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Interaction of Wnt/β-Catenin and Notch Signaling in the Early Stage of Cardiac Differentiation of P19CL6 Cells

Binhong Li,[1] Zhuqing Jia,[1] Tao Wang,[2] Weiping Wang,[1] Chenguang Zhang,[1] Ping Chen,[1] Kangtao Ma,[1] and Chunyan Zhou[1*]

[1] *Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education of China, Peking University, Beijing 100191, China*

[2] *School of Biomedicine, Faculty of Medical and Human Sciences, The University of Manchester, Manchester M13 9PT, UK*

ABSTRACT

Notch and Wnt/β-catenin signaling both play essential roles and interact closely in cardiomyocyte differentiation but the mechanism of interaction is largely unknown. Here we show that activation of Notch signaling in undifferentiated P19CL6 cells promoted cardiac differentiation, indicated by upregulated expression of early cardiac markers and activated the canonical Wnt pathway, suggested by augmented nuclear translocation of β-catenin. Further activation of the Notch pathway in early differentiating cells (at day 3) inhibited expression of a specific cardiac progenitor marker Islet1 but had no influence on β-catenin translocation. Notch signaling thus played biphasic roles in the early stage of cardiomyocyte differentiation and Wnt/β-catenin signaling. Unlike Notch signaling, Wnt signaling promoted cardiomyocyte differentiation and activated the Notch pathway in either undifferentiated or early differentiating cells. Additionally, β-catenin, recombination signal sequence binding protein-Jkappa (RBP-Jκ), and Notch1 intracellular domain (NICD-1) formed a transcriptional complex which was recruited to the Hes1 promoter region, indicating direct transcriptional regulation of Hes1. We thus document a specific reciprocal interaction between these two signaling pathways during early stage cardiac differentiation of P19CL6 cells. J. Cell. Biochem. 113: 629–639, 2012.

KEY WORDS: NOTCH; WNT/β-CATENIN; ISLET1; CARDIAC DIFFERENTIATION; P19CL6 CELLS

The heart is the first organ formed from mesodermal cells during embryogenesis[Naito et al., 2005]. Cardiogenesis is a carefully orchestrated process that involves cross-regulation of several signaling pathways. Dysregulation of this process may result in congenital heart diseases that are the most common cause of birth defects in humans [Olson and Schneider, 2003]. Wnt, activin/Nodal, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Notch signaling pathways have been investigated extensively for their roles during cardiogenesis [Foley and Mercola, 2004]. However, the interplays between these signaling pathways are still largely unknown.

Notch signaling plays important roles in determining cell fate during development and is activated upon cell-to-cell contact as a result of interactions between Notch receptors and their ligands (Delta or Jagged). Ligand binding results in proteolytic cleavage of Notch receptors by γ-secretases, leading to the release of a 80-kDa intracellular domain of Notch (NICD), which then translocates to the nucleus and complexes with the transcription factor recombination signal sequence binding protein-Jkappa (RBP-Jκ) to activate the transcription of downstream target genes [Ehebauer et al., 2006; Borggrefe and Oswald, 2009]. The importance of Notch signaling in cardiogenesis is clear from the identification of mutations in several Notch signaling components which result in congenital heart defects, including aortic valve disease, Alagille syndrome, and familial forms of cardiomyopathy [High and Epstein, 2008]. Notch signaling is thought to play inhibitory roles in the process of

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Binhong Li and Zhuqing Jia contributed equally to this work.

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*Correspondence to: Chunyan Zhou, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University, 38 Xue Yuan Road, Beijing 100191, China. E-mail: chunyanzhou@bjmu.edu.cn

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cardiomyocyte differentiation [Rones et al., 2000; Nemir et al., 2006; Jang et al., 2008] although this is controversial [Li et al., 2006; Boni et al., 2008; Chen et al., 2008].

Wnt/β-catenin signaling plays pivotal roles in development of multiple tissues through regulation of cell proliferation, differentiation, migration, and gene expression [Logan and Nusse, 2004]. Wnt/β-catenin signaling pathway is centered on β-catenin. In the absence of canonical Wnt signaling, β-catenin complexed with APC and AXIN is phosphorylated by glycogen synthase kinase 3β (GSK3β) in a degradation box, which is polyubiquitinated for proteasomal degradation [Katoh and Katoh, 2007]. In the presence of Wnt ligands, binding to its receptor complex, GSK3β is inhibited, leading to stabilization of cytoplasmic β-catenin. Accumulated cytoplasmic β-catenin subsequently translocates to the nucleus and initiates target gene transcription through T-cell factor (TCF)-lymphoid enhancer factor (LEF) transcription factors [Nakamura et al., 2003; Deb et al., 2008]. Wnt/β-catenin signaling is critical for vertebrate cardiac development. Ablation of β-catenin in Isl1-expressing cardiovascular progenitor cells disrupts multiple aspects of cardiogenesis, resulting in embryonic lethality at E13 [Lin et al., 2007]. Other studies have shown a biphasic role of β-catenin in cardiac specification. The early activation of Wnt/β-catenin signaling promotes cardiac differentiation, whereas the activation of Wnt/β-catenin signaling at the later stage inhibits heart formation [Ueno et al., 2007]. It has also been shown that the canonical Wnt pathway promotes commitment of P19CL6 cells into cardiac lineage at the early stage, inhibiting further differentiation into mature cardiomyocytes at the later stage [Naito et al., 2005].

Emerging evidence has shown crosstalk between Notch and Wnt pathways. Notch1 positively and β-catenin negatively regulate the expression of cardiac transcription factors and Wnt/β-catenin signaling is negatively regulated by Notch1 in cardiac progenitor cells (CPCs) [Kwon et al., 2009]. Notch4 can redirect hemangioblasts to a cardiac fate in part through the coordinated regulation of the BMP and Wnt pathways; some components of the BMP and Wnt pathways are upregulated by Notch4 induction [Chen et al., 2008]. Although crosstalk between Notch and Wnt signaling pathways during cardiac differentiation has been reported, the nature of the interaction between the two pathways is still largely unknown. In this study, we investigated interplay between these two signaling pathways during cardiomyogenesis in a pluripotent mouse embryonic carcinoma cell line (P19CL6), a well-validated model for the dissection of pathways determining cardiomyocyte differentiation. Zero to four days post-induction are designated the early stage, and 4–12 days post-induction are designated the middle-late stages (we refer to this as the late stage) [Charron and Nemer, 1999; Liu et al., 2009, 2010]. We found that Notch1 signaling interacts with Wnt/β-catenin pathway and plays a biphasic role at the early stage of cardiac differentiation of P19CL6 cells. Activation of Notch signaling in undifferentiated P19CL6 cells upregulates the expression of early cardiac markers and augments the nuclear translocation of β-catenin. However, further activated Notch signaling in differentiating cells (day 3) has no more effect on β-catenin translocation; the expression of early cardiac markers is either inhibited or unaffected. In addition, we demonstrate that β-catenin, RBP-Jκ, and NICD-1 are in a transcriptional complex that is recruited to the Hes1 promoter region, indicating direct transcriptional regulation of Hes1.

MATERIALS AND METHODS

CELL CULTURE, INDUCTION OF DIFFERENTIATION, AND REAGENTS

P19CL6 cells were cultured as described previously [Habara-Ohkubo, 1996; Liu et al., 2009]. In brief, the cells were grown in a 60-mm tissue culture grade dish (Nunclon® Delta, Denmark) under adherent conditions with α-minimal essential medium (α-MEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin (100U/ml), and streptomycin (100U/ml) (growth medium), and were maintained in a 5% CO_2 atmosphere at 37°C. To induce cardiac differentiation under adherent conditions, a total of 3.7×10^5 P19CL6 cells were plated in a 60-mm tissue culture grade dish with 4 ml growth medium containing 1% DMSO (Sigma-Aldrich, St. Louis, MO) (differentiation medium). The medium was changed every 2 days. Days of differentiation were numbered consecutively after the first day of DMSO treatment (day 0). The number of spontaneously beating cells was counted using a DMIL microscope (Leica, Deerfield, IL) fitted with a grid system. Recombinant mouse WNT3a as an activator of Wnt pathway (100ng/ml, 24 h) (catalog no. 1324-WN) and recombinant mouse DKK1 as an inhibitor of Wnt pathway (100ng/ml, 24 h) (catalog no. 5897-DK/CF) were purchased from R&D Systems, Inc. (Minneapolis, MN). Notch ligand Jagged simulator recombinant JAG1 (188–204) (catalog no. 61298) (1 μM, 24 h) was purchased from AnaSpec Inc (Fremont, CA). LiCl (an activator of Wnt pathway) (20mM, 12 h) was purchased from Sigma-Aldrich Corporation. The concentrations of these reagents were optimized based on our pilot studies (data not shown) and previous reports [Naito et al., 2005; Deb et al., 2008].

REAL-TIME RT-PCR

Total RNA extraction from P19CL6 cells and real-time RT-PCR were performed as previously described. Transcript levels were normalized to 18S rRNA levels. The primers are listed in Table I. Each value represents the average of at least three independent experiments.

PLASMIDS TRANSFECTION AND RNA INTERFERENCE

P19CL6 cells were transfected with plasmids or synthesized siRNA for gene overexpression or knockdown, respectively. The plasmid carrying Notch1 active region NICD-1 (pCDNA3.1-NICD-1) was a gift from Dr. Martin Baron (University of Manchester). Vector (pCDNA3.1) was used as a control for pCDNA3.1-NICD-1. Non-silencer siRNA (si-Non: sense sequence: UUCUCCGAACGUGUCACGUTT; antisense: ACGUGACACGUUCGGAGAATT) and Notch1 siRNA (si-Notch1: sense: GGACCUCAUCAACUCACAUTT; antisense: AUGUGAGUUGAUGAGGUCCTT) duplexes were synthesized by Shanghai GeneChem Co., Ltd. Transfection of the plasmids or siRNA was performed using LipofectamineTM2000 or Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols, respectively. After 48 h of transfection, the cells were harvested at the indicated days of differentiation for functional assays. All experiments were performed at least three times.

TABLE I. Primers Used in Real-Time RT-PCR

Primers	Sequence (5′-3′)	Product size (bp)	Temperature (°C)
Islet1	F: CTGCTTTTCAGCAACTGGTCA R: TAGGACTGGCTACCATGCTGT	123	60
Gata4	F: CACCCCAATCTCGATATGTTTGA R: GGTTGATGCCGTTCATCTTGT	151	60
α-MHC	F: GCCCAGTACCTCCGAAAGTC R: GCCTTAACATACTCCTCCTTGTC	110	61
ß-MHC	F: ACAACCCCTACGATTATGCGT R: ACGTCAAAGGCACTATCCGTG	100	62
Wnt3a	F: TGGCTGAGGGTGTCAAAGC R: CGTGTCACTGCGAAAGCTACT	181	63
Notch1	F: CCCTTGCTCTGCCTAACGC R: GGAGTCCTGGCATCGTTGG	162	63
Hes1	F: ATAGCTCCCGGCATTCCAAG R: GCGCGGTATTTCCCCAACA	133	62
Hey1	F: CAGCCCTTCGCAGATGCAA R: CCAATCGTCGCAATTCAGAAAG	101	62
18SrRNA	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	151	60

WESTERN BLOTTING

Western blotting was performed as previously described [Liu et al., 2009]. The antibodies used included Islet1 (09218-2E7, Abnova, Taipei, China), Gata4 (sc-25310, Santa Cruz, CA), LaminB (sc-6217, Santa Cruz), GAPDH (ab-9484, Abcam, Hong Kong, China), β-catenin (sc-7199, Santa Cruz), NICD-1 (ab8925, Abcam), Hes1 (sc-13844, Santa Cruz), and horseradish peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology.

IMMUNOFLUORESCENCE STAINING

Immunofluorescence staining were performed as previously described [Liu et al., 2009]. In order to find out whether cells differentiated into cardiomyocytes, P19CL6 cells on coverslips at 0 day or 12 days induced by DMSO were incubated with mouse monoclonal antibody against α-actinin (1:200) (A7811, Sigma-Aldrich) and subsequently incubated with TRITC-conjugated goat anti-mouse IgG (1:200) (Santa Cruz). Nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). Quantitative evaluation of differentiation efficiency was performed by counting α-actinin positive cells. A minimum of five randomly imaged fields of each coverslip were counted from at least five coverslips. The percentage of α-actinin positive cells over the total number of cells counted represents the differentiation efficiency.

P19CL6 cells on coverslips treated with LiCl (20mM, 12 h) or PBS (control) were incubated with primary rabbit polyclonal antibody (sc-7199, Santa Cruz, CA) against β-catenin (1:100) and primary rat monoclonal antibody (ab79972, Abcam) against Notch1 (1:100), and subsequently incubated with TRITC-conjugated goat anti-rabbit IgG (1:200) (Santa Cruze) or FITC-conjugated goat anti-rat IgG (1:200) (Santa Cruz) for 30 min at room temperature. Nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). Immunofluorescence staining was visualized under an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

LUCIFERASE ASSAY

Luciferase assay were performed as previously described [Liu et al., 2010]. The plasmid carrying Hes1-Luc reporter gene was a gift from Prof. Diane S. Krause (University of Yale). Hes1-Luc was activated by NICD via its binding to RBP-Jκ on the Hes1 promoter. pRLCMV plasmid (Promega) was cotransfected as an internal control. Luciferase activity was measured and normalized to Renilla luciferase activity. All experiments were done in triplicates and three independent repeating experiments were performed.

CO-IMMUNOPRECIPITATION ASSAY

Co-immunoprecipitations were performed according to the manufacturer's instructions (Roche, Basel, Switzerland). The lysates derived from P19CL6 cells stimulated with LiCl (20mM, 12 h) or PBS (Con) were incubated with anti-β-catenin antibody (sc-59737, Santa Cruz) or normal IgG for 2 h with gentle rotation, and then with 50 μl of protein G-sepharose slurry (Roche) at 4°C for 1 h. Immunoprecipitates were washed three times with wash buffer and subjected to SDS–PAGE electrophoresis, then detected with anti-NICD-1 (ab8925, Abcam) antibody or anti-RBP-Jκ antibody (sc-28713, Santa Cruz).

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) experiments were performed according to the method described previously [Liu et al., 2009]. The antibodies used for immunoprecipitation included β-catenin (sc-7199, Santa Cruz), NICD-1 (ab8925, Abcam) and RBP-Jκ antibody (sc-28713, Santa Cruz). The primers used for the Hes1 promoter were 5′-TCCTCCCATTGGCTGAAA-3′ (forward) and 5′-GGCCCTGGCGGCCTCTAT-3′ (reverse), containing the RBP-Jκ binding sequence (TGGGAA) 111 bp upstream of the ATG translation start site on the Hes1 promoter. The product length is 101 bp.

CHROMATIN IMMUNOPRECIPITATION/RE-IMMUNOPRECIPITATION

Chromatin immunoprecipitation/Re-immunoprecipitation (ChIP/Re-IP) experiments were performed as previously described [Metivier et al., 2003] and essentially same as primary IPs. Bead elutes from the first immunoprecipitation were incubated with 10 mM DTT at 37°C for 30 min and then diluted at 1:50 in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1) followed by reimmunoprecipitation with the second antibodies.

STATISTICAL ANALYSIS

The data were displayed as mean ± standard deviation (SD). Comparisons between groups were analyzed by Student's *t*-test or ANOVA. The significance was analyzed with SPSS10.0 software and a P-value < 0.05 was considered to be statistically significant.

RESULTS

A GENE EXPRESSION PROFILE DURING THE CARDIAC DIFFERENTIATION OF P19CL6 CELLS

To investigate the roles of Wnt and Notch signaling, and interactions between these two pathways, an in vitro cardiac differentiation model using P19CL6 cells induced by DMSO was used. After exposure to DMSO for 12 days, P19CL6 cells differentiated into mononucleated, rhythmically contracting cells, as confirmed by

immunostaining of sarcomeric α-actinin and video record (Fig. 1A, and S1). Real-time RT-PCR and Western blotting analysis revealed a specific gene expression profile that is consistent with that previously reported. The cardiac specific markers, Islet1 and Gata4, appeared at 3 days, an early stage of differentiation (Fig. 1B,D); the structural proteins, β-myosin heavy chain (β-MHC) and α-myosin heavy chain (α-MHC), exhibited a sudden expression from 9 days, a later stage of differentiation (Fig. 1B). The sequential expression pattern indicates successful cardiac differentiation of P19CL6 cells.

We examined the expression of several key components of the canonical Wnt and Notch pathways during the cardiac differentiation of P19CL6 cells. Although the fold induction of Notch1 was low in comparison to Wnt3a, they both peaked at day 3 (3d) of differentiation and then attenuated sharply at day 6 (6d) (Fig. 1C); nucleus β-catenin (a transducer of Wnt pathway) and NICD-1 (an active intracellular domain of Notch1 receptor) were slightly elevated after DMSO induction and showed the first peak at day 3; both exhibited a second peak at day 9 or day 12, respectively (Fig. 1D). The expression of Hes1 and Hey1, the target genes of the Notch pathway, was also examined. Hes1 expression peaked at day 4 while Hey1 sustained a relatively high level from day 4 to day 9 (Fig. 1C). Although there are some slight differences of the expression pattern, the activation of Wnt and Notch signaling pathways was mainly in the early stage of cardiac differentiation, on which we therefore focused our investigation.

BIPHASIC ROLES OF NOTCH SIGNALING ON THE EARLY STAGE OF CARDIOMYOCYTE DIFFERENTIATION

Notch signaling is thought to play an inhibitory role in the process of cardiac differentiation [Rones et al., 2000; Nemir et al., 2006; Jang et al., 2008]. However, our results exhibited an abruptly upregulated expression of Notch signals, which correlated with the expression of the cardiac specific marker Islet1. To clarify whether and how Notch

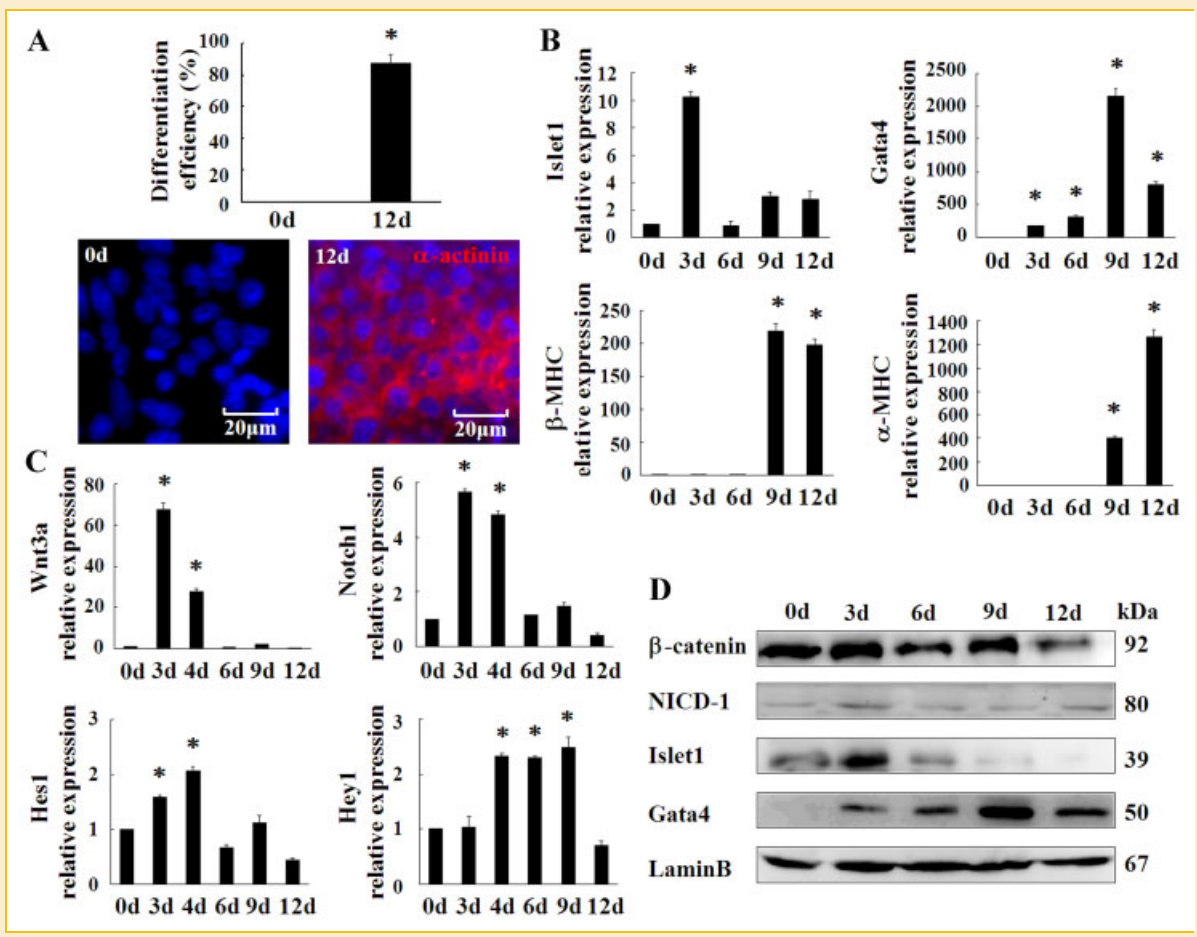

Fig. 1. Gene expression profile during cardiac differentiation of P19CL6 cells. A: Uninduced and induced for 12 days P19CL6 cells were analyzed by immunofluorescence staining with a monoclonal antibody against sarcomeric α-actinin (red in cytoplasm). Nuclei were counterstained with Hoechst33342 (blue). Scale bars are 20 μm. B, C: Real-time RT-PCR analyzed the expression of cardiac-specific genes (Islet1, Gata4, β-MHC, α-MHC), and the components of Wnt/β-catenin signaling and Notch signaling (Wnt3a, Notch1, Hes1, Hey1) with RNA extracted from P19CL6 cells at indicated time points. D: Western blotting demonstrated the expression profile of β-catenin (an effector of Wnt pathway), activated Notch1 intracellular domain (NICD-1), Islet1, and Gata4 (early cardiac-specific markers). LaminB was used as an internal control. The data represent three independent experiments. Each bar represents mean ± SD from three samples ($^*P < 0.05$, vs. the 0d). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

signaling affected cardiac differentiation at the early stage, we treated P19CL6 cells that were treated either with DMSO induction for 3 days (3d) or without DMSO induction (0d), with 1 μM JAG1, an activator of the Notch signaling pathway for 24 h, and then checked the expression of Islet1 and Gata4. JAG1 activated the Notch pathway as indicated by nuclear accumulation of NICD-1 (Fig. 2A), which was also accompanied by increased expression of Islet1 and Gata4, at both mRNA and protein levels, in uninduced P19CL6 cells. However, the expression of Islet1 at RNA level was greatly inhibited and the expression of Gata4 was not affected in cells with DMSO induction for 3 days (Fig. 2B). We then transfected 100nM specific Notch1 siRNA (si-Notch1) into P19CL6 cells with DMSO induction for 3 days to inhibit the Notch pathway that naturally peaked at this time point. As a result, the expression of Notch1 and its target genes Hes1 and Hey1 was downregulated (Fig. 2C) and the expression of Islet1 was increased, but the expression of Gata4 was not significantly affected (Fig. 2D). These results suggest that Notch signaling plays stage-specific biphasic roles in P19CL6 cells, especially on cardiac specific early marker Islet1. Notch signaling promotes Islet1 and Gata4 genes expression in uninduced P19CL6 cells which may benefit the initiation of cardiac differentiation. However when endogenous expression of Islet1 and Gata4 was abundant, further activated Notch signaling inhibited the early marker Islet1 but had no effect on Gata4, probably because Gata4 was maintained at a relative high level during the whole process of cardiac differentiation.

THE ROLES OF WNT SIGNALING ON PROMOTING CARDIOMYOCYTE DIFFERENTIATION AT THE EARLY STAGE OF CARDIOMYOGENESIS

Wnt/β-catenin signaling has been demonstrated to be necessary for primitive streak formation in vivo and for embryonic stem cell-derived mesoderm formation in vitro [Lindsley et al., 2006]. To characterize the role of Wnt/β-catenin signaling during the early stage of differentiation, a series of real-time RT-PCR and western blotting were carried out. We treated P19CL6 cells with recombinant WNT3a protein or recombinant DKK1 protein for 24 h to activate or inhibit Wnt/β-catenin signaling, respectively. Cells were subsequently examined for Islet1 and Gata4 expression. As shown in Figure 3A, the addition of WNT3a triggered activation of canonical Wnt signaling pathway as indicated by augmented nuclear import of β-catenin. Meanwhile, WNT3a dramatically upregulated the expression of Islet1 and Gata4 at day 0. Unlike Notch signaling, WNT3a upregulated the expression of Islet1 and Gata4 in cells with DMSO induction for 3 days (Fig. 3B). The application of DKK1

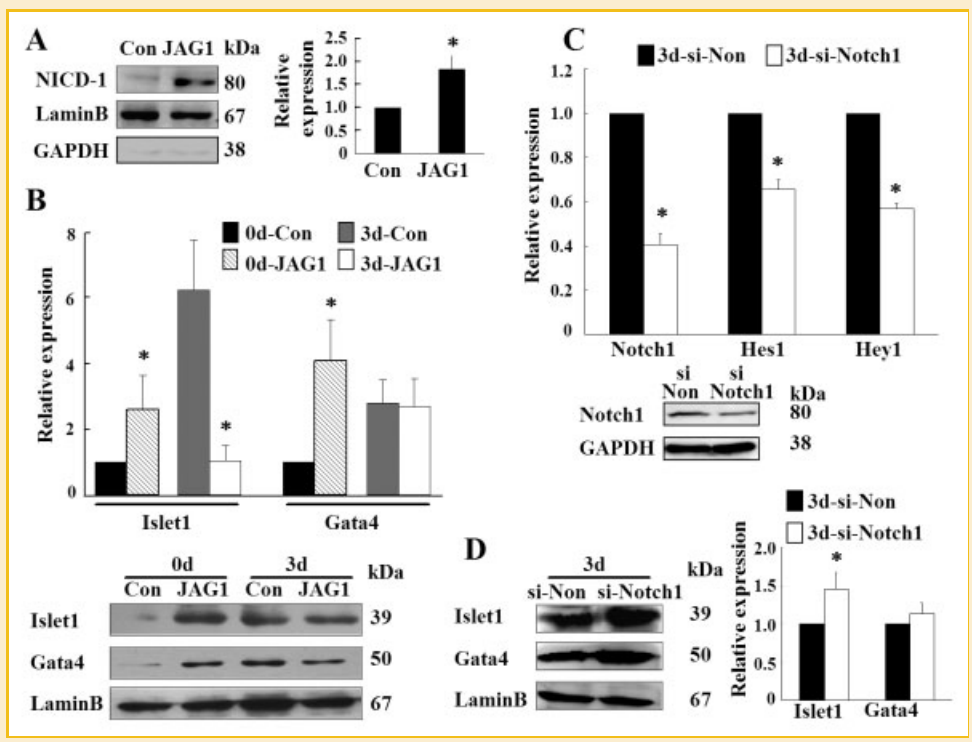

Fig. 2. The biphasic roles of Notch signaling at the early stage of cardiomyocyte differentiation. A: Western blotting was performed to detect NICD-1 level in nuclei of P19CL6 cells stimulated by JAG1. LaminB and GAPDH were used as internal controls. The densitometric analysis was performed with images of three independent experiments and the result was shown on the right of a representative image on the left. B: Real-time RT-PCR (upper) and western blotting (lower) were performed to evaluate the expression change of Islet1 and Gata4 at day 0 (0d) and day 3 (3d) in P19CL6 cells treated with or without JAG1 (1 μM) for 24 h. LaminB was used as an internal control. C: Knockdown of the Notch1 gene was performed by treating P19CL6 cells with 100nM Notch1-siRNA (si-Notch1) for 48h, Non-silencer RNA (si-Non) served as a control. Real-time RT-PCR and western blotting demonstrated the expressions of Notch1, Hes1, and Hey1 3 days after Notch1 knockdown (3d). GAPDH was used as an internal control. D: Western blotting demonstrated the expression of Islet1 and Gata4 at day 3 (3d) after Notch1 knockdown. LaminB was used as an internal control. The densitometric analysis was performed with images of three independent experiments and the result is shown on the right of a representative image on the left. Each bar represents mean ± SD from three samples ($^*P < 0.05$, vs. the control of same day).

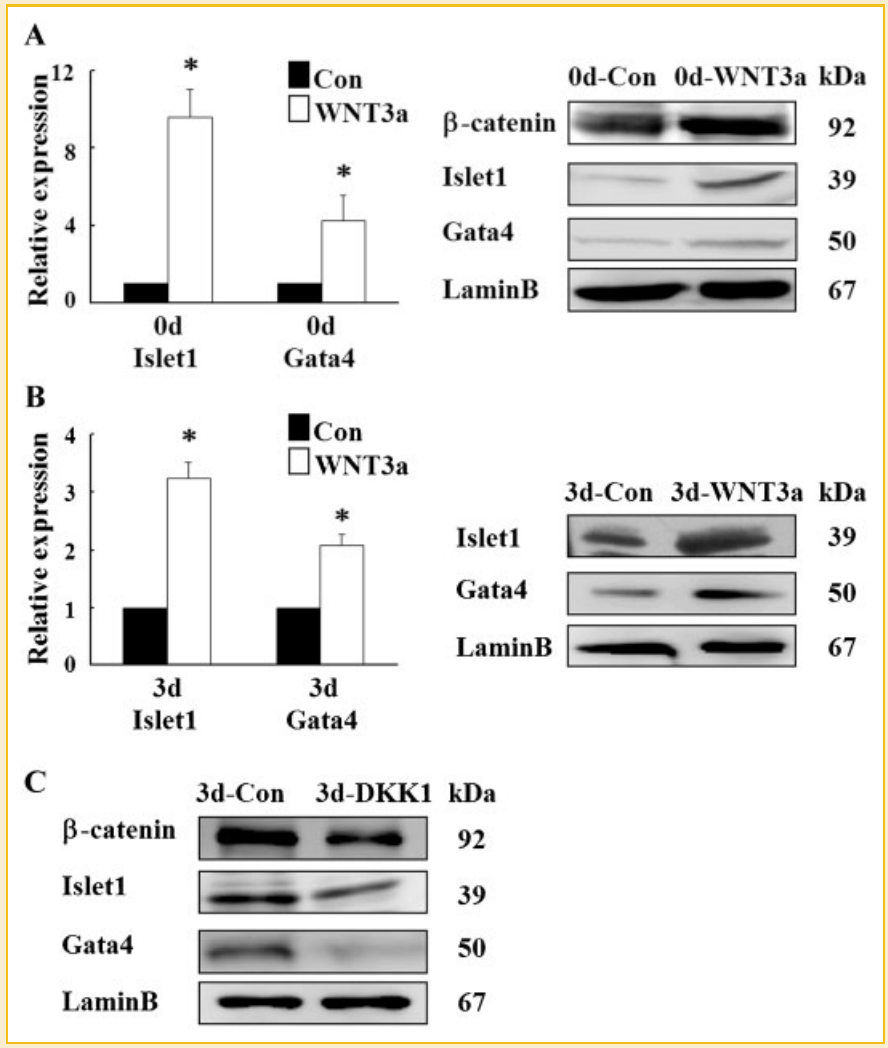

Fig. 3. The promotion roles of Wnt signaling on cardiomyocyte differentiation. A, B: Real-time RT-PCR and western blotting showed the expression level of nuclear β-catenin, Islet1, and Gata4 in P19CL6 cells treated with 100ng/ml WNT3a protein for 24 h and harvested at day 0 (0d) and day 3 (3d). C: The protein levels of β-catenin, Islet1, and Gata4 were analyzed by western blotting for P19CL6 cells treated with DKK1 (100ng/ml, 24 h). LaminB was used as an internal control. The data represent three independent experiments. Each bar represents mean ± SD from three samples ($^*P < 0.05$, vs. the control).

confirmed that the Wnt pathway behaved differently from the Notch pathway on P19CL6 cells induced by DMSO for 3 days, a visible downregulation of Islet1 and Gata4 was observed in DKK1 treated cells (Fig. 3C). These results indicate that Wnt signaling may promote cardiac differentiation at the early stage.

CROSSTALK BETWEEN WNT/β-CATENIN SIGNALING AND NOTCH SIGNALING AT THE EARLY STAGE OF CARDIAC DIFFERENTIATION

Emerging evidence suggest that Wnt signaling acts in concert with Notch pathways to regulate hematopoietic or retinal stem cells [Duncan et al., 2005; Das et al., 2008]. In our study, the components of these two pathways showed similar gene expression profiles during the process of P19CL6 cells differentiation into cardiomyocytes. Whether there were interplays between these two signaling pathways was unknown. We used LiCl or WNT3a as activators of Wnt/β-catenin signaling to treat uninduced P19CL6 cells. Real-time RT-PCR and Western blotting analysis results showed that the expression level of Notch1 was upregulated in response to LiCl or WNT3a stimulation; meanwhile, the expression of NICD-1 was also increased (Fig. 4A). In addition, LiCl and WNT3a both could activate the Hes1 promoter in uninduced P19CL6 cells more than threefold (Fig. 4B), indicating a direct regulation of Wnt/β-catenin signaling on Hes1 transcription, probably through RBP-Jκ as previously reported [Yamamoto et al., 2001]. In contrast, when Wnt/β-catenin signaling was inhibited by DKK1, the expression of Notch1 was downregulated and the quantity of NICD-1 in the nucleus was reduced (Fig. 4C). These results suggest that Wnt/β-catenin signaling could augment Notch signaling in our cardiac differentiation model.

Subsequently, we mimicked the activation of Notch pathway by transfecting plasmid pCDNA3.1-NICD-1 for 48 h in P19CL6 cells to investigate its effect on Wnt signaling. We found that the β-catenin level in the nucleus was significantly promoted by overexpression of NICD-1 at day 0 (0d), however, the nuclear accumulation of β-catenin at day 3 (3d) was not further enhanced by overexpression

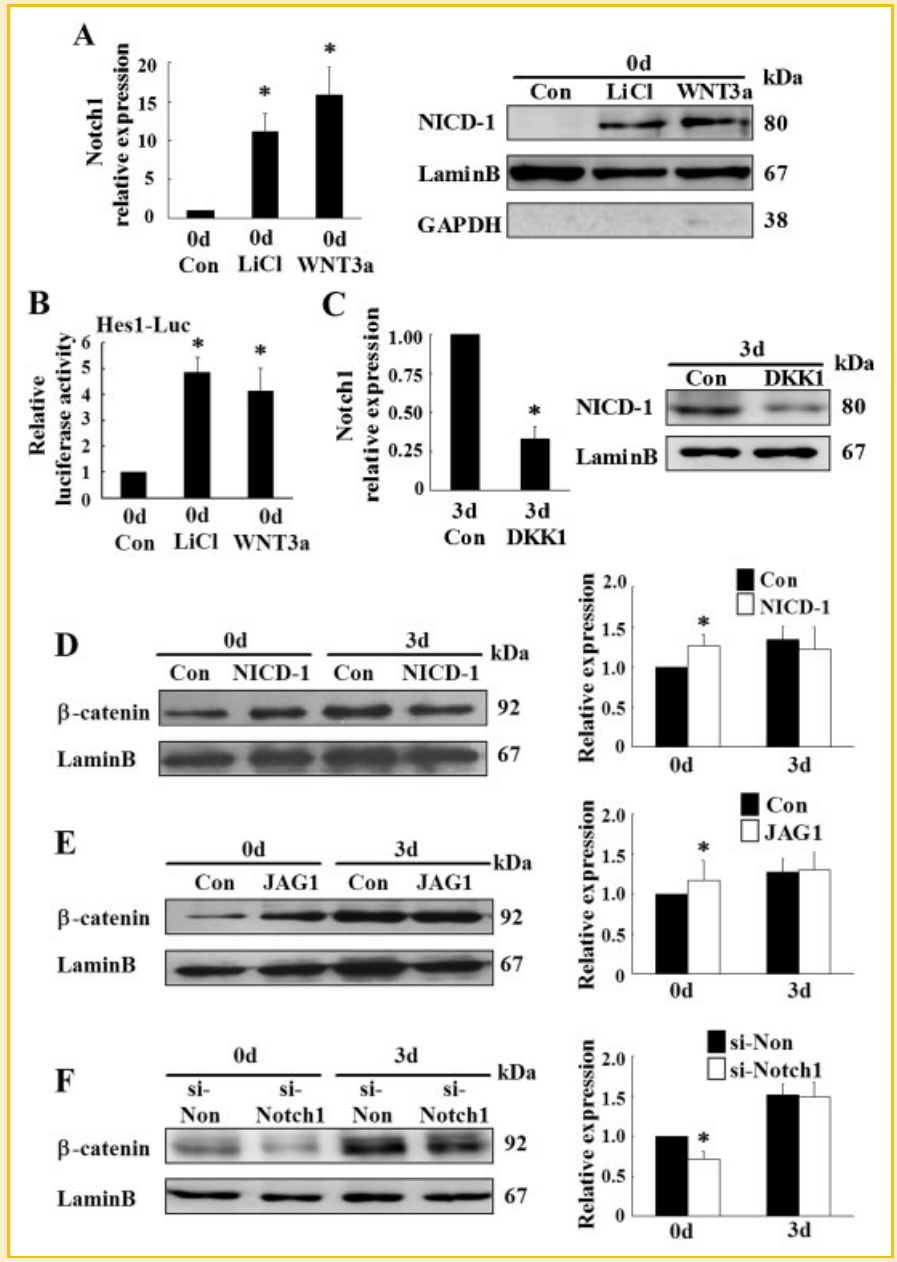

Fig. 4. Crosstalk between Wnt/β-catenin signaling and Notch signaling at the early stage of cardiac differentiation. A, C: Real-time RT-PCR and western blotting demonstrated the expression changes of Notch1 and the activated Notch1 intracellular domain (NICD-1) in response to treatment with LiCl (20mM, 12 h), WNT3a (100ng/ml, 24 h), or DKK1 (100ng/ml, 24 h) at day 0 (0d) or day 3 (3d) in P19CL6 cells, respectively. B: Luciferase assay was performed to evaluate the activities of Hes1 promoter in response to treatment with LiCl (20mM, 12 h), WNT3a (100ng/ml, 24 h). Luciferase activity was measured and normalized to Renilla luciferase activity. D, E: Western blotting demonstrated nucleus β-catenin level in uninduced or induced P19CL6 cells at indicated days after 48 h transient transfection with NICD-1 plasmid or after 24 h treatment with JAG1 (1μM). Transfection of vector (pCDNA3.1) and absence of JAG1 were used as controls, respectively. F: Western blotting was performed to detect nuclear β-catenin in P19CL6 cells treated by transient transfection with Notch1 siRNA (si-Notch1) or Non-silencer RNA (si-Non). LaminB and GAPDH were used as internal controls. The densitometric analysis was performed with images of three independent experiments and the results were shown on the right of a representative image of D, E, and F. Each bar represents mean ± SD from three samples ($^*P < 0.05$, vs. the control).

of NICD-1 (Fig. 4D). The same effect was seen in the cells treated with JAG1 (Fig. 4E). Consistently, knockdown of Notch1 expression with 100nM of siRNA (si-Notch1) indeed reduced nuclear accumulation of β-catenin at day 0, but not at day 3 (Fig. 4F). Taken together, these observations suggest that the Notch signaling may buffer Wnt signaling to keep it at an appropriate level.

β-CATENIN AND NICD-1 WITHIN A TRANSCRIPTIONAL COMPLEX BINDING ON THE HES1 PROMOTER

To understand how Notch signaling was upregulated by Wnt/β-catenin signaling, we used LiCl to activate Wnt/β-catenin signaling in P19CL6 cells. Confocal immunofluorescence using anti-β-catenin and anti-Notch1 antibodies revealed obvious subcellular colocalization of β-catenin and Notch1, after LiCl stimulation

(Fig. 5A). We further examined whether β-catenin and NICD-1 existed in a common complex by co-immunoprecipitation assay. Cell lysates prepared from P19CL6 cells cultured in the absence or presence of LiCl were precipitated with anti-β-catenin or control mouse nonimmune IgG, and then immunoblotted with anti-NICD-1 antibody or anti-RBP-Jκ antibody. We found that NICD-1 protein and RBP-Jκ protein were indeed coprecipitated with β-catenin. LiCl treatment resulted in more NICD-1 and RBP-Jκ being recruited into this protein complex (Fig. 5B).

It has been well established that the activation of Notch pathway results in nuclear translocation of NICD-1, where NICD-1 associates with the CSL [CBF1/RBP-Jκ/Su (H)/Lag1] family of DNA-binding proteins to form a transcriptional activator. Hes1 was identified as a classical Notch target gene and its expression correlated with the expression profiles of cardiac early marker Islet1, Wnt, and Notch signal molecules as showed in Figure 1. It has been reported that NICD-1 could also form a complex with β-catenin and Hes1 in neural precursor cells [Shimizu et al., 2008]. We asked whether β-catenin and Hes1 existed in one complex with RBP-Jκ. ChIP assay and ChIP Re-IP assay demonstrated that β-catenin was also recruited into the NICD-1 and RBP-Jκ complex on the Hes1 promoter in uninduced P19CL6 cells, indicating a direct transcriptional regulation of Hes1 by β-catenin (Fig. 5C,D). The schematic drawing summarized the interaction model between the Notch and Wnt/β-catenin pathways at the early cardiac differentiation stage of P19CL6. β-catenin formed a protein complex with RBP-Jκ and NICD, which bound to the promoter of Hes1 (Fig. 5E).

DISCUSSION

Notch and Wnt/β-catenin signaling participate in the regulation of almost every aspect of cardiomyocyte differentiation and heart development. In this study, we investigated the roles of Wnt and

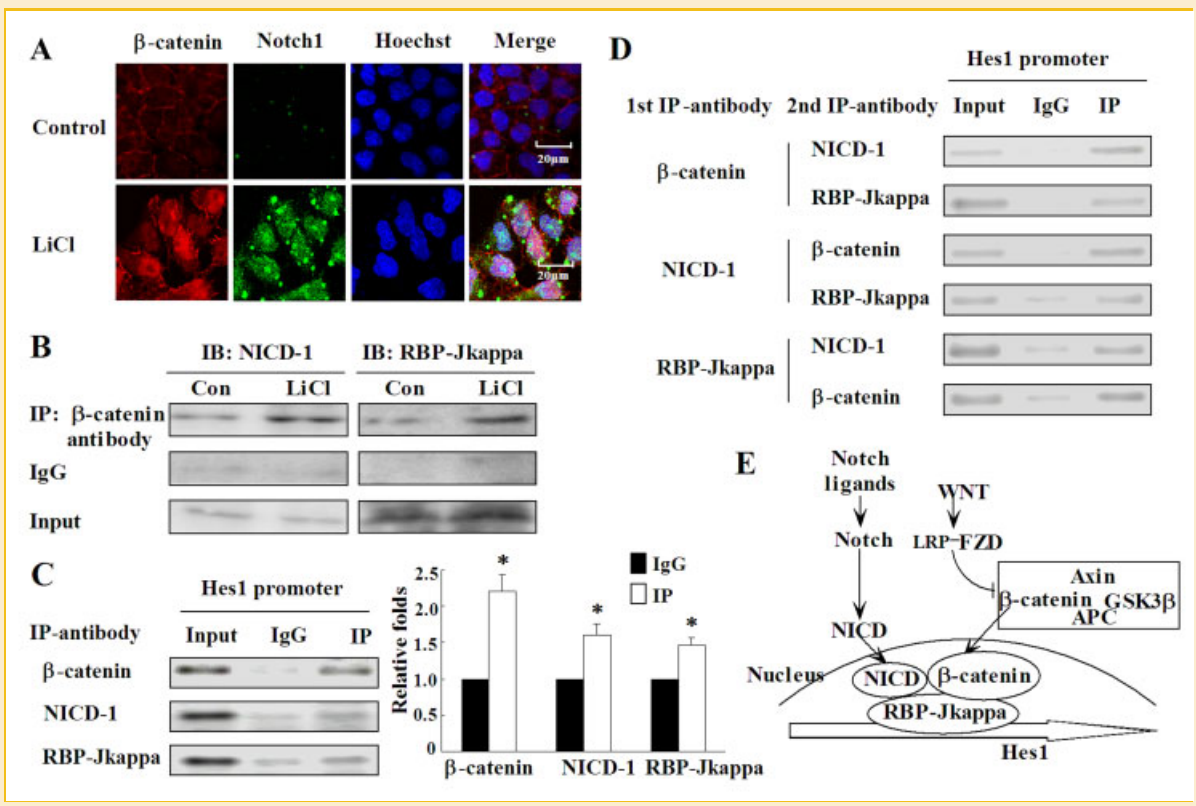

Fig. 5. β-catenin and NICD-1 within a transcriptional complex binding on the Hes1 promoter. A: Immunofluorescence staining demonstrated LiCl treatment induced the translocation of both β-catenin (red) and Notch1 (green) into the nucleus (blue) in P19CL6 cells (scale bar, 20μm). Nuclei were counterstained with Hoechst 33342. B: Co-immunoprecipitation was performed with cell lysates from P19CL6 cells treated with LiCl or PBS (control) with anti-β-catenin antibody (IP) or normal IgG as a control antibody, and then detected by anti-NICD-1 (IB, left) or recombination signal sequence binding protein-Jkappa (RBP-Jκ) antibodies (IB, right). 10% of total cell lysates were used as input. C: ChIP assays were performed with β-catenin, NICD-1, and RBP-Jκ antibodies in P19CL6 cells, respectively. The immunoprecipitated (IP) DNA fragments were amplified by PCR for the promoter region of Hes1. Input represents 10% of the total input chromatin and IgG served as a negative control. The densitometric analysis was performed with images of three independent experiments and the results are shown on the right of a representative image of (C). Each bar represents mean ± SD from three samples ($^*P < 0.05$). D: ChIP Re-IP were performed with β-catenin, NICD-1, and RBP-Jκ antibodies for the first round of immunoprecipitation; the precipitates were further immoprecipitated with NICD-1, RBP-Jκ, and β-catenin antibodies for the second round immunoprecipitation. The immunoprecipitated (IP) DNA fragments were amplified by PCR for the promoter region of Hes1. Input represents 10% of the total input chromatin and IgG served as a negative control. E: Schematic drawing summarizing our model about interactions between the Notch and Wnt/β-catenin pathways at the early stage of P19CL6 differentiation into cardiomyocytes. Augmented nuclear import of β-catenin formed a protein complex with RBP-Jκ, NICD, which bound the promoter of Hes1. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Notch1 signaling and possible interactions between the two pathways in the process of cardiac differentiation of P19CL6 cells. The results demonstrate that Wnt/β-catenin signaling promotes Notch signaling activation from day 0 to day 3 of cardiac induction, whereas Notch signaling plays a biphasic role: It augments the nuclear translocation of β-catenin in undifferentiated P19CL6 cells but has no more influence in differentiating cells (from day 4). Furthermore, our results demonstrate formation of a complex of β-catenin, RBP-Jκ, and NICD-1 on the Hes1 promoter region, suggesting direct regulation on Hes1.

Notch signaling is thought to act as an inhibitor of cardiomyocyte differentiation during development [Rones et al., 2000; Nemir et al., 2006; Jang et al., 2008]. Cardiomyogenesis is increased in RBP-Jκ-deficient ES cells [Schroeder et al., 2003]. Differentiation of ES cells into cardiomyocytes was favored by inactivation of the Notch1 receptor [Nemir et al., 2006]. However, some different roles of Notch signaling have been observed. When Notch signaling was activated by JAG1 protein, the differentiation of MSC into cardiomyocytes was enhanced [Li et al., 2006]. Notch1 signaling could initiate cardiomyocyte differentiation by direct regulation on Nkx2.5 transcription [Boni et al., 2008]. Notch4 receptor could efficiently respecify hemangioblasts to a cardiac fate, resulting in the generation of populations consisting of 60% cardiomyocytes[Chen et al., 2008]. In our study, Notch signaling exhibited biphasic roles: It upregulated early cardiac markers such as Gata4 and Islet1 in uninduced P19CL6 cells (day 0), but at the third day of induction further activation of Notch signaling inhibited the expression of Islet1 at RNA level, a well established early cardiac marker with a peaked expression at day 3 in our cardiac differentiation system. However, Notch signaling had no influence on the expression of Gata4, a marker that appeared at day 3 but peaked at day 9 of induction. It has been reported that mouse ES cells with high level of Hes1 expression tended to differentiate into mesodermal cells [Kobayashi et al., 2009]. We proposed that Notch signaling could make a decision to switch on cardiac differentiation. Meanwhile, it also played a buffering action to keep some genes expression, especially stage-specific genes, at appropriate levels. This might explain why Notch signaling had no influence on Gata4, its expression level reached a peak at day 9. As we focused our observations on the early stage of cardiac differentiation, we do not know whether the activation of Notch signaling at a later time would give different consequences.

Regarding the role of canonical Wnt pathway in heart formation and cardiomyocyte differentiation, there have been several contradictory reports depending on the model organisms used. The activation of Wnt/β-catenin pathway promoted cardiogenesis of P19CL6 cells [Nakamura et al., 2003]; canonical Wnt signaling was activated during early ES cell differentiation and was required for generation of ES cell-derived mesoderm [Lindsley et al., 2006]. Whereas it has also been reported that ectopic Wnt signals repressed heart formation from anterior mesoderm of chick embryos [Marvin et al., 2001]; Wnt antagonists Dkk-1 and Crescent specifically induced heart formation in explants of ventral marginal zone of Xenopus [Schneider and Mercola, 2001], and β-catnein promoted expansion of CPCs and negatively regulated cardiac differentiation and Notch1 [Kwon et al., 2009]. Chen et al. [2008] reported that Notch4 efficiently respecified hemangioblasts to a cardiac fate of mouse ES cells, which was required inhibition of Wnt/β-catenin pathway. It has been proposed that Wnt/β-catenin signaling has biphasic effects on cardiogenesis depending on the stage of differentiation; it displayed positive effects at the early stage and negative effects at the later stage of cardiac differentiation [Ueno et al., 2007], Nkx2.5 might involve in the early stage effect of Wnt/β-catenin signaling [Liu et al., 2009]. We provide evidences that Wnt signaling could upregulate the expression of a specific cardiac progenitor marker Islet1 and thus promote cardiac differentiation. It should be pointed that the roles of non-canonical Wnt pathway itself might be more complicated during the cardiac differentiation process of P19CL6 cells, as we found that their expression profiles are different; Wnt5a expression was peaked at day 3–4 after induction, similar to Wnt3a, while Wnt11 expressed at later stage of differentiation (unpublished data). We focused our investigation on canonical Wnt pathway in this study.

A number of reports indicate crosstalk between Notch and Wnt pathways, and have given the name "Wntch" to describe the close relationship between Wnt and Notch signaling [Hayward et al., 2008]. The addition of WNT3a increased early T-cell development via increased activation of the Notch1, possibly resulting from Notch intracellular domain stabilization [Aoyama et al., 2007]. It has been shown that GSK-3β could modulate Notch-mediated signaling through the phosphorylation of Notch intracellular domain and thus stabilize the activated Notch protein [Foltz et al., 2002]. Wnt/β-catenin signaling pathway could also affect Notch ligands. JAG1 was identified as a conserved target of Wnt/β-catenin pathway [Katoh and Katoh, 2006] and β-catenin could stimulate JAG1 transcription [Estrach et al., 2006; Rodilla et al., 2009]. It has been also reported that WNT3a alone could increase Hes1 expression in the absence of Notch ligand [Aoyama et al., 2007]. Likewise Notch signaling regulates Wnt signaling at multiple levels, but the consequences are controversial, as both positive and negative regulation have been observed. Notch signaling activated the Wnt pathway in hematopoietic progenitor cells via direct upregulation of frizzled receptor family [Zhou et al., 2009]. Notch1 negatively regulated Wnt/β-catenin signaling by control of phosphorylated β-catenin accumulation and promoted CPCs differentiation [Kwon et al., 2009]. It has been reported that NICD-1 could form a complex with β-catenin and Hes1 in neural precursor cells, while RBP-Jκ was required for the activation of Hes1 that was mediated by NICD-1 in cooperation with β-catenin [Shimizu et al., 2008]. Here we investigated the crosstalk between Wnt/β-catenin and Notch1 signaling pathways in P19CL6 cells. Our initial experimental results showed that some key components of Wnt/β-catenin pathway (Wnt3a, β-catenin) and Notch pathway (Notch1, Hes1) exhibited similar expression profiles during the cardiac differentiation of P19CL6 cells. Wnt/β-catenin signaling upregulated Notch signaling at the early stage of cardiomyocyte differentiation, not only by increasing the expression of Notch1, but also forming a transcriptional complex including NICD-1 and β-catenin, which was recruited to Hes1 promoter region. Meanwhile, Notch1 signaling played a biphasic role on Wnt signaling. The activation of Notch1 pathway increased Wnt/β-catenin signaling in undifferentiated P19CL6 cells but not in differentiating cells (day 3 after

cardiac induction). We propose that Notch signaling upregulated by Wnt/β-catenin signaling may take a buffer feedback on Wnt/β-catenin signaling. It has been found that Notch could target Armadillo, the β-catenin analog of Wnt pathway in Drosophila, for degradation to buffer the function of activated Armadillo [Sanders et al., 2009]. We provided evidence for reciprocal control of Wnt/β-catenin and Notch1 at the early stage of cardiomyocyte differentiation of P19CL6 cells. Whether reciprocal regulation between these two pathways is a synergistic or an antagonistic depends on cell-context and temporal stage.

Although the impact of Wnt/β-catenin and Notch signaling pathway on the cardiac differentiation of P19CL6 cells still requires further investigation, our findings that Notch signaling plays biphasic roles on cardiomyocyte differentiation and Wnt/β-catenin signaling at the early stage of induction may explain, in part, contradictory reports on the roles of integration between Notch and Wnt pathways on cardiac differentiation.

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