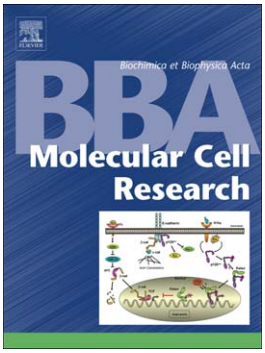

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β-cyclodextrin induces the differentiation of resident cardiac stem cells to cardiomyocytes through autophagy

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Abbreviations: β-CD, β-cyclodextrin; M-β-CD, methyl-β-cyclodextrin; HP-β-CD, hydroxypropyl-β-cyclodextrin; CSCs, Cardiac stem cells; Sca-1+, stem-cell antigen 1-positive; LDH, Lactate dehydrogenase; MI, Myocardial infarction; ABCA1, ATP-binding cassette transporter A1; 3MA, 3-methyladenine; Baf A1, bafilomycin A1; Cav-1, Caveolin-1; Flot-1, Flotillin-1; EDU, 5-ethynyl-2'-deoxyuridine

Abstract

Cardiac stem cells (CSCs) have emerged as promising cell candidates to regenerate damaged hearts, because of the potential in differentiating to cardiomyocytes. However, the differentiation is difficult to trigger without inducers. Here we reported that β-cyclodextrin (β-CD) increased the expression of cardiac transcription factors (Nkx2.5 and GATA4), structural proteins (cardiac Troponin T, cTnt), transcriptional enhancer (Mef2c) and induced GATA4 nucleus translocation in adult resident CSCs, thus β-CD could be used to enhance myogenic transition. As the differentiation process was accompanied by autophagy, we constructed the Atg5 knockdown cell line by using the Atg5 siRNA lentivirus, and the myogenic conversion was blocked in Atg5 knockdown cells, which suggested that β-CD induces the cardiomyocytes transition of resident CSCs through autophagy. Furthermore, we found that JNK/STAT3 and GSK3β/β-catenin was the downstream pathways of β-CD-induced autophagy and differentiation using the inhibitors. Moreover, β-CD performed its functions through improving intracellular cholesterol levels and affecting cholesterol efflux. Collectively, our results reveal that β-CD as a novel tool to induce myogenic transition of CSCs, which could mobilize the resident CSCs or used together with CSCs to enhance the therapy effects of CSCs on damaged hearts. In addition, the clarified molecular mechanisms supported the new targets for inducing cardiomyocyte differentiation.

Keyword: β-cyclodextrin; Resident cardiac stem cells; Differentiation; Cardiomyocytes; Cholesterol; Autophagy

1. Introduction

Heart failure characterized by the death of cardiomyocytes remains one of the top killers in the world [1]. Mobilize the heart endogenous cardiac stem cells (CSCs) differentiate into myocardial cells is a new strategy to treat heart failure [2-4]. There are various CSCs were identified including stem-cell antigen 1-positive (Sca-1^+), c-kit^+ and side-population cells [5, 6]. Among of these CSCs, the Sca-1^+ CSCs occupied the highest ratio [7]. Recent studies have shown that Sca-1^+ CSCs differentiate into cardiac muscle cells and improve heart function after injection into damaged heart in mice [8], suggesting a vital role of Sca-1^+ CSCs in regeneration of cardiomyocytes. We and others have reported that 5-azacytidine, oxytocin and sphingosylphosphorylcholine could induce myogenic transition of Sca-1^+ cells [9, 10]. To clarify the molecular of myogenic transition, more other inducers remain to be evaluated.

Cyclodextrin (CD) constitute a family of cyclic oligosaccharides comprising repetitive 6, 7, or 8 glucose units (α-, β-, and γ-CD, respectively). Among the CDs, β-cyclodextrin (β-CD) was got more attentions because the extra roles on the cholesterol. β-CD could remove cholesterol or increase intracellular free cholesterol levels by promoting cholesterol metabolism, thus it had pharmacology effects on atherosclerosis and other metabolic syndrome [11, 12]. Till now, withdraw cholesterol by β-CD contribute to the differentiation of various cells including chick cardiac cells were reported [13, 14]. However, the broad spectrum of β-CD on myogenic transition in other species and the related mechanism remains unclear.

Autophagy, an evolutionary conserved process that degrades cellular components, is crucial for maintaining and remodeling cellular homeostasis during normal cellular and tissue development [15]. Recent studies have demonstrated that autophagy is necessary for a number of differentiation processes [16, 17], including heart progenitor differentiation [18]. Furthermore, variable effects of β-CD or cholesterol on autophagy have been reported [19, 20]. Whether β-CD-regulated myogenic transition depends on autophagy is not known yet.

Autophagy performs its functions, at least in part, through the downstream signaling pathways that may be triggered by the autophagic program. Our previous studies have reported that JNK/STAT3 and β-catenin participate in cardiac stem cell differentiation to cardiomyocytes [10]. Furthermore, β-catenin activation was induced by cholesterol depletion during myogenic differentiation [21]. However, we do not know whether these two pathways located in the downstream of autophagy in β-CD-regulated myogenic transition.

The objective of this study is to evaluate whether β-CD contributes to cardiomyocyte transition of resident Sca-1^+ CSCs. We also identified the molecular mechanisms by which β-CD modulates the cardiomyocyte transition of resident Sca-1^+ CSCs.

2. Methods and materials

2.1. Reagents

β-CD, methyl-β-CD (M-β-CD) and hydroxypropyl-β-CD (HP-β-CD), 3-methyladenine (3MA), and β-actin were obtained from Sigma-Aldrich (St. Louis,

MO, USA). IMDM medium was from Invitrogen (Grand Island, NY, USA). Bafilomycin A1 (Baf A1) was from Sangon Biotech (Shanghai, China). Antibodies for total and phosphorylated GSK3β (Ser9), STAT3 (Tyr705), β-catenin (Ser33/37/Thr41), JNK (Thr183/Tyr185), Caveolin-1 (Cav-1) and LC3 were all from Cell Signaling Technology (Beverly, MA, USA). The cardiac troponin T (cTnt) and GATA4 antibodies were from Abcam (Cambridge, UK). Antibodies for ABCA1, flotillin-1 (Flot-1) were from Boster (Wuhan, China). FITC-labeled anti-Sca-1 and streptavidin were from BD Biosciences (San Jose, CA, USA).

2.2. Cell isolation, culture and treatment

Cardiac Sca-1^+ cells were isolated from adult C57BL/6 mice by magnetic cell sorting with about 90% purity, and sub-cultured on 0.2% gelatin-coated dishes with IMDM supplemented with 10% fetal bovine serum (FBS, HyClone), as described previously [22]. During the experiment, CSCs were pre-treated with medium deprived of FBS but supplement with β-CD（5 mM）for 4 h, then to normal medium when changing medium every 3 days.

2.3. Cell proliferation assay

The proliferation of CSCs and cardiac H9c2 cells was measured using the 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay kit (Ribbio Guangzhou P. R. China), according to the manufacturer's instructions. Briefly, CSCs were seeded into 24-well plates for 24 h, and incubated with EdU for 2 h. The EdU incorporation rate was expressed as the ratio of EdU-positive to total Hoechst 33342-positive cells (blue cells).

2.4. Lactate dehydrogenase (LDH) release assay

When the cells cultured on 6-well cell culture plate reached sub-confluence, the cultures were changed with the IMDM free serum with indicated concentrations of β-CD. After treatment for 4 h, the LDH release was detected by LDH cytotoxicity assay kit (Cayman, Ann Arbor, MI, USA).

2.5. Quantitative real-time PCR (QPCR)

Total RNA was extracted and reverse-transcribed into cDNA. QPCR involved the use of SYBR GreenER on the Bio-Rad PCR instrument. PCR reaction conditions followed the standard protocol. β-actin was used as an endogenous control. All qPCR reactions were performed in triplicate, and relative quantification involved the DDCt method (95% CI). Primer sequences for Nkx2.5, GATA4, cTnt and β-actin were as described [9].

2.6. Immunofluorescence staining

Immunofluorescence staining was done as reported [10]. Briefly, the Sca-1^+ enriched cells treated differently were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with anti-cTnt, anti-GATA4 or anti-Sca-1 antibodies, then FITC or IF555-conjugated secondary antibody. DAPI was used to stain the nucleus of cells. Laser scanning confocal microscopy (Leica, Wetzlar, Germany) was used for fluorescence detection. Images are representative of three independent experiments.

2.7. Western blot analysis

After treatment with β-CD for indicated times, cells were lysed by use of RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of total proteins

(30 μg) were separated by 15% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes, which were incubated overnight at 4°C with the appropriate primary antibodies (1:1000), then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Blots were exposed to the Super-Signal West Dura Extended Duration substrate (Pierce). β-actin was used as loading control. The relative quantity of proteins was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

2.8. Myocardial infarction (MI) model

C57BL/6 male mice were divided into 3 groups: Normal, MI and MI plus HP-β-CD ((n=6). MI was induced by permanent ligation of the left anterior descending (LAD) coronary artery for 14 d as described previously [9]. Mice were subcutaneously injected with HP-β-CD (2g/kg body weight or 200 ul 0.9% Nacl as vehicle control twice a week) from the second day of LAD.

2.9. Animal echocardiography

Echocardiography was performed as described previously to assess the changes in cardiac function in the three groups of mice (n=6) [9]. Systolic and diastolic functions were evaluated individually by left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS).

2.10. Histology

The heart tissue below the occlusion site were collected and fixed in 4% formalin. The fixed tissues were embedded in paraffin and 5 μm sections (n=6) were prepared. To detect the myogenic transition of Sca^+ cells, the antibodies for Sca-1 (1:100, rabbit

antibody) and cTnt (1:100, mouse antibody) were used. The FITC-conjugated anti-mouse and TRITC-labled anti-rabbit antibodies (1:200) were used as the secondary antibodies.

2.11. Flow cytometric analysis

Cells were detached using 0.25% trypsin–EDTA solution. Then cells from various groups were counted and divided into several samples (1×10^6 cells/sample). Each sample was resuspended in 100 μl cell staining buffer (BioLegend, USA) and incubated for 30 min on ice with FITC-conjugated anti-Sca-1+, after washing, the cells were stained with cTnt antibody, followed by the delight 594-labled-second antibody. Then cells were washed 3 times, resuspended in 50 μl cell staining buffer, and analyzed by flow cytometry (ImageStreamX MarkII, Amnis, USA). This experiment was performed with three independent replicates.

2.12. Lentivirus infection

To stably knock down endogenous Atg5 expression, we used lentivirus packing siRNA expression vector (pGLV3-GFP/Puro, GenePharma, Shanghai, China) to infect cells. Target cells were infected with lentivirus for 24–48 h according to the manufacturer's instruction. The RNAi oligonucleotides sequence used to knock down endogenous Atg5 expression and its negative control were as follows: Atg5 siRNA, 5'-GCAGAACCATACTATTTGCTTCTC-3' Negative control (NC) siRNA, 5'-TTCTCCG AACGT GTCACGT-3'

2.13. Cellular Cholesterol Quantitation Analysis

Cellular free cholesterol content was measured using a commercially available

quantitation kit (Applyge Technologies Inc, Beijing, China) following the manufacturer's instructions.

2.14. Statistical analysis

Data are reported as mean ± SEM and analyzed by one-way ANOVA followed by a Holm – Sidak post-hoc analysis. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. β-CD increased the expression of cardiomyocyte markers in resident Sca-1$^+$ CSCs

To clarify the function of β-CD on cardiomyocyte transition, we treated CSCs with different concentrations of β-CD, then changes in morphology were observed and the supernatant was collected for LDH detection. We observed that β-CD more than 5 mM decreased cell numbers (Fig. 1A) and had toxic properties to cells (Fig. 1B). Then β-CD at 5 mM was selected to analyze its roles on differentiation.

β-CD induced the transcription of GATA4, Nkx2.5, cTnt and Mef2c at 14 days (Fig. 1C). Furthermore, immunofluorescence assay verified the increasing of cTnt and GATA4 in protein levels (Fig. 1D, E), and showed that β-CD induced the translocation of GATA4 to the nucleus (Fig. 1D), which might be another index for cardiomyocyte differentiation [23].

3.2. β-CD induced the differentiation of resident Sca-1$^+$ CSCs to cardiomyocytes

To exclude the possibility that β-CD upregulated the expression of these genes without increasing differentiated cardiomyocytes, the frequency of cardiac marker-positive cells was estimated by flow cytometric analyses (FACS). The

proportion of Sca-1$^+$ cell used for the experiment reached 95±2%. The Sca-1 and cTnt double positive cells in β-CD-treated CSCs were about 5.1±1.2-fold to the control (Fig.2A). Then western blot and immunofluorescence staining assays were used in CSCs treated with or without β-CD, and got the similar results as FACS (Fig. 2B, 2C).

If β-CD enhanced the proliferation of pre-existing cardiac marker-positive cells, the cardiac markers would also be upregulated. To exclude this interference, we detected the role of β-CD on the cell proliferation. As shown in Fig. 2D, β-CD decreased the proliferation of Sca-1$^+$ cells and did not affect rat H9c2 cardiomyocytes. These results indicate that β-CD induces the myogenic transition of CSCs at 14 days.

We used the MI model to analyze the function of β-CD *in vivo*. The cardiac function detection showed that HP-β-CD rescued the decreased ejection fraction (EF) and fractional shortening (FS) caused by MI (Fig.2E). To investigate the changes of endogenous CSCs after MI in the hearts, the expression of Sca-1 and cTnt were detected. We observed that Sca-1$^+$ cells were located among the cardiomyocytes and inner the blood vessels. Furthermore, more cTnt/Sca-1 double positive cells appeared in mouse hearts treated with HP-β-CD (Fig.2F), suggesting that HP-β-CD could protect the hearts and also promotes differentiation of resident Sca-1$^+$ CSCs to cardiomyocytes *in vivo.*

3.3. β-CD enhanced autophagy during inducing differentiation

Autophagy plays an important role in myocardial differentiation [24]. When autophagy is induced, a small cytosolic ubiquitin-like molecule called LC3 will be

recruited by autophagosomes. LC3-II as a lipidated form of LC3 (form I) was widely used as an autophagy index with increasing requirements [25]. To evaluate the effect of β-CD on CSCs autophagy, we examined the expressing level of LC3-II, and found that LC3-II levels increased in a time dependent manner in CSCs treated with β-CD (Fig. 3A). However, an increase in autophagosomes could result from autophagy stimulation or autophagic efflux inhibition. To discriminate between these two possibilities, we used two tools of autophagy combined with β-CD. As shown in Fig. 3B, LC3-II protein levels were markedly attenuated by 3-methyladenine (3MA), the blocker of autophagy initiation, which suggested that β-CD induced the initiation of autophagy. Bafilomycin A1 (Baf A1) was used to disrupt autophagy efflux by disturbing the internal acidic environment of lysosome, and thereby leading to accumulation of LC3-II (Fig. 3B). Our data showed that LC3-II protein levels were increased further by Baf A1, suggesting that β-CD did not disrupt the autophagy efflux.

3.4. β-CD induced autophagy-dependent myogenic transition

Autophagy could affect the cell differentiation. Atg5 is important to autophagy, to understand the relationship between autophagy and differentiation, lentiviral-mediated stable ablation of Atg5 was performed in CSCs with its siRNA. When the lentiviral entered into CSCs (Fig. 4A), the siRNA effectively silenced the expression of endogenous Atg5 in CSCs (Fig. 4C), at the same time, LC3-II protein levels was markedly attenuated because autophagy was inhibited (Fig. 4B). QPCR results showed that β-CD stimulated transcription of GATA4, Nkx2.5, cTnt and Mef2c at 14

days was attenuated by Atg5 knockdown (Fig. 4D). Immunofluorescence assay showed that the cTnt expression and GATA4 nuclear translocation induced by β-CD also inhibited in CSCs with Atg5 knockdown (Fig. 4E). Our data support that β-CD induced CSCs differentiation was regulated by autophagy.

3.5. Involvement of JNK/STAT3 in β-CD-induced myogenic transition of CSCs

We have reported that JNK/STAT3 pathway participated in sphingosylphosphorylcholine induced cardiomyocyte transition of CSCs [10]. To analyze the involvement of JNK/STAT3 in β-CD induced cardiomyocyte transition, we detected changes of JNK/STAT3 in CSCs treated with β-CD or vehicles. The results showed that β-CD significantly increased phosphorylation of JNK and STAT3 at various time periods (1 h, 4 h, 7 days, 14 days) (Fig. 5A). The phosphorylations of JNK and STAT3 induced by β-CD were attenuated after autophagy was disrupted by 3MA, Baf A1 or Atg5 knockdown (Fig. 5B, C). Thus, autophagy induced differentiation was through JNK/STAT3 pathway.

3.6. Involvement of GSK3β/β-catenin in β-CD-induced myogenic transition of CSCs

GSK3β/β-catenin is another pathway participated in CSCs differentiation in our previous report [10]. β-CD activated phosphorylation of GSK3β at various time periods (1 h, 4 h, 7 days, 14 days) (Fig. 6A), indicating the involvement of GSK3β/β-catenin in β-CD induced myogenic transition of CSCs. Furthermore, the phosphorylations of GSK3β/β-catenin induced by β-CD were also attenuated after autophagy was disrupted by 3MA, Baf A1 or Atg5 knockdown (Fig. 6B, C). Thus, autophagy induced differentiation also through GSK3β/β-catenin pathway.

3.7. β-CD might induce the differentiation through regulating cholesterol

β-CD can selectively exclude cholesterol from cell membranes to change the membrane composition and structure. To investigate its action patterns, we detected the changes of Flot-1 and Cav-1 in CSCs treated with β-CD, which are the markers of lipid raft. The results showed that Flot-1 was not changed by β-CD or M-β-CD (Fig. 7A), but Cav-1was deduced by β-CD (Fig. 7B), which suggested that β-CD impaired the lipid raft.

To clarify the function of cholesterol on β-CD-induced myogenic differentiation and autophagy, the changes of cholesterol contents were detected. As shown in Fig.7C, the level of free cholesterol in CSCs treated with β-CD was increased at 1 h (Fig. 7C), but decreased at 24 h (Fig. 7C), which indicated that changes of cholesterol levels might be important for β-CD to induce differentiation and autophagy.

Increased levels of intracellular free cholesterol could trigger cholesterol efflux to maintain cholesterol stability [11, 12], so we lastly detected changes of ATP-binding cassette transporter A1 (ABCA1), a protein responsible for cholesterol efflux [26]. The results showed that it was not increased until the late stage of differentiation (Fig. 7D), we deduced that ABCA1 mediated cholesterol efflux might not be the key reasons trigger autophagy which changed since 3 h of β-CD treatment, but it might have roles on cardiomyocyte differentiation.

4. Discussion

Resident CSCs could differentiate into cardiomyocytes *in vitro* and *in vivo*, which supports a potential pathway for damaged heart repair, but the differentiation of CSC

is difficult to trigger and differentiation rate is very low. Here various derivatives of β-CD were used to suggest β-CD as a new agent to enhance cardiomyocyte differentiation. β-CD could mobilize the resident CSCs or used together with CSCs to enhance the therapy effects of CSCs on damaged hearts. Although β-CD was proved to be safe as a solubilizing agent [11], and the effective concentrations for inducing differentiation are also safe, high concentration of β-CD was toxic to cells in our study. The derivatives of β-CD with higher solubility and lower toxicity might be considered as potential drug candidates for heart repair.

β-CD can selectively exclude cholesterol from cell membranes to impair the lipid raft, which exists as a flask-like caveolae or as a less easily detectable planar form [11]. The caveolae is enriched in Cav-1, while the planar form is enriched in flotillins. Here, β-CD deduced Cav-1 levels but did not affect Flot-1. Considering the report that Flot-1-predominant rafts conversed into Cav-1-enriched rafts during osteoclast differentiation [27], we deduced that the Cav-1-enriched rafts might be important than Flot-1-predominant rafts in the cell differentiation under certain conditions [28].

Recent reports showed that β-CD could transitory affect the cholesterol metabolism by increasing the solubility of cholesterol to generate free cholesterol, thus induced ABCA1 and other proteins mediated cholesterol efflux to maintain cholesterol stability [11, 12]. Our results showed that free cholesterol levels transitorily increased after β-CD supplemented, and ABCA1 increased after that was in accordance with the reports. The data verified the roles of β-CD on cholesterol metabolism in inducing myogenic transition. Cholesterol is a sterol lipid that plays

pleiotropic roles in the plasma membrane, including maintaining membrane fluidity and permeability. Despite its importance, the consequences of cholesterol during CSC differentiation have not been described. Cholesterol depletion by β-CD induced cardiomyocyte differentiation of chicken cells [29]. Based on this report, our results further detected changes of cholesterol content, and found that dynamic changes of cholesterol but not depletion was pivotal for β-CD-induced differentiation.

Autophagy participates in myocardial differentiation with cell specificity and unknown mechanisms [24]. Atg5 knockdown impaired β-CD-induced differentiation by decreasing the expression of cardiomyocyte markers. We thus concluded that autophagy was involved in β-CD-induced myogenic transition of CSCs. Although GATA4 mRNA was deduced by Atg5 siRNA, its protein level was not affected at this time point, indicating that GATA4 translocation but not protein levels were responsible for autophagy-induced differentiation. Furthermore, autophagy induced myogenic transition of CSCs is through JNK/STAT3 and GSK3β/β-catenin pathways, which have been verified to regulate cardiomyocyte differentiation in the previous report [10, 30-32]. Our results connected the downstream pathways with autophagy to explain the differentiation mechanisms furtherly.

GATA4 is an important regulator of cardiac gene expression. In unstimulated cells, GATA4 interacted with and was directly phosphorylated by GSK3β at undetermined residue(s), leading to active GATA4 export from the nucleus [33]. Inhibition of GSK3beta by Licl caused nuclear accumulation of GATA4, suggesting that GSK3β negatively regulates nuclear expression of GATA4 [34]. In our previous

study, GATA4 translocated to the nucleus when myogenic transition was induced by the sphingosylphosphorylcholine [10], which was accompanied by GSK3β inhibition. Here, we found that Atg5 knockdown impaired nucleus translocation of GATA4 and rescued the activity of GSK3β. We deduced that β-CD-induced nucleus translocation of GATA4 was promoted by Atg5-inhibited GSK3β, but the hypothesis needs to be evaluated.

In summary, our data reveal that changes of cholesterol metabolism induce the autophagy, then activated JNK/STAT3 and GSK3β/β-catenin pathways, resulting in increased expression of cardiac transcription factors (Nkx2.5 and GATA4), structural proteins (cardiac Troponin T), transcriptional enhancer (Mef2c) and induced GATA4 nucleus translocation in adult resident cardiac stem cells (Fig. 7E). Therefore, β-CD could be used to enhance myogenic transition and have potential applications in the therapy of heart diseases.

Acknowledgments

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Figure Legends

Fig.1. β-CD increased the expression of cardiomyocyte markers in resident Sca-1$^+$ CSCs. CSCs were treated with indicated concentrations of β-CD (1, 5, and 10 mM) for 4 h, then morphologic changes were observed under a phase contrast microscope (A) (Scale bar=100 μm), and the LDH activity were detected by the LDH detection kit (B). (C) CSCs were treated with 5 mM β-CD for 14 days, mRNA levels of cardiac transcript markers including CTnt, Nkx2.5, GATA4, and Mef2c were analyzed by qPCR. (D) Representative immunofluorescent images show the expression of cTnt and nuclear translocation of GATA4 in CSCs treated with β-CD for 14 days. Scale bar, 20 μm. **P<0.01; *P<0.05. n=3.

Fig.2. β-CD induced the differentiation of resident Sca-1$^+$ CSCs to cardiomyocytes. CSCs were treated with 5 mM β-CD for 14 days, then the cells were used for detection. (A) FACS analysis of Sca-1/cTnt double positive cells. (B) Western blot results showed the changes of cTnt in CSCs treated with or without β-CD. (C) Representative immunofluorescent images showed Sca-1/cTnt double positive cells. (D) EdU assay was used to investigate the effects of β-CD on cell proliferation. (E) Animal echocardiography was conducted to analyze cardiac functions at 14 d of LAD. (F) Representative immunohistochemical images showed the double staining of Sca-1 (red) and cTnt (green) to the infarct zone. DAPI (blue) was used to show the nucleus. Arrow showed the Sca-1$^+$ cells, and star showed Sca-1 (red) and cTnt (green) double positive cells. ctr, control; **P<0.01; *P<0.05. n=6.

Fig.3. β-CD enhanced autophagy during inducing differentiation. (A) CSCs were

treated with 5 mM β-CD for indicated times (1 h, 4 h, 3 days, 7 days, 14 days), then LC3 levels were detected by western blot. (B) CSCs were pretreated with autophagy inhibitors 3MA at 5 mM or Baf A1 at 50 nM for 60 min followed by β-CD treatment for 3 and 7 days separately. The levels of LC3 were detected by western blot. **P < 0.01; *P < 0.05. n=3.

Fig.4. β-CD induced autophagy-dependent myogenic transition. (A) CSCs were transfected with LV3-NC or LV3-siAtg5, the fluorescent protein GFP was detected by using Zeiss LSM700. (B and C) QPCR and western blot analysis of Atg5 in CSCs transfected with LV3-NC or LV3-siAtg5. (D) QPCR of mRNA levels of cardiomyocyte transcript factors in CSCs transfected with LV3-NC or LV3−siAtg5 combined with β-CD (5 mM) for 14 days. (E) Representative immunofluorescent images and quantified analysis on the relative levels of cTnt expressing and the nuclear translocation of GATA4 in Atg5 knockdown CSCs treated with β-CD for 14 days. Scale bar, 20 μm. NC, negative control; ctr, control. **P<0.01; *P<0.05. n=3.

Fig.5. Involvement of JNK/STAT3 in β-CD-induced myogenic transition of CSCs. (A) Western blot analysis of STAT3 (Tyr705)/JNK phosphorylation in CSCs treated with β-CD (5 mM) for indicated times. (B and C) CSCs were pretreated with Baf A1 (50 nM), 3MA (5mM) for 60 min or transfected with LV3-siAtg5, then treated with β-CD (5 mM) for 7 days, the levels of STAT3 (Tyr705) /JNK phosphorylation were obtained by western blot. **P<0.01; *P<0.05. n=3.

Fig.6. Involvement of GSK3β/β-catenin in β-CD-induced myogenic transition of CSCs. (A) Western blot analysis of GSK3β/β-catenin phosphorylation in CSCs treated

with β-CD (5 mM) for indicated times. (B and C) CSCs were pretreated with Baf A1 (50 nM), 3MA (5mM) for 60 min or transfected with LV3-siAtg5, then treated with β-CD (5 mM) for 7 days, the levels of GSK3β/β-catenin phosphorylation were obtained by western blot. **P<0.01; *P<0.05. n=3.

Fig.7. β-CD might induce the differentiation through regulating cholesterol. (A and B)Western blot analysis of Flot-1 and Cav-1 in CSCs treated with M-β-CD（5 mM）or β-CD (5 mM) for indicated times. (C) CSCs were treated with β-CD (5 mM) for indicated times (1 h, 4 h, 10 h, 24 h), then the cellular cholesterol content was measured using a commercially available quantitation kit. (D) Western blot analysis of ABCA1 in CSCs treated with 5 mM β-CD for indicated times. **P<0.01; *P<0.05. n=3. (E) The graphic image shows that β-CD promotes the differentiation of Sca-1^{+} cells through a process of autophagy.

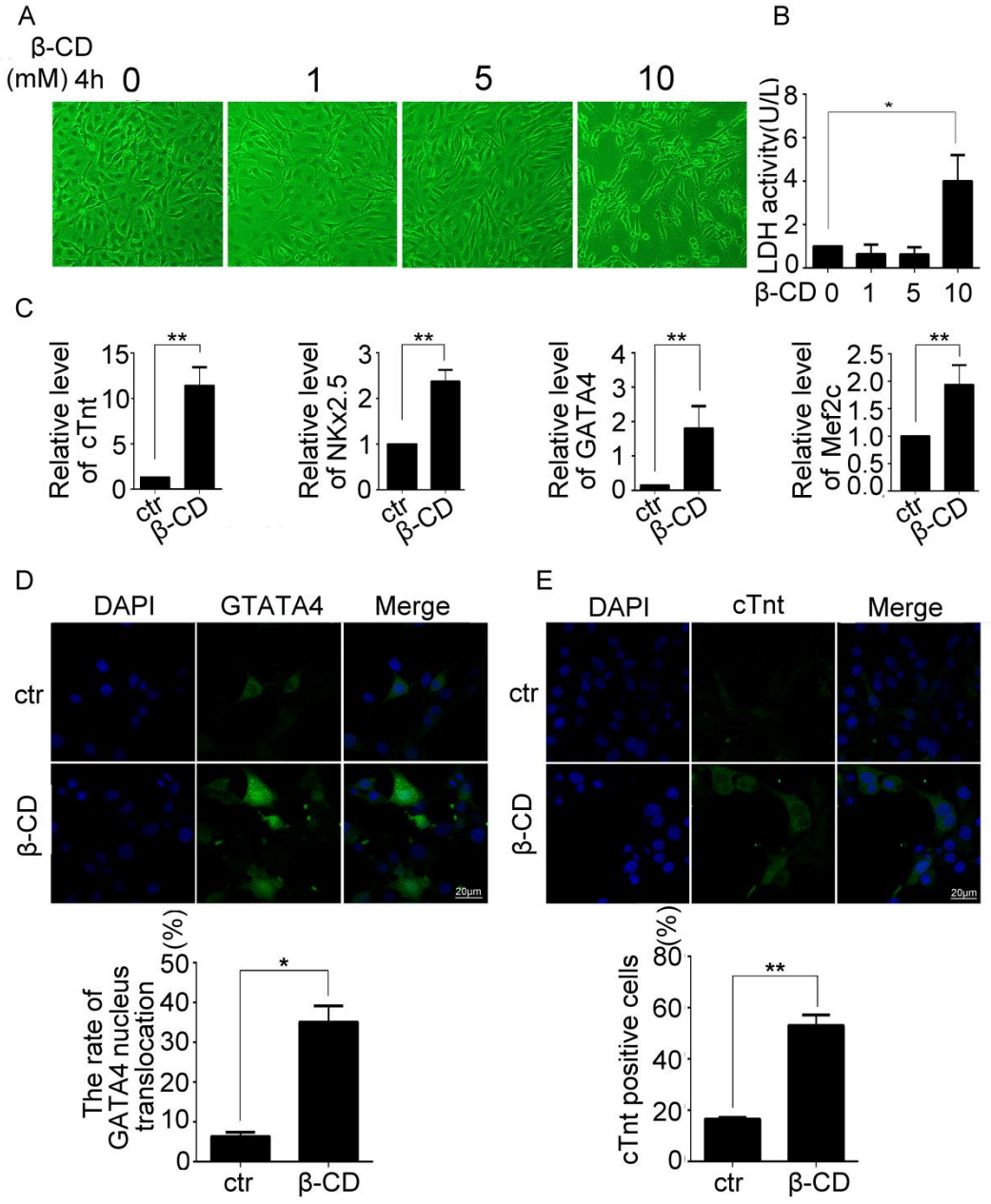


Fig. 1

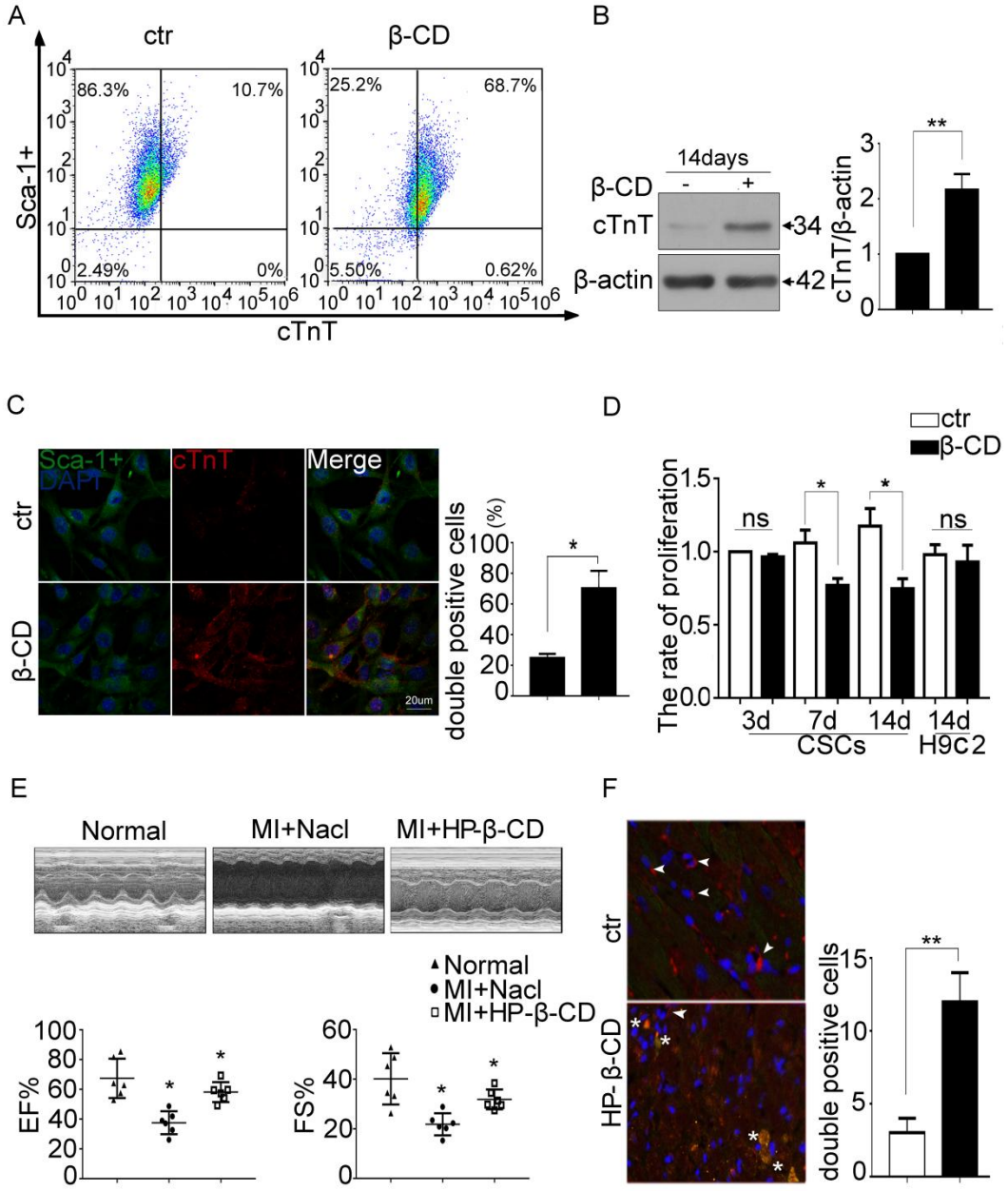


Fig. 2

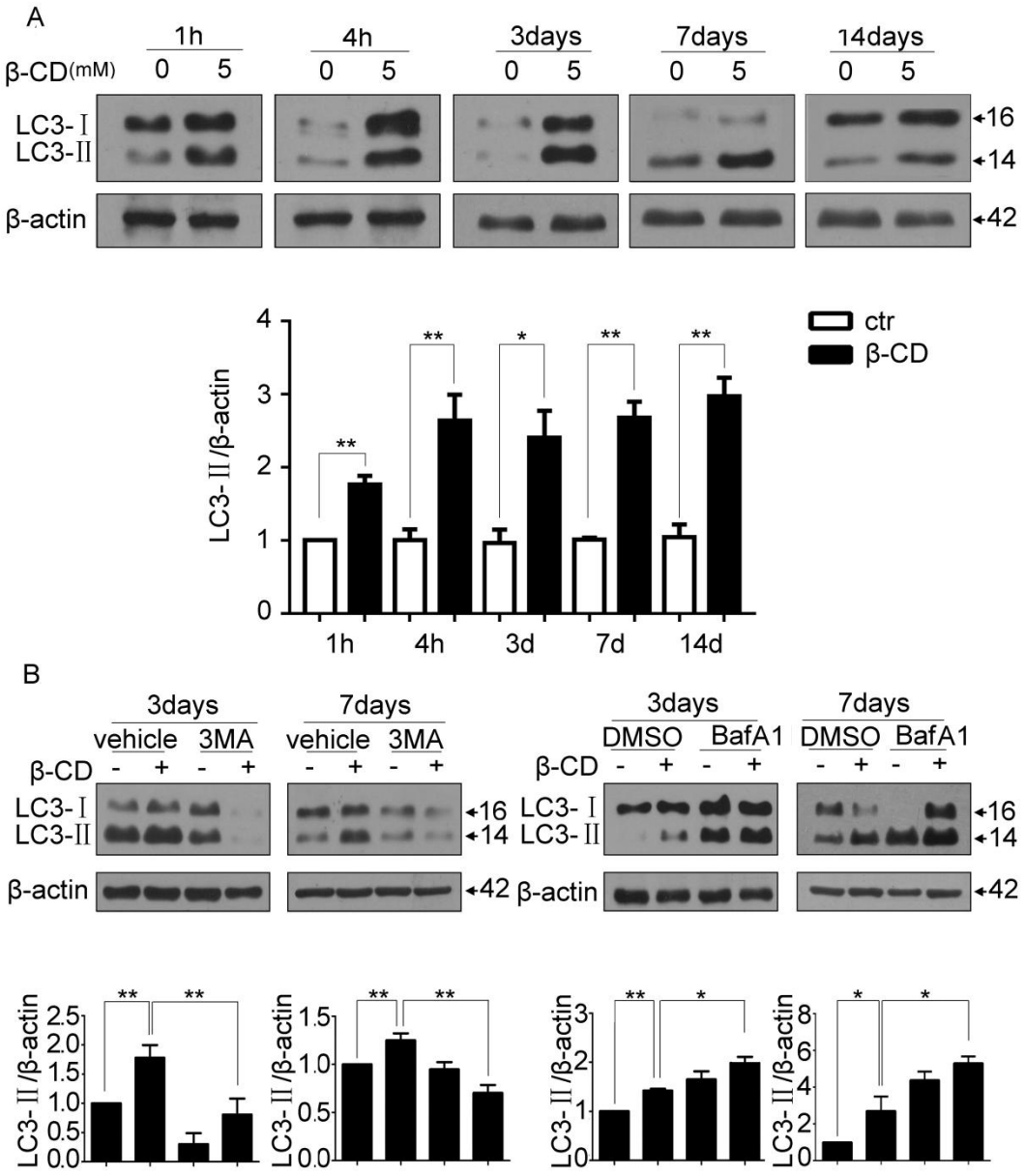


Fig. 3

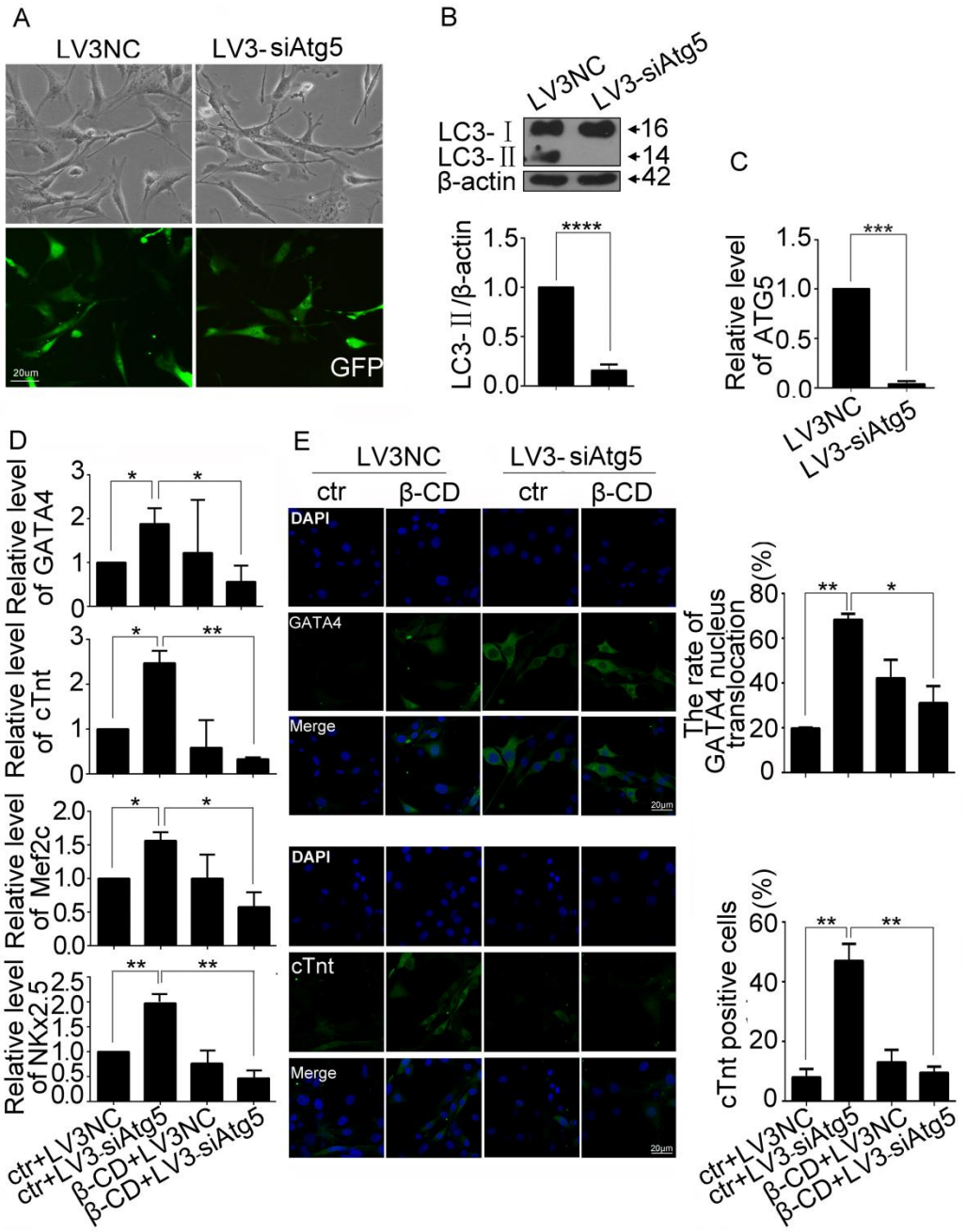


Fig. 4

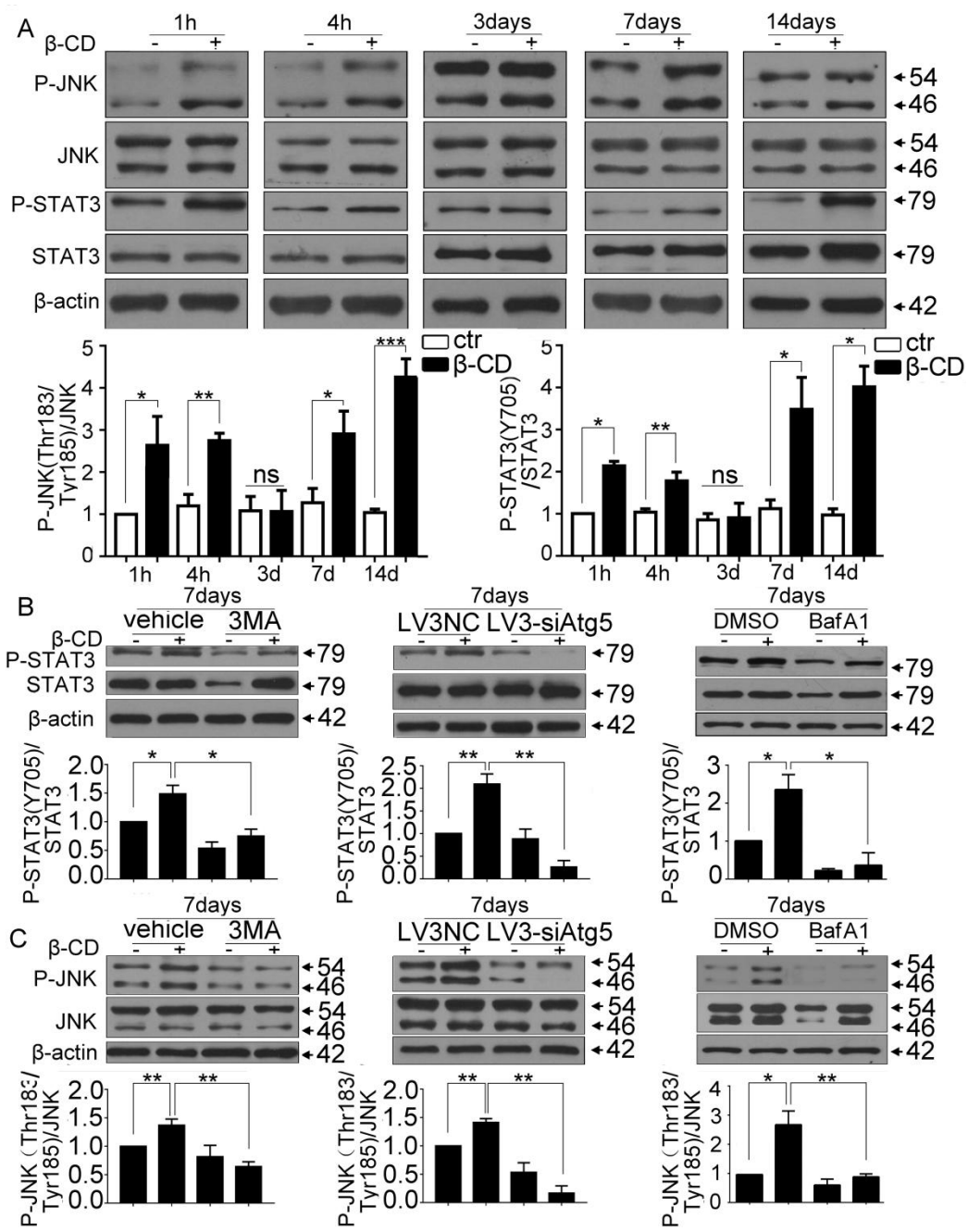


Fig. 5

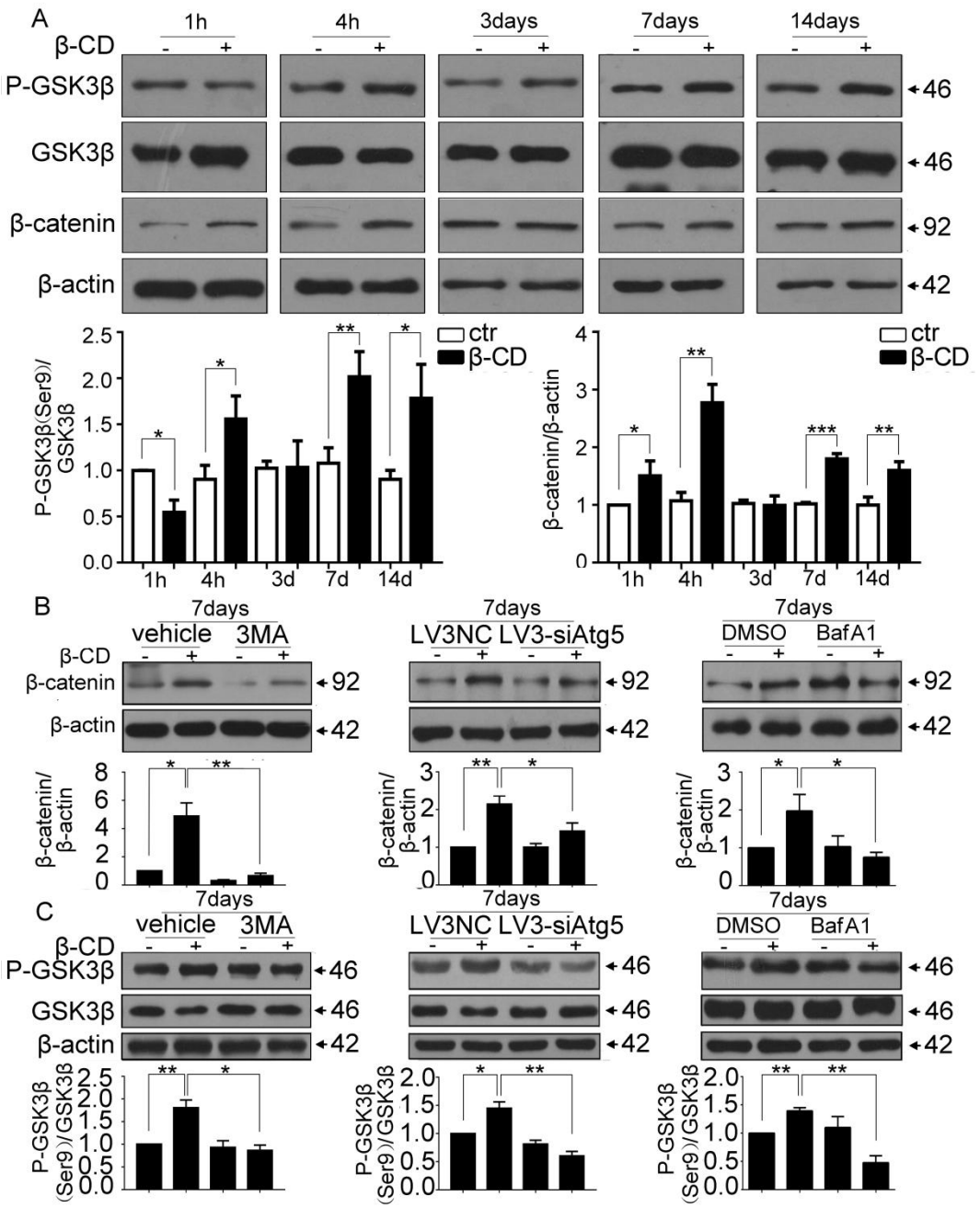


Fig. 6

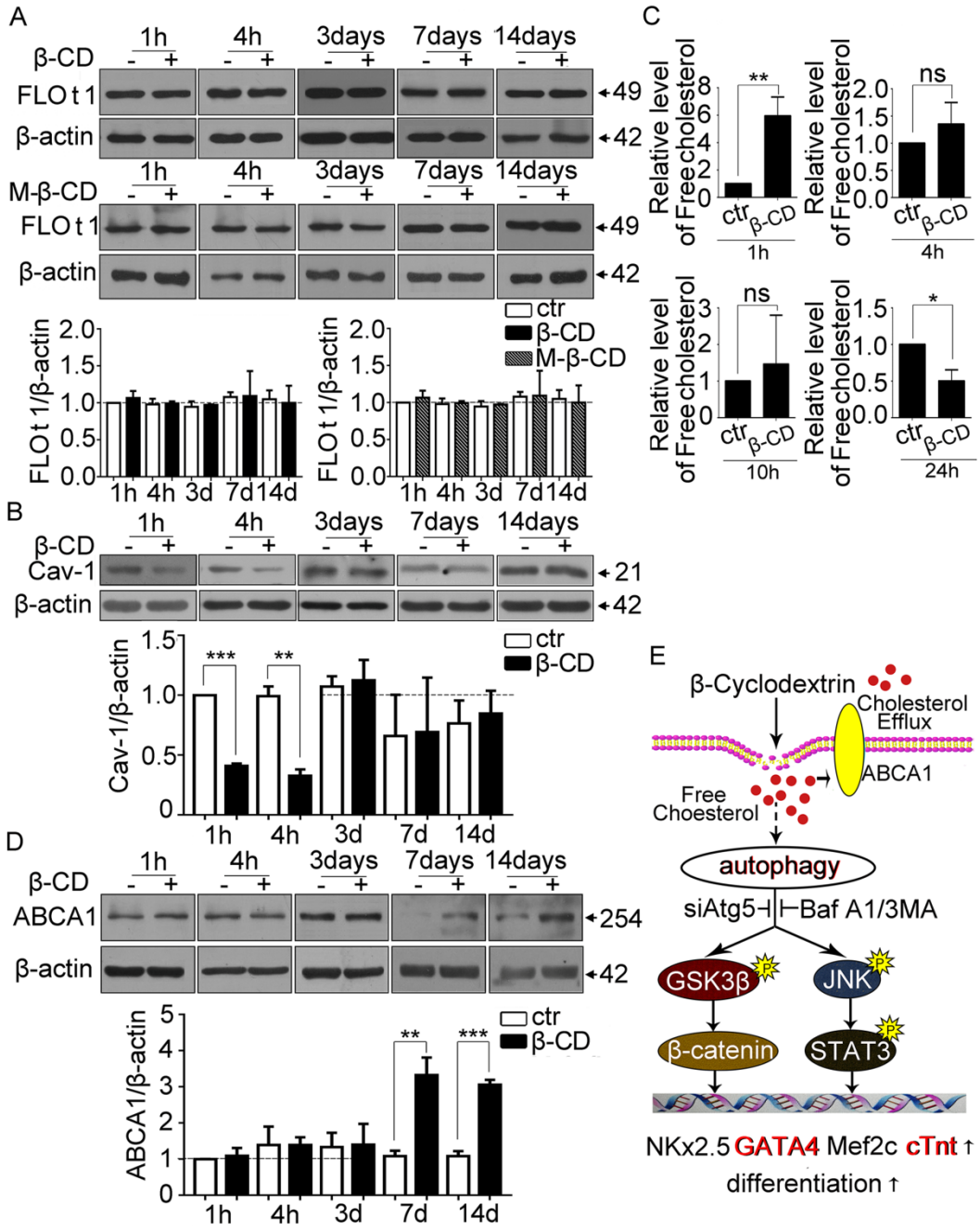
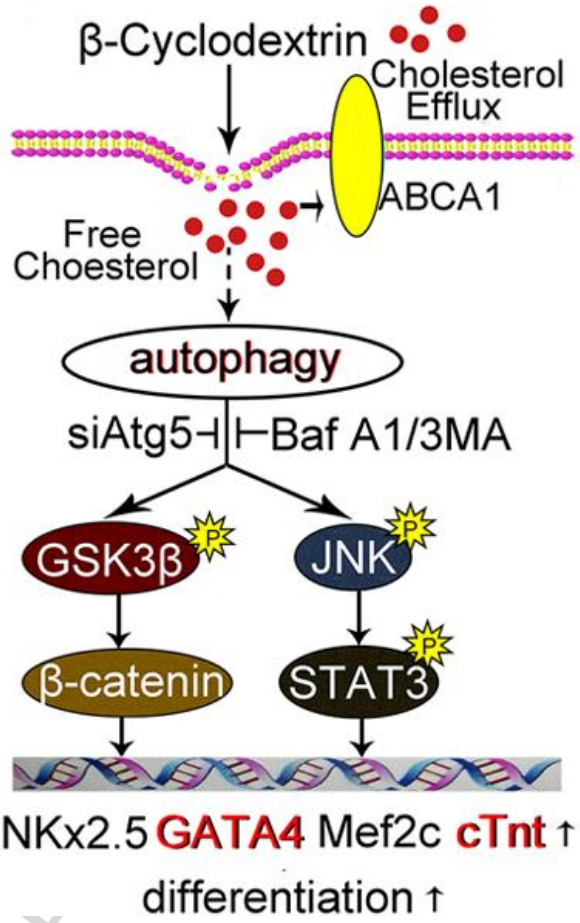

A
β-CD
1h 4h 3days 7days 14days
FLOt1
β-actin
49
42
M-β-CD
FLOt1/β-actin
ctr
β-CD
M-β-CD
1h 4h 3d 7d 14d
B
Cav-1
21
Cav-1/β-actin
***
**
C
Relative level of Free cholesterol
ctr
β-CD
1h
4h
10h
24h
ns
*
D
ABCA1
254
ABCA1/β-actin
E
β-Cyclodextrin
Cholesterol Efflux
ABCA1
Free Choesterol
autophagy
siAtg5
Baf A1/3MA
GSK3β
JNK
β-catenin
STAT3
P
NKx2.5 GATA4 Mef2c cTnt ↑
differentiation ↑

Fig. 7

Graphical Abstract



Highlights:

1. β- CD induced cardiomyocyte transition of resident sca-1^+ cardiac stem cells.
2. The induced cardiomyocyte transition is autophagy dependent.
3. JNK/STAT3 was the downstream pathway linking autophagy to differentiation.
4. β-catenin was another downstream factor linking autophagy to differentiation.
5. β-CD performed its function by increasing the free intracellular cholesterol.