression vectors and the 4xCSL promoter fused to luciferase for 48 h and analyzed for luciferase activity. (C) In the same samples of (B), expression of Myc-NICD and HA-Snail and its mutants was analyzed by immunoblot. β-actin served as a internal control.

As a result, Snail inhibited NICD-mediated transcriptional activation and MAML1 recovered decrease in NICD-mediated transcriptional activation by Snail (Fig. 3A). When increasing amount Snail was transfected with same amount of MAML1 transfection, the transcriptional activation of 4xCSL promoter was decreased by Snail dose-dependently (Fig. 3B). On the contrary, when increasing amount of MAML1 transfection with same amount of Snail transfection, the transcriptional activation of 4xCSL promoter was increased by MAML1 dose-dependently (Fig. 3C). According to the results, Snail inhibited NICD-mediated transcriptional activation via acting like its co-repressor in contrary to its co-activator MAML1.

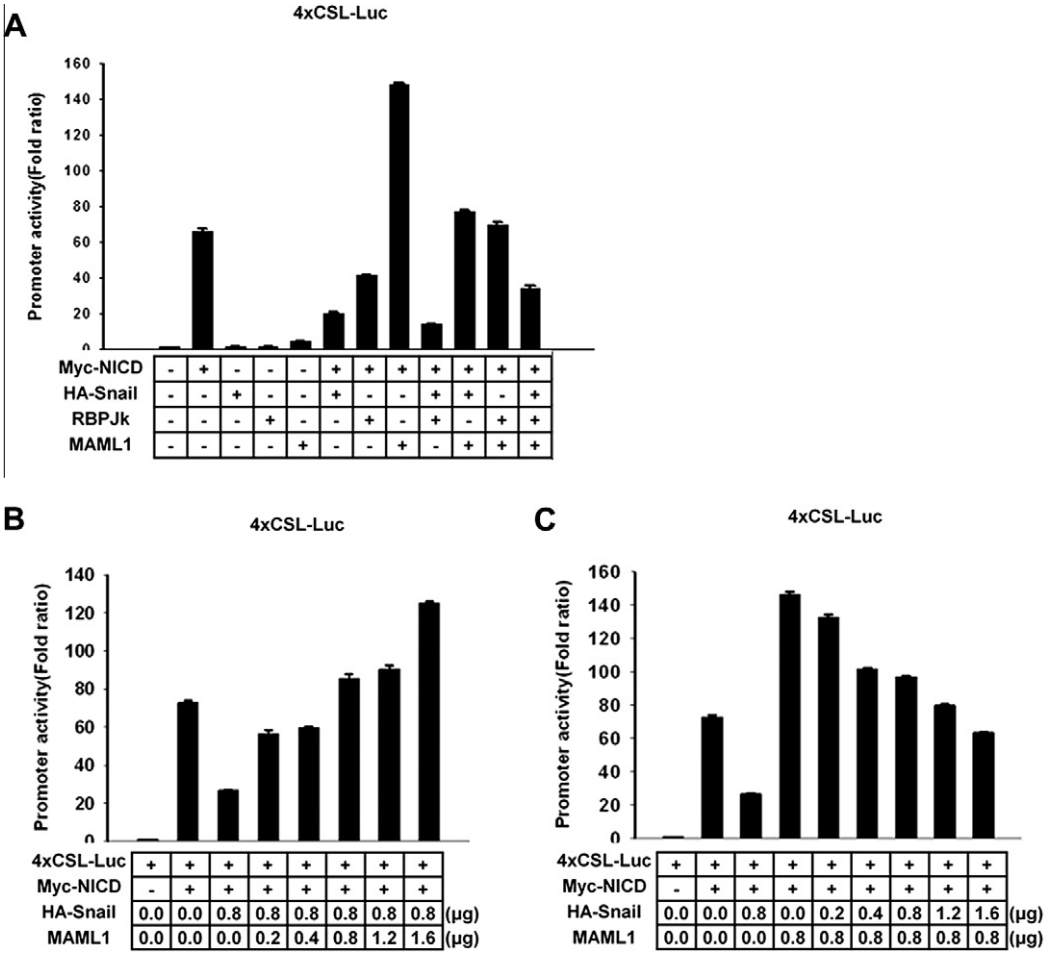

Fig. 3. Snail inhibits NICD-mediated transcriptional activation via competing with MAML1. (A) Hep3B cells were transfected with Myc-NICD, HA-Snail, Flag-RBPJk, and/or Myc-MAML1 expression vectors and 4xCSL promoter fused to luciferase for 48 h and analyzed for luciferase activity. (B) Increasing levels of MAML1 blocked inhibition of NICD transcriptional activation by Snail dose dependently. Hep3B cells were transfected with Myc-NICD, HA-Snail, and/or Myc-MAML1 (0, 0.2, 0.4, 0.8, 1.2 and 1.6 μg) expression vectors and 4xCSL luciferase reporter for 48 h and analyzed for luciferase activity. (C) Snail inhibited NICD and MAML1-mediated transcriptional activation in dose dependently. Hep3B cells were transfected with Myc-NICD, HA-Snail (0, 0.2, 0.4, 0.8, 1.2 and 1.6 μg), and/or Myc-MAML1 expression vectors and the 4xCSL reporter for 48 h and analyzed for luciferase activity.

3.4. Snail suppresses the formation of NICD-MAML1 complex by competing with MAML1

When interaction between NICD, MAML1 and Snail was studied by immunoprecipitation, NICD interacted with both MAML1 and Snail while either MAML1 or Snail only interacted with NICD (Fig. 4A). Also, when immunoprecipitation was performed by NICD, MAML1-NICD interaction was decreased whereas Snail-NICD interaction was increased under same MAML1 and increasing Snail expression condition (Fig. 4B).

In Immunofluorescence result, NICD, RBPJk, and Snail appeared strong fluorescence in same location of nucleus. This showed that NICD, RBPJk, and Snail co-localized in same nuclear foci (Fig. 4C). However, MAML1 did not have fluorescence in the nuclear foci where both NICD and Snail did, showing that MAML1 did not co-localize with NICD/Snail complex (Fig. 4D). According to the results, Snail inhibited interaction between NICD and its co-activator MAML1 resulting in inhibition of NICD-mediated transcriptional activation.

4. Discussion

In several cancers, it is reported that up-regulation of Notch1 signaling resulting in increase of NICD are related to tumor development [5,8,11–14]. Overexpression of Snail is associated with tumor invasiveness and malignancy increase in many kinds of cancers including HCC [1,11,15,16]. NICD is known to induce EMT and tumor cell invasion by up-regulating Snail mRNA transcription [8]. In addition, COX-2 is decreased under the condition of inhibiting Notch1 expression and in turns, Snail expression is decreased [17]. However, the relationship between NICD and Snail in protein level had not been studied yet.

In our recent research, we elucidated that NICD and Snail are novel binding partners and Snail ubiquitination and degradation is induced by NICD [9]. According to another our study, in cells with co-expression of NICD and Snail, Snail inhibits transcriptional activation of p21 promoter, NICD target gene, and p53 rescued transcriptional repressive effect of Snail [11]. However, molecular and transcriptional regulation mechanism by interaction between NICD and Snail has barely discovered.

In this study, we found that Snail inhibits NICD-mediated transcriptional activation via competing with MAML1, NICD co-activator, in NICD/CSL/MAML1 complex. Especially, Snail binds to ANK domain of NICD in order to disrupt the binding between ANK domain and MAML1. In this reason, Snail down-regulates the transcription of genes such as p21, Hes1, and Hes5 which have promoter with CSL motif where NICD/CSL/MAML1 complex binds to.

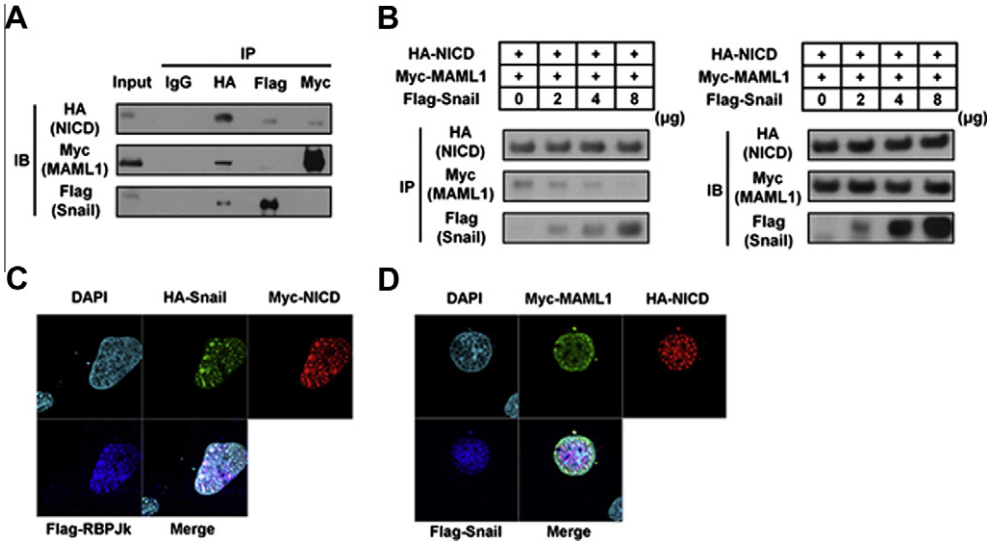

Fig. 4. Snail inhibits the formation of NICD-MAML1 complex via competing with MAML1. (A) Hep3B cells were transfected with HA-NICD, Myc-MAML1, and Flag-Snail and then were co-immunoprecipated using anti-HA, anti-Myc, and anti-Flag antibodies, respectively, and assessed by immunoblot, indicating the interaction between NICD, MAML1, and Snail protein. IgG served as a negative control. (B) Increasing levels of Snail blocked the interaction between NICD and MAML1 by Snail dose dependently. Hep3B cells were transfected with HA-NICD, Myc-MAML1, and/or Flag-Snail (0, 2, 4 and 8 μg) and then were co-immunoprecipated using anti-HA antibodies, respectively, and assessed by immunoblot, indicating the interaction between NICD, MAML1, and/or Snail protein. (C) Hep3B cells were transfected with HA-Snail, Myc-NICD, and Flag-RBPJk expression vectors and then stained with anti-HA, anti-Myc, and anti-Flag antibodies. Cellular localization of Snail (green), NICD (red), RBPJk (blue) was examined. (D) Hep3B cells were transfected with Flag-Snail, HA-NICD, and Myc-MAML1 expression vectors and then stained using anti-Flag, anti-HA, and anti-Myc antibodies. Cellular localization of Snail (blue), NICD (red), MAML1 (green) was examined. Nuclei were stained with DAPI (light blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In conclusion, we investigated Snail inhibited NICD-mediated transcriptional activation of NICD target genes via physical interaction. This result implies that Snail expression is up-regulated by NICD, but also Snail acts to inhibit the transcriptional activity of NICD, and two proteins regulate each other by negative feedback mechanism in transcription level.

Authors' contributions

Conception and design: H. S. Kim, S.-O. Lim, G. Jung.

Development of methodology: H. S. Kim, S.-O. Lim.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Jung.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-S. Kim, H. Jeong.

Writing, review, and/or revision of the manuscript: H. S. Kim, H. Jeong.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. S. Kim,

Study supervision: G. Jung.

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