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The status of MAPK cascades contributes to the induction and activation of Gata4 and Nkx2.5 during the stepwise process of cardiac differentiation

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ABSTRACT

Cardiac differentiation in vitro is a complex, stepwise process that is rigidly governed by a subset of transcription factors and signaling cascades. In this study, we investigated the cooperation of cardiac-specific transcription factors Gata4 and Nkx2.5, as well as mitogen-activated protein kinase (MAPK) cascades. P19 embryonic carcinoma cells were induced into spontaneously beating cardiomyocytes utilizing a two-step protocol that comprised an early stage and a late stage of differentiation. During early-stage differentiation in suspension culture, P19 cells aggregated to form embryoid bodies (EBs), and the *Gata4* and *Nkx2.5* genes were induced. However, Gata4 expressed at the early stage of differentiation was incapable of activating downstream gene expression, as it was localized in the cytoplasm and prone to degradation. After EBs were plated for late-stage differentiation in adherent culture, the MAPK cascades were highly activated and contributed to the activation of Gata4 and Nkx2.5. Specifically, we revealed that p38 signaling participated in regulating the localization and stabilization of Gata4 and Nkx2.5. Additionally, the JNK cascade regulated late-stage cardiac differentiation; JNK kinase reduced Gata4 stabilization and conversely alleviated Nkx2.5 degradation by direct interaction and phosphorylation of Nkx2.5. Finally, we found that the C-terminal domain of Nkx2.5 was required for its stabilization under conditions of oxidative stress and JNK activation. Overall, our results indicated that the induction and activation of Gata4 and Nkx2.5 during early- and late-stage cardiac differentiation was closely associated with the function of the MAPK signaling cascades.

1. Introduction

Pluripotent stem cells can acquire a cardiac fate with the help of growth factors and signaling molecules, as well as genetic manipulations. However, cell differentiation in chemically defined culture media often results in inconsistencies in cell culture and heterogeneous populations of cells, which impede the success of cell-based therapies for cardiac damage. It is of the utmost importance to understand the precise mechanisms of cardiomyogenesis and then optimize the induction methods that can generate spontaneously beating cardiomyocytes [4].

Cardiac differentiation of stem cells in vitro is an intricate, stepwise process, which has been shown to undergo multiple consecutive stages identified by the temporal expression of specific markers [5]. A hierarchical gene regulatory network governs the progression of cardiac commitment [1,3,5]. The conversion of pluripotent stem cells toward a cardiac fate can be characterized by the sequential expression of transcription factors such as *Brachyury T* and *Mesp1* for mesoderm formation and *Nkx2.5*, *Gata4*, *Mef2c*, and *Hand1/2* for cardiac mesoderm/cardiac progenitors. *Gata4* and *Nkx2.5* are the earliest gene markers for heart precursor cells in all vertebrates. The synergistic function of Gata4, Nkx2.5, and Mef2c triggers the cardiac specification followed by maturation. During cardiac maturation, the cells begin to express cardiac structural and contractile genes, such as *cTNT*, *α-Actinin*, *Mhc6*, and *Mhc7*. In addition, the stepwise clearance of repressive roadblocks, such as Sox2, Msx1, and Cdx2/1, also contributes to the acquisition of a cardiac cell fate [6].

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Distinct signaling molecules exert different effects depending on cell status and the stage of differentiation. Numerous signaling pathways are involved in the multi-step process of cardiac differentiation, such as TGF-β, Wnt, and Notch. These signaling pathways have been reported to participate in the cell fate decision at crucial steps of cardiac differentiation, exhibiting eminent stage-specific and dose-dependent effects [1–3]. Wnt signaling is one of the most versatile signaling pathways that acts in different spatial and temporal patterns during heart development. It is well characterized that the canonical Wnt cascade exerts biphasic effects on cardiac differentiation, both during its early and late stages [7]. Likewise, the TGF-β and Notch pathways have been found to facilitate and/or inhibit cardiac specification at different stages of the process [8,9]. Each signaling pathway activates a unique set of downstream genes and/or transcription factors when in a specific cellular context.

Mitogen-activated protein kinases (MAPKs) comprise a family of protein kinases relaying extracellular signals from the cell membrane to the nucleus via a series of sequential phosphorylation events. Diverse cellular events ranging from cell proliferation to cell apoptosis are regulated by MAPK signaling. The MAPKs consist of three well-characterized branches: ERK, p38, and JNK. Signaling through each of these MAPK branches is initiated by diverse stress and mitogenic stimuli at the cell membrane or within the cytoplasm and leads to different cellular responses [10,11].

In this study, we elucidated the roles of MAPK cascades in the stepwise process of cardiac differentiation. We focused on the MAPK-mediated regulation of the expression and function of cardiac-progenitor-cell genes *Gata4* and *Nkx2.5*. Our results showed that suitable inhibition of the p38 pathway facilitated the expression of *Gata4* and *Nkx2.5* during the early stage of differentiation. However, Gata4 protein at this early stage was incapable of transactivating the expression of cardiac contractile genes. In addition, MAPK activation was required for the stabilization and function of Gata4 and Nkx2.5 at the late stage of cardiac induction. Notably, Nkx2.5 was identified as a major target of the JNK cascade in its facilitation of cardiac differentiation. Collectively, the different status of MAPK cascades at the early and late stages of cardiac differentiation modulate the induction and activation of Gata4 and Nkx2.5, respectively.

2. Materials and methods

2.1. Reagents and plasmids

SB203580, SP600125, PD98059, *Z*-VAD-FMK, MG132, and hydroxychloroquine (HCQ) were purchased either from MedChem Express or Selleck Chemicals (Houston, TX, USA). Cycloheximide (CHX) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Gata4 (sc-25,310), Nkx2.5 (sc-376,565), phosphorylated Gata4 (Ser261, sc-377,543), and Actin (sc-47,778) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies specific for phosphorylated and total p38 (#8690, #9215), ERK (#4695, #4370), or JNK (#9252, #4668) kinases, as well as histone H3 (#4499) and LC3(#4108), were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for the Flag-tag (F3165) and His-tag (SAB1305538) were from Sigma-Aldrich (St. Louis, MO, USA). Myosin heavy chain antibody MF-20 (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) was generously provided by Dr. Qin Lu (Key Laboratory of Molecular Cardiovascular Sciences, Peking University, China). Specific antibodies against phospho-Ser (ab9332) and phosphorylated Gata4 (Ser105, ab5245) was purchased from Abcam (Cambridge, UK). The His-JNK2 plasmid was purchased from Sino Biological Inc. (Beijing, China) and the plasmids encoding full-length Nkx2.5 and C-terminal defective mutant Nk202 were purchased from Genechem (Shanghai, China).

2.2. Cell culture and cardiac differentiation

P19 embryonal carcinoma cells were cultured in α-minimal essential medium (α-MEM) (ThermoFisher Scientific, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Waltham, MA, USA), penicillin (100 U/mL), and streptomycin (100 U/mL) in a 5% CO_2 atmosphere at 37 °C. P19 cells (5×10^5 cells/mL) were placed into 60-mm bacterial-grade plastic dishes in the presence of 1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to initiate cell aggregation and cardiac differentiation. The time point at which the cells formed embryoid bodies (EBs) was taken as day 0. On day 4 of cardiac induction, EBs were plated on tissue culture-grade dishes for adherent culture. Spontaneous contractions of EBs were usually observed from day 8 and the ratio of spontaneously contracting EBs to the total number of plated EBs was counted at indicated days. The rat embryonic ventricular myocardial H9c2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1500 mg/L $NaHCO_3$ and supplemented with 10% FBS.

2.3. Reverse transcription of RNA and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, San Diego, CA USA) and 2 μg RNA was used for reverse transcription. Then, the resulting cDNA underwent real-time PCR as previously described. SYBR Green real-time Master Mix (Toyobo, Osaka, Japan) and the ABI7900 Real-Time PCR system were used (Applied Biosystems, Foster City, CA, USA). Target-gene expression levels were normalized by the expression of the 18S ribosomal subunit as an internal control gene, by calculating the ratio of Target gene/18S expression ($2^{-\Delta\Delta Ct}$). Primers for the reverse transcription and real-time PCRs are listed in Supplementary Table S1.

2.4. Western blotting

The cells were washed with cold phosphate-buffered saline (PBS) and lysed on ice in modified RIPA buffer (50 mM Tris-HCl at pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, and 150 mM NaCl) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 2 mM EDTA) and phosphatase inhibitors (10 mM β-glycerophosphate, 1 mM Na_3VO_4, and 5 mM NaF). The extracts were centrifuged at 12,000 ×*g* for 10 min at 4 °C, and the supernatant fractions were collected. The protein content in the supernatant was measured using a Bicinchoninic (BCA) Protein Assay Kit (Beyotime, China). The proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, USA). The membranes were then blocked in 5% non-fat milk for 1 h and incubated with primary antibodies specific to the target proteins and species-specific secondary antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence (ECL) detection kit (Vigorous, Beijing, China) according to the manufacturer's instructions.

2.5. Immunofluorescence

EBs at day 3 and P19 cell clusters at day 6 were disassociated using the cell detachment solution Accutase (ThermoFisher Scientific, Carlsbad, CA, USA). Cell pellets were replated on glass coverslips and cultured for 12 h. Subsequently the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then washed with PBS. Next, the cells were treated with PBS containing 10% BSA and 0.1% TritonX-100 for 30 min at room temperature and then incubated with primary antibody against Gata4 overnight at 4 °C. A FITC–conjugated secondary antibody was used to visualize cellular morphology. Nuclei were counterstained with Hoechst33342 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. Immunofluorescence was visualized under an Olympus FV1000

confocal laser-scanning microscope. To display the subcellular localization of Flag-Nkx2.5 and Flag-Nk202, H9c2 cells transfected with these plasmids were stimulated with 400 μM H_2O_2 for 4 h and subsequently stained with primary antibody against the Flag-tag.

2.6. Plasmid transfection

H9c2 cells were seeded into 12-well plates 24 h before transfection. Constructs encoding full-length Nkx2.5, C-terninal defective Nk202, and His-JNK2 were transfected using Lipofectamine2000 (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The total amount of DNA was kept constant using the pcDNA3 plasmid.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed on the cultured P19 cells using the EZ-ChIP kit (Millipore, Los Angeles, CA, USA) according to the manufacturer's instructions. Briefly, EBs or cell clusters were dissociated with Accutase and then diluted to a single-cell suspension at 1×10^6 cells per mL. Subsequently, cells were fixed with 1% formaldehyde for 10 min at room temperature. Cells were washed twice in ice-cold PBS, resuspended in SDS-lysis buffer, and sonicated until the cross-linked chromatin was sheared to an average DNA fragment length of 100–800 base pairs. Immunoprecipitation assays were performed using 5 μg of Gata4 or Nkx2.5 antibodies and normal IgG as a negative control. The precipitated DNA was amplified by real-time PCR. The primers used to amplify the promoters of target genes were specific for natriuretic peptide A (Nppa, also known as atrial natriuretic factor, Anf): forward, 5′-GAGCGCCCAGGAAGATAACC-3′ and reverse 5′-GCCAGCAGAAGAT GCCCTTT-3′.

2.8. Co-Immunoprecipitation (Co-IP)

H9c2 cells were transfected with Flag-Nkx2.5 and His-JNK2 and then scraped into ice-cold PBS and pelleted by centrifugation. Next, the cells were lysed in modified RIPA buffer with orbital shaking for 30 min at 4 °C. The lysates were incubated with specific antibody or normal IgG (negative control) overnight at 4 °C with gentle rotation on an orbital shaker. Then, they were incubated with 50 μL of protein G-agarose (Roche, Indianapolis, IN, USA) at 4 °C for 4 h with gentle orbital shaking. Immunoprecipitates were washed three times with wash buffer and subjected to SDS-PAGE and detection with specific antibodies.

2.9. Statistical analysis

All data are expressed as the mean ± standard deviation (SD) of at least three independent biological replicates. Error bars indicate the SD value. The unpaired Student's *t*-test with the significance level set at $P < 0.05$ was used to determine statistical significance for each assay.

3. Results

3.1. Gata4 activation in the stepwise process of cardiac differentiation

In this study, we used a classical, two-step procedure to generate spontaneously contracting cardiomyocytes from P19 embryonal carcinoma cells. P19 cells were first supplemented with DMSO in suspension to aggregate and form EBs. EBs recapitulate many aspects of cell differentiation during early embryogenesis. Subsequently, the cell aggregates were plated for adherent cultivation for another 4 days with DMSO. The generation of cardiomyocytes was demonstrated by their rhythmic beating activity that usually commenced at around day 8–10. The numbers of spontaneously beating EBs progressively increased after 8 days of induction. Cardiac differentiation of P19 cells underwent several stages identified by the temporal expression of specific markers (Fig. 1A and B). In summary, the entire process of cardiac induction can be divided into two major stages: early- and late-stage differentiation. During suspension culture, P19 cells underwent early-stage differentiation; cardiac precursors began to appear and the cells expressed the earliest cardiac markers such as *Gata4* and *Nkx2.5*. During adherent culture, stem cells achieved late-stage differentiation, where they further specialized into mature cardiomyocytes expressing genes like *Myh6* and *Myh7* that encode cardiac contractile proteins (Fig. 1A). In terms of protein levels, Gata4 and Nkx2.5 were first observed at day 2–4 of cardiac induction. However, cardiac myosin heavy chain first emerged on day 8 and further increased over the following periods (Fig. 1B).

The cooperative action of Gata4 and Nkx2.5, two crucial cardiac transcription factors, triggers the expression of cardiac contractile genes such as *Myh6* and *Myh7*. Although the expression of *Gata4* and *Nkx2*.5 occurred from day 2, their downstream genes started to express only at the late stage of cardiac induction (Fig. 1A). In light of this delay, we speculated that the Gata4 and Nkx2.5 proteins that were expressed at the early stage did not activate downstream target genes. Subsequent ChIP assays revealed that the recruitment of Gata4 protein to the promoter of *Nppa* greatly increased during the stage of adherent cultivation (Fig. 1C). *Nppa* is a downstream gene of Gata4 and Nkx2.5 and is indicative of differentiated cardiac tissue [12]. The distribution of the Gata4 protein also changed over the course of cardiac differentiation; while it was predominantly found in the cytoplasm at the early stage of differentiation, it was completely localized to the nucleus at the late stage (Fig. 1D). Taken together, our results demonstrated that the status of the Gata4 protein was entirely different at the early and late stages of cardiac differentiation.

3.2. Gata4 activation is associated with MAPK cascades

It is well known that post-translational modifications such as phosphorylation can effectively alter the expression and activity of Gata4 [13]. The MAPK cascade is a critical biochemical signal that mediates Gata4 phosphorylation. In this study, we investigated the temporal activation of MAPKs, specifically ERK, JNK, and p38 kinases. As shown in Fig. 2A, the p38, ERK, and JNK cascades during cardiac differentiation shared a similar activation pattern with biphasic kinetics. High levels of phosphorylated p38, ERK, and JNK kinases mainly appeared and were maintained at the late stage of differentiation. In parallel, phosphorylation of Gata4 at Ser105 and Ser261 was mainly observed during late-stage differentiation (Fig. 2B). To investigate the degradation pattern of Gata4, P19 cells at different stages were treated with the protein synthesis inhibitor cycloheximide (CHX) and subjected to lysis at various time points. We observed that the Gata4 protein expressed at the early stage of differentiation was easily degraded (Fig. 2C) and late-stage Gata4 had a significantly longer half-life. Taken together with other evidence such as Gata4 localization, our results indicated that Gata4 status during cardiac induction can be divided into two major stages and was regulated by MAPK-mediated phosphorylation. At the early stage of differentiation, the majority of Gata4 protein was localized to the cytoplasm and was sensitive to degradation. Comparatively, Gata4 expressed at the late stage was fully activated by phosphorylation and was able to translocate into the nucleus and fulfil its transcriptional function.

3.3. The effects of MAPK inhibition on early-stage differentiation

We investigated the effects of the MAPK cascades on cardiac differentiation by treating P19 cells at the early stage of differentiation with different doses of MAPK inhibitors and calculating the number of spontaneously contracting EBs on day 12. As shown in Fig. 3A, the ERK inhibitor PD98059 only weakly affected differentiation efficiency. A high dose of the JNK inhibitor SP600125 decreased the generation of spontaneously beating EBs. In comparison, the p38 inhibitor SB203580 exerted dual effects in a dose-dependent manner; a low dose facilitated cardiomyogenesis, while a high dose inhibited cardiomyogenesis of P19

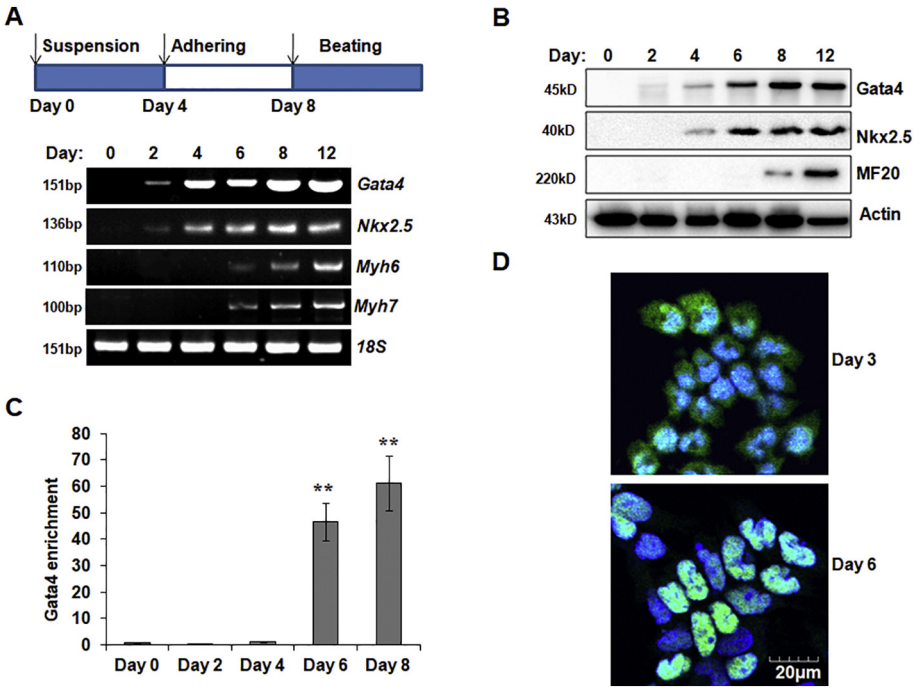

Fig. 1. The functional status of Gata4 activation across the stepwise process of cardiac differentiation. (A) Schematic diagram of P19 cell differentiation process with DMSO induction. Cells were harvested at depicted days, and mRNAs were analyzed by RT-PCR to display the relative expression alteration of cardiac transcriptional factors (*Gata4* and *Nkx2.5*) and contractile myosin heavy chain (*Myh6* and *Myh7*). 18S ribosomal RNA was utilized as an internal control. (B) Representative western blot analysis exhibits the expression profile of cardiac transcriptional factors (*Gata4* and *Nkx2.5*) and cardiac myosin heavy chain MF20 in protein level during the process of cardiac induction. (C) Differentiating P19 cells were subjected to ChIP experiments using anti-Gata4 antibody. The immunoprecipitated DNA fragments were amplified by real-time PCR for *Nppa* promoters containing Gata4 binding sites. Each bar represents the mean ± SD for at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control. (D) Immunostaining of differentiating P19 cells with specific anti-Gata4 antibody shows the subcellular localization of Gata4 at the day 3 and 6 of cardiac differentiation. Nuclei were counterstained with Hoechst33342.

cells (Fig. 3A). Further investigation suggested that day 1–2 of differentiation were more sensitive to inhibition of the p38 pathway (SB203580 treatment) (Fig. 3B). We treated P19 cells with 5 μM SB203580 for 4 days in suspension culture and then subjected the resultant EBs to real-time PCR to examine specific target genes. As shown in Fig. 3C, a low dose of SB203580 increased the expression of *Mesp1* and *Brachyury T*, which are specific markers of mesoderm formation and maturation. SB203580 treatment also enhanced the expression of *Gata4* and *Nkx2.5*, the two markers of cardiac precursor cells. In contrast, the background expression of cardiac contractile genes *Mhc6* and *Mhc7* was greatly repressed by SB203580. Western blots also confirmed that a low dose of SB203580 facilitated Gata4 expression, although Gata4 protein was still retained in the cytoplasm rather than in the nucleus (Fig. 3D). These results indicated that limited activation of the p38 kinase was required for the induction of mesoderm and cardiac precursors. Our results also validated that Gata4 expressed at the early stage of differentiation did not trigger downstream gene expression.

3.4. The effects of MAPK inhibition on late-stage differentiation

Since the MAPK cascade remained highly activated at the late stage of differentiation, we investigated the effects of MAPK inhibition on late-stage cardiac differentiation. As shown in Fig. 4A, the inhibitors of p38, ERK, and JNK kinases exhibited repressive effects on cardiac differentiation. Of all the MAPK inhibitors, the p38 inhibitor (10 μM) exerted the strongest repression, as evaluated by the decreased number of beating EBs and the expression of cardiac myosin heavy chain (Fig. 4A and B). The JNK inhibitor had more repressive effects than the ERK inhibitor. We also found that MAPK inhibition affected the expression of Gata4 and Nkx2.5 at both the mRNA and protein levels (Fig. 4C and D). The p38 inhibitor directly decreased the amount of Gata4 and Nkx2.5 proteins. In contrast, the JNK inhibitor preferentially decreased the protein abundance of Nkx2.5 rather than that of Gata4 (Fig. 4C). Collectively, our results revealed that the activation of the MAPK cascades, especially the p38 signaling pathway, was required for the late stage of cardiac differentiation.

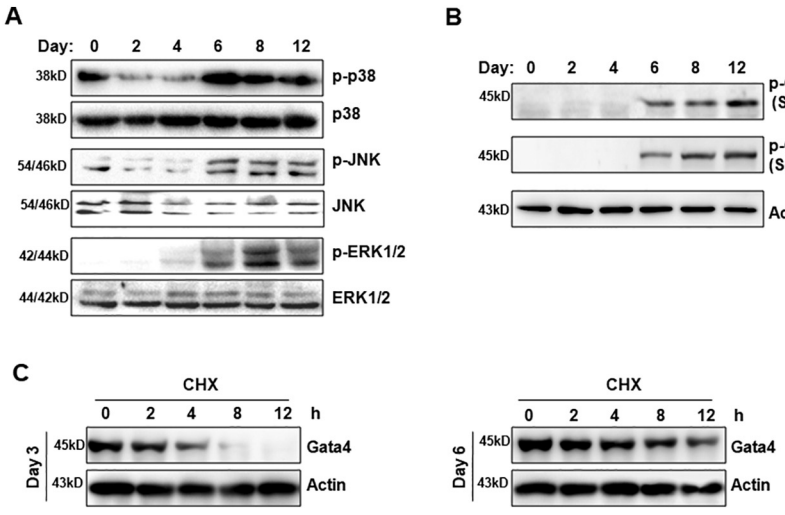

Fig. 2. The link of Gata4 activation with MAPK cascades during cardiac differentiation. (A) Representative western blot analysis shows the activation of MAPK cascades during cardiac induction. (B) The cell lysates were extracted from differentiating P19 cells at the indicated time points and subjected for western blot with antibodies to assess the phosphorylation of Gata4 at Ser105 and Ser261. (C) The protein stabilization of Gata4 expressing at the early and late stage of cardiac induction was estimated. P19 cells at the indicated time points were treated with 20 μM cycloheximide (CHX). 12 h later, cells were collected and subjected for western blot to detect Gata4 protein.

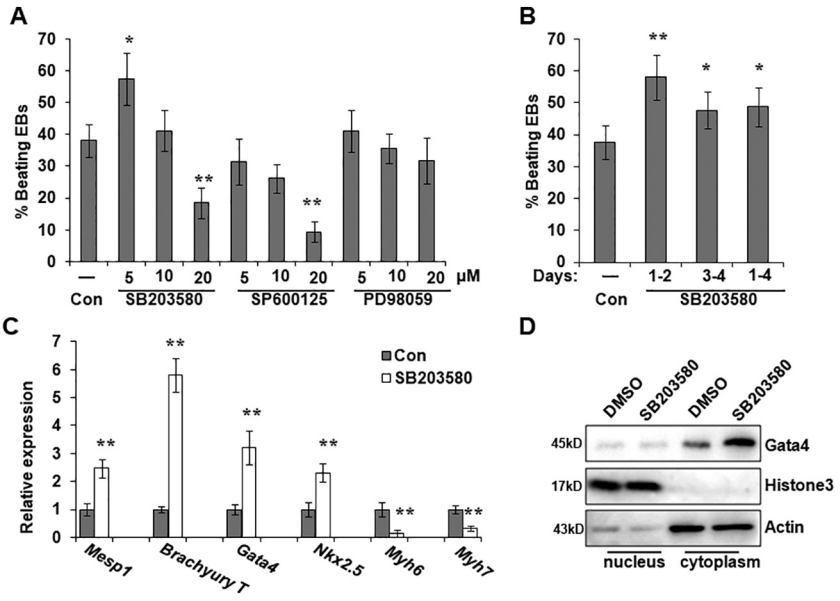

Fig. 3. MAPK inhibition affects the early-stage differentiation. (A) During the early stage (day 1–4), P19 cells were treated with different dosages of MAPK inhibitors, p38 (SB203580), ERK (PD98059), and JNK (SP600125). The number of spontaneously contracting EBs was calculated at day 12 for at least three independent experiments. (B) The effects of treatment with 5 μM SB203580 on beating cell inducing efficacy for at least three independent experiments at different periods of suspension induction on the final differentiation efficiency. (C) After treatment of 5 μM SB203580 for 4 days of suspension induction, EBs were subjected for real-time PCR to illuminate the effects on the expression of serval genes associated with developmental stages. Data shown were analysis for at least three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control. (D) P19 cells were treated with 5 μM SB203580 for 4 days of suspension induction, cytoplasmic and nuclear proteins were separated to estimate the abundance of Gata4. Histone H3 is used as an internal control for nuclear, and Actin is used as an internal control for cytoplasm.

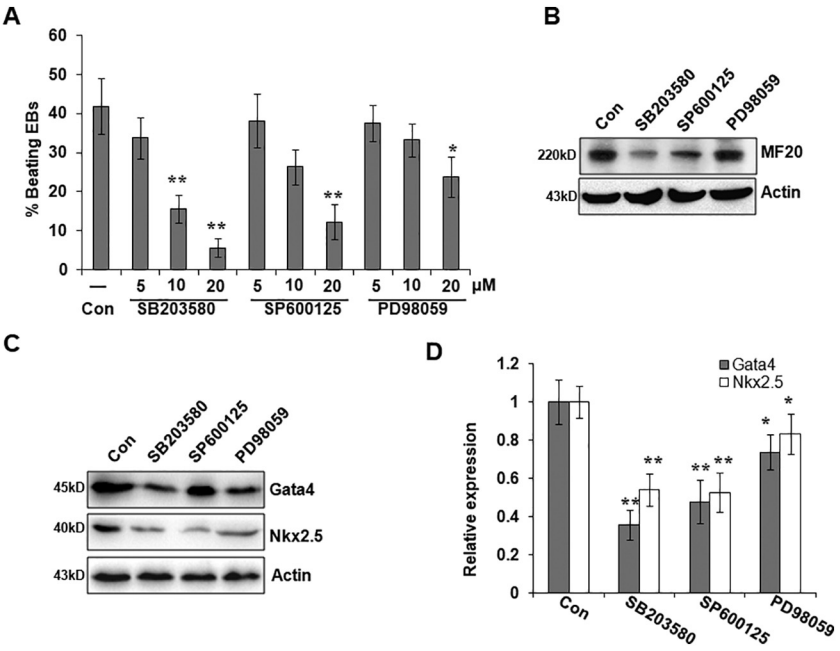

Fig. 4. MAPK inhibition affects the late-stage differentiation. (A) During the late stage (day 5–8), P19 cells were treated with different dosages of MAPK inhibitors. The number of spontaneously contracting EBs was calculated at day 12 for at least three independent experiments. (B) P19 cells were treated with 10 μM MAPK inhibitors (SB203580, SP600125, and PD98059) during days 5–8 of cardiac induction. The expression of MF20 was examined at day 12. (C, D) P19 cells were treated with 10 μM MAPK inhibitors (SB203580, SP600125, and PD98059) during days 5–8 of cardiac induction. The protein and mRNA levels of Gata4 and Nkx2.5 were examined at day 8. Values are expressed as means of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control.

3.5. The p38 cascade regulates Gata4 and Nkx2.5 activation

As reported in sections 3.1 and 3.2, Gata4 was mainly activated at the late stage of differentiation. Subsequently, we tested whether the p38 kinase affected Gata4 and Nkx2.5 activation during late-stage differentiation by examining the cellular distribution of the Gata4 and Nkx2.5 proteins in the presence or absence of the p38 inhibitor SB203580. As shown in Fig. 5A, SB203580 strongly reduced the abundance of Gata4 protein in the nucleus and mildly enhanced the cytoplasmic localization of the Nkx2.5 protein. To ascertain the effect of SB203580 on Gata4 and Nkx2.5 stabilization, P19 cells at day 6 were exposed to CHX together with SB203580 and subjected to western blotting. We found that SB203580 treatment accelerated the degradation of Gata4 and Nkx2.5 (Fig. 5B). These results indicated that p38 signaling positively modulated the activation of Gata4 and Nkx2.5 at the late stage of cardiac differentiation.

3.6. The JNK cascade regulates Nkx2.5 activation

As reported in section 3.4, we found that JNK inhibition reduced the protein levels of Nkx2.5, but not Gata4. We further examined the effects of the JNK inhibitor SP600125 on the protein stabilization of Gata4 and Nkx2.5. As shown in Fig. 6A and B, SP600125 exerted completely different effects on the protein stabilization of Gata4 and Nkx2.5. SP600125 enhanced Gata4 stabilization but conversely accelerated Nkx2.5 degradation. Taking this finding together with the JNK-inhibitor result from section 3.4, SP600125 seemed to exert dual effects on Gata4, repressing its expression but facilitating its stabilization. Meanwhile, we found that SP600125 treatment augmented the amount of Nkx2.5 protein in the cytoplasm (Fig. 6C). ChIP assays further unveiled that SP600125 treatment was detrimental to Nkx2.5 recruitment to the promoter of the downstream *Nppa* gene (Fig. 6D). In comparison, Gata4 recruitment to *Nppa* promoter was relatively insensitive to SP600125 treatment. Taken together, our results indicated that Nkx2.5 was an important target of the JNK-mediated regulation of late-stage

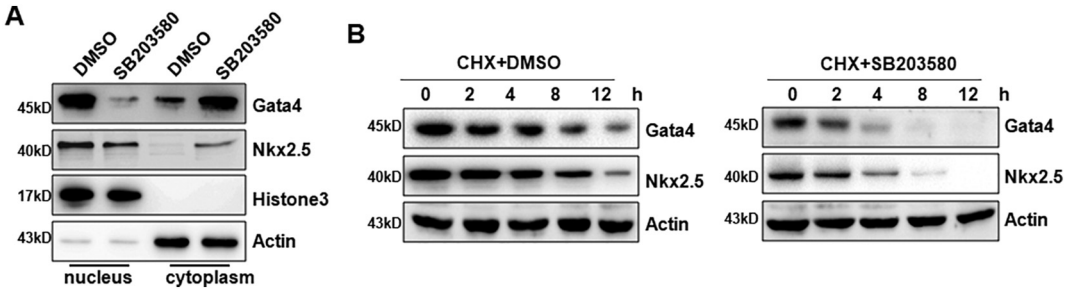

Fig. 5. p38 cascade modulates the localization and stabilization of Gata4 and Nkx2.5. (A) After P19 cells were treated with 10 μM SB203580 during days 5–8 of cardiac induction, cytoplasmic and nuclear proteins were separated to estimate the abundance of Gata4 and Nkx2.5 at day 8. (B) P19 cells were treated with 10 μM SB203580 during days 5–6. Subseqently, P19 cells were adminstrated with 20 μM CHX. 12 h later, cells were collected and subjected for western blot to detect the protein amount of Gata4 and Nkx2.5. The blots shown are representative of three independent experiments.

cardiac differentiation.

3.7. The functional interaction between JNK2 and Nkx2.5

Based on the functional association of JNK signaling and Nkx2.5, we hypothesized that these proteins might directly interact with each other. To test this, we performed Co-IP assays in H9c2 cells co-transfected with His-JNK2 and Flag-Nkx2.5 plasmids. As shown in Fig. 7A, His-JNK2 co-immunoprecipitated with Flag-Nkx2.5, implicating an interaction between these two proteins. We further questioned whether the JNK signaling pathway activated Nkx2.5 by modifying its phosphorylation status. To test this, Nkx2.5 was immunoprecipitated and the phosphorylation status of its serine residues was investigated. As shown in Fig. 7B, the phosphorylation levels of Nkx2.5 were elevated in those cells with His-JNK2 overexpression. Furthermore, JNK2 overexpression reduced the degradation of Nkx2.5 and prolonged its half-life (Fig. 7C). Collectively, these results indicated that JNK signaling could modulate Nkx2.5 activation via phosphorylation.

3.8. The C-terminal domain of Nkx2.5 affects its stabilization under oxidative stress

It is well known that the transcriptional activity of Nkx2.5 is regulated via its N-terminal activation domain and its binding activity through the homeodomain. Nkx2.5 is a transcription factor of modest function due to the inhibitory effect of its C-terminal domain [14,15]. Accordingly, a C-terminal defective Nkx2.5 mutant, Nkx202, achieves a higher transcriptional activity than the full-length Nkx2.5 (Fig. 8A). Intriguingly, His-JNK2 overexpression accelerated the degradation of Nkx202 (Fig. 8B). Moreover, Nkx202 was susceptible to degradation upon stimulation with increasing doses of H_2O_2. In parallel, the protein levels of Nkx2.5 that were initially upregulated following stimulation with mild doses of H_2O_2 subsequently declined in the presence of high-concentration H_2O_2 (Fig. 8C). Data from immunofluorescence assays indicated that Nkx202 was rapidly shuttled into the cytoplasm after H_2O_2 exposure, while full-length Nkx2.5 remained in the nucleus (Fig. 8D). Taken together, these results indicated that the C-terminal domain of Nkx2.5 is required for its stabilization under conditions of oxidative stress and JNK activation.

Next, we investigated the precise mechanisms of Nkx202 degradation under oxidative stress. As oxidative stress usually utilizes the JNK cascade to modulate cell responses and protein stabilization, we tested whether JNK inhibition could block Nkx202 degradation. As shown in Fig. 8E, JNK inhibition effectively reversed H_2O_2-induced Nkx202 degradation. Given that there are multiple methods by which the cell degrades proteins, we further tested the effects of blocking the ubiquitin-proteasome system, caspase, or autophagy pathways, to identify the specific degradation pathway responsible for clearing Nkx202. As shown in Fig. 8F, HCQ, an inhibitor of the autophagy pathway, effectively ameliorated H_2O_2-induced Nkx202 degradation. At the same time, activation of autophagy, which was indicated by accumulation of LC3 isoform II, was also ecilited when the oxidative stress was

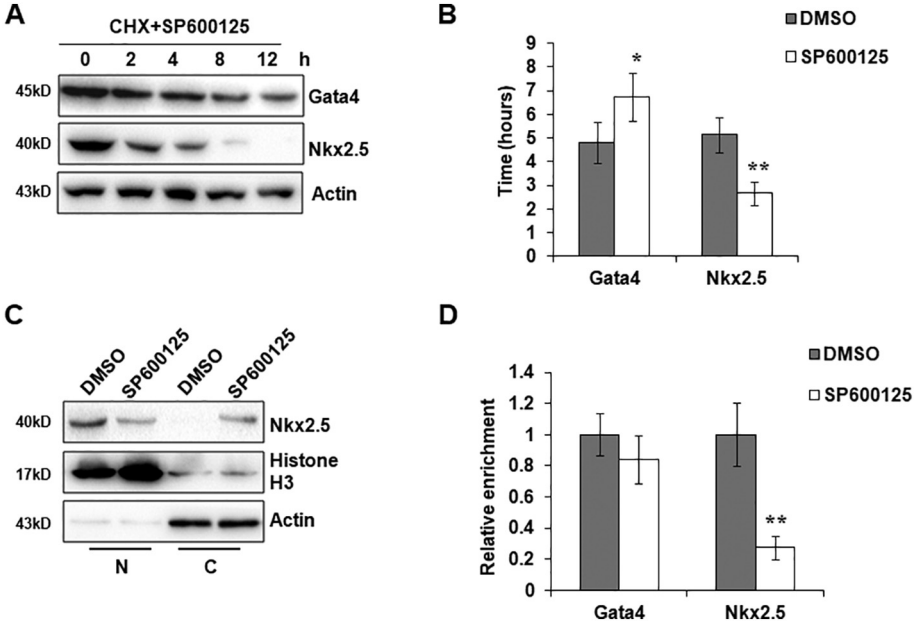

Fig. 6. JNK cascade modulates Nkx2.5 activation. (A) The effects of JNK inhibition on the stabilization of Gata4 and Nkx2.5. P19 cells were treated with 10 μM SP600125 during days 5–6. Subseqently, P19 cells were adminstrated with 20 μM CHX. 12 h later, cells were collected and subjected for western blot to detect the protein amount of Gata4 and Nkx2.5. (B) The decay curves of Gata4 and Nkx2.5 in the presence and absence of SP600125 were determined by densitometric calculation. The average values were analyzed from at least three experiments. (C) Differentiating P19 cells were treated with 10 μM SP600125 during days 5–8. Western blot analysis was used to detect the subcellular localization of Nkx2.5. Representative images were shown from three independent experiments. (D) After treatment with 10 μM SP600125 during days 5–8, P19 cells were subjected to ChIP experiments using anti-Gata4 and anti-Nkx2.5 antibodies. The immunoprecipitated DNA fragments were amplified by real-time PCR for the *Nppa* promoter containing Gata4-Nkx2.5 binding sites. Each bar represents the mean ± SD for at least three experiments. * $p < .05$, ** $p < .01$ vs the control.

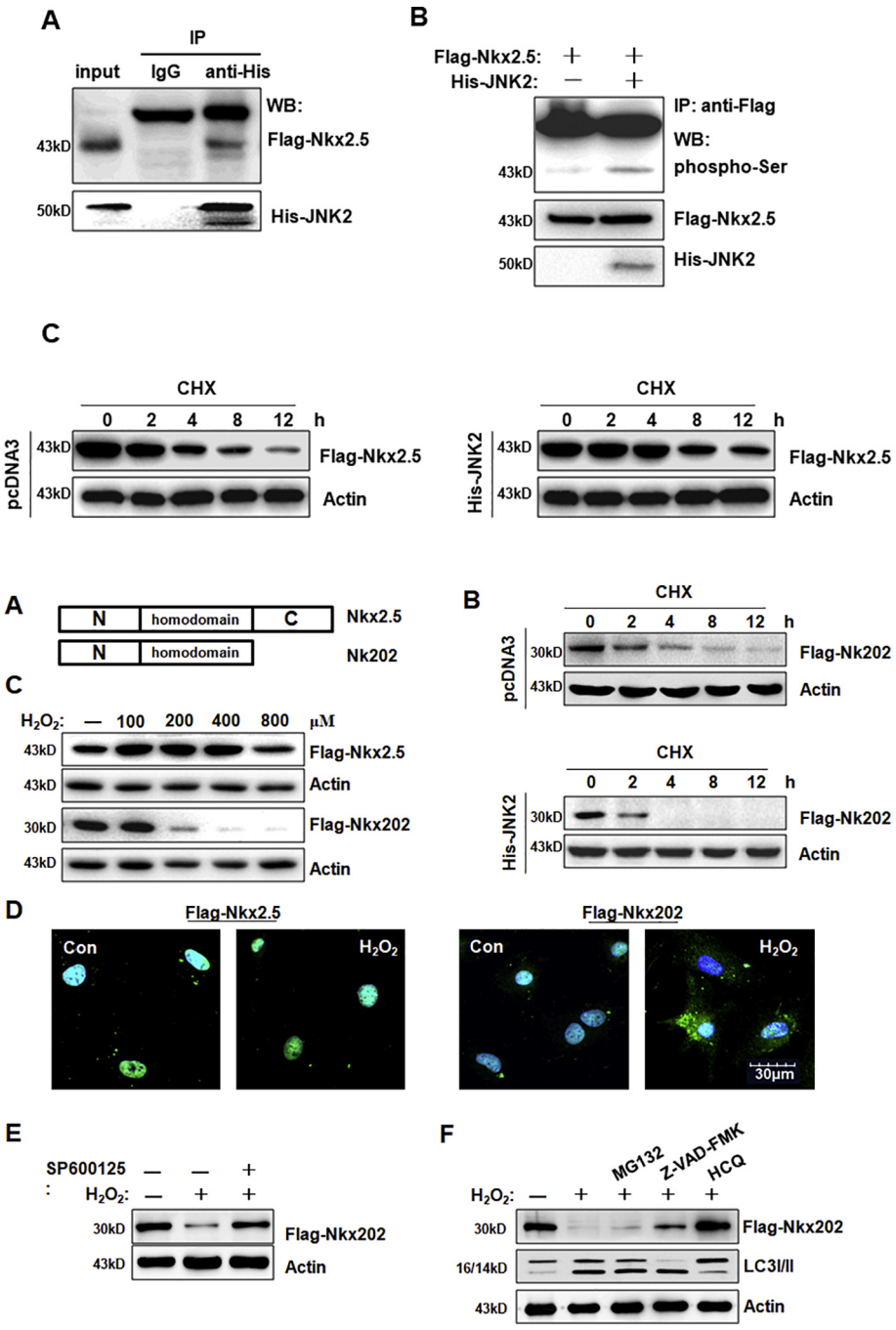

Fig. 7. JNK cascade modulates the phosphorylation and stabilization of Nkx2.5. (A) Immunoprecipitation assay shows the functional interaction of Flag-Nkx2.5 and His-JNK2. H9c2 cells were transfected with expression vectors for Flag-Nkx2.5 and His-JNK2. Cell lysates were subjected to immunoprecipitation with anti-His antibody or normal IgG as a negative control, and the precipitates were subjected to immunoblot analysis with anti-Flag antibodies, respectively. 5% of the whole cell lysate was loaded for the input control. (B) H9c2 cells were transfected with Flag-Nkx2.5 plasmid together with pcDNA3 or His-JNK2. 48 h later, Nkx2.5 protein was immunoprecipitated by ani-Flag antibody and further subjected for immunoblot analysis with antibody against phosphorylated serine. (C) 24 h later after cotransfection with Flag-Nkx2.5 and His-JNK2 plasmids, H9c2 cells were treated with 20 μM CHX for indicated periods. Western blot results showed changes of the half-life of exogenous Flag-Nkx2.5 protein. Data shown are representative images of three independent experiments.

Fig. 8. The C-terminal domain contributes to the stabilization of Nkx2.5 under oxidative stress. (A) Schematic diagram of full-length Nkx2.5 and C-terminal defective Nk202 protein structures. (B) JNK2 overexpression accelerates Nk202 degradation. 24 h later after cotransfection with Flag-Nkx202 and His-JNK2 plasmids, H9c2 cells were treated with 20 μM CHX for indicated periods. Western blot results showed changes of the half-life of exogenous Flag-Nkx202 protein. (C) Full-length Nkx2.5 exhibited more resistance to H_2O_2 stimulation than C-terminal defective Nk202. 24 h later after transfection with Flag-Nkx2.5 or Flag-Nkx202, H9c2 cells were exposed to increasing dosages of H_2O_2 for 12 h. Cell lysates were analyzed by western blot with ani-Flag antibody. (D) The localization of Nkx2.5 and Nk202 under oxidative stress. H9c2 cells were transfected with Flag-Nkx2.5 or Flag-Nkx202 plasmids. 24 h later, cells were exposed to 400 μM H_2O_2 for 4 h and then fixed for immunofluorescence analysis with anti-Flag antibody. Nuclei were counterstained with Hoechst33342. (E) JNK inhibition blocks Nk202 degradation. 24 h later after transfection with Flag-Nkx202 plasmid, H9c2 cells were exposed to 400 μM H_2O_2 for 12 h in the presence or obsence of 20 μM SP600125. (F) Autophagy inhibition alleviates Nk202 degradation. 24 h later after transfection with Flag-Nkx202 plasmid, H9c2 cells were exposed to 400 μM H_2O_2 for 12 h in the presence of 10 μM MG132, 20 μM *Z*-VAD-FMK, or 10 μM HCQ. Cell lysates were analyzed by western blot with ani-Flag and LC3 I/II antibodies. The blots shown are representative of three independent experiments.

presented (Fig. 8F). Taken together, these findings showed that oxidative stress activated the JNK cascade and induced protein degradation of the C-terminal defective Nkx202 in an autophagy-dependent manner.

4. Discussion

In vitro cardiac differentiation, recapitulating the embryonic development of the heart in vivo, is a progressive process. Stem cells undergo a stepwise, committed differentiation from pluripotent status to mesoderm to cardiac mesoderm and cardiac progenitors to functionally mature cardiomyocytes [5]. Therefore, the elaborate regulation of cardiac differentiation requires stepwise integration of transcription factors and signaling pathways [1–3]. In this study, we revealed that MAPK cascades regulated cardiac differentiation by affecting the expression and activation of the cardiac-specific transcription factors Gata4 and Nkx2.5.

It has been demonstrated that several signaling pathways exert temporal effects on cardiomyogenesis. For instance, the canonical Wnt and Notch pathways have developmental stage-specific, biphasic, opposing roles over the course of heart development. Wnt signaling favors mesoderm induction at the early stage of differentiation but imposes negative effects on late-stage cardiac differentiation after EB formation by enhancing the expression of hematopoietic/vascular genes [7]. Another finding elucidated that Wnt cooperates with Bmp signaling to establish a mesendoderm pattern by repressing Sox2. Following conversion of mesendoderm to mesoderm, inhibition of Wnt restricts the

expression of anti-cardiac regulators *Msx1* and *Cdx2/1*, which is a prerequisite for cardiac specification [6]. A previous study showed that Wnt and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells; the expression of *Isl1*, *Nkx2.5*, and *Baf60c* were controlled by Wnt cascade, while Bmp signaling preferentially modulated *Gata4*, *SRF*, and *Mef2c* [16]. Diverse types of Wnt signaling inhibitors and activators have been confirmed to accelerate cardiomyogenesis at specific phases [17,18].

In this study, we used a two-step protocol to induce cardiac differentiation in P19 embryonal carcinoma cells. During early-stage differentiation, P19 cells were induced in suspension culture to form EB-like cell aggregates and differentiate into cardiac progenitors, as verified by the emergence of *Gata4* and *Nkx2.5* gene expression. Our results indicated that these cardiac transcription factors expressed at the early stage were largely inactive in transcription function. We observed that on day 3 of cardiac induction, the Gata4 protein was localized to the cytoplasm rather than the nucleus. Meanwhile, the phosphorylation of the Gata4 protein at defined serine residues was relatively lower, which made it more susceptible to degradation. Gata4 at the early stage of differentiation was scarcely recruited onto the promoters of its downstream target genes. In contrast, the Gata4 protein expressed at the late stage of differentiation showed markedly different properties and participated in the transcriptional activation of mature cardiac genes. Therefore, we speculated that the transactivation of cardiac-specific transcription factors Gata4 and Nkx2.5 took place in the early stage of differentiation, while the transactivation of mature cardiac genes took place at the late stage.

In a co-culture preparation with neonatal rat cardiomyocytes, mesenchymal stem cells were induced to obtain a cardiac phenotype. During the early period of cardiac specification, cells showed a diffuse pattern of Gata4 and Nkx2.5 protein expression in the cytoplasm, and there was no expression of cardiac antigens. After long-term co-culture, the Gata4 and Nkx2.5 transcription factors accumulated in the nucleus, and these cells also expressed the terminal cardiac differentiation marker [19]. Coincident with this previous finding in mesenchymal stem cells, our results indicated that differentiation of stem cells toward a cardiac fate is preceded by the activation and nuclear translocation of the Gata4 and Nkx2.5 transcription factors. Gata4 expressed in the early stage of differentiation was remained in a quiescent condition and it was insufficient to activate downstream genes. We further postulated that specific signals can trigger the conversion of Gata4 from inactive to active status, thereby evoking late-stage differentiation.

Our results illustrated that MAPK cascades contributed to the activation of Gata4 and Nkx2.5 at the late stage of cardiac differentiation in P19 cells. The mammalian MAPK family includes three major members: ERK, p38, and JNK. The ERK cascade is described as a general regulator of cell growth and differentiation in response to mitogenic stimuli, such as growth factors, peptide hormones, and neurotransmitters. JNK and p38 kinases have generally been identified as stress kinases responsive to diverse stressors, such as inflammatory cytokines, oxidative stress, radiation, and osmotic stress. MAPK cascades are involved in numerous biological events, including cell growth, cell survival, cell differentiation, and migration. Several studies have reported that MAPK cascades played important roles in cardiac differentiation of embryonic stem cells, induced pluripotent stem cells, and embryonal carcinoma cells. p38, ERK, JNK cascades exert different effects on the stepwise process of cardiomyogenesis [10,11]. Specifically, several lines of evidence have suggested that the p38 cascade is a crucial signal during cardiomyogenesis [20–26].

Our results combined with a previous study suggested that the activation pattern of the three major MAPK pathway members in P19 cells is coincident with the early and late stages of cardiac differentiation [22]. During early-stage differentiation, MAPK cascades are inactive, with complete activation of p38, ERK, and JNK kinases only being observed at the late stage of cardiac differentiation. In fact, the effect of p38 signaling at the early stage of differentiation is still highly controversial. Multiple studies demonstrated that p38 activity controlled the switch of embryonic stem cells to either cardiomyogenesis or neurogenesis [20,23–25]. The *p38α*-deficient embryonic stem cells spontaneously differentiated into neurons, and cardiomyogenesis was blocked. Paradoxically, there are also opposite evidences showing that a p38 inhibitor accelerated directed cardiac differentiation in a developmental stage-specific and dose-associated manner [10,21,26]. In line with this last finding, our results showed that the limited activation of the p38 cascade at the early stage of differentiation facilitated the commitment of cells to mesoderm and cardiac mesoderm status. Consistent with the studies in human embryonic stem cells, our results from P19 cells also revealed that the effects of SB203580 (p38 inhibitor) on cardiac differentiation were dose-dependent; a low dose of SB203580 enhanced cardiomyogenesis, whereas a high dose did the opposite. Importantly, the low dose of SB203580 effectively increased the expression of *Gata4* and *Nkx2.5*, while it reduced the background expression of the mature cardiac genes *Mhc6* and *Mhc7*. Moreover, this low dose of SB20580 upregulated the Gata4 protein but sequestered it in the cytoplasm. These data also validated our hypothesis that cardiac transcription factors expressed at the early stage of differentiation are insufficient to activate the expression of cardiac contractile genes.

Previous studies have shown that the p38γ signal modulated Nkx2.5 phosphorylation and nuclear translocation [27]. In addition, p38-mediated Gata4 phosphorylation was shown to be involved in inducible cardiac gene expression [13,28]. In line with these findings, our data showed that the p38 cascade played a critical role in the activation of Gata4 and Nkx2.5 at the late stage of cardiac differentiation. Specifically, SB203580 treatment during adherent culture directly decreased the levels of Gata4 and Nkx2.5 protein. SB203580 also reduced the accumulation of Gata4 and Nkx2.5 in the nucleus to different extents. Furthermore, SB203580 treatment accelerated the degradation of Gata4 and Nkx2.5, indicating a role of p38 signaling in regulating Gata4 and Nkx2.5 stabilization. Taken together, our results indicated that p38 signaling positively modulated the activation of Gata4 and Nkx2.5 at the late stage of cardiac differentiation.

We also found that the JNK cascade regulated Gata4 and Nkx2.5 in different ways. A previous study from our research group proposed a positive role of the JNK cascade at the early stage of differentiation via caspase-mediated depletion of the transcription factor Nanog [29]. In our current study, we revealed the detailed roles of the JNK cascade on Gata4 and Nkx2.5 activation at the late stage of cardiac differentiation. Compared to Gata4, Nkx2.5 is a major downstream target of the JNK signaling pathway to promote late-stage cardiac differentiation. JNK decreased Gata4 stabilization, while it alleviated Nkx2.5 degradation by phosphorylating Nkx2.5. Nkx2.5 maintained protein stabilization under the stimulation of elevating concentrations of H_2O_2. Nkx2.5 degradation after treatment with high-concentrations of H_2O_2 could attribute to the indirect effects of JNK activation.

It is well known that the cardiac transcription factors Gata4 and Nkx2.5 are mutual cofactors that act via physical interaction [30,31]. The transcriptional machinery of the Gata4-Nkx2.5 complex plays a vital role in the cardiac gene program associated with embryonic development and postnatal hypertrophy. Additionally, the anti-apoptotic activity of Gata4 and Nkx2.5 was shown to be required for the homeostasis and survival of cardiomyocytes in the adult heart [32,33]. In this study, we revealed the differences between Gata4 and Nkx2.5 in terms of their response to JNK signaling. Our data hints that Gata4 and Nkx2.5 can play distinct cellular roles under different physiological and pathological circumstances. Moreover, we showed that the C-terminal domain of Nkx2.5 was indispensable for its stabilization under conditions of oxidative stress and JNK activation. Although the C-terminus of Nkx2.5 is recognized as an inhibitory domain for the protein's transcription function, our results highlighted a novel function of this domain of Nkx2.5.

Our results suggested that the the early and late-stage differentiation triggers the expression and activation of cardiac specific

transcription factors, respectively. This process is tightly linked with the status of MAKP cascade. Why MAPK cascade is mainly activated at the late stage of cardiac induction. There are several possible mechanisms. Firstly, the upstream molecules might determine the stepwise activation of MAPK cascade. For instance, a pioneering study showed that a constitutively activation in embryonic stem cells of GTPase Rac1, a molecular switch transducing intracellular signals from growth factors and activating p38 signal, severely compromised cardiac cell differentiation [34]. Whereas its temporal expression in cardiac progenitors improved the propensity of differentiating into beating cardiomyocytes. Another precedent study suggested that ErbB4 receptor signal contributes to the activation of p38γ during cardiomyogenesis [27]. Secondly, suspension and adherent cultures have great impacts on cell adhesion, communication, and motility, which can converge to modulate MAPK cascade. It was well interpreted that EB adhesion before the occurence of cardiac progenitors expressing *Nkx2.5* and *Tbx5* greatly inhibits cardiac differentiation [35]. However, the precise mechanisms remain to be clarified.

5. Conclusions

Our results provide evidence that the sequential activation of MAPK pathways contributes to the stepwise process of cardiac differentiation. Moderate inhibition of p38 signal at the early stage of cardiac induction facilitates the expression of cardiac transcription factors Gata4 and Nkx2.5. The activation of p38 signal at the late stage is required for cardiac transcription factors to trigger the expression of cardiac structural and contractile genes. JNK kinase prefers to activate Nkx2.5 rather than Gata4 to facilitate the late-stage cardiac differentiation. JNK signal enhances the protein stabilization of full-length Nkx2.5, but not the C-terminal defective Nkx2.5 mutant.

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Competing interests

The authors declare no conflicts of interest.

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