BMP signaling in skeletal muscle and bone

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Chapter 4

Id3 is induced by Wnt/β-catenin signaling and mediates Wnt induced proliferation and osteoblast differentiation in C2C12 cells

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Abstract

Canonical Wnt signalling plays important roles in regulating cell proliferation and differentiation. In this study, we report that inhibitor of differentiation (Id)3 is a Wnt-inducible gene in mouse C2C12 myoblasts. Wnt3a induced Id3 expression in a β-catenin- dependent manner. Bone morphogenetic protein (BMP) also potently induced Id3 expression. However, Wnt-induced Id3 expression occurred independent of the BMP/Smad pathway. Functional studies showed that Id3 depletion in C2C12 cells impaired Wnt3a-induced cell proliferation and alkaline phosphatise activity, an early marker of osteoblast cells. Id3 depletion elevated myogenin induction during myogenic differentiation and partially impaired Wnt3a suppressed myogenin expression in C2C12 cells. These results suggest that Id3 is an important Wnt/β-catenin target gene in myoblast cell fate determination.

Key words: Wnt/β-catenin, Id3, cell proliferation, myoblast

1. Introduction

Wnts are a family of growth factors controlling multiple biological processes such as embryogenesis, organogenesis and tumorigenesis [1; 2; 3; 4]. In the presence of Wnt ligand, Wnt receptor frizzled (Fz) and its co-receptor low-density lipoprotein receptor-related protein-5 or 6 (LRP-5/6) recruit Axin and GSK3β to the plasma membrane, together with the scaffold protein Disheveled (Dvl) [5; 6]. The membrane association of Axin and GSK3β disrupts the β-catenin destruction complex, resulting in accumulation of β-catenin in the nucleus, where it triggers target gene activation by displacing transcriptional repressors from DNA-bound LEF/TCF [7; 8]. In recent years, canonical Wnt signalling pathway is identified to be crucial for bone formation and bone homeostasis. The mutations in LRP-5 profoundly affect skeletal development and result in low bone mass [9; 10]. The Dickkopf-1 (Dkk-1) resistant LRP5V171 mutation leads to high bone density [11]. Conditional deletion of the β-catenin gene in osteoblasts leads to reduced bone-mass in vivo [12]. Canonical Wnt signaling is also reported to be involved in myogenic differentiation of mouse myoblast cells [13].

Id (inhibitor of DNA binding) proteins are helix-loop-helix (HLH) proteins, which lack basic region adjacent to the HLH domain that is essential for specific DNA binding in other bHLH proteins [14]. Id proteins repress bHLH proteins by binding and interfering with DNA interaction of HLH proteins. As direct target genes of bone morphogenetic proteins (BMPs), Id proteins regulate cell fate [15; 16]. In this study, we found canonical Wnt/β-catenin signaling could induce Id3 expression independent of BMP/Smad signaling. Loss of function studies in C2C12 cells demonstrated that Id3 plays a pivotal role in canonical Wnt3a-induced cell fate determination.

2. Materials and Methods

2.1 Reagent and plasmids

GAPDH antibody (Sigma), Id3 antibody (C-20, Santa Cruz), P-Smad1 (#9511 Cell signaling) and Smad1 (SC-7965 Santa Cruz), Myogenin (ab1835, Abcam); BMP response element (BRE)-Luc reporter, β-cateninWT/SY plasmids were previously descrambled [17; 18; 19]. Dominant negative Lymphoid enhancer binding factor (LEF)-1 was cloned by polymerase chain reaction (PCR) into pcDNA3.1 vector.

2.2 Cell culture

Mouse myoblast C2C12, NIH3T3, C3H10T1/2, control and Wnt3a expressing L cells and HEK293T cells were maintained in growing Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) at 37 ºC in a humidified incubator with 5% CO2. C2C12 myoblasts were seeded at a concentration of $0.35X10^6$ cells per well in 6-well plates and cultured for 24 hours to reach 100% confluence (day 0). To induce myogenic differentiation, cells were washed in PBS and cultured in low glucose DMEM supplemented with antibiotics

and 2% heat-inactivated horse serum, referred to as differentiation medium (DM). All of the experiments were carried out from day 0 to day 6 after the induction of differentiation and cells were examined for myogenin expression.

2.3 Lentiviral transduction

Lentiviruses were produced by transfecting pLV-bc-CMV (for cDNA expression) plasmids together with helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV into HEK293T human epidermal kidney cells. Cell supernatants were harvested 48 h after transfection and were either used to infect cells or stored at -80 °C. C2C12 were infected and maintained in puromycin medium as stable cells. Mouse Id3 shRNA (#1: TRCN0000071438; #2: TRCN0000071439) MISSION® shRNA Lentiviral Transduction Particles were purchased from Sigma mission library, Inc (USA). PLKO.1 control shRNA was served as control.

2.4 Transient cell transfection and luciferase activity assay

Transient transfections and reporter assays were performed in triplicates as previously described [20]. In all reporter assays, a β-galactosidase expression plasmid was co-transfected and served as a control to correct for transfection efficiency. The experiments were performed in triplicates.

2.5 QRT-PCR (quantitative real-time–PCR)

Total RNA was isolated using NucleoSpin® RNA II kit (BIOKÉ, Netherlands) reagent. 1μg RNA was reverse-transcribed using the RevertAid™ First Strand cDNA Synthesis Kits (Fermentas). Quantitative real-time PCR was accomplished with SYBR Green incorporation (Applied Bioscience) using a StepOne Plus real-time PCR system (Applied Bioscience). Results were normalized to those obtained with GAPDH. Primers used for QRT-PCR were:

mGAPDH forward, 5'-AACTTTGGCATTGTGGAAGG-3'
mGAPDH reverse, 5'-ACACATTGGGGGTAGGAACA-3'
mAxin2 forward, 5'-GGTTCCGGCTATGTCTTTGC -3'
mAxin2 reverse, 5'-CAGTGCGTCGCTGGATAACTC -3'
mId1 forward, 5'-ACCCTGAACGGCGAGATCA-3'
mId1 reverse, 5'-TCGTCGGCTGGAACACAT-3'
mId3 forward, 5'-ACCTCCCGAACGCAGGTGCT-3'
mId3 reverse, 5'-ATGCCCTCAGGCTTCCGGCT-3'

2.6 Western blotting

Western blotting was performed as previously described using standard techniques[21]. Antibodies used for immunoblotting are mentioned above.

2.7 ALP assays

Histochemical examination of alkaline phosphatase (ALP) activity in cells was performed as previously described using naphtol AS-MX phosphate (Sigma) and fast blue RR salt (Sigma) [22].

2.8. MTT cell viability assay

Cell viability was determined using the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Promega, Madison, WI) as follows. Treatment medium was replaced with fresh treatment medium containing 20 µl/ml of the Cell Titer 96 Aqueous One Solution and incubated for 10 min at 37 °C after which optical density was measured at 490 nm using a microplate reader. The quantity of soluble formazan product, as measured by the amount of absorbance, was directly proportional to the number of viable cells.

3. Results

3.1 Wnt3a induces Id3 expression

Id1 and Id3 belong to helix-loop-helix protein family. Both Id1 and Id3 are direct target genes of BMP/Smad1/5/8 signaling pathway. In C2C12 cells, transient stimulation of Wnt3a induced Id3 gene expression in a dose dependent manner (Fig. 1A), whereas the Id1 expression was not affected (Fig. 1B). The time course experiments showed that Id3 induction by Wnt3a was similar to Axin2, the direct downstream target gene of canonical Wnt/β-catenin signaling pathway (Fig. 1C). Western blotting also showed Wnt3a induced Id3 protein expression in C2C12 cells although it was not that strong as compared to BMP-induced Id3 expression (Fig. 1D). Similar observation was obtained in NIH3T3 and C3H10T1/2 cells (Fig. 1E).

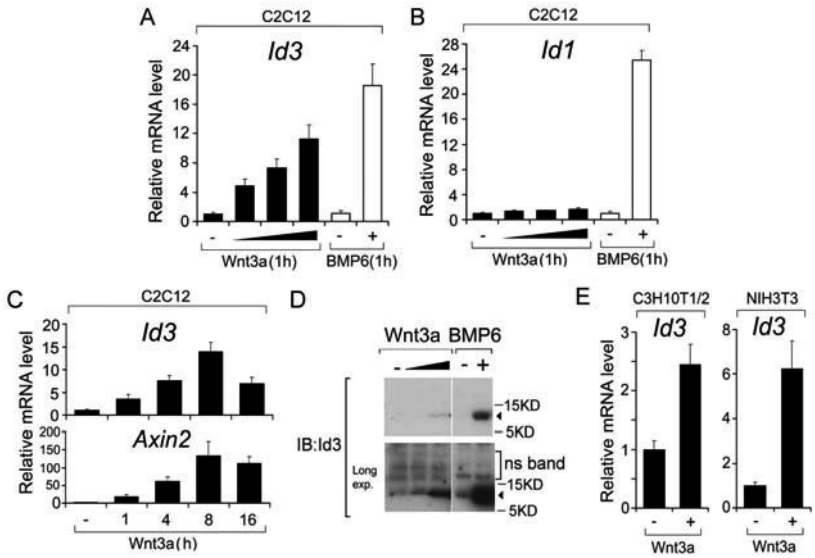

Fig1. Wnt3a induces Id3 expression. (A and B) C2C12 cells were stimulated with increasing doses of Wnt3a conditional medium (CM). One hour after stimulation, Id3 and Id1 expression was measured by q-PCR. Cells with BMP6 (50 ng/ml) treatment were employed as positive control. (C) time-course induction of Id3 and Axin2 by Wnt3a CM is analyzed by q-PCR in C2C12 cells. (D) Western blot showing endogenous Id3 expression induced by 1 h treatment of Wn3a CM (1:6 or 1:3) or BMP6 (50 ng/ml) in C2C12 cells. (E) q-PCR analysis of Id3 expression in NIH3T3 and C3H10T1/2 cells treated with or without Wn3a CM (1:3).

3.2 Wnt3a induces Id3 expression via canonical Wnt/β-catenin pathway

The data presented above suggests the canonical Wnt/β-catenin may mediate Id3 induction by Wnt3a. To verify this, we infected C2C12 cells with lentivirus expressing wild type β-catenin (WT) or constitutively active β-catenin (S33Y). Q-PCR analysis showed Id3 expression was elevated in β-catenin-stably expressed C2C12 cells (Fig. 2A). Furthermore, we found Wnt3a was unable to induce Id3 expression in the cells transfected with dominant negative form of LEF1 that lacks N-terminal β-catenin binding domain (Fig. 2B). This observation is consistent with the fact that Id3 promoter region has TCF/LEF binding site (see Discussion). Taken together, these results indicate that β-catenin mediated transcription is required for Id3 induction by Wnt3a.

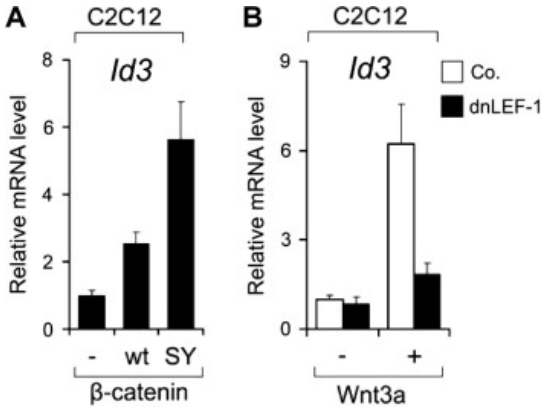

Fig2. Canonical Wnt/β-catenin pathway is required for Wnt3a induced Id3 expression in C2C12 cells. (A) C2C12 cells were infected with control lentivirus (−) or lentivirus expressing β-catenin wild type (WT) or constitutively active form of β-catenin (SY). Id3 expression was measured by q-PCR. (B) Wnt3a CM induced Id3 expression was analyzed in control or Dominant Negative LEF-1 (dnLEF-1) expressing C2C12 cells.

3.3 Wnt3a-induced Id3 expression is not dependent on BMP signaling activation

Id3 expression was additively induced by Wnt3a and BMP in C2C12 cells (Fig. 3A). It has been reported that BMP4 and BMP6 are induced by Wnt signaling in cancer cells [23; 24]. To test whether Wnt3a induced Id3 expression occurs via BMP signaling activation, we next examined whethet Wnt3a can activate a BMP selective Smad1 phosphorylation and Smad1-dependent transcriptional reporter activity in C2C12 cells. As shown in Fig.3B, Wnt3a treatment did not potentiate BMP2-induced phosphor-Smad1. By using BMP/Smad responsive reporter BRE-Luc, we found Wnt3a did not induce BRE-Luc activity, although BMP6 strongly induced it (Fig. 3C). In addition, in BRE-Luc stably expressed C2C12 cells, Wnt3a had no significant effects on BMP2 or BMP6 induced BRE-Luc activity (Fig. 3D). To summarize, Wnt3a induced Id3 expression does not occur through the activation of BMP/Smad pathway.

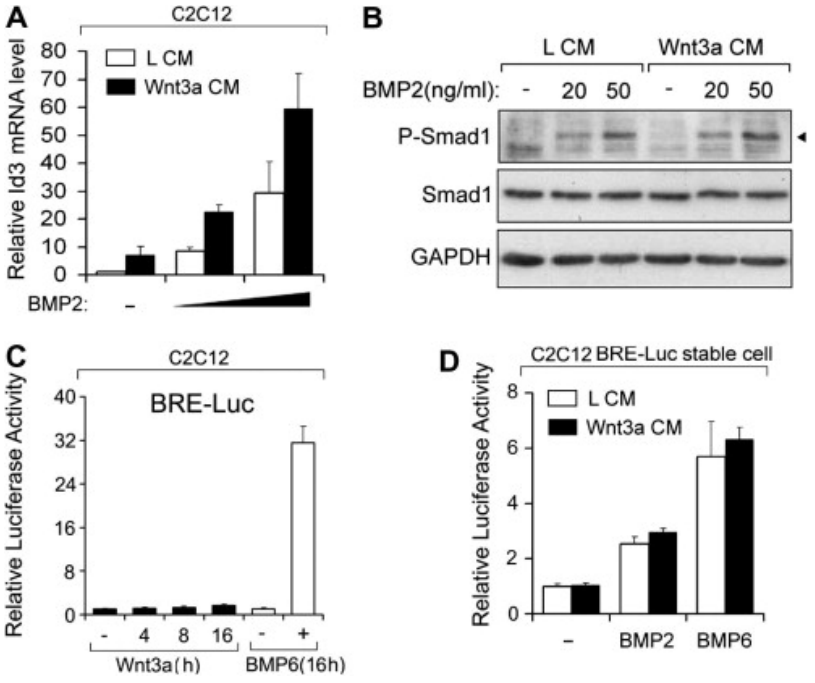

Fig3. Wnt3a induced Id3 expression is not through activation of BMP pathway. (A) C2C12 cells were stimulated with BMP2 (low dose: 20 ng/ml; high dose: 200 ng/ml) and Wnt3a CM as indicated for 10 h. Id3 expression was analyzed by q-PCR. (B) Western blot analysis of BMP pathway activation in C2C12 cells with 1 h treatment of BMP2 and Wnt3a CM as indicated. (C) BRE-Luc transfected C2C12 cells were stimulated with Wnt3a CM. as indicated. Cells were harvested for luciferase assay. (D) BRE-Luc stable expressed C2C12 cells were stimulated with Wnt3a CM in combination with BMP2 (50 ng/ml) or BMP6 (50 ng/ml) for 10 h. Cells were harvested for luciferase assay.

3.4 Id3 mediated Wnt3a-induced cell proliferation and ALP activity in C2C12 cells

Canonical Wnt pathway is important in multiple cellular processes. In C2C12 cells, Wnt3a can induce both cell proliferation and convert myoblast differentiation into osteoblast-like cells. ALP is an early marker for oseoblast differentiation. We next tested whether Id3 is required these responses. By using two independent Id3 shRNAs, we constructed C2C12 stable cell lines with Id3 depletion (Fig. 4A). As measured by cell counting and cell viability assay, Wnt3a-induced cell proliferation was impaired upon Id3 depletion (Fig. 4B and 4C). Moreover, Wnt3a-induced ALP activity was severely blocked in Id3-depleted cells (Fig. 4D and 4E). In addition, Id3 depletion enhanced myogenin level in C2C12 cells under muscle differentiaton

condition (Fig. 4F). We also observed Wnt3a repressed myogenin in the presence of or in the absence of Id3, though on response to Wnt stimulation, samples with Id3 depletion showed more myogenin than control samples (Fig. 4G). We could only conclude that Id3 partially mediates Wnt3a repression on myogenin expression.Taken together, Id3 mediated Wnt3a-induced C2C12 cell proliferation and conversion of myoblast into osteoblast-like cells. Id3 partially mediates Wnt3a repressive effect on myogenin expression.

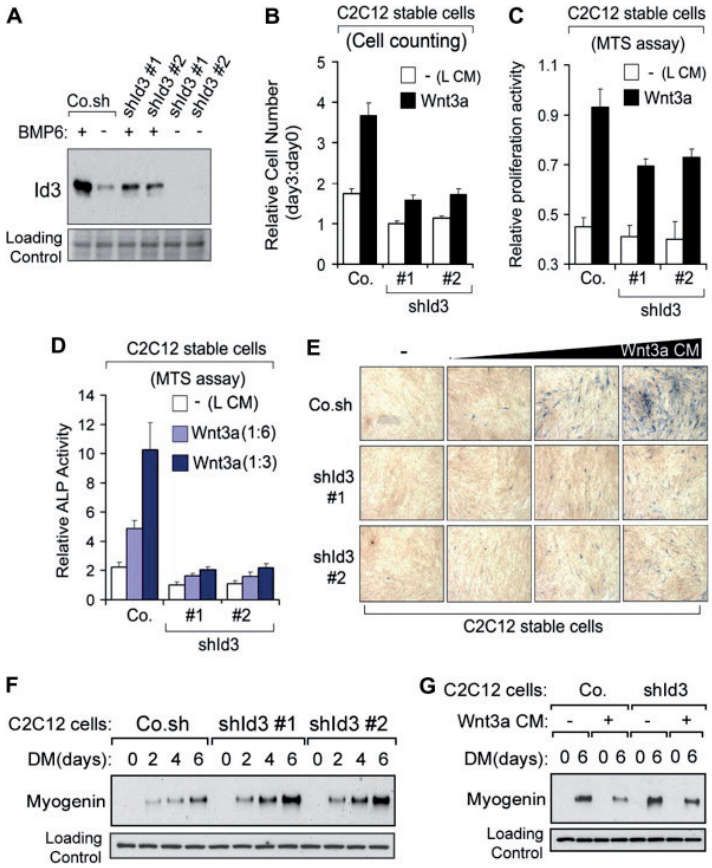

Fig4. Id3 depletion impaired Wnt3a induced cellular proliferation and ALP activity in C2C12 cells. (A) Western blot of Id3 expression in Id3 depleted C2C12 cells. 1 h BMP6 (50 ng/ml) was employed as positive control. (B and C) C2C12 cells were cultured in growing medium (5% FBS in L control CM or Wnt3a CM). Cell number was counted at day 0 and day 3 (B); MTS activity was measured at day 0 and day 3 (C). (D and E) C2C12 cells stably expressing Id3 shRNA #1, #2 was analyzed for Wnt3a-induced ALP activity (D) the histochemically stained cell was shown in (E). Data show the mean and SD of triplicates. Co.sh, control non-targeting shRNA. (F), C2C12 stable cells were culture in differentiation medium (DM) for 0–6 days as indicated, cells

were harvested for anti-myogenin immunoblotting. (G) Control and Id3 depleted C2C12 cells were cultured in differentiation medium (DM) with or without Wnt3a treatment for 6 days, cells were harvested for anti-myogenin immunoblotting.

4. Discussion

In this story, we showed that expression of Id3 can be induced by canonical Wnt signaling in C2C12 cells. Q-PCR and Western blot analysis demonstrated that Id3 mRNA level and protein is promoted by Wnt3a stimulation. Although previous studies reported that the BMP signaling can be increased by Wnt stimulation [25], the evidence that Id1 expression was not induced suggests that BMP/Smad is not likely to be involved in the Id3 induction by Wnt. To confirm this, experiments were performed to examine BMP signaling in C2C12 cells. Both BMP specific reporter and BMP induced Smad phosphorylation was barely affected by Wnt3a stimulation. To further understand the mechanism underlying Wnt-induced Id3 expression, we infected C2C12 cell with lentivirus expressing β-catenin, the essential factor of canonical Wnt signaling. Expression of Id3 was promoted in β-catenin ectopic expressed cells and was even higher in cells expressing stabilized β-catenin, indicating β-catenin mediated transcription is involved in Id3 induction by Wnt. To further consolidate this result, we specifically blocked β-catenin mediated transcription by expressing dnLEF-1 in C2C12 cells. This indeed impaired Wnt3a induced Id3 expression. These results together indicate β-catenin mediated transcriptional induction of Id3 by Wnt. Since TCF/LEF binding site (5′-CTTTGAA-3′) is relatively conserved. A sequences alignment uncovered high similar motif in Id3 promoter region (−300 bp prior to transcriptional start site) but not in corresponding region of Id1 promoter, suggesting potential regulatory machinery. Future work is required to ascertain the binding to TCF/LEF transcriptional factors to this conserved site.

We next examined the functional meaning underlying. Study through loss of function showed Id3 is required for canonical Wnt signaling promoted C2C12 cell proliferation. In osteoblast differentiation essay, Id3 was required for Wnt3a-induced ALP activity, the early marker for osteoblasts. We also observed Id3 antagonized skeletal muscle directed differentiation of myoblast C2C12 cells.

We observed an additive stimulatory effect on Id3 expression by co-stimulation with BMP and Wnt. Previously, Wnt and BMP were shown to act synergistically to promote osteoblast differentiation in vitro and in vivo [26; 27], Whether Id3 plays a role in new bone formation downstream of Wnt and BMP awaits further investigations.

Whereas the induction of ALP by Wnt3a is independent of BMP signaling, BMP –induced ALP in C2C12 cells relies at least in part on Wnt/β-catenin signaling and is associated with increased Wnt expression [28]. Consistent with this finding, we found that Id3 depletion reduced ALP activity that was initiated by BMP (data not shown). Id1 has been shown to activate β-catenin in Akt-dependent manner [29]. It will be interesting to examine whether Id3 can do the same.

In conclusion, our results demonstrate that upon Wnt3a stimulation of C2C12

cells, β-catenin signaling is activated and mediates the induction of Id3 expression. Wnt-induced Id3 expression plays a critical role in promoting cell proliferation and the conversion of myoblasts into osteoblast-like cells. We provide a new mechanism by which canonical Wnt signaling regulates cell proliferation and differentiation.

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