Biochimica et Biophysica Acta 1793 (2009) 300–311

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

WNT signaling promotes *Nkx2.5* expression and early cardiomyogenesis via downregulation of *Hdac1*

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ARTICLE INFO

Article history:
Received 1 April 2008
Received in revised form 17 August 2008
Accepted 28 August 2008
Available online 11 September 2008

Keywords:
P19CL6 cell
Cardiomyogenesis
Epigenetic
Nkx2.5
HDAC1
WNT

ABSTRACT

The cardiac transcription factor NKX2.5 plays a crucial role in cardiomyogenesis, but its mechanism of regulation is still unclear. Recently, epigenetic regulation has become increasingly recognized as important in differentiation and development. In this study, we used P19CL6 cells to investigate the regulation of *Nkx2.5* expression by methylation and acetylation during cardiomyocyte differentiation. During the early stage of differentiation, *Nkx2.5* expression was upregulated, but the methylation status of the *Nkx2.5* promoter did not undergo significant change; while the acetylation levels of histones H3 and H4 were increased, accompanied by a significant reduction in *Hdac1* expression. Suppression of *Hdac1* activity stimulated cardiac differentiation accompanied by increased expression of cardiac-specific genes and cell cycle arrest. Overexpression of *Hdac1* inhibited cardiomyocyte formation and downregulated the expressions of *Gata4* and *Nkx2.5*. Mimicking induction of the WNT pathway inhibited *Hdac1* expression with upregulated *Nkx2.5* expression. WNT3a and WNT3 downregulated the expression of *Hdac1*, contrary to the effect of SFRP2 and GSK3β. Cotransfection of β-*catenin* and *Lef1* significantly downregulated the expression of *Hdac1*. Our data suggest that WNT signaling pathway plays important roles in the regulation of *Hdac1* during the early stage of cardiomyocyte differentiation and that the downregulation of *Hdac1* promotes cardiac differentiation.

1. Introduction

Early cardiomyocyte differentiation involves three stages: the precardioblast, the cardioblast and the cardiomyocyte stage [1]. Cardiac lineage commitment occurs between the first and second stages upon expression of cardiac transcription factors such as NKX2.5, GATA4, MEF2C, and dHAND, in which NKX2.5 plays a central role in both embryo heart development and stem cell cardiomyogenesis [2]. Targeted disruption of murine *Nkx2.5* resulted in embryonic lethality due to arrested looping morphogenesis of the heart tube and decreased expression of a subset of cardiac muscle-specific genes [3]. Overexpression of *Nkx2.5* can initiate cardiac differentiation in ES cells aggregated in the absence of DMSO [4]. GATA4, bone morphogenetic proteins (BMP), fibroblast growth factors (FGF) and the WNT signaling pathway have all been documented to regulate the expression of *Nkx2.5*. However, the precise molecular pathways by which cardiac regulators act on *Nkx2.5* and coordinate cardiac differentiation is still largely unknown [5].

Epigenetic regulation has recently become important in development and differentiation research [6]. At times of genomic reprogramming, considerable dynamic changes in chromatin architecture, especially DNA methylation and histone acetylation, are closely linked to differentiation and development in mammals [7,8]. Cardiomyogenesis is a complex process associated with dramatic changes in epigenetic modification and regulation of some cardiac genes by methylation and acetylation [9]. However, methylation levels are considerably lower in early embryo development and at times of genomic reprogramming [10]. As DNA methylation of a gene promoter represses transcription and recruits other repressive chromatin-modifying activities, reprogramming might hypomethylate a specific gene promoter, initiating gene expression during these stages of development [11]. This suggests that *Nkx2.5*, as a CpG island-rich gene, can potentially be regulated by DNA methylation or histone acetylation as both modifications occur together in regulating gene expression.

Histone acetyl transferases (HATs) and histone deacetylases (HDACs) loosen or compact chromatin structure, respectively, and their cooperation establishes and maintains specific patterns of histone acetylation for global gene expression [12]. In mammalian cells there are three classes of HDACs, class I, II and III, in which class I HDACs comprise HDAC1, 2, 3 and 8 [13]. In addition to regulating histone acetylation, HDAC1 can itself regulate cell proliferation and differentiation in embryonic development, as global deletion of *Hdac1* in conditional null *Hdac1* mice resulted in death by embryonic day 9.5.

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doi:10.1016/j.bbamcr.2008.08.013

The cardiac-specific deletions of the *Hdac1* and *Hdac2* genes resulted in neonatal lethality, accompanied by cardiac arrhythmias, dilated cardiomyopathy and upregulation of genes encoding skeletal muscle-specific contractile proteins and calcium channels [14]. Despite growing knowledge of the mechanisms of HDAC-dependent regulation in embryo development, very little is known about the role of HDAC1 in cardiomyogenesis.

The activities of HDACs are tightly controlled and precisely regulated by multiple mechanisms. The activities of most HDACs are regulated by protein–protein interactions, post-translational modifications as well as subcellular localization. Less studied, but perhaps equally important, is the regulation of some HDACs by control of transcriptional expression [15], especially in cardiac development. Of the three most important signaling pathways for cardiomyogenesis (BMP, FGF and WNT), WNT may play a role in the regulation of HDAC1 expression. WNT signals exhibit developmental stage-specific and biphasic effects on cardiomyogenesis and hematopoiesis [16–18]. One study, revealing a balance between WNT signaling and HDAC activity that correlated with the ratio of proliferation to differentiation, suggested that there is a potential relationship between the WNT/β-catenin pathway and HDAC1 expression [19]. A complete understanding of how HDACs are regulated will contribute not only to our knowledge of chromatin structure and gene expression control, but offer useful insight into approaches for developing therapeutic methods through HDAC modulation. In this study, we used mouse embryonic carcinoma stem cells, P19CL6 cells, to examine the mechanism of HDAC1 function and regulation during the differentiation of P19CL6 cells into cardiomyocytes.

2. Materials and methods

2.1. Cell culture and induction of differentiation

P19CL6 cells were cultured as described previously. In brief, the cells were grown in a 60-mm tissue culture grade dish with marked grids (Nunclon® Delta, Denmark) under adherent conditions with α-minimal essential medium (α-MEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) (growth medium) and were maintained in a 5% CO_2 atmosphere at 37 °C. To induce differentiation under adherent conditions, P19CL6 cells were plated at a density of 3.7×10^5 in a 60-mm tissue culture grade dish with the growth medium containing 1% DMSO (differentiation medium). The medium was changed every 2 days. Days of differentiation were numbered consecutively after the first day of the DMSO treatment, day 0. The number of spontaneously beating cells was counted using a DMIL microscope (Leica, Deerfield, IL) fitted with a grid system. Stable P19CL6 cell lines expressing *Hdac1* were generated using Lipofectamine2000 (Invitrogen). The cells were then plated and grown in medium under the selection of G418 (400 μg/ml, Sigma). After 2–3 weeks selection, G418 resistant colonies were visible. Five colonies were isolated for further culture and analysis.

2.2. RT-PCR and real-time RT-PCR

To extract total RNA from P19CL6 cells, Trizol Reagent (Invitrogen) was used after the cells were washed three times by ice-cold PBS. An equal amount of total RNA (4 μg) was used for first-strand cDNA synthesis using oligo-dT primer and 0.25 U/μl M-myeloblastosis virus reverse transcriptase XL (Promega) in a reaction volume of 25 μl according to the manufacturer's instructions. Synthesized first-strand cDNA (1 μl) was used for each PCR reaction. For RT-PCR, Taq DNA polymerase (Promega) and GeneAmp PCR System 9700 (Applied Biosystems) were utilized. The following thermal profile was used for all PCR experiments: 95 °C for 5 min, and then appropriate cycles at 94 °C for 30 s, annealing temperature (Table 1) for 30 s and 72 °C for 30 s, and terminated by a final extension at 72 °C for 8 min. The amplified PCR products were separated in 2% agarose gel and visualized under a 302 nm UV light. The figures shown are representative of multiple independent experiments ($n\geq3$). The gels are scanned using Image-Quant software (Kodak 1D V3.53) and semi-quantitated with Multi Gauge V3.0 (Fujifilm). Each value presents the average of at least 3 independent experiments. In real-time RT-PCR experiments, SYBR® Green Real-time Master Mix (TOYOBO) was utilized. The same thermal profile was used as for RT-PCR, and the PCR products were subjected to a melting curve analysis and to conventional agarose electrophoresis to exclude synthesis of nonspecific products. To quantify each experiment, a Ct value was quantified using a standard curve for the specific gene and relatively quantified using 18s RNA as an internal reference control. Finally, it was normalized to the average expression levels of the undifferentiated samples, and the normalized ratios were used to indicate up- and downregulation.

Table 1
Primers used in RT-PCR and real-time PCR

Primers	Primer sequences (5′–3′)	Product size(bp)
Hdac1	F: GGGCACCAAGAGGAAAGT	332
	R: CTCCCGTGGACAACTGA	
Nkx2-5	F: CACCCACGCCTTTCTCAGTC	361
	R: CCATCCGTCTCGGCTTTGT	
Gata4	F: AAGGTACCTGGACTTTGCCTGTTGGGG	230
	R: CTAAGCTTACTGCGGCTGAGCCTCGG	
Anf	F: CCATCACCCTGGGCTTCT	461
	R: TTTCCTCCTTGGCTGTTATCTT	
Oct4	F: ATCACTCACATCGCCAATCA	126
	R: TGTAGCCTCATACTCTTCTCGTT	
Id1	F: CCAGAACCGCAAAGTGAG	200
	R: GATCGTCGGCTGGAACAC	
18SRNA	F: GTAACCCGTTGAACCCCATT	151
	R: CCATCCAATCGGTAGTAGCG	
Gapdh	R: ATCTTCCAGGAGCGAGACC	392
	F: CCTTCCACAATGCCAAA	
AR1	F: CAGTCTTGGGAGCTCAAGACTAACC	255
	R: CAGATCCCCAAGCTTACTAGCAACTAC	
AR2	F: CTGCTCATCCATCAGCCAGACGAAGA	357
	R: GAAAGATAAGCTGCAACTATCACCCGG	
AR3	F: CTGGGTCCTAATGCGGGTGGCGTCTC	246
	R: AACCCTCTGCTGTGTGGCCTTGTATCT	
Nkx2.5 promoter	F:AATGGTACCAGACTGGGTAAGTAGCCTTGA	818
	R: TAATTCGAACTGCCTTGTGCTCTGAAA	

2.3. Western blotting

Total cellular protein and nucleic protein were prepared as previously described [20]. Protein concentration was determined using the BCA Protein Assay kit (Pierce). Proteins (30 μg) were subjected to 10%–12% SDS polyacrylamide gel electrophoresis and subsequently transferred onto Hybond ECL membranes (Amersham). After washing with 0.1% TBS-T, membranes were incubated for 1 h at room temperature in blocking buffer (5% skimmed milk in TBS-T) and then incubated with appropriate antibodies (1:500 dilution; Histone H3 histone H4, histone H2b, acetylated histone H3, acetylated histone H4 and acetylated histone H2b antibodies are from Upstate; HDAC1, 2, 4, 5 antibodies are from SAB; others are from Santa Cruz) overnight at 4 °C. After washing with TBS-T, membranes were reacted with horseradish-peroxidase-conjugated anti-rabbit antibody (1:2000 dilution, Santa Cruz) for 2 h at room temperature. After further washing with TBS-T, detection was performed using ECL Western blotting detection reagents (Amersham) and Hyperfilm ECL (Amersham).

2.4. Luciferase assays

P19CL6 cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum. All transfections were performed in twelve-well plates using Lipofectamine2000

(Invitrogen) according to the manufacturer's protocol. The total amount of DNA was kept constant using pcDNA3.1/β-gal plasmid DNA. The pRSVluc plasmid (Promega) cotransfected with the LUC reporter plasmid was used as an internal control. Luciferase activity was measured in a microplate luminometer (FOLAR star reporter assay system, BMG), normalized to *Renilla* luciferase activity. Fold activation represents a comparison of the ratio of firefly/*Renilla* luciferase activity for each condition with that of the reporter vector control which was arbitrarily set at 1. Each value presented is the average of triplicate samples and is representative of multiple independent experiments ($n \geq 3$). The data were statistically analyzed using the unpaired, two-tailed Student's *t*-test, and differences were considered significant when $p < 0.05$.

2.5. Plasmids construction and transfection

The plasmid construct ANF-luc was a kind gift from Dr. Mona Nemer (Institut de Recherches Cliniques de Montreal, Montreal). *Nkx2.5* plasmid was obtained from Dr. Ilona S. Skerjanc (University of Western Ontario, Canada). AR1, AR2 and AR3 active regions were generated by PCR and cloned into pGEM®-T Easy Vector (Promega) for sequencing. DNA sequences encoding NKX2.5 N (AAs 1–121), H (AAs 122–202) and C (AAs 203–318) were generated by PCR and cloned into pGEX 4T-2 or pGEX 5X-1, for the GST pull-down experiment. Wild type *GSK-3*β and mutant *S9A GSK-3*β were kindly provided by Dr. Thilo Hagen (University of Nottingham, United Kingdom). *Id1* plasmid was a gift from Dr. Peter Lengyel (Department of Molecular Biophysics and Biochemistry, Yale University). *Wnt3*, *Wnt3a* and constitutively active β-*catenin* plasmids were kindly provided by Dr. Roel Nusse (Stanford University School of Medicine). pCG-LEF1-HA plasmid was a kind gift from Dr. Klaus Wolff (Department of Experimental Dermatology at the University of Vienna). *sFrp2* plasmid was kindly provided by Dr. Chingjin Chang (Institute of Biological Chemistry, Academia Sinica, Taiwan). *Hdac1* plasmid was a kind gift from Dr. Yongfeng Shang (Department of Biochemistry and Molecular Biology, Peking University Health Science Centre). *Oct4* plasmid was kindly provided by Dr. Changsheng Lin (Stem Cell Research Centre, Peking University Health Science Centre).

Gene overexpression and knockdown were carried out generally by transfection of the plasmids or siRNA using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. After 48 h transfection, the cells were harvest for functional assays. All transfections were performed at least 3 times.

2.6. Immunofluorescence

Cultured cells on coverslips were induced for 12 days by DMSO and then fixed with 4% paraformaldehyde for 10 min at room temperature. Following rinsing with PBS, cells were permeabilized with 0.3% Triton-X 100 (Sigma) for 15 min and then blocked with normal goat serum for 30 min. In order to detect if cells differentiated into cardiomyocytes, cells were incubated overnight at 4 °C with primary antibodies with mouse monoclonal antibodies against α-actinin (1:200, Sigma). After rinsing with PBS, cells were incubated with TRITC-conjugated goat anti-mouse IgG (1:200) for 30 min at room temperature and examined under fluorescence microscopy. Nuclei were counterstained with Hoechst 33342 (Sigma).

2.7. Flow cytometry analysis

Cell cycle analysis was performed by flow cytometry (FACS). Briefly, cultured cells were trypsinized into single cell suspensions and fixed with 70% ethanol for 30 min on ice. RNA was degraded by incubation with 100 g/ml RNase (Sigma) for 1 h at 37 °C. DNA was labeled with 20 g/ml propidium iodide (PI, Sigma) and DNA content was assessed by FACS Calibur (Becton Dickinson) using the ModiFit LT v2.0 software.

2.8. DNA methylation analysis

Genomic DNA was isolated with the Genomic DNA isolation Kit (Beyotime) and bisulphite treatment was carried out as described before [11]. Briefly, 2 μg of DNA was diluted into 50 μl with TE buffer (pH 8.0), then denaturated for 10 min at 37 °C with 5.5 μl of 2 M NaOH. 55 μl of freshly prepared hydroxyquinone (10 mM, Sigma) and 520 μl of freshly prepared sodium bisulphite (40.5%, pH 5, Sigma) were added, mixed, and incubated under mineral oil at 50 °C for 16 h. Modified DNA was purified using DNA purification resin according to the manufacturer's instruction (Qiagen) and eluted into 100 μl of water. Modification was completed by NaOH treatment (final concentration, 0.3 M) for 10 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20 °C. Modified DNA was amplified with Platinum® *Taq*DNA Polymerase (Invitrogen). The PCR fragments amplified were gel-purified and cloned using pGEM-T easy vector system (Promega). Five clones were sequenced to assess the level of methylation at each CpG site.

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed according to the method described previously [20]. In brief, crude nuclei of induced P19CL6 cells were prepared and the lysates were immunoprecipitated with nonspecific rabbit IgG, anti-acetylated-H4 and HDAC1 antibodies for 12 h at 4 °C. Immune complexes were incubated with Protein A-Sepharose CL-4B (Amersham Biosciences) for 2 h at 4 °C; the washed immune complexes containing DNA were then eluted and the precipitated DNA amplified by PCR with the primers listed in Table 1. The products were resolved on 2% gels.

2.10. Immunoprecipitation and GST pull-down assays

Immunoprecipitations were performed according to the manufacturer's instructions (Roche). The lysates derived from HeLa cells cotransfected with Flag-Hdac1 and Nkx2.5 were incubated with anti-FLAG antibodies (Sigma) or normal IgG for 2 h by a gentle rotation, and then with 50 μl of protein G-sepharose slurry (Roche) at 4 °C for 1 h. Immunoprecipitates were washed three times with the lysis buffer and subjected to SDS-PAGE electrophoresis, then detected with anti-NKX2.5 (sc-14033, Santa Cruz) antibody. GST-NKX2.5 protein was coupled to glutathione-Sepharose beads (Pharmacia) and then incubated with cell lysates extracted from HeLa cells overexpressing HDAC1 in binding buffer. After four washes with GST binding buffer, beads were boiled in SDS sample buffer to elute bound protein which was subsequently resolved by SDS-PAGE electrophoresis and analyzed by western blotting using anti-HDAC1 antibodies.

2.11. Statistic analysis

The data were expressed as means ± S.D. Comparisons between groups were analyzed by Student's *t*-test or ANOVA. The significance was analyzed with SPSS10.0 software and a p-value < 0.05 was considered to be statistic significant.

3. Results

3.1. Nkx2.5 gene expression was upregulated during cardiomyogenesis but no significant change in methylation status was observed in the promoter region

Nkx2.5 was originally identified as one of the marker genes for cardiac differentiation. *Nkx2.5* gene is first expressed in the mouse embryo at 7.5 dpc (days postcoitum, dpc), reaches a high level of expression during the whole development process, and maintains a

certain level in the adult heart [21]. As a first step in investigating the regulation of *Nkx2.5* gene expression, we analyzed the expression level of *Nkx2.5* during P19CL6 cell differentiation. Semi-quantitative RT-PCR and real-time RT-PCR analysis with RNA extracted from P19CL6 cells treated with DMSO showed a gradual increase in *Nkx2.5* expression with time (Fig. 1A and B). The expression of *Gata4*, another cardiac developmental marker gene and a co-activator of *Nkx2.5*, was upregulated at the same time; α-MHC, a marker for cardiomyocytes, was expressed at day 4 after DMSO induction and exhibited an increase coinciding with the upregulation of *Nkx2.5* and *Gata4* (Fig. 1A). Subsequently, we examined the relationship between upregulated *Nkx2.5* expression and DNA demethylation in the promoter region of *Nkx2.5*. The mouse *Nkx2.5* upstream regulatory region contains multiple enhancers and repressors orchestrating spatio-temporal expression in distinct populations of cardiomyocytes during embryonic development. This gene has three transcriptional

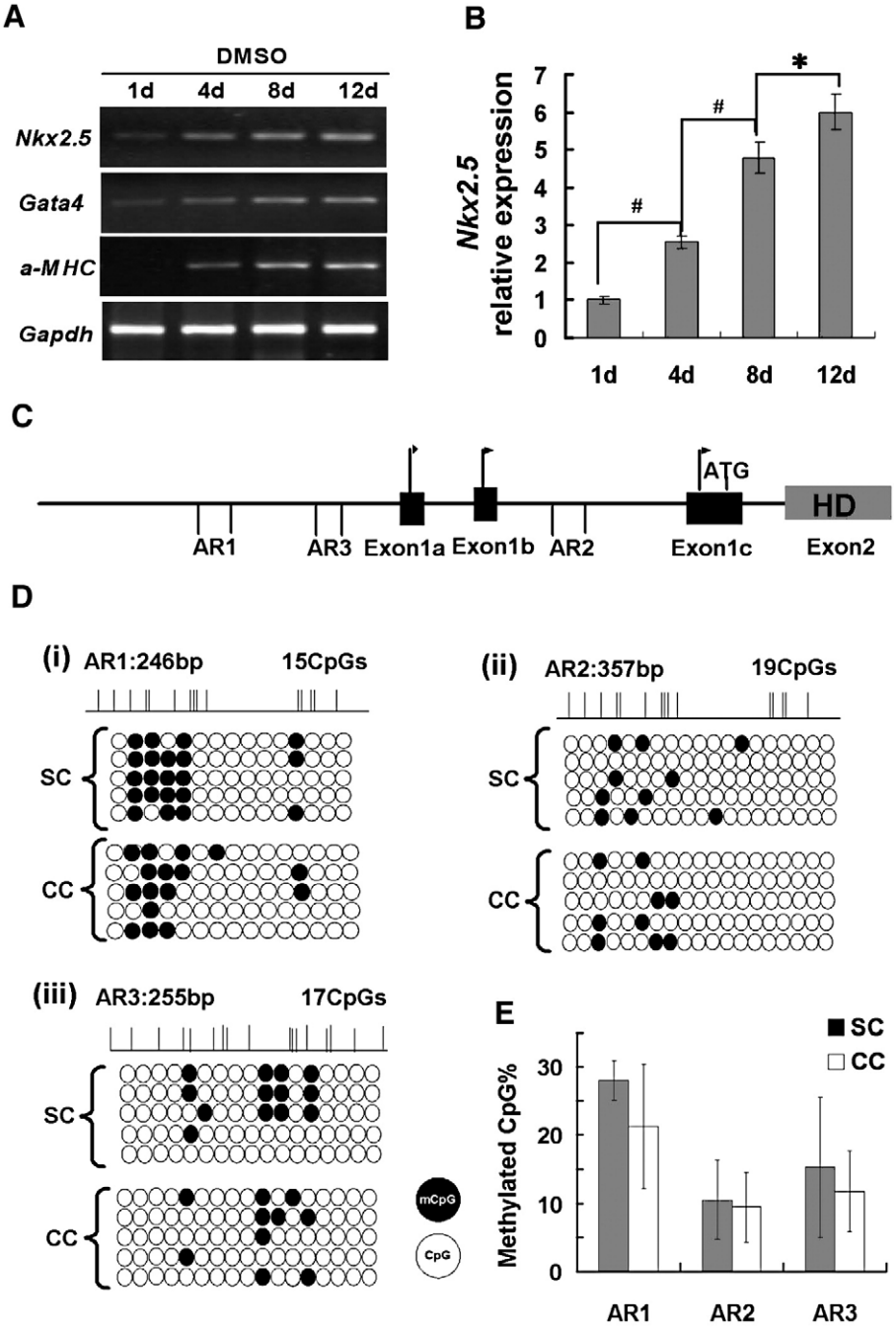

Fig. 1. The expression of *Nkx2.5* increased but activating regions of *Nkx2.5* gene underwent no significant CpG site methylation change during P19CL6 cells differentiation. (A) RT-PCR demonstrating expression of *Nkx2.5*, *Gata4*, and α-*MHC* at days 1, 4, 8 and 12 post-induction (a representative image of at least 3 experiments). (B) Real-time PCR data confirmed the semi-quantitative RT-PCR results (the data represent the average of 3 independent experiments, each experiment was performed in triplicates, $^{*}P<0.05$, $^{\#}P<0.01$). (C) The *Nkx2.5* gene structure showing three promoters and one ATG site, and the three CpG island-rich sections examined of the activating regions, AR1 (−9436U, −7363D), AR2 (−3050U, −1976D) and AR3 (−5870U, −4059D) respectively. (D) 246 bp AR1 (i), 357 bp AR2 (ii) and 255 bp AR3 (iii) regions of *Nkx2.5* gene in uninduced P19CL6 cells and differentiated cardiomyocytes were cloned into the pGL3-basic plasmid respectively for bisulphite sequencing. The positions of CpG sites in each region are labeled with a short erect line in the consensus sequence above the chromatograms. SC: uninduced P19CL6 cells; CC: cardiomyocytes. Methylated (solid dot) and unmethylated (hollow dot) CpG sites were determined by bisulphite sequencing. (E) The percentage of methylated CpG sites was not significantly different between groups ($P>0.05$ SC versus CC in each AR group; bars represent SD; $n=5$).

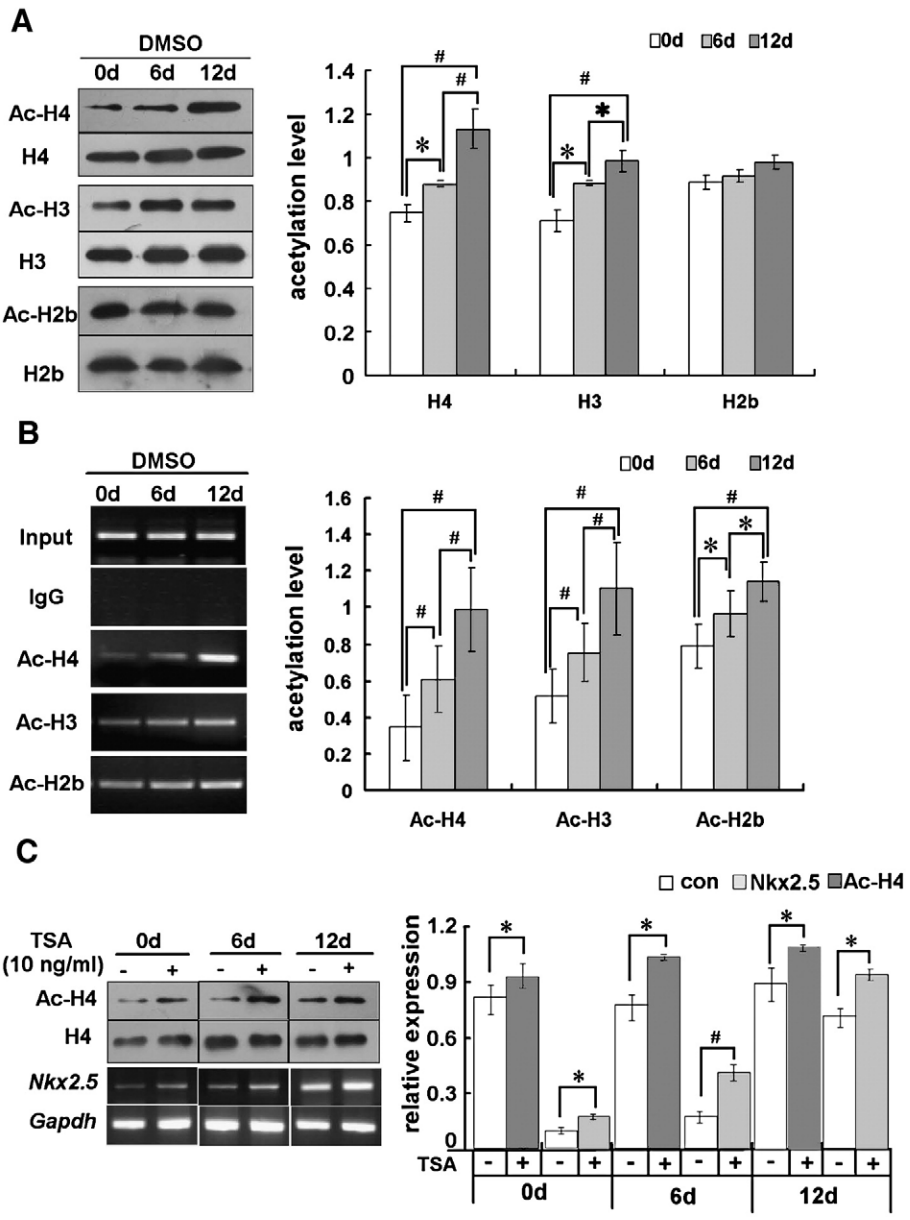

Fig. 2. Total histones and histones at *Nkx2.5* promoter underwent hyperacetylation during cardiomyocyte differentiation. (A) Western blotting was performed with total proteins from P19CL6 cells at different days to detect the acetylation level of histone H2b, histone H3 and histone H4, respectively, and total histones as the control. (B) ChIP assays were performed with P19CL6 cells at differentiation days 0, 6 and 12. Immunoprecipitated DNA fragments were amplified by PCR at the promoter regions of *Nkx2.5*. Input represents 10% of the total input chromatin. Rabbit preimmune serum (IgG) was used as a negative control. (C) 10 ng/ml TSA was added to the induced P19CL6 cells at days 0, 6 and 12 for 24 h. Western blotting was performed to detect the acetylation level of histone H4, using total histone H4 as a control; RT-PCR demonstrated the expression of *Nkx2.5*, *Gapdh* as an internal control (the representative images of above experiments are shown on the left; the semi-quantitative analysis data are shown on the right, representing the average of at least 3 independent experiments, $^{*}P<0.05$, $^{\#}P<0.01$).

start sites and one translational start site, two alternatively spliced exons and three introns, more than five activating regions and three inhibitory regions, generating three different transcripts in different tissues and stages during heart development (Fig. 1C) [22]. We therefore examined the CpG site methylation status of the three most important CpG island-rich activating regions, AR1 (−9436U, −7363D), AR2 (−3050U, −1976D) and AR3 (−5870U, −4059D) before and after induction with DMSO. The PCR products (245 bp, 357 bp and 255 bp) containing 15, 19 and 17 CpG sites respectively, were inserted into the pGL3-basic plasmid for bisulphite sequencing (Fig. 1D). To our surprise, we found that before DMSO induction the percentage of methylated CpG sites in the AR1 region was 28%, compared with 21.3% after induction (Fig. 1E), a change that was not statistically significant. As for AR2 and AR3, the percentages decreased from 10.5% to 9.47% and from 11.7% to 10.6%, respectively; both were not significant (Fig. 1E). Taken together, the methylation status of these activating regions underwent no significant change in spite of over 80% of cardiac differentiation of the P19CL6 cells, suggesting that there must be alternative mechanisms of regulation.

3.2. Histone proteins surrounding the Nkx2.5 promoter were hyperacetylated during cardiomyogenesis with reduced expression of Hdac1

To determine whether histone modification was involved in cardiomyogenesis, we examined the overall histone acetylation level. As shown in Fig. 2A, the degree of acetylated histone H4 (Lys8) as well as acetylated histone H3 (Lys9) gradually increased

during DMSO induction. On the other hand, the acetylation level of histone H2b (Lys5), a cell cycle-associated protein, displayed no significant change at day 6 and day 12 post-induction compared with day 0 (Fig. 2A). Our data suggested that P19CL6 cells underwent extensive acetylation on H3 and H4 histones during cardiomyogenesis, which likely promoted the expression of differentiation-associated genes.

To determine whether hyperacetylation of histones H3 and H4 occurred on the *Nkx2.5* promoter during cardiomyogenesis, we performed chromatin immunoprecipitation (ChIP) assays at different days during differentiation. As shown in Fig. 2B, histone H4 (Lys8) and histone H3 (Lys9) acetylation gradually increased at the *Nkx2.5* promoter at day 6 and day 12 of differentiation, whereas the level of histone H2b (Lys5) acetylation displayed less significant changes during the induction process. It seemed that the upregulation of *Nkx2.5* expression during cardiomyogenesis could be mostly attributed to histone acetylation.

When treated with TSA, a potent histone deacetylase inhibitor, for 24 h at day 0, 6, and 12 of the induction, histone H4 (Lys8) acetylation as well as *Nkx2.5* expression were both increased (Fig. 2C), indicating that suppression of HDAC enzymatic activity enhances cardiac differentiation concomitantly with stimulation of cardiac gene expression. These results further indicate that the upregulation of *Nkx2.5* during cardiomyogenesis is tightly associated with the selective induction of histone hyperacetylation at the promoter regions of this gene.

As the degree of histone acetylation is determined by the balance between HATs and HDACs, to understand the cause of the shift in this balance, we compared the mRNA and/or protein levels of HDAC isoforms before and after differentiation in P19CL6 cells. Interestingly, the mRNA level of *Hdac1* decreased during the differentiation process (Fig. 3A), whereas mRNAs of other *Hdacs*, including *Hdac3* and some class II *Hdacs* (*Hdac5* and *6*), were only slightly reduced or unchanged (data not shown). Accordingly, the protein level of HDAC1 was markedly decreased during cardiomyogenesis (Fig. 3B), suggesting that increase of histone acetylation is probably due to decreased *Hdac1* expression. The protein levels of HDACs 2 and 4 exhibited relatively insignificant changes in the early stage of differentiation (day 6 compared to day 0), whereas a marked reduction occurred later (day 12 compared to day 6, Fig. 3B). No significant changes were observed for HDAC5. We therefore speculate that the histone acetylation initiated at the early stages of differentiation at least partly resulted from downregulation of *Hdac1* expression. However, we cannot exclude the participation of other mechanisms, for example, augmented p300 and the inactivation of class II HDACs via phosphorylation by CaMK followed by nucleocytoplasmic shuttling [23].

To further elucidate whether the changes of *Hdac1* expression were associated with cardiac differentiation, we examined the effects of *Hdac1* knockdown using siRNA and overexpression. Knockdown of *Hdac1* expression reduced its protein level by 80% and was accompanied by enhanced acetylation of histone H4. This led to increased expression of *Nkx2.5* (Fig. 3C). In contrast, overexpression of *Hdac1* led to a decreased level of *Nkx2.5* expression (Fig. 3D). Furthermore, ChIP assay demonstrated that recruitment of HDAC1 to the *Nkx2.5* promoter was decreased during induction (Fig. 3E).

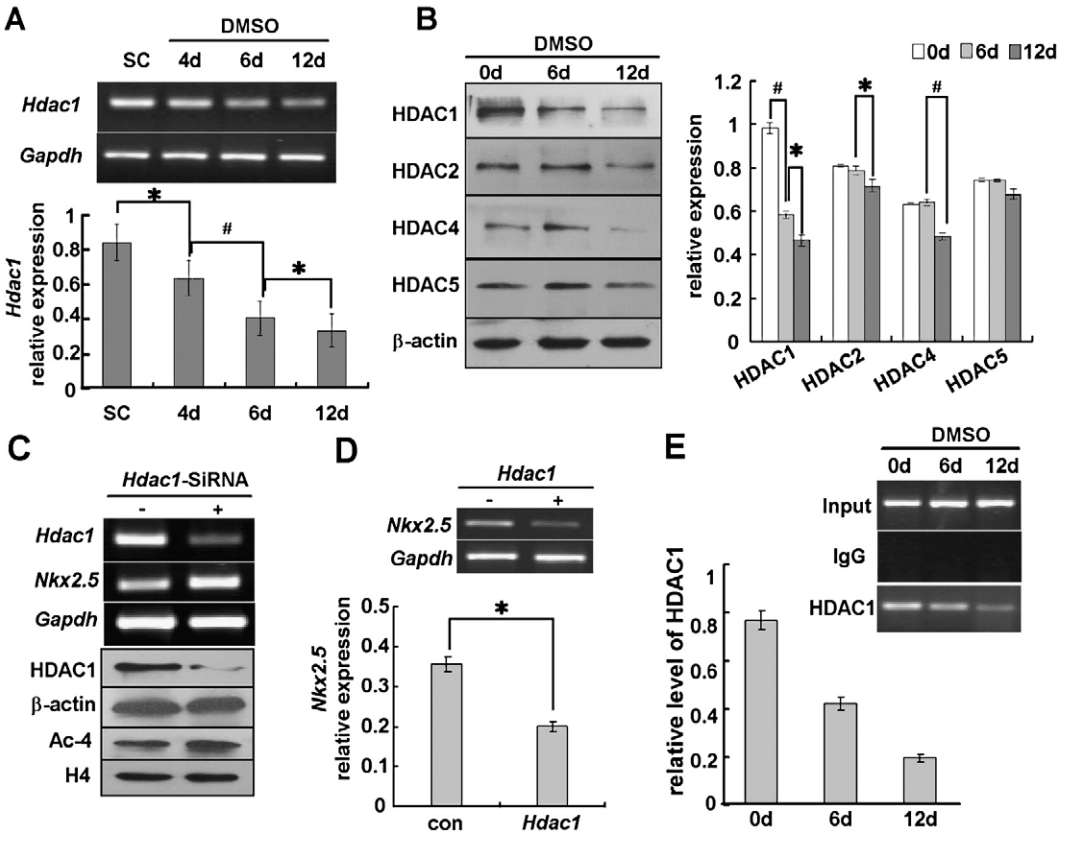

Fig. 3. Downregulation of *Hdac1* promoted *Nkx2.5* expression and histone H4 acetylation. (A) RT-PCR was performed to detect the *Hdac1* transcription level (332 bp PCR product) before and in the process of induction, *Gapdh* was used as an internal control. (B) Whole-cell lysates were subjected to Western blotting and analyzed using antibodies against HDAC1, HDAC2, HDAC4 and HDAC5 respectively, with β-actin as an internal control. (C) Knockdown of the *Hdac1* gene by 100 mM *Hdac1*-siRNA for 48 h in P19Cl6 cells. RT-PCR demonstrated the expressions of *Nkx2.5* and *Hdac1*, with *Gapdh* as an internal control; Western blotting demonstrated that total HDAC protein and total acetylated histone H4 change after *Hdac1* knockdown; *Gapdh* and histone H4 were used as controls, respectively. (D) RT-PCR demonstrated the effect of overexpression of *Hdac1* on *Nkx2.5* expression by transfection of 2 μg *Hdac1* plasmid. (E) ChIP assay detected the recruitment of HDAC1 to the *Nkx2.5* promoter. Anti-HDAC1 antibody was used to pull down the chromatin and *Nkx2.5* promoter was amplified by PCR (818 bp product). All the experiments were performed at least 3 times. The semi-quantitative analysis data represent the average of the experiments, $^{*}P<0.05$, $^{\#}P<0.01$ versus the controls.

Together, these results indicate that histone acetylation modulates the expression of *Nkx2.5* during cardiac differentiation.

3.3. Overexpression of Hdac1 inhibited the P19CL6 cell differentiation

Treatment of aggregated P19 cells with trichostatin A induces the entry of mesodermal cells into the cardiac muscle lineage, and overexpression of *Hdac4* inhibits cardiomyogenesis [8]. Downregulation of *Hdac1* stimulates adipocyte and osteoblast differentiation [24]. Our results indicated that the downregulation of *Hdac1* might be essential for cardiomyogenesis. To directly elucidate whether the changes in *Hdac1* expression were associated with P19CL6 cell differentiation, we investigated whether *Hdac1* overexpression could affect P19CL6 cell differentiation. Under differentiation conditions, *Hdac1*-overexpressing P19CL6 cells displayed inhibited cardiomyogenesis (Fig. 4A). The differentiation efficiency was less than (50+6)% in *Hdac1*-overexpressing cells, compared to (77±9)% in normal P19CL6 cells (Fig. 4B). This was accompanied by depressed mRNA levels of *Nkx2.5*, *Gata4* and β-MHC, compared with control cells (Fig. 4C). In addition, as observed microscopically, nearly all the differentiated cardiomyocytes of the control cultures beat synchronously (video S1). In contrast, the *Hdac1*-overexpressing cells showed reduced differentiation and irregular uncoordinated beating (video S2). All these data indicate that overexpression of *Hdac1* indeed depresses expression of cardiac genes and suppress cardiomyogenesis.

3.4. Canonical Wnt pathway downregulated Hdac1 expression through β-catenin/LEF1

Subsequently, we wanted to understand the detailed mechanism underlying the downregulation of *Hdac1* expression. Undifferentiated P19CL6 cells exhibited a high level of *Hdac1* expression. The presence of DMSO may trigger the activation of some signaling pathways, leading to downregulated *Hdac1* expression. Current evidence shows that WNT, BMP and FGF signaling pathways are key upstream factors that drive cardiac differentiation. WNT signals exhibit developmental stage-specific and biphasic effects on cardiac development. WNT signals promote cardiomyogenesis, but in the late stages of development inhibit cardiomyocyte differentiation [16,17]. Furthermore, it has

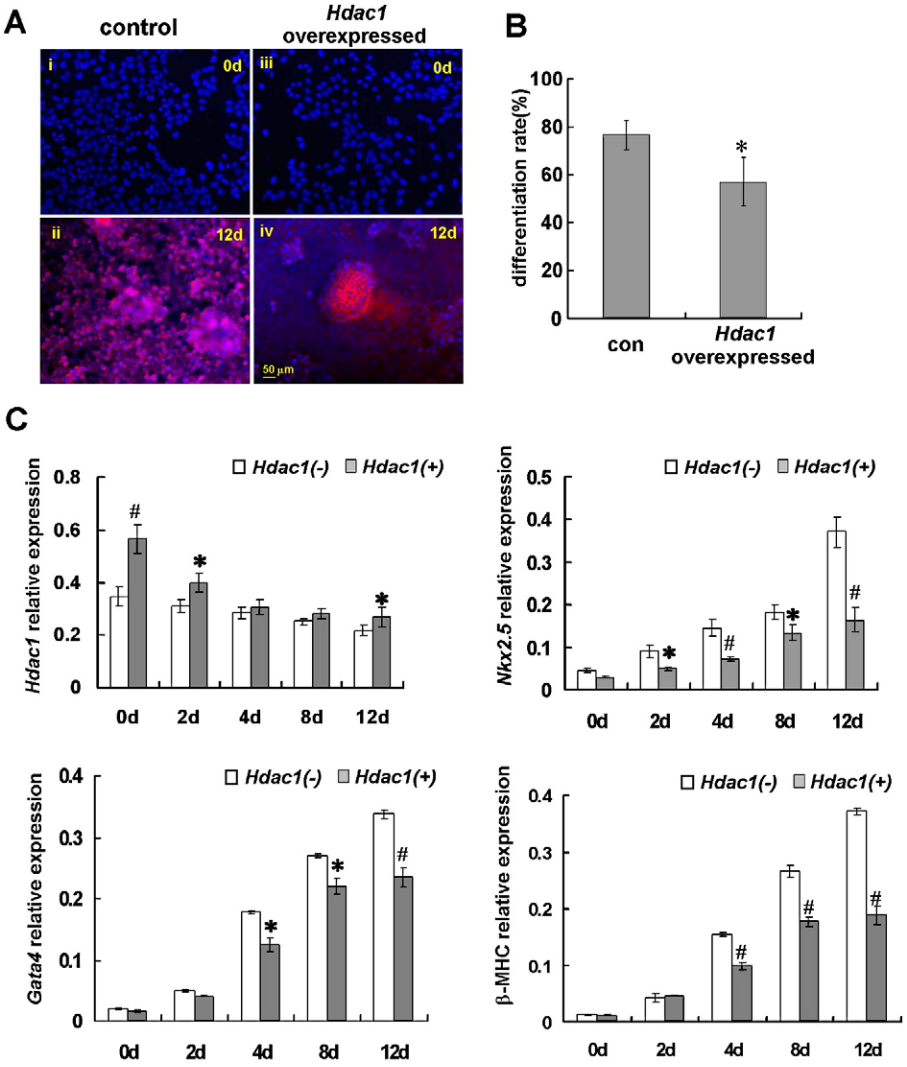

Fig. 4. Overexpression of *Hdac1* inhibited P19CL6 cells differentiation. (A) Phase microscopy and immunofluorescence analysis of P19CL6 cells with (iii, iv) or without (i, ii) stable transfection of *Hdac1* at day 12 during differentiation. Immunostaining was performed with an anti-α-actinin monoclonal antibody (Red, in cytoplasm) together with Hoechst33342 (Blue, in nuclei). Scale bar: 50 μm. (B) Differentiation rate of *Hdac1* overexpressed P19CL6 cells decreased significantly compared with the normal ones. A number of beating fields were counted randomly in four quadrants of the dish with marked grids (550 grids on a dish, 96 grids were counted, 3 dishes in normal P19CL6 control group and *Hdac1* overexpressed P19CL6 group, respectively. *$P<0.05$ versus the control group). (C) RT-PCR demonstrated the expression of *Nkx2.5*, *Gata4* and β-*MHC* in P19Cl6 cells during induction following *Hdac1* overexpression, with *Gapdh* as an internal control (the data represent the average of 3 independent experiments, each experiment was performed in triplicates. *$P<0.05$, #$P<0.01$, versus *Hdac1* untransfected cells.

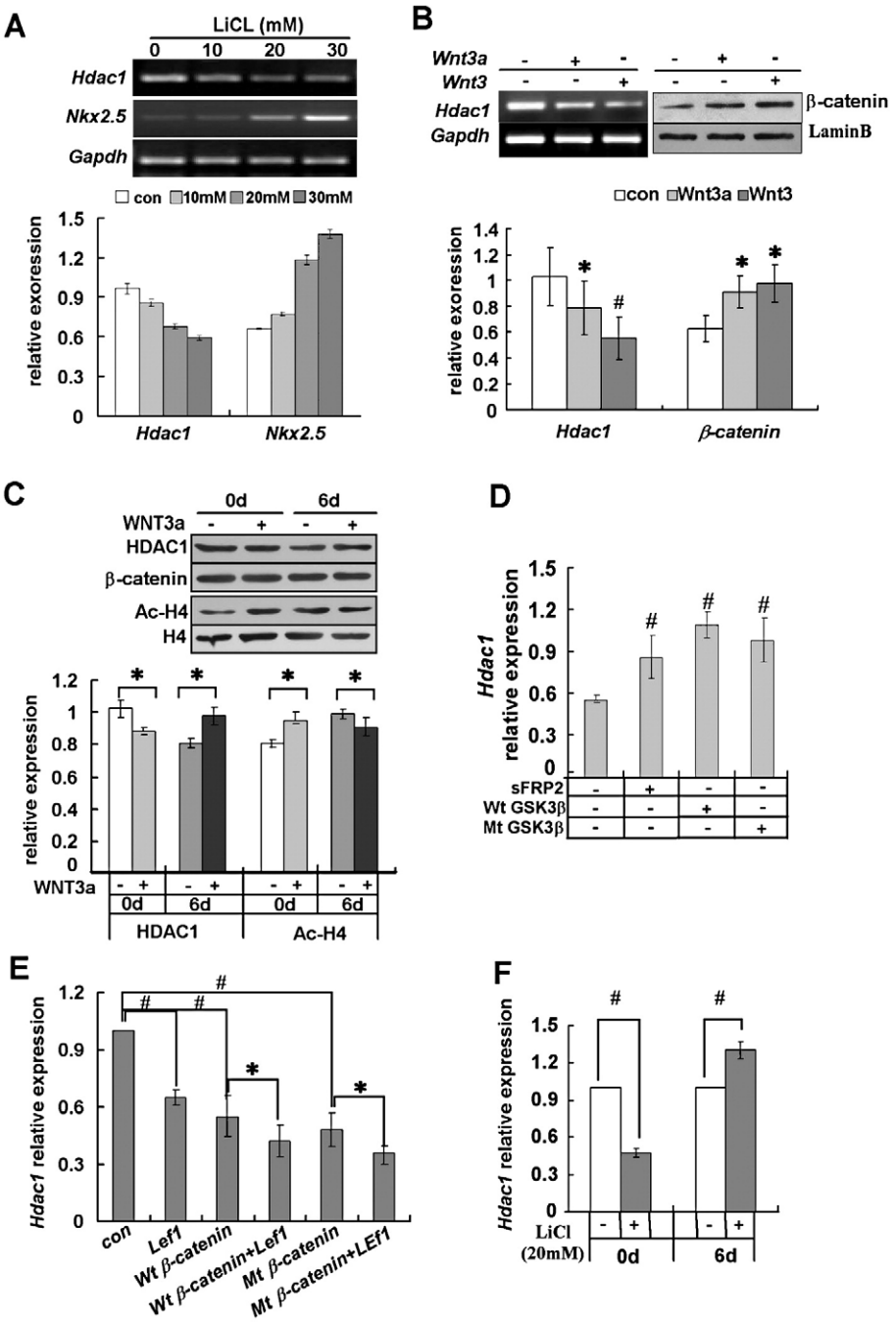

Fig. 5. Canonical Wnt signaling downregulated HDAC1 expression through the β-catenin/LEF1 pathway. (A) RT-PCR indicated expression of *Hdac1* and *Nkx2.5* in P19CL6 cells treated with LiCl at different concentrations for 48 h. (B) Effects of *Wnt3a* and *Wnt3* overexpression by plasmids transfection on *Hdac1* expression (RT-PCR, *Gapdh* as an internal control) and β-catenin nucleic translocation (Western blotting of the nucleic protein, Lamin B as an internal control). (C) Western blotting demonstrated the change of HDAC1 protein and histone H4 acetylation in P19CL6 cells treated with 50 ng/ml WNT3a peptide for 48 h at day 0 and day 6 during the induction. (D) Semi-quantitative RT-PCR demonstrated the effect of overexpression of wild type and mutant GSK-3β as well as sFRP2 plasmids on the expression of *Hdac1*; (E) Real-time RT-PCR indicated the effect of cotransfection of wild type or mutant β-*catenin* with *Lef1* on expression of *Hdac1*. (F) Real-time RT-PCR indicated the bilateral effects of LiCl on expression of *Hdac1* at day 0 and day 6 during the differentiation of P19CL6 cells. All the experiments were performed at least 3 times. The semi-quantitative analysis data represent the average of the experiments, $^{*}P<0.05$, $^{\#}P<0.01$, versus the controls.

been reported that transcriptional activation of some other class I HDACs could be regulated by the Wnt/β-catenin signaling pathway [25], implying the possibility of regulation of HDAC1 expression by WNT signals.

To verify this, we first mimicked the activation of WNT signaling pathways by treating undifferentiated P19CL6 cells with LiCl, and found that *Hdac1* expression was repressed along with increased expression of *Nkx2.5*, both in a dose-dependent manner (Fig. 5A). *Hdac1* expression was also depressed when overexpressing *Wnt3* and *Wnt3a*, in parallel with an augmented nuclear import of β-catenin (Fig. 5B). Addition of recombinant WNT3a protein also significantly attenuated *Hdac1* expression, accompanied by increased acetylation of histone H4 at day 0; while reversed effects were observed at day 6 (Fig. 5C).

WNT3a is thought to act via the canonical pathway, ultimately leading to the accumulation of β-catenin in the nucleus. β-catenin

binds to the LEF/TCF family of transcription factors to regulate gene expression. LiCl simulates the WNT signaling pathway via liberating GSK-3β-mediated repression and in turn facilitating the translocation of β-catenin into the nucleus. As expected, an increase of *Hdac1* expression was observed when overexpressing *GSK-3*β, an antagonist for the WNT/β-catenin pathway by suppressing β-catenin; the same effect was observed with the constitutively activated *GSK-3*β; when WNT signaling was blocked by overexpression of *sFrp2*, a competitive antagonist of WNT receptor fizzled [26], *Hdac1* expression was also significantly promoted (Fig. 5D). In addition, β-*catenin* or *Lef1* alone repressed *Hdac1* expression, but there was a significant decline in *Hdac1* expression when cotransfected with β-*catenin* and *Lef1* (Fig. 5E), the same effect was seen in combined transfection with *Lef1* and a constitutively activated β-*catenin*. Therefore, our results indicate that the activation of GSK-3β contributes to increased *Hdac1* expression in undifferentiated cells.

Strikingly, in the late stage of P19CL6 cell differentiation (day 6 of induction) mimicking WNT signaling by treatment with 20 LiCl led to an increase in the expression of *Hdac1* (Fig. 5F). Accordingly, addition of WNT3a protein at day 6 of differentiation also caused an increase in *Hdac1* expression in parallel with decreased acetylation level of histone H4 (Fig. 5C). In addition, this biphasic effects were also observed in the course of differentiation in ES cells (data not shown). All these data indicate that, at the beginning of cardiac differentiation, WNT signaling represses *Hdac1* expression through the β-catenin/LEF1 pathway, but in the late stage it has an opposite effect, coinciding with its biphasic effects on cardiomyogenesis.

3.5. HDAC1 was essential for maintaining the self-renewal and undifferentiated phenotype in undifferentiated P19CL6 cells

Why do undifferentiated P19CL6 cells exhibit a high level of *Hdac1* expression? A link has been found between HDAC1 and the cell cycle in cancer cells [27]. We speculated that abundant HDAC1 is required for proliferation of P19CL6 cells or ES cells. We therefore performed flow cytometric analysis to detect cell proliferation. Normal P19CL6 cells and *Hdac1*-knockdown cells showed a marked difference in proliferation (Fig. 6A). In normal P19CL6 cells, the average percentage of S phase cells was (43.40±5.02)%, but was only (27.06±4.36)% in the *Hdac1*-knockdown cells ($p<0.01$), suggesting a proliferation-linked function of HDAC1.

HDAC1 may also be required for maintenance of the undifferentiated status. We found that overexpression of *Hdac1* was associated with upregulation of *Id1*, an inhibitory transcription factor for myogenesis and cardiomyogenesis, which is degraded upon differentiation. Meanwhile, *Hdac1* silencing led to downregulation of *Id1* significantly (Fig. 6B). Interestingly, *Id1* also exerted similar positive effect on *Hdac1* expression (Fig. 6C). However, HDAC1 did not seem to have a relationship with OCT4, a transcription factor that plays an essential role in maintaining pluripotency of cells, as overexpressing either *Hdac1* or *Oct4* had no effect each other (Fig. 6C).

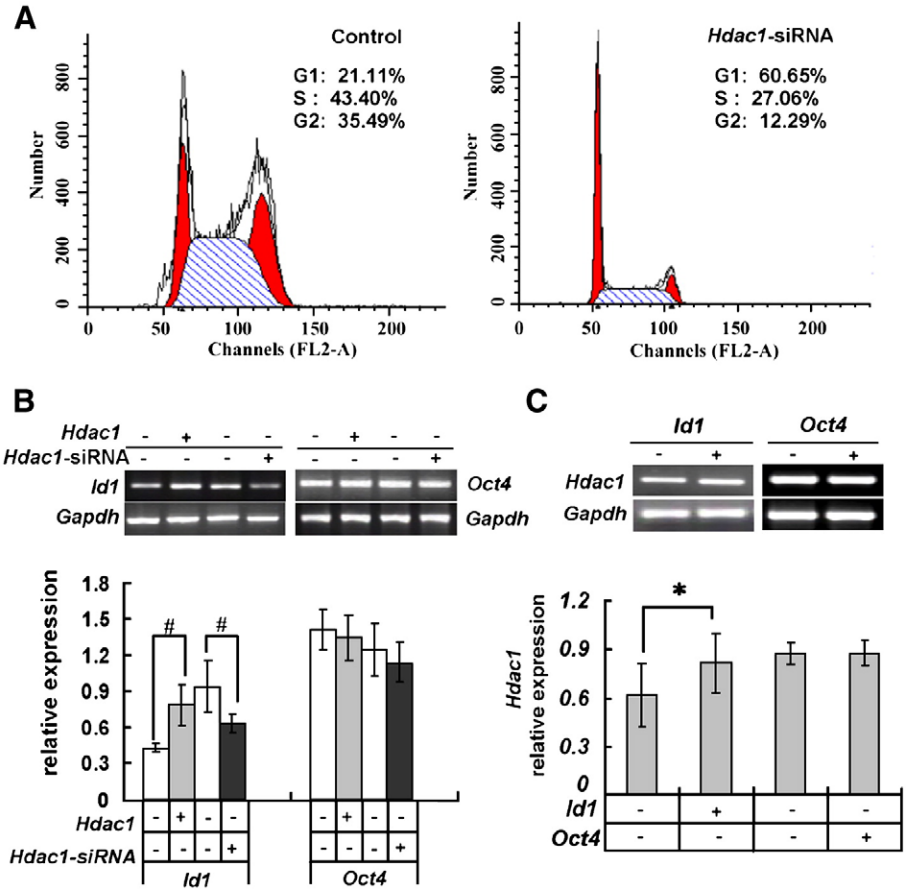

Fig. 6. HDAC1 maintained the self-renewal and undifferentiated phenotype of undifferentiated P19CL6 cells. (A) FACS showed the effect of *Hdac1* knockdown by siRNA on cell cycle. The S phase of *Hdac1* knockdown P19CL6 cells declined significantly versus to normal cells ((27.06±4.36)% versus (43.40±5.02)%, $^{\#}P<0.01$, $n=3$, each sample was tested in triplicates). (B) RT-PCR demonstrated the effects of *Hdac1* and *Hdac1* knockdown to *Oct4* and *Id1* ($^{\#}P<0.01$ versus the controls, respectively. $n=3$). (C) RT-PCR demonstrated the effects of *Id1* and *Oct4* on *Hdac* expression ($^{*}P<0.05$, versus the control; $n=3$).

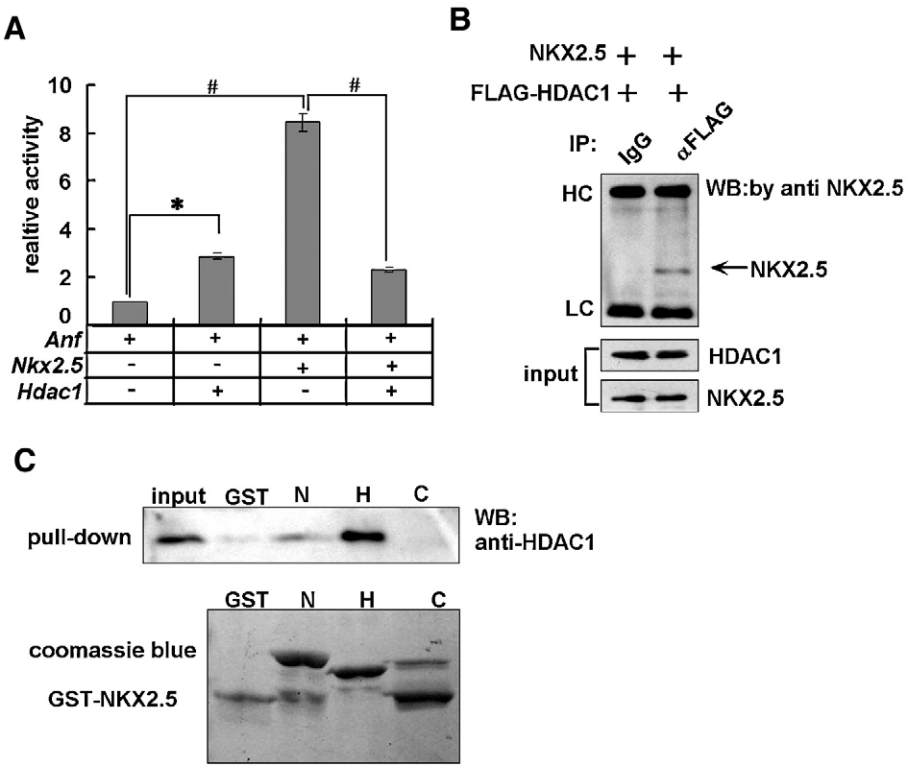

Fig. 7. HDAC1 repressed the transcriptional activity of *Nkx2.5*. (A) pANF-luc represents the rat ANF–638 bp construct upstream from the transcriptional start site. The *Nkx2.5* and *Hdac1* plasmids were transfected alone or together with pANF-luc into the P19CL6 cells for 24 h and transcriptional activity detected by Dual-Lucy Assay ($^{*}P<0.05$ and $^{\#}P<0.01$, $n=3$). (B) Cell lysates were prepared from HeLa cells transfected with 2 μg of FLAG-*Hdac1* and *Nkx2.5* as indicated. Immunocomplex was precipitated with anti-FLAG or, as control, mouse nonimmune IgG. The immunoprecipitates were resolved by 12% SDS-PAGE electrophoresis, transferred to a nitrocellulose membrane and probed with anti-NKX2.5 antibody. HC, heavy chain; LC, light chain. (C) Mapping of the NKX2.5 domains interacting with HDCA1. Bacterially-expressed GST fusion proteins with different fragments of NKX2.5 as indicated, were bound to GST–sepharose and incubated with nuclear extracts from HeLa cells overexpressing HDAC1. Material bound to the various GST fusions was analyzed by SDS–PAGE and Western blotting. N, N-terminal domain; H, homeodomain; C, C-terminal domain of NKX2.5.

Therefore, these data indicate that a role of HDAC1 in undifferentiated P19CL6 cells is to maintain self-renewal and the undifferentiated phenotype.

3.6. The transcriptional activity of Nkx2.5 was repressed by HDAC1

It has been reported that HDACs act as transcriptional repressors to inhibit GATA2, RUNX2, MEF2, PPARγ and other transcriptional factors, impairing the development of diverse tissues. Both NKX2.2 and NKX3.2 are able to interact with HDAC1. Thus, we speculated that there might be a potential interaction between NKX2.5 and HDAC1. To determine whether *Nkx2.5* transcriptional activity is repressed by HDAC1, we cotransfected *Nkx2.5* and *Hdac1* into P19CL6 cells and investigated its transcriptional activation effects on the *Anf* promoter. Consistent with a previous report [28], *Nkx2.5* alone could active the *Anf* promoter by at least 9 folds. However, when cotransfected with *Hdac1*, the activation decreased significantly (Fig. 7A). In addition, we examined the interaction between HDAC1 and NKX2.5 by GST pull-down and Co-IP assay. Interestingly, HDAC1 physically associated with NKX2.5 mainly via its homeodomain and partly via its N-terminal region (Fig. 7B and C). These results indicate that HDAC1 is able to repress the transcriptional activity of NKX2.5 via direct interaction.

4. Discussion

NKX2.5 is a crucial regulator of cardiomyogenesis, regulating expression of a number of cardiac-specific genes. *Nkx2.5* is expressed in early cardiac progenitor cells prior to cardiomyogenesis and throughout adulthood [29]. Epigenetic changes of the *Nkx2.5* promoter were thought to contribute to the spatial and temporal patterns of *Nkx2.5* expression through DNA demethylation and post-translational histone modifications. The present study was performed to examine the effect and exact mechanism of DNA methylation and histone acetylation on the *Nkx2.5* promoter during P19CL6 cell differentiation into cardiac myocytes.

Sequential events of demethylation and de novo methylation are believed to occur during differentiation. It is well documented that there are correlations between the DNA methylation status of OCT4 and the differentiation status of embryonic cells [30]. Strikingly, though a marked augmentation of *Nkx2.5* expression occurred during cardiomyogenesis, we did not detect significant alteration of demethylation in associated CpG islands of three DNA segments located in the *Nkx2.5* promoter. In undifferentiated P19CL6 cells (low level expression of *Nkx2.5*) and ES cells (no expression), the *Nkx2.5* promoter exhibited a hypomethylated status, at least in detected CpG islands, to a similar extent as in differentiated cardiomyocytes. Therefore, the potential role of DNA demethylation in the control of *Nkx2.5* expression in a tissue- and time-specific manner remains in question. Likewise, the CpG islands in the muscle determination gene *MyoD* were not methylated in nonexpressing tissues such as the brain [31]. Previous documents indicated that different genes may possess variable and specialized DNA modification patterns. Nevertheless, we have not excluded the possibility that P19CL6 cells, as committed embryonic carcinoma cells, have undergone a demethylation event leading to activation of the *Nkx2.5* promoter.

In contrast, histone modifications, notably acetylation and deacetylation, appear to be crucial for the regulation of *Nkx2.5* expression at the transcriptional level. Our results demonstrate that histone proteins on the *Nkx2.5* promoter were gradually hyperacetylated during DMSO-induced differentiation, in parallel with a dramatic

decrease in *Hdac1* expression. In addition, we found that TSA enhanced cardiomyogenesis and cardiac marker gene expression, suggesting that prevention of endogenous HDAC enzymatic activity is important for the execution of the cardiomyogenic program. In differentiated P19CL6 cells, total HDAC enzymatic activity was significantly decreased, accompanied by an increase of hyperacetylation of histones H3 and H4. It seems that histone acetylation spreads over broad genomic areas resulting in large regional effects rather than affecting *Nkx2.5* expression alone. The downregulation of total HDAC enzymatic activity during cardiomyogenesis is probably due to decreased *Hdac*1 expression. Using ChIP, we found that recruitment of HDAC1 to *Nkx2.5* promoter regions was markedly reduced after DMSO treatment. Overexpression of *Hdac1* attenuated cardiomyogenesis and *Nkx2.5* expression whereas knockdown of *Hdac1* by RNAi promoted cardiomyogenesis with an accompanying increase in *Nkx2.5* expression. Although, we could not establish the relationship between the HDAC1 and differentiation rate of P19CL16, the expression and recruitment Hdac1 correlated well with the expression of cardiac-specific gene *Nkx2.5* (Figs. 3A, E and 1A, B). Taken together, our results suggest that HDAC1 is a critical factor regulating *Nkx2.5* expression in a defined manner during cardiomyogenesis.

Covalent modifications of the histone tails regulated by HATs and HDACs have been proposed to act as epigenetic marks that impart transient or permanent "cellular memory" of the transcriptional activation state of specific genes [32]. HATs facilitate chromatin opening by acetylating the N-terminal tails of nucleosomal histones, thus promoting active transcription. Conversely, HDACs deacetylate lysine residues and induce compaction of chromatin, making the access of transcription factors to nearby promoters more difficult and thereby resulting in gene silencing. As coregulators, HDACs have been recently implicated in coordinating the activation and repression of genes involved in the differentiation process. For instance, *Hdac1* knockdown promoted adipogenesis whereas *Hdac1* overexpression attenuated adipocyte differentiation in 3T3-L1 cells. Furthermore, HDAC1 and members of the class II HDACs inhibit *MyoD* and *Mef2*, respectively, blocking myogenesis. In addition, it has been reported that HDAC1 and HDAC3 physically interact with RUNX2 and suppress its transcriptional activity in osteoblast differentiation.

Class II HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) have been shown to interact with MEF2C and SRF, and overexpression of *Hdac4* inhibits cardiomyogenesis, as shown by the downregulation of cardiac muscle gene expression. Furthermore, most *Hdac5-Hdac9* double knockout mice died owing to heart defects, suggesting a role for class II HDACs in heart development [33]. TSA added to aggregated ES cells at a later stage (day 7) promoted differentiation by activating GATA4 by acetylation. It has been reported that TSA alone is sufficient to induce cardiomyogenesis in the absence of DMSO [8]. Our results provide further evidences that a member of class I HDACs, HDAC1, is a repressor of cardiomyogenesis.

Individual HDACs may play differential roles in developmental stages of cardiamyocytes. In terminally differentiated cardiomyocytes, the class II HDACs repressed the pro-hypertrophic gene program, whereas class I HDACs seemed to suppress anti-hypertrophic gene expression. TSA treatment consistently inhibited hypertrophic growth of post-mitotic cardiomyocytes and reactivation of fetal genes such as ANF. Thus, more studies are required to investigate the underlying reasons why HDAC1 exhibits biphasic effects on ANF expression at different developmental stages.

Though class II HDACs exerted a repressive effect on cardiomyogenesis, post-translational modification could override the repression. Treatment with DMSO increased intracellular Ca^{2+}, thereby activating CaMK [34]. Class II HDACs phosphorylated by CaMK shuttled from the nucleus to the cytoplasm, activating MEF2C and other transcriptional activators. However, class I HDACs did not contain the phosphorylation sites that are conserved and exclusively located in the N-terminals of HDACs 4/5/7/9 [15]. Importantly, we observed that the expression of *Hdac1* was decreased during cardiomyogenesis; its transcriptional downregulation reduced its inhibitory effect. Subsequent experiments demonstrated that the WNT signaling pathway may contribute to the downregulation of *Hdac1*. Overexpression of *Wnt3a* or combined transfection with *Lef1* and β-*catenin* also evoked the decrease of *Hdac1* mRNA, whereas SFRP2 upregulated the expression of *Hdac1*. Early stage treatment with LiCl, which binds and inhibits GSK-3β and then activates WNT signaling selectively via the β-catenin/LEF1 pathway, also triggered a dramatic inhibition of *Hdac1* expression. Strikingly, addition of LiCl upregulated the expression of *Hdac1* and inhibit *Nkx2.5* expression. Previous reports have provided substantial evidence to demonstrate that WNT/β-catenin signaling promotes cardiac differentiation at early developmental stages and inhibited it at later stages. Except for the finding that WNT3a inhibited BMP2/4 expression at the late stages of differentiation, other associated mechanisms in relation to the antagonistic effects of WNT signaling on cardiomyogenesis still remained obscure. Based on our data, we present a hypothesis that the biphasic effect of WNT/β-catenin signaling could function via modulation of *Hdac1* expression. Taken together, we, for the first time, provide a possible mechanism through which the WNT/β-catenin/GSK-3β signaling pathway accelerates cardiomyogenesis by downregulating *Hdac1* expression at an early stage of cardiomyogenic differentiation.

We also noticed that the *Hdac1* alone could activate the ANF promoter significantly, but the mechanism is still unclear. Previous reports demonstrated that inhibition of HDACs could block myocardiac hypertrophy [35,36], in which the ANF played important roles. We propose that the HDACs may have some direct or indirect effects on ANF.

In addition, we also observed that overexpression of *Hdac1* in undifferentiated P19CL6 resulted in a significant augmentation of *Id1* expression, a transcriptional repressor blocking myogenesis and cardiomyogenesis by binding to MyoD, GATA4 and NKX2.5 and inhibiting their binding to DNA [37]. *Oct4* expression was unaffected. Further investigation indicated that there existed a reinforcing regulatory loop for *Id1* and *Hdac1* to upregulate expression of each other. Combined with the evidence that knockdown of *Hdac1* induced G1 cell cycle arrest, our data indicates that HDAC1 is important for stem cells to maintain the self-renewal and undifferentiated phenotype.

Previous research revealed that a direct interaction existed between p300 and NKX2.5 [38]. The homeodomains of NKX2.2 and NKX3.2 have been suggested to mediate the direct interaction with HDAC1 [39]. In this study, we have found that HDAC1 can interact with NKX2.5 and repress its transcriptional activity. This result implies that NKX2.5 may mediate the recruitment of HDAC1 to repress certain target genes in the absence of induction signals. It is feasible to postulate that NKX2.5 may switch its coregulator(s) from HDAC1 to p300 upon differentiation signals, leading to hyperacetylation of histone proteins at the promoters of cardiac genes as well as ANF during cardiomyogenesis. Together, we have provided more information about the complexity and pleiotropism of HDAC1 modulating cardiomyogenesis.

Acknowledgements

We are grateful to Dr. Yunzeng Zou, Fudan University Shanghai, for P19CL6 cells; Dr. Jason Wong, University of Cambridge, UK for his kind help in the preparation of this manuscript. We thank Professors Yongfeng Shang and Weiguo Zhu, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University, for their helpful suggestions during the work. This work was supported by the National Natural Sciences Foundation of China (30400242) and the 111 Project of Ministry of Education of China (B07001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.08.013.

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