Histone modifications interact with DNA methylation at the GATA4 promoter during differentiation of mesenchymal stem cells into cardiomyocyte-like cells

Hao Xu^{*†‡}, Qin Yi^{*†‡}, Chunmei Yang^{*†‡}, Yue Wang^{*†‡}, Jie Tian[§] and Jing Zhu^{*†‡}

^{*}Pediatric Research Institute, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Chongqing 400014, China, [†]China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing 400014, China, [‡]Chongqing Key Laboratory of Pediatrics, Chongqing 400014, China and [§]Cardiovascular Department (Internal Medicine), Children's Hospital of Chongqing Medical University, Chongqing 400014, China

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Abstract

Objectives: A previous study of ours confirmed that Islet-1 specifically induces differentiation of MSCs into cardiomyocytes, and that one of the mechanisms underlying that process is regulation of histone acetylation. Here, we further explore the mechanism of MSC differentiation into cardiomyocytes from the perspective of interactions between epigenetic modifications.

Materials and methods: We used lentiviral vectors to overexpress Islet-1 in MSCs, and ChIP-qPCR, MSP and BSP were performed to detect levels of histone acetylation/methylation and DNA methylation in the GATA4 and Nkx2.5 promoters. To further explore relationships between these epigenetic modifications, we used 5-aza or TSA to interfere with DNA methylation and histone acetylation, respectively, and detected effects on the other two modifications.

Results: Histone acetylation level increased and its methylation level decreased at GATA4 and Nkx2.5 promoters; DNA methylation level was reduced at the GATA4 promoter but did not change at the Nkx2.5 promoter. Furthermore, 5-aza increased histone acetylation level and reduced its methylation level at the GATA4 promoter but had no effect on the Nkx2.5 promoter; TSA reduced histone methylation and DNA methylation levels at the GATA4 promoter, but it only reduced histone methylation level at the Nkx2.5 promoter.

Conclusions: Histone acetylation/methylation and DNA methylation were both involved in regulating GATA4 expression, but Nkx2.5 expression was not regulated by DNA methylation. These three modifications had high correlation with each other during regulation of GATA4 and produced a regulation loop at the GATA4 promoter.

Introduction

Cardiovascular disease has always been one of the human diseases with the highest lethality, primarily due to irreversible myocardial damage. Myocardial cells have no self-renewal ability; however, researchers have found that stem cells with self-renewal and multi-directional differentiation abilities can differentiate into functional myocardial cells *via* induction in an *in vitro* or *in vivo* environment to replace damaged myocardium for treatment. This replacement therapy based on stem cells has shown great potential (1,2). Recently, researchers have found that several types of stem cells can be used for replacement therapy, such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) (1). An increasing number of studies have confirmed that MSCs are one of the most promising types of seed cells for treating cardiovascular disease because they are easily available and are low in immunogenicity (3).

MSCs have multi-directional differentiation potential, and their specific differentiation into cardiomyocyte cells is the key to cardiovascular disease therapy. There are several methods for inducing MSC differentiation into cardiomyocyte; for example, 5-aza can induce MSC differentiation into cardiomyocytes (4,5), and the co-culture of MSCs with cardiomyocytes can induce MSC

Correspondence: Jing Zhu, Children's Hospital of Chongqing Medical University, Box 136, No. 3 Zhongshan RD, Yuzhong district, Chongqing 400014, China. Tel.: +86 13983833810; Fax: 023-63622754; E-mail: 1686598427@qq.com

differentiation (6,7), but the mechanism underlying this process is unclear. Studies have shown that histone acetylation and deacetylation play important roles in MSC differentiation into cardiomyocytes, and the histone acetyltransferases (HATs) Gcn5 and P300 (8,9) and the histone deacetylases (HDACs) HDAC1 and HDAC2 (10,11) are key in the regulation of cardiac-specific gene expression. However, other studies indicate that HATs and HDACs lack cardiac-specific gene-binding sites, preventing them from specifically binding to cardiac-specific genes and directly regulating their expression (12,13). Our earlier study found that Islet-1, a cardiac progenitor cell marker that contains a DNA-binding site and two LIM domains, is crucial for cardiac development and cardiomyocyte differentiation. Islet-1 can guide HATs to specifically regulate the expression of cardiac-specific genes to induce MSC differentiation into cardiomyocyte-like cells (14).

It is known that in the process of regulating gene expression, there are other epigenetic modifications in addition to histone acetylation: for instance, DNA methylation and histone methylation play very important roles. DNA methylation is best known for its role in gene silencing through a methyl group (CH₃) that is added to the 5' carbon of cytosine bases in the promoters of genes, leading to the suppression of transcription (15). Histone methylation plays an important role in gene silencing and heterochromatin formation (16). Studies have shown that epigenetic modifications do not work independently; rather, there is cross-talk between them, and they regulate gene expression through synergistic or antagonistic interactions with each other (17). Thus, we hypothesized that the regulation of cardiac-specific genes is controlled by the interactions of these epigenetic modifications during the complex differentiation process.

In this study, we overexpressed Islet-1 to induce MSC differentiation into cardiomyocyte-like cells and then detected CpG methylation, H3K9 acetylation and H3K9 methylation levels in the promoter regions of the cardiac-specific transcription factors Nkx2.5 and GATA4. Subsequently, we changed the levels of these modifications one at a time and observed the changes in the other two modifications. Our data indicate that CpG methylation, histone acetylation and histone methylation in the GATA4 promoter work together to regulate GATA4 expression and that these modifications interact with each other. However, Nkx2.5 is not regulated by CpG methylation, and there is no correlation between CpG methylation and H3K9 modification at its promoter. These results suggest that, during Islet-1-induced promotion of MSC differentiation into cardiomyocyte-like cells, the three epigenetic modifications are involved in regulating different cardiac-specific

transcription factors in different ways: Nkx2.5 is regulated by H3K9 acetylation and H3K9 methylation, while GATA4 is regulated by all tested modifications, and these modifications interact with each other at its promoter region.

Materials and methods

Cell culture and lentiviral vector transfection

C3H10T1/2 cells (University of Chicago Molecular Oncology Laboratory, Chicago, IL, USA) were grown in DMEM supplemented with 10% foetal bovine serum (FBS; Merck Millipore, Darmstadt, Germany). Lentiviral vectors containing Islet-1 or GFP (MOI=20) (GENECHEM, Shanghai, China) and polybrene (5 µg/ml) were added to the C3H10T1/2 cells. The culture medium was replaced by DMEM with 10% FBS after 24 h of incubation at 37 °C in 5% CO₂. Fluorescence microscopy (BX51; Olympus, Tokyo, Japan) was used to observe GFP expression after 3 days. Transfection efficiency was detected by flow cytometry (BD Canto II Flow Cytometer; BD Biosciences, San Jose, CA, USA). Cells were divided into three groups: blank (C3H10T1/2), control (lentiviral vector with GFP) and LV-islet-1 (lentiviral vector with Islet-1). Based on the duration of the transfection, the LV-islet-1 group was divided into four subgroups: LV-islet-1-1w, LV-islet-1-2w, LV-islet-1-3w and LV-islet-1-4w.

Patch clamp

Cells were superfused with normal Tyrode's solution (mmol/l) (136 NaCl, 5.4 KCl, 1.0 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, 5.5 glucose and 1.8 CaCl₂, using 2 mmol/l NaOH to adjust the pH to 7.4) (Sigma-Aldrich, St. Louis, MO, USA), and their whole-cell membrane currents were recorded using a patch-clamp amplifier (Axon Multiclamp 700B; Molecular Devices, Silicon Valley, CA, USA). The membrane currents were recorded under different voltages when a hyperpolarization pulse with the amplitude of -120 to +50 mV in a 10 mV space and duration of 300 ms was applied to the cells. To further evaluate the nature of the individual ion channels, selective ion channel blockers were added into the extracellular fluid: tetraethylammonium (TEA), 4-aminopyridine (4-AP) and BaCl₂ for potassium channels, NiCl₂ for T-type calcium channels and tetrodotoxin (TTX) for sodium channels. All currents were recorded using the Axon Multiclamp 700B patch-clamp amplifier, processed with a Digidatal 320A converter (AMPI, Jerusalem, Israel) and the pCLAMP 9.2 analysis software

system (Axon; Molecular Devices), and analysed using Clampfit 9.2 software (Axon; Molecular Devices).

EGCG, 5-aza and TSA treatment

EGCG (120 µmol/l) (Sigma-Aldrich), 5-aza (10 µmol) (A2385; Sigma, St. Louis, MO, USA) and trichostatin A (TSA, 300 nmol) (T1952; Sigma) were added to the LV-islet-1 group after transfection for 24 h, and treatment was applied for 12, 24 and 48 h, respectively. Based on the treatment duration, cells were divided into four subgroups: LV-islet-1 + EGCG/5-aza/TSA-1w, LV-islet-1 + EGCG/5-aza/TSA-2w, LV-islet-1 + EGCG/5-aza/TSA-3w and LV-islet-1 + EGCG/5-aza/TSA-4w.

Total RNA extraction and real-time PCR

Cells were isolated using an RNA extraction kit (RP120; BioTeke, Beijing, China). cDNA was generated using the PrimeScript RT reagent kit (Takara, Dalian, Liaoning, China). Then, cDNA was amplified with gene-specific primers and a SYBR Green dye kit (Takara). The primer sequences of cardiac-specific genes and controls were designed as follows: Nkx2.5 (forward) 5'-GAGCCTGGTAGGGAAAGAGC-3', Nkx2.5 (reverse) 5'-GGTGGGTGTGAAATCTGAGG-3', GATA4 (forward) 5'-GACTACCACCACGCTGT-3', GATA4 (reverse) 5'-ATT CAGGTTCTTGGGCTTCC-3', Mef2c (forward) 5'-ATC CCAGTGTCCAGCCATAA-3', Mef2c (reverse) 5'-AGACCGCCTGTGTTACCTG-3', TNNT2 (forward) 5'-CCGATGGAGAGAGAGTGGAC-3', TNNT2 (reverse) 5'-TTCTTTGAGGGAAATCAGC-3', GJA1 (forward) 5'-AGGTGGACTGCTTCCTCTCA-3', GJA1 (reverse) 5'-ATCGCTTCTTCCCTTCACG-3', β-actin (forward) 5'-GGAGATTACTGCCCTGGCTCCTA-3', β-actin (reverse) 5'-GACTCATCGTACTCCTGCTTGCTG-3'. The analysis of relative mRNA expression was carried out using the $2^{-\Delta\Delta C_t}$ method, and β-actin was used as an endogenous housekeeping gene to normalize the mRNA levels.

Genomic DNA extraction and methylation-specific PCR (MSP)

Genomic DNA was isolated using a genomic DNA purification kit (TIANGEN, Beijing, China) and was modified using the EZ DNA Methylation-Gold Kit (ZYMO RESEARCH, Orange, CA, USA) according to the manufacturer's recommendations. The primers for methylation-specific PCR (MS-PCR) were designed as follows: methylation-specific primers, Nkx2.5 (forward) 5'-ATTAGGTGACGTAGAATTGTTCTGTC-3', Nkx2.5 (reverse) 5'-CGCCTCTCTACCTAAATATAACG-3', GATA4-site 1 (forward) 5'-TTTGGGATTTTTTTGAGATT

TTTAC-3', GATA4-site 1 (reverse) 5'-AAATTACTATTACTATTACCCCGCC-3', GATA4-site 2 (forward) 5'-GGGTTTATAGGTATTGACGTCGA-3', GATA4-site 2 (reverse) 5'-GATAAAAACTACAAAACGCCGAA-3', GATA4-site 3 (forward) 5'-CGGGTTGTATTGAGGGT AGAAC-3', GATA4-site 3 (reverse) 5'-TAAAAAATCACGTACAAAACGAA-3'. The unmethylation-specific primers were Nkx2.5 (forward) primer 5'-TAGGTGATGTAGAATTGTTTGTGT-3', Nkx2.5 (reverse) primer 5'-CCACCTCTCTACCTAAATATAACAC-3', GATA4-site 1 (forward) 5'-TTTGGGATTTTTTTGAGATTTTAT-3', GATA4-site 1 (reverse) 5'-CAAATTACTATTACTATTACCCAC-3', GATA4-site 2 (forward) 5'-AGGGTTTATAGGTATTGATGTTGA-3', GATA4-site 2 (reverse) 5'-CCAATAAAAACTACAAAACACC AAA-3', GATA4-site 3 (forward) 5'-GTTGGGTTGTAT TGAGGGTAGAAT-3', GATA4-site 3 (reverse) 5'-TAAAAAATCACATACAAAACAAA-3'. The PCR conditions were 95 °C for 5 min, 95 °C for 20 s, 30 cycles for 20 s at 95 °C and 30 s at 60 °C, 72 °C for 20 s, and 75 °C for 5 min. The PCR products were run on 2% agarose gels, stained with ethidium bromide and evaluated using UV light.

Protein extraction and Western blotting

Proteins were extracted from the cells using RIPA Reagent (P0013B; Beyotime Biotech, Shanghai, China) containing 1% PMSF (ST506; Beyotime Biotech) to prevent protein degradation. Protein concentrations were measured with the BCA method (P0012 BCA; Beyotime Biotech). Protein samples were mixed with 5× buffer for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples in loading buffer were boiled for 5 min before being loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were cut according to the markers and incubated in 5% non-fat milk-PBST for 1 h on a shaker at room temperature to block non-specific protein binding. The primary antibodies used in our study were an Islet-1 rabbit monoclonal antibody at a dilution of 1:2000 (EP4182; Epitomics, San Francisco, CA, USA) and a β-actin monoclonal mouse antibody at a dilution of 1:2000 (A5441; Sigma), which was used as a control. Membranes were incubated with primary antibodies overnight at 4 °C and then washed in PBST three times for 10 min before being incubated with the corresponding secondary antibody. Positive bands were detected by chemiluminescent reactions (Millipore). The image collection and densitometry analyses were performed with Quantity One version 4.6.2 analysis software.

Chromatin Immunoprecipitation (ChIP)-qPCR assay

Formaldehyde (1%) was added to the samples to cross-link protein–DNA complexes. ChIP trials were conducted using a ChIP assay kit (Merck Millipore, DA, Germany). After cross-linking, the DNA was fragmented by sonication (UCD-200; Diagenode, Liège, Belgium), which consisted of 25 cycles of 30 s each with an interval of 30 s to cool down. Then, protein–DNA complexes were precipitated with an anti-histone H3 (acetyl K9) antibody and an anti-histone H3 (dimethyl K9) antibody. DNA was extracted using a DNA purification kit (Merck Millipore). The experiment contained both a positive control group (precipitated with an anti-RNA polymerase II antibody) and a negative control group (precipitated with normal mouse IgG). The amount of extracted DNA was determined by qPCR. The primers were designed as follows: Nkx2.5 (forward) 5'-ACCGCCTGGGTGATAGAC-3', Nkx2.5 (reverse) 5'-CCCTCCCGAGATTGAA-GAT-3', GATA4-site 1 (forward) 5'-GTAAATCAAGC CAGAGTGCG-3', GATA4-site 1 (reverse) 5'-GAAAAT CCCAGAGGAAGGAC-3', GATA4-site 2 (forward) 5'-GCTACAGGGAGTGATGAGAAG-3', GATA4-site 2 (reverse) 5'-CACCAGCCCAGGAGTTTAT-3', GATA4-site 3 (forward) 5'-TTGGCAGAGCAGCAAACCG-3', GATA4-site 3 (reverse) 5'-CGCAGGACTCTTCCCAA AGC-3'.

Bisulphite sequencing PCR (BSP) Assay

DNA was extracted from the cells using a DNA extraction kit (AP-MN-MS-GDNA-250; Axygen, California, USA) and then treated with bisulphite (ZYMO RESEARCH). The PCR products were gel extracted (AP-GX-250; Axygen) and ligated into a plasmid vector using the pMD18-T Vector (D101B; Takara). Plasmid-transformed DH5 α bacteria were cultured overnight, and the plasmid DNA was isolated (Axygen). At least 10 separate clones were chosen for sequence analysis.

Immunofluorescence

Cells (1×10^5 /well) were plated in 24-well plates on $1 \times 1 \text{ cm}^2$ glass coverslips. They were later fixed in 4 °C acetone for 15 min. Following three washes in

PBS, the cells on glass coverslips were blocked with goat serum (1:20) and washed again, and then primary anti-cardiac troponin T monoclonal (1:400; Abcam, Cambridge, MA, UK) and anti-connexin 43 polyclonal (1:100; Abcam) antibodies were added for overnight incubation at 4 °C. The cells were then washed with PBS, and secondary antibodies (1:150; CoWin Bioscience, Beijing, China) conjugated to Cy3 were added followed by incubation for 1 h at 37 °C. DAPI was then added for 3 min. After the final wash, images were acquired using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Statistical analysis

Each experiment was repeated at least three times. All data are expressed as the mean $\pm$ SD, while the statistical evaluations were performed using independent samples by applying *t*-test, continuity correction chi-square test and one-way ANOVA. SPSS 17.0 software (SPSS Inc., Armonk, NY, USA) was used for statistical analysis. A value of $P < 0.05$ was considered to be statistically significant for all analyses.

Results

Islet-1 induced MSC differentiation into cardiomyocyte-like cells

To confirm that Islet-1 could induce MSC differentiation into cardiomyocyte-like cells, we constructed a lentiviral vector containing Islet-1, and the transfection efficiency was 84.3% (Fig. 1a). Furthermore, Western blotting indicated that Islet-1 was highly expressed in the LV-islet-1 group (Fig. 1b). These results ensure the reliability of the subsequent experiments. After transfection with the lentiviral vector containing Islet-1, compared with the blank and control groups, MSCs in the LV-islet-1 group developed into fibroblast-like cells and were arranged in the same direction (Fig. 1c). The cardiac-specific transcription factors GATA4, Nkx2.5 and Mef2c and the cardiac-specific transcripts TNNT2 and GJA1 were detected by FQ-PCR following transfection with Islet-1, and the expression of these genes increased

Figure 1. Islet-1 induces the differentiation of C3H10T1/2 cells into cardiomyocyte-like cells. (a) Transfection efficiency (84.3%) was detected by flow cytometry. (b) The protein expression of Islet-1 was detected by Western blot; Islet-1 was highly expressed in the experimental group. (c) Morphological changes of C3H10T1/2 cells transfected with the lentiviral vector containing Islet-1 observed under a microscope. No difference was observed between the blank group (magnification, 10 $\times$) and the control group (magnification, 10 $\times$), while the LV-islet-1 group developed into fibroblast-like cells that were arranged in the same direction (magnification, 10 $\times$). (d) FQ-PCR was used to detect the expression patterns of cardiac-specific transcript factors and genes. The expressions of Nkx2.5, GATA4 and Mef2c increased gradually and were highest in the LV-islet-1-3w group; the expressions of TNNT2 and GJA1 were highest in the LV-islet-1-4w group. All of these gene expressions were inhibited by treatment with EGCG (* $P < 0.05$, compared with the blank group, # $P < 0.05$, compared with the LV-islet-1 group).

gradually with increased transfection duration; additionally, the expression of early transcription factors was highest in the LV-islet-1-3w group; the expression of the later markers TNNT2 and GJA1 was highest in the LV-islet-1-4w group; and these genes were inhibited by EGCG (histone acetyltransferase inhibitor) (Fig. 1d). Subsequently, we detected the expression of cTnT and Cx43 by immunofluorescence. After transfection with Islet-1, MSC expressed cTnT and Cx43 expression on the membrane was significantly increased, but the expression of cTnT and Cx43 was not detected in LV-islet-1 + EGCG group (Fig. 2). We examined whether the membrane ion channel activities typical of mature cardiomyocytes were present in the cells transfected with the Islet-1 vector by detecting the *I_{k1}*, *I_{ks}*, *I_{Na}*, *I_T*-*Ca* and *I_{to}* currents in these cells. Although they were relatively weak compared with the positive control, these currents were detectable after transfection with Islet-1 (Fig. 3). No membrane currents were detected in the control group (data not shown). These results confirmed that Islet-1 can indeed induce MSCs to differentiate into functional cardiomyocyte-like cells and that changing the histone acetylation level can block MSC differentiation.

The presence of CpG methylation is correlated with H3K9 modification at the GATA4 promoter

As primary epigenetic modifications, histone acetylation, histone methylation and DNA methylation play important roles in gene expression. The question arose: do these modifications work independently, or do they influence each other during Islet-1-induced MSC differentiation into cardiomyocyte-like cells? To resolve this question, we detected these modifications in the promoter regions of cardiac-specific transcription factors after transfection with Islet-1. First, we found 1 CpG island in the *Nkx2.5* promoter region (51–219 bp) and 3

CpG islands in the *GATA4* promoter region (site 1: 542–884 bp, site 2: 1329–1489 bp and site 3: 1700–1945 bp) using the online software MethPrimer (Fig. 4a). We then evaluated H3K9 acetylation/methylation and CpG methylation at these sites. MSP showed that DNA methylation levels at *GATA4* site 2 decreased gradually with increased transfection duration, and the levels were lowest in the LV-islet-1-3w group. Additionally, *GATA4* site 3 was hypomethylated, while the levels at *GATA4* site 1 and the *Nkx2.5* promoter had no change (Fig. 4b,c). BSP showed that, compared with the blank and control groups, the DNA methylation levels of *GATA4* site 2 in the LV-islet-1-3w group were reduced to 91% (Fig. 4d).

Subsequently, we used ChIP-qPCR to detect the variability of H3K9 acetylation/methylation in each group. We found that H3K9 acetylation levels at the *Nkx2.5* promoter and *GATA4* promoter site 2 increased gradually with increased duration of transfection, with the highest acetylation in the LV-islet-1-3w group; H3K9 methylation levels at the *Nkx2.5* promoter and *GATA4* promoter site 2 demonstrated the opposite tendency, and methylation was lowest in the LV-islet-1-3w group (Fig. 5). In addition, the levels of histone modifications at the *GATA4* site 1 and site 3 exhibited no obvious changes (Fig. 5a). These results suggest that these modifications correlate with each other at the *GATA4* promoter and that CpG methylation does not correlate with histone modifications at the *Nkx2.5* promoter.

5-aza changed the levels of H3K9 acetylation and H3K9 methylation at GATA4 promoter site 2

Previous results have shown that during MSC differentiation into cardiomyocyte-like cells, the three modifications did not work independently but were correlated with each other. The question then becomes: what are

Figure 2. Expression of cTnT and Cx43 detected by immunofluorescence (magnification as shown in the box). The expression of cTnT and the membrane localization of Cx43 were significantly higher in the LV-islet-1 group than in the blank and control groups, and the expressions were inhibited by treatment with EGCG.

Figure 3. Transmembrane electric currents were detected in the cells transfected with Islet-1 using a whole-cell patch-clamp technique. All currents were detected in cultured neonatal cardiomyocytes as a positive control. The presence of the individual ion channel currents was further verified using selective ion channel blockers as shown in the individual panels. (a) Inward-rectifier potassium current (I_{K1}) was inhibited by $BaCl_2$. (b) Delayed-rectifier potassium current (I_{Ks}) was inhibited by TEA. (c) Sodium current (I_{Na}) was inhibited by TTX. (d) T-type calcium current (I_{T-ca}) was inhibited by $NiCl_2$. (e) Transient outward potassium current (I_{to}) was inhibited by 4-AP.

the relationships among these modifications? We added 5-aza after transfection with Islet-1 to observe the changes in H3K9 acetylation/methylation at the *Nkx2.5* and *GATA4* promoters. The Islet-1 levels were not changed, indicating that 5-aza does not affect Islet-1 expression (data not shown). MSP showed that CpG methylation in the *Nkx2.5* promoter did not change in the LV-islet-1 + 5-aza group compared with the LV-islet-1 group, but CpG methylation at the *GATA4* promoter site 2 was decreased significantly in the LV-islet-1 + 5-aza group compared with the LV-islet-1 group (Fig. 6a). BSP showed that *GATA4* site 2 CpG methylation levels of the LV-islet-1 + 5-aza 3w group were reduced to 77% compared with the blank and control groups (Fig. 6b).

ChIP-qPCR showed that H3K9 acetylation levels in *GATA4* promoter site 2 were higher in the LV-islet-1 + 5-aza group than in the LV-islet-1 group, and the H3K9 methylation levels in this region were lower in the LV-islet-1 + 5-aza group than in the LV-islet-1 group (Fig. 7a,b). However, these histone modifications exhibited no differences at the *Nkx2.5* promoter between the LV-islet-1 + 5-aza group and the LV-islet-1 group (Fig. 7a,b).

After adding 5-aza, we detected the expression of *Nkx2.5* and *GATA4* by FQ-PCR, and these data showed that the expression of *GATA4* in the LV-islet-1 + 5-aza group was significantly higher than in the LV-islet-1 group, while the expression of *Nkx2.5* did not differ between the LV-islet-1 + 5-aza group and the LV-islet-1 group (Fig. 7c). These results suggest that changes in CpG methylation levels could change H3K9 acetylation and H3K9 methylation levels at the *GATA4* promoter. *Nkx2.5* is not affected by 5-aza, which indicates that DNA methylation is not involved in regulating the expression of *Nkx2.5*.

The HDAC inhibitor TSA could change the levels of H3K9 methylation and CpG methylation in GATA4 promoter site 2

We added TSA, which TSA does not change Islet-1 levels (data not shown), after transfection with Islet-1, to observe the changes in H3K9 methylation and CpG methylation in the *Nkx2.5* and *GATA4* promoters. First, ChIP-qPCR was used to detect the H3K9 acetylation/methylation levels in the *Nkx2.5* and *GATA4*

Figure 4. Patterns of variation of DNA methylation levels in the GATA4 and Nkx2.5 promoter after the transfection of a lentiviral vector containing Islet-1. (a) Schematic representation of the GATA4 and Nkx2.5 promoters 2.0 kb upstream of the transcription start sites. There are three CpG islands in the GATA4 promoter (site 1: 542–884 bp, site 2: 1329–1489 bp and site 3: 1700–1945 bp) and 1 CpG island in Nkx2.5 promoter (51–219 bp). (b) MSP was used to detect methylation levels in the GATA4 and Nkx2.5 promoters. No differences between the groups were observed at the GATA4 site 1 or the Nkx2.5 promoter, GATA4 site 3 showed hypomethylation, and the methylation levels at GATA4 site 2 decreased gradually. (c) Quantitative presentation of DNA methylation levels at GATA4 site 2 and the Nkx2.5 promoter. The levels were lowest in the LV-islet-1-3w group compared with the blank group (* $P < 0.05$ compared with the blank group). (d) BSP was used to verify the LV-islet-1-3w group methylation levels at GATA4 site 2. The levels were reduced to 91% after the transfection of the lentiviral vector containing Islet-1.

promoters. Compared with the LV-islet-1 group, the H3K9 acetylation levels in the LV-islet-1 + TSA group were significantly increased at the Nkx2.5

promoter and GATA4 promoter site 2, whereas the H3K9 methylation levels were decreased in these regions (Fig. 8).

Figure 5. ChIP-qPCR was used to detect the patterns of histone acetylation/methylation levels of the GATA4 and Nkx2.5 promoters after transfection of the lentiviral vector containing Islet-1. (a) The band densities of histone acetylation levels and the methylation levels of the GATA4 and Nkx2.5 promoters are shown in the left and right panels, respectively. The histone acetylation levels of the GATA4 site 2 and Nkx2.5 promoters increased gradually, while the histone methylation levels decreased gradually. Histone acetylation and methylation were not changed at GATA4 site 1 or site 3. (b) ChIP-qPCR showed that the histone acetylation levels at GATA4 site 2 and Nkx2.5 promoter were highest in the LV-islet-1-3w group, whereas histone methylation levels in these regions were lowest in the LV-islet-1-3w group (* $P < 0.05$ compared with the blank group).

Figure 6. Patterns of DNA methylation levels in GATA4 site 2 and the Nkx2.5 promoter after transfection of the lentiviral vector containing Islet-1 and 5-aza treatment. (a) MSP results showed that 5-aza does not affect DNA methylation levels at the Nkx2.5 promoter. These levels decreased gradually in GATA4 site 2 compared with LV-islet-1 group and were lowest in the LV-islet-1 + 5-aza-3w group. (b) BSP was used to verify the LV-islet-1 + 5-aza-3w group methylation levels at GATA4 site 2, and the levels were reduced to 77% after transfection with the lentiviral vector containing Islet-1 and 5-aza treatment.

Subsequently, MSP showed that CpG methylation levels at the GATA4 promoter site 2 were significantly lower in the LV-islet-1 + TSA group than in the LV-islet-1 group, whereas these levels at the Nkx2.5 promoter exhibited no differences in the LV-islet-1 + TSA group (Fig. 9a). BSP showed that CpG methylation at the GATA4 promoter site 2 was reduced to 84% in the LV-islet-1 + TSA 3w group compared with the blank and control groups (Fig. 9b).

Finally, we detected the expression of Nkx2.5 and GATA4 by qPCR, and the expression levels of GATA4 and Nkx2.5 in the LV-islet-1 + TSA group were significantly higher than in the LV-islet-1 group (Fig. 9c).

These results suggest that changing the H3K9 acetylation levels at the GATA4 promoter could lead to changes in the H3K9 methylation and CpG methylation in this region. Furthermore, TSA could alter the H3K9 methylation levels in the Nkx2.5 promoter but could not affect CpG methylation levels, indicating that CpG methylation was not correlated with H3K9 modification at the Nkx2.5 promoter.

Discussion

As a pleiotropic transcription factor, Islet-1 targets multiple downstream genes in temporally and spatially

Figure 7. ChIP-qPCR was used to detect the variability of histone acetylation/methylation levels at the GATA4 and Nkx2.5 promoters after transfection of the lentiviral vector containing Islet-1 and 5-aza treatment. (a) ChIP-qPCR showed that the histone acetylation levels at GATA4 site 2 and the Nkx2.5 promoter were highest in the LV-islet-1 + 5-aza-3w group, whereas histone methylation levels in these regions were lowest in the LV-islet-1 + 5-aza-3w group ($*P < 0.05$ compared with the blank group). (b) The comparison of histone acetylation/methylation levels in GATA4 site 2 and the Nkx2.5 promoter between the LV-islet-1 group and the LV-islet-1 + 5-aza group. Histone acetylation levels at GATA4 site 2 were significantly higher in the LV-islet-1 + 5-aza group than in the LV-islet-1 group, whereas histone methylation levels were significantly lower. Histone acetylation/methylation at the Nkx2.5 promoter did not differ between the LV-islet-1 group and the LV-islet-1 + 5-aza group. (c) FQ-PCR was used to compare GATA4 and Nkx2.5 expression between the LV-islet-1 group and the LV-islet-1 + 5-aza group. The expression of GATA4 in the LV-islet-1 + 5-aza group was higher than in the LV-islet-1 group, and the expression of Nkx2.5 did not differ between the LV-islet-1 group and the LV-islet-1 + 5-aza group ($^{\#}P < 0.05$ compared with LV-islet-1 group).

Figure 8. ChIP-qPCR was used to detect the patterns of histone acetylation/methylation levels at GATA4 site 2 and the Nkx2.5 promoter after transfection with the lentiviral vector containing Islet-1 and TSA treatment. (a) ChIP-qPCR showed that histone acetylation levels in GATA4 site 2 and the Nkx2.5 promoter were highest in the LV-islet-1 + TSA-3w group, whereas histone methylation levels in these regions were lowest in the LV-islet-1 + TSA-3w group (* $P < 0.05$ compared with the blank group). (b) The comparison of histone acetylation/methylation levels at GATA4 site 2 and the Nkx2.5 promoter were significantly higher in the LV-islet-1 + TSA group than in the LV-islet-1 group, while histone methylation levels in these regions were significantly lower.

specific manners. Islet-1 plays an important role in the development of the pancreas (18), progenitors of a majority of hindlimb cells (19), vertebrate retina (20), and inner ear (21); in motor column formation (22); and in the transition from sensory neurogenesis to subtype specification (23). Additionally, as a cardiac progenitor cell marker, Islet-1 is expressed in the second heart field and is very important in the development of the heart. A previous study has reported that Islet-1 could promote the cardiac differentiation as a transcription factor (24), and our previous study confirmed that histone acetylation was involved in this process, but the mechanism was not clear. In this study, we further explored histone methylation and DNA methylation in addition to histone acetylation. We also sought to explain the mechanism of these epigenetic modifications involved in this process.

Numerous studies have confirmed that epigenetic modifications play important roles in cardiac disease and in MSC differentiation into cardiomyocytes (25–27), but most of these studies have focused on certain types of modifications, and they do not discuss the relationships between modifications. We believe that during MSC differentiation into cardiomyocyte, discussing each modification separately may reveal its mechanism more profoundly but will not truly reveal its role in MSC differentiation. Studies have confirmed that there are correlations between epigenetic modifications (17,28–30). For example, Li-Peng Wu *et al.* have found that the HDAC inhibitor depsipeptide could reduce CpG methylation levels in the P16 promoter region to activate the

expression of P16 (17). Therefore, we believe that epigenetic modifications affect the direction of MSC differentiation through the interactions among these modifications.

In this study, we found that H3K9 acetylation/methylation and CpG methylation, which are involved in regulating the expression of Nkx2.5 and GATA4, may act in different ways during Islet-1-induced MSC differentiation into cardiomyocyte-like cells. For instance, during the regulation of Nkx2.5 expression, CpG methylation is not changed at the Nkx2.5 promoter, which indicates that CpG methylation may not be involved in regulating Nkx2.5 expression, and this result was consistent with a previous study (31). Furthermore, the Nkx2.5 expression pattern is consistent with the H3K9 acetylation levels in the Nkx2.5 promoter and contrasts with the H3K9 methylation levels, indicating that these two histone modifications are antagonistic to each other at the Nkx2.5 promoter. Our data show that GATA4 expression was regulated by the three modifications and that these modifications influenced each other. For example, our data indicated that at the GATA4 promoter, H3K9 acetylation antagonizes H3K9 methylation and CpG methylation, and CpG methylation is synergistic with H3K9 methylation. Therefore, we believe that during Islet-1-induced MSC differentiation into cardiomyocyte-like cells, these three types of modifications do not regulate all genes at the same time but instead possess some selectivity. In addition, we believe that GATA4 promoter site 2 (1329–

Figure 9. Changes in DNA methylation levels in GATA4 site 2 and the Nkx2.5 promoter after transfection with the lentiviral vector containing Islet-1 and TSA treatment. (a) MSP results showed that TSA does not affect DNA methylation levels at the Nkx2.5 promoter. These levels decreased gradually at GATA4 site 2 compared with the LV-islet-1 group and were lowest in the LV-islet-1 + TSA-3w group. (b) BSP was used to verify the LV-islet-1 + 5-aza-3w group methylation levels at GATA4 site 2, and the levels were reduced to 84% after transfection with the lentiviral vector containing Islet-1 and TSA treatment. (c) FQ-PCR facilitated the comparison of GATA4 and Nkx2.5 expression between the LV-islet-1 group and the LV-islet-1 + TSA group. The expression levels of GATA4 and Nkx2.5 in the LV-islet-1 + TSA group were higher than in the LV-islet-1 group ($^{\#}P < 0.05$ compared with LV-islet-1 group).

1489 bp) is the key site for regulating GATA4 expression.

Research has shown that 5-aza inhibits DNA methylation; possible mechanisms for this process may be that 5-aza induces a loss in the ability of DNA to accept methylation *via* its integration into the DNA(32) or that 5-aza reduces DNMT activity by reducing DNMT expression (33). In this study, we observed that 5-aza not only reduced CpG methylation levels at the GATA4 promoter but also increased H3K9 acetylation levels and

decreased H3K9 methylation levels in this region. Based on related studies, we believe that the mechanism of this phenomenon may involve 5-aza reducing DNMT activity, which reduces the binding of DNMTs to the GATA4 promoter region, thereby decreasing CpG methylation levels in this region; methylated CpG islands could induce methyl-CpG binding proteins (MBDs) to bind to this region, and MBDs such as MBD1, MBD2 and MeCP2 could recruit HDACs to form complexes (29,34–36). Therefore, the reduction of

CpG methylation levels at the GATA4 promoter decreases the binding of HDACs to this region, followed by an increase in H3K9 acetylation levels. In addition, acetylation and methylation have a competitive relationship at H3K9 (17,37), and therefore, H3K9 methylation levels decrease with increasing H3K9 acetylation levels.

In contrast, we used TSA to enhance H3K9 acetylation levels in the GATA4 promoter, and we observed that CpG methylation levels were decreased in this region. We hypothesized that H3K9 acetylation was not affected by CpG methylation directly but was instead the result of altered H3K9 methylation. Studies have indicated that methylated histones can induce the binding of heterochromatin protein 1 (HP1) to this region, and HP1 recruits DNMTs to form a compound that can methylate DNA (38,39). Therefore, we speculate that the mechanism by which TSA reduces DNA methylation levels may involve TSA increasing H3K9 acetylation levels at the GATA4 promoter and decreases in H3K9 methylation levels due to the competition between H3K9 acetylation and H3K9 methylation. Then, binding of the HP-DNMT complex to this region was reduced with decreasing H3K9 methylation levels, and CpG methylation levels in this region were eventually reduced. It has previously been suggested that the reduction in CpG methylation levels could further increase H3K9 acetylation levels in this region. Therefore, the three modifications produce loop regulation of the GATA4 promoter.

Our data also show that 5-aza could not affect the levels of the three modifications in the Nkx2.5 promoter and that TSA could change H3K9 acetylation/methylation levels but could not affect CpG methylation levels in this region. These results confirmed the following points: (1) During Islet-1-induced MSC differentiation into cardiomyocyte-like cells, DNA methylation is not involved in the regulation of Nkx2.5 and is not correlated with H3K9 modifications. (2) These three epigenetic modifications are involved in the regulation of different cardiac-specific transcription factors in different ways.

In summary, this study first confirmed that H3K9 acetylation/methylation and CpG methylation are all involved in regulating GATA4 expression, whereas Nkx2.5 is not regulated by CpG methylation, and that these epigenetic modifications are highly correlated with each other during the regulation of GATA4. We discuss the possible mechanisms underlying the interactions of these epigenetic modifications with each other. However, clearly identifying those mechanisms requires further experimental evidence, which will be our focus in the next phase of our work. This study explores the mechanism of MSC differentiation from the perspective of inter-relationships among epigenetic modifications, lays an

experimental foundation for the further application of MSCs in replacement therapy and provides a new approach to exploring the specific differentiation of MSCs.

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Conflicts of interest

No competing financial interests exist.

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