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Beta-catenin modulates the level and transcriptional activity of Notch1/NICD through its direct interaction

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ABSTRACT

Wnt and Notch1 signaling pathways play an important role in a variety of biological processes including embryonic induction, the polarity of cell division, cell fate, and cell growth. Although there is evidence that the two main signaling pathways can modulate each other, the precise mechanism is not completely understood. This report shows that β-catenin can regulate the level and transcriptional activity of the Notch1 and Notch1 intracellular domain (NICD). The in vivo and in vitro results demonstrate that β-catenin binds with Notch1 and NICD, for which its Armadillo repeat domain is essential. It was further demonstrated that β-catenin could upregulate the level of Notch1 and NICD, possibly by competing the common ubiquitin-dependent degradation machinery. In addition, β-catenin enhanced the transcriptional activity of NICD on the hairy and enhancer of split 1 (HES1) and CSL through its C-terminal transactivation domain. This effect of cooperative regulation by β-catenin could also be observed in bone morphogenetic protein 2 (BMP2) induced osteogenic differentiation of C2C12 cells. β-catenin coexpression with NICD enhanced the alkaline phosphatase (ALP) activity in C2C12 cells compared with either β-catenin or NICD expression alone. Culturing C2C12 cells on Delta-1 coated dishes together with Wnt3-conditioned media induced noticeable increases in ALP staining, verifying that employed physiological levels of NICD and β-catenin are sufficient to induce ALP activation. Furthermore, effects of β-catenin on Notch1 were dramatically diminished by overexpressed LEF1. Overall, our data suggest that β-catenin can act as a switching molecule between the classical TCF/LEF1 mediated pathway and NICD mediated pathway.

1. Introduction

Wnt signal transduction involves in a variety of cellular processes including cell fate decision, cell-to-cell communication, proliferation, migration, polarity, and gene expression [1]. In the normal cell, the content of β-catenin, a major component of canonical Wnt signaling, is kept low in the cytoplasm through the ubiquitination/proteosome-mediated degradation by the association with adenomatous polyposis coli (APC)/axis inhibitor 1 (Axin)/glycogen synthase kinase 3β (GSK3β) complexes [2]. In the presence of Wnt signaling, β-catenin is relieved from the destruction complex including APC/Axin/GSK3β, and the degradation pathway of β-catenin by the ubiquitination machinery is inhibited. In this condition, β-catenin accumulates in the cytoplasm and the nucleus where it interacts with the LEF1/TCF family transcription factors (LEF1, TCF-1, -3 and -4) [3].

Notch protein is a member of conserved type I transmembrane receptors that play an important role for determining the cell fate during development. The maturation and activation of Notch signaling requires sequential processing including S1, S2, and S3 cleavage steps within the Notch receptor [4]. The cleavage of Notch1 into NICD is performed through S3 processing of the Notch1 receptors after the interaction of ligand such as Jagged 1–3 and Delta 1–2. In this condition, NICD translocates to the nucleus and combines with a highly conserved transcription factor, CSL (referred to as RBP-Jκ and CBF-1 in mammals, Suppressor of Hairless (Su(H)) in *Drosophila*) and activates the transcription of the target genes, HES and HES-related repressor protein (HERP), which are two families of basic helix–loop–helix transcription factors [5]. Notch signaling is delicately regulated in a receptor level as well as in a ligand or NICD level by interaction with several proteins.

Notch signaling crosstalks with several signaling pathways including Ras/MAP kinase pathway [6], TGF/BMP pathway [7,8] and

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Wnt pathway, and, thereby, the Notch pathway expands the response from limited stimuli. Some studies have suggested that Notch signaling and Wnt/β-catenin signaling meet in several steps. In a pluripotent human embryonal carcinoma cell line and embryonic stem cells, the Notch and Wnt signaling genes are expressed together, and it is believed that the two signaling processes are closely related [9]. Genetic analysis of Notch shows that Notch signaling represses Wingless (Wg) signaling in *Drosophila* at a stage preceding the cell fate decision [10]. In addition, the Notch receptor interacts with Wg without producing NICD [11], and also with Dishevelled, a Wnt signaling component [12]. Notch signaling encounters the crosstalk with Wnt signaling not only at the receptor level but also at the cytoplasm and nucleus levels. NICD has been reported to interact with LEF1, a transcriptional component of Wnt signaling, and has been suggested to function as a coactivator for LEF1 [13]. Presenilin and GSK3β are other proteins acting to meet Notch signaling and Wnt signaling at the cytoplasmic level. Presenilin has the activity of Notch cleavage into NICD, and is involved in the stability of β-catenin [14]. Likewise, GSK-3β is involved in Wnt/β-catenin signaling and Notch signaling. β-catenin can be phosphorylated by GSK3β and regulated by the proteosome-dependent degradation machinery [2], and Notch2 is also associated with GSK3β, which inhibits the Notch2 activity [15].

At these several levels, crosstalks between Notch and Wnt signaling have been observed, but the outcome of this coordination is still controversial. The precise mechanism on the regulation of the protein levels and transcriptional activity of Notch and β-catenin is not completely understood. This study examined whether the protein level and transcriptional activity of Notch1 are regulated by β-catenin, and whether LEF1 can negatively regulate the effect of β-catenin on Notch1. The results show that Notch1 and NICD directly bind to the arm repeat domain of β-catenin, resulting in reciprocal increases in the levels of Notch1 and β-catenin. We also found that β-catenin up-regulates the expression of HES1, the Notch1 target gene, and the transcriptional activity of Notch1 on CSL and HES1 promoter. In addition, coexpression of Notch1 and β-catenin increased the alkaline phosphatase activity of C2C12 cells, whereas LEF1 diminished such an effect.

2. Materials and methods

2.1. Plasmids

The C-terminal myc tagged constitutively active form of Notch1 (ΔEN1-myc) and Flag tagged NICD were kind gifts from Raphael Kopan (Washington University, St. Louis). The generation of the Green fluorescent protein (GFP) tagged full length and deletion constructs of β-catenin in pEGFP-C1 and HA tagged LEF1 were described in our previous report [16]. A HA tagged ubiquitin construct was used for the ubiquitination assay. The HES1-Luc, CSL-Luc, and TOPFLASH-Luc constructs were used for the luciferase reporter assays.

2.2. Antibodies and reagents

A peptide antibody (Ab) specific to green fluorescent protein (GFP) was purchased from Clontech, BD Biosciences. A polyclonal Ab (anti-cleaved Notch1 (Val-1744) or anti-NICD) recognizing cleaved Notch1 only when cleaved between Gly-1743 and Val-1744, but not the full-length Notch1 or Notch1 cleaved at the other positions, was purchased from Cell Signaling Technology, Inc. The polyclonal Ab recognizing full-length Notch1 was purchased from Santa Cruz Biotechnology. The antibody to β-catenin, and Flag were purchased from Sigma. The Myc and HA epitope were detected using the media from 9E10 and 12CA5 hybridomas. The Lipofectamin plus reagent was purchased from Invitrogen. The luciferase reporter assay kit was purchased from Promega. The protein A- or G-sepharose beads were purchased from Amersham Biosciences. The Tri reagent was purchased from MRC Inc. The AccuPower® RT PreMix was purchased from Bioneer.

2.3. Cell culture, transfection and reporter assays

All tissue culture media and antibiotics were purchased from Invitrogen. The MEF cells and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The transient transfections were performed using the calcium phosphate method or Lipofectamine Plus reagent according to the manufacturer's instructions. For the reporter assays, the cells were plated into 24-well plates 1 day before transfection. 24 h or 36 h after transfection, the cell lysates were analyzed for their luciferase activities using a luciferase reporter assay kit. The pCMV β-galactosidase plasmid was used as the internal control for the transfection efficiency. The experiments have been performed as triplicate, and at least three results from independent experiments were included in an analysis.

2.4. Immunoprecipitation and immunoblot analysis

After transfection, cells were lysed in an ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 μM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and cleared by centrifugation. For immunoprecipitation analysis, the resulting supernatants were immunoprecipitated using the appropriate antibodies and pulled down with protein A- or G-Sepharose beads. The beads were washed extensively, and the bound proteins were resolved by SDS-PAGE, and analyzed by immunoblot analysis. Immunoblot analysis was performed as described previously [17]. The protein bands were quantified using the TINA 2.09 software program (Raytest).

2.5. RNA extraction and RT-PCR

The total RNA was isolated from the cells using the Tri reagent, as recommended by the manufacturer, and the yield and purity of RNA was estimated spectrophotometrically using $A_{260/280}$ ratio. cDNA was prepared using the AccuPower RT PreMix and oligo $(dT)_{20}$ primers from 1 μg of total RNA. The primers used in PCR analysis were as follows: HES1 forward, 5′-GCA CAG AAA GTC ATC AAA GCC-3′; HES1 reverse, 5′-TTG ATC TGG GTC ATG CAG TTG-3′; β-actin forward, 5′-TCA CCC ACA CTG TGC CCA TCT ACG A; β-actin reverse, TGA TGA CCT GGC CGT CAG GCA GCT C-3′. β-actin was used as a loading control of PCR analysis. All experiments have been performed at least three times independently, and representative results were shown.

2.6. Alkaline phosphatase staining

After three or four days treating C2C12 cells with the Wnt3a conditioned media and/or BMP, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 5 min at room temperature, and washed 3 times with distilled water. For alkaline phosphatase staining, 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) Liquid Substrate System was added to cells and incubated for approximately 15 min at room temperature, washed 3 times with distilled water, and air-dried.

2.7. Wnt3a conditioned medium preparation

In order to collect the conditioned medium from the cultures of Wnt3a producing L cells, the cells were seeded in a 100 mm dish containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum. 24 h after seeding, the medium was changed to Dulbecco's modified Eagle's medium without FBS, and the cells were cultured for

24 h. Wnt3a conditioned medium (Wnt3a-CM) was then harvested, centrifuged at 1000 *g* for 10 min, and filtered through a nitrocellulose membrane. The activity of Wnt3a-CM was assayed on the normal L cells by examining the β-catenin level.

2.8. Delta extracellular domain preparation

HEK293 cells were transfected with Delta-1$^{ext\text{-}IgG}$ and cultured with G418 for selection of clones secreting fusion proteins. Then, generated conditioned medium was passed through Protein A column (Peptron Inc.) for purification. The column was washed with PBS and bound protein was eluted with 100 mM glycine, pH3.0 and neutralized with 1 M Tris, pH 8.0. Purified Delta-1$^{ext\text{-}IgG}$ was identified by SDS-PAGE followed by background staining of gels with kit (EZBiopaQ, Co., Ltd.) and a western blot.

3. Results and discussion

3.1. β-catenin binds to Notch1 and NICD in vivo and in vitro

Even though there is a report showing that Notch is associated with β-catenin in *Drosophila* [18], it is unclear whether Notch and NICD directly bind to β-catenin and how these proteins affect each other in mammalian cells. First of all, we examined whether the endogenous Notch1 and β-catenin can be co-immunoprecipitated in mammalian cells. As shown in Fig. 1A, it was found that endogenous Notch1 and β-catenin were specifically immunoprecipitated with the counterpart antibody for β-catenin or NICD in HEK293 cells, respectively. In order to confirm their association, GST pull down assay was performed using lysates from the ΔEN1 overexpressing HEK293 cells with bacterially expressed GST-β-catenin. As shown in Fig. 1B (left panel), ΔEN1 were pull-downed by GST-β-catenin. Although the interaction between Notch1 and β-catenin was apparent in a cellular context, it was unclear whether the two proteins bind directly or not. Therefore, an in vitro GST pull down assay was performed using in vitro translated β-catenin and GST-NICD. The result demonstrated the existence of a direct association between β-catenin and NICD (Fig. 1B, right panel). The association of Notch1 and β-catenin shown by GST-pull down assays was weak to a similar extent as shown by others in *Drosophila* [18], but the binding intensity was more strong in vivo than in vitro. This suggests that another protein and/or posttranslational modification may be involved in enhancing the interaction between β-catenin and NICD in vivo. In order to determine the binding domain of β-catenin on the Notch1, the immunoprecipitation assay was performed on a Notch1 with the deletion constructs of β-catenin. β-catenin consists of an amino-terminal domain of 130 amino acids, 12 Armadillo repeat domains constituted of 42 amino acids, and a carboxy-terminal domain of 100 amino acids. In Fig. 1C, deletion constructs of β-catenin used in this experiment were shown — arm construct, N-terminal deletion

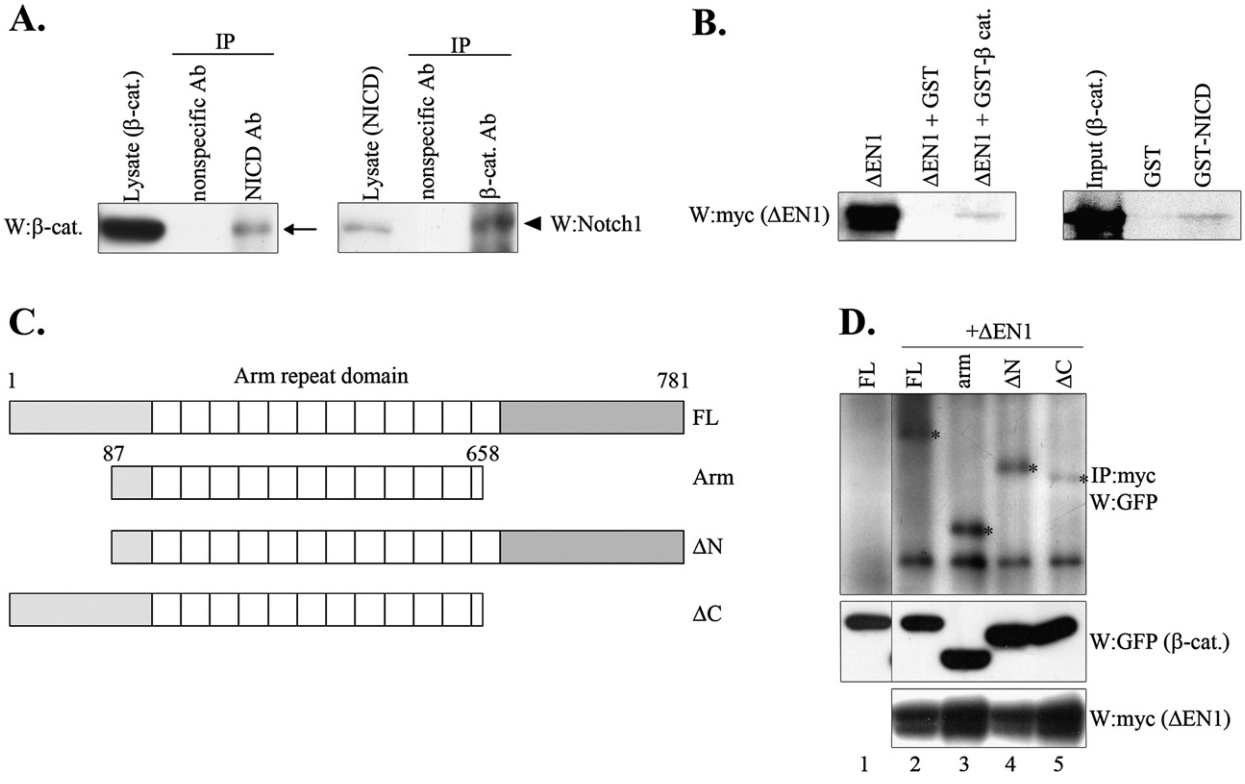

Fig. 1. Notch1 binds to β-catenin in vivo and in vitro. (A) Enodogenous binding of Notch1 and β-catenin. The binding of Notch1 and β-catenin was detected by immunoprecipitating the lysates from HEK 293 cells with Notch1 antibody (Ab) or β-catenin Ab followed by western blotting using β-catenin Ab or Notch1 Ab. An arrow in left panel and an arrowhead in right panel indicate β-catenin and Notch1, respectively. (B) Notch1 interacts with β-catenin in vitro. The HEK293 cells were transfected with the expression plasmid of myc-tagged ΔEN1. The extracts were incubated with GST or GST-β-catenin, and the bound proteins were analyzed by western blotting using myc Ab. The total lysates were loaded in the far left lane, corresponding to 1/35 of the input (left panel). In the right panel, in vitro transcripted and translationed synthetic β-catenin from the pBluscript-SK-β-catenin plasmid incorporating radiolabeled S^{35} was used in GST-pull down assay. The radiolabeled β-catenin was incubated with GST or GST-NICD and bound proteins were subjected to electrophoresis. The autoradiograph of bound protein is shown (right panel). (C) Schematic representation of the β-catenin constructs. FL indicates the construct containing full length β-catenin. Arm indicates the construct containing only 12 arm repeats domain (amino acids 87–658). ΔN indicates the construct containing arm repeats and C-terminal domain (amino acids 87–781). ΔC indicates the construct containing arm repeats and N-terminal domain (amino acids 1–658). (D) Notch1 binds to the Arm repeat domain of β-catenin. In order to determine the binding domain of β-catenin with Notch1, the HEK293 cells were transfected with each 5 μg of myc-tagged ΔEN1 with or without the β-catenin deletion constructs. Immunoprecipitation was performed with myc Ab, and western blotting was performed with GFP Ab (top panel). The expressions of β-catenin deletion constructs or ΔEN1 were identified by GFP Ab (middle panel) or myc Ab (bottom panel). Asterisks indicate bands of full-length or each deletion construct of β-catenin which was immunoprecipitated by ΔEN1.

construct and C-terminal deletion construct, respectively. As a result of immunoprecipitation, ΔEN1 was able to bind each deletion constructs of β-catenin (Fig. 1D). Together, these data provide the first biochemical evidence that Notch1 and β-catenin interact each other directly, and a central arm repeat domain of β-catenin is essential for the interaction between Notch1 and β-catenin.

3.2. The levels of Notch1 and β-catenin are stabilized reciprocally possibly due to the inhibition of ubiquitination/proteosome-dependent degradation

During the above immunoprecipitation assay, it was observed that the protein levels of NICD or ΔEN1 were higher when the cells were co-transfected with β-catenin than those transfected with NICD or ΔEN1 alone without β-catenin (data not shown). This phenomenon suggests that β-catenin may affect the level of Notch1. In order to determine the effect of β-catenin on the level of Notch1, we examined whether β-catenin affect on the levels of NICD or ΔEN1. In HEK293 cells, a constant amount of NICD or ΔEN1 was transfected along with increasing amounts of β-catenin. The levels of NICD or ΔEN1 were increased by β-catenin in a dose-dependent manner. In addition to the full-length β-catenin, the deletion constructs of β-catenin had the same effects on the levels of NICD or ΔEN1 (Fig. 2A). Likewise, the level of full-length and deletion constructs of β-catenin was increased by ΔEN1 augmentation (Fig. 2B). To further examine the effect of Notch1 or β-catenin overexpression on endogenous level of each other, the endogenous levels of Notch1 or β-catenin in HEK293 cells were determined by overexpressing the increasing amounts of the counterpart protein. As shown in Fig. 2C, the effect is bona fide exist in endogenous status. Therefore, we can conclude that there was a positive correlation between the exogenous/endogenous levels of Notch1 and β-catenin. Increased levels of β-catenin by Notch1 or NICD are worthy of notice. As GSK-3β is a key enzyme for regulating the levels of β-catenin in the cytoplasm and is known to affect Notch as well [15], the direct association between Notch1 and β-catenin may induce conformational changes and create steric hindrance to GSK-3β.

It has been documented that the stability of Notch1 can be regulated by some E3 ligases including Sel10 and Deltex, which regulate the levels of NICD via polyubiquitination and proteosome degradation in the cytoplasm [19–21]. We hence examined whether the increased levels of NICD or ΔEN1 by β-catenin is attributed to the reduction of NICD or ΔEN1 ubiquitination. In the HEK 293 cells,

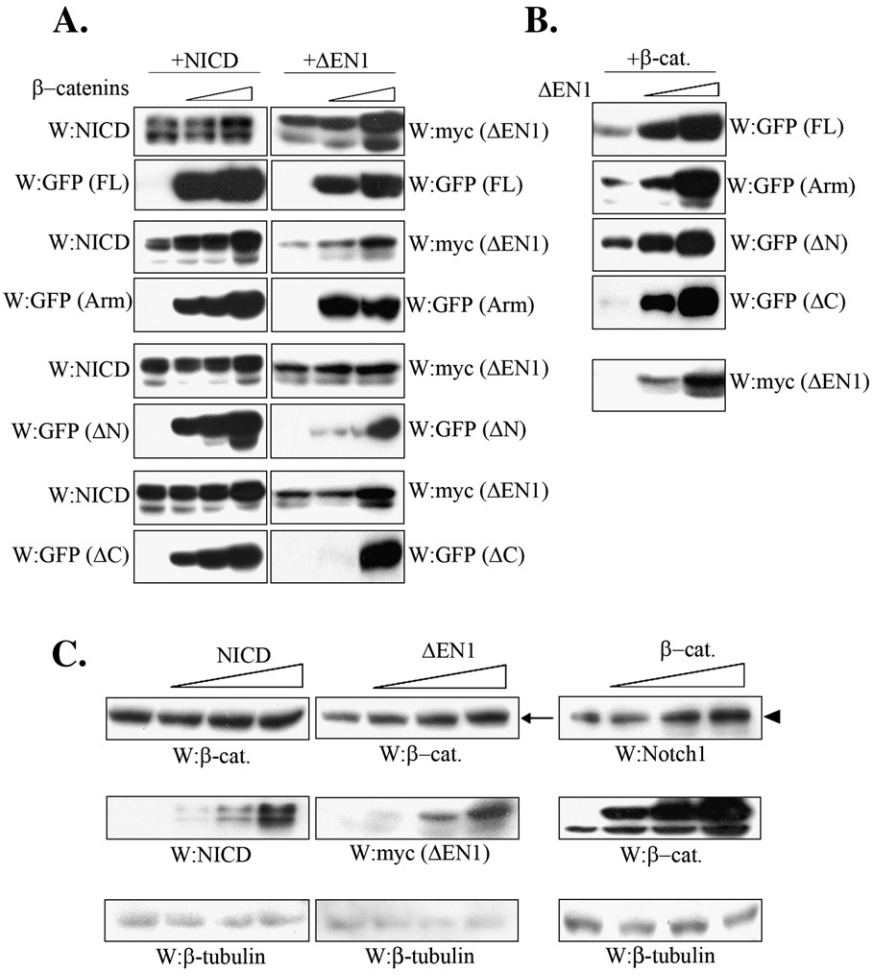

Fig. 2. The levels of Notch1 and β-catenin are stabilized reciprocally. (A) The exogenous expression of β-catenin increased the level of Notch1 in a dose-dependent manner. The HEK293 cells were transfected with the constant amount of either flag-tagged NICD or myc-tagged ΔEN1 in the presence of an increased amount of full-length β-catenin. The altered level of Notch1 by β-catenin was analyzed by western blotting using NICD Ab (left panel) or myc Ab (right panel). The expression of β-catenin was identified by GFP Ab (bottom panel). (B) The exogenous expression of Notch1 increased the level of β-catenin in a dose-dependent manner. The HEK 293 cells were transfected with the constant amount of GFP-tagged β-catenin in the presence of an increased amount of myc-tagged ΔEN1. The altered level of the β-catenin by ΔEN1 was analyzed by western blotting using GFP Ab (top panel). The expression of ΔEN1 was identified by myc Ab (bottom panel). (C) The endogenous level of Notch1 and β-catenin were increased reciprocally. The HEK293 cells were transfected with the flag-tagged NICD or myc-tagged ΔEN1 or GFP-tagged β-catenin. In order to determine the level of each endogenous protein, each lysate was analyzed by western blotting using β-catenin Ab (left and middle panel) or Notch1 Ab (right panel). β-tubulin was used as a loading control (bottom panel). An arrow in left and middle panel and an arrowhead in right panel indicate endogenous β-catenin and endogenous Notch1, respectively. Results are representative of at least three independent experiments.

Notch1 and ubiquitin were co-transfected with or without β-catenin. As a result, the ubiquitination of NICD or ΔEN1 was noticeably decreased by coexistence of β-catenin (Fig. 3). This strongly suggests that β-catenin can stabilize NICD or ΔEN1, which decreases the probability of ubiquitin-mediated degradation of NICD or ΔEN1. Overexpressed β-catenin can compete with Notch1/NICD for GSK-3β and permits Notch1/NICD less degradable by GSK-3β-primed ubiquitination/proteosome-mediated proteolysis. Given the estimation that β-catenin directly associated with NICD is less than 5% of β-catenin input, this possibility may explain the dramatic effects of over-expressed β-catenin on the levels of both exogenous and endogenous Notch1/NICD, or vise versa. In other words, it is possible that β-catenin and Notch1 can be both processed for degradation by common ubiquitination machinery, suggesting that overexpression of either one sequesters the degradation machinery away from the other. In contrast to our data, Hayward et al. [18] reported that the full length Notch could reduce the levels of Armadillo whereas NICD expression results in the increased accumulation of Armadillo^{s10}, which is the constitutively active form of Armadillo, and has no obvious effects on the endogenous Armadillo levels in *Drosophila*. This discrepancy may be due to species differences or to different Notch1 constructs used.

3.3. The transcriptional activity of Notch1 on HES1 promoter and CSL reporter can be increased by β-catenin

In the presence of Notch ligand, NICD cleaved from the Notch1 receptor can translocate into the nucleus and be associated with a CSL transcription factor. NICD functions as a transcriptional activator to express the target genes of Notch signaling including HES1, HERP families [5], and others. Therefore, the stabilized Notch1 and NICD may have more chance to activate the transcription of the target genes. In order to determine whether the β-catenin can affect the transcriptional activity of Notch1, luciferase assay was performed using two well established systems – HES1 or CSL reporter that contains 4 binding sites for CSL – to evaluate the NICD-dependent transcriptional activities. The ΔEN1 were transfected in MEF cells with or without β-catenin, and the transcriptional activity of the Notch-dependent HES1-Luc and CSL-Luc reporter were measured. In MEF cells, the transcriptional activity of Notch1 on HES1-Luc and CSL-Luc was synergistically increased in the presence of β-catenin (Fig. 4A). In consistent with our results, Estrach et al. [22] reported that co-expression of NICD and β-catenin increased the Hes-1 reporter activities, and Espinosa et al. [15] showed that Wnt-1 overexpression led to the up-regulation of Hes-1 promoter. We next examined which domain of β-catenin is responsible for the increased transcriptional activity of Notch1. Interestingly, arm and C-terminal deletion construct of β-catenin had a significantly decreased effect on HES1-Luc and CSL-Luc activity compared to the full-length β-catenin (Fig. 4B). In contrast, a N-terminal deletion construct of β-catenin had a slightly decreased effect on HES1-Luc activity compared to the full-length β-catenin and showed a decreased effect on CSL-Luc activity but to a lesser extent compared to the arm and C-terminal deletion construct (Fig. 4B). Therefore, these results suggest that β-catenin is involved in increasing the transcriptional activity of Notch1, and that the C-terminal domain of β-catenin is required for the increasing the transcriptional activity of Notch1. The increased transcriptional activity of NICD by β-catenin implies that β-catenin may recruit more transcriptional coactivators and/or, alternatively, accelerate the release of negative regulators such as HDAC to increase the target gene expression of Notch1. It should be noted that the C-terminal region of β-catenin is known to be the major transactivation domain and has also been identified as a CBP binding site [23]. On the other hand, N-terminal region of β-catenin is known to be the minor transactivation domain and the binding domain of p300 [24]. The inability of the Arm and C-terminal deletion mutants of β-catenin to increase the transcriptional activity of NICD suggests that the Arm repeat domain of β-catenin is responsible for bound to NICD but may fail to recruit transcriptional co-activators such as CBP/p300. In this standpoint, it is of great interest to note that the Arm and C-terminal deletion mutants of β-catenin increased the levels of Notch1 but did not increase the transcriptional activity of NICD, suggesting that the mere increased levels of NICD in the nucleus may not be proportional to its increased transactivational activity. Based on the results in Fig. 4A and B, it was assumed that β-catenin can potentiate NICD-mediated gene expression by cooperation with NICD and increase the expression of the Notch1 target gene. In order to demonstrate this assumption, HES1 mRNA level was analyzed by RT-PCR. In the MEF cells, Notch1 were transfected with or without β-catenin. As a negative control, an empty flag vector was transfected into the cells (lane 1 in Fig. 4C). The mRNA level of HES1 was increased more by Notch1 and β-catenin together (lane 4 in Fig. 4C) than by Notch1 (lane 2 in Fig. 4C) or β-catenin alone (lane 3 in Fig. 4C).

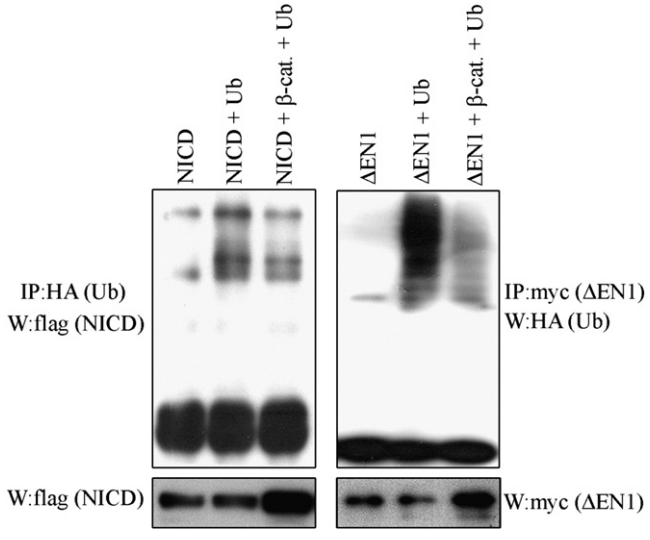

Fig. 3. The ubiquitination of Notch1 and NICD are decreased by β-catenin. The altered ubiquitination of NICD and ΔEN1 by β-catenin was determined using flag-tagged NICD or myc-tagged ΔEN1 as substrate in the in vivo ubiquitination reactions in the presence or absence of GFP-tagged β-catenin. Notch1 ubiquitination was determined by immunoprecipitation using the HA antibody (left panel) followed by western blotting using the either flag Ab or HA Ab or by immunoprecipitation using the anti-myc Ab (right panel). The expression of NICD and ΔEN1 was detected using flag Ab and myc Ab (bottom panel).

3.4. LEF1 diminishes the effects of β-catenin on Notch1-mediated transcriptional activation

LEF1 functions as an important transcription factor in Wnt/β-catenin signaling pathway. In the presence of Wnt signaling, the accumulated β-catenin in cytoplasm and nucleus interacts with LEF1. In this study, it was observed that the overexpression of wild type LEF1, which can interact with β-catenin, decreased the endogenous levels of Notch1 in a dose-dependent manner (left panel of Fig. 5A). On the other hand, the overexpression of dominant negative LEF1, which cannot interact with β-catenin, slightly increased the endogenous levels of Notch1 (right panel of Fig. 5A). These results led us to postulate that the expression of wild type LEF1, capable of binding β-catenin, can interfere with the interaction between Notch1 and β-catenin, and, thereby, reverse the inhibited ubiquitination/proteosome-mediated Notch1 degradation, resulting in its decreased level. Based on this postulation, we hypothesized that a novel intermolecular mechanism reveals a competitive inhibitory function of LEF1 for Notch1 and β-catenin. In order to confirm this, the luciferase assay was performed using HES1-Luc, CSL-Luc, or TOPFLASH-Luc system.

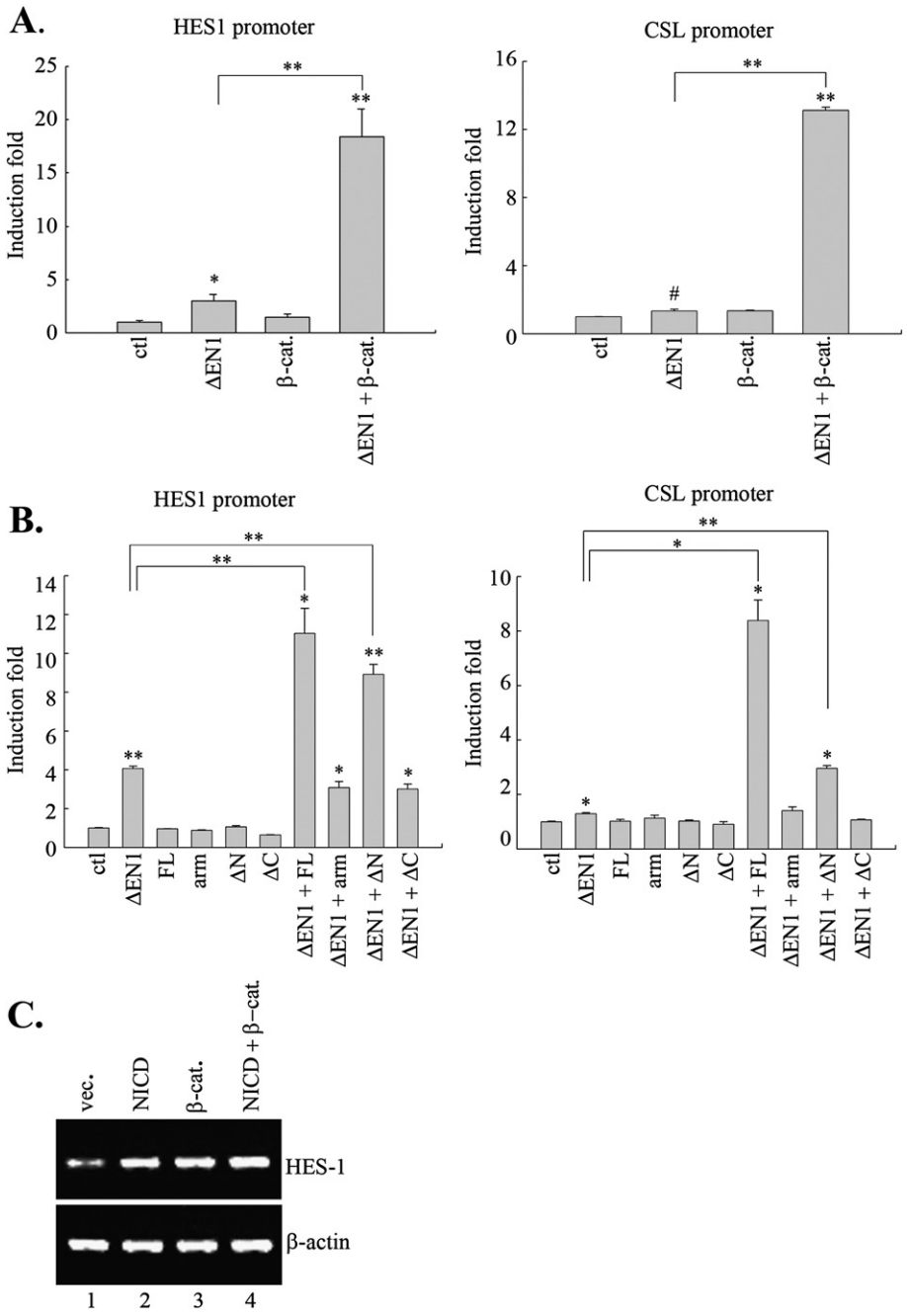

Fig. 4. The Notch1-dependent transcriptional activities and mRNA levels of HES1 are augmented by β-catenin. (A) β-Catenin increases the transcriptional activity of Notch1 on HES1 or CSL promoter. The MEF cells were transfected with ΔEN1 together with or without β-catenin in the presence of HES1-Luc reporter or the CSL-Luc reporter. The CSL-Luc reporter contains 4 copies of CSL binding consensus sequences. The relative luciferase activity ± S.E. was shown. Experiments were performed at least three independent cultures. (B) The effect of β-catenin deletion constructs on the transcriptional activity of Notch1. The MEF cells were transfected with ΔEN1 together with or without the β-catenin deletion mutants in the presence of the HES1-Luc reporter or the CSL-Luc reporter construct. The relative luciferase activity ± S.E. was shown. Experiments were performed at least three independent cultures. *, $P<0.05$; **, $P<0.001$; #, $P<0.07$. (C) β-Catenin increases the mRNA level of the HES1. The MEF cells were transfected with the flag-tagged NICD and/or flag-tagged β-catenin, and the mRNA level of HES1 was examined by RT-PCR using HES1 specific primers. β-actin was used as the control of RT-PCR.

The MEF cells were transiently transfected with the HES1-Luc or CSL-Luc reporter and Notch1 in the presence or absence of β-catenin and/or the LEF1. As shown in Fig. 5B, the increased transcriptional activity of Notch1 on HES1 and CSL by β-catenin was inhibited by LEF1. On the other side, the MEF cells were transiently transfected with the TOPFLASH-Luc reporter and β-catenin in the presence or absence of Notch1 and/or LEF1. Notch1 inhibited the increased transcriptional activity of LEF1 with β-catenin. Previous report indicates that the ability of Notch1/NICD to augment the activity of LEF1 occurs only on certain promoters, LEF-OT and Xtwin, but not on 7xLEF-Luc, WISP-1 and Cyclin D1 [13]. Our results show that there was no such augmentation by NICD on the transcriptional activation of LEF1 on the TOPFLASH reporter, which harbors 6 synthetic LEF1 binding sites. These data let us assume that the increased expression of HES1 by the cooperation of Notch1 and β-catenin may be decreased by LEF1. To determine this assumption, RT-PCR was performed. In the MEF cells, Notch1 and β-catenin were transfected with or without LEF1. It was identified that the increased HES1 mRNA by Notch1 and β-catenin cooperation was diminished by LEF1 overexpression, as shown in Fig. 5C (lanes 3 and 4).

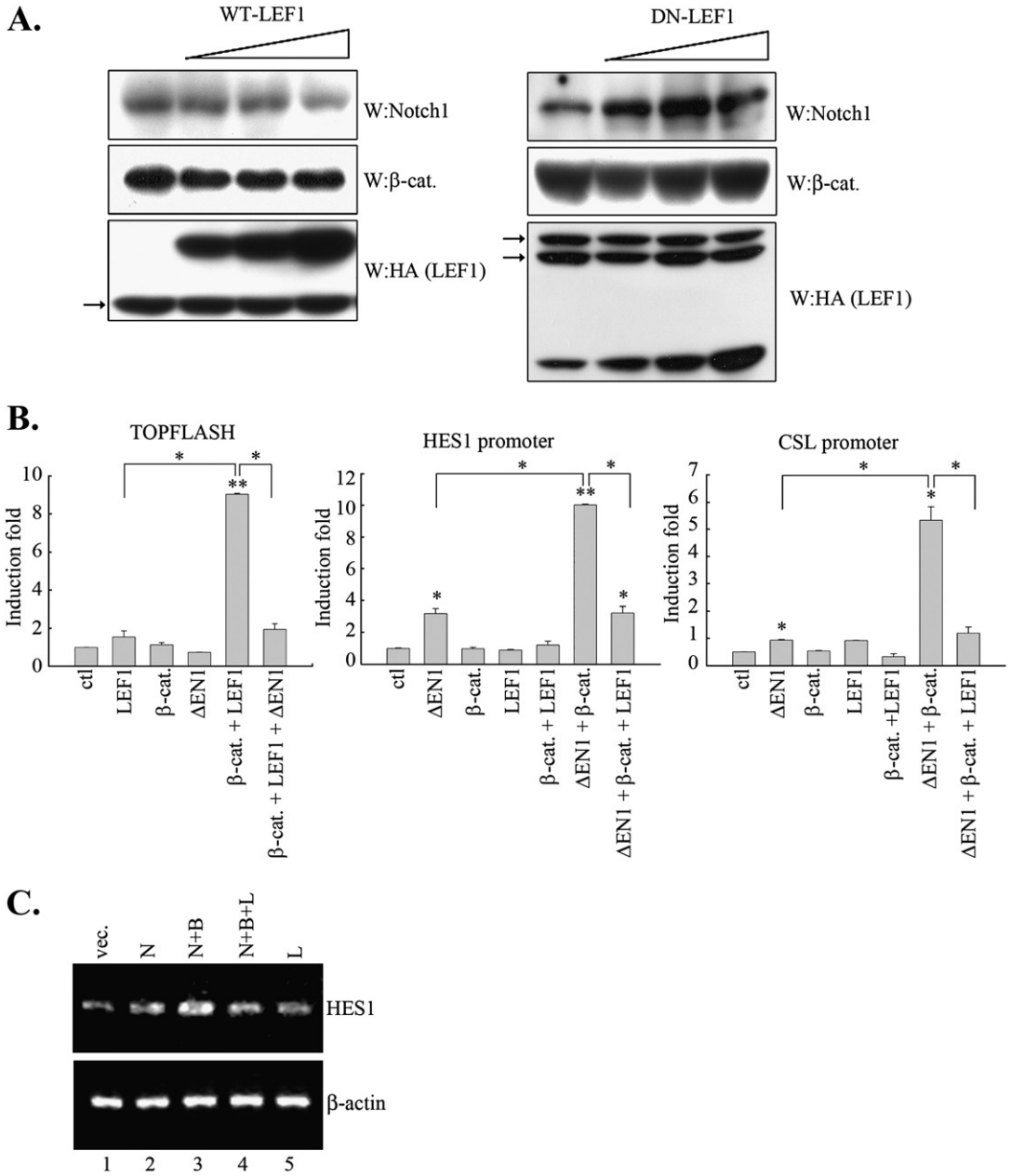

Fig. 5. LEF1 negatively regulates the effect of β-catenin on Notch1. (A) Endogenous Notch1 protein level is decreased by wild type LEF1 but not by dominant negative LEF1. The HEK293 cells were transfected with HA-tagged wild type or dominant negative LEF1. The endogenous protein was determined by detecting the lysates with Notch1 Ab (top panel) or β-catenin Ab (middle panel). The expressions of LEF1 were detected with HA Ab (bottom panel). Arrows indicate the loading control. (B) Notch1 and LEF1 have a competitive inhibitory effect on the transcriptional activity of HES1, CSL, or TOPFLASH promoter. The MEF cells were transfected with ΔEN1 together with or without β-catenin in the presence of the HES1-Luc reporter, CSL-Luc reporter, or TOPFLASH-Luc reporter. The TOPFLASH reporter contains LEF1 binding consensus sequences. Relative luciferase activity ± S.E. was shown. All the experiments were performed in three independent cultures. *, $P<0.05$; **, $P<0.01$. (C) LEF1 decreases the mRNA level of HES1 induced by Notch1 and β-catenin. The MEF cells were transfected with flag-tagged NICD and β-catenin with or without LEF1, and the mRNA level of HES1 was examined by RT-PCR.

3.5. BMP-2 induced alkaline phosphatase activation is potentiated by Notch1 and β-catenin, which is diminished by LEF1

The canonical Wnt signaling pathway is the most important regulator of bone formation that occurs through the activation of β-catenin signaling [25]. On the other hand, the effect of the Notch signaling pathway on bone formation is controversial [26,27]. This study examined the biological function of cooperative crosstalk between Notch and β-catenin by determine the effect on BMP-2 induced alkaline phosphatase (ALP) activation which is a marker for osteoblast differentiation. First, we employed physiologically increased levels of Wnt and Notch signaling effectors by using Wnt3-conditioned media (CM) and Delta-1 coated dishes, respectively. The effects of Wnt3-CM on β-catenin were confirmed by western blot analysis (Fig. 5B) and TOPFLASH reporter assay (Fig. 5A), whereas those of Delta-1 on Notch signaling was confirmed by HES1 reporter assay (Fig. 5A). ALP activation was observed by culturing the C2C12 cells in Wnt3-CM in the presence or absence of BMP-2 (Fig. 5C). Culturing cells on Delta-1 coated dishes alone did not induce any ALP activation. However, culturing cells on Delta-1 coated dishes together with Wnt3-CM induced noticeable increases in ALP staining, especially in concave outside regions (Fig. 5C). Second, in order to examine the effects of Notch1 and β-catenin more closely, the C2C12 cells were transfected with NICD and/or β-catenin, cultured to confluence, and then changed to a medium containing Wnt3a conditioned media (Wnt3a-CM) in the presence of BMP-2. In Figs. 6C and 7A, ALP

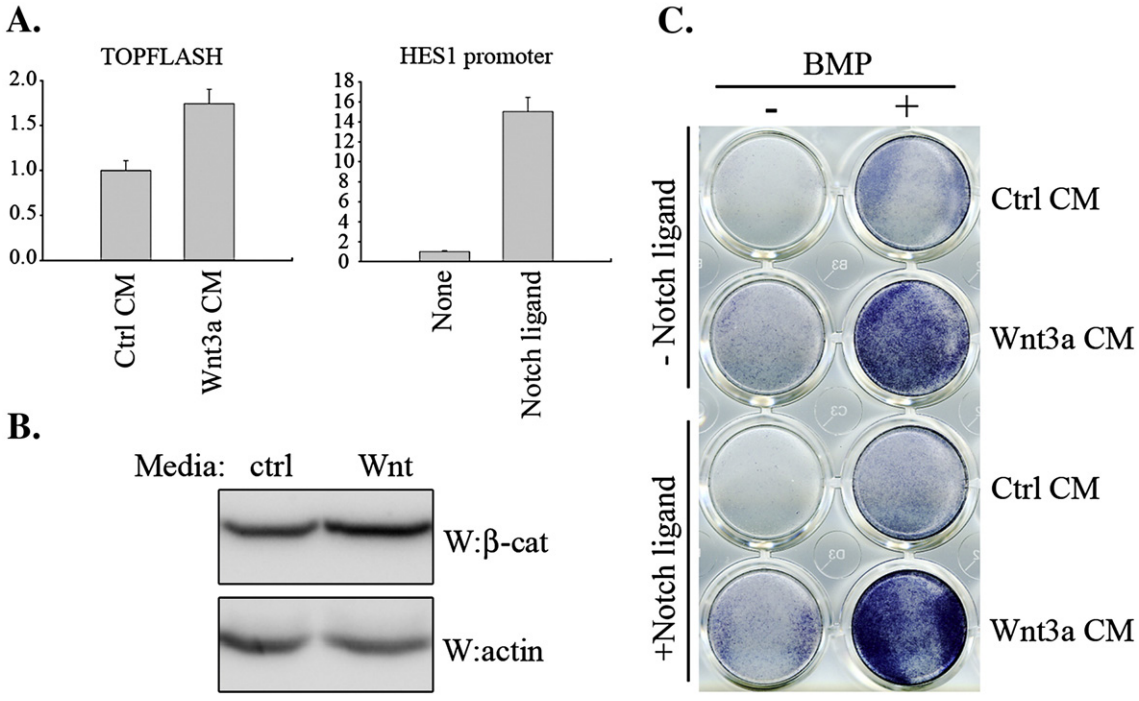

Fig. 6. BMP-2 induced alkaline phosphatase activation by Notch ligand and Wnt conditioned media. (A) The effect of prepared Wnt3a conditioned media and Notch ligand was confirmed by assaying transcriptional activities of TOPFLASH and HES1 promoter, respectively. Wnt3a conditioned media was treated after transfecting TOPFLASH reporter gene. For Notch ligand assay, prepared Notch ligand was pre-coated before cell plating, and then cells were transfected by HES1 promoter. 1 day after transfection, cells were harvested, and luciferase activity was determined. Relative luciferase activity ± S.E. was shown. (B) The MEF cells were treated with either control or Wnt3a conditioned media for 1 day, and the level of β-catenin was determined by western blotting with β-catenin Ab. (C) The C2C12 cells were treated as indicated and were incubated in the presence or absence of BMP-2 (25 μg/ml) for 3 days. After incubation, cells were stained for ALP and staining was visible as dark culture wells on the photos shown.

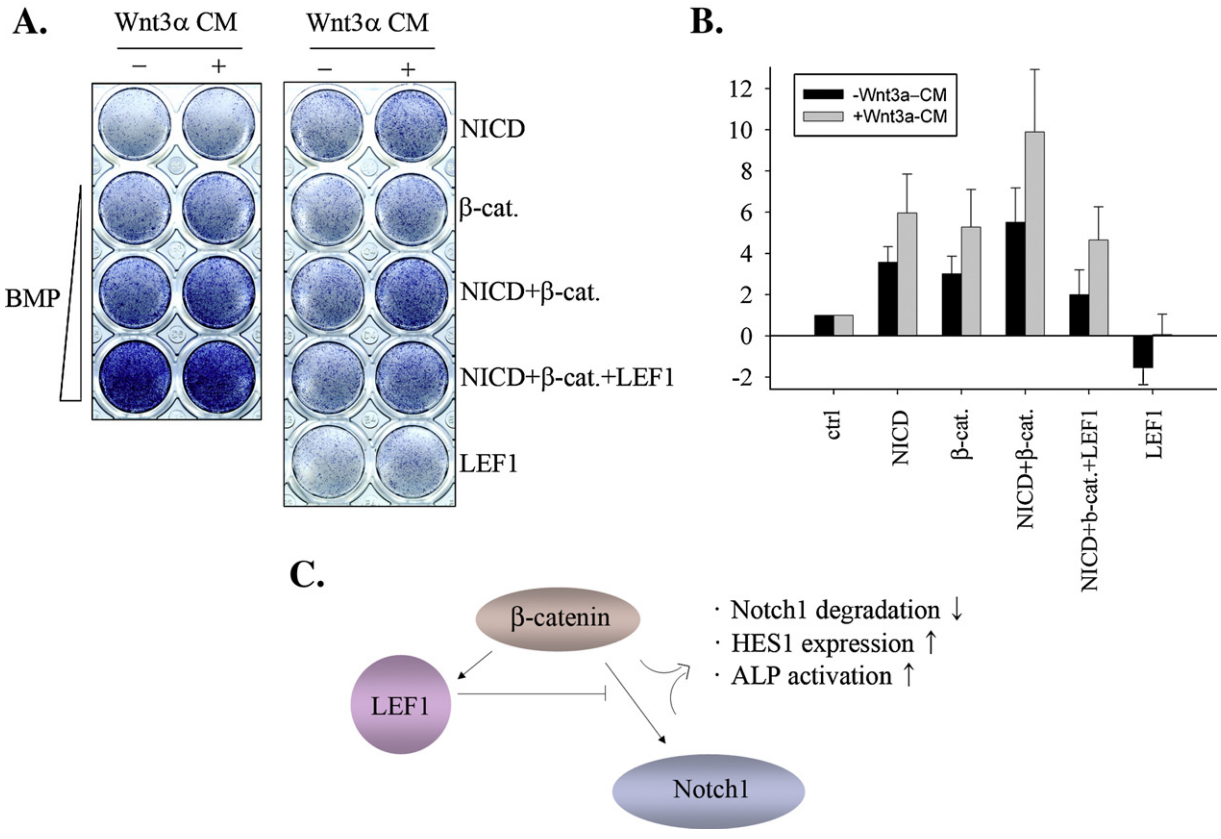

Fig. 7. BMP-2 induced alkaline phosphatase activation by Notch1 and β-catenin. (A) The C2C12 cells were transfected with the indicated expression plasmids and were treated with BMP-2 (25 μg/ml) for 3–4 days in the presence or absence of Wnt3a conditioned media, respectively. The transfected C2C12 cells were stained for ALP. Staining was visible as dark culture wells on the photos shown. The left panel indicates the concentration of the control for BMP-2. (B) Densitometric quantification of ALP staining in three independent results. The data are represented as the mean ± S.E.M. (C) A proposed model.

activation was observed by NICD overexpression (3.57±0.78 fold increase compared with control without Wnt3a-CM and 5.96±1.88 with Wnt3a-CM). In addition, co-transfection of β-catenin with NICD enhanced the effect of NICD on ALP activation (5.51±1.66 fold increase compared with control without Wnt3a-CM and 9.90±3.02 with Wnt3a-CM). Compared with the ALP activity of the C2C12 cells without Wnt3a conditioned media, the ALP activity of the C2C12 cells with Wnt3a-CM induced an approximate 1.6 fold increase. This suggests that β-catenin has an additive effect on the ALP activation by NICD, which can occur from Wnt3a-CM that increases the level of stable β-catenin within the cell. Interestingly, there was a difference between Wnt3a-CM and no Wnt3a-CM when there is β-catenin. This indicates that Wnt3a-CM induce some favorable changes for ALP activation, other than increased levels of stable β-catenin. Possible candidates include GSK-3 and Axin. In particular, overexpression of LEF1 inhibited the ALP activity approximately 50%. In accordance with the competitive inhibition of LEF1 on Notch1 and β-catenin, as shown in Fig. 5, LEF1 had a negative effect on the ALP activation by NICD and β-catenin. Along this line, it was of great interest to note that the overexpressed LEF1 did not induce any noticeable ALP activation even in the presence of the Wnt3a-CM. This suggests that stabilized β-catenin, which is induced by a Wnt signal, affects the osteogenesis to a different extent depending on its intra-cellular partnership, i.e. LEF1 or NICD. In particular, the negative effect of overexpressed LEF1 suggests the possibility that an abnormal amount of LEF1 in cells may induce the negative feedback of β-catenin-induced signaling strength. From this result, we propose the model that the stability and the transcriptional activity of Notch1 were increased by interaction with β-catenin and these effects are supposed to be competed by LEF1 (Fig. 7C). Following results can support the competition among these molecules: Interaction of Notch1 and β-catenin is occurred through arm domain of β-catenin that is well known as a binding domain with LEF1. Furthermore, our results of in vivo and in vitro pull-down assay showed that both Notch1 and NICD could interact with β-catenin, suggesting that NICD contains the minimal binding site for β-catenin. Interestingly, a previous report showed that the transactivation domain of NICD physically interacts with the LEF1 HMG domain [13]. Thereby, competition on interaction between Notch1 and β-catenin by LEF1 may be caused by overlapping of either interaction sites in β-catenin toward Notch1 and LEF1 or that in NICD toward β-catenin and LEF1.

The effects of Notch on bone formation are very controversial. For example, several groups reported that osteoblastic differentiation can be stimulated by Notch [26,28], whereas there are also accumulated reports showing that Notch inhibits osteoblast differentiation [27,29–31]. The discrepancy in results may be due to the use of different cell lines and Notch constructs or to different expression methods (i.e., transient vs. stable transfection; retroviral vs. adenoviral system). Our results also showed that culturing cells on Delta-1 coated dishes alone did not induce any ALP activation but NICD overexpression induced moderate ALP activation. This suggests that high levels of NICD are prerequisite for ALP activation.

As Notch signaling pathways can interact with Wnt signaling at different levels, the outcome for the convergence of two signaling pathways can be different depending on what Notch constructs or which component in Notch1 signaling pathway are used. For example, many Wnt signaling molecules bind at different regions of Notch. GSK-3β directly binds at C-terminal of the Notch2 ankyrin repeats [15], and Dishevelled binds within a broad region C-terminal to this domain and reduces the Su(H)-independent activity of Notch [12]. Furthermore, LEF-1 binds at the C-terminal transactivation domain of NICD [13], and our results showed that β-catenin interacts with NICD. The NICD construct we used contains full length NICD, whereas an OPA/PEST domain is deleted in ΔEN1, a constitutively active form of Notch1. Our results showed that both NICD and ΔEN1 interact with β-catenin and promote HES1 expression. However, the usage of Notch1 constructs deleting different parts in Notch1 may explain the discrepancies for the effects of Notch1 on Wnt pathway published by others.

Accumulating evidence suggests that β-catenin interacts not only with its classical binding partners in the nucleus, TCFs/LEF1 but also with other transcriptional factors under certain circumstances, and affects other signaling pathways and cellular processes. For an example, β-catenin binds directly to a FOXO transcription factor, which is enhanced in response to oxidative stress [32]. In addition, the Wnt3 conditioned media stimulate proliferation in the androgen receptor (AR)-positive prostate cell lines mainly through AR-dependent mechanisms rather than classical TCF-dependent mechanisms [33]. Furthermore, there are many reports on the crosstalk between Wnt and Notch signaling pathways as well as possible feedbacks from each other. This research provides unique and more advanced contribution on the crosstalk between Wnt and Notch signaling pathways. Future experiments should also include more NICD downstream targets to generalize our model. However, based on our results, it is believed that β-catenin activates NICD-dependent gene expression through its direct interaction rather than by forming a trimeric complex with NICD and LEF1. Depending on the partnership of β-catenin, stabilized β-catenin can affect the different profiles of the downstream target genes and, thereby, modulates cellular processes to a different extent.

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