

ITGB1 promotes apoptosis in H9C2 cells

Weizhe Liu^{1,a}, Juanjuan Zhang^{2,3,b}, Aiying Li^{1,2,c,*}

¹Department of Biochemistry and Biology, College of Basic Medicine, Hebei University of Chinese Medicine, Shijiazhuang, China

²Hebei Key Laboratory of Chinese Medicine Research on Cardio-Cerebrovascular Disease, Shijiazhuang, China

³Key Laboratory for Health Care with Chinese Medicine of Hebei Province, Shijiazhuang, China

^a1024773@163.com, ^bwindy8968@163.com, ^caiyingli1963@163.com

Weizhe Liu and Juanjuan Zhang contributed equally to this work (co-first-author).

*Corresponding author

Abstract: Myocardial infarction is a heart disease with high mortality rate. The research on the cardiomyocyte apoptosis is helpful to the prevention and treatment of myocardial infarction. As a cell surface receptor, the function of ITGB1 gene in cardiomyocytes is not clear. This study aimed to analyze the function and molecular mechanism of cardiomyocyte derived ITGB1 in myocardial apoptosis. To analyze cardiomyocyte apoptosis, TUNEL staining was performed with ITGB1 overexpression and knockdown in H9C2 cells. Apoptosis-related markers were also detected using Western blot. In addition, apoptosis-related signaling pathways were detected to elucidate the molecular mechanism regulated by ITGB1. Our results indicated that ITGB1 promoted cardiomyocyte apoptosis by inhibiting the Notch1 and Smad2/3 signal pathways. These data highlight the potential usefulness of ITGB1 as a potential target for preventing myocardial infarction.

Keywords: ITGB1, H9C2 cells, Cardiomyocyte apoptosis, Myocardial infarction

1. Introduction

Ischemic heart disease, especially myocardial infarction is the prominent cause of morbidity and mortality worldwide [1]. Apoptosis is considered the main mechanism leading to myocardial infarction [2,3]. Increased apoptosis level is observed in the blood sample and heart tissue of patients with acute and subacute myocardial ischemia [4,5]. Numerous apoptosis-related factors were proved to be accumulated in the heart tissue after myocardial infarction or associated with the severity of myocardial infarction [6]. The circulating markers of apoptosis, sFas and sTNFR1, were elevated in patients with acute myocardial infarction [7]. Proapoptotic factor TNF- α showed significant correlations with infarct size and LV-dysfunction in myocardial infarction patients [4]. Meanwhile, Simvastatin improved cardiac function after myocardial infarction by up-regulated Bcl2, which is able to regulate intrinsic pathway-induced apoptosis [8]. The above reports reveal the close connection between apoptosis and myocardial infarction. The consideration of the therapeutic implication via targeting apoptosis in myocardial infarction is obvious. But presented studies are insufficient to clarify the complex interplay between apoptosis and myocardial infarction, further pointing out the mechanism of apoptosis is helpful to identify effective targeted therapy.

Integrins play an important role in the regulation of various genes related to cell growth, cell adhesion, apoptosis, thrombocytopoiesis, and hypertrophy [9]. As a key member of integrins family, ITGB1 (Integrin beta1) plays a variety of roles in the cardiovascular system. It is reported that ITGB1 is required for embryonic and postnatal vascular development [10,11]. ITGB1 also participates in assembly of the basement membrane, differentiation, stem cell survival, cardiac hypertrophy, cardiac function and failure [12]. The absence of ITGB1 resulted in impaired growth of blood vessels into the infarcted myocardium and reduced cardiac function [13]. In addition, the whole body knockout heterozygous mice of ITGB1 showed β -adrenergic receptor-induced increased TUNEL positive cells and fibrosis in heart tissue, but the effect and mechanism of ITGB1 on apoptosis have not been deeply interpreted [12]. In ITGB1 heterozygous knockout mice, percentage of fractional shortening and ejection fraction, Peak left ventricular developed pressure and left ventricular end-diastolic pressure were significantly lower than wild type mice after L-isoproterenol infusion, which implied ITGB1 play a

crucial role in beta-adrenergic receptor-stimulated myocardial remodeling^[12]. However, it has not been studied whether the ITGB1 expressed by cardiomyocytes could regulate myocyte apoptosis or not.

In this work, we interpreted the function of cardiomyocyte derived ITGB1 on cardiomyocyte apoptosis. We utilized rat cardiomyocytes (H9C2 cell line) to explore the possible mechanism of apoptosis. ITGB1 promoted cardiomyocyte apoptosis mediated by the Notch and Smad2/3 pathway.

2. Materials and Methods

2.1. Cell Culture

The H9C2 cells were obtained from the Procell Life Science&Technology Co., Ltd (Wuhan, China). Cells were cultured in Dulbecco's Modified Eagle Medium (Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Grand Island, NY, USA) and 1% antibiotic-antimycotic at 37 °C in a humidified incubator (5% CO₂). In hydrogen peroxide stimulation experiment, cells were incubated with medium containing different concentrations of H₂O₂ (100 and 500 μmol). After cultured for another 2 h, cells were harvested for further use.

2.2. ITGB1 siRNA transfection

The ITGB1 siRNA sequences were purchased (GenePharma, Shanghai, China). Lipofectamine 3000 (Thermo Fisher Scientific) was used as transfection reagent. For the transfection, ITGB1 siRNA was incubated for 68 h at 37 °C in 5% CO₂. The sequences of siRNA-944 sense (3'-5') and antisense (5'-3'): GCACCAGCCCAUUUAGCUATT and UAGCUAAAUGGGCUGGUGCTT, respectively. The sequences of siRNA-1474 sense (3'-5') and antisense (5'-3'): CCAGACGGAGUAACAAUAATT and UUAUUGUUACUCCGUCUGGTT, respectively. The sequences of siRNA-1612 sense (3'-5') and antisense (5'-3'): CCAAUAAGGAGUCUGAAATT and UUUCAGACUCCUUAUUUGGTT, respectively.

2.3. TUNEL assay

H9C2 cells were cultured to study the apoptotic cell death. The TUNEL assay was performed following the manufacturer's instructions of the TUNEL assay kit (Servicebio, Wuhan, China). The staining was visualized under Leica fluorescence microscope (Leica, Wetzlar, Germany).

2.4. Western blot analysis

The molecules were incubated with primary antibodies of ITGB1 (1:2000, ab179471, Abcam), HA (1:4000, ab9110, Abcam), Cleaved caspase3 (1:1000, #9661, Cell signaling), Smad2 (1:2000, ab480855, Abcam), Smad3 (1:1000, ab480854, Abcam), phospho-Smad3 (1:1000, #9520, Cell signaling), Bax (1:1000, #GB11690, Servicebio), Bcl2 (1:1000, #26593-1-AP, Proteintech), Notch1 (1:1000, #3608, Cell signaling), Jagged1 (1:1000, #SC-390177, Santa Cruz, American), Hes1 (1:1000, #11988, Cell signaling), GAPDH (1:10,000, #60004-1-Ig, Proteintech), and β actin (1:10000, #66009-1-Ig, Proteintech) at 4 °C overnight. These proteins were then incubated with the secondary antibodies of HRP-conjugated anti-rabbit IgG (1:20000, #ZB-2301, Beijing zhongshan Jinqiao Biotechnology Co., Ltd, China) and anti-mouse IgG (1:20000, #ZB-2305, Beijing zhongshan Jinqiao Biotechnology Co., Ltd, China) for 1 h. The immunoreactive bands were examined with ECL (Invitrogen, American) reagents and the OmegaLumC multifunction imaging system (Aplegen, American).

2.5. Statistical analysis

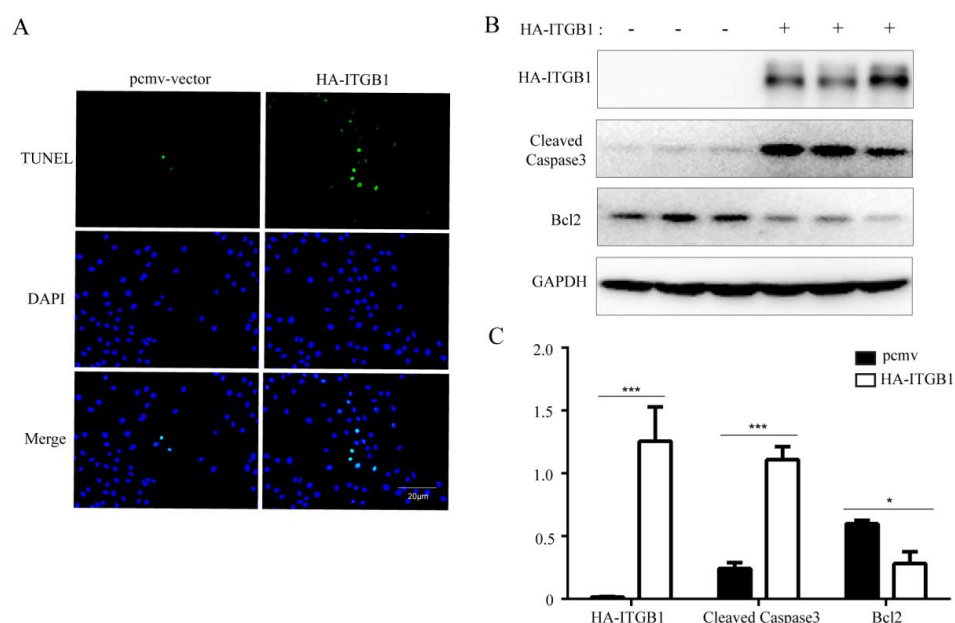
All quantitative variables were expressed as the mean ± SD. Statistical significance was determined using t-test or two-way ANOVA with Tukey's multiple comparisons test. All of the reported *P* values were two tailed, and a value of *P* < 0.05 was considered statistically significant. The Graph Pad Prism 6 software was used for all statistical analyses.

3. Results

3.1. ITGB1 overexpression promoted H9C2 cell apoptosis

Myocyte apoptosis and necrosis are two forms of myocardial death of myocardial infarction, among which apoptosis is a death manner regulated by genes [14-16]. Therefore, we focused on the regulatory function of ITGB1 in cardiomyocyte apoptosis. Due to the diversity of cell types in heart tissue, it was not convenient to study the function of genes in cardiomyocytes. Therefore, the H9C2 cell line (the rat embryonic cardiomyocyte line) was used to study the regulatory function of ITGB1 on cardiomyocyte apoptosis.

TUNEL staining results showed that compared with the control group, apoptotic cells increased significantly in the HA-ITGB1 transfection group (Figure 1A). As shown in Figure 1B and 1C, the results of western blot showed that apoptosis-related markers cleaved caspase3 was dramatically increased after ITGB1 transfection. Meanwhile, Bcl2 protein was reduced in the ITGB1 transfection group compared with the control group. Therefore, both the TUNEL staining and western blot results showed that ITGB1 could promote cardiomyocyte apoptosis.



(A) The TUNEL staining was performed in H9C2 cells with pcmv-vector or HA-ITGB1 transfection. (B) Cell apoptosis-related markers, including cleaved caspase3 and Bcl2, were detected by western blot. (C) Quantitative analysis of the western blot. * $p < 0.05$, *** $p < 0.001$.

Figure 1: ITGB1 promoted cardiomyocyte apoptosis in H9C2 cells.

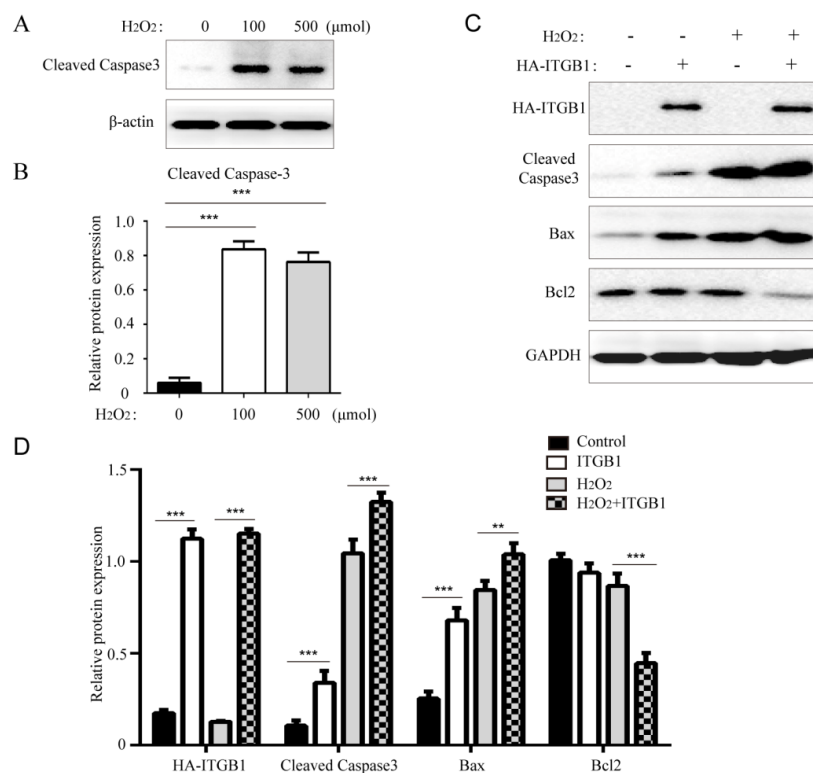
3.2. ITGB1 promoted H₂O₂ induced apoptosis in H9C2 cells

Oxidative stress is the major denominator of many cardiac diseases [17,18]. In acute myocardial infarction, oxidative stress induces the generation of severely damaging cardiomyocytes [19,20]. Our results showed that both 100 μ M and 500 μ M H₂O₂ induced significant cell apoptosis (Figure 2A and 2B). As shown in Figure 2C and 2D, the levels of proapoptotic proteins (cleaved caspase3 and Bax) were increased after ITGB1 transfection, whereas the antiapoptotic protein Bcl2 was decreased. In addition, Cells treated with H₂O₂ also showed increased cleaved caspase3 or Bax, and the ITGB1 gene exacerbated H₂O₂ induced apoptosis in H9C2 cells. These results showed that ITGB1 accelerated the apoptosis of H9C2 cells with H₂O₂ treatment.

3.3. ITGB1 knockdown inhibited apoptosis in H9C2 cells

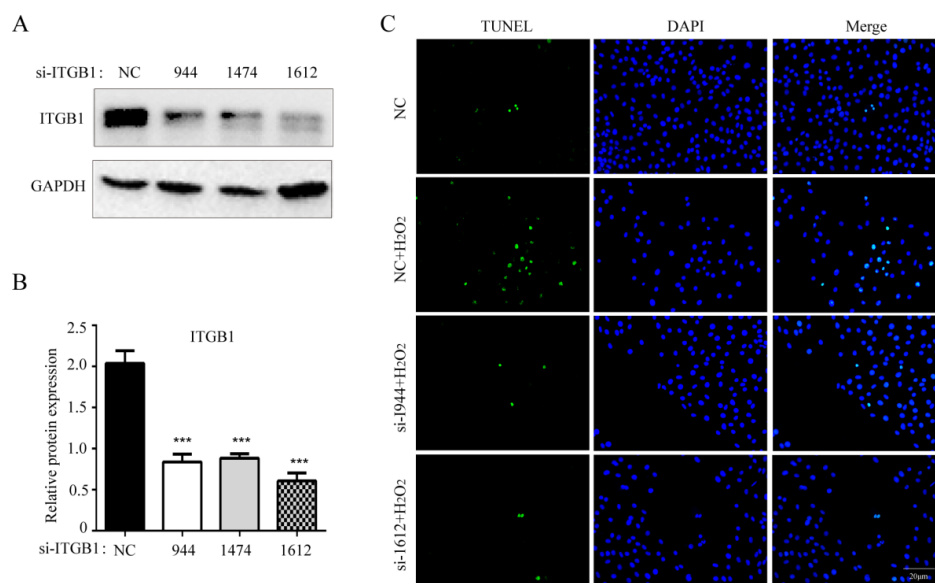
To study the effect of endogenous ITGB1 gene on cardiomyocyte apoptosis, we prepared small interfering RNA of ITGB1 to silent the gene expression. As shown in Figure 3A and 3B, all the three siRNA sequences could effectively disturb ITGB1 expression. Among these siRNA sequences, siRNA-1612 showed the best knockdown efficiency. The effect of siRNA sequence on H₂O₂-induced

cardiomyocyte apoptosis was examined by TUNEL staining. The results showed that ITGB1 knockdown inhibited H₂O₂-induced cardiomyocyte apoptosis (Figure 3C).



(A) and (B) Lysates of H9C2 cells were analyzed with the cleaved caspase3 antibodies. (C) Apoptosis-related markers were detected by western blot using their antibodies. (D) Quantitative analysis of the western blot. ** $p < 0.01$, *** $p < 0.001$.

Figure 2: ITGB1 promoted H₂O₂ induced apoptosis in H9C2 cells.

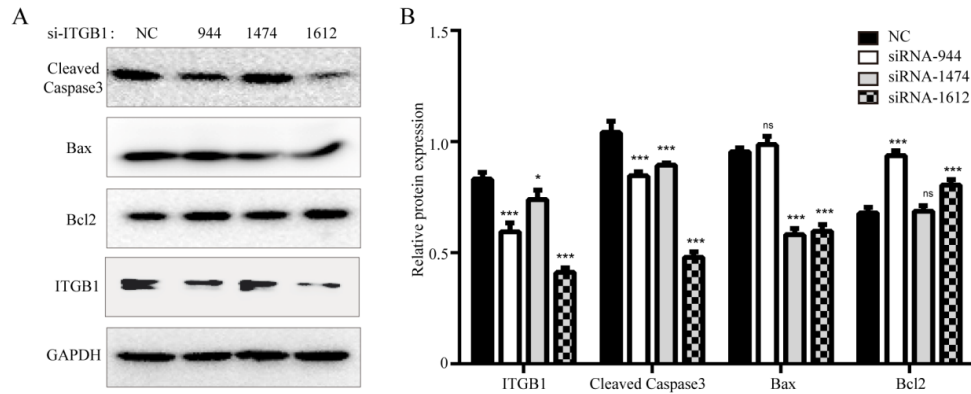


(A) Lysates of H9C2 cells transfected with NC or ITGB1 siRNA were immunoblotted with the ITGB1 antibody. (B) Quantitative analysis of the western blot. (C) The TUNEL staining was performed after NC or siRNA transfection with or without 100 μM H₂O₂ treatment. *** $p < 0.001$.

Figure 3: ITGB1 knockdown suppressed cardiomyocyte apoptosis in H9C2 cells.

3.4. ITGB1 knockdown regulated apoptosis-related markers in H9C2 cells

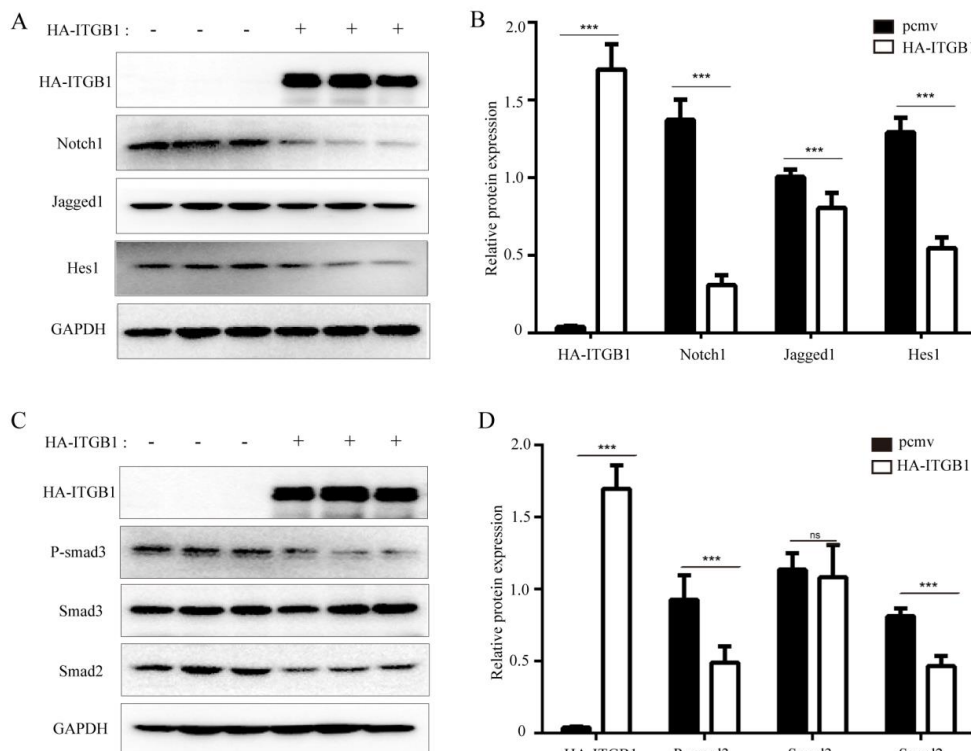
Western blot was performed to analyze the function of endogenous ITGB1 on the apoptosis-related markers (Figure 4A and 4B). The results exhibited that siRNA-944 and siRNA-1612 effectively interfered with ITGB1 expression. However, the interference effect of siRNA-1474 was not obvious at this time. Cleaved caspase3 and Bax protein expression were reduced after ITGB1 knockdown. Meanwhile, Bcl2 protein level was increased in the ITGB1 knockdown group. Therefore, both the TUNEL staining and western blot results with endogenous ITGB1 knockdown showed that ITGB1 could promote cardiomyocyte apoptosis.



(A) Apoptosis-related markers were detected by western blot using their antibodies. (B) Quantitative analysis of the western blot. * $p < 0.05$, *** $p < 0.001$.

Figure 4: ITGB1 knockdown regulated apoptosis-related markers in H9C2 cells.

3.5. ITGB1 promoted apoptosis by inhibiting Notch1 and Smad2/3 signaling pathway



(A) Notch1 signaling pathway related genes, including Notch1, jagged1, and Hes1 were detected by western blot using the antibodies. (B) Quantitative analysis of the western blot. (C) Smad2/3 signaling pathway was detected by western blot using p-Smad3, Smad3, and Smad2 antibodies. (D) Quantitative analysis of the western blot. *** $p < 0.001$.

Figure 5: ITGB1 inhibited the Notch1 and Smad2/3 signaling pathway in H9C2 cells.

As a transmembrane receptor, Notch1 has extracellular domain (NECD) and intracellular domain (NICD). Studies had validated the Notch1 pathway as an anti-apoptotic pathway in multiple cell types [21,22]. So we then examined whether ITGB1 regulated Notch1 signaling pathway. Western blot showed when ITGB1 protein expression increased, Notch1 signaling molecules, including Notch1, Jagged1, and Hes1, decreased significantly (Figure 5A, 5B). So ITGB1 suppressed the Notch1 signal pathway in H9C2 cells.

The Smad2/3 signaling pathway is closely correlated with cell apoptosis [23,24]. After transfection of ITGB1 plasmid in H9C2 cells, phosphorylated Smad3 was decreased markedly, while total protein levels of Smad3 remained unchanged (Figure 5C, 5D). Different from Smad3, the total protein level of Smad2 was decreased with ITGB1 overexpression. Therefore, the above results showed that ITGB1 inhibited both the Notch1 and Smad2/3 signal pathway in H9C2 cells.

4. Discussion

Myocardial infarction and hypertrophy are two different processes, but they are inextricably linked [25]. Myocardial infarction generally includes apoptosis or necrosis of cardiomyocytes. Apoptosis is a programmed death manner, which is regulated by genes [26]. Necrosis is a passive death caused by some acute stimuli, accompanied by release of cell contents [27]. After cardiomyocytes death through these two forms, the survival and prognosis of the patients depend on the number and function of the remaining living cardiomyocytes. Acute myocardial infarction will lead to compensatory myocardial hypertrophy [25]. Moderate myocardial hypertrophy is beneficial for the heart tissue to adapt to the injury and exercise its function. However, excessive myocardial hypertrophy will lead to serious heart disease. So myocardial infarction is closely related to myocardial hypertrophy [28]. Through ITGB1 overexpression and RNA interference experiments, we found that ITGB1 promoted the process of apoptosis. This study focuses on the regulatory function of ITGB1 on cardiomyocyte apoptosis. Moreover, we previously detected that ITGB1 could inhibit autophagy (data not shown), so ITGB1 might mediate the balance between autophagy and apoptosis

The Smad2/3 signaling pathway is involved in many cellular processes in mature organisms and developing embryos, including cell growth, differentiation, apoptosis, dynamic balance, and other cellular functions [23,24]. Smad2/3 is located mainly in the cytoplasm. After phosphorylation, P-Smad2/3 translocates into the nucleus to exercise the function of transcription factors and promote the expression of a series of downstream target genes [23]. We did not detect whether the activation of Smad2/3 by ITGB1 depended on the TGF beta receptor. In our study, ITGB1 was found to inhibit Smad2/3. Interestingly, the regulatory mechanisms of Smad2 and Smad3 by ITGB1 were different. The results showed that ITGB1 could inhibit the total protein expression level of Smad2. However, ITGB1 had no significant effect on the protein expression level of Smad3 but inhibited its phosphorylation level. Therefore, ITGB1 has molecular specificity in the regulation of Smad family members.

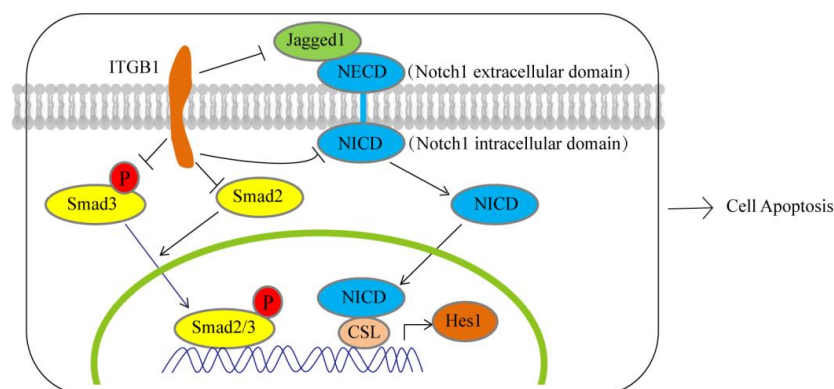


Figure 6: Schematic representation for ITGB1-mediated myocyte apoptosis.

In conclusion, this paper studied the role of ITGB1 in myocardial infarction. It was found that ITGB1 could inhibit the protein expression levels of Notch1 and jagged1, resulting in inhibiting the protein level of downstream Hes1. Notch1 pathway was inhibited, resulting in the expression changes of apoptotic genes. In addition, ITGB1 could also inhibit the protein level of Smad2 and the phosphorylation of Smad3, resulting in the decreased level of p-Smad2/3 in the nucleus, and finally leading to apoptosis (Figure 6).

Myocardial infarction seriously endangers human health and life. Therefore, understanding the pathogenesis of acute myocardial infarction and looking for effective therapeutic drugs plays a very important role. This study shows that ITGB1 promotes cardiomyocyte apoptosis through the Notch pathway and Smad2/3 pathway, accelerating the process of myocardial infarction. In the later stage, we could try to design some molecularly targeted drugs for ITGB1 to inhibit the function of ITGB1, eventually leading to inhibition of cardiomyocyte apoptosis and preventing myocardial infarction.

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Conflict of interest

The authors declare no conflict of interest.

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