Original Research

The effects of β-catenin on cardiomyogenesis via Islet-1 and MLIP ubiquitination

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Impact Statement

Mesenchymal stem cells (MSCs) can treat myocardial injury–related diseases by differentiating into cardiomyocytes. It has been shown that Islet-1 can perform effectively in developing the heart. We have previously found that the pivotal factor in histone acetylation regulation in this process is Islet-1. In this study, we show that decreasing β-catenin regulates the ubiquitination of two proteins, upregulates Islet-1 and accelerates MLIP degradation, reducing the amount of free Islet-1 binding to MLIP and increasing the amount of binding to GCN5 in the nucleus. Therefore, the transcriptional activity of Islet-1 is significantly activated, inducing C3H10T1/2 cells to differentiate into the myocardium. β-catenin and Islet-1 are key factors to induce myocardial differentiation. Further knowledge of biochemical pathways, including molecular signaling pathways, can provide more insights into the myocardial differentiation mechanism of MSCs. This study provides the groundwork of application of MSCs in the clinical treatment of heart disease.

Abstract

Mesenchymal stem cells (MSCs) can treat myocardial injury–related diseases by differentiating into cardiomyocytes. Islet-1 plays an essential role in cardiac maturation. We have discovered that Islet-1 plays a crucial role in the histone acetylation regulation in this process. In addition, to increase GATA4/Nkx2.5 expression, Islet-1 may bind to Gcn5 and then guide Gcn5 to the GATA4/Nkx2.5 promoters, thereby facilitating the differentiation of MSCs into cardiomyocytes. Islet-1 is an important factor in the maturation of the heart. We have previously found that the pivotal factor in histone acetylation regulation in this process is Islet-1. Furthermore, Islet-1 and Gcn5 may boost GATA4/Nkx2.5 expression, which in turn promotes cardiomyocyte differentiation from MSCs. But the molecular mechanism of Islet-1 binding to GCN5 has not been elucidated. In this study, we found that the competitive binding relationship between Islet-1 and MLIP and GCN5 affected myocardial differentiation. The key enzymes of ubiquitination modification of MLIP and Islet-1 are UBE3C and WWP1, respectively. When short hairpin RNA (shRNA) was used to inhibit β-catenin expression, we found that the expression of UBE3C was upregulated, modifying MLIP ubiquitination and reducing its expression, and it upregulated Islet-1 by inhibiting the expression of WWP1. By using the chromatin immunoprecipitation (ChIP) and luciferase reporter system, we found that when MLIP binds to Islet-1, it significantly inhibits the transcriptional activity of Islet-1. In summary, our results show that decreasing β-catenin regulates the ubiquitination of Islet-1 and MLIP, affecting their expression, reducing the amount of Islet-1 binding to MLIP, and increasing the amount of binding to GCN5 in the nucleus. Therefore, the transcriptional activity of Islet-1 is significantly activated, inducing C3H10T1/2 cells to differentiate into myocytes. Further knowledge of biochemical pathways, including molecular signaling pathways, can provide more insights into the myocardial differentiation mechanism of MSCs.

Keywords: C3H10T1/2 cells, cardiomyogenesis, Islet-1, MLIP, ubiquitination, β-catenin

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Introduction

The process of heart development is intricate and very dynamic. The heart is the first organ to form in a mouse embryo, beginning at about embryonic day 6.5 (E6.5), for the duration of gastrulation, when primitive cardiac progenitor cells (CPCs) migrate and undertake lineage determination.[1] There are two distinct cell populations that make up the heart throughout development: the first heart field (FHF) and the second heart field (SHF).[2] The left ventricle (LV) and atrium are formed by the FHF, which also makes up the cardiac crescent and the primitive heart tube. The expansion of the primitive heart tube is dependent on the SHF, which acts as a cell reserve for future heart growth. The SHF is responsible for the development of the right atrium, ventricle, and outflow tract (OFT). In addition, distinct genetic markers for each cardiac field have been identified. Tbx5, for instance, is unique to the FHF, while Islet-1, Fgf10, Tbx1, and Fgf8 define the SHF.[3]

ISL LIM Homeobox 1 (Islet-1) is a protein-coding gene that codes for a transcription factor from the LIM/homeodomain family. In both mice and humans, Islet-1 is crucial for heart development.[4] Islet-1-positive adult stem cells in mice and humans likely function in cardiac repair and regeneration.[5] Islet-1 identifies the proliferating, undifferentiated progenitors of the dorsal/medial heart field.[6] These progenitors cannot divide, survive, or migrate into the developing heart without Islet-1. At E10, islet-1-null mice undergo embryonic death. Their hearts lack the outflow tube and right ventricle from the SHF, and they have much less atrial tissue. Understanding the elements that control Islet-1 expression is crucial given its crucial function in cardiomyogenesis.

Moreover, Muscle-enriched A-type Lamin-interacting Protein (MLIP) is a newly described heart-specific nuclear protein. Huang *et al.*[7] demonstrated that MLIP expression is restricted to cardiomyocytes. Previous researchers further characterized MLIP as a transcription cofactor of the LIM/homeodomain transcription factor Islet-1. MLIP physically interacts with Islet-1 and represses its transcriptional activity.[8]

During embryogenesis and adult tissue homeostasis, Wnt/β-catenin signaling directs several cellular procedures, involving the cell cycle, stem cell self-renewal, differentiation, and organogenesis.[9–11] Multiple investigations, using both chick and mouse embryos and cell culture methods, have shown that blocking Wnt signaling induces cardiogenic mesoderm. Anterior endoderm-derived Wnt antagonists Dickkopf1 and Crescent promote cardiogenic mesoderm development in chick embryos.[12] Ablation of β-catenin in endodermal tissues employing a Cytokeratin19 promoter-driven Cre (K19-Cre) recombinase caused ectopic heart development in mouse embryos.[13] Increased GATA4(pos)/Sca-1(pos)-resident cardiac progenitor cell differentiation is one mechanism by which β-catenin depletion attenuates postinfarct LV remodeling, as determined by Zelarayán *et al.*[14] Under strictly controlled circumstances, Lian *et al.*[15] guided cardiomyocyte development from human pluripotent stem cells by manipulating Wnt/β-catenin signaling. Human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) were focused toward cardiomyocyte differentiation by inhibiting the Wnt/β-catenin pathway, as reported by Van den Berg *et al.*[16] Myocardial development was discovered to be promoted in rat bone mesenchymal stem cells (BMSCs) through the downregulation of Wnt/β-catenin signaling by Lv and his colleagues.[17,18]

This group's prior research has shown that Islet-1 increased the expression of the protein general control nondepressible 5 (Gcn5) and improved Gcn5's ability to bind to the promoters of NK2 homeobox 5 (Nkx2.5) and GATA binding protein 4 (GATA4).[19] Nonetheless, the underlying biochemical mechanism of these interactions is still unclear. In this study, we preliminarily proved β-catenin regulates the interaction between Islet-1 and Gcn5 and MLIP in the regulation of cardiomyocyte differentiation. The particular enzyme responsible for controlling Islet-1 and MLIP as well as the molecular mechanism of protein modification was then further examined in this work. This makes clear the crucial intervention element for more study. These results form the basis for boosting MSC differentiation rates and encouraging the use of MSCs in therapeutic applications.

Materials and methods

Cell culture and lentiviral vector transfection

The growth of C3H10T1/2 cells (University of Chicago Molecular Oncology Laboratory, Chicago, IL, USA) was accomplished in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). GENECHEM created lentiviral vectors and miR-34a mimics, miR-34a-3p mimics, and miR-34a-5p mimics (Shanghai, China). Serum-free media and polybrene were combined to a target concentration of 8 μg ml^{-1} in a 24-well plate before transfection. According to the multiplicity of infection (MOI) value, the correct quantity of lentivirus was added to each well. After culturing for 48 h, the cell culture medium in the culture dish was replaced with 200 μg/mL of puromycin screening medium. A six-well plate was used to continue cultivating the cells. After the cells expanded, they were frozen. The cell transfection efficiency was verified (Supplemental Figure S4).

Total RNA extraction and real-time polymerase chain reaction (PCR)

A PrimeScript RT reagent kit (Takara, Dalian, Liaoning, China) was used to convert total RNA samples into complementary DNA (cDNA) after they had been extracted with a kit (RP120; BioTeke) for RNA extraction. Using gene-specific primers and a SYBR Green dye kit, the cDNA samples were then amplified (Takara). The primers were created in accordance with Supplemental Table S2.

Protein extraction and Western blotting

RIPA Reagent was used to extract proteins from cells (P0013B; Beyotime Biotech, China). The membranes were sectioned and treated in 5% nonfat milk–phosphate-buffered saline–Tris for 1 h as indicated by the markers. Overnight at 4°C, the membranes were treated with primary antibodies (Supplemental Table S1), after which they were washed

three times for 15 min with phosphate-buffered saline (PBS). The membranes were then subjected to incubation with the secondary antibody of interest. Chemiluminescent processes (Millipore, USA) were used to identify positive bands.

Immunofluorescence

The cells were blocked with goat serum after being fixed in acetone at 4°C for 15 min (1:20), followed by adding anti-connexin 43 polyclonal and anti-cardiac troponin T monoclonal (both from Abcam, Cambridge, UK) antibodies and incubating overnight at that temperature. After that, secondary antibodies were added and incubated for 1 h at 37°C using CoWin Bioscience (Beijing, China). Then, DAPI was applied for 3 min. With the use of a fluorescent microscope, images were captured (BX51; Olympus). Supplemental Table S1 lists the antibodies that were used.

Co-immunoprecipitation (Co-IP) assay

We used a Co-IP assay kit (Merck Millipore, DA, Germany) to perform co-immunoprecipitation, and we used a BCA (bicinchoninic acid) assay to determine the relative amounts of protein in the cell lysates. Islet-1 was pulled down using an anti-Islet-1 antibody from Abcam, while MLIP was pulled down using an anti-MLIP antibody from Boster Biological Technology (Pleasanton, CA, USA). Anti-IgG antibodies were employed as a negative control. Protein samples were investigated by Western blotting with antibodies to examine for their presence of proteins that attract Islet-1 or MLIP. Supplemental Table S1 lists the antibodies that were used.

Chromatin immunoprecipitation (ChIP)-quantitative PCR assay

Protein-DNA systems were crosslinked for 5 min at 25°C in PBS containing 1% formaldehyde, and then glycine (0.125 M) was added to stop the reaction. Sonication (UCD-200, Bioruptor) was used to break the crosslinked material into smaller pieces; the process included 25 cycles of 30 s each with 30-s cooling intervals. Antibodies used in conjunction with the ChIP test kit (Merck Millipore) are mentioned in Supplemental Table S1. One percent of the initial chromatin was utilized as input, and two groups were formed: those precipitated with normal mouse IgG and with an anti-RNA polymerase II antibody. Key primers are included in Supplemental Table S2.

Luciferase reporter assay

pGL3-CM vectors (Promega Corporation, London, UK) or pMIR-REPORT Luciferase Vectors (Promega Corporation) were used to clone the 3′-UTR (untranslated region) sections including amplified sites (wild type, Wt) and mutant sites (mutant type, Mut). Subsequently, the plasmids were transfected into the corresponding experimental group cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Pittsburgh, PA, USA). Then, a dual-luciferase reporter assay device was used to quantify the luciferase activity (Promega, Madison, WI, USA). Primers are detailed in Supplemental Table S2.

Pulse-chase analysis

After being separated by centrifugation, the cells underwent two PBS washes. With a cell coverage of 5×106 cells/mL, the cells were resuspended and cultured in methionine-free DMEM for 30 min. At 37°C for 60 min, 100 μCi/mL of [^{35}S] methionine was then added and labeled. At 0, 120, 240, and 360 min, the cells were washed and grown in DMEM complete media with methionine (10 mM) and cysteine (5 mM). Following the tracking process, the cells were centrifuged, given two PBS washes, and then submitted to immunoprecipitation analysis.

Mitochondrial status detection

After treatment of each group of cells, 0.1 nM TMRM, 5 μM MitoSox-Red, and 5 μM MitoTracker™ RedCMXRos (Invitrogen Corporation, NY, USA) were added. The mitochondrial membrane potential, mitochondrial number, and mitochondrial reactive oxygen species (ROS) level were all measured after 30 min of incubation with a probe staining solution at 37°C. Rhod2-AM staining solution (5 M) was applied to each batch of cells, and after 2 h on ice and 30 min at 37°C, the amount of mitochondrial calcium was detected. The cells were stained, then washed in PBS, resuspended, and sent through a flow cytometer for analysis. An ATP test kit was used to detect ATP (ab83355; Abcam).

Statistical analysis

Unless otherwise specified, all numbers in this article are presented as mean ± SD. There were a minimum of three replicates for each experiment, and therefore, the data presented here should be considered reliable. Using GraphPad Prism, we checked for statistically significant differences between groups using one-way analysis of variance (ANOVA) and then evaluated those differences using the Tukey–Kramer HSD (honestly significant difference) post-test. P values were used to define statistical significance: n.s., $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$.

DIANATools website: http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/page&view=software; accessed 12 February 2020.

Ubibrowser website: http://ubibrowser.ncpsb.org/ubibrowser/; accessed 8 February 2020.

Results

Knockdown of β-catenin significantly increases the binding between Islet-1 and GCN5

A total of three short hairpin RNA (shRNA)-β-catenin lines were constructed and evaluated. Cells expressing each shRNA were shown to have lower levels of β-catenin expression than the control group. However, shRNA-β-catenin#3 produced the highest levels of β-catenin knockdown (Supplemental Figure S1A, B). Therefore, the subsequent studies are based on shRNA-β-catenin#3 for its significant interference effect. The cell morphology of the shRNA-β-catenin group showed changes on the 7th and 14th days after obtaining the stable strain, and the cells showed fibroblast-like growth and arrangement (Supplemental Figure S2A). In addition, the

expression levels of Nanog, Sox2, cTnT, and Connexin43 in the indicated groups were identified through Western blotting. On the 7th day, the expression levels of Nanog and Sox2 in the shRNA-β-catenin group were significantly decreased (Supplemental Figure S2C and Supplemental Figure S1C, D), while cTnT and Connexin43 expression levels were greatly elevated (Supplemental Figure S2C and E, F). The early cardiac transcription factors GATA4 ($p < 0.05$) and Nkx2.5 ($p < 0.01$) also had elevated mRNA levels (see Supplemental Figure S2B). The subsequent results suggest that myocardial differentiation markers are markedly upregulated 7 days after stable suppression of the β-catenin cell line has been achieved. Therefore, the 7th day was chosen as the time point for the follow-up study.

After determining the 7th day as the best time point, we further studied mitochondrial structure, ATP release, and the expression of myocardial structural proteins on the 7th day. We checked the mitochondrial state and ATP content. The shRNA-β-catenin group had considerably greater membrane potential, mitochondrial number, mitochondrial reactive oxygen species (ROS), and ATP than the shRNA-NC group (Supplemental Figure S2D to H). Immunofluorescence analysis then revealed that cTnT and Connexin43 expression levels were considerably greater in the shRNA-β-catenin group than in the shRNA-NC group (Supplemental Figures S3A and S1G, H). These results show that knocking down β-catenin in C3H10T1/2 cells causes them to differentiate into myocardial cells and boosts their metabolic rate. Meanwhile, we confirmed that β-catenin overexpression significantly inhibited C3H10T1/2 cell differentiation into cardiomyocytes (Supplemental Figure S3B to D).

Islet-1 and Gcn5 formed a combination in C3H10T1/2 cells when Islet-1 expression was highly induced.[20] We first showed that the protein and mRNA levels of Gcn5 in C3H10T1/2 cells were unaffected by β-Catenin (Figure 1(A) and (D)). And the expression of Islet-1 increased gradually at the protein level in the shRNA-β-catenin group (Figure 2(A)). Then the Co-IP assay showed that the interactions between Gcn5 and Islet-1 were significantly changed. Detection of different enriched regions in Co-IP assays determines the enrichment efficiency of immunoprecipitation. The more enriched regions detect the more efficient experiments among other results. In this paper, we formally detect enrichment efficiency of immunoprecipitation assays by detection of differentially bound regions and enriched regions (Figure 2(A)). The ratio of Islet-1 to Gcn5 binding was higher in the shRNA-β-catenin group. Islet-1 binding to the promoter regions of Nkx2.5 and GATA-4 was increased in the shRNA-β-catenin group (Figure 2(B) and (C)).

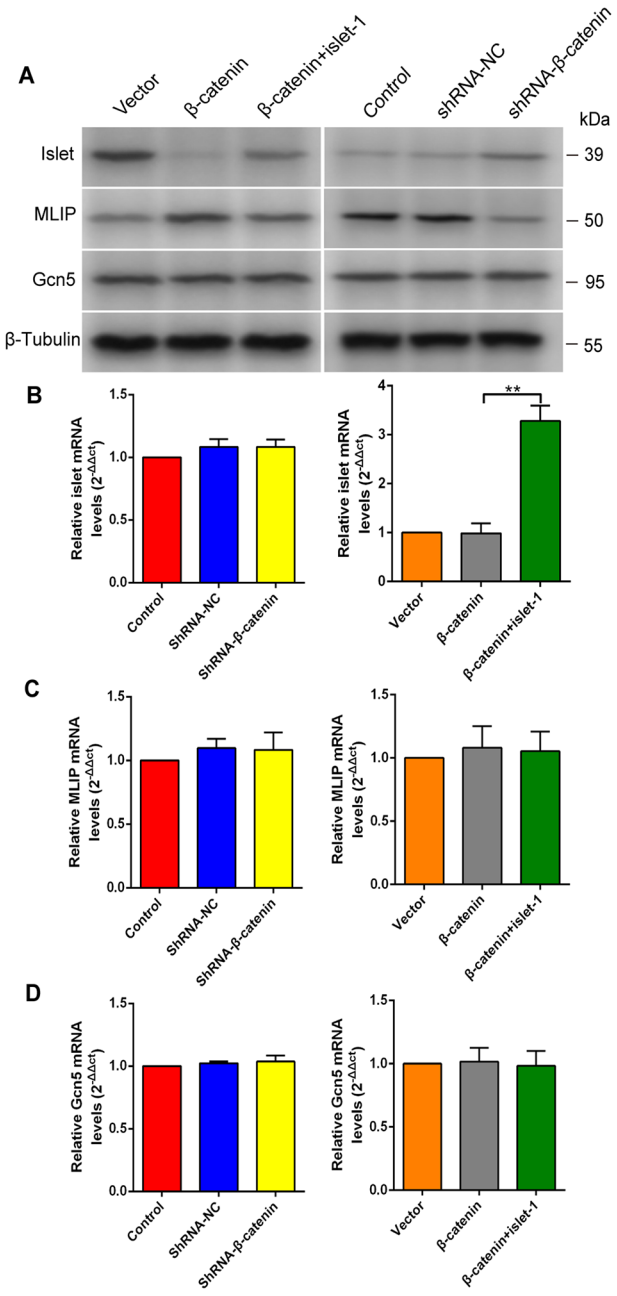

Figure 1. β-catenin affects the expression of Islet-1, GCN5, and MLIP. β-catenin was stably knocked down in C3H10T1/2 cells using a lentiviral vector and was overexpressed in C3H10T1/2 cells using a lentiviral vector. The expression of Islets, MLIP, and Gcn5 (A) in the indicated groups was detected by Western blotting. The mRNA levels of Islets (B), MLIP (C), and Gcn5 (D) in the indicated groups were measured by qPCR. The results are expressed as means ± SD of three individual experiments that, for each condition, were performed in triplicate. *$p < 0.05$, **$p < 0.01$. (A color version of this figure is available in the online journal.)

Knocking down β-catenin significantly changes the binding between Islet-1 and MLIP

In addition, MLIP is known as a regulation factor in critical conditions for cardiac gene expression and cardiac hypertrophy. MLIP could inhibit Islet-1-mediated MEF2C transcriptional activity. Therefore, Western blotting was used to determine the MLIP expression levels in the specified populations. Overexpression of β-Catenin in C3H10T1/2 cells led to a dramatic increase in MLIP protein (Figure 1(A)). Furthermore, the mRNA level of MLIP was not significantly altered by catenin overexpression (Figure 1(C)). Although MLIP transcription was significantly different between the control and shRNA-NC groups, MLIP protein levels were reduced in the shRNA-β-catenin group.

The results of this experiment demonstrated that the protein levels of Islet-1 and MLIP were dramatically altered in C3H10T1/2 cells as a result of β-catenin regulation. Thus, we investigated the relationship between MLIP and Islet-1 and Gcn5 in this cell model. The Co-IP assay showed that

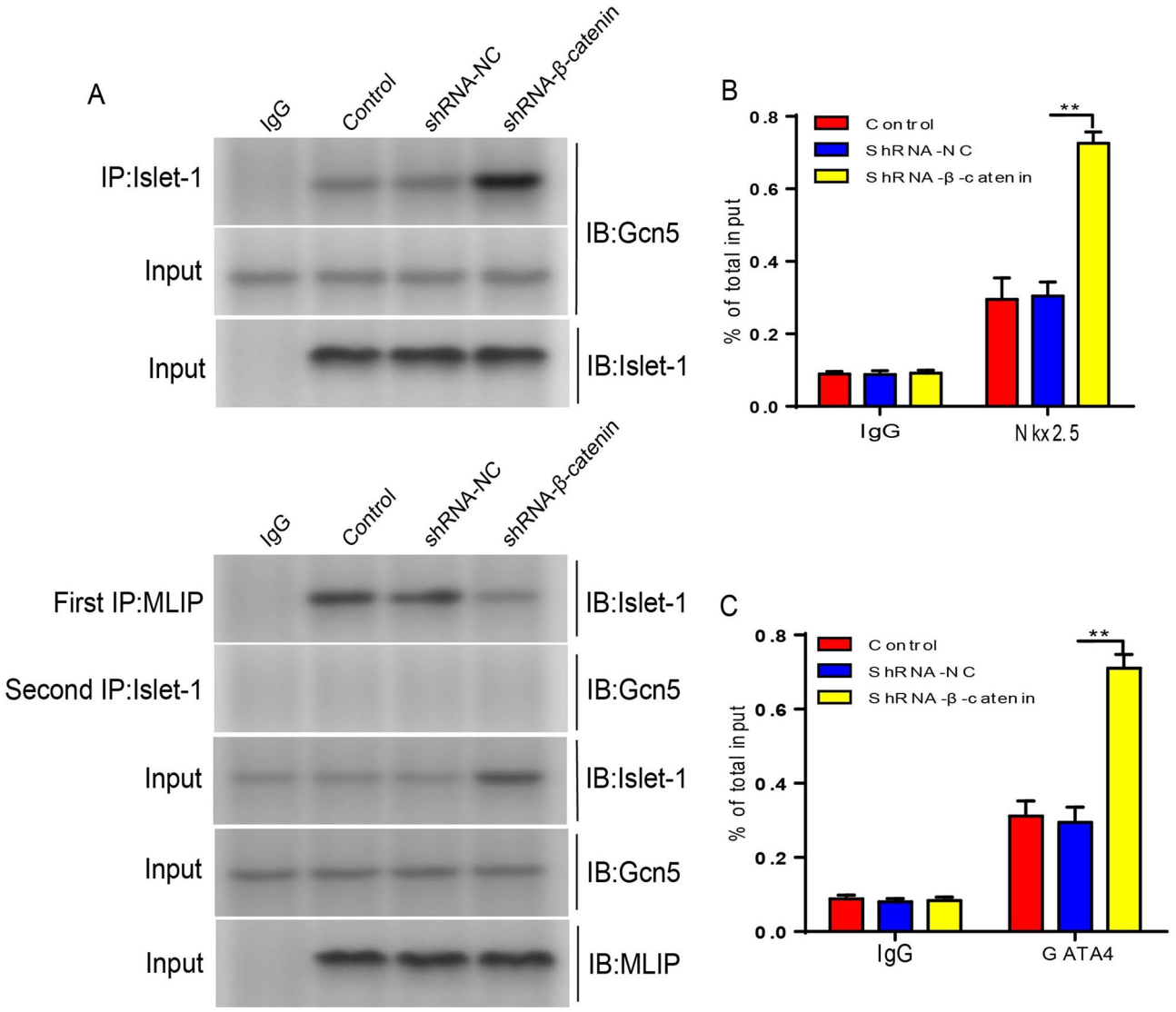

Figure 2. β-catenin affects the binding relationship of Islet-1, GCN5, and MLIP. β-catenin was stably knocked down in C3H10T1/2 cells using a lentiviral vector. On the 7th and 14th days after obtaining the stable strain, the cell lysates were immunoprecipitated with an anti-Islet-1 antibody, an anti-MLIP antibody, or control IgG followed by the Co-IP assay to determine the interaction of Gcn5 and Islet-1 (A). After the cell extracts from the indicated groups were subjected to immunoprecipitation with the anti-MLIP antibody and immunoblotting with the anti-Islet-1 antibody, the cell lysates were immunoprecipitated with the anti-Islet-1 antibody again, and Gcn5 was detected by immunoblotting (A). Islet-1 interaction with the promoter regions of Nkx2.5 (B) and GATA4 (C) was determined by the ChIP assay in C3H10T1/2 cells. The results are expressed as mean ± SD of three individual experiments that, for each condition, were performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$. (A color version of this figure is available in the online journal.)

the interaction between MLIP and Islet-1 was significantly changed (Figure 2(A)). In the shRNA-β-catenin group, the binding of Islet-1 to Gcn5 increased, and that of Islet-1 to MLIP decreased. In each group, Islet-1 could not bind to Gcn5 after binding to MLIP (Figure 2(A)). In the shRNA-β-catenin group, Islet-1 binding to the promoter regions of GATA-4 and Nkx2.5 was enhanced (Figure 2(B) and (C)). In conclusion, knockdown of β-catenin may further affect the binding relationship between Islet-1 and MLIP and Gcn5 by regulating the expression of Islet-1 and MLIP.

β-catenin knockdown changes the ubiquitination level of MLIP and Islet-1

Therefore, whether β-catenin regulates their expression by affecting the posttranscriptional modification process of Islet-1 and MLIP remains unclear. To confirm that after knocking down β-catenin the degradation of Islet-1 and MLIP plays crucial roles in their expression changes, we used isotopic labeling to detect the half-lives of MLIP and Islet-1 in cells. For protein half-life detections, an appropriate loading control is necessary. For this purpose, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are most commonly used to check protein expressions. The total protein expression was found to be consistent, while the protein expression of β-catenin and β-actin was observed to be different. However, the protein level of GAPDH was stable in all cells. We concluded that total protein was the most appropriate internal control in different stages of cardiomyogenesis.[21]

Knocking down β-catenin in C3H10T1/2 cells shortened the half-life of MLIP. The Islet-1 protein showed a prolonged half-life and increased protein stability (Figure 3(A) to (C)). This result further confirms that knocking down β-catenin significantly regulates the stability of Islet-1 and MLIP proteins.

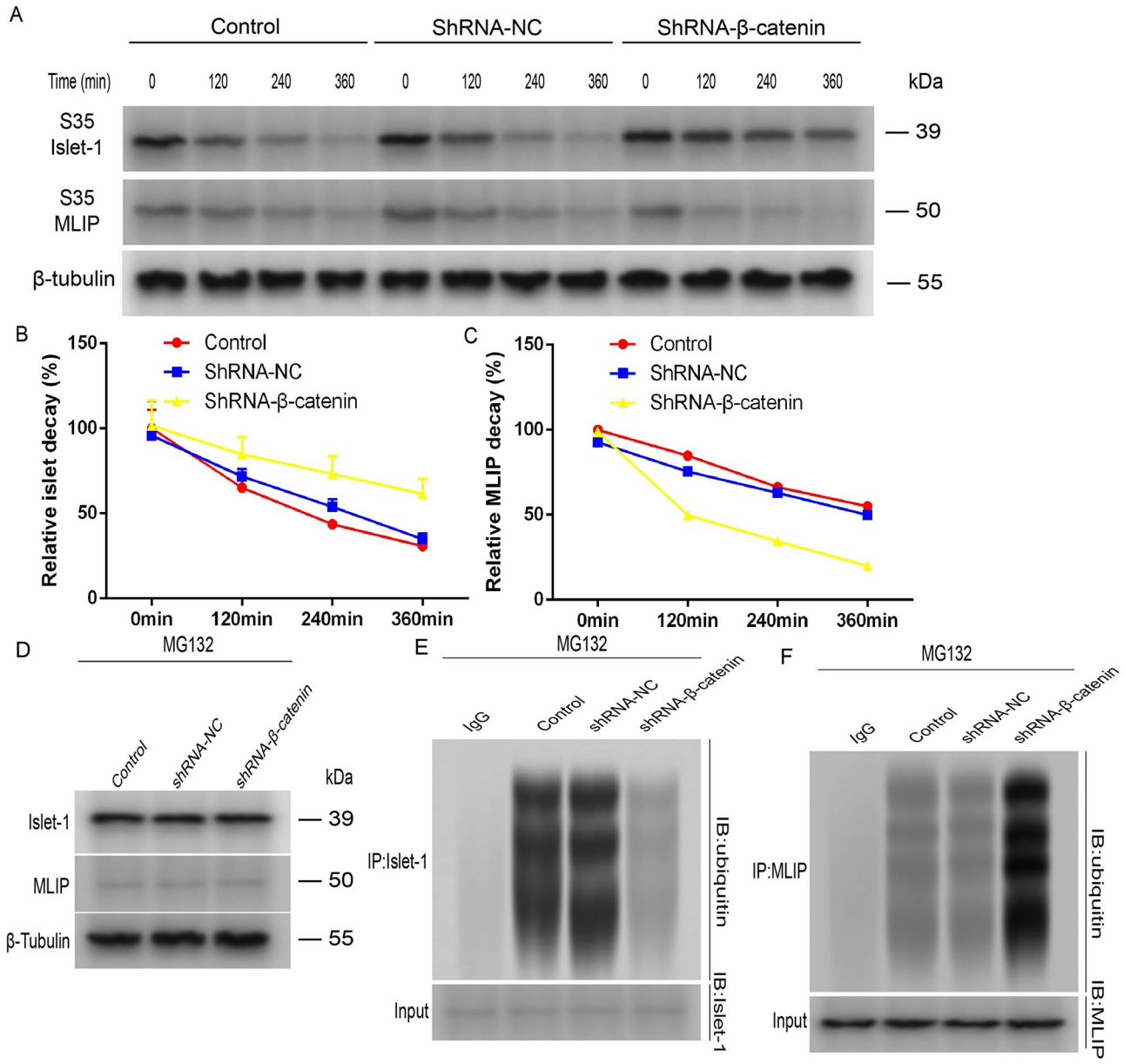

Figure 3. β-catenin affects the ubiquitination of Islet-1 and MLIP. β-catenin was stably knocked down in C3H10T1/2 cells using a lentiviral vector. The half-lives of MLIP and Islet-1 were detected by pulse-chase analysis (A–C). After knocking down β-catenin for 7 days, the cells were treated with MG132 (10 μM) for 24 h. The expression of Islet-1 and MLIP (D) in the indicated groups was detected by Western blotting. The cell extracts from the indicated groups were subjected to immunoprecipitation with an anti-Islet-1 antibody (E) or anti-MLIP antibody (F), followed by immunoblotting with an anti-ubiquitin antibody. The results are expressed as the mean ± SD of three individual experiments that, for each condition, were performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$. (A color version of this figure is available in the online journal.)

Presently, protein degradation occurs primarily using two pathways: the lysosomal degradation pathway and the ubiquitin-mediated proteasome degradation pathway. The ubiquitin-mediated pathway is a specific protein degradation pathway that is strictly controlled by time.[22] Ubiquitination is a cascade of enzymatic reactions in which E3 ligases determine the specificity of the ubiquitome.[23]

Therefore, we speculated that the difference in protein expression might be related to the ubiquitination modification of the target protein. In each group of cells, because of the addition of MG132, the expression levels of Islet-1 and MLIP did not change significantly (Figure 3(D)). In the shRNA-β-catenin group, the ubiquitination level of Islet-1 decreased significantly (Figure 3(E)) and the ubiquitination level of MLIP increased significantly (Figure 4(F)). According to the experimental results, we speculated that the knockout of β-catenin might increase the ubiquitination level of MLIP, leading to MLIP degradation and downregulation of its protein level. The ubiquitination level of Islet-1 in the cells of the shRNA-β-catenin group decreased, inhibiting Islet-1 degradation.

β-catenin directly regulates the E3 ubiquitin ligase WWP1 and acts on Islet-1 to affect ubiquitin modification

Previous studies found that knocking down β-catenin not only promotes MLIP ubiquitination but also directly inhibits

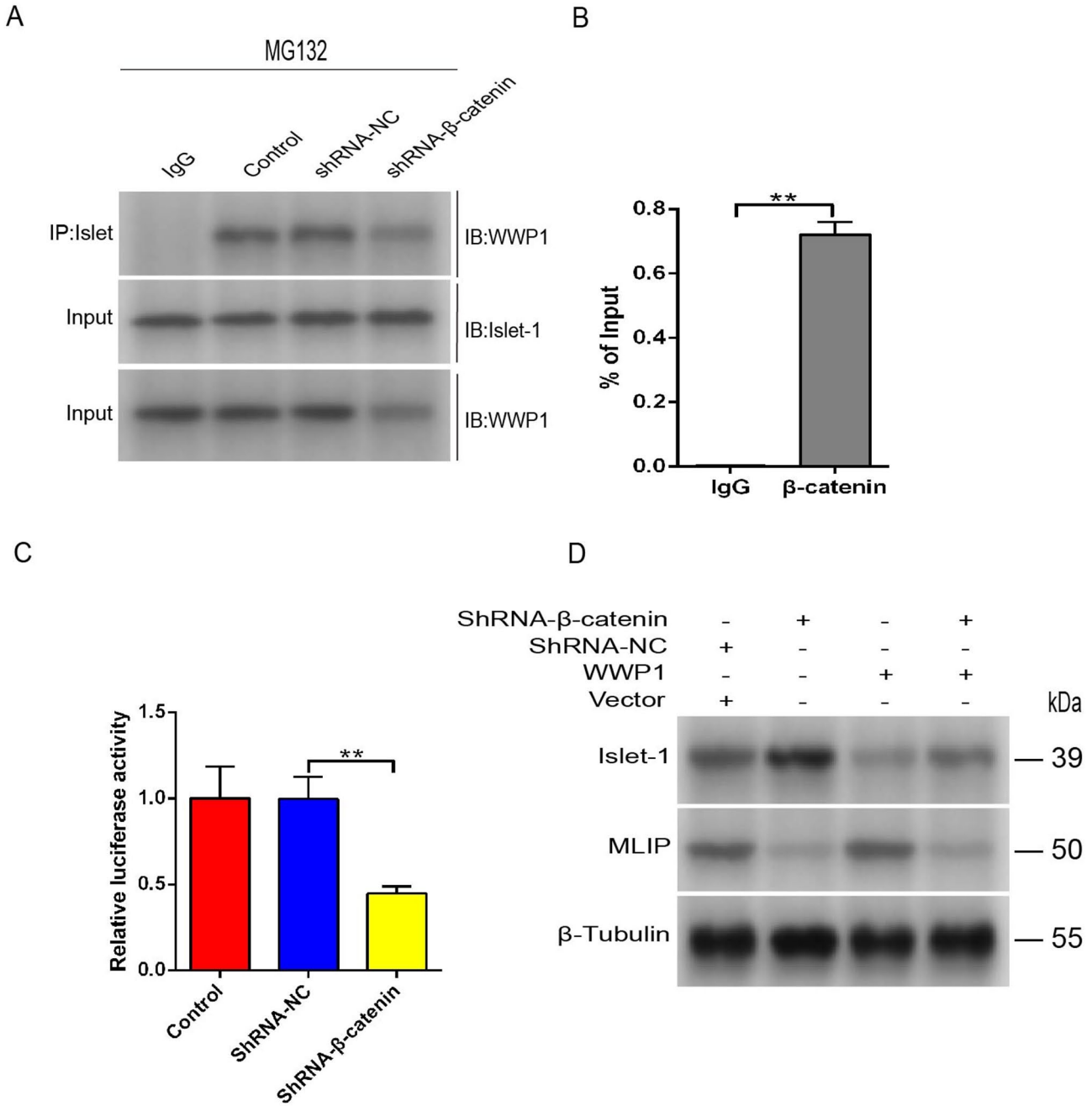

Figure 4. The E3 ubiquitin ligase WWP1 acts on Islet-1 to affect its ubiquitin modification. β-catenin was stably knocked down in C3H10T1/2 cells using a lentiviral vector. The tissue lysates were immunoprecipitated with an anti-Islet-1 antibody or control IgG followed by the Co-IP assay to determine the interaction of Islet-1 and WWP1 (A). The interaction of β-catenin with the WWP1 promoter region was detected by the ChIP assay (B). WWP1 promoter activity was measured by the luciferase assay (C). β-catenin was stably knocked down or WWP1 was overexpressed in C3H10T1/2 cells using a lentiviral vector. The expression of Islets and MLIP (D) in the indicated groups was detected by Western blotting. The results are expressed as mean ± SD of three individual experiments that, for each condition, were performed in triplicate. *$p < 0.05$, **$p < 0.01$. (A color version of this figure is available in the online journal.)

Islet-1 ubiquitination. What is the connection between Islet-1 ubiquitination and β-catenin? Beta-catenin plays the central role in the Wnt signaling pathway, as it is considered a central component for the regulation of cell proliferation and differentiation. During protein modification by ubiquitination, ubiquitination ligase E3 plays a key role.[24] Therefore, to investigate the regulatory mechanism of the ubiquitination modification of the two proteins, we used the Ubibrowser website to predict the E3 ubiquitination ligase of the two proteins (Supplemental Figure S7A). Islet-1 primarily exists in the nucleus. So we screened the ubiquitinating enzymes in the database and identified the WW Domain involving E3 ubiquitin Protein Ligase 1 (WWP1) in the nucleus and that the transcription factor of WWP1 ubiquitinating ligase is most likely TCF1, which is the cotranscription factor of β-catenin.[25] After knocking down β-catenin, we confirmed the binding relationship between WPP1 and Islet-1 by Co-IP (Figure 4(A)). Moreover, in the shRNA-β-catenin group, both WWP1 protein and mRNA levels were drastically decreased (Supplemental Figure S8A, B). The promoter

activity decreased significantly (Figure 4(C)). ChIP experiments showed that β-catenin directly bound to the promoter region of WWP1 (Figure 4(B)). Hence, β-catenin affects WWP1 expression by regulating its gene transcription and the ubiquitination of Islet-1. Prior studies showed that the regulatory network of Wnt pathway effector β-catenin is required for the cytoplasmic stabilization or degradation of other transcription factors such as WWP1. The mechanism studied for enhancing nuclear β-catenin or silencing the expression of WWP1 is more complicated and less characterized. However, we analyzed that silencing of β-catenin inhibits different Wnt target genes that ultimately decline the WWP1 expression.[26] To confirm the above conclusion, we overexpressed WWP1 after β-catenin knockdown in C3H10T1/2 cells. β-catenin knockdown and WWP1 overexpression reversed the Islet-1 upregulation (Figure 4(D)).

β-catenin regulates the E3 ubiquitin ligase UBE3C and acts on MLIP to affect ubiquitin modification

Next, we analyzed MLIP as described above and found that the Ubiquitin Protein Ligase E3C (UBE3C) likely regulated the ubiquitination of MLIP through screening. Although UBE3C is observed lower in rank than other top-ranked E3 ligases, it binds to promoters directly and upregulates the expression of UBE3C (Supplemental Figure S7B). We then verified that knocking down β-catenin significantly upregulated the binding level of MLIP to UBE3C (Figure 5(A)). In addition, the protein and mRNA levels of UBE3C increased (Supplemental Figure S8C, D), but its promoter activity did not change significantly (Figure 5(B)). Thus, β-catenin does not directly affect the transcriptional activity of its promoter to regulate UBE3C expression.

β-catenin regulates MLIP ubiquitination through the TCF7/miR-34a/UBE3C axis

Accumulating evidence demonstrates that the UBE3C gene is regulated by many miRNAs, hsa-miR-34a-5p is a spliced form of miR-34a, and its target gene is UBE3C.[27] Therefore, we sought to determine whether the β-catenin pathway affects UBE3C expression through miR-34a. We used DIANATools to mine the possible transcription factors of these miRNAs and found that miR-34a is regulated by the transcription factor TCF7L2, which functions as the auxiliary transcription factor of β-catenin. Reduced levels of miR-34a were seen in C3H10T1/2 cells after β-catenin was knocked down (Figure 5(D)). RNA interference (RNAi) technology has been advanced in stages of clinical viability and is especially preferred for transcriptional regulators including β-catenin. For this purpose, RNAi technology is used as promising approach for cardiac diseases, as it has potent to adequately silence β-catenin at the mRNA level, which results in alteration of miR-34a expression. Furthermore, these studies suggest that direct RNAi-mediated silencing of β-catenin alters expression of miR-34a; not only do they possess cotranscription factor, they also induce protein expression of E3 ubiquitin ligase UBE3C.[28]

We discovered that miR-34a-5p-mimic and miR-34a-mimic reversed the suppression of β-catenin in UBE3C overexpression after transfection of miR-34a with various splicing bodies (Figure 5(C) and (E)). Through the use of luciferase reporter genes, we were able to determine that UBE3C is a miR-34a-5p target gene (Figure 5(F)). ChIP assays revealed that the cotranscription factor of β-catenin, TCF7L2, regulates miR-34a expression (Figure 5(H)). Thus, we found that β-catenin regulates miR-34a expression through the cotranscription factor TCF7L2, thereby regulating the expression of the E3 ubiquitin ligase UBE3C. In addition, UBE3C directly ubiquitinates MLIP and accelerates the degradation of MLIP.

To verify this finding, we inhibited UBE3C expression in C3H10T1/2 cells after β-catenin knockdown. Reversing the downregulation of MLIP was achieved by the reduction of β-catenin in C3H10T1/2 cells and UBE3C (Figure 5(G)).

Discussion

The differentiation of MSCs into cardiomyocytes is a complex and orderly process in which MSCs change from the initial undifferentiated state to high expression of myocardial-specific genes, show cardiomyocyte-like cell morphology, and finally differentiate into cardiomyocytes with electrophysiological functions. Presently, the mechanism of this process mainly involves direct interactions among cells, signaling pathways, epigenetic modification, and paracrine signaling.[29] Demonstration of the molecular mechanisms underlying cardiomyogenesis is a prerequisite for understanding the altered interactions present between transcription factors WWP and Islet-1. In this research, we provide prominent and multifaceted roles of Islet-1 and WWP by following the enrichment efficiency of Islet-1. These results showed that the expression of transcriptional regulator Islet-1 is detected in developing cells and is restricted in mature islet cells. The gene regulatory network of Islet-1 and its functions in cardiomyogenesis has been found to be ambiguous, while the great details for understanding the cellular and molecular modes of action still need further investigations.[30]

Wnt/β-catenin signaling promotes the preservation of pluripotency in mouse embryonic stem cells (mESCs), and it also promotes the development of primed cells (i.e. epiblast stem cells [EpiSCs]) in the direction of the mesendoderm. The Wnt signaling pathways demonstrate a significant role in determining the destiny of MSCs.[31]

Ubiquitin is an 8.5-kDa globular protein that binds to the amino group of the protein it is targeting.[32] There are seven lysine residues in the ubiquitin molecule, and the destiny of the protein that has been "tagged" depends on which lysine residue is present. A series of enzymes, involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase, work together to promote the ubiquitination of their respective targets. The ubiquitin-proteasome system (UPS) is essential in many different types of regulated biological activities, including differentiation. Myocardial remodeling, familial cardiomyopathies, chronic heart failure, and ischemia-reperfusion damage are all areas where the UPS is increasingly being recognized as playing a role in heart disease. The UPS is an important factor in the heart and has the potential to be a therapeutic target in cardiac illness, making it crucial to understand its precise involvement in heart development and disease. Our study helps to understand the mechanism of ubiquitination on myocardial differentiation induced by β-catenin. We found two key E3 ubiquitin ligases for

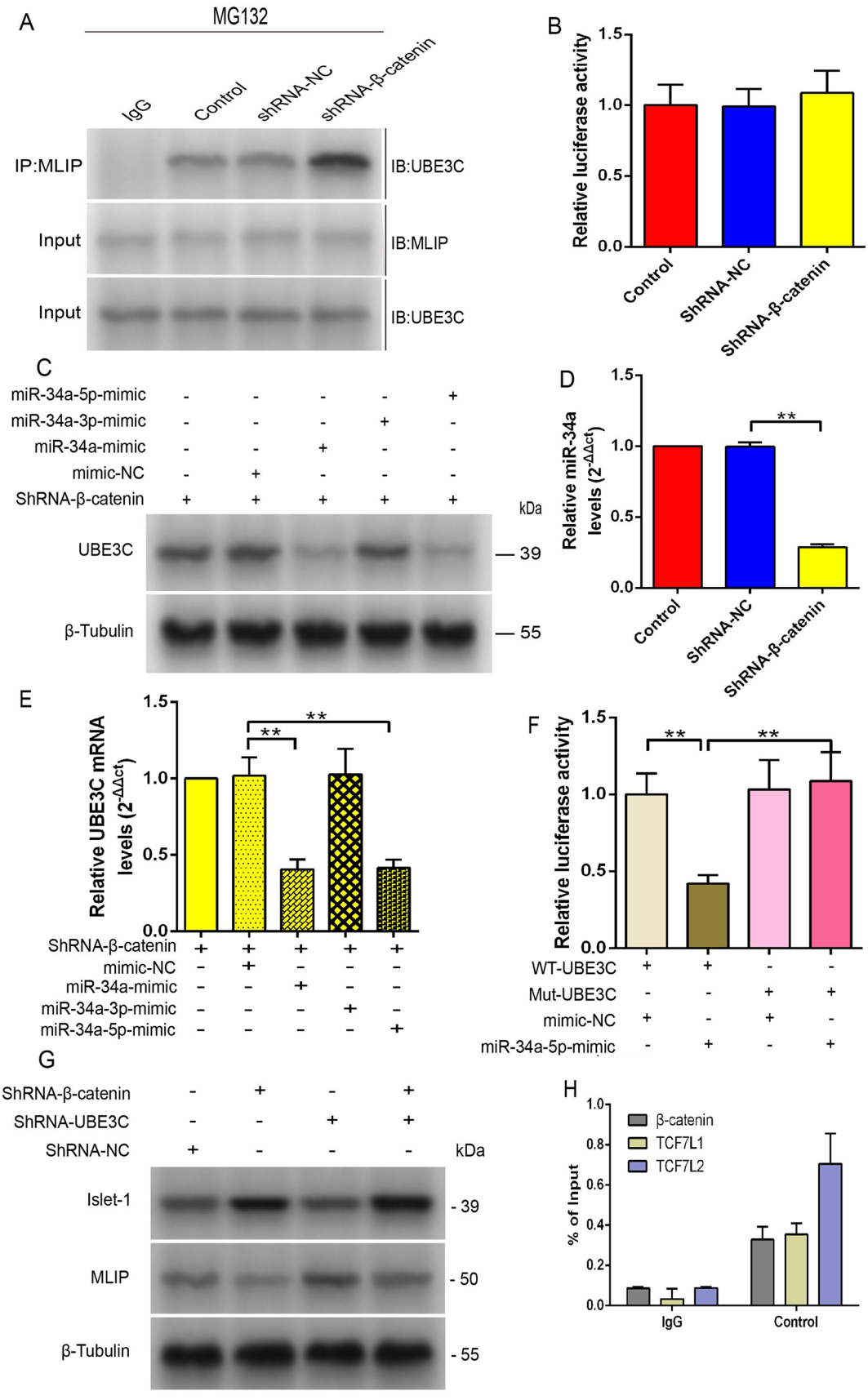

Figure 5. The E3 ubiquitin ligase UBE3C acts on MLIP to affect its ubiquitin modification. The tissue lysates were immunoprecipitated with an anti-MLIP antibody or control IgG followed by the Co-IP assay to determine the interaction of MLIP and UBE3C (A). UBE3C promoter activity was measured by the luciferase assay (B). UBE3C expression in the indicated groups was detected by Western blotting (C). The expression of miR-34a (D) and UBE3C (E) in the indicated groups was detected by qPCR. Luciferase assay (F) and ChIP assay (H). β-catenin or UBE3C was stably knocked down in C3H10T1/2 cells using a lentiviral vector. The expression of Islets and MLIP (G) in the indicated groups was detected by Western blotting. The results are expressed as mean ± SD of three individual experiments that, for each condition, were performed in triplicate. *$p < 0.05$, **$p < 0.01$. (A color version of this figure is available in the online journal.)

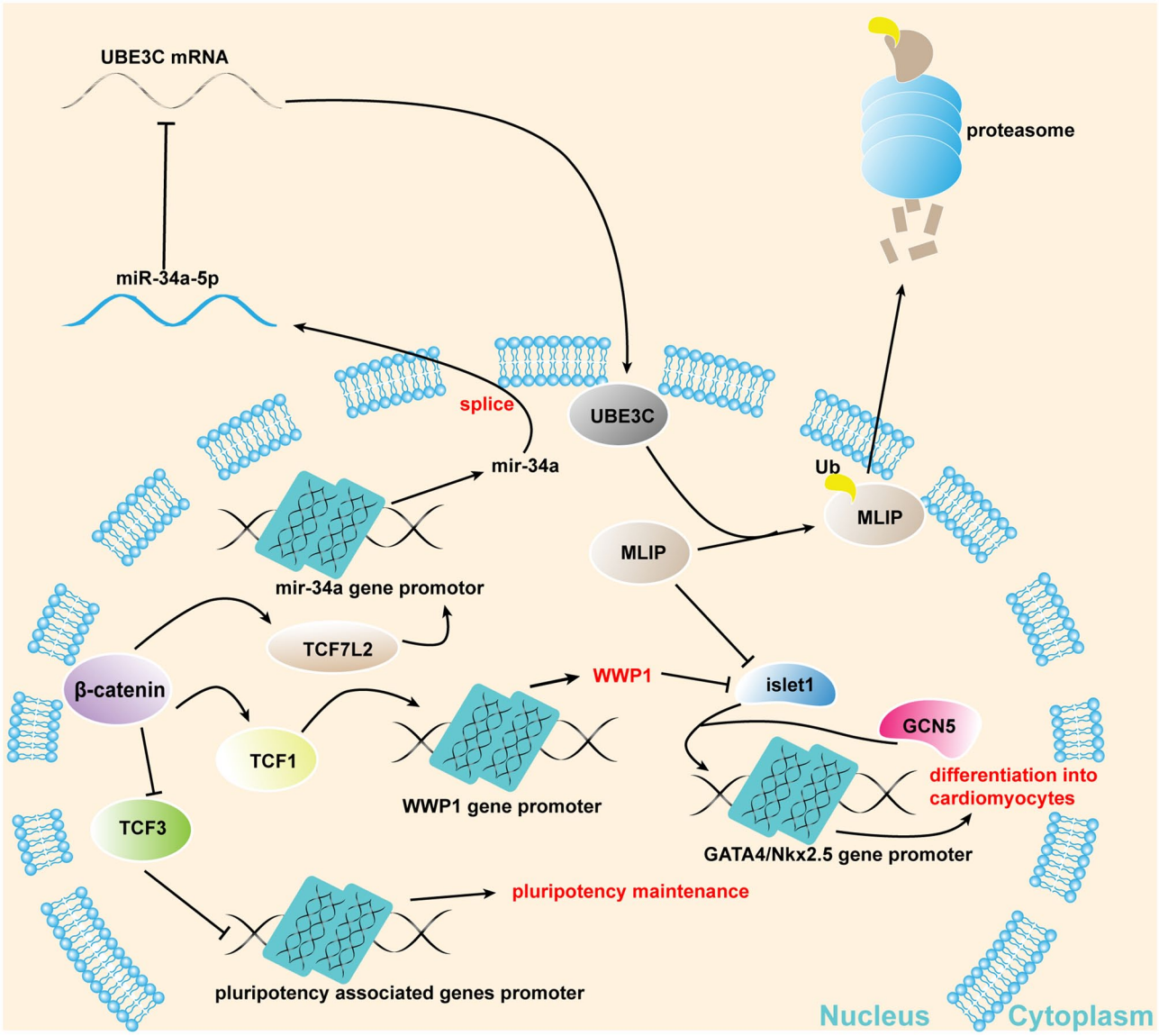

Figure 6. Mechanistic diagram of the Wnt/β-catenin pathway in pluripotency maintenance and myocardial differentiation of C3H10T1/2 cells. (A color version of this figure is available in the online journal.)

regulation of the ubiquitination of Islet-1 and MLIP. The ubiquitination status of Islet-1 and MLIP also has been implicated to regulate their function transition. However, the types and mechanisms of ubiquitination of Islet-1 and MLIP need to be deeply studied.

Our prior research has shown that Islet-1 is critical for regulating histone acetylation. A complex between Gcn5 and Islet-1 may exist.[33] In addition, it has been proposed that Islet-1 increases GATA4/Nkx2.5 expression by interacting with Gcn5 and directing it to the GATA4/Nkx2.5 promoters, thereby speeding up the differentiation of MSCs into cardiomyocytes. It seems that Islet-1 exerts its regulatory influence only in cardiac tissue via a mechanism involving histone acetylation and the production of early transcription factors.[8,19] Islet-1 binds to GCN5, although the chemical mechanism by which this occurs is unclear. Understanding the molecular mechanism through which Islet-1 plays a crucial role in heart development might pave way for a technique for differentiating MSCs into cardiomyocytes.

Here, to explore the molecular mechanism of Islet-1 binding to GCN5, we selected the C3H10T1/2 cell line that has been used in our previous studies on myocardial differentiation. This cell line was isolated from the embryonic fibroblast epithelium of C3H mice.[34] As mouse embryonic fibroblast epithelial cells, they are often used as a cell model to investigate the differentiation procedure of osteogenesis[35] and adipogenesis.[36] First, we established the model of inducing cardiac differentiation by downregulation of β-catenin and found that inhibition of β-catenin may initiate cardiac differentiation. Our study helps to understand the mechanism of early myocardial differentiation induced by β-catenin in C3H10T1/2 cells. Islet-1 and MLIP expression was shown to be correlated with β-catenin and to be cooperatively controlled.

In our study, we found that the competitive binding relationship between Islet-1 and MLIP and GCN5 affected myocardial differentiation. And we speculated on the mechanism by which β-catenin regulates the myocardial differentiation of C3H10T1/2 cells (Figure 6). It was observed that the key

enzymes of ubiquitination modification of MLIP and Islet-1 are UBE3C and WWP1, respectively. β-catenin knockdown reduces the transcription of miR-34a mediated by TCF7L2, leading to the downregulation of miR-34a-5p levels. In addition, the target gene of miR-34a-5p is UBE3C, whose regulation leads to upregulated UBE3C mRNA levels and increased UBE3C expression. When UBE3C expression increases, its ubiquitination modification of MLIP is upregulated, leading to MLIP degradation and downregulation of its protein level. At the same time, β-catenin reduces the transcription level of the E3 ubiquitination ligase WWP1 through TCF1 and downregulates the ubiquitination modification of Islet-1, inhibiting the ubiquitin-mediated protein degradation of Islet-1 and promoting the stability of the Islet-1 protein. Thus, the binding of MLIP to Islet-1 gradually decreases, and free Islet-1 can then bind to Gcn5, mediating the increased transcription of GATA4 and Nkx2.5 related to cardiomyocyte differentiation and mediating the differentiation of C3H10T1/2 cells into cardiomyocytes.

However, cardiogenesis is a tightly regulated and complex process, and the available evidence suggests that the relative importance of signaling via the canonical versus non-canonical Wnt pathways in cardiac specification varies depending on the species and specific model systems. Therefore, in the follow-up study, we will try to make a comparative study in different cell lines. More valuable clues should be provided for understanding the mechanism of myocardial differentiation induced by β-catenin.

Conclusions

We show that decreasing β-catenin regulates the ubiquitination of two proteins, upregulates Islet-1 and accelerates MLIP degradation, reducing the amount of free Islet-1 binding to MLIP and increasing the amount of binding to GCN5 in the nucleus. Therefore, the transcriptional activity of Islet-1 is significantly activated, inducing C3H10T1/2 cells to differentiate into the myocardium. β-catenin and Islet-1 are key factors to induce myocardial differentiation. Further knowledge of biochemical pathways, including molecular signaling pathways, can provide more insights into the myocardial differentiation mechanism of MSCs. This study provides the groundwork for application of MSCs in clinical treatment of heart disease.

AUTHORS' CONTRIBUTIONS

JZ: Funding acquisition, Supervision. JT: Supervision. LY: Conceptualization, Data curation, Writing—original draft and editing. MX: Methodology. BT: Software. HX: Validation. LY: Visualization. QY: Project administration. YZ: Investigation. XZ: Investigation.

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SUPPLEMENTAL MATERIAL

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